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Edited by

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Foreword

The new generation of therapeutics, including immunotherapy, probiotics, and vaccines act through complex biological processes, often involving multiple organ systems in the human body. Traditional 2D cell cultures and animal testing models cannot meet this new demand. This has catapulted the rapid advancement of human microphysiological systems or popularly known as human organs-on-chips to better predict the efficacy and side effects of these new classes of therapeutics.

Organs-on-Chips technology epitomizes the true integration of biological and engineering sciences. It combines tissue engineering and microfluidic technologies to culture human cells and tissues under biomimetic microenvironments so that they can recapitulate the physiological functions of different organ systems. At the same, they must be manufacturable and fulfill the functions of an experimental testing platform in terms of multiplexing and measurement capabilities to realize their potential in human disease modeling and drug testing. Hence, researchers developing human organs-on-chip devices, no matter whether you are a biologist or a microfluidic technologist, often need to learn and acquire complementary knowledge from another discipline domain. This can be a daunting task, especially for students or researchers new to the field, to sieve through the large body of research work in microfabrication, fundamental physics of microfluidics, tissue engineering, and biology, to identify and assimilate relevant information for the establishment of a human organs-on-chips model.

I am delighted that students and researchers now have a textbook to jumpstart their learning on the guiding principles to establish human organs-on-chips. This textbook provides comprehensive coverage on the design and fabrication of microfluidic devices, physicochemical factors affecting cells cultured in microfluidic devices as well as various cell culture configurations and cellular assays compatible with microfluidic devices. There are also individual chapters devoted to specific organ systems, which will provide insights on how the design of the organ-on-chips devices is tailored to reproduce tissue architectures and functions of that organ system. This book also touches on issues related to the commercialization of human organs-on-chips as well as data handling capability using AI technology, which will likely drive future developments of human organs-on-chip

technologies and translation. Therefore, I anticipate that this textbook will interest and benefit readers from a wide range of backgrounds, including both students to biological and medical R&D practitioners looking to adopt this technology to advance their research.

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Preface

“Principles of Human Organs-on-Chips” aims to cover all aspects of microfluidic organ-on-a-chip systems, from fabrication to applications and commercialization. Organ-on-a-chip models are created to mimic the structural, microstructural, and physiological functions of human organs, providing the potential to bypass some cell and animal testing methods. These are useful platforms with widespread applications, frequently in drug screening and pathological studies. This book offers a comprehensive and authoritative reference on microfluidic organs-on-chips, spanning all key aspects from fabrication methods, cell culture systems, and cell-based analysis to dedicated chapters on specific tissue types and their associated organ-on-a-chip models, as well as their use as disease models, drug screening platforms and more.

“Principles of Human Organs-on-Chips” helps materials scientists and biomedical engineers to better understand the specific requirements and challenges in the design and fabrication of organ-on-a-chip devices. This book also bridges the knowledge gap between medical device design and subsequent clinical applications, allowing medical professionals to easily learn about related engineering concepts and techniques. It starts with the techniques and materials for the fabrication of microfluidic devices and then explains the fundamentals of cellular biology aspects. After presenting detailed chapters on the applications for different tissues and organs, it highlights the future of the field and the chances for commercialization.

This project intends to bring all the information together in one major reference, providing an in-depth understanding of the fundamentals and applications of organ-on-a-chip systems. It is not only a textbook for students in biomedical engineering, chemical engineering, materials science and engineering, cell biology, biotechnology, and medical courses at advanced undergraduate and graduate levels but also a reference tool for research and clinical laboratories. The knowledge of the contributors added an extra value to this book project toward an in-depth source for organ-on-a-chip system fundamentals and strategies. It is believed that the breadth of the collaborations in this book can overcome many existing problems and limitations.

Masoud Mozafari, PhD

CHAPTER 1

Techniques and materials for the fabrication of microfluidic devices

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1.1 Introduction

Microfluidics, a technique characterized by the engineering manipulation of fluids at the submillimeter scale, has emerged in recent decades as a promising enhancement in diagnostics and biological research [1]. According to the definition from National Cancer Institute, microfluidic devices, also known as miniaturized total analysis systems (μ TASs) or lab-on-a-chip technologies, offer advantages of small working volumes and reduced reagent amounts while enabling high scalability and dynamic control over the cell culture microenvironment. When microfluidic devices are applied in biological research, these advantages allow the high throughput of biological experimentations [2]. Organ-on-a-chip (OoC) is the biomimetic system built on microfluidic chips and was selected as one of the “Top Ten Emerging Technologies” in the World Economic Forum [3]. The microenvironments in the OoCs are designed to mimic that of specific organs by recreating a physiological environment with dynamically controlled chemical and mechanical stimulation as facilitated by a combination of cell biology, tissue engineering, and biomaterial technologies [4]. OoCs representing different human organ systems have also been connected within a single platform, termed body-on-a-chip technology, to recapitulate the interorgan communication in vivo [5,6]. This development in OoC technology further advances the potential of OoC systems as a model to predict human tissues response at an organism level to various stimuli, including drugs and environmental influences, in contribution to reducing, replacing, and refining the use of animal models and to develop personalized medicine [7–9]. In this chapter, we discuss the theories governing the physics of microfluidics which affects the microenvironment of cells and thus their behaviors and functions in OoC. We then discuss considerations for the fabrication of microfluidic devices in OoC applications.

1.2 Theory of microfluidics

This section discusses theories governing fluid flow and mass transport in microfluidic devices. Understanding of the physics of microfluidics, which applies to fluids contained in devices in the microscale (at least one order smaller than 1 mm), has grown in tandem with the advance in fluid dynamics. The fluid phenomena that dominate liquids are drastically different at the submillimeter scale [10].

The Navier–Stokes equations, which encompass the law of conservation of momentum in fluidics, explain the space–time evolution of the velocity field of a particular fluid in a confined volume [11].

$$\rho \left(\frac{\partial \mathbf{u}}{\partial t} + \mathbf{u} \cdot \nabla \mathbf{u} \right) = -\nabla p + \mu \nabla^2 \mathbf{u} + \mathbf{f}, \quad (1.1)$$

The inertial acceleration and the forces are represented on the left and right sides of the equation, respectively. On the left side, describing acceleration, $\frac{\partial \mathbf{u}}{\partial t}$ represents the velocity change with respect to time (local change), and $\mathbf{u} \cdot \nabla \mathbf{u}$ represents the velocity change with respect to space (convective change). On the right side, describing forces, $-\nabla p$ denotes the pressure force, $\mu \nabla^2 \mathbf{u}$ denotes the viscous force, and $\mathbf{f}$ denotes the body forces (such as gravitational force). The continuity equation (which is the law of mass conservation in fluid) is described in Eq. (1.2).

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{u}) = 0, \quad (1.2)$$

With the assumption that the fluid is incompressible (i.e., the density is constant over space and time, thus $\partial \rho / \partial t = 0$) and Newtonian (i.e., the viscosity does not vary with the flow), these equations can be further simplified. Overall, the velocity field of an incompressible, Newtonian fluids is described in Eq. (1.3).

$$\begin{cases} \left(\frac{\partial \mathbf{u}}{\partial t} + \mathbf{u} \cdot \nabla \mathbf{u} \right) = -\frac{1}{\rho} \nabla p + \nu \nabla^2 \mathbf{u} + \mathbf{f} \\ \nabla \cdot \mathbf{u} = 0 \end{cases}, \quad (1.3)$$

where $\mathbf{u}$ [m/s] represents the velocity field of the fluids, ρ [kg/m³] represents the density of the fluids, p [Pa] represents the pressure field, ν [m²/s] represents its kinematic viscosity, and $\mathbf{f}$ [m/s²] represents an external acceleration field due to forces such as gravity or electrostatics. By substituting dimensionless quantities for existing dimensional quantities, including $\nabla^* = L \nabla$, $\mathbf{u}^* = \frac{\mathbf{u}}{U}$, $t^* = \frac{t}{L/U}$, $p^* = \frac{pL}{\rho \nu U}$ and $\mathbf{f}^* = \frac{\mathbf{f}}{f_0}$, where L denotes the characteristic length of the geometry (i.e., hydraulic diameter in a micro-channel), U denotes the typical velocity of the fluid, and f_0 denotes the typical intensity of the acceleration field, the velocity field equations can be nondimensionalized:

$$\text{Re} \left(\frac{\partial \mathbf{u}^*}{\partial t^*} + (\mathbf{u}^* \cdot \nabla^*) \mathbf{u}^* \right) = -\nabla^* p^* + \nabla^{*2} \mathbf{u}^* + \frac{f_0 L}{U^3} \mathbf{f}^*, \quad (1.4)$$

Here, Re is a dimensionless variable termed as the Reynolds number, defined as $Re = \frac{UL}{\nu}$. The Reynolds number (Re) describes the ratio of the inertial force to the viscous force. Re is proportional to L (length of the system) and U (velocity of fluid). As such, scaling down the dimension and velocity reduces Re , and microfluidic systems are often characterized as low Re . The magnitude of Re indicates the flow patterns: laminar and turbulent.

1.2.1 Laminar and turbulent flows

Flow patterns are determined by the magnitude of the Reynolds number (Re). Fluid flow becomes laminar (i.e., smooth and streamlined) at small length scales and low flow rates, which is the characteristics of microfluidic flows [12]. Laminar flow is typically characterized as $Re < 2000$ [13,14]. In contrast, flows become turbulent (i.e., irregular and convective) at high Re , which is typically characterized as $Re > 4000$ [13,14].

At high Re , the left side of the velocity field equation (Eq. 1.4) dominates. As Re is a similarity parameter of the ratio between inertial force and viscous force, the viscous force becomes negligible in comparison to inertial force. Therefore, the viscous term ($\nu \nabla^2 \mathbf{u}$ in Eq. 1.3) becomes negligible. The flow is governed by the equation: $\frac{\partial \mathbf{u}}{\partial t} + (\mathbf{u} \cdot \nabla) \mathbf{u} = -\frac{1}{\rho} \nabla p + \mathbf{f}$. Under this assumption, the velocity field ($\mathbf{u}$) is unstable and nonlinear. This flow pattern is known as turbulent. At low Re , the left-hand side of Eq. (1.1) is considered small. Eq. (1.1) is reduced to $0 = -\frac{1}{\rho} \nabla p + \nu \nabla^2 \mathbf{u} + \mathbf{f}$. This relationship suggests that the viscous stress tensor ($-\frac{1}{\rho} \nabla p + \nu \nabla^2 \mathbf{u}$, a linear approximation of the stress around a point in the flow) is parallel to the sum of the body forces ($\mathbf{f}$). As a result, the flow is driven toward the same direction as the external force but not perpendicular to that direction. Such flows form parallel streamlines, which is known as laminar flow. The fluids form parallel layers of flow along the channel, and the mixing occurs only diffusively.

The laminar flows at the microscale result in a stable gradient of biological molecules that can be regulated both spatially and temporally [15]. Biological phenomena such as angiogenesis, invasion, and migration are driven by concentration gradients of a variety of biochemical signals [15,16]. Microfluidic systems can be employed to study these complex physiological processes experimentally. For example, to recapitulate these processes, stable three-dimensional (3D) biochemical concentration gradients can be generated in a microfluidic system by changing flow velocity and channel geometry with microvalves and micropumps [17].

1.2.2 Diffusion in microfluidics

The diffusion of the fluid results in mass transport. The diffusion–advection equation gives the space–time evolution of a concentration field c [kg/m^3] for some molecular species,

$$\frac{\partial c}{\partial t} - D\nabla^2 c + (\mathbf{u} \cdot \nabla)c = 0, \quad (1.5)$$

where D [m^2/s] is the diffusion coefficient. The Peclet number (Pe) can be defined as follows:

$$\text{Pe} = \frac{LU}{D}, \quad (1.6)$$

Pe represents the relative importance of convective transport to diffusive transport. Pe is typically low in microchannels due to low flow rates (U) and the small length scale of interest (L). As such, mixing takes place primarily through diffusion in microchannels [18].

Diffusion is a statistical transportation phenomenon that occurs when a concentration gradient of a solute exists in a fluid. Fick's Law of Diffusion is given as

$$\mathbf{J} = -D \cdot \Delta n, \quad (1.7)$$

where D is the diffusion coefficient, n is the particle density or concentration, and Δ is the Laplace operator. The diffusion time (t) is the time taken for a molecule to travel a distance (x) by diffusion, which is described as,

$$t = \frac{x^2}{2D}, \quad (1.8)$$

This equation suggests that the reaction time is proportional to the square of the rate-limiting distance for diffusion-limited reactions. It implies the reaction rate in a 100 μm -diameter microreactor is faster than in a 10 cm-diameter flask by a factor of 1,000,000. Such enhanced reaction rate is one of the major motivations to develop microfluidic systems for rapid and high throughput chemical reaction.

Diffusion is intrinsic because particles move over time due to Brownian motion, and this results in a constant average particle concentration across the volume eventually. As in Brownian motion, the statistical movement of a single molecule in a fluid is random. Collisions among solute and solvent particles (or molecules) are the driving force of diffusion. The Einstein–Smoluchowski relationship governs the motion of a particle

$$x = \sqrt{2Dt}, \quad (1.9)$$

where x is the average distance traveled between molecule collisions after a time interval of t .

In laminar flows, the diffusion happens across the parallel streamlines. The flows form parallel streamlines, and the velocity of the flow is perpendicular to the direction of diffusion. Therefore $(\mathbf{u} \cdot \nabla)c$ becomes zero. This assumption reduces Eq. (1.5) to Fick's second Law, which can be used to describe the kinematics of diffusion in microfluidics:

$$\frac{\partial C}{\partial t} = D \cdot \nabla^2 C, \quad (1.10)$$

where C denotes the chemical concentration, and D denotes the diffusion coefficient of the molecule in interest. The path of a particle between two collisions can be represented by a straight line and the average of which is known as the mean free path. Because the change in the magnitude and direction of molecular velocity in a collision is random, the distribution of the solute is homogeneous. Diffusive flux (J), or the number of molecules crossing the unit surface over the unit time, is proportional to the concentration gradient. The negative symbol in this equation (Eq. 1.7) represents the fact that the diffusion occurs in the opposite direction of the solute concentration gradient, determining the direction of the diffusive transport. The diffusion coefficient (D) is the proportionality constant. As a result, the diffusion coefficient is measured in the unit of m^2/s . Einstein–Stokes' equation can be used to approximate the diffusion coefficient D ,

$$D = \frac{k_B T}{6\pi\eta r_H}, \quad (1.11)$$

where k_B is the Boltzmann's constant, T is the temperature, η is the dynamic viscosity of the solvent, and r_H is the hydrodynamic radius of the molecule for diffusion of spherical particles through the fluid.

These two characteristics—laminar flow and the dominance of diffusion—allow the fluid to be treated almost as a solid in microfluidics; fluids in the microfluidic systems can be physically positioned, and their flow can be delicately regulated [19]. As a result, OoCs with surface modifications have been developed to position-specific biological cues at specific points in a device. These OoCs with surface modifications have the potential to provide various advantages, including tissue architectural recapitulation, drug and signaling component diffusion kinetics, and

physiological flow conditions [20]. As such, since their inception in the early 2000s, OoCs have demonstrated remarkable success in recapitulating the physiological activities of in vivo tissues that were not achievable using standard cell culture models [8,9].

1.2.3 Surface tension

The important physics of microfluidics is not only about the flows in microchannels but also about the interface between multiple fluids and/or surfaces at microscales. Surface tension is caused by a discrepancy of intermolecular tensions among the molecules of fluids and solid surfaces. The effect of surface tension can be exemplified by the behaviors of a lightweight needle on the surface of the water. When the needle is gently placed on the surface of the water, surface tension may prevent the needle from sinking even when the density of the needle is greater than that of water. However, when a downward force is applied to the needle, the surface tension is removed, and the needle may sink instantly.

Microscale devices, in addition to having a low Re , have a high surface area to volume ratio that can cause the surface tension to become the dominant force [21]. The Bond number (Bo) represents the contrasts of gravitational force to surface forces,

$$Bo = \frac{\Delta\rho g L^2}{\sigma}, \quad (1.12)$$

where $\Delta\rho$ denotes the difference in mass density between the two fluids, g denotes the gravitational acceleration, L denotes a characteristic length scale, and σ denotes the interfacial tension between the two types of fluidics in contact. Gravitational forces in microfluidic applications are often insignificant (characterized as $Bo < 1$) because of the small length scale (L) in microscale.

Within the fluid, surface tension is generated by cohesive forces on the surface of the fluid. Outside of the fluid, capillary action is caused by adhesive forces between the fluids and the walls. At microscales, capillary forces are difficult to overcome, providing a useful way to transport fluids in a microscale. When multiple types of fluids co-flow through a microchannel, the interface between fluids can be curved by surface tension to balance the usual pressure difference. The Young–Laplace equation governs this relationship,

$$\Delta p = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right), \quad (1.13)$$

where Δp denotes the pressure difference, γ is the surface tension, and R_1 and R_2 are the radii of curvatures of the interface of the fluids. If the radius of curvature is small enough, as it is in microfluidic devices, this equation suggests that an interface can withstand high pressure differences across it. In a circular channel, the equation is reduced to be

$$\Delta p = \frac{2\gamma \cos\theta}{a}, \quad (1.14)$$

where Δp denotes the pressure difference, γ denotes the surface tension, θ notes the contact angle, and a denote the radius of curvature of the tubing.

The fluid motion caused by surface tension gradients has been reported as early as 1855 [22]. Fluid flow can be driven in microchannels due to the pressure difference caused by the formation of a curved boundary. Capillary action is an important driving force of paper-based microfluidic systems [23,24], where surface tension plays an important role in the flow at microscale.

1.3 Materials consideration for organ-on-a-chip devices

Appropriate considerations must be given to material selection when developing microfluidic devices for OoC applications. Necessary considerations for the selection of materials include biocompatibility, sterilization tolerance, optical transparency, and mechanical properties.

1.3.1 Biocompatibility

In the context of OoCs, biocompatibility refers to the ability of the material to support cellular activities [25]. Not all materials are suitable for cell culture in vitro. Biocompatible surfaces need to promote cell adhesion, spreading, and cellular functions [26]. Traditionally, glass substrates are used as cell culture surfaces because they are optically transparent and thus ideal for visualization under microscopes. Unfortunately, glass substrate does not promote adequate cell attachment, and coating of extracellular matrix is usually required [27].

Plastic tissue culture surfaces became the predominant material for cell culture only after the accidental discovery by Falcon Plastic Company (Brookings, SD, USA). Falcon Plastic Company found that the oxygen plasma treatment of the polystyrene (PS) produced an optimal surface for cell culture [28]. Today, pretreated polymer substrate is the preferred material for cell-culture applications. Hacking and Khademhosseini provided a comprehensive overview of the effect of materials on cell response and

behavior [29]. Commercially available OoC devices are usually produced using polystyrene. Some OoC companies, including Mimetas, AIM Biotech, Ibidi, and TissUse, produce devices out of plastics [30]. Polydimethylsiloxane (PDMS), a common material used to fabricate microfluidic and OoC devices, is broadly considered to be noncytotoxic. It is popular within the OoC community because it facilitates the fabrication of complex geometries at the microscale. However, PDMS does not promote cellular adhesion, proliferation, and functions. Nevertheless, PDMS can be effectively coated with proteins to promote cell attachment [31].

1.3.2 Sterilization tolerances

Proper sterilization of devices is essential to develop OoC without micro-organismal contamination. The selected material must tolerate at least one of the sterilization techniques. Wet heat (autoclaving), dry heat (flaming, baking), solvents (ethanol), and radiation (ultraviolet light) are commonly available sterilization techniques [32]. X-rays, gamma rays, or ethylene oxide are also less available in academic research settings, yet possible options.

For example, PDMS can withstand a variety of sterilization techniques. Moolman and colleagues used autoclave (120°C for 15 min) to sterilize their PDMS devices (bonded to glass substrate) [33]. Before cell seeding, Huh and colleagues sterilized their PDMS device using ultraviolet (UV) irradiation [34]. Our group have sterilized PDMS channels by flushing with 70% ethanol for 90 min before cell seeding [35]. Plastic devices (e.g., polystyrene) is not autoclavable. Ethylene oxide or ethanol are suitable sterilization options for plastic devices. Polymethyl methacrylate (PMMA) is also a popular material employed to fabricate microfluidic devices. PMMA cannot tolerate autoclaving. Sterilization by 70% ethanol is more suited for PMMA, although prolonged exposure to ethanol (>1 h) may cause crazing or microcracks on the surface of the PMMA, which may compromise optical clarity [36]. Users should also consider sterilization methods for other components that come into direct contact with the devices [37]. These components may include tweezers, tubing, adaptors, containers, and paper towels.

1.3.3 Optical transparency

Observation of OoCs is typically performed using optical and fluorescent microscopes, which requires the device to be transparent. The transparency of the material does not guarantee the transparency of the device due to the surface roughness of the materials. One of the most common materials for microfluidics is PDMS. PDMS is a material with excellent transparency to

optical wavelength. Coincidentally, the method of fabrication (i.e., soft lithography) that popularized the use of PDMS for microfluidics allowed to produce microchannels with low surface roughness which translated to good optical transparency. The transparency of the microchannels can be compromised by the method of fabrication even when a transparent material is employed. For example, micromilling of PMMA creates surface roughness within the microchannels that interfere with the observation under optical microscopy. 3D printing of transparent material can be employed to fabricate microchannels, which may also result in translucent surfaces due to the inherent mechanism of 3D printing (e.g., fused deposition modeling (FDM) and selective laser sintering) that inadvertently produce surface roughness [38]. When optical transparency is required, consideration must be given to both the material and the method of fabrication.

1.3.4 Mechanical property

Some OoC devices can mimic *in vivo* mechanical stimuli. For example, organs, including the lung, blood vessels, and intestinal tract, are not stationary *in vivo* but experience cyclic motions that are vital for their functions. Huh et al. demonstrated one of the representative OoC device for on-chip mimicry of mechanical stimulus, known as lung-on-a-chip [34]. Elastomeric materials (PDMS) were used to accomplish this motion, allowing the device to mimic the stretching found in the lung.

Elastomeric materials can also be used to make tubing connections more convenient. The inlets and outlets of PDMS microfluidic devices are commonly created by punching a hole with a biopsy punch. The connection tubing can be directly pushed into the inlet and outlets. The elastomeric property of the PDMS ensures a proper seal that prevents leakage. When rigid materials are employed, additional considerations (e.g., adaptors, O-rings) are required to ensure proper sealing between the tubing and the device. Connecting different chips is of particular importance when the interactions among multiple organs are studied using multiple, independent units representing different organs [35,39].

1.4 Fabrication techniques and their materials

This section summarizes existing fabrication techniques for microfluidic devices and discusses the appropriate materials for each technique. We categorize fabrication techniques into three categories: subtractive manufacturing, formative manufacturing, and additive manufacturing [40].

Each fabrication technique is materials-specific; it is not always possible to repurpose a particular fabrication technique for any materials.

1.4.1 Subtractive manufacturing

Subtractive manufacturing is the process where materials are selectively removed from a stock material to form a desired shape. Several methods have been developed to remove materials selectively from a stock material. We discuss some of the most frequently used methods for fabricating microfluidic devices.

1.4.1.1 Examples of subtractive manufacturing

Bulk micromachining

Early microfluidic devices in the 1990s were primarily fabricated using subtractive manufacturing, partly because few fabrication techniques were capable of fabricating microscale features back then. Researchers have relied on the well-established bulk micromachining technique developed for semiconductor and microelectromechanical systems (MEMS) [41–43]. Because bulk micromachining in MEMS uses silicon and glass substrates, early microfluidic devices also consisted of silicon and glass substrates. Bulk micromachining mainly relies on a photolithography prior to chemical etching to generate the microfluidic features [43]. Briefly, the fabrication consists of the following three steps: (1) photolithography, (2) etching, and (3) bonding. Firstly, a thermal oxide layer is deposited on the substrate (usually silicon) (Fig. 1.1a(i)). Next, a layer of photoresist is deposited on the thermal oxide (Fig. 1.1a(ii)). A photomask is positioned over the photoresist and exposed to UV light (Fig. 1.1a(iii)). The areas exposed to UV light are chemically modified and are removed during the development. After the exposure to the UV light, the substrate is soaked in an etchant (Fig. 1.1a(iv)). The etchant only removes the silicon oxide layer in the areas not covered by the photoresist. Next, the photoresist layer is removed, and the substrate is etched again using an etchant that attacks the exposed silicon substrate (Fig. 1.1a(v)). After removing the silicon oxide layer, the substrate is bonded to another flat silicon substrate (by anodic bonding or fusion bonding) to form the channels (Fig. 1.1a(vi–ix)) [44,45]. Early iterations of microfluidic channels are usually made of silicon and glass fabricated by bulk micromachining. These devices were applied for electrophoresis [46,47] and PCR amplification [42]. While bulk micromachining is mostly suited for glass and silicon substrates, alternative etching processes are available to etch polymer substrates [48].

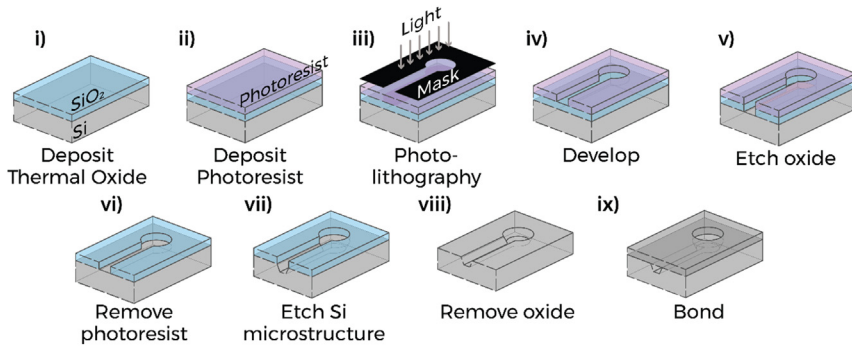


Figure 1.1 Subtractive manufacturing employed for microfluidic fabrication. Illustration highlighting the various steps involved in bulk micromachining. (From Ching et al. [40].)

Laser ablation

Bulk micromachining is applicable to silicon and glass substrates but not effective to work with polymeric substrates. With the commercialization of laser ablation apparatus, researchers investigated the potential of laser ablation in the fabrication of microchannels. Laser-cutting machine is an example to employ laser ablation that removes materials from a polymeric film selectively; laser-cutting works by focusing a laser beam to cut or emboss features on materials (Fig. 1.2a,b). After using a laser cutter to cut or emboss features on polymeric materials, the channel features are sealed to create closed channels. Several methods of sealing have been explored. Patko et al. demonstrated a laser cutter to create fluidic features on a double-sided tape and subsequently sandwich the double-sided tape with fluidic features between two rigid substrates to form a fluidic device [49]. In this method, the double-sided tape served as an adhesive to create a sealed bond between sheets of polymeric films. Thompson et al. used a laser cutter to cut features on a polyester film and subsequently laminated (i.e., thermal bonding) the patterned sheet between other polyester films [50]. Klank et al. explored the use of plasma activation and solvent-assisted bonding techniques to seal multiple layers of engraved features on PMMA [51].

Cutting plotter/Xurography

Apart from the laser ablation of polymeric thin films to create the channel features, researchers explored the use of a cutting plotter to remove features from polymeric thin films. A cutting plotter uses a digitally controlled blade to cut a piece of material (i.e., paper, polymeric film) to form features on

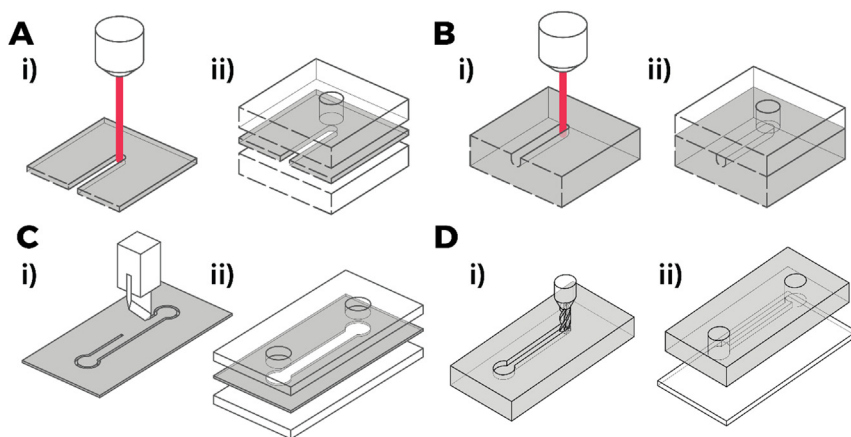


Figure 1.2 Subtractive manufacturing employed for microfluidic fabrication. Illustration highlighting the various steps involved in (A) laser ablation of thin-film, (B) laser embossing, (C) xurography, and (D) micromilling. (From Ching et al. [40].)

them. Briefly, a thin film is secured onto an adhesive mat and fed into a cutting plotter machine. The outlines of the microfluidic channels are drawn in the cutting plotter software. The design of cutting patterns is thereafter patterned on the substrate with the blade (Fig. 1.2c(i)); the blade is digitally controlled to cut the outline according to the input design. After removing the cut film from the adhesive mat, the film is sealed between substrates to obtain the microchannels (Fig. 1.2c(ii)).

This technique to use a cutting plotter to create features on thin-film substrates is called xurography. Xurography is an alternative to laser ablation since some polymers generate harmful fumes (e.g., polyvinylchloride, polycarbonate, polytetrafluoroethylene, Teflon). For example, Hizawa et al. fabricated microfluidic devices by cutting fluoropolymer films. These fluoropolymer films are generally not suited for laser ablation as they may give off harmful fumes. After cutting the channel outline with a cutting plotter, the channels were laminated and sealed using heat and pressure [52]. Additionally, the same technology has been expanded to create multilayer channels. At the moment, the most advanced cutting plotters can produce features as small as 20 μm [53].

Micromilling

Researchers have investigated the use of micromilling to remove materials from a stock bulk material selectively to fabricate microfluidic channels [54]. Milling is a machining technique in which rotary cutters are used to

remove material from the workpiece by controlling the motion of the cutter (Fig. 1.2d). Micromilling is primarily suitable for stiff materials with high elastic moduli, as they allow the cutting tool to effectively shave off the swarf (through shear deformation and fracture) from the bulk material. Micromilling can cut features as small as 100 μm . Polymeric materials with high elastic moduli are generally suitable in micromilling (e.g., PMMA, polyethylene, polyvinyl chloride, polypropylene (PP), acrylonitrile butadiene styrene (ABS)) [55]. Although micromilling is not recommended for elastomeric materials with a low elastic moduli (e.g., PDMS, silicone, or hydrogels), some researchers have examined the effects of cryogenically cooling of PDMS to increase its elastic modulus during micromilling to fabricate microfluidic devices [56].

Owen et al. used micromilling to create microchannels on the faces of Lego bricks (made of ABS). These microchannels on Lego bricks were then sealed with transparent adhesive polyethylene sheets to make microfluidic devices. They have made microfluidic modules with various functions that can be put together to construct microfluidic devices [57]. Micromilling is an adequate rapid prototyping tool for small-scale manufacturing of microfluidic devices. However, it should be noted that micromilling features with lateral dimensions of $<100\ \mu\text{m}$ might come with challenges. Micromilling bits smaller than 100 μm in diameter are not readily available due to their susceptibility to fracture. Inherent surface roughness caused by the micromilling bit should also be taken into consideration as it may impede the fabrication of devices with high optical transparency. Guckenberger et al. provided a coherent review on protocols, tips, insights, and considerations for practitioners interested in using micromilling to create microfluidic devices [54].

1.4.1.2 Discussion on subtractive manufacturing

Materials for subtractive manufacturing

Bulk micromachining is generally suited for manufacturing silicon, glass, and quartz substrates. These processes also require dedicated facilities (e.g., cleanroom) and specialists for the operation of the equipment. Many researchers favor devices made of polymers mainly due to their ease of fabrication, glass fluidic devices are still irreplaceable for some applications (e.g., applications that involve elevated temperatures or organic solvents). Bulk micromachining remains the ideal fabrication method to produce glass microfluidic devices. While such setups are not readily available to all researchers, commercial companies such as Elveflow, Dolomite, Citrigene,

Chipshop, and Darwin Microfluidics offer services for fabricating glass and quartz fluidic devices. These businesses also provide services for custom-built fluidic devices based on customer requirements.

Accessibility of subtractive manufacturing

Bulk micromachining does not offer rapid fabrication processes and is not suitable for rapid prototyping. On the other hand, laser ablation, xurography, and micromilling are rapid and cost-effective fabrication methods highly suited for rapid prototyping. A laser cutter and micromilling machine are often available for <10,000 USD, and a desktop cutting plotter of <500 USD has been used for xurography. These tools have, therefore, become accessible to academic research settings. These fabrication methods are compatible with a wide array of polymeric materials. A quick design-to-prototyping cycle is another crucial benefit of these fabrication methods. In most cases, users may create a prototype in merely a few hours. Importantly, polymeric films that are irreplaceable in *in vitro* cell culture (e.g., polystyrene and polycarbonate) can be easily converted into microfluidic devices using these rapid subtractive manufacturing methods [26], providing opportunities to develop cell-laden devices for OoC applications.

1.4.2 Formative manufacturing

1.4.2.1 Examples of formative manufacturing

Formative manufacturing refers to a manufacturing technique to shape materials using a mold. Whitesides, Quake and other researchers have pioneered to develop microfabrication methods collectively termed soft lithography. Soft lithography in microfluidics refers to the process of casting PDMS replicas on photolithography-generated master molds [58]. Soft lithography does not only refer to one fabrication method but refers to a collection of techniques that uses microfabricated elastomeric PDMS stamps, which includes replica molding, microcontact printing (μ CP), microtransfer molding, micromolding in capillary, solvent-assisted micromolding, phase-shifting edge lithography, nanotransfer printing, decal transfer lithography, and nanoskiving [59]. Since its introduction, soft lithography has become the popular choice to fabricate microfluidic devices due to its simplicity, cost-effectiveness, and rapid turnaround time in comparison with bulk micromachining. Additionally, PDMS is optically transparent and biocompatible, making it favorable for applications in OoCs. This section discusses three notable formative manufacturing processes used to fabricate microfluidic devices: soft lithography, embossing, and injection molding.

Soft lithography

Soft lithography has been arguably the most popular technique for fabricating microfluidic devices in academic settings over the past 20 years [60,61]. Fig. 1.3a describes a typical workflow for fabricating PDMS devices via soft lithography. The production of the master mold (Fig. 1.3a(i-iv)) is followed by the casting and bonding of elastomeric polymer (Fig. 1.3a(v-viii)). A silicon wafer is first spin-coated using SU-8 (a negative photoresist). Variations in the rotation speed, acceleration, and viscosity of SU-8 allows

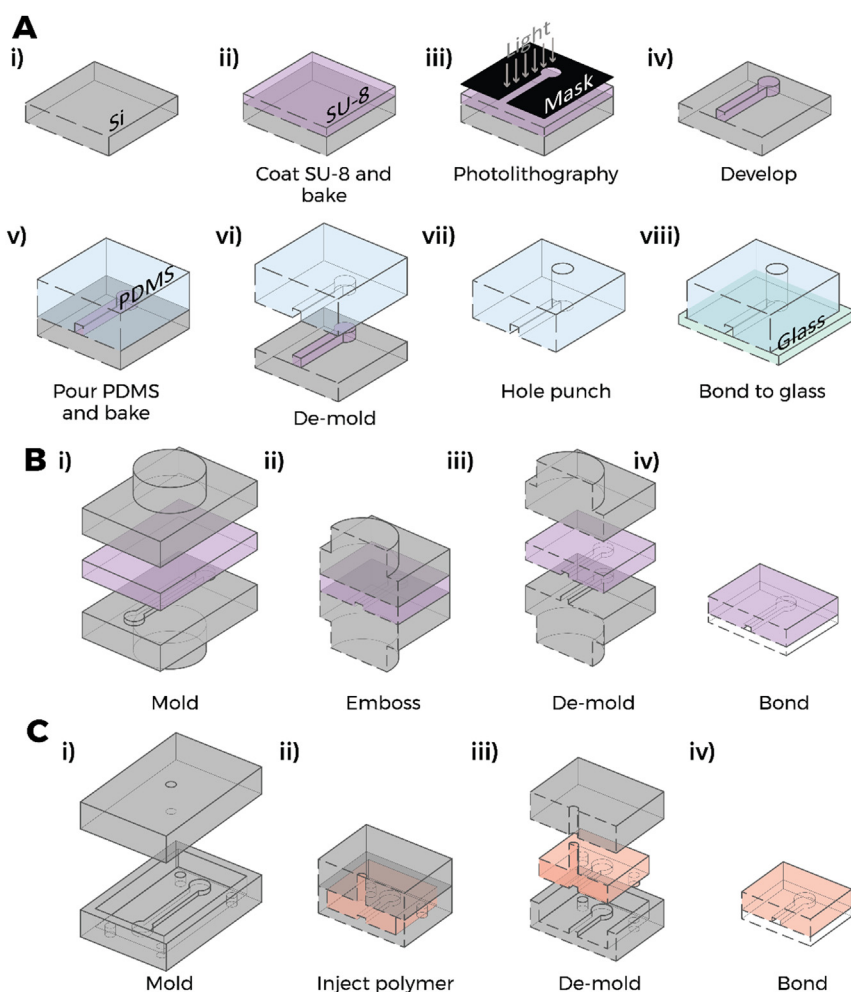


Figure 1.3 Formative manufacturing techniques employed for microfluidic fabrication. Illustration highlighting the various steps involved in (A) soft lithography, (B) embossing, and (C) injection molding. (From Ching et al. [40].)

for the precise control of the thickness of the SU-8 coating [62]. The thickness of the SU-8 coating determines the height of the fluidic channel. After coating, the photoresist is then soft-baked (5–10 min at 65°C) to partially eliminate the solvent and firm the photoresist before UV exposure. After soft-baking, a photomask is placed over the photoresist coating, and UV light (350–400 nm) is applied. Two types of photomasks are usually employed: (1) a rigid photolithography mask (or photomask) made of glass or quartz substrate patterned with a thin chromium layer, and (2) a soft photomask made of a plastic film printed with black ink at a high resolution (20,000 dpi). The rigid photomask typically has higher resolutions (1 μm) than the soft photomask (10 μm). As SU-8 is a negative photoresist, the sections of SU-8 exposed to UV light are crosslinked; the sections not exposed to UV light remain soluble in a developer solution. The photoresist is heated for postbaking after UV exposure to speed up the polymerization of SU-8. By immersing the entire substrate in a developer solvent (i.e., 1-methoxy-2-propyl acetate), the unpolymerized sections of the SU-8 are eventually eliminated. After this step, an optional baking is performed to complete the mold creation. Prior to PDMS replication, the mold is normally salinized (e.g., octadecyl trichlorosilane, trimethylchlorosilane) to facilitate the demolding of cast PDMS.

Uncured PDMS is cast over the mold after salinization, followed by degassing to remove bubbles in a low-pressure chamber. The uncured PDMS in the mold is then heated to speed up the curing. Cured PDMS is easily removed from the mold to obtain a micropatterned PDMS stamp. A biopsy punch is used to create access ports for connecting tubing to the PDMS replica. Finally, the channels are formed by bonding the PDMS stamp to a substrate. The surfaces of both PDMS and another substrate (such as glass) are activated using oxygen plasma (or air plasma) to convert $\text{Si}-\text{CH}_3$ to $\text{Si}-\text{OH}$, making them suitable for covalent bonding to form $\text{Si}-\text{O}-\text{Si}$ bonds. To complete the sealing, the sealed device is oven-baked at 65°C to accelerate the formation of $\text{Si}-\text{O}-\text{Si}$ bonds via dehydration reaction.

The most popular type of master mold for replica molding of PDMS has been SU-8 master molds. Other surfaces can, however, be used for PDMS replica molding too. Hwang et al. provided a comprehensive evaluation of atypical molds used for PDMS casting [63]. The use of PDMS as a mold to cast rigid polymers (including SU-8) has also been reported [64]. It has also been established that 3D-printed master molds or sacrificial templates can be used to cast a secondary material [65,66]. To create microchannels in PDMS and other substrates, water-soluble polyvinyl alcohol (PVA) has been employed frequently [67,68].

4.2.1.2 Embossing

Embossing is a process to transfer micropatterns on the master mold to other polymers. Embossing does not require materials for device fabrication to be in a liquid state. Embossing of polymers usually occurs when polymeric materials are malleable without complete melting. Rigid polymers are commonly used for hot embossing. Fig. 1.3b shows the use of embossing to create microfluidic devices. Stamp embossing, roller embossing, UV embossing, and thin-film lamination are examples of different processes of embossing to produce microfluidic devices [69–73].

Injection molding

Injection molding is yet another formative manufacturing process for fabricating microfluidic devices. Injection molding involves injecting molten polymers into a mold, followed by cooling and hardening the polymers. Four essential steps are involved in microinjection molding. First, the two-part mold is aligned to create a mold cavity (Fig. 1.3c(i)). The heated thermoplastic is then injected into the mold cavity (Fig. 1.3c(ii)). The cast object is then removed from the mold after cooling (Fig. 1.3c(iii)). Finally, the micropatterned polymer surface is sealed with another substrate to form microchannels [74]. Once the master mold is created, injection molding allows rapid manufacturing of multiple copies of the device, making it suitable for industrial fabrication.

1.4.2.2 Discussion on formative manufacturing

Materials for formative manufacturing

Formative manufacturing requires materials that can exist in a malleable state (i.e., liquid state, semi-molten state). The material candidate must conform to the shape of the mold before undergoing solidification to form the desired features on the material. It is preferable to use a material that can first exist in the liquid state in soft lithography. PDMS is arguably the most widely used material in soft lithography although other silicone alternatives are available. PDMS is a castable liquid before crosslinking, which can faithfully take the shape of the mold. Several advantages continue to make PDMS an attractive choice. PDMS is biocompatible, optically transparent, gas permeable, and inexpensive. Furthermore, due to the elasticity of PDMS, it enables the extension of functions (such as valves, pumps, and actuators), allowing the creation of devices with unique dynamic actuation combined with cell cultures [34]. Such capabilities are of paramount importance in the application of OoCs. There are drawbacks to the use of

PDMS. For example, nonpolar solvents (e.g., benzene and pentane) are not compatible with PDMS devices. PDMS is also known to absorb small molecules or protein from the cell culture medium. Such absorption can potentially alter the behavior of the cells cultured in the microfluidic devices [75,76]. Furthermore, PDMS may adsorb small drug molecules, which affects drug bioavailability in OoC devices used for drug testing [39].

Because of the drawback of PDMS, thermoplastics are also preferred for cell culture applications [7]. Thermoplastics such as polystyrene and polycarbonate, which are substrate materials widely used in *in vitro* cell culture, are ideal candidates [28,77]. Crucially, these materials can be effectively applied in injection molding and embossing techniques. The material is usually heated to a molten state for injection molding and forced into a mold. Thermoplastics are ideal for this fabrication because they do not require a high temperature ($>1000^{\circ}\text{C}$) to reach a molten state. On the other hand, hot embossing does not require the material to reach a fully molten state. The polymer is usually heated to just above its glass transition temperature for transferring the pattern into the softened polymer.

Accessibility of formative manufacturing

Formative manufacturing requires steps that are conceptually distinct from subtractive manufacturing. Subtractive manufacturing entails procedures that alter the fluidic device directly. In contrast, formative manufacturing necessitates an additional process of constructing the mold. The replicas from these master molds can be reused multiple times with little variation. Importantly, the quality of the replicas depends solely on the quality of the mold. Therefore, it is important to ensure that the molds are fabricated with good dimensional tolerance. The construction of the master mold is usually the most expensive step in the fabrication process in formative manufacturing. In soft lithography, fabrication of a SU-8 mold can cost from a few hundred to several thousand dollars, depending on the complexity and the resolution of the feature size. Once the mold is obtained, however, replica molding of PDMS can be carried out with relative ease and at a low cost. Hwang et al. published an exhaustive review of novel molds used in PDMS casting [66]. Additionally, dissolvable or removable structures served as molds for the formation of microchannels. For instance, it has been demonstrated that sacrificial microfibers can be used as templates to fabricate PDMS microfluidic devices [78,79]. It was also demonstrated that 3D-printed master molds or sacrificial templates could be used to cast a secondary material [65,66]. For the fabrication of

microchannels in PDMS and other materials, water-soluble PVA has been widely used [67,68].

Molds for embossing and injection molding usually cost in the range of 10,000 USD. As a result, these processes are only cost-effective for high-volume production (>10,000 devices). Embossing and injection molding also require dedicated machineries that are not readily available in most research setting. These processes are often outsourced to specialists with dedicated facilities. Although metal molds that can withstand heat and pressure are preferred for embossing and injection molding, researchers have shown to perform embossing using 3D-printed templates as molds to fabricate microfluidic devices. Hamed et al. demonstrated the use of 3D-printed embossing molds to create fluidic devices with cellophane films [80].

1.4.3 Additive manufacturing

Additive manufacturing is a process that creates shapes by sequential addition of materials, typically in a layer-by-layer manner. The notion of additive manufacturing, in which a 3D object is constructed layer by layer, is not new. The first evidence of additive manufacturing dates back to 10,000 years ago when buildings were constructed brick by brick [81]. Computer-controlled automation of fabrication, on the other hand, is a comparatively recent invention [82]. With increasing affordability of 3D printers over the past years, 3D printers have become a commodity found in many research settings. Researchers have since begun to harness 3D printing as a tool to fabricate microfluidic devices. While there are many types of 3D printing techniques, we focus on three main techniques that are usually applied in fabricating microfluidic devices for OoC applications: (a) light-assisted 3D printing, (b) inkjet 3D printing, and (c) extrusion-based 3D printing.

1.4.3.1 Examples of additive manufacturing

Light-assisted 3D printing

In light-assisted 3D printing, 3D objects are constructed layer by layer using light to polymerize a photocurable resin (or photorein). The operating principle of a digital light projection processing (DLP) printer (a subset of light-assisted 3D printers) is illustrated (Fig. 1.4a). Light-assisted 3D printers usually take an inverted configuration, with the printed object facing downward while attached to the build plate. Briefly, the light source exposes a pattern to the photorein in the vat, and the photorein exposed to the light polymerizes to create a layer. After polymerizing one layer, the

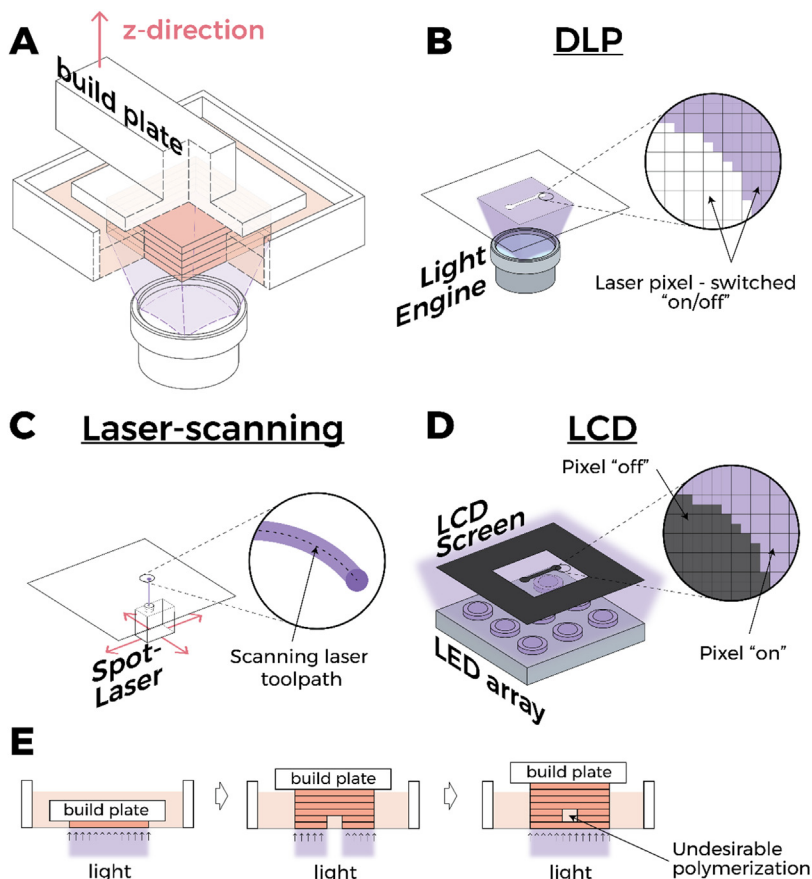


Figure 1.4 Light-assisted 3D printing of microfluidic devices. Illustration highlighting the working principles of (A) digital light processing (DLP) 3D printer, (B) laser-scanning 3D printer, and (C) liquid crystal display (LCD) 3D printer. (E) Illustration of layer-by-layer fabrication of light-assisted 3D printing. (From Ching *et al.* [40].)

build plate moves upwards (in the z -direction), leaving a gap for the next layer. The following light pattern is exposed to polymerize the next layer of photoresin. These steps are repeated over many layers to build a 3D object. There currently exist several technologies to control the exposure pattern. DLP, laser-scanning, and liquid crystal display (LCD) are some common methods readily available in the mass consumer market.

The light pattern in a DLP printer is controlled using a light engine (Fig. 1.4b). A laser source and a digital micromirror device (DMD) are contained within the light engine. DMD is a dynamic mask made up of microscopic-sized mirrors arranged in a matrix on a semiconductor chip. A

single light source (laser) is shone on the DMD; by rapidly toggling these tiny mirrors, light can be directed to the bottom of the resin vat at specific coordinates. Each individually toggled micromirror represents one pixel (Fig. 1.4b), and the pixel pitch (i.e., the distance between the center of two pixels) represents the smallest achievable feature size in the horizontal direction (i.e., xy -resolution). Unlike DLP printers, laser scanning 3D printers use a spot laser that pans across the vat to polymerize the desired pattern. Laser-scanning printers generally print slower than DLP printers due to the additional time needed to draw the exposure pattern for each layer. LCD printers use an LCD screen as a photomask (Fig. 1.4d) [83]. A light source (usually an LED array) is positioned below the LCD screen. Pixels on the LCD screen are selectively switched on or off to create the desired exposure pattern.

Optimizing materials and operating conditions are required when 3D printing microscale fluidic devices with a light-assisted 3D printer. Due to the nature of light-assisted 3D printing, channel clogging may occur. Clogging of channels is typically attributed to undesirable polymerization that occurs within the channel void. Evacuating the unpolymerized photoresin can also be difficult (Fig. 1.4e) [84]. To avoid channel clogging, it is necessary to carefully tune the photoresin and light source. Gong et al. for example, have successfully built fluidic devices with a cross-sectional dimension of $<20\text{ }\mu\text{m}$ by adequately selecting the optimal photoresin and light source parameters [85,86]. Another approach by Noriega et al. involved careful tuning of the optical dose of each individual pixel. For example, when printing the overhanging feature (Fig. 1.4e(iii)), the optical dose directly below the channel void was reduced to prevent undesired polymerization. Using this approach, Noriega et al. successfully printed channels with excellent resolution ($15\text{ }\mu\text{m} \times 15\text{ }\mu\text{m}$ in cross-section) and complex channel designs (e.g., valves) [87].

Advanced light-assisted printing techniques, such as two-photon polymerization (2PP) (or direct laser writing (DLW)), also exist commercially. At the nanoscale scale, DLW provides superior voxel resolution [88]. However, DLW often needs a lengthy print time with a limited print volume in exchange for the enhanced resolution [89]. As a result of these limitations, there are only a few examples of DLW being used to manufacture whole microfluidic devices [90–92].

Extrusion-based 3D printing

Extrusion-based printing utilizes multiple layers of extruded material through a nozzle to create 3D objects (Fig. 1.5a). Extrusion-based 3D

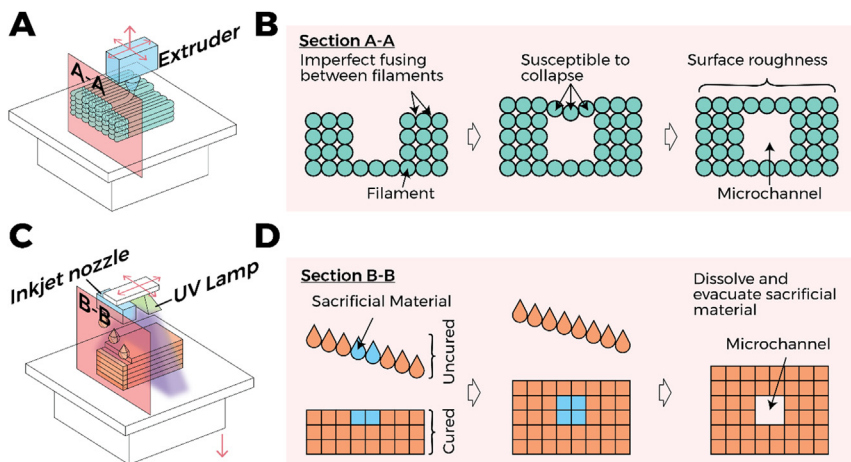


Figure 1.5 Extrusion-based and inkjet technology for 3D printing of microfluidic devices. Representative illustration highlighting the fabrication of microfluidic devices using (A, B) extrusion-based 3D printer and (C, D) inject 3D printer. (From Ching et al. [40].)

printing is currently the most popular 3D printing technology in the mass consumer market. The original FDM patent has expired, leading to the formation of an open-source development community [93]. The open-source initiative led to the introduction of a wide array of inexpensive FDM printers available to the public. In FDM, a solid thermoplastic filament is fed into the print head and heated to a semi-molten state before being extruded through the nozzle. When exposed to ambient temperature, the molten material spontaneously hardens upon extrusion. Direct ink writing (DIW) is another extrusion-assisted 3D printing technology in which liquid precursors are pneumatically extruded through a nozzle loaded in syringes. DIW 3D printing can be used in conjunction with UV irradiation and heating to accommodate a variety of solidification mechanisms for liquid precursors used in 3D modeling.

However, extrusion-based printers may not be ideal for fabricating microfluidic devices despite their inherent mechanism. Printing of microchannels using an FDM printer is illustrated (Fig. 1.5b). Firstly, since the dimension of each extruded filament is typically large ($>150\ \mu\text{m}$, subject to the nozzle diameter), it sets a limit to the attainable microscale channel sizes. Secondly, in extrusion-based printing, filament strands are laid sequentially next to one another. As such, printed filaments frequently fail to form a uniform bond with their partnering filaments, resulting in leakages and nonuniform inner surfaces [94]. In addition, during the printing of the

overhanging layer to cover the microchannels, the overhanging filaments are susceptible to collapse due to gravity, leading to unsuccessful microchannel fabrication. Lastly, FDM printing of microfluidic devices resulted in high surface roughness relative to devices printed with DLP and inkjet-assisted printers [38]. The sequential printing of filaments next to one another creates undulating surfaces that compromise the smoothness and optical transparency of the surface. Unless there are specific objectives only achievable by extrusion printing (such as incorporating cell-laden hydrogel ink by DIW), other modes of 3D printing may be preferred to perform fabrication of microfluidic devices.

Inkjet 3D printing

Inkjet 3D printing utilizes a photoresin jetted through an inkjet nozzle, which is then polymerized under UV light (Fig. 1.5c). They are frequently referred to as multiple-jet (MJ) printers or polymer jet printers due to their ability to accommodate multiple nozzles for printing multiple polymeric materials. The capability to print multiple materials offers a unique advantage when fabricating microfluidic devices using inkjet printers. The void in the channel is first printed with a sacrificial material that can be dissolved in postprocessing (Fig. 1.5d). Several variants of the MJ printing are currently patented (e.g., Stratasys, 3D Systems). At the moment, low-cost MJ printers are yet to appear, and the majority of users rely on proprietary printers and materials. Crucially, dissolving and evacuating the sacrificial material can be challenging due to the slow evacuation rate of the narrow microchannels (with the cross-sectional dimensions on the order of 100 μ m). At present, the high cost of MJ printers (>100,000 USD) and proprietary photoresin ($\sim$ 1000 USD/L) make them less popular choice in fabricating microfluidic devices than other desktop 3D printers. Nonetheless, several groups have demonstrated the fabrication and applications of millifluidic devices fabricated using MJ printers [95–98].

1.4.3.2 Discussion on additive manufacturing

Materials for additive manufacturing

Light-assisted 3D printing requires the use of photocurable materials. Photoresin generally consists of three basic classes of ingredients: (1) monomer, (2) photoinitiator, and (3) photoabsorber. When exposed to light, the photoinitiator converts photolytic energy to form reactive species (i.e., radical or cation). Subsequently, the reactive species drives the chain growth of the monomers via radical or cationic reactions [99]. The function

of a photoabsorber is to control the penetration depth of the incident light via light absorption [100]. Different types of photoresins are commercially available. For the fabrication of OoC devices, the materials must be non-cytotoxic. Unfortunately, commercialized photoresin consists of proprietary additives that are cytotoxic and unsuitable for cell culture [101]. Folch, Nordin, Woolley, Miller, and other researchers have reported self-formulated photoresin with polyethylene glycol diacrylate (PEGDA) as the primary monomer [85,86,102–104]. High molecular weight PEGDA is ideal for cell culture application as they are bioinert. These works have identified several combinations of biocompatible photoinitiators and photoabsorbers suitable for creating microfluidic devices for cell culture.

Extrusion-based 3D printing requires printing material that can behave like a liquid upon extrusion from the printer nozzle. Immediately after extrusion, the material should behave like a solid material to avoid spreading. For instance, in FDM printing, a plastic filament is first heated to a semi-molten state to permit extrusion through the nozzle. When the printed material is in ambient temperature after extrusion, the printed thermoplastics spontaneously harden without spreading. Numerous thermoplastics (e.g., ABS, PS, polycarbonate, polycaprolactone (PCL), polylactic acid, polybutylene terephthalate, and polyglycolic acid) are demonstrated for FDM printing [105]. However, barring few exceptions such as PCL, the extrusion temperatures of FDM-compatible thermoplastics are $>150^{\circ}\text{C}$. As such, FDM is typically not compatible with simultaneous printing of cell-laden inks in proximity.

Similar requirements for the printable inks apply to DIW printing. Firstly, the printing material should permit effective extrusion of ink from the nozzle. It is preferable to use fluids with shear-thinning properties to achieve effective ink extrusion. Shear-thinning is the non-Newtonian behavior of fluids, whereby the viscosity of the fluid decreases under shear strain. When shear stress is introduced, the viscosity of the extruded ink temporarily drops, allowing the ink to be extruded from the nozzle. Secondly, the extruded ink should retain the extruded shape without spreading. Inks with sufficiently high yield stress can withstand the shear force against deformation by gravity [106]. Room temperature vulcanizing (RTV) silicone is one example of an ink that exhibits these two characteristics (i.e., shear-thinning and high yield stress), which can be effectively applied for DIW-based fabrication of microfluidic devices. RTV silicone has been demonstrated to fabricate an entire microfluidic device [107] or part of the microfluidic device [108,109]. Additionally, extrusion-based

printers are used to print sacrificial structures to create microchannels [68,110]. Lin et al. developed a method for printing fugitive inks that are evacuated after casting a secondary extracellular matrix (ECM) using DIW-printing [111,112].

Lastly, inkjet-assisted printing (such as those from Stratasys and 3D Systems) requires photoresin. While these companies offer several resin formulations, the formulations are usually proprietary. Nonetheless, some resins are transparent for the ease of in-channel observations. Some companies have also developed biocompatible photoresins suitable for cell culture [105,113]. However, the customizability remains the major challenge for such commercial inkjet-assisted 3D printing for the fabrication of microfluidic devices of OoCs.

Printer resolution versus channel dimension in additive manufacturing

In additive manufacturing of microchannels, printer resolution (as specified by the manufacturers) is largely different from the attainable channel dimension. As illustrated in Figs. 1.4e, 1.5b, and d, the attainable channel dimensions are generally larger than the printer resolutions specified by manufacturers of the printers. Minimum channel dimensions using extrusion and inkjet-based printers are $>100\text{ }\mu\text{m}$ [37,89]. Improving the channel dimensions (i.e., obtaining small channels), our group has developed a hybrid method that combines DIW printing with laser-ablated PMMA sheets and achieved the channel dimension of $\sim 30\text{ }\mu\text{m}$ (in both lateral and vertical dimensions) [108]. Applying this hybrid technique also enabled the production of microfluidic devices with good optical transparency using PMMA. At present, only light-assisted 3D printers (e.g., DLP) can readily produce channel dimensions (in both lateral and vertical dimensions) less than $100\text{ }\mu\text{m}$ [85,87,113,114].

Material transparency versus device transparency in additive manufacturing

The transparency of the material is a physical property that allows light to pass through the material without appreciable scattering. While many 3D printing inks are optically transparent in nature, 3D printing tends to introduce surface roughness that can drastically compromise optical transparency, which impedes in-channel observations under a microscope. Macdonald et al. studied different methods of 3D printing and found that DLP printers produced microfluidic devices with the least surface roughness

with the smallest disruption to optical transparency [38]. The inherent surface roughness cannot be neglected in different mechanisms of 3D printing [115]. Furthermore, the surface topology may have crucial effects on cellular growth and response. These considerations should be taken into consideration when employing 3D printers for the fabrication of microfluidic devices [116].

Rapid prototyping

The key benefit of additive manufacturing is in its rapid design-to-prototype cycle, albeit limitations in attainable channel resolution and optical transparency. Computer-aided design files of the 3D-printed microfluidic devices can be shared online and made available to researchers with publications. Researchers with the same printer (or similar class of printers) can download the files and fabricate the devices in-house. We and other groups have previously reported the development of 3D-printable, open-source microfluidic components (such as pumps) [36,117,118]. 3D printing of microfluidic devices using DLP printers allows rapid prototyping. Noriega et al. have demonstrated the printing of microfluidic devices within minutes [87]. Continuing development of specialized 3D printers and resin for microfluidic devices should pave an avenue for rapid, efficient, and low-cost fabrication of microfluidic devices for research communities.

1.5 Outlook

This chapter discussed the materials and fabrication of microfluidic devices for OoC applications. Numerous fabrication techniques have enabled the development of OoCs. Since its inception, OoCs have been adopted in the research communities including etiology, toxicology, and pharmacology; they have been promoted in pharmaceutical and cosmetic industries [7,119]. Soft lithography with PDMS has been a widely used fabrication technique for OoC devices. In addition to the simplicity in fabrication, the presence of established fabrication protocol and well-documented results for cell culture has continually made PDMS the preferred choice for OoC developers. Although soft lithography using PDMS offers a reasonably short design-to-prototype cycle (within a week), it often requires dedicated cleanroom facilities for the fabrication of a master mold, which may not be ideal in resource-poor settings. Easily accessible fabrication methods with a short design-to-prototype cycle are desired. The versatility of the device can be enhanced to meet its specific requirements in iterative development.

3D printing is a viable candidate of a fabrication method that can offer a fast design-to-prototype cycle, decreasing the time scale of production from days to merely hours.

Future fabrication of OoCs may involve the combination of microfluidics and bioprinting technologies. Bioprinting allows spatial control of the cell culture on OoCs to enhance their physiological relevance. There has been the development of microfluidic devices to create fluidic interfaces with bio-printed constructs. Some examples of such development include fabricating a microfluidic system that allows perfusion culture of bioprinted endothelialized myocardium tissue [120] and direct printing of hepatic spheroids into microfluidic bioreactors [121]. To simplify the fabrication process, a one-step fabrication platform has been developed to 3D-print a microfluidic chip followed by bioprinting of endothelialized liver cell constructs [122].

In fabricating the OoCs for industrial applications, the developers need to design an adequate OoC platform to obtain biological readouts. Optical transparency of the OoC is desired because microscopy readouts using immunofluorescence have been one of the common biological assays to distinguish different cell types. The ease of retrieving biological samples from the OoC during experimentation should be another important design consideration. The recovery of tissue samples from the OoC is often necessary for next-generation sequencing and other quantifiable histochemical assays to allow reading of molecular signatures that have been validated to define diseased and healthy cell types. Aiming to achieve the next generation OoCs serving for various industrial applications, the developers of OoCs need to balance ease of fabrication, time and cost required for the design-to-prototype cycle, scalability, and feasibility of OoCs to perform targeted cell culture.

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CHAPTER 2

Critical factors affecting cells behavior in microfluidic chips

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2.1 Introduction

The unique advantages of microfluidic systems and their ability to control various effective parameters have revolutionized the human approach to cell biology, which will pave the way for the availability and widespread use of high-efficiency human organs-on-chips. Cell and its internal structures having the micrometric physical dimensions can adapt well to the microfluidic environment since it was tailored to well-suit to various aspects of live cells to mimic the related natural milieu. The study of cell biology in microfluidics is an interdisciplinary field in which the biochemical, physicochemical, and biomechanical aspects together with the physical scale of cells should be considered. The microfluidic systems are highly beneficial in cell biology as they can lead to rapid responses, prevent reagent wastage,

easy automatization, and the ability to adjust according to the cellular microenvironment. Also, by switching from macro- (Petri dishes and well plates) to microscale much more precise control can be applied over various patterns, biomechanical, spatial, and temporal gradients. Taking advantage of this unique control over the influencing factors and microenvironment, the high throughput microfluidic chips can be achieved, the first step is to fully and accurately understand the mechanisms and factors affecting cell behavior in a microfluidic environment. In summary, it can be claimed that the vast majority of factors influencing cellular behavior in the microfluidic environment can be studied under the domain of the cellular microenvironment and its components. In this chapter, we try to introduce the critical factors which may affect the natural cell behavior and its function in the microfluidic chip environment and to perform a detailed study on the cellular microenvironment. The goal is to maintain cells viable without any considerable changes in their function and keep them alive as long as possible to monitor their interaction and behaviors toward any possible treatment or intended stimuli.

2.2 Cellular microenvironment

The full peripheral three-dimensional (3D) conditions of cells' milieu, including all physical, chemical, and biological cues having any direct or indirect effect on cells' behavior and its later clinical outcomes are defined as a cellular microenvironment that has crucial importance in determining the biological processes. The 2D culture systems are not able to fully provide and maintain the total microenvironment that can assist the production of microtissue with complex physiological relevance, also they cannot perfectly simulate *in vivo* microtissues' performance [1], for example, the generation of *in vivo*-like bile canaliculi structures by HepG2 hepatocytes just can be done in the 3D microenvironment. The cellular microenvironment can include various components and properties such as proteins, soluble molecules like growth factors, extracellular matrix, possible interactions with similar or dissimilar cells, cytokines, hormones, reactive species, and mechanical forces. It also has some physicochemical characteristics such as temperature, pH, osmolality, and oxygen and carbon dioxide level [2,3]. Fig. 2.1 schematically represents the cellular microenvironment and any internal and external factors influencing its behavior.

The main microenvironmental factors (MEFs) in 3D culturing includes (1) biochemical or compositional characteristics, (2) geometrical or spatial

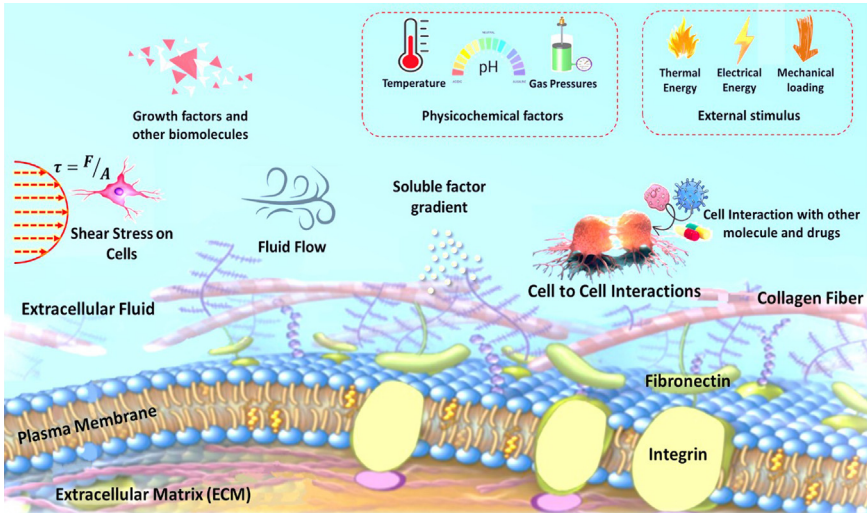


Figure 2.1 Cell microenvironment and internal and external factors possibly influencing its behavior. Cell microenvironment and internal and external factors possibly influencing its behavior. Extracellular matrix (ECM) structure including the plasma membrane, extracellular fluid, integrins, fibronectins, and collagen fibers. The existence of other possible components in the cellular microenvironment such as shear stresses, flows, cell-to-cell interactions and their possible interactions with a variety of molecules and drugs, and chemical cues like growth factors and soluble factors. Physicochemical factors including temperature, pH, and partial gas pressures, and external stimuli like thermal, electrical energies, and mechanical forces.

and temporal condition, and (3) biophysical, biomechanical, and force condition [4,5]. These MEFs and microenvironmental cues can considerably affect the cellular phenotype such as apoptosis, proliferation, differentiation, and gene and protein expression [6]; simultaneously cells can also affect the microenvironment. In addition, the extracellular matrix (ECM) has a key role in the cellular microenvironment as it can coordinate the interaction of soluble factors and their availability, circulation, and transduction; also it can intercede and adjust the mechanical and electrical fields [7]. Hence, the emulation of natural conditions of native ECM is of crucial importance.

2.2.1 Biochemical microenvironment

The chemical composition, cell adhesion ligands, cytokines, growth factors, hormones chemokines, soluble factors, and other biomolecules are studied under the biochemical microenvironment. The combination of these MEFs

generates complex signaling pathways that determine the cell's fate, and the main signaling pathways in the biochemical microenvironment are soluble factor signaling and endocrine signaling. The soluble factor signaling regulates by autocrine and paracrine processes in which diffusion plays a crucial role. The autocrine—paracrine signaling is of substantial importance *in vivo*, hence firstly it should be carefully investigated *in vitro*. Unfortunately, the influence of secreted endogenous factors *in vitro* is considerably inhibited in canonical culture platforms. In multiwell plates, the extensive air—liquid interface raises the convective flows with the continuous mixture, leading to fluid disruption and alteration of the diffusion-based accumulation. Hopefully, microchannels provide a favorable microenvironment with the potential to highly control the secreted factors and their effects [8]. In the case of endocrine signaling, the convective transport of hormonal signals controls the process. It has been known for a long time that these biochemical signals alter cellular migration [9,10], and migration due to biochemical or biophysical stimulus takes place in a 2 or 3-dimensional environment with subsequent cell movement in a physical substrate.

The biochemical microenvironment includes proteins that produce the extracellular matrix, growth factors released by cells, and other biochemical factors that most of them adhere to the ECM until activation. The ECM assists and mediates the signaling cascades that resulted from both biochemical and biophysical cues. Also, both extracellular and intracellular types of biochemical and biophysical signals can influence the directionality (persistence) and speed of migration [11]. Apart from the ECM effects, the cell culture medium is of crucial importance since it acts as a main biochemical microenvironment of the culture, consisting of vitamins, carbohydrates, salts, amino acids, and other components in an aqueous solution. The essential components of the cell culture medium were discovered by Eagle about 60 years ago, which is still used as basal media for cellular culturing and can also be used in microfluidic cell culture systems. In addition, the basal media must be supplemented with other components to ensure improved cell growth and division, such as growth factors and hormones. Traditionally fetal bovine or calf serum was utilized to maintain the required components, but recently the serum-free media is preferred to ensure the exact similarity in experiments. Lam et al. [12] fabricated a high-throughput microfluidic environment that can control multiple MEFs, including proteins, nutrients, dissolved gases, growth factors, and seeded cell populations to detect and analyze the dental bacteria growth and biofilm generation. This high-throughput microfluidic system includes numerous

(up to 128) incubation chambers to accomplish parallel cultivation and determination of related biochemical factors. Also, microfluidic systems have substantial potential in the manipulation of the extracellular chemical microenvironment [13]. In microfluidic systems to investigate the interaction between biological cells and their niches, the dynamic biochemical signals can be controlled via programmable pumps. Na et al. [14] introduced an innovative microfluidic control system including an external feedback control system with a Y-shaped microfluidic chip having a “Christmas tree” inlet, and the effect of vibrations was mitigated through the Proportional Integral Derivative controller. Overall, considering the influential role of the biochemical microenvironment in cell fate, the microfluidic systems should be carefully designed and controlled to cover all the requirements.

2.2.2 Biophysical microenvironment

Any physical and structural factors such as cell morphology, degradability, matrix mechanics, electrical conductivity, and ECM nano-topographical features together with any internal or external forces exerted on the cell structure such as cell mechanical attachment and anchoring by surface integrins, fluid shear force, matrix tension/pressure, matrix hardness, and cell surface tension lead to the generation of a dynamic 3D physical microenvironment. These biophysical parameters can substantially influence physiological processes. For example, due to fluid shear force in blood vessels which is sensed by endothelial cells, these cells’ functions encounter some changes such as morphological transformation, transcription, proliferation, barrier function, and actin cytoskeleton rearrangement [15]. Some microfluidic systems are specially designed to investigate the effect of fluid shear forces and biophysical parameters; for instance, an integrated microfluidic chip with a high-throughput and easy operation was utilized to study drug toxicity in five different fluid shear stress gradients by Feng et al. [16].

Force and substrate physical property (pliability) is one of the most influential MEFs, especially in 3D cell cultures and microfluidic chips that determine the formation of physiologically relevant constructs. Most of the available 3D cell culture platforms can be categorized from substrate physical property into three main groups; (1) pliable and soft environments with hydrogel bases such as alginate, agarose, chitosan, fibrin, hyaluronan, and collagen ones, (2) stiff environments with high modulus of elasticity like synthetic microporous scaffolds and constructs made from polystyrene, poly(lactic-co-glycolic acid) (PLGA), poly-L-lactide acid (PLLA), and polycaprolactone (PCL), (3) the constructs that lack cell-exogenous

material interaction like scaffold-free 3D formats such as spheroids or cellular aggregates. It was seen that increasing gel rigidity in the first group led to improved proliferation and inhibition of differentiation due to focal adhesions [17]. Unfortunately, the first group environments usually have not a defined geometry and are unable to impose any physical constraints on the size of the aggregates. The second group having above the physiological range pliability seems to be unable in providing the optimum biophysical cues, but they have been successful since, in addition to the kind of material itself, the morphology and form can affect the pliability. In addition to pliability, on the microscale, surface tension can prevail against gravity, and viscosity forces can dominate inertia. The dominance of viscosity can cause flow lamination with anticipated streamlines. Due to surface tension and resultant pressure difference, fluid pumping through microscale channels can be done against gravity and is known as passive-pumping that makes it possible to mimic almost any intricate case [18].

Mechanical forces are regarded as another important biophysical cue. In cellular mechanics, the impact of any mechanical forces derived from the extracellular matrix was thoroughly studied and proved that integrin-cytoskeleton-mediated force transduction can influence cell behavior. Usually, when cells are exposed to a specific extension of force, they show some conformational changes and evolution binding kinetics. Also, these mechanical forces can affect both the proliferation and differentiation of cells and influence the biological pathways [19]. In this regard, molecular pathways and reactions are of special interest among academics and are studied in the context of mechanical forces with the use of available schemes in the mechanobiology and mechano-transduction fields. Also, due to various processes in biology, cells can induce stress forces to the matrix through cell morphogenesis, differentiation, tissue remodeling, and even normal physiological activities. The extent of this stress depends on the matrix rigidity and many receptor-mediated adhesions of cells in the microenvironment consequently, these features affect the matrix contraction [20]. The matrix contraction triggers the intracellular tension leading to the production of stress fibers or acinus in a cell. For the case of more rigid matrixes which inhibit further contraction, this can be led to the increment of some cell functions [5] like endothelial organization [21] and may inhibit some others.

2.2.3 Physicochemical microenvironment

Despite the substantial impact of physicochemical microenvironment on cell behavior, unfortunately, it has not been thoroughly investigated yet.

The pH, oxygen tension, CO₂ and O₂ gas concentration, temperature, and osmolality are among the main physicochemical parameters in the cellular microenvironment. These physicochemical properties are critical in the preservation of cell cultures. Usually, these properties are regarded as inherent to the *in vivo* environment and remain fixed and unperturbed throughout the normal cellular experiment unless the physicochemical properties themselves were under investigation. It was reported that extraordinary pH (low extracellular pH) and oxygen tension (hypoxia) values could lead to miscellaneous pathologies such as cancer metastasis [22]. It was also reported [23] that osmotic fluctuations affect cell cycle checkpoints and high osmolality leads to provoked DNA damage. Also, renal cells can not favorably sense and treat DNA damages under increased osmolality conditions [24]. High NaCl osmolality caused DNA damages and breaks with impairing its repair both in cell culture and *in vivo*. It was also shown that as soon as the NaCl levels reduced to typical values the DNA damage response rapidly activated [25].

Temperature is another decisive physicochemical parameter that can highly influence cellular behavior, it was seen that [26] hyperthermia transforms the intratumor surroundings into acidic, hypoxic, and nutritionally deprived environment due to vascular damage and prevents the thermal damage recovery, and it interferes with the development of thermal tolerance. Moreover, hyperthermia has a great potential to destroy tumor tissue in temperatures that exceed the cytotoxic threshold (42.5°C) and can lead to cell death in tumor tissue with a selective sparing of the normal surrounding tissue.

The gas concentration also plays a determinant role in regulating cell response in the microenvironment, Wildbrett et al. [27] reported that insufflation with CO₂ or He gases could lead to substantial changes of intra- and extracellular parameters, such as cell proliferation, oxidative phosphorylation to produce ATP, cell proliferation, and the onset of apoptosis. In this regard, it is also known that gas partial pressures within the cell microenvironment are among the crucial modulators of cell pathophysiology. Two kinds of gases are of special importance in the cell microenvironment, respiratory (O₂ and CO₂) and gasotransmitters gases (CO, NO, H₂S). Oxygen as the most influential gas in the cell microenvironment has a physiological partial pressure from a maximum of ~13% in the arterial endothelium to 2%–5% in cells of normal tissues, and to lesser than 1% in tumor cell [28], most cellular investigations are done at ~19% O₂ pressure [29]. It should be noted that because of the wide and various range of oxygen content in tissues and organisms, each type of cell must be investigated

under its homeostatic physiological level of oxygenation. Despite the numerous studies about the disadvantages of hypoxia, hyperoxia can also have induced some types of risks for acute lung injury patients and newborns receiving critical care [29]. Meanwhile, CO₂ may promote both hypoxia and hypercapnia/acidosis in tissue and cellular media. Also, hypercapnia/acidosis plays an important role in tumor pathophysiology leading to proliferation, invasiveness, and metastasis [30]. Apart from O₂ and CO₂, the other gases also show a significant impact on cellular functions. Overall, the partial pressures of all gases in cell culture systems must be accurately controlled to have values and fluctuation rates corresponding to the cellular model under study. In this regard, microfluidic chips with a high potential to control the microenvironmental parameters maintaining the optimized gas pressures are promising techniques to reach near to ideal cell culture systems.

2.2.4 Geometrical microenvironment

One of the important factors in designing a successful microenvironment with satisfactory functionality is multidimensionality as almost all the tissue components possess multidimensional order and orientation that justifies the necessity to emulate the full geometrical, temporal, and spatial conditions. All the tissue components and microorgan structures should obey the required dimensional and geometrical characteristics, and the microenvironment should have the potential to fully maintain it [31]. In this regard, microwells having diverse geometries can maintain tunable confined structures to control cell differentiation and proliferation. Also, 3D porous structures are highly interested since porosity considerably affects cellular behavior. Microporosity can enhance bone regeneration even more than potent growth factors [32] and enhance ECM secretion, tissue growth, cellular infiltration, molecular delivery, and cell differentiation [33]. Utilization of modern bioprinting and 3D printing systems make it possible to attain any desired geometries and structures such as microgrooved collagen substrates, hydrogels with gradient porosities, and hierarchical structure of tissues and cells from macro to the micro- and nanoscales with porous and fibrous structures that can considerably affect the cellular behaviors [34].

2.3 Components of cellular microenvironment

2.3.1 Extracellular matrix

The ECM plays a crucial role in the physiology, function, and fate of living cells, and it has a fundamental role in cell biology. The ECM character

including its fibrous structure [35,36], nonlinearity [37], mechanical properties, rigidity, and viscoelasticity [38] can considerably affect the cellular behavior. In addition, it is known that ECM composition (including specific adhesion receptors) and its stiffness have a significant impact on the cellular response [21,39]. Moreover, any cell-induced strain or forces can affect the ECM and realign its fibers. ECM having both direct and indirect signaling pathways can communicate with cells both by direct binding to cell surface receptors or indirectly through noncanonical growth factors [40]. The ECM stiffness as a decisive factor enables cells to sense and respond to external force stimulus, which is known as mechanotransduction [41,42]. Fig. 2.2 shows the interaction between stem cells and ECM, affecting cellular behavior. The interaction of cells with ECM is mediated through integrins that can signal bidirectionally and transmit mechanical forces that are produced in pathological processes. These integrin-mediated interactions (direct or indirect) are in relation to growth factor signaling and the complex cytoskeleton of cells. The existent complex interplay

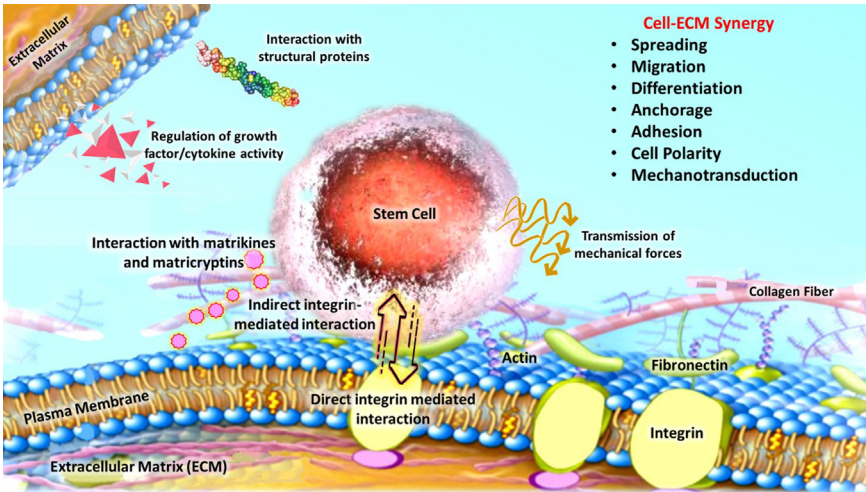


Figure 2.2 *The interaction between stem cells and ECM.* The interaction between stem cells and ECM, affecting cellular behavior. The cell-ECM interactions are mediated through integrins (direct or indirect), and they are related to growth factor signaling and complex cellular cytoskeleton. The resultant complex interplay manipulates gene expression, interacts with proteins, and regulates growth factor and cytokine activity. Stem cells trigger via the receptors on the cell membrane and exhibit various responses like differentiation and gene expression, cell spreading, migration, polarity, adhesion, and anchorage. These responses are due to a combination of soluble matrix-binding factors with other elements and finally decide the cell fate.

manipulates gene expression, interacts with proteins, and regulates growth factor and cytokine activity. The stem cells trigger via the receptors on the cell membrane, and in presence of ECM structure, they exhibit various responses like differentiation and gene expression, cell spreading, migration, polarity, adhesion, and anchorage. Also, these responses originated from a combination of soluble matrix-binding factors with other elements, which finally decide the cell fate [38,43].

Due to reciprocal interaction between cell and ECM, any variations in ECM component or its related parameters lead to substantial changes in ECM function and its biomechanical, biochemical, and physical properties, which finally cause the generation of a chaotic network, cellular failure, and homeostasis [44]. ECM can influence many cellular functions, including organoid formation, proliferation, differentiation, spreading, growth, and migration. ECM can also affect cancer progression, its differential invasion, migration, and metastatic potential, Lugo-Cintrón et al. [45] used a microfluidic model to show the influence of 3D heterogeneous ECMs on the migration behavior of human breast cancer cells. It was found that ECM variations can lead to an increment of migrating cells and migration distance, improved secretion of metalloproteinases (MMPs), and increased matrix remodeling.

The interaction between stem cells and ECM usually occurs through integrins and other receptors, also limited communications are done by nonintegrin receptors. ECM has a key role in stem cell therapy since “anoikis” as a type of apoptosis initiates when the interactions between stem cells and ECM are interrupted. Reconstructing these interactions leads to improved cell viability and promotion of function recovery [46]. ECM as a dynamic construct with permanent contact with the neighboring environment has a substantial role in the stem cell niche and regulating its behavior [47,48]. Han et al. [49] showed that neural stem cells (NSCs) could be guided in a 3D ECM microenvironment using quantitative real-time polymerase chain reaction; results show the considerably improved NSCs’ differentiation. Microfluidic systems have a substantial potential to control and regulate ECM and its components. In this regard, Allazetta et al. [50] utilized a droplet microfluidic technology to generate an artificial ECM microgel with the capability to adjust its stiffness, composition, dimension, etc., according to specific cell type. Overall, ECM and its related parameters have a decisive role in designing a successful microenvironment to achieve high throughput microfluidic designs that mimic *in vivo* conditions.

2.3.2 Adjacent cells

Cells in humans and other biological systems are always subject to the effects of similar and dissimilar peripheral cells. The resultant cell-to-cell interactions and their dynamics impact the function and morphological characteristics of multicellular tissue [51] and it is known as one of the key aspects in tissue development [52]. Cells can communicate with adjacent cells in two ways, either directly (cell–cell contact) or indirectly (interceded by soluble factors) [7]. Any possible disorder in direct cell–cell communications may induce deviant cellular behavior and pathologies [53], even if it can impact tumor development [54] and cause metastatic manner in cancer [55], these direct communications are controlled by signaling pathways and microenvironmental cues [56]. Numerous coculturing studies proved the role of cell–cell communications and interactions significantly influence cell function and its behavior [57]. Shahabipour et al. [58] reported that coculturing of osteogenic and angiogenic cells in 3D hydrogel could improve cell interactions, leading to improved angiogenic and osteogenic properties. In another study [59] about the tumor–endothelial interaction, it was seen that these cellular communications can lead to further tumor progression and may induce resistance against cancer therapy. Lugo-Cintrón et al. [45] showed that coculturing of fibroblasts and human breast cancer cells in the microfluidic system increased cancer cell migration. It was shown that [60] pneumatic microvalve controlled microfluidic chips can simultaneously have precise control on numerous cell manipulation, enabling to coculture different cell lines. The considerable impact of intercellular communications and its complex nature lead to the development of microfluidic coculture systems with various setups, including microfluidic cell trap arrays [61], valved microfluidics [62], and droplet microfluidics with high efficiency and single-cell resolution. These microfluidic coculture systems facilitate the realization of cellular interactions and reduce the complexity of analyzing these communications.

2.3.3 Soluble factors

Under *in vivo* conditions, cells are constantly exposed to various soluble factors which are among the pivotal components of the tissue microenvironment. Cells in microfluidics are surrounded by many soluble factors in the aqueous microenvironment, including basic nutrients necessary to tissues' metabolism and life, such as oxygen, carbon, glucose, amino acids, fatty acids, macromolecules, and micronutrients [63]. Amid all these basic

nutrients, oxygen is of more importance and because of low solubility, it can be easily depleted. The inefficiency of oxygen in the microenvironment leads to unsuccessful tissue engineering and is one of the major obstacles in the construction of complex 3D tissues, so modern oxygen-generating biomaterials were developed [64]. It is known that oxygen concentration (oxygen tension) according to cell type can have a big impact on cellular behavior, for instance, hypoxia (low oxygen tension) in human embryonic stem (hES) cells improves the preservation of proliferative, pluripotent population of these cells [65]. In a tumor environment, hypoxia can trigger some events which finally lead to the further spread of aggressive-heterogeneous-tumor cells and clones with lethal phenotypes [66]. It was also proved that targeting hypoxia in cancer therapy can conquer hypoxia-associated resistance issues [67]. Moreover, according to cell type and its specification, hypoxia can have a significant impact on controlling the immune response and inflammation [68], and in heart regeneration, it can lead to improvement of cardiomyocytes proliferation [69].

In addition to basic nutrients, soluble signaling molecules are among the key factors in the cellular microenvironment, they are usually categorized into three main groups as hormones, growth factors, and cytokines, and the other forms can be salt ions, neurotransmitters, exosomes, or microvesicles containing lipids, DNA, and microRNA (miRNA). These soluble factors as crucial peptides enable cellular receptors' binding, leading to the transduction of either preventive or permissive signals to trigger differentiation and/or proliferation processes [70]. Despite the basic nutrients, signaling molecules encounter some challenges during delivery and synthesis procedures especially in complex 3D structures, and this issue can be intensified in presence of various cells leading to further dynamicity in the system [71].

The most widely studied soluble signaling molecules in microfluidics are growth factors [72,73], and it is known that [74] microfluidic channels may accelerate the accumulation of endogenous signals. Each specific cell has its specific growth factor milieu that should be considered in the design of the microenvironment, lots of growth factor groups were studied in 3D cell culture systems, including nerve (NGFs), epidermal (EGFs), fibroblast (FGFs), vascular endothelial (VEGFs), transforming (TGFs), hepatocyte (HGFs), basic fibroblast (bFGFs), platelet-derived growth factors (PDGFs), and bone morphogenetic proteins, whether in aqueous media or immobility condition in ECM, these growth factors are thoroughly controlled in time and space with concentration gradients in the environment. Their distribution, concentration, and biological activity have a critical role in

cellular behavior, for instance, culturing of NSCs in a microfluidic chip device with an osmotic pump under a continuous fluid flow maintaining growth factor concentration gradients of EGFs/bFGFs showing that lower concentration of growth factors can induce differentiation, while by removing growth factors NSCs proliferation occurs and cells can differentiate into neurons or glia [72]. Lyu et al. [75] constructed a microfluidic chip maintaining the concentration gradient for osteogenic growth factors to treat tendon-to-bone interface injuries and it is also able to regulate the activity of stem cells. This chip can successfully represent the complex three-layer structure of the tendon-to-bone interface as proved by histology and immunohistochemistry experiments.

2.4 External stimuli

Synthetic cell microenvironments can be designed to respond to external stimuli like light, mechanical forces, electricity, temperature, and magnetic fields, this is usually done in four- dimensional cell microenvironments. Natural cells *in vivo* have the potential to sense and respond to a range of physical stimuli from any stress and strain field to acoustic, magnetic, electrical, and thermal fields. These factors were categorized under physical cues or external stimuli that are far different from biophysical cues. Some of these external stimuli need to be intermediated by ECM to stimulate cells. The effectiveness of these factors depends on their sources and position, for instance, blood cells are under continuous impact of stress and strains fields due to blood flow. Also, heart and lungs' cells always encounter cyclical tensile stress and strain forces, while bone and cartilage cells experience compressive stress and strains. The mechanical fields affect cell behaviors and functions, and they mainly depend on cell type and loading parameters (e.g., amplitude, loading type, waveform, frequency, and duration) [76,77]. The utilization of microfluidic channels makes it possible to accurately control the shear stress magnitude and mechanical forces. These microfluidic systems revealed that tensile stressing and straining can enhance fibroblasts' alignment and proliferation [78]. Shear stresses has also beneficial effects on endothelial cells (ECs), these cells can switch mechanical stimuli into intracellular signals that in turn affect cellular functions, such as apoptosis, proliferation, migration, and remodeling, as well as gene expression [79]. In addition, shear stresses transform the genetic program of ECs leading to interference with cell death, signal transduction, and inhibition of ECs' apoptosis [80]. These stress and shear forces can also

positively affect the cardiomyogenic differentiation [81] and remodeling [82]. Horner et al. [83] showed that dynamic compressive strains could have a considerable impact on the multiphenotypic differentiation of mesenchymal stromal cells (MSCs). There are many other examples in the literature illustrating the effect of mechanical forces on cells which largely depend on cell type and mechanical loading properties.

In addition to mechanical forces, the effect of other physical fields such as electrical, magnetic, acoustic, thermal, and light was investigated on cells. It was shown that electricity could regulate cell proliferation, migration, organization, and differentiation. Electrical stimulation can promote the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), and it was shown that bone-related markers and genes were considerably up-regulated by electrical stimulation [84]. Electrical discharge plasma application can lead to apoptotic cell death of cancer cells [85]. Electrical stimulations can trigger numerous intracellular signaling pathways, regulate the intracellular microenvironment, and consequently influence cell migration, proliferation, and differentiation [86]. This positive function can be utilized in biocompatible conductive materials and modern microfluidic systems.

Unfortunately, acoustic, magnetic, and thermal fields are not thoroughly investigated in microfluidic systems and many investigations must be done to achieve a full comprehension of the related mechanisms and effects. Despite the safe nature of magnetic fields that are used in magnetic resonance imaging systems and the fact that it has not any considerable adverse effects on 3D cultured cells, this safety largely depends on the magnitude of the field hence necessary precautions must be taken. Meanwhile, the application of magnetic fields seems very promising in microfluidic systems since the high magnetic fields have a substantial potential to coordinate the mitotic spindle during mitosis and align fibronectins and collagen fibers in polymerization processes. This physical stimulus can be used in magnetic cell sorting and alignment, and it has the potential to pave the way toward more sophisticated and smart microfluidic systems. Acoustic fields can also shift the normal cellular behavior, it was found that [87] cell viability and membrane permeability severely relies on the exposure condition of the acoustic field. It was far known that sound waves could have therapeutic effects [88–90]. Moghaddam et al. [91] reported that low-intensity ultrasound waves can considerably improve spermatogonia's proliferation and colonization in stem cells. Ohlsson et al. [92] introduced a high throughput microfluidic continuous flow method by utilization of acoustic impedance to separate

bacteria from blood in sepsis diagnosis. Soft cells and tissues can be considerably affected and deformed by acoustic fields due to acoustic radiation forces [93,94]; also, sharp edges in microfluidic structures can induce acoustic radiation forces [95]. The possibility of thermal fields' existence in the body as an isothermal system is low and the temperature variations in the cell microenvironment can alter cell behavior and function. Some biochemical reactions that are related to enzyme catalysis were proven to be temperature sensitive [96], temperature variations can be used in the cell-selective treatment of disease [97], and it can considerably affect gene expression [98] and tumor metabolism [99]. It can be easily deduced that the application of external thermal fields has a great potential to evolve the cell microenvironment and affect its behavior in microfluidic systems, and this subject is widely investigated in cancer therapy [100,101]. The recent introduction of biocompatible multifunctional theranostic nanoparticles has enabled the simultaneous sense and regulation of temperature to target tumor cells [102–104]. There are limited studies about this new technology, but it can facilitate the way toward designing smart microfluidic systems. Light is another factor that can alter cell behavior. Huang et al. [105] designed a microfluidic chip that can induce five different UV radiation doses at one experiment session on NIH/3T3 cells. It was found that UVB radiation considerably damages the cells, especially at high UV doses. Also, microfluidic photobioreactors can be utilized to investigate various aspects of light applications, and these novel systems can fully imitate light-dark cycles or any complex dynamic light conditions [106]. Exposure to light can vary influences from beneficial to adverse, for instance, vitamin D production, and inhibiting some kinds of cancer are among the positive effects while DNA damages, photoaging, immune suppression, and skin cancers are among the side effects. Hence, it is of crucial importance to design the microfluidic systems in an accurate manner to adjust the desired conditions according to experiment and natural in vivo conditions.

2.5 Summary

Recently, the microfluidic chip as a must-have device for many biological fields attracted much attention, and the unique advantages of microfluidic systems and their ability to control various effective parameters have revolutionized the human approach to cell biology and will pave the way for the widespread use of high-efficiency human organs-on-chips. These microfluidic chips have numerous benefits including three-dimensional

structure, better in vivo mimicking, close to the natural microenvironment, time consumption, increased productivity, and its microscale dimension similar to natural conditions. To attain reliable results that are of eminent importance in biological investigations all the related factors and mechanisms affecting cellular behavior must be thoroughly controlled and considered. In general, most of the factors influencing cellular behavior in the microfluidic chips can be studied under the domain of cellular microenvironment and its related components. In this regard, the critical factors that may affect the natural cell behavior and its function in the microfluidic chip environment were studied in this chapter. Firstly, the cellular microenvironment that includes all physical, chemical, and biological cues along with their direct or indirect effect on cells' behavior and responses are introduced. Then biochemical (chemical composition, cell adhesion ligands, cytokines, growth factors, hormones chemokines, soluble factors) and biophysical cues (cell morphology, degradability, matrix mechanics, electrical conductivity, cell adhesion and anchoring, surface integrins, fluid shear force, matrix tension/pressure, matrix hardness) as the most effective parameters in cell fate were studied. Also, physicochemical factors and geometrical microenvironment as effective parameters in cell behavior were explained. The extracellular matrix as the main actor in physiology, function, and fate of living cells was thoroughly discussed, also the cell-to-cell interactions, soluble factors, and the possible effects of external stimuli were considered. Due to these numerous effective parameters in cell biology and their intricate reciprocal interactions, it is of paramount importance to design the microfluidic systems accurately to attain the exact desired conditions according to experiment and natural in vivo conditions. All these factors and any possible parameters should be considered to attain reliable, high throughput, close to the natural microenvironment, and optimized microfluidic chips.

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CHAPTER 3

Cell-based assays on microfluidic chips

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3.1 Introduction

As from the advent of a miniaturized gas chromatography analyzer on a silicon wafer by Terry et al. [1] and most importantly as from the conceptual work on miniaturized total chemical analysis systems by Manz et al. [2], micrototal analysis systems or lab-on-a-chip technology has found a wide variety of applications in many biotechnological areas, developed new biomedical reagents, and improved cell and biochemical assays. In general,

the term microfluidic refers to the precise manipulation and control over small amounts of liquids at a micrometer scale. This technology is characterized by high interdisciplinarity and receives inputs from numerous scholars in life sciences, which, in turn, lead to the development and application of microfluidics in many (bio)technological areas, like clinical diagnostic [3], proteomics [4], cell and tissue engineering [5], pharmacology [6], and environmental monitoring [7]. In particular, a large number of microfluidics-based cell assays have been developed to conduct advanced studies concerning different cell behaviors, including cell growth, differentiation, adhesion, and migration under tightly controlled microenvironments. In light of the recent progress, microfluidic platforms have been growingly utilized to design complex multicellular environments and elucidate cell–cell and cell–extracellular matrix interactions. In this chapter, cell-based assays on various microfluidic platforms have been reviewed. Also through this chapter, we attempt to highlight the applications of recent cell assays based on microfluidic devices.

3.2 Significance of cell-based assay

The effects of different physicochemical stimuli on cells play a key role in many medical and pharmaceutical fields and represent an expanding area of research known as “cell-based assay.” One of its interesting applications is in the drug discovery and development processes. In the first step to identify leads of therapeutic potential, extensive screening of large compound libraries is conducted against specific targets expressed in immortalized cell lines. G-protein coupled receptor, for instance, is screened by detecting the intracellular Ca^{2+} concentration via fluorescence [8]. Additionally, a variety of cell-based absorption, distribution, metabolism, excretion, and toxicity assays is considered to understand if the resultant leads can present the required pharmacokinetic features and cause any toxicity. Therefore, the most important reason behind developing cell-based assays is a tendency to design tests that can impart data indicative of biological responses; in the best possible way, results should be consistent with those of animal or human studies. Cell-based assays can extract new information unlikely to get from other methods [9]. Of note, they can be advantageous over ligand binding assays in terms of throughput, costs, and data quality [10]. Although biochemical assays rely on a specific target, cell-based assays enable a potential compound to enter into interaction with various targets. In fact, biological living cells are considered the final targets of all drugs so applying them to experimental models appears

to be more productive than evaluating distinct genes or proteins. Moreover, cell-based assays can focus on multiple targets and demonstrate the influence of a whole transduction pathway. New target sites, as well as signaling pathways, can also be explored in these assays. Involving diverse cell lines from certain tissues facilitates the screening of drugs against several types of cancer [11]. More importantly, employing microfluidic technologies in a combination of cell-based assays can open new avenues for engineering small volumes of liquids in microchannels, which is not only user-friendly but also produce sophisticated results [12]. Different types of microfluidic cell-based assays are described in the next section.

3.3 Microfluidic cell-based assay systems

3.3.1 Cell-based assays on continuous-flow microfluidics

Continuous-flow microfluidics refers to manipulating the fluid flow via constructed microchannels without losing continuity. In other words, this type of microfluidic technique involves continuous flows in the microchannel controlled by external mechanical pumps [13,14] or by electrokinetics combined with capillary forces [15–18]. Due to its convenient implementation, continuous-flow microfluidics is most commonly used for simple biochemical [19–21] and biomedical applications [22–24]. For evaluating glycerol secretion, Clark et al. cultured adipocytes on glass cover slips and then transferred them into the cell chamber of the perfusion chip, which was then coupled with an online fluorescence-based enzyme assay [25]. Song et al. developed a continuous-flow microfluidic device (Fig. 3.1) for sorting stem cells and their differentiation progenies. To assess sorting performance, an on-chip cell count besides an off-chip hemocytometric analysis was carried out by analysis of videos obtained during experiments and trypan blue assay, respectively [26].

3.3.2 Cell-based assays on droplet microfluidics

In contrast with continuous-flow microfluidics, droplet-based microfluidic systems address the generation and manipulation of separate droplets by using multiphase flow of immiscible fluids inside the microchannels [27–29]. This technique enables the transportation, mixing, and analysis of each droplet as a microreactor [30,31]. In view of parallel processing and experimentation, droplet-based microfluidics show capacity for augmenting throughput and scalability with no increase in the size or complexity of the microdevices. Recently, it has found a lot of applications in materials

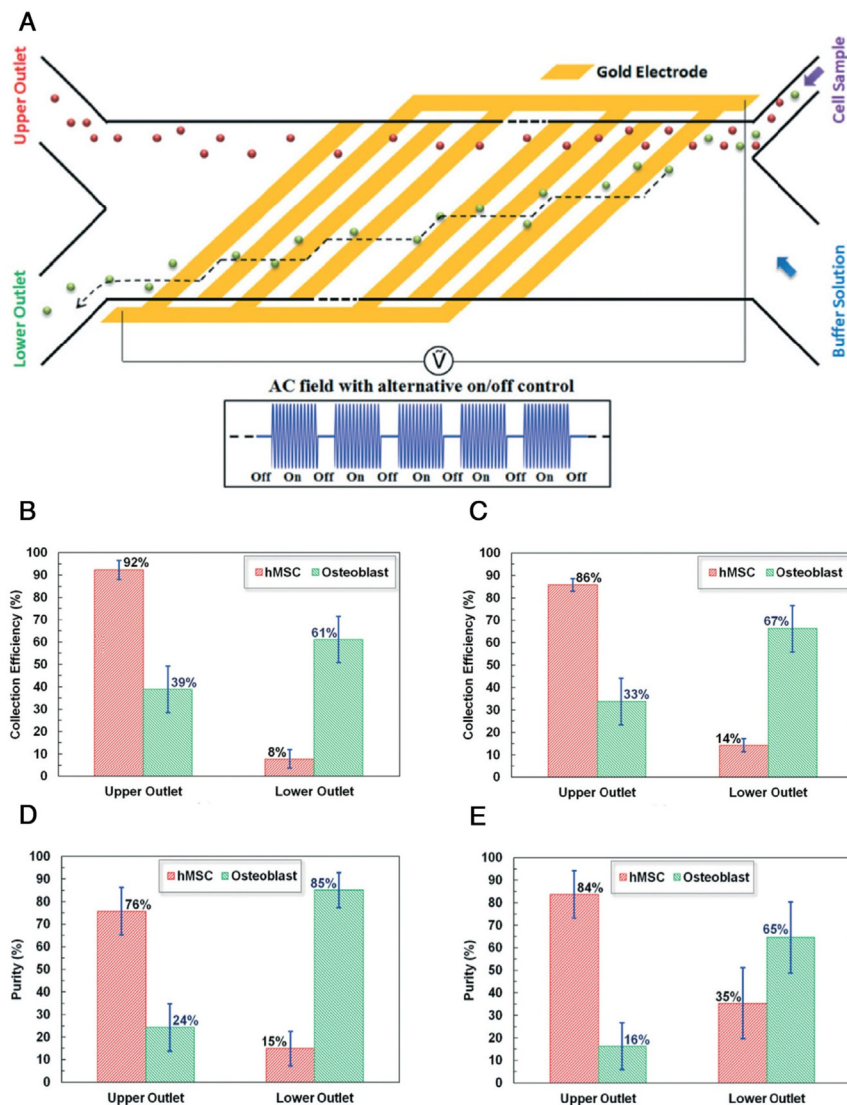


Figure 3.1 Schematic representation of the continuous flow dielectrophoresis-based microfluidic cell separation. In its design, there was a microchannel connected to two inlets (cell sample and buffer solution) as well as two outlets. Also, the device included an array of oblique interdigitated electrodes inclined at 45 degrees relative to the flow direction. These electrodes were placed on the floor of the microchannel. To generate dielectrophoresis, an AC field was used with alternating on/off control between the electrodes (a) Collection efficiency of stem cells and osteoblasts at various outlets measured from an on-chip count (b) and an off-chip count (c) Purity of human mesenchymal stem cells and osteoblasts at various outlets measured from an on-chip count (d) and an off-chip count (e) The collection efficiency and purity were calculated at a flow rate of $1.8 \mu\text{L min}^{-1}$ ($0.3 \mu\text{L min}^{-1}$ and $1.5 \mu\text{L min}^{-1}$ for the sample and buffer inlets, respectively) and an AC voltage of 7.2 V peak to peak (at 3 MHz). (Reprinted from Ref. [26]. Open Access 2021 Royal Society of Chemistry.)

synthesis [32–35], biochemical analysis [36–39], and biomedical engineering [40–43]. To measure the expression of a gene reporter (GFPS65T) as well as the fluorescent label (dextran Texas red) simultaneously, Baret et al. compartmentalized single *Bombyx mori* BM5 cells in nanoliter droplets. Applying this device (Fig. 3.2A), they could successfully provide dose-response profiles and calculate EC50 values using small numbers of cells [44]. Du et al. reported a microfluidic droplet system based on a sequential operation droplet array (Fig. 3.2B). A549 cells were cultured in an oil-covered two-dimensional droplet array chip, which was a couple with a tapered capillary connected with a syringe pump. The entire system was capable of multiple droplet manipulations, that is, liquid metering, aspirating, depositing, mixing, and transferring, along with complex multistep operations for drug combination screening, that is, long-term cell culture, medium changing, schedule-dependent drug dosage and stimulation, and cell viability testing [45].

3.3.3 Cell-based assays on digital microfluidics

Digital microfluidic technique involves manipulation of discrete microdroplets on the surfaces of two-dimensional planar chips by applying the electric control of electrode arrays instead of handling the droplets in the channels. The main mechanism underlying this droplet-based technology is electrowetting-on-dielectric that alters the interfacial tension between the droplet and the dielectric layer, thus, changing the contact angle of the drop in case of exerting an electric field across the droplet [46]. Accordingly, there is no dispersion because of diffusion, almost no or insignificant cross-mixing, and facile fluidic manipulation [47]. Considering the merits of digital microfluidic, such as the isolation of each droplet from the surrounding environment, convenient intelligent control with real-time feedback, and automatic analysis [48], its applications in point-of-care testing diagnostics [49,50] and many other biomedical areas [51–54] are of high interest. For cell-based assays, numerous research studies have been conducted [51,55–57]. For example, Bender et al. constructed a digital microfluidic device allowing for the formation of spheroids by using the hanging drop method, encapsulation of the spheroids in the collagen gel, and the treatment of spheroids to migration-modulating substances. This system was intended to mimic the three-dimensional (3D) tissue microenvironment and measure cell invasion, which plays a pivotal role in tissue growth, wound healing, and tumor progression [56]. Another study by Leclerc et al. described a fucosylation inhibition assay on a digital microfluidic platform (Fig. 3.3a–c) with the

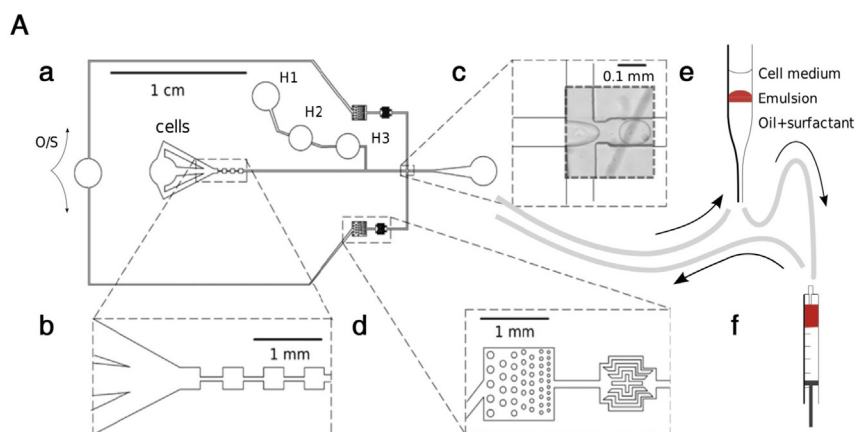


Figure 3.2 Schematic representation of the microfluidic device for coencapsulating cells and hormone 20E (A) The emulsion was prepared by mixing oil and surfactant with the flow-focusing device, and accordingly cells were cocompartmentalized with 20E. In this design, the three different concentrations of hormone 20E traveled through three distinct syringes shown by the inlets H1, H2, and H3 (a) Zoomed representation of the series of constrictions responsible for splitting cell clumps (b) Zoomed representation of the flow-focusing junction with a micrograph of an encapsulated cell (c) Zoomed representation of the set of filters that serve to prevent dust from transferring to the device (d) After collecting the emulsion, it was incubated for 24 h at room temperature and subsequently formed as a sandwich structure between a layer of medium and the oil/surfactant mixture (e) After incubation, the emulsion was flushed back into the original chip by a syringe for laser-induced fluorescence measurement (B) The device consisted of a fused-silica capillary with a tapered tip (inner diameter $\sim 120\ \mu\text{m}$ and outer diameter $\sim 150\ \mu\text{m}$) used for liquid handling, liquid metering, droplet assembling, reagent addition, and liquid transferring. The capillary was connected to a syringe pump. The nanowell array chip and reagent reservoirs were fixed on an x–y–z stage controlled by a homemade LabVIEW program. The nanowell array chip was composed of a poly(dimethylsiloxane) (PDMS) substrate (thickness $\sim 0.5\ \text{mm}$). The nanowell array and PDMS holding layer were responsible for oil containment. (A) Reprinted from Ref. [44]. Copyright 2021 Elsevier. Schematic representation of the semiclosed droplet array system (B) Reprinted from Ref. [45]. Copyright 2021 American Chemical Society.)

integration of an image-based analysis to obtain dose–response curves with associated IC₅₀ values (Fig. 3.3d–g). In their work, proof-of-concept testing was carried out for a new fucosylation inhibition assay by the fluorogenically labeled synthetic disaccharide, MU- β -LacNAc. This disaccharide was found to be the desired probe for fucosylation by *H. pylori* FucT, with the capacity for high-throughput purposes. This design used guanosine diphosphate (GDP) (guanosine 5'-diphosphate) as a test inhibitor, which subsequently underwent serial dilution on the device [57].

3.3.4 Cell-based assays on paper microfluidics

In general, paper-based microfluidic devices consist of several nitrocellulose fibers [108,109] or hydrophilic cellulose [58,59] to transfer the fluid from an inlet to an exact outlet via imbibition. The manufacturing of such devices is based on technologies like wax printing [60,61], photolithography [62,63], plasma treatment [64], etc. As paper-based microfluidics has an extremely low cost, easy fabrication and operation, favorable flexibility, appropriate lightness and portability, convenient integration with other devices, and low thickness [65], it has been applied in many areas of research, including point-of-care disease diagnostics [66–68], food inspection [69–71], and environmental monitoring [72–74]. Patterned barrier on a paper device in 96- and 384-microzone format can be considered an alternative to traditional multiwell plates [75]. Modified cell culture array on a paper device can be successfully used as a high-throughput platform for screening compounds or drugs [76], which suggests the capacity of paper microfluidics for cell-based assays. Park et al. reported paper microfluidics for determining the concentration of *Salmonella Typhimurium* and *Escherichia coli* on the smartphone. To this aim, anti-*Salmonella Typhimurium* and anti-*Escherichia coli* conjugated latex particles were preloaded in each paper microfluidic channel. As the device was dipped in the *Salmonella* solutions, the antibody-conjugated submicroparticles drove the immunoagglutination of the latex particles (Fig. 3.4). The amount of immunoagglutination was measured by detecting Mie scatter from the digital images acquired at an optimized angle and distance with a smartphone. The detection limit in their study was at the single-cell level [77].

3.4 Applications of microfluidic cell-based assays

Cell-based assays play a central role in many fields, such as pharmacology, cell biology, and tissue engineering. Recent advances in microfabrication and microfluidic technologies have introduced different lab-on-a-chip

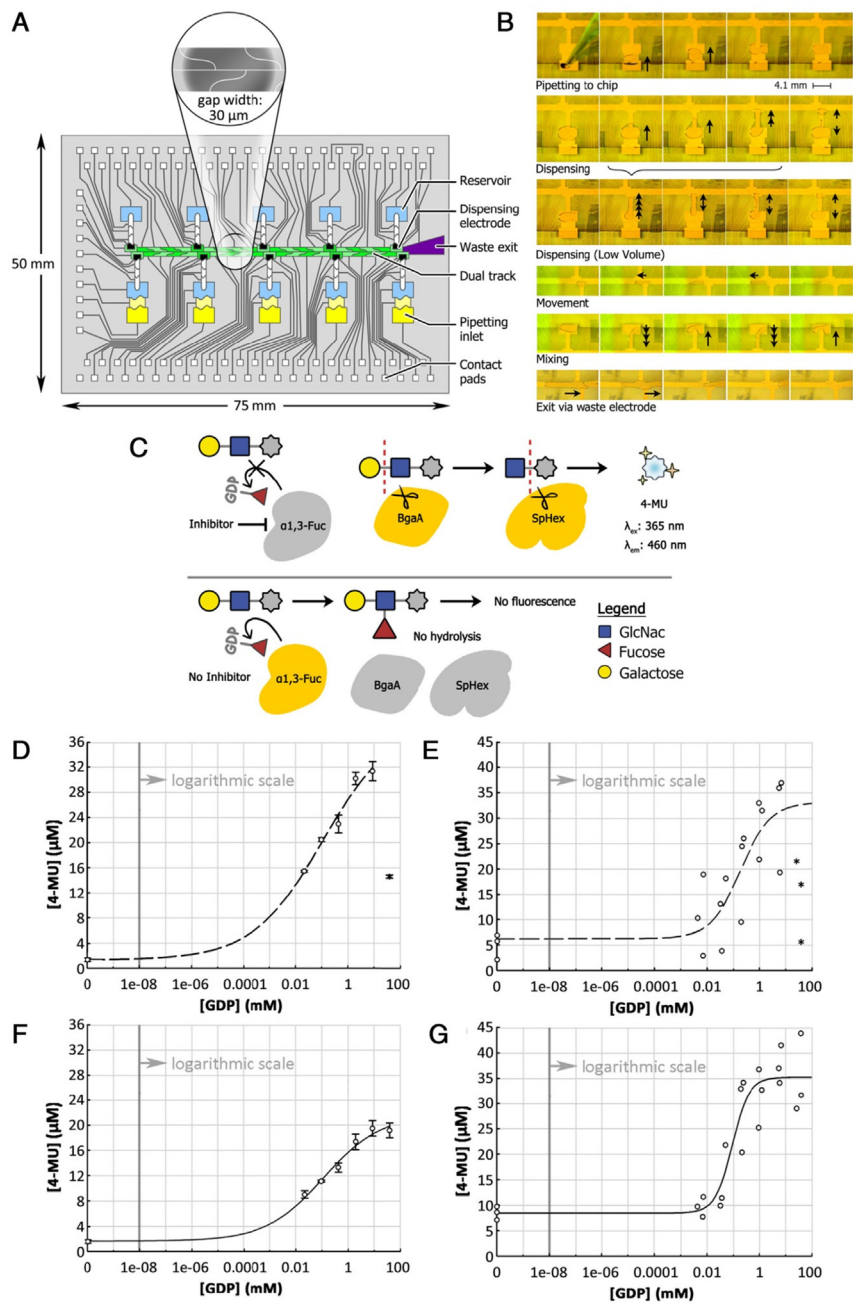


Figure 3.3 The schematic representation of digital microfluidics (DMF) for a fucosyl-transferase inhibition assay. The device layout of the microfluidics chip consisted of 103 electrodes, including a waste electrode collecting unused fluids, two adjacent

devices with several functionalities for cell culturing. Microfabrication techniques are employed for the mass fabrication of chip-based devices with functional high-density arrays. Furthermore, the use of microfluidic devices for cell assays brings important advantages, including elevated throughput, accurate liquid manipulation in the nanoliter range, and decreased sample volume. Fewer cells are required for each experiment, and thus this platform is useful, effectively saving a lot of resources for conducting experiments on rare cells, like stem cells or primary cells. On-chip culture systems provide culture environments that have features at microscale lengths. Such an on-chip culture environment can be designed to favor the *in vivo* cell microenvironment in comparison to traditional cell cultures. A number of on-chip culture systems with several functionalities have been investigated thus far. Prokop et al. created a device for cell culturing in microfluidic channels and could successfully culture cells for five days [78]. Hung et al. fabricated a device that possessed discrete cell loading and media perfusion inlets for efficient cell collection and perfusion with no cell loss [79]. Additionally, the design allowed for cell passaging by trypsin, with cell culturing over two weeks [79]. Up to now, numerous cell types, such as human hepatocytes, fibroblasts, osteoblasts, stem cells, and breast cancer cells, have been successfully cultured on-chip [80]. Microfluidic cell culture arrays might involve concentration gradient generators in an attempt to



tracks connecting each section, and 10 reservoir electrodes. At the edge of the chip, every electrode was linked to a square contact pad by means of 70- μm wide lines. Five reservoirs contained pipetting inlets with the ability to pull droplets upon actuation. The zoomed view shows a skewed-wave electrode on the track, with an average of interelectrode gap size of 30 μm (a) Step-by-step representation of the various droplet operations conducted on the device (b) Top and bottom images represent a fucosylation assay; top: the β -galactosidase gene of *Streptococcus pneumoniae* (BgaA) and *N*-acetylhexosaminidase gene of *Streptomyces plicatus* (SpHex) sequentially acted upon synthetically methylumbelliferated disaccharide (MU- β -LacNAc) and hydrolytically cleaved it into its monosaccharide components to produce fluorescent 4-methylumbelliferone (4-MU). Bottom: fucosylation by an $\alpha(1,3)$ -fucosyltransferase ($\alpha(1,3)$ -Fuc) led to a structure not recognizable by the glycosidases, preventing hydrolysis by BgaA and SpHex, and lowering fluorescence significantly (c). Testing of fucosylation assays on a digital microfluidic device (d and f) and in a well plate (e and g); on the microfluidic device, the IC_{50} was $0.089 \text{ mM} \pm 0.092$. The well plate indicated the IC_{50} of $0.015 \text{ mM} \pm 0.029$. Curves show the inhibition of $\alpha(1,3)$ -fucosyltransferase from *Helicobacter pylori* (FucT) by GDP before (d and e) and after glycine dilution (e and g). The IC_{50} values after glycine dilution were fairly comparable to each other at $0.114 \text{ mM} \pm 0.086$ in well plates and $0.093 \text{ mM} \pm 0.037$ on DMF devices. (Reprinted from Ref. [57]. Copyright 2021 AIP Publishing.)

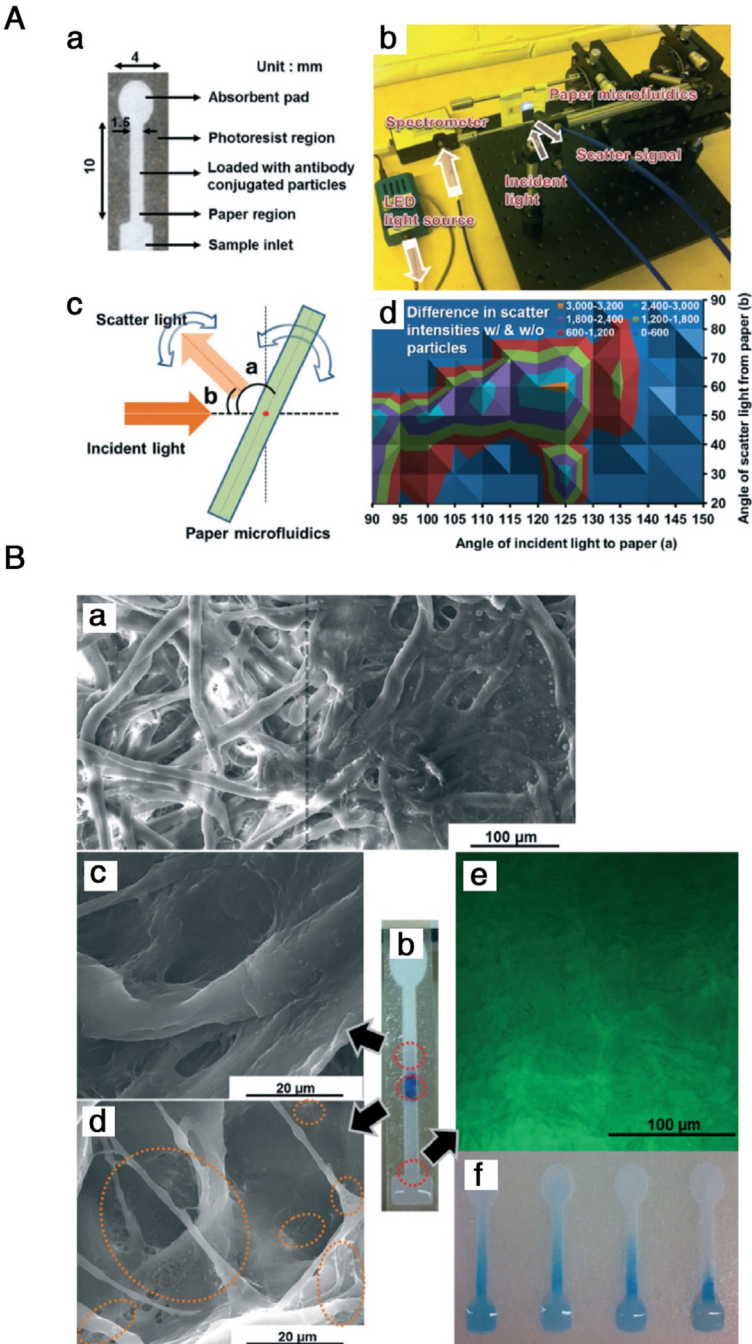


Figure 3.4 Detection of submicroparticles using a microfluidic system (A) A single-channel paper microfluidic device was loaded with antibody- or bovine serum albumin (BSA)-conjugated particles at the center of each channel. The device had an

treat them with a single compound at a variety of concentrations [81,82]. Constructing a chip with a high-density cell array, King et al. carried out a real-time evaluation of gene expression by applying green fluorescent proteins as a reporter [83]. Cells were exposed to multiple stimuli at the same time in a single experiment, and around 5000 measurements were made. A different device was proposed by Kim et al. for cell culturing at a range of flow rates simultaneously [84]. Cell patterning and long-term cellular differentiation were also reported using on-chip perfusion systems [85]. A 3D combinatorial cell culture array device was made by Liu and Tai for cell-based screening of multiple compounds at once on a microfluidic platform [86]. In this section, many applications of microfluidic cell-based assays are reviewed.

3.4.1 High-throughput screening

Systematic screening and large data play a pivotal role in pharmaceutical research and simplify the analysis of complex reactions, interactions, and systems. Systematic screening results in a successful resolution to massive data for chemical [87], biochemical [88], and cell-based assays [89]. High-throughput experimentation is an advantageous technology, which provides a reliable and cost-effective platform. Recent attempts have dealt with

absorbent pad, which appeared as a circular area that allowed the sample solution to travel continuously from the inlet and through the channel until the absorbent pad was filled (a) A benchtop system was utilized for optimizing Mie scatter detection from paper microfluidics (b) The measurement of light scatter intensities was carried out while altering the angle of incident light to paper (angle a) and the angle of light scatter from paper (angle b) (c) The results were exhibited as a contour plot, where every data point was regarded as the scatter intensity difference between the wet paper microfluidic device in the presence or absence of particles versus the angles of light irradiation and scattering detection (d) Behavior of submicroparticles, cells, and proteins while flowing through the paper microfluidics (B). Scanning electron microscopy (SEM) images show confinement of submicroparticles and their potential for immunoagglutinate within paper fibers (a) Image of the boundary between the paper channel (left) and the photoresist layer (right), where the pores between the cellulose fibers have been almost full of photoresist materials (b) Approximately, there were no antibody-conjugated particles away from the central particle loading point (c) whereas immunoagglutinated particles were confined at this loading point (d) Confocal microscopic image presented viability of *Salmonella* on the paper microfluidic device (e) An image of the paper microfluidics loaded with Bradford-stained BSA at 1000, 500, 75, and 25 $\mu\text{g/mL}$ (from left to right) showed that free antigens could travel through the paper easily. (Reprinted from Ref. [77]. Copyright 2021 Royal Society of Chemistry.)

developing microfluidic devices that can serve as inexpensive tools for high-throughput cell-based assays [90–92]. Pharmaceutical companies all over the world build extensive and growing libraries of various compounds, largely in view of the simplicity in which combinatorial chemistry is applied for the synthesis of numerous compounds [93]. If the screening of compounds must be conducted with higher throughput at lower costs, the miniaturization of such assays is inevitable, leading to the consumption of far lower volumes of chemicals, solvents, and cells [94]. Johnson et al. make a microfluidic device with potential applications in high-throughput screening [95]. The system, which is intended to take the place of conventional, plate-based screening platforms, consists of a glass microchip with integrated “sipper” capillaries responsible for “sipping” fluids, such as compounds, dyes, buffers, or negative controls, from plate wells, while simultaneously drawing solutions, such as enzyme, substrate, or cell suspensions, from integrated, on-chip wells in a continuous manner (Fig. 3.5). The resultant mixtures travel through a microchannel, which also functions as an incubator, and into a downstream detection point, where recordings of fluorescence signals occur. The whole process is conducted serially so that each chip takes a large number of cell suspensions, compounds, and dyes, which subsequently empty, and the system fills with the buffer solution again. This device has been shown with capabilities of performing experimentation from enzymatic assays to cell-based calcium flux assays with notably higher throughput and better reproducibility than conventional platforms [96]. The platform described earlier is commercialized by Caliper Life Sciences.

3.4.2 Expression and activity of ion channels

Ion channels are membrane-spanning proteins that allow for the selective diffusion of ions across the cell’s and organelle’s membrane. These integral proteins play a key role in cell-to-cell communication and take part in many cellular events, such as muscle contraction and brain function, thus, receiving much attention in biology fields. There are several ion channel-related diseases in which ion channels are considered interesting drug targets. The gold standard means of evaluating ion channel activity is patch clamp, known as an electrophysiological technique [97–99], which focuses on single-channel recording with microsecond temporal resolution. Yet, it takes a notoriously labor-intensive and low-throughput process and has limitations in the number of patch-clamped cells per unit time, thus, posing a challenge to massive data generation.

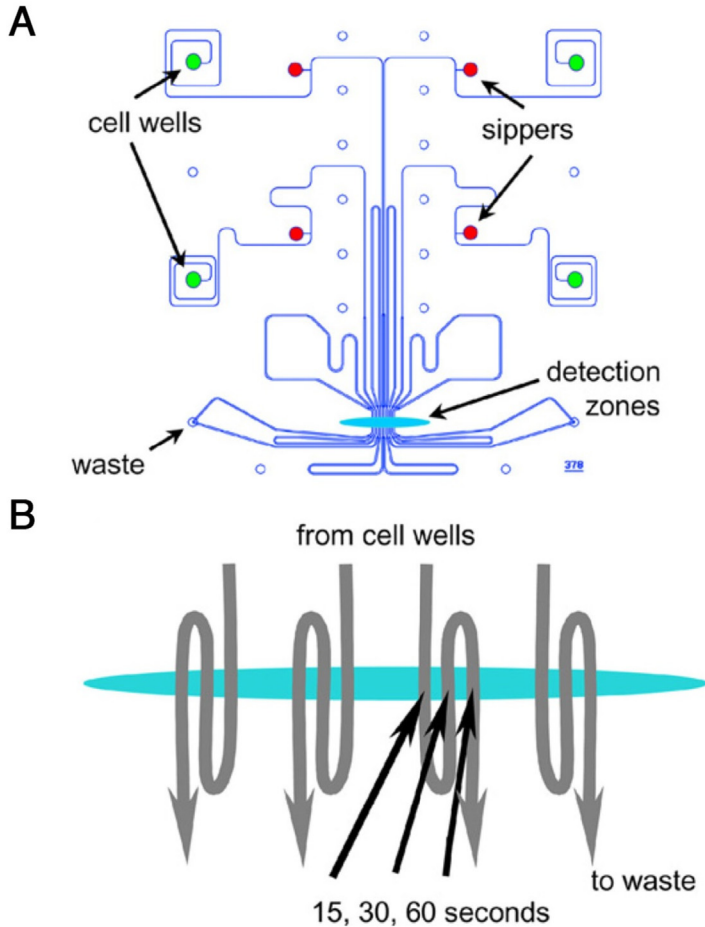


Figure 3.5 Schematic representation of the microfluidic device used for detection of agonist-induced calcium flux. Fluid was drawn from the four cell wells (a, center of blue spirals) applying vacuum at the waste port. Platelets were subjected to agonist aspirated from a 384-well plate (sipper, red circles). Platelets and agonist were mixed via diffusion while sample as well as compound traveled continuously from the sipper junction into the fluorescence detection zone (a, b). Flow rate was a function of both applied vacuum and sample viscosity. This microfluidic device was designed to flow four samples at the same time via the detection zone three times, leading to agonist-platelet incubation times of 15, 30, and 60 s (b). The cell-agonist incubation time to detection was defined by the flow rate besides the distance from the sipper junction to the detection zone. (Reprinted from Ref. [96]. Copyright 2021 Elsevier.)

Increasing the number of data points per patch-clamped cell by combining microfluidics to patch clamp is the first way to improve throughput [18,100,101]. This approach is developed by Orwar et al. and then commercialized by Celectricon. The system setup is built around a microfluidic chip made of silicone elastomer and glass (Fig. 3.6a). It contains several sample wells linked to an open volume by means of microfluidic channels, which are tightly packed at the exit. Liquids are drawn through the channels and into the open volume by exerting pressure on the wells. These fluids join together thickly, leading to a single laminar flow that spreads in the open volume (Fig. 3.6a). The resulting flow comprises several discrete zones with clearly described chemical environments related to the loading of the wells. Due to the low Reynolds number of the setup, only diffusive mixing occurs between surrounding environments [102]. In the open volume, cells are patch-clamped and undergo scanning across the various environments after translocation to the channel exits. The result includes dose-response curves obtained at very stable conditions, besides a number of dose-response scans from the same cell. Furthermore, it is feasible to capture prolonged recording times from one cell, even up to hours, by applying the well-defined force that emerges from the flow and carries a particular effect on the cell-pipette system. Such force contributes to the stabilization of the patch-clamped cell, as well [101]. In reference to the gold standard, a microfluidic system is a higher-throughput approach with less sample consumption and no risk of cross-contamination, because the devices are disposable. It is used in many stages of drug discovery and evaluation of receptor functionality [103,104].

The other, exclusively microfluidics-based approach to enhance throughput has been introduced by Lee et al. [105,106]. Similarly, cells are patch-clamped in a $2\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$ microfluidic channel (Fig. 3.6b). The delivery of the cells from sample reservoirs is carried out via microfluidic channels toward an array of small channels, in which a vacuum is applied to trap the cells, leading to a whole-cell configuration. Drugs are transported through the same microchannel as the cells but have distinct input wells. Although this approach does not produce data with the same quality as the conventional patch-clamp, potential applications have emerged, including massive parallelization [107].

3.4.3 Cellular chemotaxis: responding to chemical gradients

Chemotaxis refers to the directional cell migration upon exposure to chemical gradients of molecules known as chemoattractants. This cellular process is of utmost importance in a large number of biological processes,

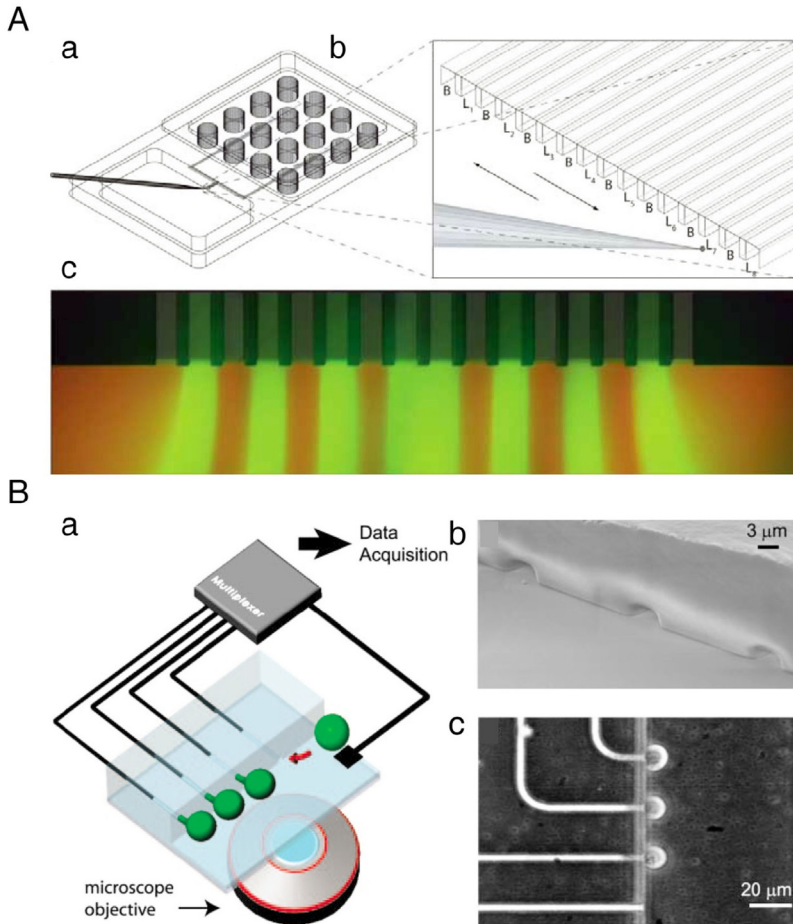


Figure 3.6 Design of the cell-based bar-code reader (A) Schematic representation of a 16-channel device (a) and a pipette with a patch-clamped cell outside the channel outlets (b) In this design, the cell was subjected to various solutions, which can be regarded as a bar code. A fluorescence micrograph for the collimated stream of various dye solutions at the exit into the open volume (B) Negative pressure to recording capillaries was used to trap cells. Attached cells underwent deformation, protruding into the capillaries. Patch-clamp recordings were conducted by inserting AgCl electrodes in every capillary and in the main chamber. The resultant signals were fed through a multiplexing circuit and into the data acquisition system. Also, the device was bonded to a glass coverslip to achieve optical monitoring (a) An SEM image of the device following bonding showed three recording capillary orifices (b) Darkfield optical microscope image of trapped cells in these orifices. (A) Reprinted from Ref. [9]. Copyright 2021 Elsevier. *Fabrication of patch-clamp array on a microfluidic device* (B) Reprinted from Ref. [106]. Open Access 2021 *Proceedings the National Academy of Sciences of the United States of America (PNAS)*.)

such as wound healing, immune response, and morphogenesis [108–110]. Many techniques, including various kinds of chambers or puffer pipettes, have been made to examine chemotaxis [111–116]. Most of the methods are not suitable for this particular purpose because the resulting gradients are in macroscopic-scale environments, nonlinear, and dependent on time uncontrollably. Employing a microfluidic chip for gradient generation has allowed the precise spatiotemporal control of the chemical environment surrounding cells in chemotaxis studies [117–119]. A steady time-independent gradient is built by the microfluidic network. Then, solutions in the presence and absence of chemoattractants are added to the network. While the two solutions travel down the network, they are divided, reunited, and mixed many times, leading to a growing number of channels and concentrations. As the network comes to the end, the channels are brought together, forming a concentration gradient from one side to the other. At this point on a single, wide microchannel, cells can be subjected, and their response can be investigated. This technique has been applied by Jeon et al. to explore neutrophil chemotaxis in gradients of interleukin-8. This study has shown the successful use of microfluidics for chemotaxis investigation in various research fields [82,120–122], which suggests the possibility of screening for drugs with modulatory effects on chemotaxis.

3.4.4 Simple cell-based platforms

Today, lab-on-a-chip technology is being vastly assessed for in vitro models of different diseases/medical conditions or for predictive toxicology. Considering the nature of biological systems, of great importance are cell-based models, including single-cell arrays as well as complex 3D cell culture systems. There are two fundamental variables in in vitro models, both cell type and source, which determine the exact representation of the (patho) physiological states observed in vivo, besides the reliability and validity of experimental studies. Thus far, great examples are primary cells of the liver, heart, and brain. Yet, culturing primary cells tends toward a decrease in specificity because of significant changes in the cell culture environment [123]. The microfluidic systems can better mimic the cellular microenvironment and enhance cell handling, positioning, and analysis. However, they present some problems, such as low-volume liquid handling capabilities, high reagent consumption, and high operation cost. Liu et al. developed a cell culture device composed of an integrated combinatorial mixer and 3D microfluidic networks in an attempt to screen the combinatorial impacts of multiple-compound exposure on cultured cells [124].

The design included a $1\text{ cm} \times 1\text{ cm}$ chip with a three-input combinatorial mixer as well as eight individually isolated microculture chambers [124]. Culturing stem cells in microfluidic networks is also important, as current methods used for isolating and culturing stem cells cannot direct their differentiation into relevant cell types in a predictive manner to construct in vitro models of disease for drug–target interactions or toxicity studies [125]. Microfluidic cell-based assays can be another option for the control of cell fate in vitro through the enhancement of the spatiotemporal cell behavior, particularly due to their greater regulation of biochemical and biophysical extracellular factors [126,127]. Yet, such platforms have not developed very much and required a more intensive focus to achieve an optimal design for controlling cell fate by means of environmental factors.

One main application of microfluidic cell culture platforms is the evaluation of DNA damage in cancer models, usually analyzed by the multistep comet electrophoresis assay, which deals with damage and repair at the single-cell level. Wood et al. suggested a simple method for measuring high-throughput DNA damage that gives data regarding multiple lesions and pathways. The technique was based on single cells trapped by gravity into a microwell array with DNA damage. This device could also provide novel functionalities, such as multiplexed labeling for parallel single-cell assays and DNA damage measurement in cell aggregates [128]. Another interesting achievement is droplet technology for drug discovery that involves encapsulation of single cells within droplets and appears to be consistent with high-throughput screening and cell sorting [129]. Brouzes et al. developed a droplet-based microfluidic technology with the capacity for high-throughput screening of single mammalian cells. Conducting a droplet cytotoxicity assay enabled the quantitative scoring of cell viability. Then, an optically coded droplet library was made to screen drug libraries for cytotoxic effects on cells. Besides this application, the droplet microfluidic device can be used for combinatorial screening and facilitating small sample analyses [130].

3.4.5 Toxicity analysis

As for toxicity studies, the data resulting from these models help predict the safety issues of the new drug candidates. The unpleasant toxicity of the drug is the main cause of underlying failure in modern drug development [131]. Microplate- and micronucleus-based assays largely serve to investigate cytotoxicity, genotoxicity, drug–drug interactions, and metabolite-mediated toxicity. Such tests could be performed by applying

microfluidic platforms [132–134]. The same designs are implemented to go beyond the assessment of toxicity in cells/organs, to reconstruct the metabolism of a drug, and to explore systemic responses *in vitro*. The given pharmacokinetic data for early toxicity form a holistic view of toxicology *in vitro*, to be conducted before more costly *in vivo* studies. Cell culture analog [135], for example, has evolved into an “animal-on-a-chip” platform at the microscale level that serves to integrate cocultures of various tissues and fat cells with physiological fluid arrays on a single chip [136,137]. The chip acts as a surrogate predictor of animal studies for assessing chemical exposures, which can be attained if only time-consuming and costly conventional toxicity studies are carried out. This chip technology paves the way for robust *in vitro* toxicology models that estimate the pharmacokinetic profiles of drugs and specific systemic toxicity in humans, other than animals [138]. Therefore, the adoption in pharma is viable, when both high-throughput and high-content assays can be controlled, which implies the significance of accurately designed and integrated microfluidic systems.

3.4.6 Three-dimensional cell culture systems

3D cell culture is more advantageous than growing cells in a flat layer because it can (a) alter cell morphology, (b) cause more realistic drug response in view of elevated resistance, (c) generate phenotypic heterogeneity, and (d) mimic the tumor microenvironment. Apart from controversies on *in vivo* testing, a large number of cancers still have no qualified animal models. In reference to growing cells on 2D culture systems, cells seeded on 3D culture platforms bear more close resemblance to *in vivo* cell–cell interactions and tumor heterogeneities [139]. On the other hand, 3D cell culture is more complex and expensive than monolayer cultures. However, recent advances in chip technologies and microfluidic systems have facilitated the use of more feasible and affordable platforms for 3D cell culturing and experiments. The use of micropatterned substrates, for instance, can be a viable approach for precisely controlling the size and morphology of the multicellular aggregates (microtissues), which involves the proper supply of nutrients to cells as well as the elimination of undesired metabolites. In this regard, the development of microgroove substrates coated with temperature-responsive film can form harvestable tissue constructs with controlled size and shape. Therefore, if this template is integrated with microfluidic devices, it can act as tissue model for drug discovery and tissue engineering [140]. As another possibility, microtissues with a diameter within the range of 250–350 μm can be fabricated in microfluidic devices by cell photoencapsulation in polyethylene glycol

hydrogels. Also, the microtissues can be evaluated using multiplexed parallel analysis [141]. Methods based on continuous-flow lithographic encoding can be employed to produce multifunctional particles and accordingly improve current approaches for multiplexed analysis, which includes complex or costly processes [142]. All these efforts have focused on the evaluation of the 3D model response to various drugs and their combinations. Moreover, other new microfluidic systems with enhanced functionalities are created to increase data quality from in vitro assays [143,144]. In addition to giving a deeper insight into the spatiotemporal factors that regulate cell fates, the main feature of these platforms is to develop them as high-throughput devices with enough accuracy for drug discovery.

3.5 Conclusions

Cell-based assays are central techniques in a broad range of research studies, such as pharmaceuticals, cell biology, and tissue engineering. Different advancements were made to apply cells for conducting assays in cellular experiments, which, in turn, culminates in a wealth of information regarding the complex behavior of cell–cell interactions and molecular components of the cells. Since high-throughput screening is in great demand, conventional techniques, including well plates and robotics, have been constantly manipulated and enhanced to meet the demand. Yet, the use of these techniques is limited due to their associated disadvantages, such as poor liquid handling properties, difficult scale-up of well plate density, and high costs of equipment and operations. Microfluidics becomes apparent as a promising technology to conduct cell-based assays in a high-throughput fashion. Microfluidic devices are advantageous in terms of high-throughput, mass fabrication, and reagent consumption, cell density, and cost. With all this immense potential, the field of the cell-based assay on microfluidics is still young with some commercialized products described above. Altogether, microfluidics will play a leading role in the advancement of cell-based assays for the development of a more precise and higher quality target in (bio)technological areas.

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CHAPTER 4

Cell culture techniques in microfluidic chips

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4.1 Overview on cell culture

Over the past decades, cell culture has played an essential role in the development of biology and medicine. As a model system, cell culture has had many applications for comprehending the cellular functions as well as mechanisms of diseases production and progression in preclinical studies in various biological fields such as tissue engineering, genetic engineering, cancer research, vaccine production, and drug research [1]. For the first time, in 1907, Ross G. Harrison et al. applied the in vitro cell culture method during a research on the origin of nerve fibers in a kind of frog. They successfully could cultivate the fibers in lymph fluid for several weeks [2,3]. After that, the cell culture methods constantly have undergone changes and improvements over time for monitoring the function of different types of cells. Nowadays, cell provision sources for in vitro culture include primary cells, secondary cell lines, and more recently, personalized patient-tissue-derived cells [4]. In this connection, more than 3600 cell lines of 150 different species have been developed [5]. According to early culture approaches, cells are seeded on a planar substrate exposed to a culture medium for a determined period of time, inside an incubator to control temperature and CO₂. One of the key steps in the development of cell culture methods to better mimic the microenvironmental and microstructural conditions of the body is the transition from two-dimensional (2D) to three-dimensional (3D) techniques [6]. However, both conventional 2D and 3D culture methods do not allow efficient imitation of dynamic processes present in vivo due to the lack of continuous systems. In such methods, the processes, including nutrients supply, gas exchange, and metabolite removal, proceed in a static manner against continuous dynamic conditions inside the body, that consequently affect the phenotype and function of cells. Also, dynamic monitoring of the involved parameters in cellular vitality and behavior is hardly practical in these systems [7]. On the other hand, using animal models, which is a solution to achieve more reliable results in cell studies, in addition to ethical issues, is costly and time consuming and also has incompatibilities in different species. Cancer studies, for example, have shown that animal models have limitations in mimicking processes such as intertumoral heterogeneity, carcinogenesis, and progression in human cancers [4]. In the following of advances in biotechnology regarding cell culture approaches, one of the valuable tools is microfluidic culture platforms that can greatly overcome the limitations of conventional 2D and 3D methods and remove the need for animal models [4]. Microfluidics as a state-

of-the-art technology has been developed since the 1990s for culturing various cell types from single cells to complex 3D tissue models [8,9]. Microfluidic systems allow the monitoring of the cellular vitality, function, and behavior by enabling the dynamic precise control over culture micro-environmental conditions like temperature, pH, medium and gas exchange, interconnections between different tissues, and rapid manipulation of related parameters via perfusion flow as well as integrated components such as microchannels, microvalves, microreservoirs, micropumps, microelectrodes, and microsensors [10,11]. Thanks to such integrated components, different processes such as cell positioning, seeding, trapping, culturing, mixing, coculturing, and variables detection can be handled at the same time on a small chip in microfluidic devices [8,12]. The micron dimensions present in microfluidic systems are consistent with the dimensions of cellular structures within the body [13]. These systems also provide the opportunity to perform culture processes automatically instead of manual and robotic handlings that not only facilitate processes but also reduce potential external contaminants [7,8]. In addition, the integration of biosensors with microfluidic culture systems by providing real-time data recording and consequently a deep understanding of cellular behavior under various dynamic conditions has played a significant role in the development of this field [14]. In this regard, various methods are employed for cell culture in microfluidic systems, and the choice of the appropriate method depends on the type of cell and the purpose of the study. But in general, in all techniques, various microenvironmental factors are involved in the successful culturing and cell health and growth in microfluidic systems. This chapter first deals with these factors and then discusses cell culture techniques in microfluidic systems.

4.2 Microenvironmental factors affecting cell culture

In general, most cells of multicellular organisms, form 3D arrangements of cell populations and interact homeostatically with the microenvironment and neighboring cells [15]. Cellular health and function are strongly influenced by biochemical and biomechanical features of microenvironment [16]. Therefore, understanding the microenvironmental factors involved in cellular behavior such as cell–extracellular matrix (ECM) interactions, cell–cell communications, nutrients and oxygen supply, pH and temperature of culture medium, shear stress on cells, and material of device helps to control and regulate culture conditions and simulate *in vivo* microenvironment as much as possible.

4.2.1 Cell–ECM interactions

The ECM is a 3D fibrous network of nearly 300 types of protein and carbohydrate molecules in three groups of collagens, glycoproteins, and proteoglycans [17]. As a noncellular component of the tissue microenvironment ECM plays crucial roles in cells' structural and biochemical behaviors including, morphogenesis, adhesion, proliferation, differentiation, maturation, and responses to growth factors dynamically and reciprocally [18,19]. Actually, some ECM molecules activate the extracellular signaling pathway that is detected by cells and affect their behavior. In this regard, ECM acts as a structural support for adherent cells through collagens that are the most abundant proteins of ECM as well as the body. Also, the interaction of ECM with special cell surface receptors such as integrins induces cell adhesion on the ECM [20]. Integrins as transmembrane receptors interact with ligand glycoproteins that are present in ECM including laminin, fibrinogen, fibronectin, and vitronectin. As well, interactions between ECM and integrin activate intracellular signaling cascades that are responsible for expressing genes that involved in regulating cellular functions including survival, proliferation, differentiation, and apoptosis [21]. Besides, glycosaminoglycans (GAGs) such as heparin, keratin, and hyaluronan are long linear polysaccharides that covalently linked to a core protein and constitute proteoglycans, store large amounts of water in their chains, and through binding, affect the availabilities of soluble factors. GAGs also have a role in compressive resistance of ECM [17,22]. Nevertheless, according to the importance of ECM for cells, although many cells secrete their own matrix compounds, in vitro cultures require artificial ECM to provide structural support for cells and mimic the natural microenvironment of in vivo at both conventional and microfluidic-based cultures.

4.2.2 Cell-to-cell communications

Interaction between neighboring cells is one of the crucial features of the cell microenvironment, which not only dynamically affects cellular behavior, but also plays a vital role in tissue and organs generation and functions [23]. Cell–cell communications principally occur both directly through gap junctions, tight junctions, and desmosomes as well indirectly by secreting signaling molecules (mainly cytokines) via receptor-mediated mechanisms. In addition, cell–cell synapses and extracellular vesicles such as exosomes are involved in intercellular interactions [24]. Therefore, it should be considered that the ECM plays an important role in cell–cell communication as well [9].

Including molecules that play a key role in cell–cell adhesion are cadherins. Binding between cadherin molecules through hemophilic interaction creates a strong cell cohesion and compaction between cells [8]. Cell–cell interactions are of great importance in cell proliferation and migration as well as tissue configuration and homeostasis [25], and it is necessary to design cell culture platforms that can mimic cell–cell communication similar to *in vivo* conditions especially in coculturing applications, spheroid, organoid, and microtissue formation purposes.

4.2.3 Nutrients and oxygen supply

Cells need nutrients and oxygen for survival and cellular functions. In cell culture platforms *in vitro*, suppling of nutrients is performed by a medium containing glucose, some amino acids and vitamins, inorganic salts, and a serum that contains attachment and growth factors as well as hormones [2]. Besides, to reduce contaminations like the gram-positive and gram-negative organisms, a mixture of antibiotics including penicillin-streptomycin is added to the culture medium. Depending on the different needs of various cell types and culture platforms, other essential compounds can be added to the culture medium. In traditional culture techniques, it is necessary to change the culture media in a drop-by-drop way through hand-operated pipetting to supply nutrients and remove waste products [26]. However, in microfluidic platforms, culture medium and oxygen are injected into the microchannels through an inlet port of the device. Reciprocally, there is an outlet port for ejecting the medium containing the metabolic products. Using unique features of such systems, the medium flow and perfusion of oxygen can be accurately optimized like *in vivo* conditions [13]. The critical point in this regard is to prevent the creation of bubbles during the injection of the culture medium into the device. Cells become dehydrated and die if exposed for a long time to air bubbles. Also, it should be noted that in microfluidic systems, the culture medium may be consumed more rapidly compared to conventional methods due to the small volume and also significantly higher surface-to-volume ratio. These parameters change cell proliferation and subsequently increase the concentration of produced metabolites by the cells [27,28]. In addition, the consumption and availability of medium by cells depend on cellular own parameters such as type, size, and population [29]. On the other hand, physical features of the culture medium fluid such as flow type, rate, and viscosity have significant effects on cell viability especially in long-term cultures using a perfusion flow system for renewing of medium [27]. Therefore, it is necessary to take

considerations in the design of devices, in terms of material and geometry. With respect to this, besides advances in improving the culture medium in terms of nutrients and reduction of contamination from bacteria, yeast, and mold, many efforts have been made to develop the appropriate materials of the culture substrates, as they mainly have a significant role in oxygen permeability required for cells. In addition, environmental variables such as temperature and pH are also important factors in cell culture, which are explained in the following sections [28,29].

4.2.4 pH of medium

pH is one of the main influential variables of the cell culture medium. It is known that as the structure and function of most proteins are dependent on pH levels, it plays an important role in multiple cellular processes such as cell growth, proliferation, and metabolism [30,31]. The optimum pH for most cells is close to 7.4, which may vary slightly in some cell lines [32]. In a culture medium, pH tends to acidify due to the processes of cellular metabolism and the production of carbon dioxide and lactate by cells [33]. Therefore, to maintain the stability of the culture medium, it is necessary to adjust the pH to the physiological range (~ 7.3 – 7.4) [34]. Virtually pH regulation in culture media is achieved by a natural buffering system and/or exogenous chemical buffers. The natural buffering system is based on the combination of acidic gaseous CO_2 with basic HCO_3^- . To provide CO_2 gas in these systems, an incubator with the ability to maintain the air atmosphere containing 5%–10% CO_2 is utilized. The supply of bicarbonate is also obtained by adding the right amount of HCO_3^- salt (for example NaHCO_3) to the culture medium. A chemical buffering system uses zwitterions such as HEPES [1], PIPES, and MES, which cover a wide pH range without the need for a controlled gaseous atmosphere [33,35]. It is better to note that the natural buffering system is nontoxic and cost-effective while chemical buffering aids despite superior buffering capacities are relatively expensive and for some cell lines are harmful at high concentrations due to producing toxic compounds like hydrogen peroxide under visible light [2,35]. In microfluidic systems, due to the very small amount of culture medium, the effect of buffering processes and pH adjustment of the medium is more important. In conventional systems, the color change of the phenol red indicator in culture media or pH meters equipped with ion-selective glass electrodes are commonly used to determine the pH changes. However, due to dimensional limitations in microfluidic devices, the use of conventional methods for direct pH

measuring is difficult. Alternative methods in these systems include the embedding of pH microelectrodes or sensors in the microfluidic device [32]. For instance, electrochemical sensors such as electrolyte–insulator–semiconductor or feedback-based ion-sensitive field-effect transistors sensors are used for direct and real-time monitoring of pH. Furthermore, optical sensors that are based on the detection of optical properties in pH indicators have received more attention. These optical properties include fluorescent intensity or light absorption of indicators during environmental pH changes. Optical sensors are less susceptible to ions and other compounds in the culture medium than electrochemical sensors. Also, the flow rate generally does not affect the performance of these sensors [36]. In this regard, the indicator can be common phenol red or pH-sensitive fluorescent dyes that are added to culture media [32]. It should be noted that for cell culture applications the sensors should be biocompatible, nondestructive, and sufficiently sensitive in such scale [37]. Moreover, pH-sensitive hydrogels have been used to generate and control microvalves in microfluidic microchannels. In such systems, by changing the pH of the flow, the hydrogel swells or collapses, causing microvalves opening or closing. Therefore, these pH-sensitive hydrogels can be useful for controlling the pH of the environment [38].

4.2.5 Temperature of culture medium

One of the microenvironmental factors that are important in cell viability and function is the temperature of the culture medium. Temperature affects the chemical reactivity of compounds, as well as the kinetic and thermodynamic of reactions. For example, the temperature has a great effect on membrane potential and the efficiency of enzymatic reactions [11]. Cells are sensitive to excessive changes in temperature, so increasing the temperature by affecting cell adhesion may cause cell death whereas lowering the temperature slows down the activity of cells and makes them softer [29,39]. Therefore, it is necessary to adjust the temperature of the culture medium close to the physiological temperature of the body, typically at 37°C. In culture platforms without a portable heating system, significant temperature variations may occur during moving cells for imaging and other analysis purposes, which may damage the cells. Also, in static culture systems, temperature variations are inevitable during replacing the culture medium [39]. Fortunately, in microfluidic culture platforms, the dynamic condition and allowing the use of precise temperature microsensors and high-resolution real-time monitoring reduce these variations. To measure

temperature variations in such systems, various types of thermometers that are in direct contact with the culture medium, such as resistance temperature detectors, microscale thin-film thermocouples, resonant thermal sensors, and bimaterial microcantilever thermometers or remote control thermometers like infrared thermography and luminescent thermometry are utilized [11]. Also, there are several methods for controlling the temperature in microfluidic systems. For example, in systems where the heat source is located outside the device, fluid is preheated to control the temperature. Furthermore, a printed wiring board can be used as a heating system under the microfluidic device [40].

4.2.6 Shear stress

Shear stress imposed on the cell surface due to laminar flow is a mechanical factor affecting cell function and behavior in microfluidic platforms. Cellular differentiation, proliferation, growth kinetics, gene expression, and many other functions are influenced by shear stress [29,41]. In microfluidic devices, the fluid flow rate and viscosity as well as the geometric structure of microchannels including length, width, and height are involved in the shear stress levels applied to the cells [7]. Indeed, at smaller scales of the channels, the ratio of surface forces to volume is predominant which increases the shear stress. High shear stresses, even if they do not induce cell death, will result in transformations in shape, growth, cell interactions, and nutrient consumption that will invalidate the data obtained from the experiments [27]. By all means, the maximum amount of tolerable shear stress varies in different cell types. In vivo conditions, cells that are in contact with body fluids are inevitably exposed to shear stress. On the other hand, although the shear force is generally considered a stress for cells, it is necessary in some cases, for example, maintaining endothelial cell functions, which are constantly under blood shear stress. Therefore, employing microfluidic microchannels to mimic the vascular system and monitor the shear stress variations on cultured endothelial cells has been useful. For intense, it has been proven that the reduction of shear stress on human umbilical vein endothelial cells in a microfluidic platform leads cells to reorganize the actin skeleton and change the phenotype [42]. In addition, investigation of osteoblasts has demonstrated the morphology and cytoskeleton changes as well as responses of calcium signals using a microfluidic culture system [43]. In general, directly quantitative assessment of shear stress on cells is challenging because of the complex response of cells to external stress stimuli, and mostly for estimation of fluid shear stress, particle velocity profile or

energy dissipation rate is measured around the cellular microenvironment. The effect of shear stress on cell physiology is also reported by evaluating changes in cellular behaviors including viability, morphology, gene expression, protein production, and calcium uptake using various tools such as fluorescent cell sensors [44].

4.3 Microfluidic systems for culture based on substrate material

The material of the substrate used in the manufacture of microfluidic devices can affect the fate of cultured cells. The characteristics of the substrate materials used in microfluidic devices such as flexibility and optical properties are factors that determine the geometry of the device as well as the method required for the fabrication of the microfluidic platforms [45]. In addition, physical factors such as topography, roughness, stiffness, and water permeability significantly affect cellular behaviors. For instance, smooth materials improve epithelial cells' growth, attachment, and spreading, but for osteoblast cells, rough-surface materials show better results [46]. Oxygen permeable materials are also preferred because of reducing hypoxia, especially in multicellular cultures [12]. Moreover, the hydrophilicity of the substrate material is a key parameter that has an impact on the uniform growth and proliferation of cells as well as inhibits the attachment of cells on the surface [7]. Biocompatibility and nontoxicity of the material are other crucial parameters for cell vitality likewise. In this regard, early in development, microfluidic devices were made of nonpolymeric materials such as glass or silicon, but then polymer devices came into focus and grew rapidly. Various polymers have been used in this field but polydimethylsiloxane (PDMS) has received the most attention for culture applications. Paper-based platforms have also been developed in this area [10,29]. Therefore, the microfluidic systems used for cell culture can be divided based on the substrate material, which will be discussed in the following.

4.3.1 Glass-based devices

Glass is one of the first materials in the fabrication of microfluidic culture substrates. The advantage of glass-based systems even over polymers is superior optical properties that make them suitable for high-resolution Raman microscopy, thermal lens microscopy, and other imaging techniques. As the provided electroosmotic flow on glass surfaces is

reproducible and reliable, glass-based systems can be used for a long time and also multiple times. High hardness to prevent deformation of microchannels walls, thermal stability, biological inert nature, and capability of surface modification are other unique properties of glass that make it suitable as a microfluidic culture substrate. However, glass-based microfluidic systems, due to lack of oxygen permeability, lead to hypoxic conditions for cells that are threatening to cells but can also be used in hypoxia studies [13,47]. Approaches used to fabricate glass devices include micro-machining, embossing, laser-based microfabrication techniques, and electrochemical or ultrasonic-assisted methods. However, as achieving 3D microstructures through machining of glass is practically difficult, the general method of creating microchannels in glass devices is photolithography in combination with wet etching by acid solutions such as hydrofluoric acid [45].

4.3.2 Paper-based devices

One of the simple and relatively inexpensive microfluidic platforms is paper-based devices. Paper is made by pressing cellulose micro and nano-fibers that can be considered a mimic of the ECM [46]. So it is an appropriate choice for cell culture applications such as cell differentiation, single-cell monitoring, and disease model mimic due to biocompatible, porous, fibrous, and flexible nature of paper. Plus, depending on the application, properties of paper such as cellulose composition, pore size, dimensions, and surface properties are adjustable [48]. For example, porosities, which also facilitate conditions for cell growth and chemical surface modification, can be tuned to allow controlling of the nutrients and oxygen supply. Moreover, topography and stiffness of paper that affect cellular behaviors through affecting intracellular cytoskeleton formation and cellular adhesion, as well as secretion of ECM, can be manipulated for specific purposes. Also, surface modification of paper, which is necessary for cell adhesion and proliferation, can be performed using biomolecules such as peptides and nucleotides by printing methods, initiator chemical vapor deposition, corona discharge surface, and so on [46]. After all, to manipulate the fluid flow in such microfluidic systems, hydrophobic/hydrophilic interfaces are formed on paper substrates. There are various approaches to create hydrophobic/hydrophilic patterns on paper, including lithography, printing methods, plasma treatment, and cutting methods. Among them, wax printing is the most widely used method, which mainly is based on printing wax on filter or chromatographic papers. In paper-based devices,

single layers of papers can be stacked to create 3D structures named microfluidic paper-based analytical devices that provide a better simulation of *in vivo* conditions. Different papers with unique properties as well as different cells suspension can be used in each layer of these structures to achieve multifunctional functions [13,48]. Also, in particular platforms, paper can be used along with other materials like PDMS or hydrogels to improve immobilization and mechanical support, respectively [46,48]. Despite the advances in the field of paper-based microfluidic devices, the use of this platform for cell culture faces challenges. It is necessary to adopt standardization in culturing protocols for these platforms. In addition, surface modification of paper-based systems is not as simple as conventional plate-based culture methods because of some weak mechanical properties. Also, exposure to the culture medium breaks down the hydrogen bonds of cellulose molecules and consequently reduces the efficiency of paper as a supporting scaffold for cells. On the other hand, optical properties such as auto-fluorescence interference make it difficult to perform related cellular analysis [48]. Finally, in the commercial papers manufacturing process, some additives are used that may be released into the medium over culture time, causing cytotoxicity [46]. Following as mentioned, via chemical modification methods and manipulation of physical properties, these limitations can be removed to some extent, but in general, overcoming some of these limitations requires more studies in the field of paper-based systems.

4.3.3 Polymer-based devices

The most common materials used in microfluidic device fabrication are polymers. Among the various polymers as biocompatible substrates for microfluidic platforms, PDMS is widely used due to its good physical properties. As a thermoplastic polymer, PDMS has multiple advantages for use in microfluidic cell culture platforms. The manufacturing process of the PDMS is easy and cost-effective, and also its flexibility allows the design of various geometries and microarray structures as well as pneumatic micro-valves and microperistaltic pumps. For fabricating PDMS-based microfluidic systems, there are several methods, where soft lithography is the most common one for cell culture purposes. In addition, PDMS has good optical transparency, which makes it very suitable for live-cell real-time monitoring and imaging [10,28]. In addition, a feature that makes the PDMS highly suitable for cell culture applications is its gas permeability. Indeed, in PDMS-based platforms, the controlling of O_2 and CO_2 concentrations can be easily performed, which results in improved cell viability. Despite these

advantages, PDMS porous hydrophobic nature leads to absorb small hydrophobic compounds of culture medium such as lipids. Accordingly, to maintain stability, the culture medium must be replaced regularly. In addition, the permeable nature of PDMS causes rapid evaporation of the culture medium, which is crucial in microfluidic systems due to the small volume of the medium. Evaporation of the medium, while restricting the supply of nutrients to the cells, causes bubbles in the system and subsequent lysis of the cells. Using a humidifier in culture incubators or media reservoirs on microfluidic chips can solve this problem. Moreover, curing at the appropriate temperature is required to obtain fully crosslinked PDMS; otherwise, some noncrosslinked monomers of the polymer may release into the culture medium and interact with the hydrophobic components of the cell membrane, leading to cytotoxicity [27,28]. Actually, PDMS is a two-part fluid of liquid silicon rubber and a curing agent at room temperature with a very low glass transition temperature and needs to cure at appropriate thermal conditions to turn a full-crosslinked polymer [27]. Another way to avoid the monomers releasing into the culture medium and mutually reduce the absorption of small hydrophobic molecules by PDMS is chemical surface modifications. Methods for surface modification of PDMS include plasma oxidation treatment, UV/ozone treatments, coating of surface with polar functionalities, grafting to other polymers such as PEG, and covalent bonding to hydrophilic materials such as glass, to name a few [45,49]. Besides, cell adhesion can be significantly improved by coating the PDMS with ECM proteins such as laminin, fibronectin, and Matrigel. On the other hand, to prevent cell attachment in undesired places of microfluidic devices including valves, PDMS can be treated with compounds such as nonionic copolymers [28]. In addition to PDMS, another common polymer is poly(methyl methacrylate) (PMMA), which is fabricated by soft-lithography technique as well. However, as is the drawback with PDMS as well, this polymer swells exposed in strong solvents and affects fluid flow in microchannels. Alternative chemically resistant polymers in this regard are polytetrafluoroethylene and cyclic olefin copolymer (COC), which are fabricated by the hot-embossing technique [50]. Other polymers for microfluidic devices are PMMA, polycarbonate, polystyrene, polypropylene and polyvinyl chloride, and polyimide, which are handled by injection molding, mechanical micromilling, lithography, photolithography, etc. methods for microfluidic device fabrication [27,45]. Nonetheless, PDMS is still the best option in terms of permeability for cell culture

applications so that it is known for example the oxygen diffusion of PMMA and COC is three orders lower than of PDMS in these materials [29].

4.4 Cell culture techniques in microfluidic systems

There are several categories of cell culture methods in microfluidic systems. Basically, conventional cell culture techniques are divided into two categories of 2D and 3D in terms of the dimensions of formed cellular organizations. Both of these techniques have been used for culture in microfluidic platforms as well (Fig. 4.1). Also, from other points of view, 3D culture methods in microfluidic systems include two groups of matrix-free and matrix-based methods. On the other hand, depending on the type of culture medium flow, these systems are divided into continuous-based and droplet-based microfluidic devices. In the following, each of these techniques will be explained in detail.

4.4.1 Two-dimensional cell culture platforms

Conventional static 2D cell culture platform is the most popular classical method for the cultivation of cells in a monolayer array. This method principally has been used for adherent cell cultivation since almost one century ago in which a flat glass or plastic (commonly polystyrene) surface of culture flask, Petri dish, or multiwell microplate as a mechanical support is used for seeding cells on, aiming to simulate the cell attachment on ECM [51]. For improving cell health and adhesion, the surface of the plate can be

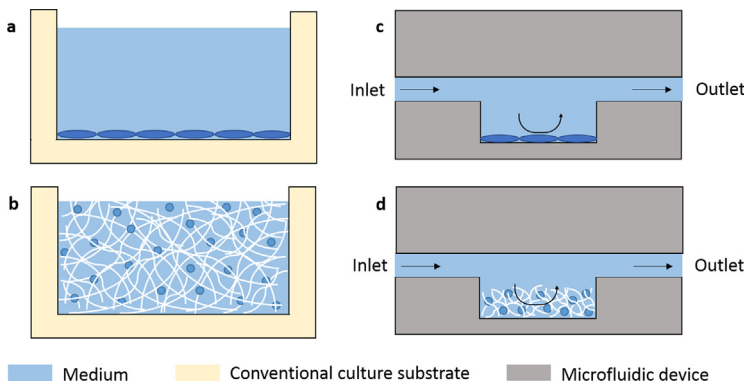


Figure 4.1 Cell culture techniques. (a) Conventional 2D culture, (b) conventional 3D culture (matrix-based), (c) 2D culture in microfluidic device (continuous flow-based platform), and (d) 3D culture in microfluidic device (matrix-based continuous flow-based platform).

easily modified by functionalizing compounds such as special proteins. 2D culture methods allow to assay large quantities of cells, are cost-effective, and have simple protocols, and it is easily possible to observe the cells morphology, viability as well as cellular density and distribution by direct imaging. Also, the sampling out for various analyses is simply done on these platforms [5]. But among these, the most important advantage of 2D culture methods is being universally known, with abundant available literature and publications for scientists to compare the past results [52]. On the way to the development of biotechnology, the conventional 2D cell culture platform in the early 2000s underwent evolution through combination with microfluidics [53]. This development not only resulted in to benefit from high throughput and multitask culture platforms but also the combination of these platforms with biosensors allowed for accurate control and monitoring of culture conditions such as temperature and shear stress [7]. For example, electrochemical impedance sensors as a strong tool are used to monitor some cellular features such as adhesion, proliferation, or differentiation via measuring the electrical impedance of cell layers in 2D culture platforms especially for determining transepithelial resistance in epithelial cell lines [54]. Digital microfluidic methods in the 2D culture have also been developed in recent years for seeding, growth, and detachment of adhesive cells on new surfaces [55]. The potential of microfluidic 2D culture systems has been proven as a powerful tool in the study of cancer progression and tumor metastasis [56]. In miniaturized 2D culture using microfluidics, the medium distributes continuously, and also the toxic products of cell metabolism are removed by the fluid flow in a dynamic manner. Therefore, there is no need to repeatedly replace the culture medium through pipetting for supplying nutrients and removing metabolites such as what is used in the static method. Despite the extensive popularity and usefulness, 2D culture methods have a major limitation due to the inability to accurate reflection of the natural 3D structures of the body components and microenvironments. Indeed, in 2D culture, platforms lacking organized cell–cell and cell–ECM interactions disrupt many vital functions of the cells as migration and differentiation [7]. Also, missing the polarity in 2D changes the cells' various phenomena such as gene expression and splicing, apoptosis, etc. In 2D culture methods, cells are forced to attach to a 2D surface of the substrate with limited binding sites and defined stiffness, which confines the geometry and morphology of the cell as well as limits the surface area of the cell that is exposed to the culture medium. As a result, on one hand, the cell access to the chemical gradient

of ingredients is limited to only a fraction of each cell; on the other hand, the monolayer array of cells gives them unlimited access to ingredients unlike conditions within the body [1,5]. Consequently, the implementation of 2D culture methods in some studies may yield misleading results [7]. Various alternative techniques have been proposed to mitigate some of these limitations. For instance, 2.5D cultivation using the sandwich culture method, which includes adding ECM-like layers on the bottom and top of the monolayer of cells, can improve cell polarization problems. This method is useful for culturing hepatocytes that are not highly viable under 2D culture conditions [16]. Efforts have also been made to improve 2D culture plates. Nevertheless, over the past decades, there has been a growing tendency to design 3D culture platforms in microfluidic instead of 2D formats due to as mentioned limitations.

4.4.2 Three-dimensional cell culture platforms

It has been found that increasing the dimensions from 2D to 3D significantly affects cell physiology. For example, the natural phenotype of chondrocytes for matrix synthesis is lost in 2D culture as opposed to 3D manner [57]. Also, culturing cells in 3D spheroids shows more resistance to anticancer therapeutics compared to 2D platforms, due to differences in proliferative gradients, cell secretions, hypoxia, etc. [58]. There are many examples in the literature related to different cellular responses to various phenomena in 2D versus 3D that are beyond the scope of this discussion. Indeed, the most important advantage of cell culture in 3D over 2D models is that cells can better imitate the natural 3D conditions of the body because cell–ECM and cell–cell interactions more reflect the *in vivo* conditions. Unlike 2D culture, in which cells are forced to polarize due to the attachment on the planar surface through integrins, in 3D platforms the random distribution of integrins causes cell adhesion to the scaffold or matrix in a multiorientation form similar to the *in vivo* conditions [59]. Also, the morphological aspects are closer to the natural forms in the body compared to 2D flat cultures. In 3D structure, cells can aggregate together to form spheroids or organoids in a culture medium or a support matrix. As well, it is possible to mimic tissue-like structures [15]. In general, the superiority of 3D culture over 2D in various aspects including cellular morphology, proliferation, differentiation, migration, survival, phenotype, response to physical stimuli and drugs, gene expression, and protein synthesis has been proven [13]. However, despite all the advantages, 3D culture systems suffer some drawbacks. One of the challenges in using the

conventional 3D culture methods is the limitation in Imaging and the use of microscopic techniques due to the opacity of 3D tissues and cell aggregates. Moreover, in common 3D cell culture, many phenomena such as the dynamic spatiotemporal gradient of nutrients and metabolic products as well as the tissue–tissue interface are associated with challenges [16]. For example, 3D culture systems do not fully mimic vascularization in tumor models [50]. In this regard, the combination of 3D culture with microfluidic technology provides the possibility of high throughput real-time analysis and visualization by providing integrated organ-on-a-chip and body-on-a-chip systems. Microfluidic-assisted 3D culture platforms are superior to traditional formats in many respects, including a complex multicellular microenvironment, appropriate vasculatures, and dynamic medium exchange [7].

4.5 Conventional matrix-free 3D culture methods with microfluidics

Thanks to most cells' ability to secrete their own ECM components and accompany neighboring cells to form 3D aggregations, scaffold-free culture methods have been developed to produce 3D spheroid and organoid microstructures [54]. Due to the medium gradient in spheroid structures, different stages including proliferating, hypoxic, quiescent, and apoptotic occur as a mimic of *in vivo* microenvironment [15]. Cell–cell interactions play a crucial role in the formation of such structures. Scaffold-free methods have been widely used to production of multicellular spheroids and the association of microfluidics with these techniques developed a niche especially in tumor studies for both long-term culture and drug screening in lab-on-a-chip devices [54,60]. In this regard, a number of matrix-free 3D culture methods in conjugation with microfluidics including hanging-drop, liquid overlay, and magnetic levitation have been developed (Fig. 4.2).

4.5.1 Hanging-drop

As a scaffold-free method, hanging drop culture was introduced by Keller in 1995 for embryonic stem cell cultivation [61]. How this method works is that firstly drops of the cell suspension are dropped on the undersurface of the culture substrate and then the substrate is inverted. Due to greater adhesion force between suspension and surface than suspension weight, droplets form and cells settle in the bottom of droplets at the liquid–air interface owing to gravity [60]. Accordingly, cell spheroids are formed

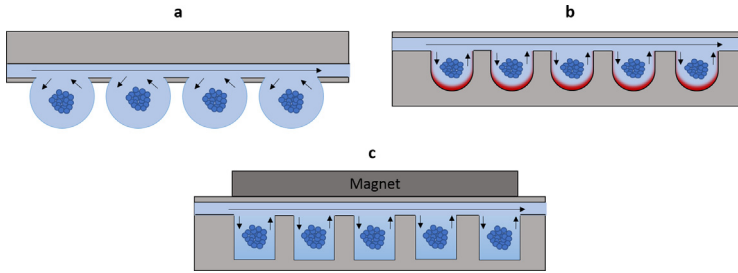


Figure 4.2 Matrix-free conventional 3D cell culture methods in combination with microfluidic technology. (a) Hanging drop, (b) liquid overlay, and (c) magnetic levitation.

because of cell–cell interaction promotion that dominates over cell–substrate interaction [8]. By adjusting the density of the seeded cells, spheroids of the desired size can be obtained. Simplicity of the hanging drop culture platform as well as the high reproducibility rate for producing a 3D spheroid per droplet allows to be used for numerous cell line cultivations [15]. Despite the successes in this area, one limitation of the conventional hanging drop technique is the difficulty of refreshing the medium within droplets [16]. Furthermore, this method is not suitable for long-term uses because of the completion of low volume medium within the droplets through cell consumption [62]. Also, despite the fact that the liquid–air interface facilitates the exchange of gases, on the other hand, it evaporates extensively [8]. Additionally, for larger volumes of droplets (>50 μL), surface tension cannot hold the droplets [13]. Using hanging drop cell culture routes with microfluidics can overcome some of these issues. In the dynamic environment of a microfluidic device, the droplet size can be adjusted by controlling the inflow and outflow. Also in Interconnected hanging droplet arrays, the medium can easily be transferred between droplets [63]. For example, embryoid bodies have been cultured using hanging drop arrays on a chip-based microfluidic device with the ability of medium exchange through continuous flow in droplet arrays and direct high throughput characterization [64]. Moreover, a medium microreservoir can be used in devices to overcome evaporation and maintain the humidity [8]. Apart from that, hanging drop culture has been used in the production of a highly versatile analytical platform for real-time observing of metabolism in human colon cancer microtissue spheroids in combination with microfluidics and multianalyte biosensors. In this regard, interconnected arrays of hanging drop networks were designed in a microfluidic PDMS

chip to culture and monitoring of multiple cell types in 3D spherical conformation. Also, it was used in biosensors implemented in the ceiling of hanging drop substrate based on platinum electrodes embedded in glass plug-ins and functionalized with a hydrogel containing lactate and glucose oxidase enzymes for sensing glucose and lactate changes of cultured cells. Taking advantage of such technology, new insights can be made into organ-on-chip experiments [65]. In addition, the hanging drop technique has been used in finger-actuated microfluidic pumping devices. Using these devices enables to refresh the culture media repeatedly by pushing the buttons without needing any other external equipment. This platform can be an alternative to manual or robotic arms-aided pipetting [66].

4.5.2 Liquid overlay

Liquid overlay culture method was developed by Costăchel et al. and Yuhas et al. in the 1970s [62]. This technique is based on the seeding of cells on a modified surface of the substrate to avoid cell adhesion on the surface and encourage cell–cell spheroidal aggregations. Generally, U-shaped or V-shaped designed wells are used to culturing, and modification of the surface is done by covering it with nonadhesives polymers such as agar, agarose, and poly(2-hydroxyethyl methacrylate) [8]. This method is relatively simple and cost-effective and also very common in tumor studies specially matrix-free multicellular tumor spheroids [60], but its main drawback is the difficulty of controlling the number and size of formed spheroids [15]. Better control over the cell aggregates can be achieved using this method along with microfluidics. For example, drug testing on tumor spheroids (Human hepatoma cells) has been done using liquid overlay culture on a PDMS-based biochip. The surface of the substrate was covered by a thin layer of nonadherent agarose hydrogel. Human hepatoma cells were seeded on the surface of hydrogel and the formation of tumor spheroids was performed for 5 days. Using the electrodes set below the hydrogel, real-time changes in impedance were measured for monitoring the size and number of tumor spheroids in a label-free manner [67].

4.5.3 Magnetic levitation

Another scaffold-free technique for culturing of spheroids or single cells is the magnetic levitation method. This method is based on the levitation of magnetized cells using a nonadhesive surface toward the liquid/air interface during the application of an external magnetic field to boost cell–cell adhesion and forming cell spheroids [18]. Cells in the magnetic levitation

method can be cultured in two routes of positive or negative magnetophoresis based on how the magnetic field is applied. In positive magnetophoresis which cells move toward the region with high magnetic field strength, magnetic agents such as nanoparticles are used for labeling of cells to induce levitation because not possessing magnetic properties inherently [68]. These nanoparticles including iron oxide, gold, and poly-lysine attach to the cell membrane by electrostatic interactions [69]. However, it is known that in the positive magnetophoresis route, due to the drawback of removing the nanoparticles, there is a risk of cytotoxicity and DNA damage [70]. So recently negative magnetophoresis has been further developed as a label-free method in which a paramagnetic salt solution or a ferrofluid-contained medium is used instead of cell labeling. Therefore, due to the low magnetic properties, cells escape from strong magnetic fields to accumulate in lower magnetic field regions [68]. The integration of magnetic and microfluidic methods, known as magnetofluidics, possesses several advantages. In these systems, micro or nanoscale magnetic labels can be used inside microchannels or culture fluid, while applying an external magnetic field with a small magnet. In addition, the magnetic field allows various manipulations on the cells by easily passing through various components of the microfluidic device with different materials of glass, plastic, metal, and liquid media [68]. Labeled or label-free magnetofluidics have been widely used for cell sorting and separating applications. For instance, label-free separation of HeLa and blood cells have been performed using a water-based biocompatible ferrofluid medium in a PDMS device [71]. Therewith, this approach is appropriate for applications that require cell culture under weightlessness conditions while allowing real-time monitoring [72]. Negative magnetophoresis method to simulate weightlessness has been applied for long-term cell culture in levitation microfluidic device using an aqueous solution containing gadolinium as a paramagnetic medium. This strategy allows the weightlessness study on living structures from single cell to coculture clusters in a device as well as the opportunity for real-time imaging [73]. Magnetic levitation technique has been used for multicellular mesenchymal stem cells culture, bronchiole culture, tumor models mimicking, adipose tissue formation, etc. for various research purposes [18,60].

4.6 Matrix-based 3D culture in microfluidics

Matrix-free culture methods, despite advantages in terms of culture and growth of cellular spheroids and organoids in 3D forms, lack the structural

support of the ECM components. Therefore, matrix-based methods in which cells are embedded into the matrix are used to better mimic the cell microenvironment in interaction with the ECM [4]. For this purpose, for the better imitation of ECM compositional and mechanical features like *in vivo*, various compounds, whether as solid scaffolds, hydrogel, or microbeads have been developed that can generally be classified into three categories of synthetic matrices, natural biopolymer matrices, and matrices based on components derived from decellularized tissues. Synthetic scaffolds can be made of bioceramic materials including calcium phosphates or synthetic polymers such as polyethylene glycol and poly(lactic-co-glycolic acid). As well, natural biopolymers for ECM mimicking including gelatin, alginate, cellulose, chitosan, collagen, and hyaluronic acid are derived from different natural species and often made in the form of hydrogels [21,60,74]. In the meantime, the category of decellularized extracellular matrices (dECM) has recently been considered. Decellularization technique involves removing cellular components from native or regenerated tissues to obtain remnant ECM with natural composition and morphology [17]. Various commercial ECMs are available in this area, and the most common is Matrigel for 3D cell cultures. Matrigel is a basement membrane protein mixture including collagen, laminin, perlecan, entactin, expression of matrix metalloproteinases (MMPs), and growth factors derived from tumor [13]. In cell culture applications, the matrix must be able to assist cell growth by transporting gases and nutrients, as well as be adaptable to shear stresses caused by the optimization of structural features. The physical and biological properties of matrices, such as stiffness, porosity, charged moieties, stability, adhesiveness and biocompatibility have great importance in more effectively mimic of ECM conditions. Reciprocally, the matrix applies mechanical forces to the cells, exerts influence on biochemical signals, and through mechanotransduction affects the expression of genes [60]. Therefore, synthetic matrices that lack the essential compounds of ECM for cell adhesion and growth need to be modified with hormones, growth factors, or other biologically molecules such as integrin-binding peptides like arginylglycylaspartic acid (RGD) promote cellular functions. Also, hybrids of two or more polymers such as Matrigel-PEG hydrogel have been used for achieving more improved properties such as stiffness and rigidity [18,60]. Among various materials, natural biopolymers and ECM-derived compounds due to biocompatibility and high susceptibility to be remodeled by cells have been extensively used as support material in culture platforms [60].

4.6.1 Hydrogel matrices

The optimized features of hydrogels including pore size, gradients, fiber thickness, and cell seeding make them outstanding choices for use as 3D cell culture matrices in microfluidic systems. With over 95% water in their structure, hydrogels can provide a cell–liquid interface while exhibiting the properties of solid-like materials [18]. Hydrogels are a network of cross-linked hydrophilic polymer chains so that cells can be seeded within this network through the gelation process and the cell-laden microcapsules can be formed in microfluidic channels [50]. Crosslink formation and gelation of hydrogels are done by chemical stimuli as well as physical processes including radical reactions, temperature, and photon triggers [8]. As cells grow and proliferate, they fill the interstitial space of the hydrogel network and easily migrate toward other cells and form 3D organizations through attachment together. The permeable and porous structure of the hydrogels network allows the cells to easily access the culture medium and oxygen [13]. Hydrogels derived from natural ECM possess good cell binding and mobility properties as well as sustain physiological cell functions but often do not have sufficient mechanical stability. On the other hand, the mechanical properties of synthetic hydrogels are easily controllable, but to create better conditions for cell binding and behavior, they need to be modified with different moieties [9]. It is also possible to obtain hybrid hydrogels by mixing different hydrogel materials with the aim of improving physical and biological properties [18]. Various hydrogels have been used as 3D cell culture matrices in microfluidic devices, some of which are listed in [Table 4.1](#).

4.7 Microfluidic platforms for cell culture based on fluid flow type

4.7.1 Continuous flow-based culture in microfluidics

Continuous flow-based culture in microfluidic systems implies the fabrication of microstructures within fluid microchannels for seeding and culturing of 3D cell aggregations under medium continuous perfusion [82]. In such systems, laminar flow occurs because viscous forces within a fluid are higher than the internal force (Reynold number <3000) [10]. Thanks to constant laminar flow and high-density microfluidic components, culture media perfuse continuously and uniformly like in vivo vascularization within a network of microchannels in a controllable manner [26]. The strength of this method is the easy access of cells to accurate concentration gradients of medium and avoiding the accumulation of metabolites

Table 4.1 Some hydrogel materials as matrix for cell as well as tissue culture in microfluidic devices.

Hydrogel	Microfluidic device material	Cell line/tissue	References
Collagen	PDMS	Primary keratocyte cells	[75]
Gelatin	PMMA	Human colorectal adenocarcinoma cells, human liver cancer cells	[76]
Fibrin	PDMS	Human adipose-derived stem cells, human umbilical vein endothelial cells	[77]
Chitosan	PDMS	Myocardial sections isolated from murine embryo	[78]
Agarose	PDMS	Bovine articular cartilage	[79]
Alginate	PDMS	Mesenchymal stem cells	[80]
Poly(ethylene glycol)	PDMS	Human lung adenocarcinoma epithelial cells	[81]

produced by cells due to continuous change of medium [6]. In these systems, compounds such as hydrogels are used for coating or filling microchannels as a matrix for cells [8]. One of the important factors in continuous flow-based culture is maintaining the constant flow for a long time, which is possible by commercial syringe pumps or gravity-driven and surface-tension-driven flow systems [83]. Using automatic equipment to continuously direct the culture medium in microchannels has also been developed in this field. For example, an auto-perfusion system in spiral microfluidic culture device for medium exchange has been developed to compare with the conventional membrane filtration method. The use of this method for culturing Chinese hamster ovary cells has provided a higher cell concentration, viability, and a more regular morphology than conventional culture methods [84]. Moreover, a PDMS-based perfusion culture device has been developed for continuous long-term culturing and monitoring of the nonadherent yeast cells' growth rate through electrical impedance spectroscopy and high-resolution microscopy methods. This platform also can be useful in nutrition and cell metabolism studies [85].

4.7.2 Droplet-based culture in microfluidics

As an emerging branch of microfluidics, in droplet-based technology, highly uniform slugs or discrete aqueous droplets are generated and

manipulated inside the microfluidic devices using immiscible phases, generally water-in-oil emulsions. Using this technology, cell or cells can be mixed and encapsulated within a well-confined microenvironment of single droplets like a bioreactor. The principle of this method is based on the disruption of interfacial tension between dispersed and continuous liquid phases via applying a microscale force to achieve discontinuous liquid phase droplets. Hydrodynamic force-based strategy is a passive method for droplet generation, which consists of T-junctions or Y-junctions, flow focusing, and coflowing devices [8,10]. In T-junctions devices, the droplets are produced by applying the continuous phase shear force to the dispersed phase via the orthogonal meeting of dispersed and continuous phases flow in an intersection of two microchannels [10,86]. Flow focusing-based devices use compressing of the dispersed phase placed in the middle microchannel by continuous phase within side microchannels to produce droplets. At the end of each microchannel, there is a microsize hole through which the phases are forced to pass. Because of continuous phase shear stress and flow force due to the entry of the dispersed phase into a larger open microchannel, the continuous phase fills the open microchannel and the dispersed phase is broken in the outlet hole. In coflowing devices, a capillary containing the dispersed phase is placed in the center of a larger channel containing the continuous phase, and droplets are produced by viscous shear force from the continuous phase. Beyond that, other strategies based on pneumatic pressure, optical techniques, and electrical methods such as dielectrophoresis (DEP) and electrowetting on dielectric (EWOD) are active methods that use an external actuation source to produce droplets [50]. For example, in the DEP technique, droplets are generated by pulling the droplets from a fluid reservoir. Also, EWOD devices are equipped with electrode planes, which when activated lead to wetting of the microchannels with fluid and forming a finger of fluid between the electrodes. Switching off the electrodes cause to surface of microchannels being hydrophobic and breaking the finger from the reservoir and consequently forming the droplets [87]. In each method, the factors influencing the formation of droplets and their size are different, but in general, the geometry of microchannels, fluid surface tension, viscosity, and velocity as well as the type of flow in terms of being laminar or turbulent are important factors [50]. The immiscible fluid phase in droplet-based techniques generally consists of materials such as mineral oils with biocompatible surfactants. In water-in-oil formats, the outer oil layer act as a selectively permeable barrier for small molecule transportation. Besides, to improve

nutrition supply and gas exchange for encapsulated cells for long-term culturing purposes, water-in-oil-in-water double emulsion formats have also been designed, in which the outer aqueous phase ensures the sufficient supply of nutrients and permeability of gases. On the other hand, amphiphilic biocompatible surfactants are commonly used for the stabilization of the interfaces. Surfactants help to reduce the destructive forces imposed on the droplets and maintain their stability by regulating the viscous shear forces and interfacial tension between the continuous and dispersed phases. Contrary to continuous microfluidic devices, droplet micoreactors are isolated by the liquid–liquid interface from the surrounding fluid, instead of being surrounded by solid walls. Thus, the contact of the trapped cells in the droplets with solid chips, which may be toxic to some kinds of cell lines, is minimized. In addition, the isolated environment of the droplets avoids other possible contaminants [8,50]. Using droplet-based microfluidics for cell culture, a large number of reactions and data acquisition can be performed simultaneously without the need to increase the size of the device while it is possible to independently control each drop. The capability of producing thousands of droplets per second in this method allows the study of cellular characteristics at specific time points as well based on the kinetic behavior of cells [87]. Also, the scalable volume of droplets allows single cells confinement for accurate studies such as single-cell population heterogeneity, transcriptomes, and genome sequencing assessments [88]. In addition, precisely positioned spheroids in an array obtained with the benefit of this method can be used for toxicity tests and high throughput drug screening [8]. As well, encapsulating cells with droplet-based microfluidics allows for more efficient investigation of cell–cell and cell–ECM interactions and various other cellular behaviors in microtissues. Commonly, cell culture in such systems is performed in conjugation with hydrogels or other supporting biomaterials [87]. In this regard, cell-laden ECM microbeads have been developed using droplet-based microfluidic systems (Fig. 4.3) [9].

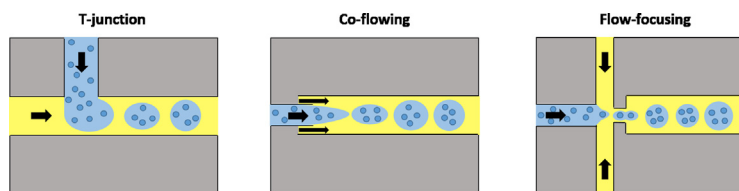


Figure 4.3 Droplet-based microfluidic devices for cell culture.

4.8 Digital microfluidics

As a state-of-the-art technique, digital microfluidics (DMF), which are the modern version of EWOD systems, enable to manipulation of picoliter to microliter-sized discrete liquid droplets on a planar hydrophobic surface of microelectrode arrays through an electric field [88,89]. DMF culture consists of a closed format or two-plate configuration platform in which droplets are sandwiched in the space between two parallel plates. Also, the actuation electrodes mostly are installed on the bottom plate that is coated by an insulating material, and on the top plate, there is a continuous ground electrode that connects to the electrode arrays at the bottom. The top plate is usually made of optically transparent conductive material for imaging purposes. Both plates are covered with a hydrophobic material to minimize friction during droplets' movements. The controlling factor of the droplets' movements in these systems is the conjunction of liquid dielectrophoretic and electrowetting forces. By controlling the electric field via adjusting the voltage, it is possible to manipulate most droplet functions such as metering, merging, splitting, dispensing, and mixing [8,90]. DMF by providing stable medium conditions is a suitable method for cell culture within droplets [55]. In addition, by enabling automation, DMF reduces the need for routine manual workflows [89]. A noteworthy point in this regard is that according to some studies the electric field in DMF has negligible effects on cell growth, proliferation, viability, morphology, and gene expression, which makes that a suitable platform for 2D and 3D cultures [10,58]. However, the high voltage values in DMF can cause loss of dielectric properties of the insulation coating and consequently reduce the lifetime of the chip. Therefore, efforts continue to minimize the effects of the electric field on cells and chips. In this regard, a DMF platform with 3D microstructures on a chip in a semiclosed configuration has been designed for single-cell trapping and culturing for a long time. Aiming to reduce the effects of electrical voltage, the use of gas-soluble silicone oil with low evaporation temperature as well as a fluorinated surfactant reduced the voltage to four times less than the normal value by facilitating the movement of droplets. Evaporation of oil at the physiological temperature of 37°C also allows cells respiration in long-term cultures. As well, drug sensitivity tests using breast cancer and normal model cells have demonstrated the high potential of this system for single-cell studies [91]. Other than this, single-cell-omics deep analysis has been performed with the exploitation of DMF in conjunction with laser-cell lysis and a selection

algorithm based on artificial intelligence. This platform via providing the opportunity to select the desired cells for sequencing, connecting single-cell sequences to phenotypes based on images, and analyze sequencing by multiple omics methods without the need for cell separation enables to collecting multiomics data of single cells for genomics, transcriptomics, and proteomics studies [92]. Moreover, droplet cell culture has been used in combination with the DMF method for cytotoxicity studies on human Jurkat T-cells as a type of suspension cell [93].

4.9 Coculture, organs-on-a-chip, and organoids-on-a-chip

To achieve whole tissue models for various study purposes, coculture of different cells in microfluidic systems has been developed. In coculture setup, two or more different types of cells are grown while they have some degree of contact with each other, aiming to study natural interactions between them like *in vivo* [94]. So, cell–cell and cell–ECM interactions are very important concepts in coculturing. Taking the advantage of coculture setups in microfluidics has led to the emergence of organ-on-a-chip devices. To alternative animal models for preclinical tests, organ-on-a-chip devices with the ability for mimicking complex tissue models have received a lot of attention. These devices simplify bioassays by achieving realistic organ models on a chip. The organ-on-a-chip concept initially was established through coculturing of liver spheroid and neurospheres separately and connecting by a microfluidic circuit [8]. Indeed, as a complicated organ, the liver is responsible for lots of functions in the body especially in drug metabolism, and liver-on-a-chip devices that can maintain metabolic activity and phenotype of the hepatocytes are very useful to study pharmacokinetic parameters. In addition, orally administered drugs are generally absorbed by the intestine and intestine-on-a-chip devices are common in drug screening as well [13,50]. Thanks to heart-on-a-chip systems, the contractility and electrophysiological functions of the heart can be monitored in various conditions *in vitro* [50]. As well, the development of organotypic cancer tissue models, usually made of two or more cell types to mimic complicated processes within tumors such as metastasis capability and tumor angiogenesis, has been a niche in cancer studies [4]. Coculturing of cancer and endothelial cells on microfluidic chips for achieving tumor-on-a-chip models allow to mimic the heterotypic cell–cell interactions and tumor vascularity, for angiogenesis, migration, intravasation, and

extravasation studies [6,8]. Also, in recent years, due to the high outbreak of viral respiratory diseases including COVID-19 and avian influenza virus H5N1, the use of lung-on-a-chip models to better understand the mechanism of these diseases and feasible treatments has found a privileged status [95]. Also, other systems such as vessel-on-a-chip, kidney-on-a-chip, and brain-on-a-chip have been developed via culturing different cell types in microfluidic systems for various preclinical purposes [96]. Apart from this, in recent years, organoid culturing in microfluidic platforms that can simulate features such as organ development, homeostasis, and diseases has gained more attention. Unlike the organ-on-a-chip in which the cells of related organs are cultured, organoids-on-a-chip are generated from stem cells or embryonic stems [8]. Also, induced pluripotent stems (IPs) that are derived from a patient are used in organoid development for personalized treatment models. A fully functional intestine, stomach, and liver can be obtained by differentiating stem cells that express the LGR5 receptor. In addition, it is possible to reprogram the fibroblasts into stem cells using IPS culture approaches to differentiate them to different tissues including blood lineage cells, cardiomyocytes, and neurons [6]. So, organoids-on-a-chip is a promising field for preclinical tests and prediction of clinical outcomes in a high throughput manner.

4.10 Conclusion

The choice and application of appropriate cell culture techniques in microfluidic platforms depend on the goal of each study. Accordingly, platforms should be designed specifically for each cell culture target. But overall, the main purpose of microfluidic culture platforms is to bring phenomena as close as possible to *in vivo* so that cells have the most similar functions to natural conditions. In these platforms, on-chip integrated components allow to easily control over chemical and physical parameters through flowing the fluids containing target compounds in a precise desirable timing. Microfluidic systems also provide a valuable opportunity for high throughput real-time analysis of cellular phenomena. Therefore, it is expected that microfluidic systems as versatile tools can be a suitable alternative to conventional cell culture methods in various applications such as cell trapping, single-cell studies, drug screening, and tissue engineering, as well as organs-on-chip and body-on-chip investigations. However, issues including reproducibility, standardized protocols, and operator training have not yet been resolved in microfluidic platforms. Therefore, despite all

the strengths of cell culture in these systems, more optimizations are needed to achieve completely close results to *in vivo* conditions and practical widespread fulfill, in which the synergy of biotechnology, system biology, and bioinformatics can be beneficial.

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CHAPTER 5

Advances in skin-on-a-chip and skin tissue engineering

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5.1 Background

5.1.1 Anatomy and function of human skin

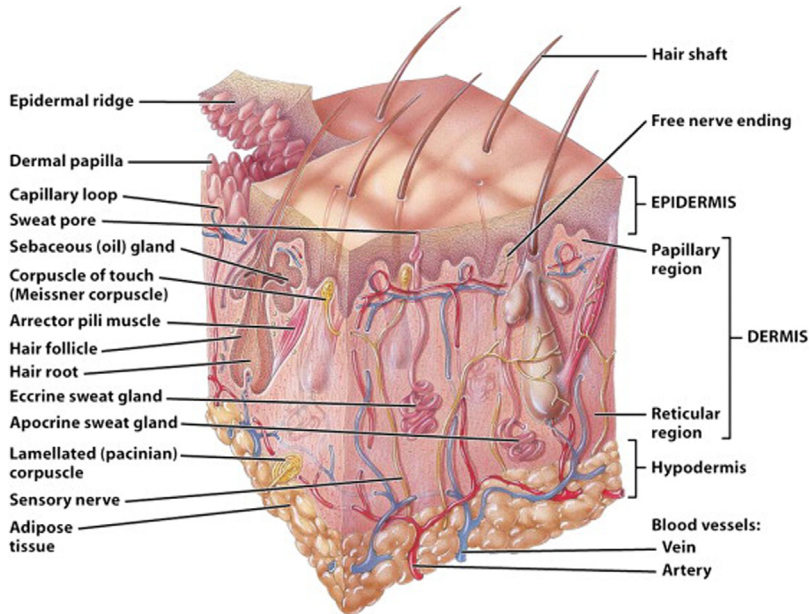
The skin is the largest organ in the human body and acts as an interphase with the external environment. It has numerous functions such as isolation from pathogens and environmental chemicals, water retention, temperature regulation, collection of sensory cues, excretion of waste, and immunologic and endocrine functions. Human skin comprises the outer epidermis (superficial epithelial layer) overlying the dermis (fibrous connective tissue) and hypodermis (adipose connective tissue). The epidermis is composed

primarily of keratinocytes (KCs) but also contains pigment-producing melanocytes (MCs), antigen-presenting cells called Langerhans cells (LCs), and slow-adapting mechanoreceptors called Merkel cells. KCs originate in a stem cell pool in the stratum basale and migrate upwards to form the stratum spinosum, stratum granulosum, stratum lucidum (only on palmo-plantar surfaces), and ultimately the laminated stratum corneum. Between the epidermis and the dermis is a semipermeable barrier called the basement membrane zone. The dermis is divided into a superficial layer called the papillary dermis and a deep layer called the reticular dermis. The dermis is composed primarily of fibroblasts (FBs) that synthesize the extracellular matrix, which provides structure and support to the dermis. The dermis also contains immune cells (mononuclear phagocytes, mast cells, and dermal dendritic cells) and vasculature. The cutaneous vasculature comprises a superficial plexus, found in the reticular dermis, and a deep plexus, found in the follicles and glands, where modified smooth muscle cells allow for shunting from arterioles to venules called glomus cells. Within the skin are several adnexal structures including eccrine sweat glands, apocrine sweat glands, sebaceous glands, and hair follicles. The cutaneous sensory nerves comprise free nerve endings that sense itch and pain (A- δ and C fibers) and specialized nerve receptors found in different parts of the body that sense pressure (Meissner's corpuscle, Ruffini corpuscle, Pacinian corpuscle, Merkel nerve endings, and Krause end bulbs). Underneath the dermis lies the hypodermis, composed of adipocytes sustained by a network of structural fibers that is responsible for thermoregulation, energy storage, and participates in endocrine metabolism (Fig. 5.1).

5.1.2 Current models to study skin

Animal models. Various animal models have been used in dermatological research to study percutaneous drug delivery, skin inflammation, wound healing, and many skin diseases, such as alopecia areata, atopic dermatitis, and psoriasis among others [2]. The closer these animal models resemble humans, the more the outcome of the study will be applicable to humans and clinical use. Depending on the type of study, different animals could be more relevant than others. Additionally, these animals are often humanized; human cells or genetic material are introduced into the animal to better resemble human physiology.

Small animals including mouse, rat, and rabbit have traditionally been used in skin research, as they are easy to breed and maintain in animal facilities. However, their skin is thinner with higher permeability than



Sectional view of skin and subcutaneous layer

Figure 5.1 Anatomy of human skin. The skin is composed of three layers: epidermis, dermis, and hypodermis. Subcutaneous arteries running in the hypodermis branch toward the surface of the skin to generate a deep vascular plexus in the base of the reticular dermis and a superficial (arteriolar) plexus in the papillary dermis, from here capillary loops extend toward the dermo-epidermal juncture. A variety of skin appendages such as hair follicles, sweat glands, and sensory appendages populate the layers. (From Gantwerker, E.A. & Hom, D.B. [1].)

human skin. The skin of larger animals, such as pigs and monkeys, is anatomically and biologically closer to human skin, but these animals pose different challenges in terms of maintenance costs, size and location of animal facilities, and length of breeding.

The epidermis of large mammals, such as humans, pigs, and primates, is thicker, while small mammals, such as mice and rabbits, have just a few layers of KCs. There is also a difference in the epidermal pigmentation between humans and other mammals in general, due to the number, distribution, and organization of MCs, their interaction with surrounding cells, and the interspecies variation of eumelanin and pheomelanin (Mills & Patterson, 2009).

Small animals usually have loose skin while humans and larger mammals have their skin firmly attached to the fascia underneath. Likewise, the dermis of large mammals is much thicker than small mammals. Human and pigs have

a very similar dermis both in terms of thickness (with a dermis to epidermis ratio of 13:1–10:1) and extracellular matrix composition and organization.

The hypodermis is much thicker in large mammals and minimal in small mammals (their dense fur assures thermal homeostasis) making the latter an unsuitable model for subcutaneous and intradermic injections. Small mammals also have a muscular layer (panniculus carnosus) within the hypodermis that allows their skin to heal mostly by contraction, while large mammals (which often lack a functional panniculus carnosus) heal prevalently by reepithelization and scarring [3]. A different composition of skin appendages and immune cells further differentiate humans and other mammals. The murine immune system is well characterized, so mice are commonly used for immunological studies. However, the development, composition, and activation of their immune cells partially differ from humans, diminishing the relevance of these studies. In humans, T cells are mainly of the subtype, while mice present mainly with T cells. Pigs also present T cells, while they have no subtype. In humans, the interstitial dendritic cells populating the dermis are CD1a⁺ and CD14⁺, which are absent in mice. In pigs CD14⁺, CD135⁺, CADM1⁺, CD4⁺, CD172a⁺ and CD209⁺ immune cells appear to be conserved [4]. Consequently, these differences limit the relevance of immunological studies in animal models.

Regarding skin appendages, many mammals lack eccrine sweat glands (such as pigs), or both eccrine and apocrine (such as mice and rats—paws excluded) and have different sensory appendages compared to humans. Moreover, many small mammals, such as mice and rats, have a patchy hair cycle that influences skin thickness locally, while in humans skin thickness is not influenced by the hair cycle.

Human skin models. Ex vivo human skin from human subjects is difficult to obtain in sufficient quantities and the use of human cadaver skin carries legal and ethical concerns. Due to these challenges, the generation and use of in vitro models of human skin have been a major focus in the field for many years. The simplest of these models are 2D cultures of single skin cell types, such as KCs, FBs (e.g., scratch assay) [5], MCs, and co-cultures of these cells in various combinations. Although 2D cultures of these cells can be useful for performing basic assays, for example, cell toxicity, they do not recapitulate the physiological 3D microenvironment and specialized skin niches, which have significant influences on cell behavior and biology. Therefore, there is a strong need for physiologically relevant human skin models, for example, tissue-engineered skin and skin-on-a-chip platforms.

5.2 Tissue engineering of human skin

In 2013, the European Union banned animal testing for beauty products [6]. Consequently, companies, such as L’Oreal, started using bioengineered human skin constructs (HSCs) to test their products before releasing them on the market. Although useful to an extent, these skin models currently lack the ability to accurately mimic the response of human subjects to the chemical compounds tested, due to their relative anatomical simplicity. We and others have demonstrated that despite this limitation, skin chips may offer a suitable tool to evaluate transcutaneous/transdermal permeability, and the diffusion to and from the systemic circulation of cosmetics, and of both topical and systemic drugs (pharmacokinetic studies) [7–11]. The 3D environment was also proven to be more physiologically relevant than conventional 2D cultures to assess the direct cytotoxicity of chemical compounds [11,12].

Furthermore, HSCs and skin chips are currently employed to recapitulate normal physiology [13,14], to mimic biological processes, such as inflammation, immune response, wound healing, and aging, to model complex diseases, such as psoriasis, and to test permeability, distribution, efficacy, and toxicity of drugs and cosmetics. The use of skin chips allows for the active perfusion of culture medium across HSCs. This is imperative for many reasons; for example, it replenishes nutrients and removes toxins from the cell surroundings, increases the endothelial and epithelial barrier function, improves cell survival and proliferation, and the cells that normally are present in the blood vessels (e.g., neutrophils) can be incorporated in the model as circulating cells. Additionally, active perfusion through the fluidic system not only has the advantage of creating microphysiological systems, but also allows for these to be equipped with a number of probes and devices for live monitoring and automated adjustment of culture conditions for high-throughput screening.

5.2.1 Simple engineered skin constructs

Currently utilized engineered human skin models comprise an epidermis with KCs in contact with a dermis with FBs, usually cultured in a simple bilayer. Exposure of the epidermis to the air–liquid interface (ALI) allows for differentiation of KCs to form a mature cornified epidermis. Still, several human skin epidermis models that comprise only KCs are available in the market. For example, EpiDerm, SkinEthic, and EpiSkin, which utilize primary human KCs [15]. Full-thickness skin models comprising primary

human KCs and FBs are commercially available as EpiDerm-FT, Phenion-FT, StrataTest, and AST-2000 [16]. These models allow for the important cross-talk between KCs and FBs. In addition to primary human KCs, foreskin KCs immortalized with high-risk human papilloma virus (HPV) [17] and hTERT [18], a.k.a. HaCaT human keratinocyte cell line (probably the most frequently used KCs for in vitro models) [19], and H9 human embryonic stem cell-derived KCs [20] have been used. These KCs cell lines have the advantage of overcoming the short life span that characterizes primary human KCs, but the modified biology makes them less than an ideal model to recapitulate human skin physiology. In fact, these cells resemble more of a cancer phenotype than healthy tissue [21–23] and their use in bioengineered models of human skin should be limited.

5.2.2 Advanced engineered skin constructs

As skin comprises numerous other cell types than dermal FBs and epidermal KCs, models that add other cells native to human skin aim to mimic skin physiology with greater fidelity and thus broaden the utility of skin-on-a-chip models. Engineering more physiologically relevant skin requires the use of advanced technologies and new cell culture methodologies to add more cell types while recreating the complex architecture that these native cells are found to be in native skin. With more complex skin models, more intricate interactions can be modeled in skin-on-a-chip devices. We will briefly describe several cell types that can be integrated into HSCs to enhance the physiological relevance of the models, an extended review on this topic can be found elsewhere [24].

Endothelial cells. A major challenge in tissue engineering is maintaining an adequate supply of nutrients and oxygen to all cells embedded in a 3D tissue scaffold. Vascularization is integral for ensuring the proper long-lasting structure and function of tissue constructs in clinical applications and the modeling of healthy tissues in vitro. Vascularized HSCs display better epidermal and dermal maturation than their nonvascularized counterparts after being grafted onto immunodeficient mice, and preexisting vascularization benefits in vivo revascularization as well [25,26]. Additionally, the presence of perfusable vasculature in engineered human skin models is a priority for these models to be used in pharmacologic studies monitoring systemic drug absorption to or from the skin. Perfusable vasculature can be connected to the microfluidic system of a skin-on-a-chip model allowing studies involving both epidermal and endothelial barrier function to be performed in conjunction, increasingly recapitulating normal skin physiology

[25,27–32]. In light of the molecular and phenotypical differences observed in the endothelial cells of different organs [33], it is important to select the appropriate EC source to generate different tissues [26].

Melanocytes. The addition of MCs to human skin models is a necessity for the creation of increasingly realistic skin constructs and further enhancing their clinical applications. MCs secrete and arrange the pigment of the skin and the hair (eumelanin and pheomelanin), which not only plays a role in UV protection but also has a significant role in its cosmetic appearance. Hyper- and hypo-pigmentation are potential adverse effects of drug treatments. Consequently, pigmented models have great utility in high-throughput pharmacologic screening studies. MCs are also the causative cell type in melanoma, a skin cancer with high systemic metastatic potential resulting in very high mortality, which further highlights the relevance of incorporating these cell types in human skin-on-a-chip models for in vitro studies. Furthermore, one of the central challenges in the use of engineered human skin models in tissue replacement therapy for the repair of wounds is hypopigmentation and discordant skin tone compared to the surrounding recipient skin [34]. Many pigmented skin models have been published, since the first model in 1986 [35–47].

Adipose tissue and hypodermis. While the main focus of the study of skin biology typically falls on the epidermis and dermis, skin function is entangled with that of the adipose tissue directly underneath it—referred to as the hypodermis or subcutaneous tissue. The hypodermis plays many important functions such as thermoregulation, mechanical protection, energy storage, and endocrine signaling. In the context of skin, hair bulbs, vasculature, and innervation to the skin, all have some component that resides in or passes through the hypodermis before reaching its final destination. Consequently, creating models that include this tissue with its overlying layers of the skin may be necessary to recapitulate normal skin physiology and further elucidate the role of adipocytes in skin physiology [48–53].

Skin appendages: hair follicles, sweat glands, mechanoreceptors. Skin has numerous specialized structures often referred to as appendages. These appendages have a highly complex cellular organization that facilitates various roles that skin plays in maintaining homeostasis. Hair follicles (HF), sebaceous glands, and sweat glands are all important in maintaining a healthy protective barrier by lubricating the skin and engaging in thermoregulation [24]. As a result of their important function, the lack of skin appendages in engineered skin constructs presents significant limitations to

their use both in *in vitro* models and in clinical applications. Inclusion of appendages like the pilosebaceous unit or eccrine glands in engineered constructs would offer advantages in drug screening, and allow for the study of stem cell dynamics, morphogenesis, lineage commitment, tissue regeneration, and wound healing.

Hair follicles are of special interest because the pilosebaceous unit houses its own regionalized stem cell niches [24,54]. These follicular stem cells have been implicated in wound healing and reconstitution of the epidermis after injury. As such, the HF may represent an important compartment of the skin in regenerative medicine [55]. Furthermore, there is a large clinical need for treatments for highly prevalent conditions like androgenetic alopecia and chemotherapy-induced alopecia. *In vitro* models that include appendages like the HF would prove critical in catalyzing studies aimed at finding solutions to these [56–58]. Most successful HF models to date typically rely on animal or chimeric models [59,60].

Eccrine sweat glands development is much less studied than the pilosebaceous unit. Still, eccrine glands are important structures involved in thermoregulation that are not reconstituted after normal wound healing. Consequently, there is a clinical need to utilize tissue engineering to reconstitute these structures for tissue replacement therapies. Most approaches to recreate a sweat gland *in vitro* rely on spontaneous self-assembly of cells in a 3D matrix [61–68].

Immune cells. As the skin has important immunological functions, to create HSCs that truly recapitulate skin physiology, HSCs must contain the resident immune cells found in human skin. The incorporation of immune cells significantly broadens the scope of study that can be undertaken utilizing HSCs. These models can be used to study both healthy skin immune processes and inflammatory processes of infectious, allergic, and other immunologically mediated skin diseases. Of the many immune cells in the human body, there are models integrated with LCs [69–72], monocyte-derived dendritic cells [73], macrophages [74], and T cells [75–81].

Innervation and sensory receptors. Another of the primary roles of the skin is to function as a sensory organ for tactile/mechanical sensation. To function in that capacity, skin has numerous subsets of peripheral nerve fibers that connect skin's sensory receptors (nociceptive, mechanoreceptive, and thermoreceptive) to the spinal cord and to the central nervous system. The role of sensory cells, neurites, and peripheral nerves in the skin is broad. They have been found to also be involved in early and late stages of the inflammatory response in the skin. Neurites influence skin cells via

neuropeptides like substance P, neuropeptide Y, and nerve growth factor, and a model for an interconnected neuro-immuno-cutaneous system that maintains skin homeostasis has gained broader adoption. Consequently, neuronal cells are also thought to play important roles in many skin conditions like psoriasis, atopic dermatitis, vitiligo and herpetic infections [82]. A major barrier for in vitro innervation of skin constructs is the relative scarcity and difficulty in the sourcing of neuronal cells, especially of human origin. Still, rudimentary in vitro innervation of human skin constructs has been achieved in a variety of ways [83–92]

Induced pluripotent stem cells (iPSCs)/patient-tailored therapies and skin organoids. With the advent of iPSC technology, it has become increasingly feasible to generate HSCs from a single patient-derived source. iPSCs have been differentiated into a number of cell types that make up natural skin: KCs, FBs, ECs, MCs, neural cells, and adipose cells. Consequently, one can leverage this technology to make increasingly complex patient-specific human skin constructs for in vitro study of disease and for clinical therapies. For example, CRISPR/Cas9-based targeted genome editing was used on patient-derived iPSC to correct recessive dystrophic epidermolysis bullosa mutations and generate HSCs displaying healthy skin integrity [93]. This represents an exciting clinical application for these technologies. Interrelated with iPSC technology is organoid technology that uses stem cells aggregated into spheroids and relies on stem cell self-assembly to recapitulate complex organ microenvironments. Organoids based on iPSCs have been used to generate full-thickness HSCs containing hair follicles due to these microstructures' inherent complexity. Organoid technology is an exciting alternative to the more prevalent manual forms of stepwise reconstruction of skin architecture in engineered constructs. Still, organoid body differentiation is not fully characterized, not entirely anatomically representative, not reproducible, and is cost-prohibitive. Therefore, the development of standardized protocols, engineering strategies, and technologies that allow for large-scale, high fidelity in vitro skin organoid body differentiation are important considerations moving forward.

5.3 Skin-on-a-chip systems and applications

Definition. Herein, we define skin-on-a-chip broadly as any skin cells coupled with a microfluidic system. We can divide the skin chip systems in literature into two main categories: those designed for the 2D cultures of

skin cell types, and those designed for the culture of 3D HSCs or explanted human skin; each category will be described at the beginning of the corresponding section.

5.3.1 2D cultures of skin cells on a chip

The microfluidic systems for 2D culture are generally shaped after a standard microscope glass slide and made of biocompatible and easy-to-cast polymers, such as polydimethylsiloxane (PDMS) and poly-methylmethacrylate (PMMA), using common soft lithography techniques. These chips are usually a few mm thick, enough to host a number of microfluidic culture channels (Fig. 5.2).

Walker et al. [94] were one of the first to culture primary KCs (KCs) within a microfluidic system. This chip was inspired by an electric circuit and the same physical principles governing electric resistance here were applied to microfluidics. The chip is composed of a series of nine microchannels, 100 μm high, 300 μm wide, and 15 mm long, containing each about 1 μL of medium, perfused through two inlets and embedded between two PDMS layers bonded to a glass slide (Fig. 5.2A). The channels are organized in a way that the flow resistance creates a linear dilution of the perfusion medium bearing a cytotoxic chemical of interest. Walker et al. validated the model by staining human KCs with nine different dilutions of calcein and confirmed the linear gradient of fluorescent intensities. The low fabrication cost and simple configuration make it a useful device for performing high-throughput dose-dependent cytotoxicity assays.

The following year, **O'Neill et al. [95]** characterized the culture of primary KCs in a microfluidic device with constant perfusion. They designed the chip resembling an electrical circuit, with four microfluidic channels with a 300 μm cross-section embedded between a casted PDMS layer and glass slides, bounded through oxygen plasma treatment (Fig. 5.2B). Each channel has a serpentine tract of different lengths that determines a specific resistance to the flow to generate multiple flow rates simultaneously. The channels terminate with a straight tract where KCs are cultured and empty in a common medium reservoir. The flow is activated by a syringe pump. The KCs are cultured on collagen patches. At 72 h of culture, KCs showed a viability between 93% (0.025 $\mu\text{L}/\text{min}$) and 99.6% (0.3 $\mu\text{L}/\text{min}$) that was maintained even at confluency, while the control (cultured in a standard 96-well plate) had a viability of 87% and would not go over 80% confluency before cells started stratifying and dying.

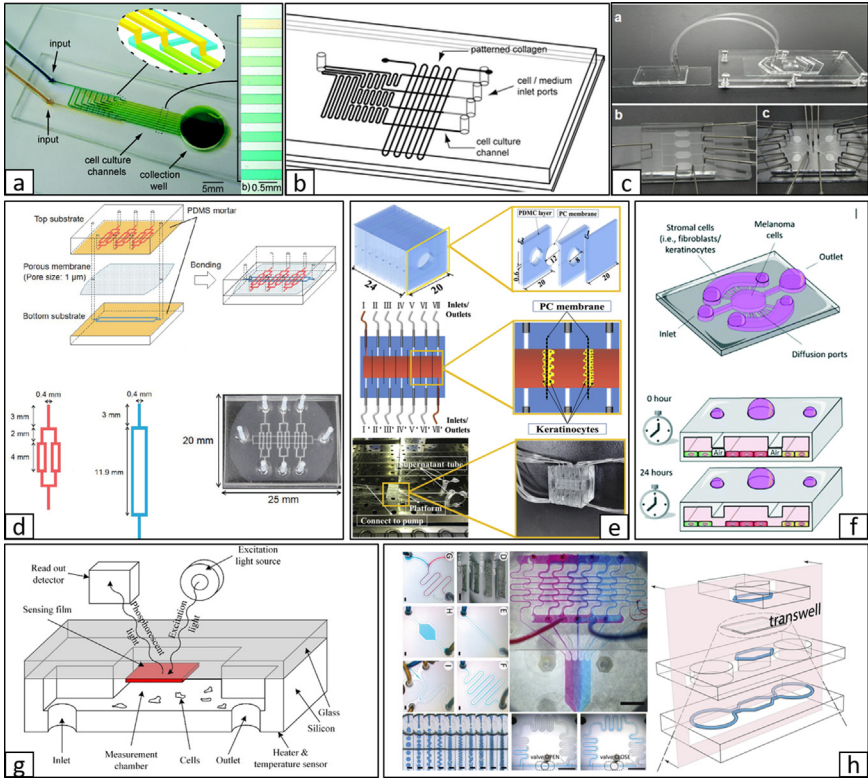


Figure 5.2 Microfluidic chips for 2D culture of skin cells. (A) Multi-channel circuit device for the culture of primary keratinocytes by Walker et al., with nine microchannels generating a range of flow resistance to create a linear dilution of the perfusion medium bearing a chemical of interest [94]. (B) Multi-channel circuit device for the culture of keratinocytes by O'Neill et al., this device is characterized by a patterned collagen coating in the cell culture area of the microchannels, which generate a range of flow resistance thanks to variations in the serpentine tract length of each channel [95]. (C) High-throughput protein microarray device by Zhang et al., a common inlet feeds four culture chambers, which are connected through a system of microchannel and valves to the protein microarray device that is able to analyze four different proteins simultaneously [96]. (D) Microfluidic chip for parallel permeation assays by Sasaki et al., with branched microchannel patterns which generate twentyfour crossing allowing for a high number of experimental replicates [97]. (E) Multilayered microfluidic chip for cytotoxicity assays by Ke et al., bearing seven culture chambers separated by polycarbonate (PC) porous membranes on which keratinocytes are cultured under continuous medium perfusion [98]. (F) Microfluidic chip to study cellular cross-talk through the coculture of melanoma cells, KCs and FBs designed by Ayuso et al., which boasts a central chamber hosting the melanoma cells connected through microchannels to two lateral semilunar chambers hosting KCs and FBs respectively [99]. (G) Microfluidic chip integrated with an oxygen sensor to monitor the metabolism of mammalian cells designed by Bunge et al. [100]. (H) 3D printed microfluidic platform integrated with fluid handling system by Ching et al., which presents microchannels with different geometries and functional properties (such as valves, flow resistors and gradient generators), integrated on a printed-circuit board to generate a fluid electrical interface within the circuit and microporous membrane for the culture of KCs under ALI conditions [101].

Zhang et al. [96] developed a microfluidic chip featuring a high-throughput protein microarray device to assess the cytotoxicity of fine particles, PM_{2.5}, on HaCaT cells at a molecular level. PM_{2.5} are defined as fine particulate matter with a size smaller than 2.5 μm and are dangerous air pollutants as they can bypass the air filtration mechanism of the human airways, reaching the alveoli and causing inflammation. For these experiments, the PM_{2.5} were isolated in Shanghai with a high-throughput air sampler. In this model, the culture chip consists of two layers of PDMS bounded together on a glass slide, containing four microfluidic culture chambers (4 mm long $\times$ 3 mm wide and 25 μm deep) coated with poly-L-lysine, connected through microchannels to a common inlet on one side, and to the protein microarray chip on the other side (Fig. 5.2C). The chip was perfused at a rate of 50 $\mu\text{L/h}$ through a syringe pump. The microarray chip is fabricated with an acrylic bottom layer covered with a 3D polymer layer with aldehyde modification (PolymerSlide G - CapitalBio, China) that binds the protein molecules, and an upper PDMS layer that is cast with the microfluidic circuit. The microarray is able to process four different samples simultaneously thanks to a system of valves that isolates the common inlet (for washing buffers and antibodies) and the specific sample inlet of each channel. After the proteins bind the chip, they can be quantified with fluorescent antibodies; the data obtained with the microarray was confirmed through enzyme-linked immunosorbent assay (ELISA). The HaCaT cells cultured in the chip were treated with PM_{2.5} for 24 h. After the treatment, the concentration of the nuclear factor of kappa B (NF- κB), IL-6, IL-1 β , NALP3, and caspase-1 was analyzed. The PM_{2.5} activated the signal transduction of NF- κB and NALP3 that upregulated the expression of IL-1 β and IL-6, inducing an inflammatory response in the HaCaT cells. NF- κB and IL-6 peaked after 6 h of treatment and then gradually decreased, while NALP3 and caspase-1 peaked after 12 h and decreased subsequently. IL-1 β peaked twice at 6 and 12 h, as it is activated by both the mentioned signal transduction pathways. Given its simplicity and cost-effectiveness, this chip proved to be a useful platform to assess cytotoxicity in skin cells.

Ke et al. [98], from the same group, developed a multilayered microfluidic chip to assess the cytotoxicity of biomass combustion soluble constituents (BCSCs) on HaCaT cells. The chip was fabricated by combining seven PDMS squared modules containing a central circular hole (8 mm diameter) connected with two channels (600 μm diameter), one inlet and one outlet, and two simple lateral modules to seal the microfluidic system (Fig. 5.2E). The modules are separated by six polycarbonate (PC)

porous membranes (3- μm pores) forming seven chambers, on which 12 layers of KCs were cultured under constant medium perfusion at a rate of 10 $\mu\text{L}/\text{min}$ and maintained using a digital syringe pump. The HaCaT cells were treated for 24 h with BCSCs dissolved in the culture medium when they reached 80% confluence. With TEM, Ke et al. demonstrated signs of cellular damage following the exposure to BCSCs: changes in cell shape, mitochondrial deterioration with ridges obscuration, and increased vacuolization of the cytoplasmatic membrane. The viability and the apoptotic rate were greatly influenced by the treatment and confirmed that the level of cytotoxicity was directly proportional to the concentration of the BCSCs. It may not be accurate to call this chip a 3D model, as claimed by the authors, as it consists of a multilayered 2D culture with no KCs stratification. Nevertheless, it is a useful platform to assess the cytotoxicity of BCSCs under microfluidic culture.

The following year, **Sasaki et al.** [97] developed a photolithography-free chip for parallel permeation assays. They used a laser-cutter to fabricate masters for substrates possessing branched microchannel patterns. The upper PDMS substrate contains three microchannel patterns, and each one branches into four microchannels, while the lower substrate contains a single double-branched channel (Fig. 5.2D). A porous polyethylene terephthalate (PET) membrane (1 μm pores) is interposed between the substrates generating 24 crossings of the microchannels, which allows for 24 experimental replicates simultaneously on a single device. HaCaT cells were cultured on the membrane until confluent and subsequently perfused on the apical side with a medium containing an irritant chemical (potassium dichromate) and a fluorescein isothiocyanate (FITC)-labeled dextran as a tracer to assess the epithelial permeability level.

Ching et al. [101] developed a microfluidic platform integrated with fluid handling that was directly 3D-printed instead of being cast on a pre-made master mold. They used a direct ink writing (DIW) 3D printer that extrudes fast-curing flexible silicone resins—such as PMMA—on different substrates to generate microchannels (Fig. 5.2H). The printed substrate is interfaced with another flat substrate to form microchannels of the desired dimension, with the smallest channel reaching the size of 32 μm of width $\times$ 30 μm of height. They were able to generate microchannels of different geometries and with functional properties—valves, flow resistors, gradient generators—with optically clear materials. They were able to print microfluidic systems on unmodified printed-circuit boards to generate a fluid–electrical interface within the circuit, and microporous membranes

for the culture of KCs in ALI conditions. This novel approach paved the way for the fabrication of a new generation of 3D-printed chips with microfeatures comparable in size and definition to the ones fabricated with traditional soft lithography, but exempt from the time and resource-consuming processes that these lithographic methods require.

Agarwal et al. [102] used a simple microfluidic chip to test the cellular reaction to flow-induced shear stress in KCs. Their device is generated by combining a standard coverslip with a molded PDMS layer containing a single straight microfluidic channel measuring $40\text{ mm} \times 1.2\text{ mm} \times 100\text{ }\mu\text{m}$, and coated with gelatin before seeding the HaCaT cells. Here the cells were exposed to a flow rate of 60, 6, and 0.6 mL/h . The authors found that the HaCaT cell could not withstand a stress of 6 dyne/cm^2 and would detach from the channel (unlike other mechanosensitive cells), such as osteoblast and endothelial cells, while when the shear stress was reduced to 0.6 and 0.06 dyne/cm^2 , significant alterations in the gross cellular morphology emerged. The exposure to shear for 10 min would induce the onset of colony reorganization, which persisted for 6 h until a dynamic balance was reached. The phalloidin staining of F-actin filaments revealed that the shear stress induced a cytoskeletal reorganization proportional to the intensity of the stress, with the formation of a dense circumferential actin network, as already described in the literature for other cells. With immunofluorescence (IF), the author demonstrated an upregulation of focal adhesion kinase that triggers downstream signaling transduction cascades, such as Ras/Raf/MEK/Erk1/2 and PI3K/Akt pathways, which further condition cellular adhesion, spreading, and migration; Erk1/2 was also upregulated upon the application of shear stress. E-cadherin and Zonula occludens-1 (ZO-1) were found to be hyper-expressed in direct relation to the intensity of the shear stress, with an increased colocalization at the cell–cell junction. Additionally, the authors observed an upregulation of nuclear lamin that suggests the transduction of the mechanical signaling to the nucleus. Overall, these findings provide useful insights into the mechanosensory responses of human KCs and will be useful to study the organogenesis of the epidermis.

Bunge et al. [100] developed a microfluidic chip integrated with a sensor to monitor the oxygen consumption of mammalian cells as a direct indicator of cell metabolism. The chip is fabricated as a rectangular silicon wafer containing a U-shaped culture chamber (width 2.5 mm , length 12.3 mm , and depth $200\text{ }\mu\text{m}$) connected to an inlet and outlet micro-channel (Fig. 5.2G). Underneath the PDMS layer, a thermometer paired with a heater regulates the temperature. The oxygen sensor consists of an

oxygen-sensitive phosphorescent film made of a polystyrene matrix coated with the sensing dye Pt(II) meso-Tetra(pentafluorophenyl)porphine activated by a led light emitting at 395 nm, and the fluorescence emitted is quantified through a built-in Raspberry Pi camera after passing through a 600 nm filter. The sensing film is located on the top of the cell culture chamber and the accuracy of the sensor is $\pm 0.8\%$ for the relative oxygen concentration of 0%–26% at temperatures between 23°C and 41°C. The holders and housing are 3D printed. As a proof-of-concept, HaCaT cells were cultured in the chip, and oxygen uptake under different temperatures was assessed. As expected, oxygen consumption was directly proportional to the culture temperature.

Ayuso et al. [99] established a microfluidic chip for the coculture of melanoma cells, KCs, and FBs to investigate their cross-talk. They used primary KCs and FBs isolated from the neonatal foreskin. The device consists of two layers of PDMS molded with traditional soft lithography techniques (Fig. 5.2F). A central circular chamber hosts the melanoma cells and is connected to two semilunar lateral chambers hosting KCs and FBs through a series of concentric narrow microchannels. Each chamber has a separate loading port. The connection channels are not treated with oxygen plasma to keep their surface hydrophobic and generate the so-called “air-walls”; this way, when seeding the cells in different chambers, the cell suspension does not reach the connection channels, ensuring the segregation of the cells. With this method, it is not necessary to physically separate the cells with hydrogels or porous membranes. They found that the coculture of these cells resulted in changes in melanoma cell morphology and growth pattern. Changes were discovered also in the chemokine secretion, and multiple secreted factors involved in the tumorigenesis were identified with multiplex analysis of the culture medium. Optical metabolic imaging also highlighted different metabolic characteristics between KCs, FBs, and melanoma cells. In addition, the presence of stromal cells induced metabolic changes in melanoma cells, underscoring the significance of the skin microenvironment on the evolution of the tumor.

5.3.2 3D full-thickness skin-on-chip models

The microfluidic chips for the culture of full-thickness HSCs typically resemble the design of those for 2D skin culture; additionally, they involve a cylindrical culture chamber paved with a porous membrane, normally made of polycarbonate (PC) or PET, and a perfusion chamber underneath connected to microfluidic channels. Fig. 5.3.

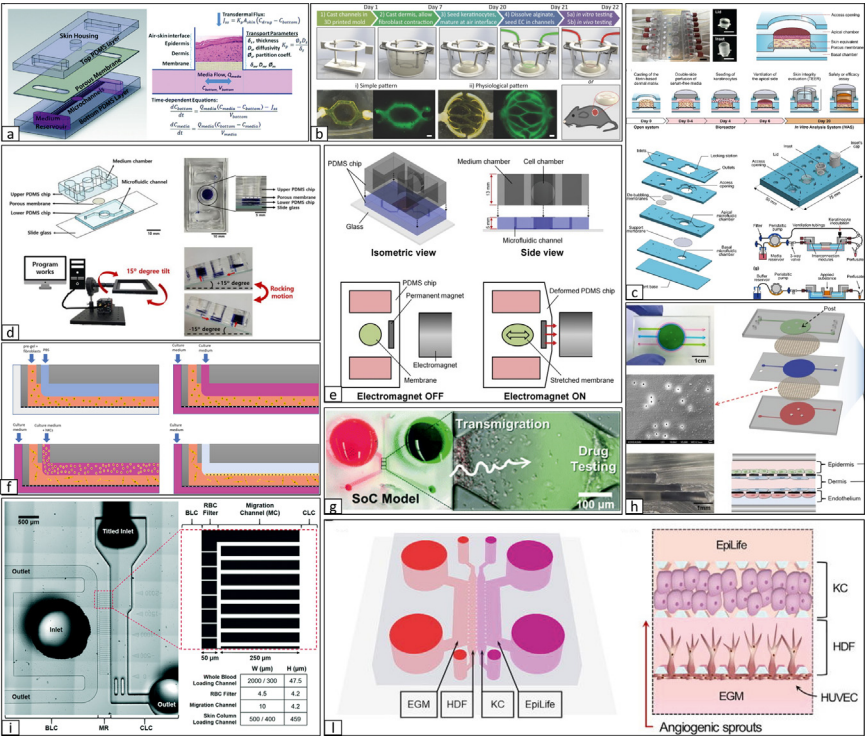


Figure 5.3 Microfluidic chips for 3D culture of skin cells. (A) Microfluidic skin chip for the long-term culture of full thickness HSCs by Abaci et al., boasting a central culture chamber connected by microchannels to two lateral reservoirs that allow for gravity-driven perfusion when the device is located on a rocking platform [7]. (B) The same group generated a device with a perfusable spatially controlled micropatterned vascular network directly integrated into the dermis using a sacrificial alginate layer to cast the channels [103]. (C) Microfluidic chip for the culture of HSCs by Sriram et al., presenting four culture chambers perfused through microfluidic channels connected to a peristaltic pump [14]. (D) Pumpless gravity driven microfluidic skin chip by Kim et al. to assess the effect of α -lipoic acid on HSCs cultured in dynamic conditions [104]. (E) Stretchable skin chip by Lim et al. to model skin aging, presenting a permanent magnet embedded in the chip wall that interact with an external magnet to induce stretching of the chip and of the HSCs hosted inside it [105]. (F) Microfluidic device employing a parallel flow method to generate full-thickness HSCs directly within the device by Valencia et al. [106]. (G) Skin chip for the study of T cell immune responses in the skin environment designed by Ren et al., which allows for the analysis of transendothelial and transepithelial migration and drug testing [107]. (H) Skin chip to model edema and inflammation by Wufuer et al., composed of epidermal, dermal and endothelial layers, separated by fibrin-coated PET porous membranes and cultured under static conditions [108]. (I) Microfluidic chip for the study of neutrophil immune response to *Staphylococcus aureus* designed by Kim et al., which presents a loading chamber for a skin biopsy and a reservoir containing whole blood (the source of neutrophils) connected through four migration channels for neutrophils [109]. (J) Microfluidic chip with gravity-driven perfusion to recapitulate skin inflammation in an angiogenesis model by Jusoh et al., presenting four channels separated by microposts and hosting medium, KCs, FBs within a collagen matrix, and endothelial cells with medium respectively [110].

5.3.2.1 Skin physiology and aging

Abaci et al. [7] in 2015 established one of the first microfluidic skin chips, which was capable of long-term culture of full-thickness HSCs. The chip consists of two rectangular layers of PDMS, generated with conventional soft lithography techniques (Fig. 5.3A). The shape is modeled after a standard microscopy glass slide, which is used as a rigid base for the chip. A polycarbonate membrane with 5 μm pores is located between the two PDMS layers. The layers are bonded together with oxygen plasma treatment. The upper layer hosts the HSC culture chamber, while the lower layer has five microfluidic channels connecting two lateral medium reservoirs. The channels, measuring 150 μm in height and width, are connected at the center of the chip to a circular chamber underneath the porous membrane that supports the skin construct. The porous membrane guarantees the circulation of nutrients between the two reservoirs and lifts the skin construct so that the epidermal layer is exposed to air, generating an ALI culture system that is essential for epidermal differentiation and homeostasis. The flow is gravity-driven with the chip tilted 15 degrees back and forth on its longitudinal axis (parallel to the microchannels) by an automated rocking platform. The microfluidic system is designed taking into account the human skin blood residence times and the flow rate is easily tunable without the need of an external pump, allowing for a physiologically relevant perfusion. Compared to a traditional 6-well transwell culture, this system reduced the culture medium and cells needed by 36 folds and is able to support human skin constructs for at least 3 weeks. They further validated the model assessing the cell viability, differentiation, and epidermal barrier integrity of HSCs, which were found comparable to traditional static cultures. They also investigated the use of this model for drug testing purposes, examining the cytotoxicity of doxorubicin. The same year, in a separate paper, they proposed alternative designs and strategies for the generation of microphysiological systems, including the skin chips, for pharmacokinetic and pharmacodynamic studies [8]. The following year, Abaci et al. proposed a different model [103], where the microfluidic system is directly integrated within the dermis (FBs in a collagen type I matrix) of HSCs. This group generated a perfusable spatially controlled micropatterned vascular network using a sacrificial alginate layer, cast in a 3D-printed mold and embedded in the dermis before the collagen polymerization occurred (Fig. 5.3B). A 3D-printed ring-shaped holder allows to position the network with an offset of 1 mm from the bottom of the HSC and compensates the architectural

alteration derived by dermis remodeling and contractions, which was quantified as 15-fold in thickness and 2-fold in diameter on average. The alginate layer is dissolved by perfusing from the inlet using sodium citrate, leaving perfusable microchannels with a cross-section of 300 μm that further contract to a diameter between 100 and 250 μm (close to the size of arterioles). With the same method, reducing the thickness of the sacrificial layer, was possible to generate microchannels with a diameter $<100 \mu\text{m}$, but the increase in pressure drop caused some degree of leakage. After generating the channels, ECs isolated either from human umbilical vein (HUVECs) or differentiated from induced pluripotent stem cells (iECs) were seeded within the microchannels to generate an endothelial barrier. The permeability of the endothelial barrier was assessed by perfusing the microchannels with a (FITC)-dextran (70 kDa) solution; after 1 h, the HUVECs and iECs vascularized channels showed a similar permeability of 0.045 and 0.018 $\mu\text{m/s}$, respectively, compared to the much higher value of the FB control, 1.6 $\mu\text{m/s}$. The formation and maturation of the epidermis were confirmed with the IF staining of the markers keratin 14 (basal), keratin 10 (suprabasal), and loricrin (cornified envelope). After establishing the model, the vascularized constructs (vHSCs) were grafted onto the back of immunodeficient mice and harvested after 5 and 14 days. On day 14, the vHSCs showed a high level of vascularization compared to controls, with a vascularized area increased by 5–6 folds compared to avascular control. It was also observed that the micropatterned vessels guided the neo-vascularization and the proliferative cells in the basal layer of the epidermis (Ki67^+) increased to 40%, while in the control just 5% of basal KCs were found to be proliferative. Overall, this model not only is a useful platform for pharmacological studies, but also addressed the important problem of the lack of a bioengineered skin graft with perfusable vasculature, potentially improving the outcomes of graft attachment and survival.

Groeber et al. [111] followed another approach to obtain perfusable vessels. They utilized a segment of decellularized porcine jejunum with a vascular component as a biological tissue scaffold onto which FBs, KCs, and ECs were seeded to create an engineered vascularized HSC. They seeded in the preformed vascular network human dermal blood endothelial cells (HDBECs), which attached in the lumen and aligned with the vessels, and were able to efficiently perfuse the regenerated skin. Their system maintained a physiologic perfusion with 120 mmHg of systolic and 80 mmHg of diastolic pressure. The proper formation and maturation of the epidermis were demonstrated with histology and IF. Their bioreactor was also

integrated with the impedance spectroscopy system, able to measure the integrity of the epidermal barrier in a noninvasive way, but the details of its fabrication and characteristics are not disclosed.

Sriram et al. [14] from **Bigliardi** group generated a dermal–epidermal bilayered skin construct within a microfluidic chip using a fibrin-based dermal matrix. The authors postulated that dynamic culture conditions created with a peristaltic pump would improve the structure and functionalities of HSCs, compared to conventional static culture systems. The chip culture system was technically complex, compared to previous models, containing four independent tissue culture and analysis units. Each unit is composed of two fluidic compartments (lower and upper) separated by a permeable porous membrane, with an apical opening to manipulate the sample and perform measurements, such as trans-epithelial electrical resistance (TEER) (Fig. 5.3C). The porous membrane supports the organotypic culture and allows the transport of the culture medium between the two compartments. The lower compartment contains a basal chamber connected to two microchannels—one inlet and one outlet—and is used to perfuse culture medium during normal culture or different liquids during in vitro assays. The upper compartment contains a first chamber 1.5 mm deep on the top of the membrane and a larger apical chamber connected to two microchannels that are used to inject the cell and hydrogel mix during the skin construct generation, to flow culture medium in the apical part of the skin construct (before the ALI culture phase), to ventilate the compartment (after ALI culture phase) and introduce probes or chemicals on the top of the skin construct during in vitro assays. The culture units are generated by assembling five microstructured layers of PMMA with different thicknesses and designs, bearing different porous membranes for tissue support (in polycarbonate—between second and third layers) and debubbling (in PTFE—on the top of the fluidic channels between the third and the fourth layers). After assembling, the five PMMA layers and the membranes are thermally bonded together. The platform is generated with computer numerically controlled (CNC) micromilling. Sriram et al. demonstrated that active perfusion and a precise control of the microenvironment enhanced the quality of epidermal morphogenesis and differentiation, as well as the epidermal barrier function. Thanks to the lack of contraction in fibrin-based dermal matrix they were able to run integrity and permeability testing directly on the chip, overcoming the need to place the samples in specific diffusion chambers for permeability testing.

Lee et al. [58] also developed an analog model where they studied the effect of different culture conditions on HSCs in a microfluidic chip, demonstrating that the survival and differentiation of HSCs depend largely on the culture environment and are different in static and dynamic conditions. The chip consists of three layers of PDMS, with the middle one bearing a permeable membrane at the center to support the skin construct and divide the tissue chamber from the medium chamber underneath. The medium chamber was connected to two reservoirs located at the sides of the chip through two microchannels on each side, and the flow was gravity driven by placing the chip on a custom-made control device. The skin construct was composed of an epidermal and a dermal layer (with FBs embedded in a collagen type I scaffold), and the bottom of the support membrane was seeded with HDBECs. As other groups showed before in different models, Lee demonstrated that cell seeding density, dermal thickness, culture medium volume, and frequency of medium refreshment affect cell viability, matrix contraction, and the strength of dermo-epidermal adhesion. These studies generated useful insights for the optimization of skin culture in microfluidic chip platforms.

The group of **G.Y. Sung** started publishing microfluidic chips models in 2017 [112,113] and in the years 2020–21 validated the model by investigating the antiaging effect of curcumin [114], α -lipoic acid [104], and co-enzyme Q10 [115] and the side effects of sorafenib [116] on a pumpless gravity-driven microfluidic skin chip.^{107–109} They generated HSCs from primary cells, containing a dermal and an epidermal layer, which were hosted in a PDMS microfluidic device. The device presents an 8 mm circular culture chamber separated through a porous membrane (0.4 μm pores) from a perfusion chamber underneath, which is connected to two microfluidic channels (inlet and outlet) measuring 200 μm , in width and 150 μm in height (Fig. 5.3D). The medium circulates between two reservoirs at the two extremities of the chip, and the flow is activated by a rocking platform. With this system, they were able to demonstrate through histology, immunofluorescence, and RNA expression, the dose-dependent enhancement of the epidermal barrier properties of coenzyme Q10, and the antiaging properties of α -lipoic acid and curcuma longa leaf extract on the epidermis. In the skin constructs treated with sorafenib, an antiangiogenesis drug used as anticancer, the authors found changes in the granular layer with thickening of the granules similarly to the alterations described in patients treated with the drug. Due to its simplicity of use, this chip has a good potential for future applications both for cosmetics and pharmacological studies.

The group of **J.H. Sung** published in 2017 a microfluidic chip for the culture of HSCs. They used the same chip design, materials, and fabrication protocol described by G.Y. Sung group previously. **Lee et al.** [117] modeled an HSC composed of dermal FBs embedded in a collagen type I scaffold, with either primary KCs from newborn skin or HaCaT cells. They used *in silico* models (COMSOL Multiphysics) to estimate the diffusion of oxygen and glucose in the chip as an aid to perfection the design of the chip. Using FITC-labelled dextran (MW 250 kDa), they studied the actual mass transport within the collagen hydrogel matrix, and with the live/dead kit (Calcein AM and ethidium homodimer-1, Invitrogen), they assessed the viability of both FBs and KCs, which was maintained after 10 days of culture in ALI conditions. In the following years, **Kwak et al.** [118] used the chip to study the mechanical and transport properties of gelatin methacrylate (GelMA) employed as a scaffold to generate the dermis in HSCs, and its influence on FBs and HaCaT cells. Using live/dead staining and fluorescence microscopy, they found that FBs were negatively influenced by the stiffness of the GelMA, showing an inverse relationship between the GelMA concentration and the elongation and overall number of FBs. Unlike FBs, HaCaT cells seemed to benefit from a higher level of matrix stiffness, with a 2.5-fold increase in adhesion and increased proliferation. **Kwak et al.** [119] recently integrated the model with HUVECs underneath the PC membrane and perfused leukocytes in the microfluidic compartment to recapitulate the immune response in skin tissue. They validated the model assessing the toxicity of doxorubicin with and without HUVECs and found a decreased FB mortality when ECs were present. Additionally, they exposed the model to ultraviolet (UV) radiation and found an increase in the neutrophil infiltration to the skin and in the cytokine secretion in the culture medium.

Lim et al. [105] developed a wrinkled skin chip by uniaxial stretching that is proposed as a model of skin aging suitable to test antiwrinkle cosmetics and medicines. As an excessive physical stimulation is thought to accelerate aging, Lim's group uniaxially stretched their HSCs by 10% in length. The constructs were hosted within a perfused chip culture platform and stretched for 12 h a day at either 0.01 or 0.05 Hz for up to 7 days, to obtain wrinkled skin constructs. One unique feature of this chip is that in correspondence to the culture chamber, on one side, there is a permanent magnet embedded in the PDMS wall that drives the chip stretching through interaction with an external electromagnet (Fig. 5.3E). They found that the wrinkling was proportional to stretching frequency, and the

samples stretched at 0.05-Hz lost the stratum corneum almost completely. Additionally, collagen IV, fibronectin, and keratin 10 expression were downregulated by the high rate stretching, indicating skin degeneration and aging.

The group of **D. Velasco** recently developed a vinyl-based microfluidic device with a novel approach to generate 3D tissues directly within the microfluidic channels and modeled a full-thickness HSC as a proof of concept [106]. They employed a parallel flow method resulting in the deposition of the dermal and epidermal layers in the upper chamber and the perfusion of culture medium in the lower chamber (Fig. 5.3F). The fluidic system consists of two parallel fluidic chambers vertically stacked and separated by a porous PC membrane (with 5 μm pores) that allows for solute diffusion. The chip is obtained by combining multiple layers: the PC membrane is sandwiched between two lower and six upper vinyl layers containing the fluidic system, which are further sandwiched between an upper layer of PDMS and an upper and a lower layer of PMMA for structural support. The novelty of the method resides in the simultaneous injection within the same channel of the hydrogel containing FBs and PBS at specific flow rates, so the gel instead of filling the channel entirely leaves free space (initially filled by PBS) to seed cells or to flow medium or air. The FBs were suspended in a fibrin hydrogel that was injected at a rate of 200 $\mu\text{L}/\text{min}$ after prefilling the chamber with PBS that was kept perfusing at a rate of 100 $\mu\text{L}/\text{min}$. The final thickness of the dermal compartment was 500 μm , the lowest found in the adult human body. After 24 h of perfusion through both upper and lower chamber, primary KCs were seeded perfusing the upper channel with a suspension of 10^7 cells/mL at a rate of 500 $\mu\text{L}/\text{min}$ for 1 min. The cells were left to attach for 6 h before restarting the perfusion of the culture medium. The preliminary data show the possibility of long-term culture, submerged or in ALI, of the tissue generated within the device. The group also published an analog study where they used HaCaT cells instead of primary KCs [120].

5.3.2.2 Pharmacokinetic and pharmacodynamic studies

Alberti et al., from Bigliardi Lab, established a microfluidic platform for in vitro high-precision skin permeability testing to overcome the limitations of the static models based on the Franz diffusion cell [9]. The chip, defined as a microfluidic permeation array (μFPA), contains a series of six independent permeation units and measures 50 $\times$ 75 mm. Each permeation unit contains a cylindrical chamber divided from a polytetrafluoroethylene

(PTFE) membrane in an upper donor chamber and a lower receptor chamber connected with the microfluidic system (300 μm cross-section), a PC inset forming the upper part of the chamber, and a debubbling unit coupled with the inlet channel. The chamber has a permeation area of 0.2 cm^2 , donor volume of up to 300 μL , and receptor volume of 28 μL . The chip is fabricated by combining three micromilled layers of either PC or PMMA, with PTFE membranes interposed between the second and third layer and the upper layer contains an auto-locking system for the inlets. As a proof of concept, the author tested the permeation of caffeine (hydrophilic), salicylic acid (amphipathic), and testosterone (lipophilic), demonstrating that the chip detects the permeant cumulative amounts with higher sensitivity and has similar or lower coefficients of variation compared to traditional methods based on the Franz cell.

In 2019, **Lukács et al.** proposed a skin chip—Microfluidic Diffusion Chamber—to monitor the transdermal delivery of drugs in skin biopsies [121]. Their chip presents a core of two layers of PDMS cast with soft lithography, with the bottom one containing a single microfluidic channel (1.4 mm wide, 1 mm high, and 60 mm long) with inlet and outlet at its extremities and boasting a central 5 mm receptor cell, which is topped either by a membrane or the skin construct. Underneath the chamber, there is an embedded heating foil that keeps the temperature within a prefixed range under the control of Arduino UNO (Arduino, Somerville, MA, USA) via a built-in proportional–integral–derivative controller function. The PDMS core is surrounded by lower and upper PMMA layers with the lower one containing the embedded heating element, and the upper one part of the donor chamber where the skin construct is located. These layers are surrounded by an upper and a lower polylactic acid (PLA) frame, and the upper frame presents in its center the donor chamber, shaped as a truncated cone with a lower diameter of 8 mm, an upper diameter of 18 mm, and a height of 6 mm. The device is therefore composed of three elements: a top donor compartment, a central interface compartment containing the skin construct, and a bottom receptor chamber connected to a syringe-driven microfluidic system that allows for medium perfusion and collection. They tested the skin barrier integrity with a 2% caffeine cream applied locally as a model of a hydrophilic drug. They postulated that further improvements and modifications of the chip will allow its industrial use in drug formulation optimization, dermal pharmacokinetics, and pharmacodynamic studies. The advantage of their chip over traditional methods is its low cost, minimal consumption of explanted skin and drugs,

and a physiologic continuous circulation. The aforementioned Microfluidic Diffusion Chamber chip was further characterized by **Tárnoki-Zách et al.** in a publication from 2021 where they compared the permeability to 2% caffeine cream of a 3D HSC and a skin biopsy [122]. The transport kinetics of caffeine resulted similar in both conditions, and after an initial sharp increase in the concentration, the diffusion of the molecule underwent a long-lasting high concentration steady state.

5.3.2.3 Inflammation and wound healing

In 2016, **Wufuer et al.** generated a model of edema and inflammation with a skin chip composed of three layers—epidermal, dermal, and endothelial—each separated by fibrin-coated PET porous membranes within a PDMS device [108]. Through soft lithography, the authors generated three layers of PDMS containing each a culture chamber and one inlet and one outlet microchannels (Fig. 5.3H). Medium was changed every day using gravity, and the device was not actively perfused with medium. TNF was perfused in the dermal compartment to initiate the inflammatory response, and the levels of several cytokines and endothelial permeability were assessed as a readout. FITC-dextran was used to assess the level of endothelial barrier permeabilization following the inflammation. The model was further validated using dexamethasone to suppress the inflammatory response.

Ramadan et al. in 2016 developed a microfluidic platform that allows for the coculture of a stratified epidermis with HaCaT KCs and a layer of dendritic cells separated by a porous membrane to measure TEER as a proxy for epidermal barrier function in response to chemical and UV stimulation [123]. The fluidic system is enclosed between two central PMMA layers separated by a PET porous membrane (0.4 μm pores) and an upper PDMS layer; the chip is bounded to a PS sheet that confers rigidity to the system. The flow is driven by a syringe pump. The group used a human leukemic monocyte lymphoma cell line (U937) as a model of human dendritic cells. Systems incorporating immune cells into HSCs in a microfluidic system like this are especially relevant for high throughput pharmacologic screening studies, the study of epidermal barrier function, interactions with potential allergens, and skin sensitization in conditions like allergic contact dermatitis.

In 2019, **Jusoh et al.** established a microfluidic chip to recapitulate skin irritation in an angiogenesis model [110]. The PDMS chip is fabricated with traditional soft lithography techniques and contains two channels with a

cross-section of 800 μm separated by three rows of microposts that form two 100 μm wide pseudo-channels containing primary human KCs and primary human dermal FBs within a fibrin matrix (Fig. 5.3J). Each channel is connected on both sides to an individual medium reservoir. HUVECs are seeded at the interface between the pseudo-channel containing FBs and the main channel. The flow is activated by gravity, placing the chip on a rocking platform. The microposts allow for the free diffusion of molecules and the crosstalk between the cultured cells. The channels were perfused with endothelial growth medium (EGM) on the HUVECs side and Epilife medium on the KCs side. The sprouting of the HUVECs in the fibrin matrix was assessed with IF staining for CD31 and collagen IV. The presence of KCs caused an average increase of 12% in the length of the sprouts and of 10% in vessel branching while it reduced the lumen diameter of 10%. Such switch in the angiogenic properties suggests a shift of the HUVECs toward a capillary-like phenotype. The authors further validated the platform testing the influence of the chemical irritants sodium lauryl sulfate (SLS) and stearyltrimonium chloride (SC) on angiogenesis, which is of particular interest given their ubiquitous presence in creams and cosmetics in general. As expected, since inflammatory processes trigger neoangiogenesis, both SLS and SC promoted vessel sprouting in all the tested concentrations (10–60 μM). These results contradict the classification of SC as nonirritating compounds and support the utility of using neo-vascularization as a readout in drug testing.

Ren et al. published a skin chip model with the capability of studying T cell immune responses allowing for quantitative studies of trans-endothelial and transepithelial migration and drug testing [107]. The chip consists of a PDMS layer combined with a glass slide (Fig. 5.3G). Three culture microchannels interface each other through a fenestrated structure. Two lateral channels host HUVECs and immortalized KCs (HaCaT). The central channel is filled with collagen gel to avoid free cell migration between the channels; the collagen also serves as a dermal scaffold and gets degraded by T cells along their migrations. The side channels are connected to two different reservoirs, each filled with culture medium supplemented with different chemicals to generate a gradient and induce chemotaxis in T cells. The chip is not actively perfused with medium. This study found that an engineered CCL20 locked dimer (CCL20LD) is able to inhibit the CCL20-dependent T cell transmigration and support the potential immunotherapeutic use of CCL20LD for treating skin diseases such as psoriasis. They also showed how the transepithelial migration of T cells in

response to a CXCL12 gradient (which physiologically induce the T cell egression from the skin) is significantly reduced by a sphingosine-1-phosphate (S1P) background, suggesting a role for S1P in T cell retention in inflamed skin; additionally, T cell transmigration is promoted by KCs stimulated by inflammatory cytokine. Therefore, this multicellular microenvironment allows quantitative studies of T cell transmigration at a single cell level in response to chemical gradients.

In 2018, **Biglari et al.** [124] proposed a dermal chip mimicking the wound microenvironment and the paracrine signaling in the early inflammation stages of normal wound healing. The chip is articulated in three interconnected microfluidic channels with multiple injection ports. The system contains a monolayer of dermal FBs in the collagen and fibrin-coated side channels, and HUVECs encapsulated in 3D Matrigel in the central channel. The channels were obtained by combining a coverslip with a single PDMS layer in which the channels (20 mm long, 500 μm wide, and 120 μm high) were cast through soft lithography. TNF treatment and triculture with macrophages were alternatively utilized to induce the wound-associated inflammation and to compare their effect on the cytokine secretion. Both methods significantly increased the IL-1 β , IL-6, and IL-8 concentrations in the supernatant, and their action was partially antagonized through dexamethasone supplementation in the culture medium. The presence of macrophages did not only increase the inflammatory cytokine concentrations but also stimulated the development of vascular structures, especially when M2 macrophages were used. The inflammatory cytokines also induced the FBs to differentiate into myofibroblasts, as it normally happens during wound healing, independently by the presence of macrophages.

5.3.2.4 Immune response to infection

J.J. Kim et al. used a skin chip platform to show that the neutrophil response to *Staphylococcus aureus* skin infections is directly proportional to bacterial load [109]. Their device contains biopsied skin microcolumns obtained with minimal invasive sampling techniques and neutrophils from a singular peripheral blood drop. Both were incorporated in a PDMS chip fabricated with classical soft lithography. The chip boasts three main components: a column loading channel (CLC), a blood loading channel (BLC), and a migration region (MR) (Fig. 5.3I). After harvesting, the skin microcolumns are transferred on the top of the device with a random orientation, in the proximity of the inlet of the CLC, and negative pressure

is applied on the other extremity of the channel so that the microcolumns get trapped within the channel. Neutrophils are studied directly in the whole blood, so they are not stressed by the isolation procedures. *Staphylococcus aureus* is added to the skin columns to induce neutrophils activation and migration. The bacterial load to integrate into the CLC is measured through optical density assessment. The MR is composed of 39 red blood cells filters, 40 migration channels, and 39 red blood cells filters with a cross-section of $4.5 \times 4.2 \mu\text{m}$, big enough to allow neutrophils diapedesis but also able to stop bigger leukocytes. The design of the chip makes it possible to quantify precisely the neutrophil migrating from the blood to the skin column. Even though there was a significant interdonor variability in terms of neutrophil response to skin infection, this study demonstrated that neutrophil response correlates with bacterial load, a preincubation step increases the number of migrating neutrophils even with low bacterial loads and an antibiotic treatment in the CLC reduces the migratory activity.

5.3.3 Multiorgan chips integrating skin

In the past decade, in the laboratories of Uwe Marx at TissUse (Berlin, Germany), Donald Ingber at Harvard University (Boston, USA), and Gordana Vunjak-Novakovic at Columbia University (New York, USA), skin constructs have been cocultured with other types of bioengineered tissues to generate integrated platforms capable of elucidating the crosstalk between different organs and recapitulate physiologic processes related to drug distribution and metabolism.

The first multiorgan chip reported in the literature was established by **I. Wagner et al. in Marx lab** [125]. They cocultured liver organoids with skin biopsies in a 96-well format. Their PDMS platform was fabricated using soft lithography and contained two tissue culture chambers (2 mm high) connected by a microfluidic system (100 μm high) perfused through an on-chip peristaltic micropump that generates a pulsatile flow with a near-physiological rate. The base of the PDMS device is bounded to a standard glass slide through low-pressure plasma oxidation. Crosstalk between skin and liver was observed during 14 days of coculture when tissues were exposed to fluid flow. Liver organoids also showed sensitivity to different concentrations of troglitazone—a toxin—during a 6-day exposure. This group provided a promising new tool for systemic assessment of drug pharmacokinetics and pharmacodynamics. In a separate study, **I. Maschmeyer et al.** improved on the skin biopsy and liver organoid model by adding kidney proximal tubule cells and intestine organoids (separately

[126] and all four combined [127]). The chip hosting four tissues has four culture chambers and an additional microfluidic circuit with two extra reservoirs for the drainage of fluid excreted by the kidney epithelial cell layer. The platform reached homeostasis within 4 days from the beginning of culture. The group proposed this model as a platform to test absorption, distribution, metabolism, and excretion of drugs. Later, **Hübner et al.** adapted this chip for the coculture of human H292 lung cancer microtissues and HSCs [128]. They tested a repeated administration of the anti-EGFR antibody, cetuximab, to obtain both toxicity and efficacy data. This drug affected both lung cancer cells and healthy basal skin KCs, inhibiting their proliferation and inducing apoptosis. Therefore, this platform is an important tool to evaluate the therapeutic index of the anti-EGFR drugs and can be useful to test other promising drug candidates. **Kühn et al.** tested the pharmacokinetic and pharmacodynamic properties of permethrin and hyperforin in the skin and liver multiorgan-chip model [129], with no substantial modifications of the chip that is now commercially available as HUMIMIC Chip2 (TissUse GmbH, Berlin).

Novak et al. from the Ingber group established an “interrogator,” which consists of a multi-organ platform supporting eight tissues: skin, intestine, liver, kidney, heart, lung, blood–brain barrier, and brain [130]. It uses liquid-handling robotics, custom software, and an integrated mobile microscope for the automated culture, perfusion, medium addition, fluidic linking, sample collection, and in situ microscopy. This system is maintained within a standard cell incubator and is able to maintain the viability and organ-specific functions of the bioengineered tissues for 3 weeks when coupled together through vascularized microfluidic channels perfused with a common blood substitute. Additionally, they tested the distribution of an inulin tracer perfused through the fluidic system. The automated system was capable of imaging cells within the chips and allowed for repeated sampling from both the vascular and interstitial compartments without alteration of medium perfusion. No skin-specific findings were reported in this publication.

The group led by **Gordana Vunjak-Novakovic** recently developed a four-organ microfluidic chip containing bioengineered heart, liver, bone, and skin both from primary and iPSC-derived cells and circulating monocytes with and without an endothelial barrier as an interface between the single culture chambers and the circulation [131]. The chip is the size of a glass slide and consists of four modular chambers customized for each organ that can be rearranged and connected in any preferred order to

simulate different scenarios. The body of the chip, including the chambers, is fabricated in polysulfone using a 3-axis CNC milling machine (Haas OM2); this material has the advantage over PDMS of not absorbing hydrophobic molecules, and thus it does not influence the medium composition and the pharmacokinetic profile of the drugs. Each chamber contains 1.5 mL of tissue-specific medium and is paved with a nylon mesh (20 μm pores) covered with HUVECs and mesenchymal stem cells acting as pericytes. Underneath the membrane, there is a reservoir for the common culture medium (12 mL in total for each chip), which is perfused through a peristaltic pump. The reservoir is connected through an inlet/outlet microchannel system with the reservoirs of the adjacent modules. After a few days of culture, the tissues reached a homeostatic state that was maintained over a period of 1 month. The model was validated comparing the cytotoxicity of doxorubicin in several conditions: organs cultured individually (isolated system), organs cultured together with the endothelial barrier (InterOrgan system) and without (mixed system). They used histological, genetic, and metabolomic assays to underscore the cross-talk between these tissues and the shifts in pharmacokinetic and pharmacodynamic properties of the drug in different conditions, concluding that the InterOrgan chip is an ideal platform to bridge in vitro and clinical studies.

5.4 Remaining challenges

Many aspects of the skin chip fabrication and culture still need substantial improvement. To set up high-throughput and reliable platforms for drug screening, it is essential to cut production and maintenance costs of the skin chips, generate more physiologically relevant HSCs, as well as implement automation and optimize simple and efficient readouts.

Miniaturization. The most intuitive first step to reduce the costs is to work toward the miniaturization of the model. Over the years, the surface area of HSCs was reduced from 4.67 cm^2 (6-well plate size) [132], to 1.12 cm^2 (12-well) [133], up to 0.33 cm^2 (24-well) [134]. In 2018 **Schimek et al.** published a full-thickness HSC in 96-well format (0.143 cm^2 area), but to achieve this size they used a decellularized bovine dermis rather than a hydrogel [135]. This approach does not allow for rapid prototyping and is unsuitable for the generation of complex models with spatially controlled vasculature and tissue architecture. Therefore, further miniaturization of hydrogel-based HSCs to go below 24-well format will greatly benefit drug screening studies.

Coculture optimization. Coculture medium is another expenditure item needing refinement: ideally, they should contain only the essential defined components to sustain normal cell physiology. At present, the media used is highly variable between research groups, poorly characterized, and comprises a mix of the different specific cell growth media rather than a balanced medium specifically formulated for each coculture condition. More effort is needed in the optimization of media composition, especially in eliminating superfluous and undefined animal-derived components. This is particularly important when the skin chips are integrated with other organs in multiorgan platforms, and the needs of numerous cell types have to be satisfied. A partial solution to this problem for multiple organ chips is to interpose endothelium in the interface between each organ-specific compartment and a shared medium reservoir, so that they could contain an organ-specific medium and a general culture medium, respectively [130].

Automation. The lack of automation in many phases of the production process is probably the biggest problem that the skin chips—and generally organs-on-a-chip—currently face. The automation is essential not only to cut costs and allow high workloads, but especially to guarantee reproducibility. A number of companies, such as Eppendorf, Sartorius, Opentrons and Beckman Coulter introduced automated bioreactors capable of autonomously culturing and passaging cells. Still, at present, there is no automated system to “close the circuit” and integrate the automated bioreactor for cell culture with 3D bioprinters and further bioreactors for culture and analysis of the skin chips.

In terms of outcome analysis, many different classical assays can be reconsidered and adapted to fit an automated high throughput setting. In the past years, many research groups have successfully integrated different kinds of sensors within the tissue culture chips to monitor a number of parameters of both the culture medium and the tissue [136,137,138]. These sensors monitored the tissue culture environmental cues, such as temperature, pH, humidity, oxygen level, glucose, and metabolism byproducts, for example, H_2O_2 , lactate, and specific molecules of interest in the culture medium, the cell behavior, for example, adhesion, detachment, proliferation and cell death, mechanical and electrical stimulations, for example, tissue stretch, relaxation, and TEER.

In 2018 **F. A. Alexander et al.** developed one of the first skin chip capable of automated live monitoring of TEER and extracellular

acidification during regular culture [139]; this chip is now commercially available and implemented with additional O₂ and temperature sensors (Cellasys).

Another very useful tool is the generation of reporter cells lines with specific fluorescence labeled proteins, the variation of which is easily detectable with high-throughput fluorescence detectors. An alternative to this technique is the use of Chromobody, in which the proteins of interest are not directly fused with fluorescent proteins but are targeted by autoexpressed Camelidae antibody VHH fragments fused with fluorescent proteins, thus protecting the functionality of the proteins of interest [140].

Some common laboratory assays such as quantitative PCR (qPCR) and ELISA are now executable in an automated high throughput setting, while others such as immunofluorescent analysis of tissue sections still need substantial human labor.

Additional advanced imaging tools seem to be promising aid for high throughput screening. For instance, the noninvasive reflectance confocal microscopy (RCM) uses near-infrared laser light to generate histological images of the superficial skin layers and is able to reach the papillary dermis. RCM is employed in some advanced dermatological centers to achieve a better visualization of the skin to serve as an aid in the differential diagnosis of melanoma when it was suspected following epiluminescence microscopy. Due to some recent technological advancements, RCM can now be employed to screen specific histological features with the aid of AI systems [141].

Bioprinting. The recreation of skin microenvironment can become prohibitively complex as one adds more cell types and specialized components. As a result, innovative methods of microfabrication like 3D-bioprinting have been leveraged to meet these challenges. Likewise, the generation of functional skin-on-a-chip models that can support the generation and maturation of small-scale skin constructs while mimicking physiologic conditions requires the use of methods that provide fine control of device design. There have been a variety of bioprinting technologies that can be classified as extrusion-based, droplet-based, and laser-based (details can be found elsewhere [142]). Additionally, some of these techniques can be executed inside a bath of support material (usually gelatin) that support the printed architecture; this method is called “freeform reversible embedding of suspended hydrogels.” Once the bioink is solidified, the support material is dissolved and washed away [143].

While still in its infancy, bioprinting has been used in a variety of applications in skin tissue engineering. Bioprinting modalities have been used to make 3D-ECM matrices synchronously imbued with growth factors to create an inductive niche with spatial cues for direct unilateral differentiation of epidermal progenitor cells into sweat gland cells [144]. In the generation of HSCs, a bioprinting system that combines inkjet and extrusion to generate full-thickness HSCs containing KCs and FBs was first developed in 2014 by **Lee et al.** in the lab of **Karande** [145]. **Baltazar et al.**, from the same group, recently developed a bioprinted HSCs containing KCs, FBs, ECs, and pericytes inside transwells and demonstrated upon transplantation onto immunodeficient mice, the inosculation of the human self-organized vasculature with the host vasculature [146]. **M. Ferrer** and his collaborators at the NIH worked on reducing the size of bioprinted full-thickness HSCs and successfully generated a 24-well size format [147]. They recently integrated ECs in bioprinted HSCs and further validated the platform to model atopic dermatitis *in vitro*. With this model, they were able to recapitulate several features of the disease, such as spongiosis and hyperproliferation of the epidermis, upregulation of AD markers, reduced TEER, and secretion of proinflammatory cytokines [148]. **Kim et al.**, in the group led by **D. Cho**, developed a bioprinted HSC within their custom-made polycaprolactone (PCL) transwell system that allowed for ALI [149]. Building on this approach, they were able to bioprint an HSC with the epidermis, dermis, hypodermis, and a perfusable vascular channel [150]. They demonstrated that through this process they could control the pattern of the perfusable vasculature contained in their constructs. Bioprinting has also been used to generate pigmented HSCs with MCs [151]. Bioprinting facilitated the deposition of MCs to generate epidermal-melanin units with predefined patterning of MCs and KCs and the generation of biomimetic hierarchical porous structures like that of native skin. Bioprinted constructs were shown to have more uniformly distributed melanin granules, a more well-developed stratified epidermal layers, and a continuous layer of basement membrane proteins.

As bioprinting becomes more widely adopted and further advances in the field are made, it will become easier to develop advanced HSCs in a variety of settings. Hybrid printing systems will make it possible to incorporate these bioprinted HSCs with custom-made printed devices, allowing for the automatized generation of skin-on-a-chip platforms. However, there are still some important considerations to be aware of in the use of bioprinting currently. One of the challenges is the availability of a

biomaterial that is both easy to print and performs as well as native collagen to recreate the dermal microenvironment biochemically. Up to this date. Currently, there is no material that satisfies both of these needs, as structural modification to allow photopolymerization negatively impacts the biological properties of these materials. Furthermore, current methods do not offer cellular levels of resolution in printing. While the microarchitecture of tissues can be better recreated by bioprinting different cell types contained in distinct bioinks in a variety of conformations, the development of intricate cell–cell spatial relations/interactions is still partly dependent on spontaneous self-assembly. Still, recent advances in combining the new generation of 3D bioprinters [152] with innovative bioinks, such as photo-cross-linkable recombinant collagens [153], have made high-resolution bioprinting up to a single-cell resolution possible [154]. Thus, in the near future, we will likely be able to achieve the construction of 3D-bioprinted skin chips with high anatomical complexity.

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CHAPTER 6

Blood vessels-on-a-chip

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6.1 Introduction

The development of a microfluidic organ model called organ-on-a-chip, which is used for bioassays and especially in drug development, is a major innovation in bioanalytical chemistry [1–6]. These organs-on-chips have allowed modeling of major organs, including the lungs and liver; however,

blood vessels are also important targets of bioassays [7–14]. Blood vessels are part of the circulatory system, which provides blood supply to all tissues. They are responsible for supplying oxygen and nutrients to and collecting waste products from the tissues. As shown in Fig. 6.1, oxygenated blood is transported from the left ventricle through the aorta, then the arterioles, and finally to the capillaries in the tissues. The arteries are the pathways that carry blood, and the capillaries are where material exchange occurs to maintain the tissue microenvironment. Deoxygenated blood returns from capillaries in the peripheral tissues through the venous system to the right atrium of the heart.

Blood vessel structure is related to function, and this is seen in their different sizes and cell types [15]. The aorta originates from the heart and has an inner diameter of 12 mm, with a thick three-layered vascular wall to withstand high-pressure blood flow. The tunica intima, which is the innermost layer of the three layers, is composed of endothelium and is in

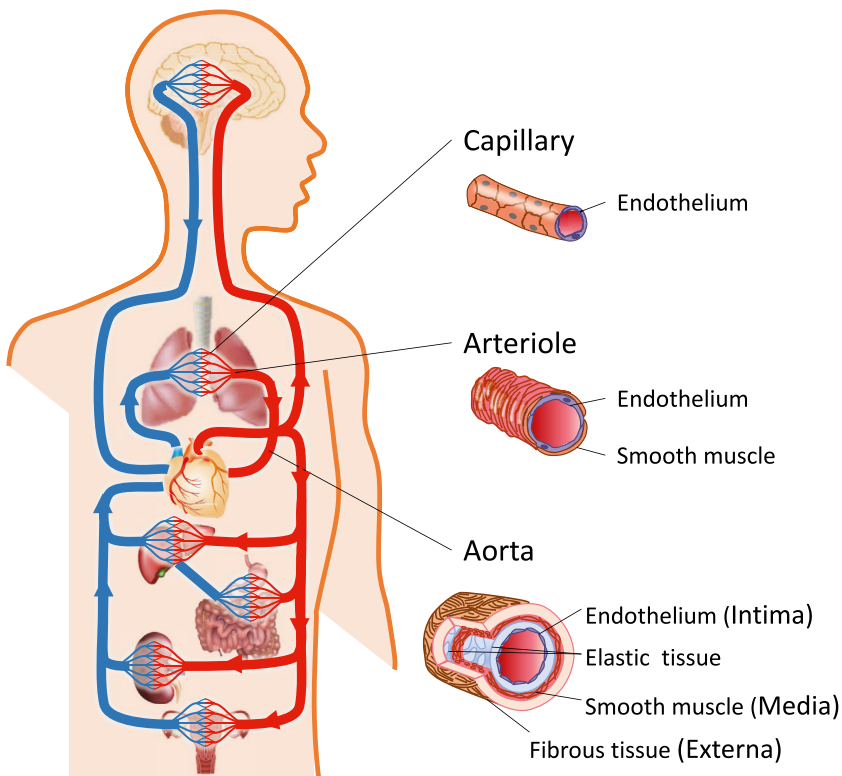


Figure 6.1 Illustration of human blood vessels.

direct contact with the flow of blood. The middle layer is the tunica media, which is composed of smooth muscle cells (SMCs) and elastic tissues. The smooth muscle is responsible for vascular constriction and vasodilation. The outermost layer is the tunica externa and is composed of fibrous tissues.

The artery branches into thinner vessels called arterioles, which have an inner diameter of 15 μm and a two-layered structure composed of endothelium and smooth muscle. The arterioles branch into capillaries that have an inner diameter of 3 μm . The capillaries consist of a layer of endothelium and branch into the tissues to create an extensive blood vessel network. There are three types of capillaries based on their tubular structures. Continuous capillaries are located in the muscles, brain, bones, lungs, and skin. Their endothelial cells (ECs) are continuously connected by adherens and tight junctions and are sometimes surrounded by pericytes. In particular, capillaries in the brain have special tight junctions that prevent the permeation of unwanted molecules as part of the blood–brain barrier. Fenestrated capillaries surround the epithelia of the kidneys, intestines, and some endocrine glands. These capillaries are named after the 50–80 nm pores and fenestrae in the walls for material exchange. Discontinuous capillaries are found in the liver, spleen, and some endocrine glands. They are notable for having gaps between the ECs. This allows blood cells to easily pass through the wall of the discontinuous capillary.

Functional abnormalities in the blood vessels are closely associated with angiogenesis, cancer metastasis, and blood vessel diseases [16]. Conventionally, blood vessels and related diseases are studied using experimental models of animals and cells cultured two-dimensionally under static conditions. However, the results obtained from animal testing are not always applicable to humans, and *in vitro* cell cultures do not make good models of vascular diseases due to dimensional differences and the absence of blood flow [17].

Recent organ-on-a-chip research has produced three-dimensional (3D) primary cultures of human cells to mimic certain parts of the human body. Using these *in vitro* models, it is possible to culture cells in an extracellular matrix (ECM) gel with mechanical stimulation that mimics actual organ microenvironments. In the past decade, many new models of vascular structures, such as the brain [18–23], lungs [24], liver [25], kidney [26,27], and placenta [28,29] have been produced.

Various ECMs and human primary cells from the aorta, pulmonary artery, umbilical vessel, and microvasculature of the skin, bladder, and uterus are commercially available as experimental materials. From the aorta to the capillaries in multiple organs, various human vascular models have

been developed by embedding cells and ECMs in microfluidic devices to be cultured with stimulation that simulates blood flow and strain, which mimic the environment of human blood vessels.

In this chapter, we introduce the progress studies have made on microfluidic vascular models in the past decade. These devices are classified by structure in [Section 2](#), and the delivery methods of mechanical stimuli are reviewed in [Section 3](#). The basic phenomena and functions of these vascular models, including permeability, angiogenesis, and thrombosis, are explained in [Section 4](#).

6.2 Design of microfluidic devices

In vitro vascular networks are mainly constructed using two distinct approaches: either ECs cultured on a microchannel surface or self-assembled vascular networks. In addition to the two approaches, an unusual method using an intact vascular network with surrounding tissue has also been reported.

6.2.1 Polydimethylsiloxane microchannels

Most microfluidic devices used for vascular modeling are made of a silicone elastomer called polydimethylsiloxane (PDMS). PDMS devices are produced using a soft lithography replica-molding method in which the devices are replicated from a reusable mold. The PDMS replica is bound to another PDMS sheet or a glass slide. An inlet hole is made and connected to a pump via tubing to infuse culture media into the microchannels. This method allows channels with various patterns to be easily created. However, microchannels fabricated using the soft lithography technique produce rectangular or square cross-sections, which do not closely mimic real microvessels in vivo that have circular cross-sections.

Fabricating microdevices with cell culture microchannels mimicking complex tissues requires a porous membrane [30] or hydrogel [31] to be incorporated into the PDMS device. In membrane-based devices, ECs are cultured horizontally on a porous membrane that separates an upper channel from a lower channel ([Fig. 6.2a](#)). Similar to conventional Transwell assays, membrane-based devices are ideally suited for examining endothelial barrier functions under various conditions.

Most hydrogel-based microfluidic devices ([Fig. 6.2b](#)) have three parallel channels: a central channel containing the hydrogel and two adjacent side channels in which the culture media flows. The central and side channels

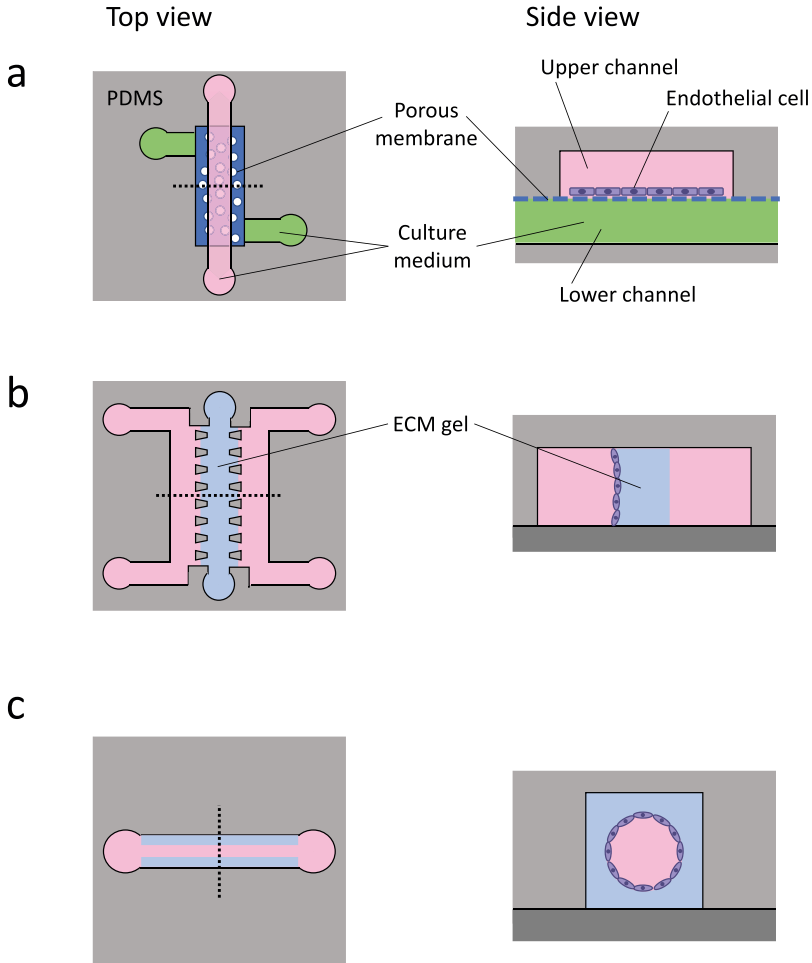


Figure 6.2 Typical microfluidic blood vessel models. Endothelial cells (ECs) were cultured (a) on a porous membrane, (b) on the sidewall of an extracellular matrix (ECM) gel in a polydimethylsiloxane (PDMS) microchannel, or (c) on the surface of an ECM gel channel. A cross-sectional view of the channel (dotted line) is shown on its right. Typical examples include the (a) permeability test [32], lymphatic model [30], blood–brain barrier (BBB) model [19], kidney model [26], and placenta model [29]. (B) Capillary model with pericytes [31], BBB model [18], tumor angiogenesis model [33], and tumor metastasis model [34,35]. (C) Artery model [36] and BBB model [20].

are divided by an array of micropillars, and the hydrogel is kept in the central channel by surface tension. In these devices, ECs are cultured on the sidewall of the ECM gel. Instead of the micropillars, the channels can be separated by a “phase guide” a small ridge that acts as a pressure barrier [37].

6.2.2 Hydrogel microchannels

In some devices, microfluidic channels are fabricated using an ECM gel (Fig. 6.2c). A simple linear channel is produced using this approach. A needle or rod is used as a template, which is physically removed by pulling it out of the surrounding ECM gel, typically collagen type I [38], fibrin [36], or gelatin methacrylate [39]. ECs are seeded on the channel surface to form a tubular monolayer of ECs [40]. In comparison to PDMS devices, hydrogel-based devices closely mimic the human extracellular environment. However, this method can only create a thick and simple linear microvascular channel because it is formed by inserting and then removing a needle or thin rod.

6.2.3 Self-assembled networks

Vascular ECs are suspended in an ECM hydrogel and cultured in a microdevice in which the cells spontaneously reconstitute self-assembled 3D vascular networks [31,34,41–43] (Fig. 6.3a). This approach does not

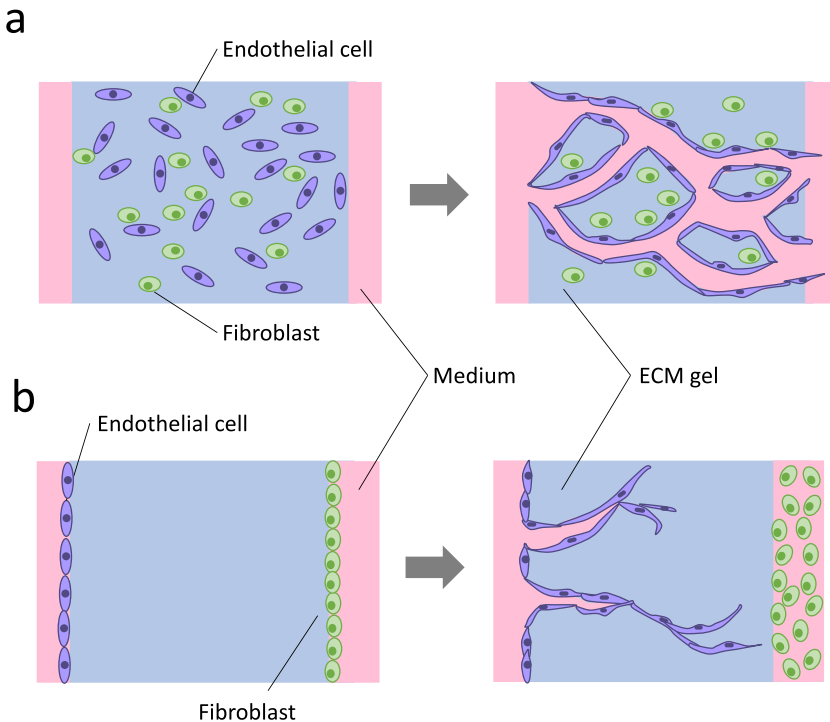


Figure 6.3 Self-assembled microvascular network in a microfluidic device. (a) Vasculogenesis on a chip. Endothelial cells (ECs) suspended in an extracellular matrix (ECM) gel spontaneously form a microvascular network. (b) Angiogenesis on a chip. ECs migrate into an ECM gel to form vascular sprouts.

require a structural skeleton to guide the vasculature, and the device design is similar to that of ECM-based devices (Fig. 6.2b). ECs and normal human lung fibroblasts (NHLFs) are suspended in a mixture of fibrinogen and thrombin, and the suspension is introduced into a gel channel and then allowed to form a fibrin gel. When cultured, ECs elongate, form vacuoles, and connect with neighboring cells to form lumens (Fig. 6.3a). This process is similar to that of vasculogenesis *in vivo*. Kim et al. found that the vasculogenic morphogenesis of human umbilical vein ECs (HUVECs) was dependent on discrete coculturing with NHLFs; HUVECs without cocultured NHLFs failed to form interconnected networks [41]. They used a chip consisting of five parallel channels: a central channel and two stromal cell culture channels separated by two additional channels containing media (medium channels). A mixed coculture of HUVECs and NHLFs in the central channel formed well-interconnected vascular networks after 5 days. However, these networks were not connected to the adjoining medium channels and could not be perfused as a result. On the other hand, when NHLFs were cultured in stromal cell culture channels, the HUVEC networks formed connections to the adjoining medium channel. The spontaneously formed capillary networks consisted of perfusable microvessels with diameters ranging between 1 and 100 μm [44]. These microfluidic devices have also been used for the development of *in vitro* angiogenesis models (Fig. 6.3b). In these models, ECs are elongated and sprout into the ECM gel. These topics are discussed in Section 4.2.

6.2.4 Intact small artery-on-a-chip

A rare example of an intact small artery-on-a-chip that used an intact tissue slice with a vascular structure obtained from an animal subject and embedded in a microfluidic device without dissociation of the cells has been reported [45]. Mouse olfactory artery segments were embedded in a microdevice and maintained under physiological conditions. This model made it possible to apply drugs to the artery mounted in the device and to visualize intact vasodilation in the transparent PDMS device.

6.3 Microfluidic cell culture systems designed for mechanical stimuli

The presence of mechanical stimuli on vessel walls due to blood flow is unique to vascular tissues and plays a crucial role in vascular systems. The distinctive characteristic of microfluidic vascular systems is the inclusion of

fluidic and stretch stimulation to cells to mimic blood flow, which is not realized in conventional models that use a culture dish. The mechanical stimuli applied to microfluidic vascular systems are classified into three categories: shear flow, stretch/strain, and compression.

6.3.1 Flow culture systems

The pumping of fluids into a microchannel is important for constructing blood vessel models in microfluidic devices because it mimics blood flow while delivering nutrients and reagents.

Several pumping methods have been reported to date. Pumping by hydrostatic pressure, which is generated by a difference in water level between the inlet and outlet, can induce continuous flow with a simple experimental setup [46]. A computer-controlled pump can be used to control both continuous and pulsatile flow at various rates. Syringe and peristaltic pumps have also been utilized (Fig. 6.4a and 6.4b). These pumps expose ECs to continuous shear stress [47], pulsatile shear stress [48,49], or both [50].

Although these pumping methods have been applied to cell experiments in microfluidic devices, commercially available pumps are generally much larger than the devices, and the downsizing of the entire system is often limited by the size of the pumps. A solution to this limitation is the use of smaller pumps. An integrated culture system consisting of a small peristaltic pump, medium reservoir, and motor controller has been reported [51]. All components, except the motor controller, are integrated into a microscope stage insert. A small microfluidic cell culture system driven by a miniaturized peristaltic pump has also been reported [52]. These pumps possess a reservoir for the culture medium, an electrical control circuit, and an internal battery; thus, there is no need for tubing and electrical connections outside the system. Another downsizing solution is the integration of pumping units into microfluidic devices [53–57]. Microfabricated peristaltic pumps offer not only space-saving advantages but also circulate media in the closed circuit similar to the bloodstream (Fig. 6.4c). Imura et al. developed a microcirculation model [58], which consisted of a pneumatic microperistaltic pump (heart), dialysis component using a dialysis membrane (renal corpuscle), and cell culture chamber (drug target) to mimic the excretion process. Sakuta et al. modified this system to form a multichannel platform, which consisted of three independent circulatory channels with a common microfabricated pneumatic peristaltic pump [57].

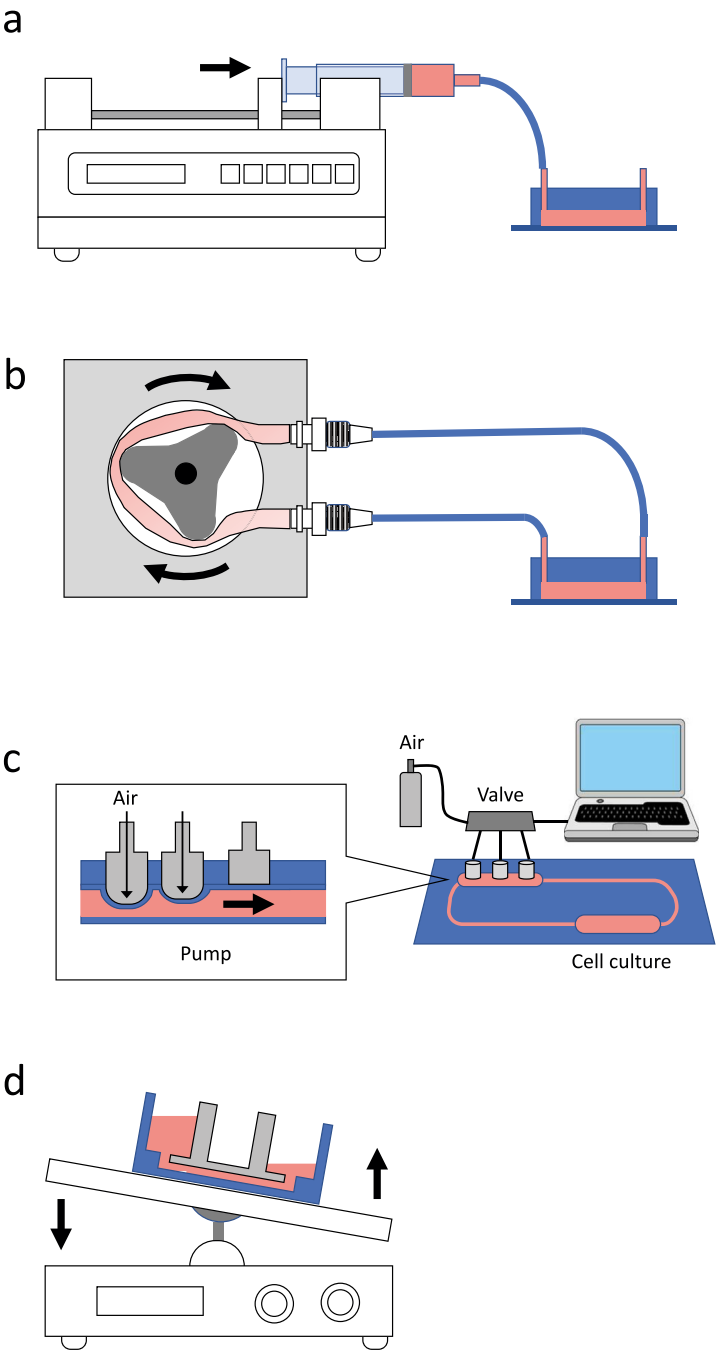


Figure 6.4 Typical pumping systems for microfluidic vascular models: (a) syringe pump, (b) peristaltic pump, (c) microfabricated pneumatic peristaltic pump, (d) gravity-induced flow system using a programmable rocker platform.

In addition to pneumatic drives, a micropump driven by the piezoelectric pins of Braille displays has also been developed, which was utilized for endothelial cell culture [53] and a palm-sized cell culture system with Braille displays has also been developed [54].

Furthermore, a pumpless microfluidic platform that utilizes gravity-induced flow and a rocking motion to recirculate cell culture medium [37,59–61] has recently attracted attention because its design makes it possible to control a large number of devices on a single rocker (Fig. 6.4d). Medium recirculation between the two reservoirs is achieved by periodically reversing the tilt direction. This pumpless design uses gravity as the driving force and offers advantages such as space-saving and user-friendliness. Additionally, by integrating all fluidic components into a single platform, the assembly and operation of the device are greatly simplified, and sterility is more easily sustained than in systems with external tubing.

Another factor to consider is that bidirectional flow differs from physiological blood flow. Wang and Shuler developed the “UniChip” fluid network design [60], which transforms reciprocating flow input into unidirectional perfusion in microchannels by utilizing supporting channels and passive valves. The design enables unidirectional perfusion with recirculation on pumpless platforms and provides an effective backflow-proof mechanism. Lee et al. also developed a novel microfluidic chip [62] that enables gravity-induced unidirectional flow by using a bypass channel with a geometry different from the main channel.

The details of these pumping systems for cell cultures were reviewed by Byun et al. [63].

6.3.2 Cell stretching systems

Cell strain is another unique mechanical stimulus induced by blood flow. Modeling of this phenomenon was achieved by incorporating a thin stretchable PDMS membrane into a microdevice as a cell adhesion surface to simulate stretching stimuli in cell culture systems. Hydrodynamic [64] and pneumatic [65] actuation are major methods for generating mechanical strain.

Zhou et al. reported the development of a microchip platform with cyclic circumferential strain stimulation to study blood vessels [64]. This device consisted of an array of microfluidic channels with widths ranging from 20 to 500 μm . The channels were covered with suspended deformable membranes, on which cells were cultured, and were stimulated by a

cyclic circumferential strain of up to 20% via hydrodynamic actuation of the fluid in the microchannels to mimic the biomechanical conditions of small blood vessels.

Sato et al. developed another microfluidic cell-stretch device [65]. This microfluidic device harbors an upper cell culture and lower control channels separated by a stretchable PDMS membrane that acts as a cell culture substrate (Fig. 6.5a). The inlet of the lower channel is connected to a vacuum pump via a digital switch-controlled solenoid valve, which allowed the simulation of a cyclic stretch at heartbeat frequency (80 bpm). One of the most famous stretching devices is the lung alveolus chip developed by Huh et al. [66]. A vacuum was applied to two side chambers where the ends of a membrane were attached (Fig. 6.5b). The membrane was attached to the center of the chamber wall; thus, the actuation did not cause any vertical motion. Instead, the membrane was stretched horizontally in the focal plane of the microscope.

6.3.3 Cell compression systems

Ahn et al. reported a method to model biomechanical-stimuli-induced blood vessel compression in vitro within a PDMS microfluidic 3D microvascular tissue culture platform with an integrated pneumatically actuated compression mechanism [67]. The device consisted of a cell culture channel and an additional upper chamber for pneumatic actuation, which applied compressive forces to the vascular channel (Fig. 6.5c). To mimic biomechanical stress, compression was applied to the vascular channel in the range of 0.5–2.5 psi with a compression control valve.

6.4 Analyzing vascular phenomena using model systems

6.4.1 Blood flow

ECs and SMCs are affected by complex mechanical stimuli, such as shear and tensile stresses, compressive strain, and interstitial flow. Mechanical stresses cause EC phenotype alteration, cytoskeletal remodeling, and vascular disease progression. Although biomechanical stress-induced vascular abnormalities are a major mechanism of disease, its pathology is poorly characterized.

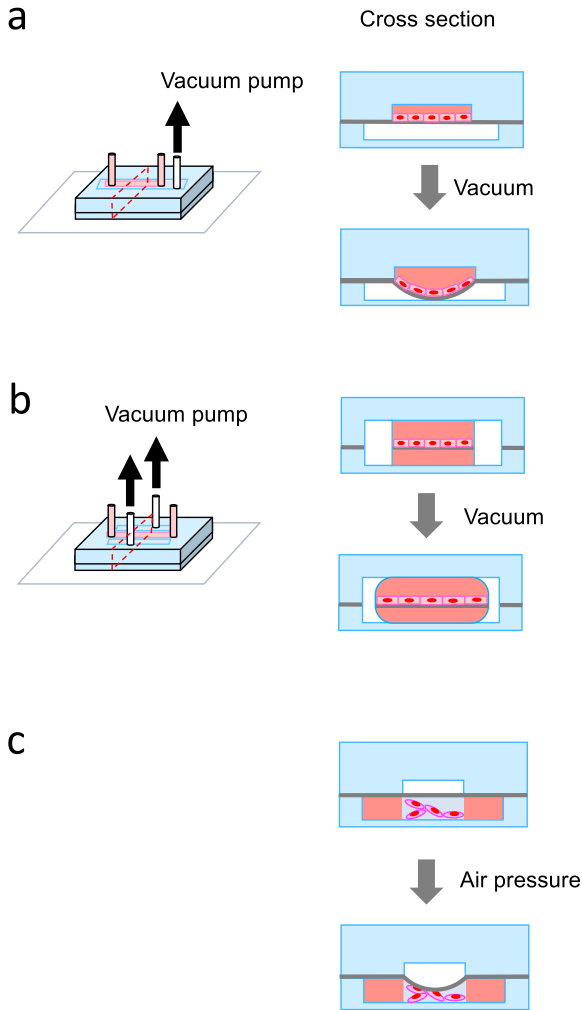


Figure 6.5 Microfluidic devices for applying mechanical stimulation on cells: (a, b) cell stretching, and (c) cell compression systems.

6.4.1.1 Fluid shear stress

Many experiments applying fluid shear stress (FSS) to ECs cultured in microchannels have been reported. FSS ranges from 10 to 70 dyn/cm² (1 dyn/cm² = 0.1 Pa) in the arteries and 1–6 dyn/cm² in the veins [68], which can be reproduced in fluidic culture microchannels using a syringe pump. Frame et al. designed one of the earliest attempts by using branching microchannels (20–50 μm in diameter) fabricated on a borosilicate glass

slide to reproduce the geometry of the arteriolar network [69]. The system could perfuse culture medium at physiological flow rates corresponding to shear stress values ranging from 0.0348 dyn/cm^2 . ECs would be cultured in a microchannel under fluidic conditions for 5 days after seeding.

Cells cultured with FSS showed different responses from those cultured without FSS. For example, FSS strengthened the EC–EC junctions [30]. The FSS-induced proliferation of SMCs was also reported. Sato et al. developed a microfluidic cell culture device for pulmonary hypertension studies that withstood high shear stresses [70]. Pulmonary artery SMCs cultured in this device were collected to analyze mRNA, and it was found that high shear stress caused a 7.5-fold increase in the transcription levels of cyclin D1, a protein involved in regulating cell cycle progression. They concluded that the abnormal proliferation of SMCs causes blood vessel closure in pulmonary hypertension.

Shear stress is changed by microvascular occlusion or the hemodynamic changes during space flight. The response of ECs to the change of the shear stress has been investigated [71]. One of the first observed EC adaptations to shear stress produced by blood flow is cell elongation and orientation along the direction of flow. Inglebert et al. found that, within 1 h of exposure to reduced wall shear stress, HUVECs reorganized their actin skeletons with a decrease in the number of stress fibers and an increase in actin recruited into the cell's peripheral band, indicating a fast change in the cell phenotype from changes in the flow rate.

6.4.1.2 Cell strain

Cell strain affects the phenotype and proliferation of ECs and SMCs. Zhou et al. reported that cell strain affects differentiation, as shown by their cell stretching device [64]. They demonstrated that human mesenchymal stem cells (MSCs), which can differentiate into osteoblasts, chondrocytes, adipocytes, or vascular SMCs, cultured with continuous cyclic stretch (CS) stimulation for more than 7 days produced different localization and alignment of MSCs when mechanical stretch was $>10\%$, and proteins in multiple signaling pathways, including SMAD1/SMAD2 and canonical Wnt/b-catenin, were detected.

Sato et al. developed a microfluidic cell stretch device [65] that exposed cells to cyclic circumferential stretch to investigate its effects on the proliferation of pulmonary artery SMCs (PASMCs) in pulmonary arterial hypertension (PAH). In this study, PASMCs were cultured in a device with CS at the same rate as that of heartbeats of the patients, and cell proliferation

and the transcription levels of related genes were analyzed. The results they found are consistent with the abnormal proliferation observed in PAH, showing stretch stress could promote cell proliferation in PAH.

Zheng et al. reported a flow and CS microdevice, which integrated two major mechanical factors in blood vessels, FSS and CS [72]. The device could simulate FSS and CS on vascular cells simultaneously or separately to mimic the hemodynamic microenvironment of blood vessels. By imposing FSS-only, CS-only, or FSS plus CS stimulation on rat MSCs and HUVECs, they found that the alignment of the cellular stress fibers varied with the type of cell and stress.

6.4.1.3 Cell compression

Solid stress is the stress exerted by the solid components of tissues and is known as one of the physical characteristics of cancer. Solid stress is directional because tissues have different structural properties depending on their location and orientation. The interaction between vascular networks and external solid stress has not been well studied, despite it being a relevant factor of many pathological conditions. Ahn et al. reported the effects of compression on vascular networks using their own devices [67]. Histological reactions to compressive forces were quantified and live/dead assays indicated the presence of a microvascular dead zone within high-stress regions while reactive oxygen species quantification exhibited a stress-dependent increase. Fluorescein isothiocyanate-dextran flow assays showed that compressed vessels developed structural failures and increased leakiness, which was confirmed by finite element analysis, indicating that these vascular tissue deformation and tissue stress distribution models were conceptually correct. This study provided a powerful in vitro method to model the microphysiological responses of microvascular tissues to compressive stress, which opens the way for further studies on external stress as a cause of vascular failure. The compressive solid-stress-induced vascular damage model has numerous applications, including the safety evaluation of clothing and wearable devices. For example, long-term compressive stresses imposed on the body from tight clothing, safety harnesses, and wearable medical devices may pose vascular damage risks.

6.4.1.4 Interstitial flow

Interstitial fluid (IF) is a liquid that surrounds cells and originates from blood plasma that has leaked from capillaries through pores and intercellular gaps. IF plays an important role in vascular development. Moya et al. reported

the development of an in vitro perfusable capillary network in a micro-device by using IF to mimic the human microvascular environment [73]. A continuous capillary network was spontaneously generated from the cells, ECM, and angiogenic stimuli. The ends of the capillary network were anastomosed with an adjacent microchannel to perfuse the capillary network.

Kim et al. developed a microfluidic 3D vascular model to investigate the role of IF in the vasculogenesis and angiogenesis of microvascular networks [43]. In this model, HUVECs were cocultured with stromal fibroblasts that triggered neovessel sprouting or spontaneous formation of a network of interconnected microvasculature that expanded into adjacent avascular areas. Angiogenic sprouting was enhanced only when the directions of the IF and sprouting were opposites. The vasculature switched between active angiogenic and nonsprouting states, depending on the IF conditions.

The effect of IF hydrostatic pressure and the concentration of vascular endothelial growth factors (VEGFs) on 3D microvascular network formation were also examined [74]. In this study, HUVECs were cultured in a series of IFs under 2, 8, and 25 mmH₂O, and it was found that IF pressure significantly enhanced vascular sprouting, network extension, and the development of branching networks. Furthermore, they demonstrated that the proangiogenic effects of IF could not be substituted by an increase in VEGF concentration. In addition, HUVECs near vascular sprouts were significantly elongated under >8 mmH₂O conditions, while Src kinase activation was detected even under two mmH₂O conditions, suggesting that the balance between the IF and VEGF concentration is critical for the regulation of 3D microvascular network formation.

6.4.2 Angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting vessels, is a major research topic in biology, especially in the fields of development, cancer, tissue engineering, and regenerative medicine. Kim et al. fabricated a microdevice to study angiogenesis with five parallel channels: a central EC channel and two stromal cell channels separated by two culture medium channels [41]. To induce angiogenic sprouting, HUVECs were seeded on the sidewalls of the fibrin gel-filled central channel, and NHLFs were cultured in the stromal channel on the opposite side to expose HUVECs to a gradient of NHLF-secreted factors (Fig. 6.3b). Within 24 h of coculture, the formation of tip cells and angiogenic sprouting was observed. The tip

cells guided the growth of the sprouts through the fibrin gel until they reached the opposite side of the gel channel, whereupon ECs formed tubular structures by day 2. The lumina around the stalk region appeared by day 3, and the lumenized vessels were connected to the other medium channels after day 4. The microdevice was used for angiogenesis experiments with cancer cell-secreted factors. Highly malignant human glioblastoma multiforme U87MG cells were seeded into the stromal channel. HUVECs invaded the fibrin gel in response to U87MG-derived factors within 24 h of coculture. Compared to the sprouts produced with NHLF, the U87MG-induced sprouts showed the incomplete formation of vascular networks with immature and poorly lumenized vessels on day 4.

Although HUVECs are used in most angiogenesis studies, Seo et al. compared the functional angiogenic ability of human aortic endothelial cells (HAECs) and HUVECs in a 3D microfluidic cell culture system [75]. In this system, ECs formed new sprouts that invaded a scaffold depending on the gradient of VEGF-A, which is an important regulator of angiogenesis. Their study showed that HAECs had a stronger angiogenic potential than HUVECs [76].

The details of angiogenesis systems were reviewed by Akbari et al. [77].

6.4.3 Endothelial permeability

The endothelial barrier maintains vascular and tissue homeostasis and regulates many physiological processes, including angiogenesis [78]. The integrity of the vascular barrier can be disrupted by a variety of soluble permeability factors, and changes in barrier function can exacerbate tissue damage during disease progression. Knowing the mechanisms of endothelial barrier function is important in understanding vascular homeostasis.

There are two types of microfluidic devices for conducting endothelial permeability tests: either a permeable porous membrane (Fig. 6.2a) or hydrogel (Fig. 6.2b) is used as culture support for ECs. Kim et al. reported a permeable membrane-based microfluidic device for nano-drug permeability testing [32]. EC monolayers showed a decrease in transendothelial electrical resistance, which indicated an increase in permeability when ECs were exposed to shear stresses of 1 and 10 dyn/cm² for 24 h. After a suspension of lipid-polymer hybrid nanoparticles was introduced into the microchannel, the nanoparticles that appeared in the other channel were monitored every 10 min. A significant increase in nanoparticle permeation across the EC layer was observed when the cells were treated with tumor

necrosis factor α (TNF- α). To compare the permeability of the endothelialized microdevice with an *in vivo* atherosclerosis model, New Zealand White rabbits were used. A 3D dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) technique using a 3-T clinical MRI scanner analyzed the *in vivo* permeability of atherosclerotic plaque microvessels. A high correlation was observed in nanoparticle permeabilities determined by *in vivo* DCE-MRI and by *ex vivo* near-infrared fluorescence imaging.

A porous membrane was also used to evaluate the endothelial permeability of the nanoparticles instead of the cultured cells. It is difficult to precisely evaluate the effects of physical parameters, such as pore and nanoparticle size, on permeability using conventional animal experiments and cell-based microfluidic bioassays because the sizes and shapes of the vascular pores are not uniform. A porous membrane with straight uniform pores was integrated into a microfluidic device for nanoparticle permeation studies to examine the effects of pore size and pressure differences across the membrane on nanoparticle permeability [79]. The experimentally determined permeability coefficient of the 1.0- μm pore membrane against the 100-nm-diameter nanoparticles in this model agreed well with the theoretical values.

Although the microvessel diameters where the material exchange takes place in peripheral tissues is approximately 10 μm , the diameters of experimental microchannels are relatively large (600–800 μm). To mimic physiologically relevant conditions, permeability tests are conducted using a self-assembled microvascular network model [21,80]. Campisi et al. reported [80] a blood–brain barrier network model composed of perfusable microvasculature with permeability lower than conventional *in vitro* models and similar to *in vivo* rat brains.

A microfluidic microvascular interstitium model that is used to study the leakage of drugs from blood vessels under *in vivo*-like fluidic conditions has been reported [81]. MRI was used to demonstrate the compatibility of this model with experimental animal and human methods. In this study, two types of contrast agents with different molecular weights were transported using the interstitium model. The obtained transport rate ratio was consistent with the ratio calculated from the diffusion coefficients of the agents.

6.4.4 Thrombosis and hemostasis

When blood vessels are damaged, proteins and other cellular materials, including collagen and tissue factor (TF), aggregate at the injury site to create a stable blood clot, which prevents excessive blood loss. At the same time, normal blood vessels should not clog and maintain normal circulation because the vascular endothelium has high antithrombotic activity [82]. However, blood clots form easily in diabetic patients because of oxidative stress on the endothelium. Although it is well known that platelet aggregation, a process essential for thrombus formation, is influenced by shear stress, the mechanisms by which shear stress activates platelets are poorly understood [83]. Microfluidic vascular devices allow suitable observation of thrombus formation because ECs are cultured in transparent microchannels under various FSS conditions, and the composition of the media can be freely adjusted to contain various testing agents of different concentrations.

Jain et al. developed a microfluidic device to predict whether a blood sample was likely to form clots [84]. The device mimicked a stenosed artery network and evaluated blood clotting with a small amount of blood under pathophysiological flow conditions. Clotting time analysis based on a computational model of thrombus formation enabled accurate *in vitro* measurements of coagulation and platelet function using patient blood samples. The device was integrated with the extracorporeal circuit of a porcine model of endotoxemia and heparin therapy. This allowed real-time monitoring of *ex vivo* clotting, which is generally more reliable than standard clotting assays.

Brouns et al. developed a vascular model composed of a microfluidic chamber coated with thrombogenic collagen and TF. This was covered with patches of human ECs to evaluate the local inhibitory roles of a discontinuous endothelium [85]. The heterogeneous formation of platelet aggregates and fibrin clots after perfusion with human blood and plasma was monitored by fluorescence microscopy. A HUVEC coverage of 40%–60% on collagen/TF coatings resulted in a strong overall delay in platelet deposition and fibrin fiber formation. Fibrin formation colocalized with the deposited platelets and was confined to the area between the ECs, indicating that the clotting process was immediately and locally suppressed.

However, there is still a need for an *in vitro* model of hemostatic responses to vascular injury that integrates all major biological, biochemical, and biophysical components of hemostasis. Sakurai et al. developed a comprehensive *in vitro* mechanical injury bleeding model composed of

endothelialized microchannels coupled with a microfabricated pneumatic valve that induces a vascular “injury” [86]. Whole blood could be perfused, and hemostatic plug formation was visualized and “in vitro bleeding time” measured. The interaction of various hemostatic components was examined, and some of the unsolved problems in hematology were answered. The effects of antiplatelet agents on clot contraction and hemostatic plug formation, the requirement of von Willebrand factor for hemostasis at high shear, the fact that hemophilia A blood leads to unstable hemostatic plug formation and changes in fibrin structure, and the importance of endothelial phosphatidylserine in hemostasis were visualized and quantitatively demonstrated.

The details of hemostasis systems were reviewed by Panchal et al. [87].

6.4.5 Coculture

Mural cells of the vascular wall, namely pericytes and vascular SMCs, are essential for vascular integrity [88]. While an EC monolayer encloses the vessel lumen, pericytes are associated with the abluminal surfaces of capillaries. Vascular SMCs, which cover larger arteries and veins, are thought to be closely related to pericytes. These cells stabilize blood vessels through physical and chemical interactions with neighboring ECs, and the loss of these cells leads to vascular leakage and hemorrhage. Microfluidic 3D vascular models that closely mimic living tissue by coculturing vascular ECs and surrounding cells have been reported.

6.4.5.1 Pericytes

van der Meer et al. developed 3D vascular tissue in a PDMS microfluidic device by introducing a mixture of HUVECs and human embryonic stem cell-derived pericytes suspended in rat-tail type I collagen [89]. The cells spontaneously formed a single long tube similar to a blood vessel. Confocal microscopy revealed a mature endothelial monolayer complete with platelet-endothelial cell adhesion molecule-1 existing at the cell-cell junction and pericytes incorporated in the tubular structures. Tube formation was disrupted in the presence of a neutralizing antibody against transforming growth factor-beta (TGF- β), which is essential for normal vascular development. In engineered microvessels, inhibition of TGF- β signaling resulted in the formation of tortuous tubes with smaller diameters, very similar to the abnormal vessels observed in patients with the vascular disease hereditary hemorrhagic telangiectasia.

Kim et al. developed a microfluidic device to investigate the interactions between ECs and pericytes during the sprouting, growth, and maturation steps of neovessel formation [31]. A mixture of HUVECs and human placental pericytes was seeded on the sidewall of a prepatterned 3D fibrin gel and allowed to sprout into the gel. The effects of pericytes coculturing on EC maturation and the formed EC network were confirmed using confocal microscopy. Compared with EC monocultures, EC-pericyte cocultured vessels showed a significant decrease in diameter, an increase in the number of junctions and branches, and a decrease in permeability. In response to biochemical factors, ECs with pericytes showed features similar to those observed in *in vivo* experiments.

van Dijk et al. developed a PDMS microfluidic device that used fibrin gel to reproduce a blood vessel with a confluent endothelium and mural cell support that optimized endothelial barrier function [90]. To generate the blood vessel with pericytes, fibrinogen mixed with pericytes was polymerized around a rod, the rod was removed to leave an empty lumen, and the subsequent lumen was seeded with HUVECs. HUVECs seeded into the ECM channel were cultured for 3 days to form a confluent monolayer. Meanwhile, pericytes that were suspended in the fibrinogen matrix were increasingly recruited to the neovessel over time. In contrast, EC coverage of the channels remained low, and it was difficult to maintain a confluent monolayer in the absence of pericytes. The presence of pericytes in the microfluidic system was essential to maintain the endothelial barrier function of the tissue-engineered vessel, as monitored and quantified by perfusion of fluorescently labeled dextran.

Bichsel et al. developed an *in vitro* 3D microvascular model that closely mimicked the human lung microvasculature to study lung vasculogenesis and vascular remodeling in terms of accessibility, functionality, and cell types [91]. To analyze their role in the generation of microvessels, lung pericytes obtained from the human distal airways were mixed with fibrin gel and seeded into a microcompartment together with HUVECs. It was found that soluble signals from the lung pericytes were necessary to establish vascular perfusion, and the pericytes migrated toward the endothelial microvessels. Cell-cell interactions in the formation of tight junctions as well as basement membrane secretions were confirmed by transmission electron microscopy and immunocytochemistry. Direct coculture of ECs and pericytes decreased the microvascular permeability from 17.8×10^{-6} cm/s to 2.0×10^{-6} cm/s and resulted in vessels with significantly smaller diameters. When phenylephrine was administered,

vasoconstriction was observed in microvessels cocultured with pericytes, but not in endothelial microvessels alone. Perfusable microvessels were also generated in the human lung microvascular ECs and lung pericytes. Lung pericytes have been shown to have a notable influence on microvascular morphology, permeability, vasoconstriction, and long-term stability in *in vitro* microvessels.

6.4.5.2 Smooth muscle cells

Arterial and venous walls are composed of three layers: (1) tunica intima, a continuous monolayer of ECs, (2) tunica media, concentrically organized SMCs with elastic fibers, and (3) adventitia, a collagenous ECM containing fibroblasts and perivascular nerve cells. Tan et al. developed 3D artificial vessels mimicking the basic architecture of the artery that contained a similar combination of ECs, SMCs, and a collagen-rich ECM. The artificial arteries were fabricated using a needle-based method in a PDMS collagen channel scaffold (330 μm in diameter). The SMC/EC bilayer was constructed to mimic the structure of an arteriole (direct coculture) or larger artery architectures (encapsulated coculture) in which the EC monolayer was covered by the SMC multilayer. Elongation and sprouting of SMCs and ECs were observed in the hydrogel under static coculturing conditions. Cells were grown to form tubular structures with an internal endothelium expressing the intercellular junction marker CD31. No significant changes in the luminal diameters were observed during the 4 day static maturation period (less than 10%). Both coculture models showed high glucose consumption rates during the initial proliferation phase followed by a quiescent maturation stage. Hasan et al. also developed native blood vessel-like perfusable structures with multilayered vascular walls [39]. They demonstrated a new method to fabricate tri-layer blood vessel-like structures in a PDMS microfluidic device using a pair of concentric needles and a layer-by-layer organization of fibroblasts, SMCs, and ECs in a photocrosslinkable 3D hydrogel. The method allows the fabrication of a range of vascular-like structures with varying thicknesses (200–540 μm) of different wall layers and varying lumen diameters (120–400 μm).

6.4.5.3 Lymphatic ECs

IF that is leaked from the blood capillaries is absorbed by lymphatic vessels. It is important to study the mass transfers that occur in peripheral tissues and the ability of the lymphatic vessels to recover IF is as essential as the vascular permeability of blood. Sato et al. developed a membrane-based

microfluidic microcirculation model containing both blood and lymphatic ECs to examine vascular permeability [30]. The device consisted of upper and lower channels that were aligned and separated with a porous membrane on which blood vascular and lymphatic ECs were cocultured adjacent to each other. It was found that fluidic culture promoted the formation of EC–EC junctions, and treatment with the proinflammatory substance, histamine, altered the localization of tight and adherens junction-associated proteins, resulting in increased vascular permeability.

Osaki et al. [92] modeled lymphangiogenesis and vascular angiogenesis in a microdevice with a collagen gel chamber. Lymphatic and blood vessels were fabricated by sacrificial molding, and human lymphatic ECs and HUVECs were seeded into the microchannels (600 μm in diameter). Experiments using this device showed that VEGF-A, B, and C antagonists are potential antilymphangiogenic and antivascular angiogenic agents. The results also suggested that vascular angiogenesis affects lymphangiogenesis. This model will be useful for studying pathologies involving vascular angiogenesis and lymphangiogenesis, including inflammation, tumorigenesis, and viral infection.

Examples of lymphatic EC monocultures instead of cocultures with vascular ECs have also been reported. Similar to angiogenesis, lymphangiogenesis has also been investigated using microfluidic devices. Kim et al. reproduced lymphangiogenesis in an ECM-containing microfluidic device composed of three parallel channels [93]. Gong et al. developed a model composed of a tubular lymphatic vessel embedded in a collagen gel chamber [94]. The vessel channel was molded with a lumen rod made of PDMS cured in a 25-gauge hypodermic needle, and it had a smaller diameter than that of Osaki's device that used a glass rod (600 μm in diameter) as a mold. Collagen type I gel (3–6 mg/mL) was polymerized around the lumen rod, the rod was subsequently removed to leave an empty lumen, and then the resulting lumen was seeded with primary human lymphatic endothelial cells isolated from a lymph node to form a lymphatic vessel with a diameter of 200–250 μm . They used this model system to analyze the effects of collagen density on cell viability. They found that softer collagen gels (3 and 4 mg/mL) produced significantly more viable vessels with maximum viability of 92% at 3 mg/mL.

The system was also used to characterize lymphatic barrier function and to model tumor microenvironments surrounding the lymphatic vessels. This technique has been applied to a breast tumor–lymphatic microfluidic model to examine the crosstalk between lymphatic vessels, MCF-7 cells,

and MDA-MB-231 cells [95]. This model showed that breast cancer cells have a significant impact on lymphatic vessels, affecting many pathways including angiogenesis, hypoxic response, metabolism and metabolite transport, epithelial-mesenchymal transition, and DNA stability and repair.

The details of blood and lymphatic vasculature on-chip platforms were reviewed by Henderson et al. [96].

6.5 Conclusions

The vascular microdevices developed to date have been outlined. Tissue-specific capillary models will be much-needed vascular devices in the foreseeable future because capillaries are structurally different from other tissues. As mentioned in the introduction, there are three types of capillaries in the body: continuous capillaries generally found in the fat, muscles, and nervous system, fenestrated capillaries found in the kidneys, endocrine glands, and small intestine, and sinusoidal capillaries found in the liver. The development of tissue-specific capillary models will make it possible to evaluate disease-specific drugs that work only in their target organs. The development of capillary models for all diseases has great potential in aiding drug discovery.

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CHAPTER 7

Liver-on-a-chip

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7.1 Introduction

Accounting for 2% of body weight, the liver is the largest organ in the human body that plays an integral role in metabolic activities. Some of these functions include the following: synthesis of serum proteins the most important of which is albumin, carrier of particles in the blood; regulation of blood cholesterol and triglycerides; glucose metabolism, involving glycogenesis, glycolysis, and gluconeogenesis; storage of some essential compounds such as iron and vitamin A; amino acid degradation, removing amino group and producing urea; metabolism and detoxification of xenobiotic; and bile production, necessary for emulsification and uptake of fat in the intestines [1,2]. Accordingly, its functionality is crucially important for maintaining the homeostasis of the human body. Therefore, studying the liver is of great importance to have a better understanding of liver function in human health.

The first reason to study the liver is drug-induced liver injury (DILI). Playing a critical role in drug metabolism, the liver is the primary site for drug-induced toxicity [3]. DILI is a consequence of improper medication that can resemble some of the liver diseases such as hepatitis, cholestasis, and fibrosis, or in some cases even result in death [4]. A study on drug development by Katini and DiMasi [5] shows that despite the increased investment in research and development, there is a decrease in the number of approved drugs. Also, Onakpoya et al. [6] studied postmarketing withdrawal of 462 medicinal products between 1953 and 2013 as a result of adverse drug reactions. They demonstrated that the leading cause of postmarketing withdrawal was hepatotoxicity. Accordingly, DILI is one of the main reasons for most drug failures in preclinical or clinical tests. Hence, it is indispensable for pharmaceutical development to design models that predict DILI more effectively.

Another health-threatening issue that attracts immense attention to study is liver diseases. Liver diseases are responsible for approximately 2 million deaths in the world annually, with cirrhosis and liver cancer as the 11th and 16th leading causes of death, respectively. Cirrhosis, the late stage of fibrosis, is mostly caused by a variety of liver conditions such as alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), hepatitis B virus (HBV) infection, and hepatitis C virus (HCV) infection [7]. These conditions also account for the main reason for chronic liver disease among the patients on the liver transplantation list in the United States [8]. Hepatocellular carcinoma (HCC) is another liver disease the development of

which is mostly caused by chronic liver diseases such as HBV and HCV and also cirrhosis [9]. HCC is the most common type of liver cancer and the third leading cause of cancer deaths [10]. Thus, as a result of the fatal consequences of liver disease, it is imperative to study liver diseases, different stages of them, and factors that cause these conditions, which indicate the urgent need to develop liver models for pathophysiological studies.

Until now, two-dimensional (2D) and three-dimensional (3D) *in vitro* models and animal models have been used for these purposes, each having its advantages and disadvantages (Table 7.1). The most commonly used models are 2D *in vitro* models, which are due to their high throughput characteristics rendering them beneficial for drug testing and discovery. However, issues such as the lack of a 3D environment and static mode make them unreliable. With the advance in biology and engineering, several methods have been developed aiming to create 3D models the most important of which are spheroids, organoids, and hydrogel/scaffold-based models. Despite this advantage and some merits that are unique to each of them, these models suffer some major setbacks: spheroids and organoids suffer from the creation of necrotic regions in their core and also a lack of reproducibility [11] and scaffold-based models experience a heterogeneity across their environment [12]. Besides, all of them lack a perfusion system and interorgan communication rendering them inefficient for mimicking human tissue or organ [12,13]. Animal models are another method to assess the efficiency or toxicity of drugs. Although these models are more realistic, the interspecies differences between humans and animals result in identifying less than 50% of DILI by animal models results [11]. Besides, animal models are mostly time consuming and expensive and of course, subjecting animals to these experiments raises ethical concerns [14,15].

According to the drawbacks of current *in vitro* and *in vivo* models, developing a new sophisticated method with a reliable outcome would be of great interest in physiological, pathophysiological, drug discovery, drug metabolism, and toxicity studies. One promising approach to address all the obstacles associated with existing models is the implementation of microfluidics devices. These miniaturized systems, so-called organ-on-a-chip (OOC), are capable of resembling the native niche of human tissues and organs by providing a multicellular, perfusion-based, 3D cell culture [16]. The idea of mimicking the organ-level function inside a microfluidic device was first proposed by Schuler et al. [17], in 2004, while they were working on capturing liver and lung interactions on a silicon chip. In 2010, Ingber et al. [18] used the term OOC in their attempt to reproduce the essential function of the alveolar-capillary interface of the human lung in a

Table 7.1 Advantages and disadvantages of common models for preclinical studies.

Model	Advantages	Disadvantages
2D cell cultures	• High throughput • Low cost	• Monoculture • Simplified architecture • Lack of ECM • Phenotype heterogeneity • Static mode • Lack of interorgan cross-talk
Animals	• More realistic compared to 2D models • Presence of blood flow • Tissue—tissue intercommunication	• Time consuming • Expensive • Interspecies differences • Unsatisfactory imaging results • Ethical issues
Organoids/spheroids	• 3D configuration • Multicellular culture • Prolonged longevity • Gradient generation • ECM Formation	• Limited size due to restricted diffusion • Simplified architecture • Irreproducibility • Static mode • Lack of interorgan cross-talk
Scaffolds	• 3D cell configuration • Multicellular culture • Prolonged longevity • Presence of ECM-like structure • Tuning scaffold properties	• Heterogeneous environment • Static mode • Lack of interorgan cross-talk
Organ-on-chips	• 3D cell configuration • Precise control over the model • Presence of ECM-like structure • Multicellular culture • Prolonged longevity • Perfusion • Gradient generation • Tissue—tissue intercommunication	• Labor-intensive • Expensive • Lack of online sensing methods • Lack of standard methods

microfluidic lung model. Since then, a lot of efforts have been made to mimic different organs such as skin [19], gut [20], heart [21], kidney [22], lung [18], and liver on a chip [23].

Liver-on-a-chip (LOC) could be a promising candidate to fulfill the requirements of the liver or liver disease models. Due to precise control over this microfluidic device, LOCs could be potentially used to assess the effect of various model features such as flow, shear stress, cell–cell interaction, and scaffold properties to create more realistic *in vitro* liver models [24–27]. As a result of advances in fabrication procedures, methods such as bioprinting could be used to enable researchers to pattern hepatic cells in the form of a liver sinusoid or liver lobule [28,29]. Besides, tuning bioink characteristics allows creating a suitable environment for cells to maintain their phenotype [24]. Organoids and spheroids could be combined with microfluidic technology to develop organoid- or spheroid-on-a-chip, which benefits the advantages of both methods [30,31]. In addition, implementing perfusion could mimic the natural transport of materials in the model. Also, a dynamic model provides the opportunity to create a unique feature of the liver: metabolic zonation [32,33]. These models could be used to assess drug toxicity and metabolism more effectively by creating a concentration of oxygen or hormones on a chip [34,35]. Moreover, liver disease and injuries could be modeled on LOCs to recreate and study different stages of the disease and also evaluate the effect of drugs on reversing or stopping its progression [36–38]. In addition, as these microfluidic devices are capable of integration to create multiorgans-on-a-chip (MOC), they could be used to investigate the interaction of the liver with other organs such as skin [39], gut [40], and kidney [16], for applications such as first-pass metabolism, and absorption, distribution, metabolism, elimination, toxicity (ADMET), off-target toxicity, and body-on-a-chip.

7.2 Liver physiology

Located in the right upper quadrant of the abdomen, the liver is the vital organ for the metabolic activity of the human body. This complex organ consists of two categories of cells: parenchymal cells (PCs) and non-parenchymal cells (NPCs) (summary is provided in Table 7.2). Hepatocytes are the hepatic PCs and the main component of the liver *in vitro* models as they are responsible for the major functions of the liver. On the other hand, NPCs include hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSECs), Kupffer cells (KCs), lymphocytes, and biliary cells—also called cholangiocytes. In this highly vascularized organ, blood is supplied through the hepatic portal vein and hepatic artery. Hepatic portal vein supplies

Table 7.2 Characteristics of important cells in liver models.

Cell	Type	Diameter (μm)	Volume (% of total)	Primary function	Markers
Hepatocyte	Epithelial cell	20–30	~ 78	• Major metabolic functions of liver, e.g., carbohydrate, nitrogen, and xenobiotics metabolism	• Albumin production • Urea formation • CYP450^a expression • MRP-2^b expression • CFDA^c staining • Staining TJ^d proteins
LSEC	Endothelial cell	6.5–11	2.8	• Permeable barrier protecting hepatocytes against blood flow • Regulate hepatic blood pressure	• CD31 expression • CD32b expression

HSC	Fibroblast	10.7 –11.5	1.4	• Fat and vitamin A storage • Producing ECM proteins	• GFAP^e Staining • CRBP-1^f expression • α-SMA^g expression • TNF-α^h expression • IL-6ⁱ expression
KC	Macrophage	10–13	2.1	• Endocytosis	

^aCytochrome P450.

^bMultidrug resistance-associated protein 2.

^cCarboxyfluorescein diacetate.

^dTight junction.

^eGlial fibrillary acidic protein.

^fCellular retinol-binding protein-1.

^gAlpha-smooth muscle-actin.

^hTumor necrosis factor-alpha.

ⁱInterleukin-6.

Adapted from Usta et al. [45].

about 75% of liver blood, which is received from the intestines and is rich in nutrients, while the hepatic artery provides the other 25%, which is oxygenated blood carried from the aorta [1,41].

The structural unit of liver tissue is called the hepatic lobule, about 10^3 of which are found in a human liver (Fig. 7.1). These hexagonal columns are composed of hepatocyte plates in a radiating pattern with discontinuous capillary branches called sinusoids, branches of hepatic artery, and portal vein wherein LSECs and KCs reside. In a liver lobule, hepatocytes are separated from sinusoids by presinusoidal space or Disse space, which is a protein-rich extracellular matrix (ECM) compartment hosting HSCs. At each apex of a lobule, there is a portal triad that consists of three conduits: a hepatic portal vein branch, a hepatic artery branch, and a bile duct branch [42–44]. Within a lobule, bloodstreams from the hepatic artery and portal vein come into contact with sinusoids and supply hepatic cells with oxygenated, nutrient-rich blood that finally will be drained into the central

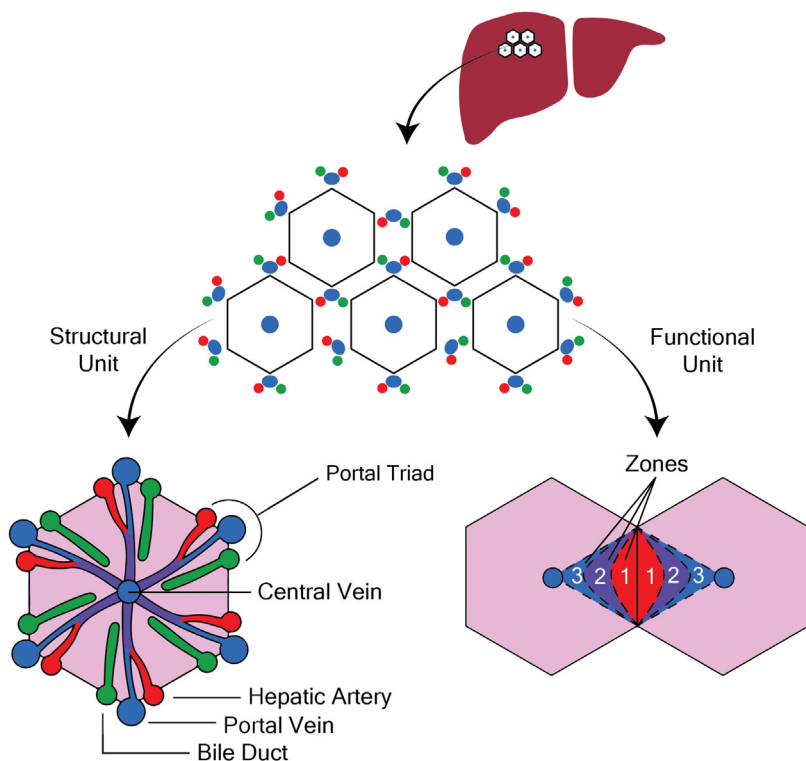


Figure 7.1 Schematic of the liver structural unit (liver lobule) and liver functional unit (liver acinus).

vein that exists at the center of each lobule. Also, the bile, excreted by hepatocytes, flows in the opposite direction in the bile duct and will be collected in the gallbladder [41].

Another important definition in studying the liver is acinus, which is considered as the functional unit of the liver (Fig. 7.1). In liver acinus, PCs and NPCs form an irregular oval-shaped space between two central veins of two adjacent liver lobules [46]. This region is commonly described by three zones due to the variation in the received oxygen and nutrient. Hepatocytes in the peripheral region of the liver lobule compose the first zone, called the periportal region. They are usually responsible for oxidative metabolisms as they receive most of the nutrients and oxygen. The region near the central vein, poor in oxygen and nutrients, is called the perivenous zone or zone 3 wherein lipid formation and drug metabolism mostly happen. The space between these two regions is called the transitional region or zone 2, which contains hepatocytes with intermediary metabolic function [1,47]. This variation is also observable in the composition of liver ECM. Collagen type I and III, and laminin are abundant in the periportal zone while ECM in the perivenous zone is rich in collagen type IV and VI and highly sulfated proteoglycan [46].

7.3 Considerations for higher physiological relevancy in an LOC

Due to the advances in biology and engineering, microphysiological systems (MPSs) could be promising *in vitro* models to mimic human tissue, organ, or body. However, according to the complex structure of some organs such as the liver, several issues must be considered so that the results of these models would be realistic. Therefore, this section aims to describe the necessary issues regarding the design of LOCs and how they impact the results (Fig. 7.2). Meanwhile, it must be noted that the purpose of creating a more complex system is not reaching an organ-level complexity. In fact, OOC designs aim to consider the critical features of the system and add the complexity to a level that fulfills the purpose of the study.

7.3.1 Cell–cell interactions

7.3.1.1 Improvement of hepatocyte activity

As mentioned earlier, most critical liver functions are carried out by hepatocytes. However, aside from homotypic interactions, heterotypic interactions remarkably influence hepatocytes' viability and functionality.

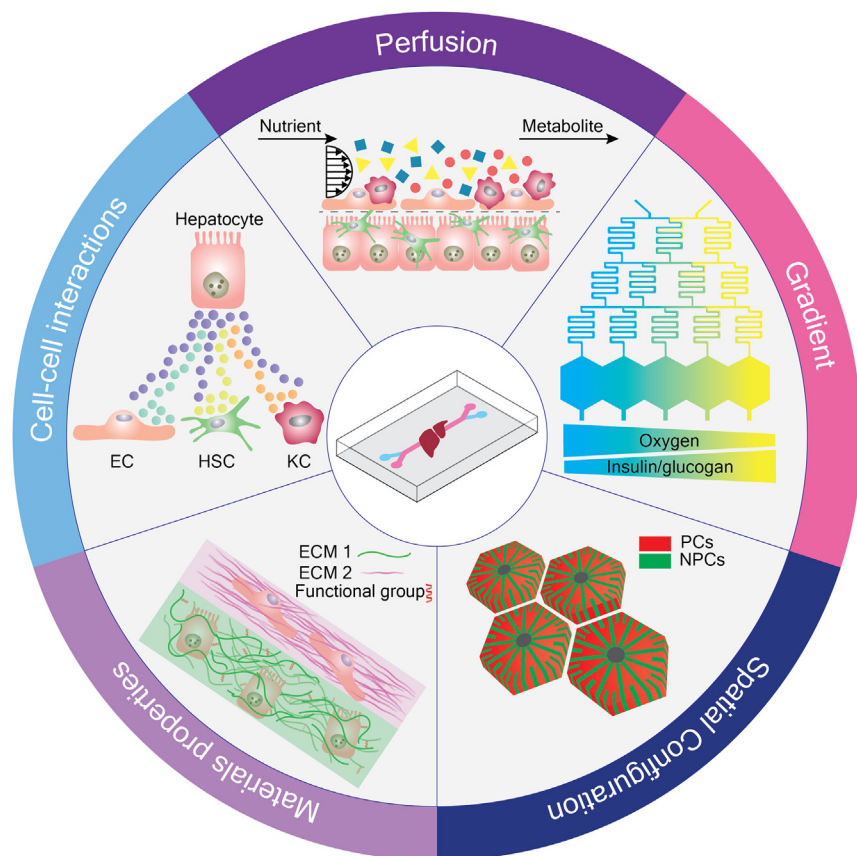


Figure 7.2 Considerations to improve physiological relevancy of LOCs.

Due to its vicinity to hepatocytes, several studies investigated the effect of HSC coculture. Lee et al. [48] studied the effect of coculture of hepatocytes with HSCs through mono- and cocultured spheroids, which were named hepatosphere and heterosphere, respectively. The scanning electron microscopy (SEM) images showed a smoother surface for the heterosphere compared to that of the hepatosphere, which indicates tighter junctions for the hepatosphere. Aside from improved morphology, urea formation and CYP450 activity were higher for the cocultured spheroids indicating a positive impact of HSCs on hepatocytes activity. In another study [25], they also explored the paracrine effect of HSCs on hepatocytes. To avoid physical contact, they cultured hepatocyte spheroids and HSCs on two connected distinct chips, and culture media from HSCs cells was

continuously transferred to the hepatocyte spheroids. Compared to the positive control, mono-culture with the flow, the spheroids did not show higher viability; however, the smoother surface of the spheroids again confirmed improved tight junctions. In addition, an elevation in albumin and urea formation was observed, which could be attributed to the fibroblast growth factor 7 (FGF7) secretion by HSCs. There are also studies showing that coculturing with HSCs enhanced ECM deposition due to their mediatory effect on hepatocytes by secretion of hepatic growth factor (HGF) and transforming growth factor-beta 1 (TGF- β 1), which ensures longevity and functionality of hepatocytes [49,50]. Other fibroblast cells such as 3T3 fibroblast have also been used to coculture with hepatocytes to upregulate their activity [51–53].

In addition, the interaction of hepatocytes with ECs is critically important for hepatocyte phenotype. Bale et al. [54] cultured LSECs with hepatocytes in a collagen gel in three different configurations—above, below, and with hepatocytes. Generally, upon coculturing with LSECs, the cuboidal morphology of hepatocytes was well preserved for a longer period and their viability was enhanced compared to the monoculture system. In setups that LSECs maintain their phenotype, albumin production was notably improved and was also stable for up to 4 weeks. CYP450 enzyme expression was also improved, but to a lesser extent compared to albumin synthesis. However, urea formation was not influenced by coculture, which was in agreement with previous studies of coculturing rat LSEC and hepatocytes [55]. Due to loss of viability and phenotype after isolation, other ECs have also been used instead of LSECs. By culturing gel-coated primary rat hepatocytes (PRHs) with gelatin-coated bovine ECs, hepatocytes maintained their cuboidal shape for up to 12 days while starting to become flattened in mono-culture after 6 days. Improved claudin-3 and MRP-2 expression and bile acid transportation in the coculture model also indicate higher morphological polarization. Increased urea and albumin formation also imply enhanced functionality [56]. Human umbilical vein endothelial cells (HUVECs) are the other source of ECs that are frequently been used. These cells play a significant role in fetal liver development; accordingly, Kehtari et al. [57] cocultured embryoid bodies with HUVECs in a polydimethylsiloxane (PDMS)-based microfluidic chip. Their results showed that the paracrine effect of these cells elevated the expression of hepatic maturation genes.

7.3.1.2 *NPCs specific functions*

Aside from improving hepatocyte morphological and functional polarization, there are some normal or disease conditions wherein NPCs play a key role. Upon activation, HSCs transdifferentiate into myofibroblast-like cells, the paracrine effect of which alters the liver environment. In this regard, incorporating them into a 3D tumor-on-chip led to their activation and as a result higher concentrations of alpha-smooth muscle-actin (α -SMA), an indicator of their activation, and epithelial-mesenchymal transition markers such as Ki-67; therefore, the coculture system is capable of resembling liver tumor more faithfully compared to hepatocyte-only culture [58]. NAFLD could also be imitated more closely by including NPCs. Creating spheroids containing HUVECs and HepG2, Lasli et al. [38] designed an LOC to recapitulate NAFLD progression. Their result demonstrated that the presence of ECs in the system increased lipid accumulation in HepG2 cells. KCs could also be included in a liver model to make advanced models for toxicology research. Creating a quadruple-cell chip, Jang et al. [59] were able to study KC-target DILI. Upon administration of JNJ-1, there was no discernible change in hepatic function while KCs depletion was confirmed by a decrease in the concentrations of IL-6 and monocyte chemoattractant protein-1. Also, the study of Verneti et al. [60] showed that in a KC-containing MPS, the addition of lipopolysaccharide resulted in the inflammation of KCs that could significantly affect the hepatotoxicity results. Thus, these results demonstrate the importance of including both PCs and NPCs in LOCs to fill the gap between in vitro models and preclinical and clinical trials.

7.3.2 Spatial configuration

7.3.2.1 *3D structure*

Besides cell–cell interactions, there are other aspects of a model that could influence cells including chemical and biochemical cues, mechanical forces, and cell–environment interactions. One of the key contributing factors in this complex interplay is a 3D compartment. 2D models fail to faithfully recapitulate cell–environment and cell–cell interactions, which hurt cellular morphology and gene expression [61]. Particularly, this is a critical issue in the case of hepatocytes as their longevity significantly decreases in 2D models. In their natural niche, hepatocytes interact in three dimensions through their three distinct domains; however, in 2D models, they are subjected to a rigid substrate on the sinusoidal domain, and instead of interacting with other hepatocytes at the lateral domain, they experience a

liquid medium. Adherence to a rigid substrate and attenuated interactions with cells and the surrounding environment result in the flattened shape of hepatocytes and adopting a different cellular pathway that leads to their dedifferentiation, and consequently, the rapid loss of viability in 2D cell cultures [62,63]. In this regard, several studies have demonstrated the positive effects of the 3D environment on specific liver functions and sensitivity toward toxicity both in monoculture [64,65] and coculture [58,66] with NPCs.

The most common type of 3D structures used in LOCs is cell-laden hydrogel models. These hydrogels are mostly fabricated from ECM proteins such as collagen, gelatin, and fibronectin. Geltrex and Matrigel are also commercial gelatinous protein mixtures in which cells are suspended and inserted into the system. In the case of bioprinting, gelatin methacryloyl (GelMA) and hyaluronic acid (HA) are generally used due to their printability and biocompatibility. The interaction of cells and these materials reduces the flattening of the cell cytoskeleton and also improves cell–cell interaction. Tuning their stiffness also helps cells maintain their phenotype [67,68]. Besides, the incorporation of GFs in them allows the regulation of the (bio)chemical cues in the cell surrounding environment and eventually improving hepatocyte functionality [69]. This is an important issue to consider in the design of LOCs because, *in vivo*, GFs are mostly bound to ECM rather than be in a soluble state. Investigating the effect of TGF β on hepatocytes, Lin et al. [70] showed that soluble TGF β resulted in the downregulation of albumin synthesis and CYP2A6 expression while TGF β absorbed on the polyelectrolyte multilayer platform improved primary human hepatocyte (PHH) activity.

Spheroids, which are scaffold-free 3D models, have also been used to recapitulate both healthy and disease conditions of the liver in LOCs [31,71,72]. They could be defined as self-aggregated 3D cells that rely on cell–cell interactions [68]. The most common methods for the formation of these cell aggregates are the utilization of hanging drop plates, micro/nanopatterned surfaces, perfused bioreactors, and low-adhesion plates [12]. Proteomic analysis of a 2D culture and spheroids from the same PHHs showed a drastic change in the proteomic signature of the PHHs in the 2D culture only after 24 h while protein expression of the PHHs cultured in spheroid form was more close to that of the fresh cells of the same donor [73]. This could be attributed to the 3D configuration, contact, and communication between hepatocytes, which result in maintaining their phenotype, prolonged longevity, and higher sensitivity compared to 2D

models [68,73]. Another advantage of spheroids is the ability of coculture of hepatocytes with NPCs in this form to improve hepatocyte functionality and create a mimetic 3D microtissue [71,74,75]. Also, they could resemble the oxygen concentration gradient in the liver lobule as peripheral cells experience different oxygen tension compared to the cells in the core of the spheroids [31].

Organoids or organ buds are other main sources of self-organized scaffold-free microtissues used in LOCs [30,76,77]. These cell aggregates are generally formed by implementing stem cells and their differentiation into the target cells to resemble organ-level function. This characteristic is notably attractive in the case of hepatocytes since PHHs, the most standard and reliable source of hepatocytes, easily dedifferentiate in an in vitro environment [78]. They also could be used to create in vitro disease models. Having obtained sample cells from patients with primary liver cancer, Broutier et al. [79] produced high-fidelity cancerous organoids that could resemble three major subcategories of primary liver cancer, and their RNA sequencing analysis showed that organoid gene expression was analogous to the respective parental tissue. Besides, like spheroids, they are capable of forming multicellular models to make fully functional organoids. Through the interaction of mesenchymal cells and ECs, Takaabe et al. [80] recapitulated organogenesis in the liver, and by incorporating induced pluripotent stem cells (iPSC) they formed vascularized 3D liver buds.

It must be noted that all these aforementioned 3D models have been used to create liver models; however, the lack of a perfusion system may hamper their reliability for long-term applications. Therefore, microfluidic devices could be a promising technology to create perfused models for proper nutrient exchange and also provide an opportunity for interorgan communication.

7.3.2.2 Precise patterning

Another important issue regarding cell arrangement in an in vitro model is spatial patterning. In any multicellular organ, different cell types are arranged in an organized structure, which is imperative for the functionality of that organ [81]. This is because, apart from the paracrine effect, other factors in the coculture system including material transport, direct contact, and junctions between neighboring cells influence the fate of cells, which renders spatial control of cells an important issue to consider [82]. Khetani and Bhatia [51] cultured hepatocytes with and without 3T3-J2 fibroblasts in a multiwell elastomeric device; each well contains 37 islands to seed

hepatocytes and places in between to seed 3T3-J2. By maintaining constant values of cell ratio and numbers, they showed that micropatterning generally improved hepatocyte activity both in mono- and coculture. Also, different patterning, which means a change in the distance between cell islands and the number of cells in each island, could affect cell functionality. Microfluidic devices could also be used to precisely pattern different cell types as laminar flow in microfluidics limits the mixing of different streams and allows precise control over their composition. Kobayashi et al. [52] prepared an anisotropic hydrogel sheet containing hepatocytes and 3T3 cells. Through three different inlets, hepatocyte-alginate suspension, 3T3 fibroblasts-alginate suspension, and spacer solution were introduced to a network of channels wherein each hepatocyte-alginate channel is sandwiched by two channels of the fibroblasts-alginate channel. Spacer solution channels created a gap to separate these sandwich structures. The extruded sheet was delivered to a gelation solution bath containing Ba^{2+} to create a stripe-patterned hydrogel sheet to be used as a liver model.

Some studies have also been dedicated to mimic liver lobule microstructure. Ho et al. [81] attempted to recreate the microstructure of the liver lobule by active patterning. After creating a stellate pattern of electrodes array on a glass, the chip was coated with collagen to improve cellular attachment. Finally, HUVECs and hepatocytes were seeded in a 2D radiating pattern employing dielectrophoresis. 95% cell viability was found in both cell types after dielectrophoresis patterning and expression of CYP450-1A1 was improved up to 43% compared to that of the non-patterned cocultured model. To create a 3D model with hepatic lobule architecture, Ma et al. [83] manufactured a multilayer PDMS microfluidic composed of a fluidic layer with a hexagonal cell chamber, channels for nutrient and cell supply, and a control layer with a pneumatic microvalve system. After preparing the device for operation, HepG2 cells were introduced to the chamber in the form of a collagen suspension whose attachment was assisted by pillar arrays to fabricate 48 hepatic chords. Then human aortic endothelial cells were added to the system to create the sinusoid-like network. Finally, a 3D coculture in a hexagonal shape was formed with improved activity over 2D and 3D HepG2 models. 3D bioprinting is another technique to seed cells in a 3D, predefined pattern. Ma et al. [29] used stereolithography to pattern human iPSC-derived hepatic cells as PCs and HUVECs and mesenchymal cells as supporting cells. In a two-step stereolithography bioprinting technique, PCs and supporting cells were patterned in an array of hepatic lobule analogs with a thickness of 200 μm to resemble the microtissue of the liver.

7.3.3 Material properties

7.3.3.1 Scaffold material

In the case of implementing a biomaterial for cell culture, biocompatibility is an unavoidable feature that must be taken into account [84]. However, as they provide the surrounding environment of the cells, their physical and biochemical properties become integral aspects of designing an in vitro model. Therefore, scaffold properties must be tuned so that they resemble the (patho)physiological niche of the target tissue [85].

Liver stiffness is a strong indicator of its condition since it gradually increases as the liver disease state progresses to fibrosis, cirrhosis, and eventually HCC [86]. This indicates that the rigidity of the hepatic cells' environment characterizes their metabolic activity. Therefore, compliance with the cell environment should be designed for its purpose [68]. As polymers are the most favorable biomaterials used for this purpose, stiffness could be tuned by polymer concentration and degree of crosslinking. For instance, Ozkan et al. [87] used 4 and 7 mg/mL collagen solutions to resemble the stiffness of healthy and cancerous liver on a chip, respectively. Matrix mechanical properties could significantly affect the diffusion of nutrients through the model and also the adhesion, migration, aggregation, and GF responsiveness of cells [88]. Desai et al. [89] studied the effect of matrix rigidity on hepatocytes by varying stiffness of gelatin-polyacrylamide gel from 75 Pa to 60 kPa. Increased rigidity profoundly impacted the cytoskeleton tension resulting in a flattened shape. It also attenuated hepatic-specific function, which was attributed to the downregulation of hepatocyte nuclear factor 4 α , a protein responsible for hepatocyte function in a 3D environment. Schrader et al. [90] observed the increased formation of actin stress fiber in HCC cell lines—associated with their dedifferentiation to mesenchymal cells—while they were cultured on a stiff polyacrylamide gel. Chemotherapy assays also showed stiffness reduced their susceptibility to apoptosis. In studying micrometastasis, Clark et al. [91] proved that matrix stiffness can determine the response of invading cells to medication and whether they will be in a quiescent or proliferative state. Matrix mechanics also could affect NPCs. Stiff matrix changed HSC morphology from round to spread-like shape with a decrease in vitamin A storage. It also led to the upregulation of α -SMA and downregulation of peroxisome proliferator-activated receptor gamma, which are indicators of HSC differentiation into myofibroblasts [92]. As anchorage-dependent cells, ECs behavior could be modulated by matrix stiffness as well. By

increasing the degree of cross-linking of the film, Chang et al. [93] showed that stiffness altered adhesion, migration, and proliferation of ECs and the effect of HGF on their behavior.

Porosity is another feature of the scaffold that could affect cells' fate. A highly porous structure facilitates the exchange of waste, nutrients, and GFs in the cell environment [41]. Besides, oxygen concentration could be affected by porosity, which is an important consideration regarding the high metabolic activity of hepatocytes [94]. An important factor about these pores is their interconnectivity, which supports the material exchange. The interconnectivity of the structure also allows better migration of cells in the cell culture environment [84]. In addition, high porosity increases the specific surface area of the structure, which is necessary for cell adhesion and improving cell seeding proficiency [95]. The size of these pores is another important factor. A study by Ranucci et al. [96] showed that although the pore size in the scaffold did not change the percentage of cell adhesion, it has a significant effect on cell morphology, polarity, and albumin production of hepatocytes after attachment. In general, interconnective pores with a size ranging from 50 to 150 μm are desirable for hepatocyte culture [95]. Utilizing the 3D printing method, Lewis et al. [97] studied the effect of macropore geometry on hepatocytes. They observed that, compared to square pores, triangular pores improved the functionality of hepatocytes, which they attributed to increased interconnectivity of the cell culture. Meanwhile, it is worth mentioning that aside from the effect of porosity on cells, cells could also alter the porous structure of the scaffold, which affects the material and drug transfer in the system. Compared to their respective controls, healthy liver cells did not change the porosity of the collagen gel while carcinoma liver cells increased ECM porosity by about 14% [87]. This change in the porosity of the ECM affects the transportation and accumulation of particles in the system.

Aside from physical properties, scaffold chemistry plays a major role in cell adhesion and differentiation. The wettability of a scaffold is the first parameter that affects cellular attachment. Generally, a hydrophilic surface increases cell adhesion and eventually cell metabolic activity [98]. Nevertheless, apart from hydrophilicity, the presence of cell-binding moieties is another contributing factor to cellular attachment. The arginine—glycine—aspartic acid (RGD) sequence is the most common motif that is used to improve cell—ECM interaction. This sequence exists in natural polymers such as collagen and gelatin. Although synthetic polymers do not usually contain this motif, this could be compensated by incorporating

RGD into their structure [99]. Lin et al. [100] cross-linked the RGD motif into a poly(ethylene glycol) diacrylate (PEGDA) hydrogel, which improved the adhesion of 3T3 fibroblasts and sustained viability of the embedded cell. To develop a bioprinted LOC, Christoffersson et al. [24] fabricated an HA-PEG-based bioink in which modification with cyclooctyne allows both cross-linking with a multiarmed azide-modified PEG and also introducing RGD motif into the system. The results illustrated that attachment of human iPSC-derived hepatocyte-like cells to cyclic RGD motif allowed the formation of large 3D structures of these cells and a considerable amount of albumin secretion, which shows the vital role of cell attachment. Galactose is another functional group that enhances hepatocyte adhesion due to the presence of asialoglycoprotein receptors on their surface. Galactose could be introduced to the material in the forms of lactose monohydrate, lactobionic acid, aminohexyl-D-galactopyranoside, 1-amino-1-deoxy-b-D-galactose, and 1-amino-1-deoxy-b-D-lactose [46]. Surface grafting of poly(styrene-co-maleic acid) and polystyrene fibers with galactose led to improved cell viability of hepatocytes in the formed spheroids and higher activity of CYP3A11, and CYP2C9 enzymes [101]. Ghodsizadeh et al. [102] also showed that compared to collagen, galactosylated collagen matrix remarkably enhanced the maturation of human embryonic stem cell-derived hepatocyte-like cells, which was proved by increased expression of proteins such as albumin, CYP1A1, and ASGR1.

7.3.3.2 Microchip material

The choice of material for manufacturing the microfluidic device is of paramount importance regarding two distinctive issues: fabrication method and physicochemical properties. The availability of a low-cost and time-efficient fabrication method enables rapid prototyping of the device without the need for complex, expensive equipment [103]. On the other hand, physicochemical properties should be taken into account as they affect cellular behavior, biochemical transportation, and the condition of the cell environment [104]. Surface chemistry can significantly influence cellular attachment in an OOC. As most materials used for microfluidic fabrication such as PDMS, thermoplastics, and glass are hydrophobic, their native surface is not a suitable environment for cellular attachment. This makes it necessary to coat the inner surface of the chip with ECM proteins or positively charged molecules [105]. Due to the diverse properties of human tissues, different cells grow on different surfaces. Indeed, substrate stiffness can influence cell migration and proliferation; therefore, it must be

tuned to mimic the tissue of the target cell [106]. By tuning the stiffness of a PDMS-based microfluidic device, Natarajan et al. [107] showed that hepatocytes cultured on a soft substrate with lower Young's modulus (2 kPa) preserved their polygonal morphology while culturing hepatocytes on a hard tissue with Young's modulus of 50 kPa resulted in an elongated, fibroblast-like morphology. The gas-permeation property of the substrate plays a major oxygen concentration, which is a key feature of a liver model due to the high metabolic activity of hepatocytes [32]. A study by Ochs et al. [108] showed substrate material could determine the equilibrium oxygen concentration in hepatocyte culture. Bale et al. [109] also illustrated that utilizing different underlying materials affects oxygen concentration and eventually long-term specific liver function. In the case of a gas-permeable substrate, ambient CO₂ penetrates through the substrate and can be dissolved into the medium and eventually affect the pH of the system [105].

In general, PDMS and thermoplastic elastomers (TPEs) are the most common materials to fabricate microfluidic devices. PDMS is an organo-silicon elastomer, which is favorable for microfluidic device fabrication due to its inert nature, gas permeability, transparency, low level of auto-fluorescence, and most importantly low-cost, straightforward, and reproducible microfabrication [105,110]. Besides, its high compliance allows creating hybrid devices and most importantly integrating valves and pumps to create perfused and high-throughput systems. However, PDMS suffers from some serious setbacks. High deformability of PDMS leads to cross-sectional deformation, which could adversely affect control of shear stress in perfused culture. Its high gas-permeation property could result in water evaporation in the system and eventually cell death due to bubble propagation and change in medium osmolarity [103,105]. Besides, the interaction of medium and PDMS results in the leaching of uncrosslinked oligomers from polymer to the medium, and also the absorption of small molecules in the polymer network renders the model unreliable and imprecise. These problems could be addressed by sol-gel coating or Soxhlet extraction [111,112]. In addition, its hydrophobic nature hampers cell adhesion, which makes surface coating of PDMS-based microfluidics with materials such as fibronectin [113], gelatin [27], and poly-L-lysine [114] a necessary pretreatment before cell seeding.

TPEs are biocompatible and generally transparent plastics including polystyrene, polycarbonate, poly(methyl methacrylate), cyclic olefin polymer, and copolymer. Due to their mechanical stability, they could be a

more reliable option for the commercialization of microfluidics. Compared to PDMS, they are more resistant to organic solvents, acids, and base, and their low gas permeation reduces water evaporation [115,116]. On the other hand, there are some problems working with TPEs. Their labor-intensive and complex manufacturing process restricts their rapid prototyping, and their bonding process is difficult due to their physicochemical properties. However, some studies have reported TPE-based microfluidic with low-cost rapid manufacturing methods to facilitate their commercialization [117–119]. Besides, like PDMS, these plastics are hydrophobic polymers that require surface modification to ensure cellular attachment. This process could be implemented by plasma and UV treatment [76,120] or dynamic coating with hydrophilic materials such as collagen [121,122].

7.3.4 Perfusion

7.3.4.1 *Material exchange*

Due to the highly vascularized structure of the liver, a perfused model could help mimic the hepatic niche. First, it provides control over nutrients, oxygen, and waste exchange [11]. In static cultures, the medium is changed on a regular basis, which leads to the accumulation of wastes and metabolites and also depletion of oxygen and nutrient in the cell-surrounding environment. Oxygen transport is of high importance in the case of hepatocytes as they are metabolically active and produce energy through oxidative phosphorylation; therefore, their high rate of oxygen uptake could create a hypoxic condition [123,124]. Besides, impaired exchange of material restricts cell number in a static model, which hampers cell–cell interaction and eventually fails to resemble the *in vivo* environment [84]. On the other hand, in a dynamic model, continuous flow supplies cells with a nutrient-rich environment and the ability to remove wastes and helps them to maintain their phenotype [125]. Tanaka et al. [126] studied the effect of a continuous medium flow on culturing hepatocytes on a microchip. The results showed that lower flow cannot supply enough nutrients and oxygen, which resulted in oxygen depletion and a decrease in cell density. Higher flow rates also were not effective and led to cell wash in the system. However, a proper flow rate markedly improves the viability and morphology of the cells in the microchip culture. Lee et al. [25] also investigated the effect of flow on their viability of hepatocyte spheroids. The results showed almost 95% viability for spheroids exposed to medium flow—with proper flow rate—while there was a significant decrease in viability of the static mode over 13 days. Studies also show that under

proper flow rate, both in mono- and cocultures, specific activities of hepatocytes such as clearance rate, expression of CYP450 enzymes, and formation of urea and albumin significantly improved [127,128].

7.3.4.2 Shear stress

Another merit of perfusion is the incorporation of shear stress into the system. Due to the transduction of mechanical cues to biological responses by cells, mechanical stimuli influence cell viability, morphology, signaling, functionality, and gene expression [129]. Hence, a flow imposing shear stress on the culture is of significance to resemble what cells experience in the human body. In a study, Tanaka et al. [26] investigated the impact of shear stress on hepatocytes. The results illustrated that low shear could affect the morphology of cells and also improve albumin synthesis. In contrast, high shear stress, by increasing the flow, resulted in lower albumin synthesis even lower than that of static models, which implies that although cells were supplied with more nutrients, high mechanical stress negatively impacts cell functionality. Wang et al. also showed implementing flow in the system improved the survival of cells in developing 3D hepatic organoids. Studying the maturation of HepaRG, Jang et al. showed that compared to a one-side channel, a two-side perfusion model promoted cell–cell interaction, bile exertion, and larger clusters of cells. This is because the two-side perfusion model can resemble the canals of Herring, wherein cells are exposed to shear from two sides.

Shear stress modulates NPCs' activity as well. Sera et al. [130] studied the effect of shear on HSC migration in a monolayer cell culture by a scratch-wound assay. Results showed that not only did the shear affect the migration distance, but also it affected the migration direction so that in downstream, HSCs migrated in the direction of the flow instead of migrating toward the wound. Besides, shear stress significantly influenced the expression of platelet-derived growth factor receptor beta in downstream. Mechanical stimuli also induce Krüppel-like factor 2 expression by LSECs that changes the expression of vasoactive agents such as endothelial nitric oxides synthase (eNOs). Accordingly, shear stress affects nitric oxide production by LSECs [131]. Also, the study of Du et al. [132] showed that upon implementing a shear flow in a quadruple-cell LOC, HGF expression of NPCs was increased by 332% and 484% in the absence or presence of hepatocytes, respectively.

Meanwhile, it is worth mentioning that in natural liver conditions, hepatocytes are not subjected to shear stress as they are protected by the

LSECs and the space of Disse [113]. Therefore, this configuration could be taken into account to avoid the negative impact of mechanical stress on hepatocyte function and morphology and simultaneously benefit from a dynamic model. By means of lithography, a set of parallel microchannels was created on a chip that enables nutrient and waste exchange while protecting hepatocyte cells from being exposed to shear [65,133]. Another method is implementing a porous permeable membrane made of materials such as polycarbonate [128], polyethylene terephthalate [134], and polyester [132]. In this configuration, hepatocytes and ECs are seeded on the lower and the upper side of this membrane, respectively, to avoid direct contact with the flow with hepatocytes.

7.3.5 Gradient

As mentioned earlier, the heterogeneity in the metabolic activity of hepatocytes depends on their spatial location in one of the three zones in liver acinus. This variation manifests itself as a gradual change in the efficiency of activities such as amino acid breakdown, glucose metabolism, xenobiotics metabolism, and lipid formation [113], and structural variations such as cell composition, cell morphology, and ECM composition [124]. One of the contributing factors in this phenomenon is oxygen tension. Lee-Montiel et al. [32] implemented a proper flow rate to create different hepatic zones separately on a chip. The results demonstrated that while the production of urea and albumin was increased in zone 1, the expression of α 1AT and CYP2E1 was more significant in zone 3. Based on the maximum oxygen uptake rate by hepatocytes, Li et al. [47] optimized flow rates in a microfluidic device to establish an oxygen tension gradient that encompasses all three zones in liver acinus. While higher mitochondrial membrane potential was observed in zone 1 due to the higher oxidative phosphorylation, higher lipogenesis led to elevated lipid storage in zone 3. This zonation affected NPCs as well. The addition of TGF- β to the medium showed higher expression of α -SMA by HSCs in zone 3, which indicates higher proliferation and activation of them in this region. Tonon et al. [135] studied the effect of oxygen gradient on the phenotype of differentiating hESCs. After the formation of hepatic endoderm (at day 8), the oxygen gradient was applied to the microchamber utilizing an oxygen-depleted stream that stimulates the hypoxia on one side of the channel while the other side is exposed to atmospheric air. On day 17, CYP3A4 measurements showed a gradient expression of this enzyme similar to the liver environment. As lactate was used as the substrate, higher glycogen was

observed in the oxygen-rich part of the chamber. Also, low oxygen concentration resulted in the downregulation of cyclin A and upregulation of cyclin D1 that indicate the inability of cells in this region to progress from the G1 to the S phase of the cell cycle.

The ratio of hormones and chemicals can also play a role in liver zonation. Due to laminar flow through microchannels, mixing is highly unlikely to happen and different streams can flow side-by-side culminating in a smooth gradient of a certain molecule [136]. Kang et al. [113] used a concentration gradient generator to induce a gradient in the insulin-to-glucagon ratio and achieve a gradient of metabolic activity on a chip. The region with higher glucagon stored a lower amount of glycogen as glucagon induces gluconeogenesis and glycogenolysis while the region with higher insulin resembles zone 3 with enhanced glycogenesis. This gradient can affect nitrogen metabolism by its impact on carbamoyl phosphate synthetase 1 activity, an enzyme that plays a major role in the production of urea from glutamine and ammonia. The results showed an elevated amount of this enzyme by glucagon and its attenuated expression as the glucagon-to-insulin ratio increased. Using the same approach for creating the aforementioned gradients, McCarty et al. [33] guided a 3-methylcholanthrene gradient on a chip to induce CYP activity and glutathione conjugation. Following allyl alcohol treatment, reduced glutathione conjugation caused higher cell death in the zone 1-like region while induced expression of CYP enzymes increased acetaminophen (APAP) digestion and eventually cellular damage in Zone 3-like region. These findings indicate that current *in vitro* models with no gradual activity over – or underestimate the results of hepatotoxicity or functionality of hepatocytes.

7.4 LOC studies

7.4.1 Physiological models

The main goal of developing OOCs is to create mimetic *in vitro* models for drug screening and toxicity. Accordingly, several designs have been proposed for LOCs to study the effects of drugs and chemicals on liver cells (summary in Table 7.3). The most studied drug for toxicity assays is APAP. Bhise et al. [31] used an extrusion-based bioprinter to print a suspension of hepatic spheroids in GelMA on a chip. APAP-treated spheroids showed a significant decrease in their metabolic activity and MRP-2 expression compared to that of the untreated culture. The metabolic activity increased

Table 7.3 Summary of the recently proposed LOC models for hepatotoxicity application.

Ref.	Cell type	Design description	Experiment period (days)	Summary of the liver model	Drug/chemical candidate	Drug/chemical treatment results
[31]	• HepG2	• Bioprinting spheroid-laden GelMA on a PDMS chamber • Reversible sealing of the chamber facilitates access to cells	30	• Secretion of liver activity biomarkers decreased per cell, but their production for the whole chamber remained in a comparable range throughout 30 days	• APAP	• Decreased hepatic activity following APAP treatment • Attenuated MRP-2 expression • Decreased nuclei density implying apoptosis
[138]	• HepG2	• Seeding the bioreactor with HepG2-contained collagen suspension • Online measurement of oxygen using oxygen-sensing beads • Online measurement of lactate and glucose using enzyme-based sensors	30	• Improved enzymatic activity detected by increased expression of CYP450 enzymes in the dynamic mode	• Troglitazone • Rotenone	• Dose-dependent mitochondrial damage due to drug treatment • TC₅₀ values of 12.5 and 285 μM for rotenone and troglitazone, respectively • Detecting mechanisms of toxicity caused by rotenone and troglitazone treatment

[139]	• PHH	• A moderate-throughput device containing 12 microsystems to culture PHHs in collagen-coated chambers	13	• Upregulation of genes related to phases 1 and 2 drug metabolism compared to the petri dish	• Midazolam • Phenacetin	• A 300-fold higher concentration of APAP, as a metabolite of phenacetin, compared to 2D model • 5000-fold and 100-fold higher concentrations of 1-OH midazolam and 4-OH midazolam, respectively, compared to the Petri dish • Detection of glucuronide conjugate of midazolam only in the LOC
[60]	• PHH • EA.hy926 • LX-2 • U937	• Sequential seeding of the four hepatic cells in a collagen matrix	28	• Self-assembly of cells in a partial sinusoidal structure	• Troglitazone • Nimesulide • Trovafloxacin • Levofloxacin	• Impaired metabolic activity, and bile efflux and induces ROS generation

Continued

Table 7.3 Summary of the recently proposed LOC models for hepatotoxicity application.—cont'd

Ref.	Cell type	Design description	Experiment period (days)	Summary of the liver model	Drug/chemical candidate	Drug/chemical treatment results
[142]	• PRH • RHSC	to manufacture a dynamic LOC • Seeding a mixture of the two types of cells to form a scaffold-free organotypic culture on a micropatterned, collagen-coated PDMS membrane • Mimicking flow pattern of hepatic lobule	14	• Detect the phases 1 and 2 drug metabolites • Increased expression of α-SMA and COL1A2 upon transdifferentiation of LX-2 cells by methotrexate • Higher albumin production and CYP enzyme expression in the LOC model • Regulation of hepatic functions by exposure to rifampin and ketoconazole	• APAP	upon troglitazone exposure • Rapid LDH^a production and cell apoptosis caused by nimesulide • Effect of LPS^b-induced inflammation of U937 on hepatotoxicity • Long-term and higher sensitivity of cells in the LOC model compared to petri dish culture • Zonal toxicity of APAP in the direction of the flow

[132]	• PMH^c • LSEC • HSC • KC	• Culturing PMH in a lower chamber with an upper chamber for perfusion • A separating porous membrane with HSCs seeded on the lower side and KCs and LSECs on the upper side	—	• Improved albumin production of hepatocytes and HGF secretion of NPCs upon perfusion • Complementary role of hepatocytes and NPCs on each other indicated by improved HGF and albumin production	• Phenacetin • Dextromethorphan	• Superior activity of CYP2D6 and CYP1A2 upon coculture and dynamic mode for digestion of dextromethorphan and phenacetin
[143]	• PHH	• Injecting a suspension of cells in collagen into the liver MPS	28	• Elevated expression of hepatocyte nuclear factor 4 α in the liver MPS • Enhanced formation of canalicular structures and polarization of hepatocytes in the microfluidic design	• Aflatoxin B₁	• Dose-dependent response to toxicity with high LDH secretion from the model while exposed to 30 μM of the drug

Continued

Table 7.3 Summary of the recently proposed LOC models for hepatotoxicity application.—cont'd

Ref.	Cell type	Design description	Experiment period (days)	Summary of the liver model	Drug/chemical candidate	Drug/chemical treatment results
[144]	• PRH	• Culturing hepatocyte spheroids in a multilayer perfusion incubator chip • Integrating active debubbler and bubble trap to avoid further connector for this purpose • Inserting a heater into the chip to avoid the use of incubator	14	• Higher urea and albumin production of the LOC compared to a laminar flow bioreactor and static models • Improved experiment success in the microfluidic design • Enhanced hepatic functionality in spheroid culture compared to the collagen-sandwich model	• Diclofenac • APAP	• Higher sensitivity of spheroids toward APAP compared to the collagen-sandwich model • Calculated IC50 values of 495.61 μM and 22.51 mM for diclofenac and APAP, respectively, in acute toxicity studies • Calculated IC50 values of 50.86 μM and 2.391 mM for diclofenac and APAP, respectively, in chronic toxicity studies

[137]	• HepG2 • HUVEC	• Inserting HepG2- and HUVEC-laden collagen suspensions into the model • Two channels with the upper one for medium/ growth factor and the lower channel for metabolites	7	• Migration of HUVECs toward the medium/ growth factor flow and form a thin line aggregation • More than 80% viability of cells and considerable hepatic activity up to 7 days	• APAP	• Time- and dose-dependent decrease in cell viability caused by APAP stimulation • Significant reduction in albumin and urea production due to mitochondria damage
[145]	• PMH	• Developing microtrench topology on the chip to mimic liver sinusoid and cord-like arrangement of hepatocytes	28	• Heparin coating, deep microtrench topology, and perfusion improved albumin and urea secretion • Perfusion affects the long-term CYP activity of the chip	• APAP • Chlorpromazine • Tacrine	• Improved correlation between IC50 and LD50 using the bioartificial liver • Higher sensitivity of the model for chronic toxicity studies compared to multiwell plate
[128]	• PHH • EA.hy926 • LX-2 • U937	• Multilayer model with BME^d- encapsulated	8	• Proving active transport of fluorescent CLF by HePG2 cells	• APAP • Omeprazole • Rifampicin • Ciprofloxacin	• Higher sensitivity of the system to drug toxicity due to the

Continued

Table 7.3 Summary of the recently proposed LOC models for hepatotoxicity application.—cont'd

Ref.	Cell type	Design description	Experiment period (days)	Summary of the liver model	Drug/chemical candidate	Drug/chemical treatment results
		HepG2 cells in the middle surrounded two polycarbonate porous membrane • The upper membrane was seeded with LX-2 cells on one side, and EA.hy926 and U937 cells on the other side • Two separate channels for artificial blood and bile flow		from artificial blood to bile flow • Superior functionality of hepatocytes by mimicking the sinusoid architecture and implementing shear flow	• Amiodarone	physiologically relevant environment • TC50 values of 9.8 mM, 1153.4 μM, and 315.1 μM for APAP, Rifampicin, and Amiodarone respectively • Better in vitro –in vivo correlation compared to 96-well plate • Illustrating the drug–drug interaction in the LOC that was similar to the PRH behavior

[28]	• HepaRG • HUVEC	• Using poly(ethylene/vinyl acetate) as the substrate material to print the chip • Printing HepaRG-laden liver dECM and HUVEC-laden gelatin to mimic liver sinusoid • Two separate channels for artificial blood and bile flow	7	• Superior biliary system in the two-channel device, compared to the only one-channel chip, proved by biliary cells' markers • Enhanced hepatic activity of the two-channel device due to proper disposal of toxins and wastes	• APAP	• Higher sensitivity of the LOC to drug toxicity proved by a significant drop in the functionality of hepatocytes
[59] ^e	• Primary hepatocyte • LSEC • HSC • KC	• A separating porous membrane with specific ECM coating for rat, dog, and human hepatic cells • An epithelial channel consists of hepatocytes	14	• Comparable or higher CYP activities both in dual- or quadruple-cell LOCs compared to freshly isolated hepatocytes	• APAP • Bosentan • JNJ-1 • JNJ-2 • JNJ-3 • Fialuridine • Methotrexate • Fasiglifam	• Detection of APAP toxicity for both PCs and NPCs • In vivo-relevant and species-specific toxicity response upon bosentan administration • Activation of HSCs by

Continued

Table 7.3 Summary of the recently proposed LOC models for hepatotoxicity application.—cont'd

Ref.	Cell type	Design description	Experiment period (days)	Summary of the liver model	Drug/chemical candidate	Drug/chemical treatment results
		overlaid with Matrigel • An endothelial channel cultured with KCs, HSCs, and LSECs • Both channels were perfused with fresh medium				methotrexate treatment • Detecting JNJ-1 toxicity toward KCs • Observing dose-dependent lipid accumulation upon fialuridine administration • Identifying interspecies differences by JNJ-2 and JNJ-3 administration • Illustrating idiosyncratic DILI upon fasiglifam administration to the chip

^a*LDH*, Lactate dehydrogenase.^b*LPS*, Lipopolysaccharide.^cPrimary mouse hepatocytes.^dBasement membrane extractant.^eIn this study LOC was made of three different species' hepatic cells: rat, dog, and human.

by 78% compared to the first day of cell culture. In the coculture of HUVECs and HepG2 in a microchip, Mi et al. [137] showed that APAP exposure caused mitochondria damage, which led to insufficient energy supply and eventually cell death. Urea and albumin production of hepatocytes were also declined; however, the decrease in urea secretion was sharper compared to albumin synthesis. This phenomenon was attributed to the formation of citrulline, an intermediate of ammonia metabolism, in mitochondria. As APAP caused mitochondrial damage, restriction of citrulline formation significantly interrupts urea production. APAP is also toxic to NPCs. A study by Jang et al. [59] in a quadruple-cell LOC showed APAP exposure reduced glutathione and ATP in both PCs and NPCs and eventually induced reactive oxygen species (ROS) generation. Verneti et al. [60] studied the toxicity of troglitazone in liver MPS. Their result showed decreased albumin and urea secretion in a dose-dependent manner. Besides, the accumulation of 5-chloromethylfluorescein diacetate (CMFDA) proved inhibition of bile efflux in the HepG2 cells. By online measurement of oxygen concentration, Bavli et al. [138] illustrated that the toxicity of rotenone and troglitazone is mostly dominated by mitochondrial damage rather than toxic metabolites. They also detected the change in the metabolic pathway of hepatocytes upon drug administration. Comparing the drug metabolism of an LOC to a Petri dish model, Jellali et al. [139] showed that hydroxylated metabolites of midazolam were significantly higher in concentration in the LOC model and the glucuronide-conjugated form of midazolam was only found in the LOC. These results illustrate the superior capability of LOC models to detect the hepatotoxicity of different drugs and their metabolic pathways.

Aside from the type of the candidate drug, Jang et al. [59] studied the effect of the cross-species differences in liver toxicity responses by using the same quadruple-cell LOC models of rats, dogs, and humans. JNJ3 is a drug that is withdrawn from development due to hepatotoxicity to both humans and dogs. Treating dog and human chips with this drug showed an increased expression of aspartate aminotransferase, alanine transaminase, and glutamate dehydrogenase, which are signs of hepatic injury that were found in the drug developmental studies. JNJ2 is another drug that was discontinued due to liver fibrosis, which was proved by an elevated level of α -SMA in rats. In accordance with the studies of rodent species, JNJ2 treatment induced α -SMA expression and decreased albumin synthesis in the rat chip. In contrast, the malfunctioning of HSCs and hepatocytes was not found in the human chip implying the inefficiency of animal models and also the importance of interspecies differences in liver toxicity.

Throughput is another important aspect of LOCs that ensures their applicability in the pharmaceutical industry. Tan et al. [140] developed an MPS called the PREDICT-96 platform, which consists of PREDICT-96 array and PREDICT-96 pump. The PREDICT-96 array provides two channels for each of the 96 LOC chambers: the upper channel for the culture of hepatocytes in a collagen sandwich and the bottom channel for medium circulation. A 384-well plate was aligned to this layer that provides access to inlets and outlets of both channels. The PREDICT-96 pump displaces the media from the outlet to the inlet to supply media circulation. Ma et al. [141] also used a microwell design to develop a high-throughput LOC to improve the efficiency of spheroid culture. In this model, HepG2 cells were inserted into arrays of V-shape concave microwells, which were coated with Pluronic F127 to avoid cell adhesion. A polyethylene terephthalate microporous membrane was also used to inhibit the negative impact of shear on hepatocytes and resemble the EC barrier. After spheroid formation, a proper perfusate enables a simultaneous culture of 1080 spheroids in an active mode. Besides, the nonadherent surface of the microwell and the reversible sealing of the device facilitates the collection and analysis of the cells.

7.4.2 Pathophysiological models

7.4.2.1 NAFLD

NAFLD, a subcategory of fatty liver diseases, begins with steatosis and its progression causes nonalcoholic steatohepatitis (NASH), and in later stages, fibrosis and eventually cirrhosis [146]. Gori et al. [65] induced hepatic steatosis in a microfluidic device by adding palmitic acid and oleic acid to the system. The accumulation of triglycerides was observed in HepG2 cells, which is an indicator of steatosis. Compared to the 2D model, the level of fat accumulation was lower in the LOC, which implies the improved activity of fatty acid β -oxidation, a pathway responsible for triglyceride catabolism in the liver. Steatosis could also be induced by drugs: a pathway that was observed in the study of Ehrlich et al. [147]. Treating HepG2 organoids on a chip with valproate and stavudine led to a fourfold and a threefold increase in intracellular lipid, respectively. Bulutoglu et al. [148] showed that oxygen deprivation was correlated with an increased level of lipid accumulation, which was attributed to the role of oxygen in FFA oxidation. Increasing the initial concentration of linoleic acid resulted in less distinct lipid accumulation along the oxygen gradient implying that the progression of NAFLD could lead to losing lipid zonation and inducing

severe situations such as cirrhosis. Lasli et al. [38] seeded HepG2/HUVEC spheroids in interconnected holes resembling the interaction of hepatic lobules. Their results illustrated that while HUVECs did not store fat, incorporating them into the system increased lipid accumulation in HepG2 cells. Also, HUVEC-containing spheroids showed a delayed recovery in response to drug treatment compared to HepG2 spheroids. Suurmond et al. [71] developed this model by incorporating KC, which resulted in increased lipid storage in diet-induced steatosis. Activation of KCs was also observed by ROS generation and upregulation of TNF- α and IL-6, which could be due to the paracrine effect of FFA-induced hepatocytes. It also implies the progression of the disease from steatosis to NASH as activated KCs are correlated with the NASH state of the disease. In contrast to the HepG2/HUVEC model, HepG2/HUVEC/KC spheroids were incapable of achieving full recovery, which shows the critical role of KCs in the progression of NAFLD. However, using HepaRG instead of HepG2 led to full recovery of KC-contained spheroid, which indicates the importance of cell source for in vitro modeling of NAFLD.

7.4.2.2 ALD

ALD is another subtype of fatty liver disease that could progress to hepatitis, fibrosis, and eventually cirrhosis [72]. Zhou et al. [36] studied the reciprocal interaction of hepatocytes and HSCs and the mechanisms involved in the activation of HSC upon alcohol liver injury. Their results showed that hepatocytes, as the initiator of this molecular pathway, uptake alcohol, which led to their alcohol injury and eventually expression of TGF- β 1. Having been exposed to TGF- β 1, HSCs were activated and upon their activation, they express TGF- β 1 as well. Also, by adding alcohol-injured hepatocytes and anti-TGF- β 1 neutralizing antibodies to HSCs, the amount of TGF- β 1 secretion by HSCs was reduced as TGF- β 1 was neutralized by an antibody that minimizes the paracrine effect of injured hepatocytes on HSCs. A study by Lee et al. [72] showed that treating mono-cultured spheroids with alcohol caused a dose-dependent surface roughness of the spheroids due to reduced cell–cell interaction. Also, the higher expression of α -SMA and the appearance of a fibrous structure in the cocultured spheroids demonstrated the activation of HSCs. Considering the accumulation of calcein as a sign of MRP-2 activity in bile transport, mono-cultured spheroids treated with ethanol were not able to retain this ability while in the cocultured spheroids, this accumulation reappeared, which was an indicator of HSCs function in the regeneration of damaged

liver tissue. Deng et al. [149] investigate the effect of ALD on NPCs in a quadruple-cell LOC. Treating with alcohol-containing perfusate dramatically reduced VE-cadherin expression implying the damage of TJs between ECs and increased permeability of the EC barrier. eNOs expression ECs also decreased, which reduced nitric oxide production of ECs. Increasing ethanol concentration also led to a higher expression of vascular endothelial growth factor and α -SMA by HSCs and also their proliferation, which indicated their activation.

7.4.2.3 Fibrosis

Hepatic fibrosis is a state of the liver that is caused by the activation of HSCs and their transdifferentiation into ECM-secreting cells. Accordingly, the deposition of ECM in the space of Disse and sinusoidal capillarization disrupt blood flow and functionality of parenchymal tissue [150]. Lee et al. [151] 3D printed an LOC containing hepatocytes, activated stellate cells, and ECs. The results showed that activated stellate cells contributed to higher expression of fibrotic markers including α -SMA, and vimentin. In addition, higher secretion of collagen type 1 was observed in the liver fibrosis model compared to the normal group. To capture the natural progress of NAFLD to fibrosis, Cho et al. [152] encapsulated hepatocytes, ECs, HSCs, and KCs in GelMA and cultured this liver microtissue with FFA to induce fibrosis. FFA supplementation led to the activation of HSCs and consequently phenomena such as neovascularization and deposition of ECM components such as fibronectin and collagen type I and III. Besides, IL-6 expression was higher, which was attributed to the interaction of activated HSCs with KCs. Compared to the steatosis state of the disease model [38], the fibrosis state was irreversible without medication, which indicates a higher level of inflammation and progression of NAFLD to fibrosis. Moreover, the hepatic fibrosis-on-a-chip model showed a dose-dependent response to pirfenidone, which implies the sensitivity of the model to the treatment method and its application for studying and treatment of hepatic fibrosis.

7.4.2.4 HBV

HBV is a member of the hepadnavirus family that replicates in the liver and encodes proteins involved in the progression of HCC. This virus enters hepatocytes, then through a cascade of events, it will be replicated and secreted from the host cell that led to the transfection of the virus [153]. Studying HBV in a microfluidic chip, Sodunke et al. [153] infected HepG2

and PRH cells with cationic lipids containing cDNA of the HBV genome and enhanced green fluorescent protein plasmid. The presence of these plasmids allows for determining 40% and 10% transfection for HepG2 and PRH cells, respectively. This difference in transfection originates from the lower receptivity of primary cells. Therefore, in another method, the recombinant adenovirus encoding HBV genome was used for PRHs. This method led to 80% transfection efficacy in PRHs and also detectable HBV replication by polymerase chain reaction. The natural initiation of viral invasion cannot be recapitulated in vitro unless using PHHs. As PHHs rapidly lose their phenotype in a 2D culture, Kang et al. [154] developed a liver-sinusoid-on-a-chip that comprises two channels with separate channels for culturing bovine aortic endothelial cells and PHHs, respectively. They inserted an HBV-containing flow at a 200–250 genome equivalent (GE) to the PHH channel that resulted in direct infection of these cells with an infection efficiency of 79%. Another problem with conventional 2D models is the excessive amount of GE, up to 500 GE of HBV per cell, required for infection. Due to a higher level of PHH polarization, Ortega-Prieto et al. [37] were able to cause infection with GE/cells value as low as 0.05 in a liver MPS. Tenofovir alafenamide treatment decreased HBV DNA replication while antigen expression was unchanged demonstrating that the sensitivity of the model is comparable with the liver niche. Besides, in the case of coculturing with KCs, these cells could not control the replication of HBV DNA and did not secrete IL-6 and TNF- α unless in response to another stimulus, lipopolysaccharide. These results suggest that HBV suppresses the innate immune response of both hepatocytes and liver macrophages.

7.4.2.5 Cancer study

HCC, the second main cause of cancer-related deaths, is mostly associated with the last stages of chronic liver diseases such as hepatitis infection, NAFLD, and ALD. LOC could be used to model liver cancer to study the involved mechanisms, different stages of the disease, and developing new drugs and therapeutic methods [155]. Chen et al. [58] studied a coculture of Hep1-6 and JS-1 stellate cells to recapitulate a liver cancer microenvironment. Activation of JS-1 cells manifested in a higher level of α -SMA in the coculture system. Besides, following chemotherapy with paclitaxel, the viability of JS-1 cells in the coculture system was improved compared to that of the mono-culture of JS-1 cells, which implies that their activation by Hep1-6 increased their resistance to paclitaxel. Feng et al. [156] cultured

liver tumor HepG2/3T3 fibroblasts-containing spheroids in an active microfluidic chip that comprises four chambers with individual valves to screen combinatorial and/or sequential effects of three different therapeutic methods. The results showed that neither magnetic nanoparticles nor RAW 264.7 macrophages could effectively penetrate the fibroblast–collagen matrix. However, a combinatorial/sequential approach of these two methods accompanied by hyperthermia treatment proved to be effective. Wheeler et al. [157] seeded breast cancer cell lines in an established hepatic tissue to recapitulate micrometastasis. Although confocal microscopy showed that cancer cells penetrated the hepatic tissue, no significant change was observed in hepatic-specific functions that resemble the first stages of micrometastasis. Incorporating NPCs into the model with MDA-MB-231 cells resulted in a nonproliferative microenvironment, which was confirmed by an enhanced level of cancer attenuation signals such as follistatin, while the interaction of NPCs with MCF7 cells elevated the levels of proinflammatory cytokines and cancer-associated factors.

7.5 MOC

Another important aspect of liver activity is its interaction with other organs such as the intestine, skin, heart, and kidney that could be captured in MOCs (Fig. 7.3). These integrated models could present several opportunities for biological studies. One is in vitro liver models with results comparable to that of the in vivo models. A study by Chen et al. [158] showed that gut MPS can regulate the functionality of the liver MPS. While some of the processes involved in the cell division in the liver were upregulated, the metabolic processes such as bile acid synthesis, steroid metabolism, and amino-acid betaine catabolism were downregulated. Developing a microfluidic gut–liver system, Lee and Sung [159] simulated the absorption of FFAs by the intestine and then their accumulation in the liver (Fig. 7.3a). Compared to the 2D gut design, a 3D model resembling intestine villi sharply increased lipid accumulation in HepG2 cells due to the higher specific surface. Also, the administration of butyrate and TNF- α confirmed that the altering expression of TJ proteins can alter the integrity of the gut barrier and eventually hepatic steatosis. De Gregorio et al. [160] connected an intestine MPS to a liver MPS through a microchannel to inquire about the effect of the gut on ethanol-induced injury in hepatocytes. Compared to direct exposure of HepG2 cells to ethanol, the administration of alcohol to the intestine chamber reduced the amount of

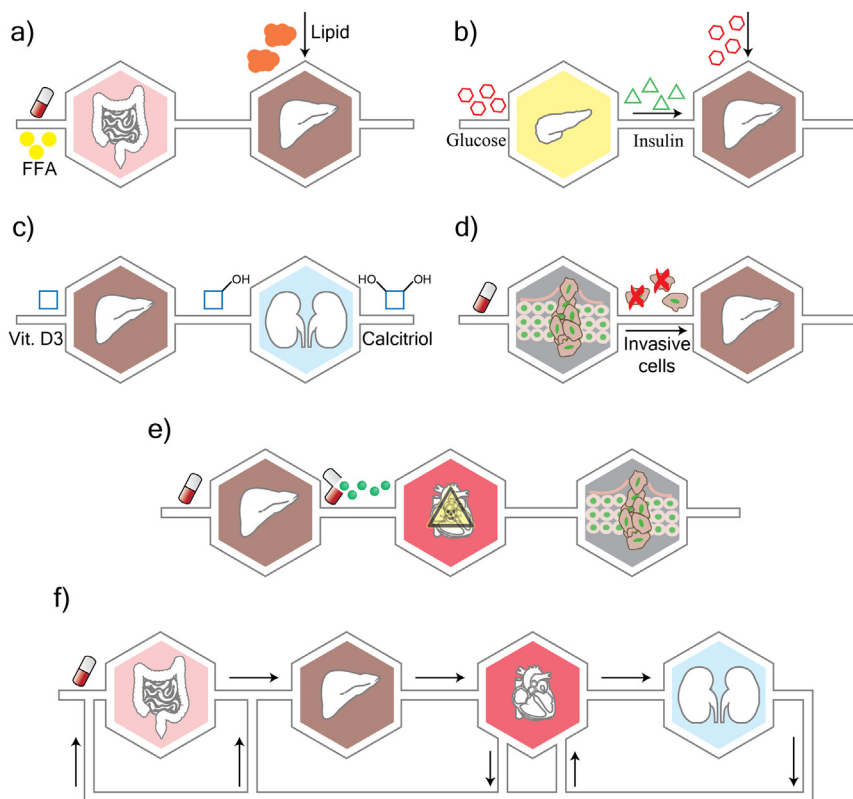


Figure 7.3 Applications of MOCs in liver studies: (a) regulation of lipid accumulation in the liver MPS by altering the diet and medication of the gut MPS, (b) regulation of glucose uptake in the liver MPS by having a pancreas MPS in the upstream, (c) transformation of vitamin D3 to calcitriol in a liver-kidney-on-a-chip, (d) recapitulating metastasis and observing the effect of anticancer drug, (e) off-target toxicity of drugs and their metabolites, and (f) discovering pharmacokinetic and pharmacodynamic behavior of drugs.

ROS expression and lipid formation in hepatocytes. Also, the presence of TJs and a network of permeability glycoproteins indicated the protective role of the intestine in alcoholic liver injury. Interaction of the liver with the pancreas is also of high importance because its malfunction cause type 2 diabetes. Bauer et al. [161] designed an MOC with pancreatic islet microtissues in the upstream and HepaRG/HSC spheroids in downstream of a microchannel (Fig. 7.3b). The cross-talk between the two cultures manifested in lower glucose concentration in the media, which is a sign of induced glucose consumption of hepatocytes due to the insulin secretion of pancreatic islets.

Another advantage of MOC systems is that they enable mimicking some biological phenomena in the human body, which are affected by the interaction of the liver with other organs. Through the interaction of a liver and kidney chamber on a chip, Theobald et al. [16] were able to recapitulate hydroxylation of vitamin D3 first in the liver and subsequently in the kidney that led to the formation of its bioactive form (Fig. 7.3c). In the preceding, it was mentioned that an LOC is applicable to study micro-metastasis; however, in an MOC it is possible to study the dissemination of the metastatic cells from tumor tissue, their circulation, and eventually invasion to healthy tissue. Designing a chip containing a liver and a cancerous intestine chamber, Skardal et al. [162] showed that after 14 days, HCT116 cells entered the circulation, and after 2–3 days, migrated in the liver chamber (Fig. 7.3d). Besides, the susceptibility of the model to drugs was confirmed by delayed migration due to marimastat treatment and a dose-dependent response to 5-fluorouracil exposure.

While the liver is the frequently used model to detect the toxicity of drugs to the human body, produced metabolites of these drugs in the liver could affect drug toxicity in other organs. Bovard et al. [163] introduced a lung/liver-on-a-chip system to study the toxicity effect of aflatoxin B1. After 72 h of exposing the lung monoculture system to aflatoxin B, a severe injury was observed in the normal human bronchial epithelial due to the activity of CYP enzymes in these cells. However, in the coculture system, high expression of CYP3A4 in HepaRG spheroids played a protective role for the lung chamber. Theobald et al. [164] designed an MOC that comprises liver and kidney chambers that enable them to detect secondary toxicity of aflatoxin B1 in the kidney chamber. This phenomenon cannot be captured in a single kidney model as this chemical must first be metabolized by CYP enzymes to become a toxic agent. McAleer et al. [165] designed a chip that integrated liver, heart, and a cancer tissue chamber (Fig. 7.3e). Using this design, they were able to detect the off-target toxicity of tamoxifen while they were treating the cancer micro-tissue. Besides, their results showed that the addition of verapamil for the treatment of multidrug-resistant cancer cells can significantly alter the cardiotoxicity of the therapeutic method and cause irreversible damage to the heart tissue. Camilly et al. [166] studied the integration of a skin surrogate membrane with heart and liver MPSs. This model enables them to compare the cytotoxicity of oral and topical administration of different drugs. Besides, they studied both acute and chronic topical administration of APAP and diclofenac. APAP caused no significant acute or chronic

toxicity either for hepatic or cardiac tissue; however, diclofenac chronic exposure caused malfunctioning of both cardiomyocytes and hepatocytes. Drug-induced skin sensitization is a phenomenon caused by the metabolism of parent drugs in the liver. This action forms reactive metabolites, which in turn stimulate an immunological response that affects epidermal cells. In a liver-immune system, Chong et al. [167] cultured liver spheroids and U937 myeloid cells in concentric microchambers that transfer material through diffusion. After proving the formation of secondary metabolites of three skin-sensitizing drugs in the liver compartment, the immunological response of the myeloid cells was confirmed by CD86 upregulation. This response was not observed in APAP since it is a nonsensitizing drug.

Finally, these integrated models could be used to predict the pharmacokinetic and pharmacodynamic behaviors of drugs. Lee et al. [168] investigated the first-pass metabolism of paracetamol by resembling the interaction of liver and intestine MPSs through a porous membrane. Their results indicate that optimizing values for liver MPS volume and gut surface area enables them to accurately predict the pharmacological effect of the drug, demonstrating the importance of the scaling ratio of different organs for MOCs studies. Herland et al. [169] developed a model to integrate gut, liver, and kidney microchambers with an arteriovenous reservoir for the systemic circulation of the blood substitute (Fig. 7.3f). Nicotine was inserted into the apical side of the gut chamber to resemble its oral administration. The bioavailability of the drug in the reservoir was comparable in quantity to the human body and the predicted pharmacokinetic parameters using this device were similar to that of the human clinical data. This indicates the capability of this device to be used in preclinical trials.

7.6 Conclusions

Sophisticated LOC models are crucially useful for the future of drug development, understanding of liver disease mechanisms, and suggesting proper therapeutic methods as they can provide different aspects of the human body. 3D structure and precise patterning help to mimic structures such as liver sinusoid and lobule. Besides, tuning the properties of the materials improves the functionality of cells and enables mimicking the disease or healthy state of the liver. Perfusion also provides material exchange and shear stress required for culturing cells. In addition, it is also possible to recapitulate liver zonation using a gradient generator. Moreover, the possibility of integrating different OOCs is useful in studying the interaction of the liver with other organs.

Aside from the accuracy of the results, the viewpoint of the end user must be taken into account while designing these models. The first concern is the ease of use that could be addressed by designing chips that do not need sophisticated equipment for operation. Besides, automation and a user-friendly interface to run the automated device allow a widespread usage of these devices by those who are not necessarily experts in the field. Another aspect that must be considered is the apparatus for online measurement. This could reduce the need for harvesting cells and operating labor-intensive and expensive analyses. Besides, this enables constant monitoring of the cell culture that eliminates the possibility of overlooked responses such as an intermittent or temporary rise in expression of certain genes that could be missed in off-chip assessments. The low throughput of LOCs is another concern that must be answered. As these models are going to be used for drug development, they are required to be high-throughput models that allow convenient and fast drug screening essays. In addition, as some of the end-users of these devices are not experts in the field of OOC, they may prefer to purchase preassembled LOC for their studies. Therefore, methods and protocols for their preservation that ensures storage and shipping of ready-to-use LOCs are required. Finally, As for any analytical method and device, standardization of the protocols and materials is necessary for the worldwide usage of these models.

Nomenclature

2D	Two-dimensional
3D	Three-dimensional
ALD	Alcoholic liver disease
Between 3D and ALD	ADMET Absorption, distribution, metabolism, elimination, toxicity
Betweenb ALD and CRBP-1CMFDA	5-Chloromethylfluorescein diacetate
Between ROS and TGFSEM	Scanning electron microscopy
CRBP-1	Cellular retinol-binding protein-1
CYP450	Cytochrome P450
DILI	Drug-induced liver injury
EC	Endothelial cell
ECM	Extracellular matrix
eNOS	Endothelial nitric oxides synthase
FFA	Free fatty acid
FGF	Fibroblast growth factor
GE	Genome equivalent
GelMA	Gelatin methacrylol
GF	Growth factor

GFAP	Glial fibrillary acidic protein
HA	Hyaluronic acid
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatic growth factor
HSC	Hepatic stellate cell
HUVEC	Human umbilical vein endothelial cell
IL-6	Interleukin-6
iPSC	Induced pluripotent stem cell
KC	Kupffer cell
LOC	Liver-on-a-chip
LSEC	Liver sinusoid endothelial cell
MOC	Multi-organs-on-a-chip
MRP	Multidrug resistance-associated protein
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NPC	Nonparenchymal cell
OOC	Organ-on-a-chip
PC	Parenchymal cell
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PHH	Primary human hepatocytes
PRH	Primary rat hepatocyte
ROS	Reactive oxygen species
TGF	Transforming growth factor-beta
TJ	Tight junction
TNF-α	Tumor necrosis factor-alpha
TPE	Thermoplastic elastomers
VEGF	Vascular endothelial growth factor
α-SMA	alpha-smooth muscle-actin

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CHAPTER 8

Lung-on-a-chip

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8.1 Introduction

The organ-on-a-chip is among the top 10 emerging technologies that provide a controlled and replicable platform for studying and testing novel methods in biomedical engineering. This technology develops from the combination of microfluidics with cell biology to biomimetics, the living system, and the microenvironment of a physiological organ for culturing living cells [1–4]. Much of our understanding of different organs has been gained from traditional models such as cell and tissue cultures or small and large animal models and tissue-engineered organoids. However, due to their inherent drawbacks, they are inadequate for investigating fundamental mechanisms in the human body. For example, animals and animal models have had an immense impact on medicine to mimic aspects of a disease found in humans. However, not only do animal studies require ethical and regulatory considerations, but also they have failed in replicating many important human diseases. Many successful scientific discoveries in animal models failed in human studies due to the huge difference in associated factors [5,6].

In conventional two-dimensional (2D) cell culture models, communication of the different cell types cannot be studied. Hence, mimicking the function, architecture, and structure of the *in vivo* tissue is not possible in 2D cell culture [7,8]. 3D cell culture models are borne out of the need for a platform to provide a suitable environment for integrating several cell types along with an extracellular matrix. Nevertheless, the main drawbacks of 3D models are the lack of mimicking the tissue's multiscale architecture, function, tissue-tissue interfaces, biological, mechanical cues, and vascular perfusion [9–12]. In an organoid culture system, which is a 3D tissue-engineered culture system, the physiological or pathological environment of tissue can be emulated. For instance, the effect of normal mechanical cues can be studied in organoid cultures while the organs' interaction in the body cannot be considered [13–16]. To solve this drawback, microfluidic devices have been incorporated into 3D cultures, and their integration with each other produces an organ-on-a-chip. As microfluidic channels can easily connect multiple organoid systems, the interaction of organs in the body can be represented by mimicking the interaction of different tissues. Hence, multichannel 3-D microfluidic cell culture chips can be used as robust disease models that mimic *in vivo* environments [17,18].

Respiratory diseases, as a common cause of illness and mortality around the world, are vast pathological conditions that can affect the respiratory tract. Respiratory diseases include obstructive lung disease (asthma, bronchiectasis, chronic bronchitis, and chronic obstructive pulmonary disease (COPD)), restrictive lung diseases (respiratory distress syndrome and neuromuscular dysfunction), tumors (malignant tumors, benign tumors), pleural cavity diseases, and pulmonary vascular diseases (Fig. 8.1) [19,20]. The number of pulmonary diseases has been increasing meaningfully during recent decades. For instance, COPD prevalence, morbidity, and mortality have increased by 39.8% since 1990 [21,22]. The final stage of treatment in these kinds of diseases is transplantation, but due to a lack of donors, this strategy has been different and limitless. As a result, new methods are essential not only for the treatment of patients but also for functional analysis and drug screening. Although different methods such as synthetic scaffolds for lung tissue engineering, decellularized lung scaffold, and *in vivo* study have been investigated to solve lung diseases, all of them have some limitations. Due to the aforementioned reasons and drawbacks of previous models, in recent years, lung-on-a-chip technology has been developed as a potential substitute for animal experiments. This strategy, with the help of new materials, microfluidics techniques, and tissue engineering, shows

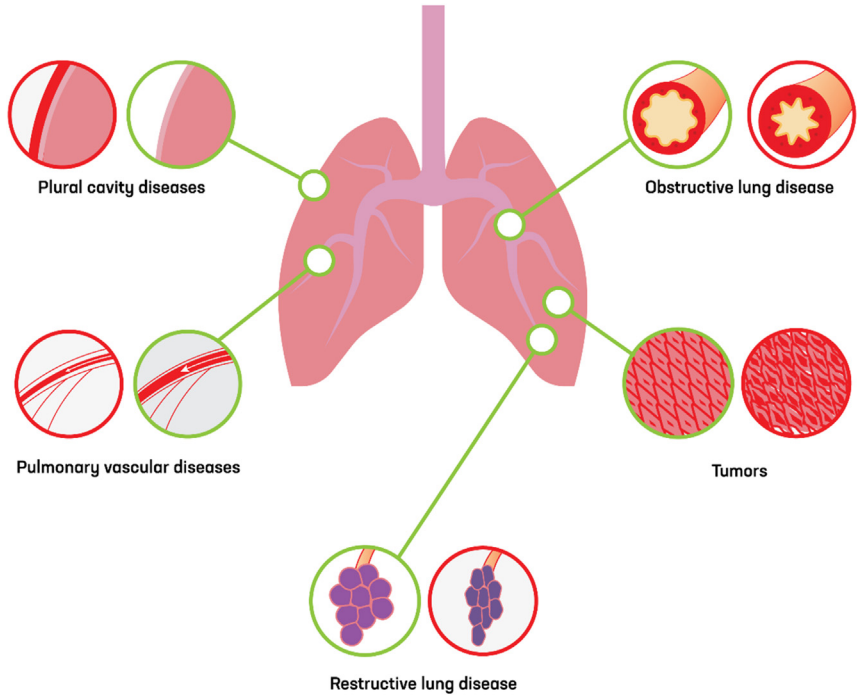


Figure 8.1 Common respiratory diseases include obstructive lung disease (asthma, bronchiectasis, chronic bronchitis, and COPD), restrictive lung diseases (respiratory distress syndrome, and neuromuscular dysfunction), tumors (malignant tumors, benign tumors), pleural cavity diseases, and pulmonary vascular diseases. Schematic changes in the anatomy of a lung showed from a healthy situation (green circle) to a diseased situation (red circle).

outstanding potential for overcoming various kinds of lung problems [23–26]. Although lung-on-a-chip technology has already shown promising results, various challenges must be overcome before it can find prevalent application in different research areas. Some recently published reviews discuss the current applications of lung-on-a-chip in respiratory diseases [27–32]. In this chapter, we discuss new technologies and biomaterials that have been used in this area.

8.2 Fabrication and system parameters

Generally, organ-on-a-chip has been developed based on progress in microfluidic technology, microelectronic, microelectromechanical system, and cell biology. The incorporation of these techniques together provides

an opportunity to culture cells in a designed microenvironment with particular mechanical, structural, and fluidic parameters. Microscale manufacturing techniques have been developed to fabricate different organ-on-a-chip. Photolithography is one of the conventional fabrication techniques that are used to produce numerous complex structures, but it is limited by the materials that can be used in this technique. Silicon and glass are two materials that are commonly used in photolithography. These materials are not suitable for cell culture. As a result, their application in organ-on-chip fabrications is limited. Ultraviolet lithography and electron beam lithography are methods that have been developed to overcome traditional photolithography limitations. Despite further developments in these techniques, low throughput and high expense limit their applications. Soft lithography resolves some limitations of conventional photolithography and allows the fabrication of micro- and nanostructures in a much easier way. Soft lithography technology is a large family of techniques used to fabricate microfluidic culture systems with reasonable control of surface features, shapes, and size of the substrates. These techniques are low cost and do not require advanced tools, so they are used vastly for rapid prototyping of organ-on-a-chip. In addition, soft lithography is highly compatible with various materials. Elastomeric materials like poly(-dimethylsiloxane) (PDMS), cyclic olefin copolymer, poly(methyl methacrylate), polyurethane, and polyimide are typically used to produce a master mold. PDMS is the most commonly used because of its biocompatibility, flexibility, optimal clearance, and gas permeability. Soft lithography has been widely used in organ-on-a-chip fabrication; however, it is a multistep process and requires several manual operations. Replica molding is a form of soft lithography used to fabricate nanoscale pattern microfluidic devices with the help of molding. The replica molding process includes designing the required pattern, fabrication of the master mold, filling the mold with a PDMS precursor and baking it, peeling off the PDMS from the master, and bonding to a glass slide. The first lung-on-a-chip in 2020 was fabricated with this method to create hollow microchannels [33,34]. Other procedures in soft lithography are listed, and more information about them is available in this review [35,36].

Additive manufacturing, also known as 3D printing, is a bottom-up method for fabricating heterogeneous structures. 3D printing is gaining attention in tissue engineering not only because of its ability to build complex structures but also its adoption of different biomaterials [37,38]. In the organ-on-chip, 3D printing facilitates the construction of microfluidic

devices with complex designs under a one-step and automated fabrication process [39,40]. Bioprinting is a rapidly growing and multidisciplinary technology that has received a lot of attention in tissue engineering. The main concept of bioprinting is to provide a three-dimensional microenvironment for cell culturing through simultaneous printing cells and scaffolds. Current bioprinting methods are listed in Fig. 8.2 [41,42]. In fact, with the help of bioprinting, a microfluidic device can be upgraded to have a microtissue. In Table 8.1, some pioneer studies of organ-on-chip and their properties are listed.

8.3 Lung-on-chip

The respiratory system is the network of multiscale organs and tissues, including the upper and lower respiratory tracts. The lung is a part of the lower respiratory tract, including the trachea, mainstem bronchus, lobar bronchus, segmental bronchus, bronchiole, alveolar duct, and alveolus. The diversity and complexity of this organ limit the lung-on-a-chip proposed models to an isolated region. For example, for having valuable and useful information, cell–blood flow, cell–cell interaction, etc., should be considered [50,51]. As a result, lung-on-a-chip could mimic the mechanical and physiological microenvironment of a native lung for researchers to study the main function of the lung. On the other hand, lung-on-a-chip models

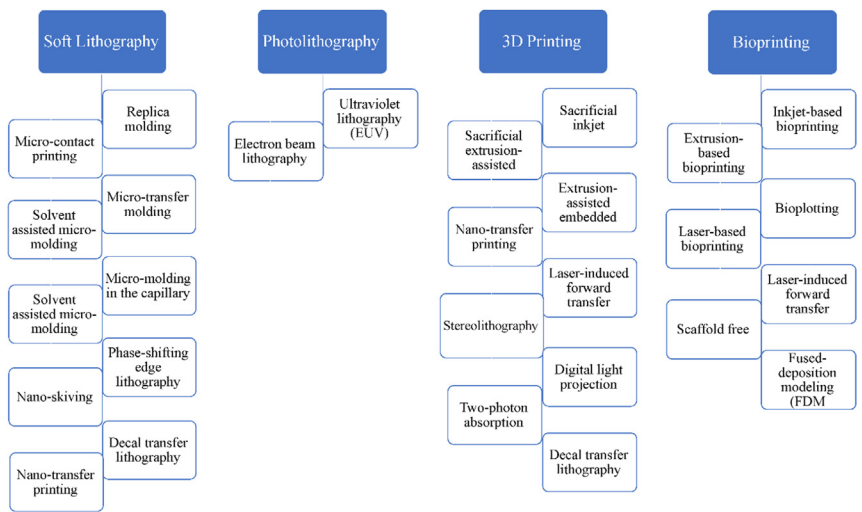


Figure 8.2 Conventional and advanced fabrication techniques have been used in organ-on-a-chip fabrication.

Table 8.1 New fabrication methods in organ-on-chip and their potency in different applications.

Method	Application	Potency	References
Photolithography			
Ultraviolet lithography (EUV)	Direct monitoring cell interactions	Provide independent culture conditions that permitted cellular migration and interactions	[43]
Soft lithography			
Replica molding	Drug development	Multiple tissue integration for in vitro study	[44]
Micromolding technique	Vascular networks	Fabrication of microchannel networks in a variety of widely used tissue engineering hydrogels	[45]
Injection molding	Preservation of preassembled liver-on-chip	Designed hypothermic storage of liver-on-a-chip for the maintenance of cell viability, morphological structure, metabolism, and biotransformation activity for up to 2 days.	[46]
3D printing			
Laser-induced forward transfer	Vascular networks	Glass centimeter-length microchannels with uniform diameters	[47]
Bioprinting			
Extrusion-based bioprinting	3D organ-like structure	Biofabrication of multimaterial structures at up to 15 times the level of current nozzle-based modalities	[48]
Scaffold-free	Drug development	Stable producing small portions of human liver tissue	[49]

have been designed for various kinds of studies, including physiological studies, pathology of lung disease, cancer studies, and drug development. It

should be mentioned that with this microdevice, a controlled system could improve long-term studies with different kinds of cells [52,53]. Recently, a microlayer of hydrogel was used in the lung-on-a-chip, and because of the existence of this microlayer, not only the growth of smooth muscle cells improved meaningfully but also their interaction with epithelial cells was outstanding [54,55]. In this regard, Young's group utilized a combination of Matrigel and type I collagen to enhance epithelial cell adhesion. In their study, they have improved a thermoplastic-based microfluidic lung airway-on-a-chip model to estimate chronic lung diseases and simulate the lung airway tissue microenvironment. For more information, the microdevice was fabricated from poly(methylmethacrylate) (PMMA) and involved three vertically stacked microfluidic chambers. In the middle of this thermoplastic device, a thin layer of hydrogel was placed. One of the most critical aspects of this microdevice was a thin layer of hydrogel that was covered with airway epithelial cells and airway smooth muscle cell culture at the top and bottom of it, respectively (Fig. 8.3).

8.3.1 Lung-on-a-chip in physiological studies

Mimicking the physiological functions provides a primitive tool for monitoring cell behaviors in an *in vivo*-like environment. Conventional lung-on-a-chip platforms reconstruct the cellular microenvironment, but advanced platforms aim to incorporate cyclic mechanical stress besides the cellular microenvironment. For example, Sellgren et al. [56,57], used all primary cells to fabricate a biomimetic microfluidic model of the human airway. With this model, researchers could culture all three primary human cell types in microfluidic devices. Researchers used a hydrophilized polytetrafluoroethylene upper membrane to improve the differentiation of the cells. This kind of membrane is not only beneficial for lung-on-a-chip research but also is more valuable for other microfluidic cell culture systems. Additionally, they found that this hydrophilized polytetrafluoroethylene membrane is suitable for long-term culture, and cells could adhere to this membrane *in situ*. Moreover, another challenge for multiple primary cells is finding a suitable culture medium. In this study, they could find a useful culture medium to culture primary airway epithelial and lung microvascular endothelial cells. Microfluidic airway models based on primary human cells in a relevant biomimetic configuration will improve physiological relevance and enable novel disease modeling and drug development studies. In another study, Stucki et al. [58,59] designed a

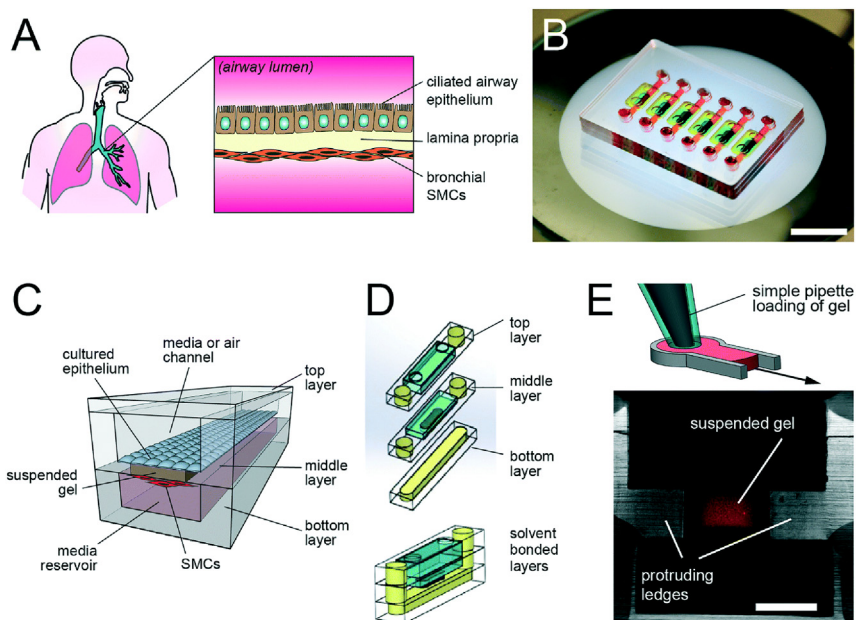


Figure 8.3 Lung airway-on-a-chip device design and fabrication. (A) The diagram of airway lumen tissue structure including a thin extracellular matrix (ECM) layer sandwiched between ciliated airway epithelium and aligned bronchial smooth muscle cells. (B) Photo of an assembled lung airway microdevice with the top, middle, and bottom compartments showed in yellow, green, and red, respectively (scale bar = 10 mm). (C) The lung airway microdevice diagram display three vertically stacked layers of PMMA with a top airflow chamber, middle suspended hydrogel chamber, and bottom media reservoir. (D) Exploded view of the three PMMA layers that contain the microdevice and assembled view of the solvent bonded PMMA layers. (E) The suspended hydrogel is introduced into the middle layer by simple pipette loading. Magnified image of device cross-section showing a hydrogel loaded with red-fluorescent microparticles, supported by surface tension between the hydrogel and the surfaces of the two protruding ledges (scale bar = 500 μm) [54]. (From M. Humayun, C.W. Chow, E.W.K. Young, *Microfluidic lung airway-on-a-chip with arrayable suspended gels for studying epithelial and smooth muscle cell interactions*, *Lab on a Chip* 18 (9) (2018) 1298–1309. <https://doi.org/10.1039/c7lc01357d>.)

breathing human primary cell alveolus-on-chip model for lung research. They fabricated a device with active and passive pumping of the medium flow that provides the cyclic mechanical stress in air-liquid interface cell culture. Primary human alveolar epithelial and lung endothelial cells are cultured in this *in vivo*-like microenvironment. In the next step, the effects of physiological stretching (similar to breathing) on cell morphology and permeability of the cells were studied. Through this study, the alveolus-on-

chip is a valuable tool to mimic several critical aspects of breathing, like breathing motion, the air-blood barrier, and the air-liquid interface (Fig. 8.4). On the other hand, reading out information from the small number of cells cultured in microengineered cell culture devices is a challenge in organ-on-chip technology. In the Mermoud et al. [60] study, the electrochemical and mechanical changes in the engineered lung-on-a-chip were controlled using microimpedance tomography that integrated into a PDMS lung-on-a-chip. The results showed this platform could be used to monitor the integrity of an epithelial barrier situated at a distance of 1 mm from the microimpedance electrodes. In addition, cyclic breathing movement of the chip and the mechanical strain applied to the cultured cells on the membrane showed amplified impedance magnitude. They suggested their platform can be used to study the mechanical properties of lung cells and tissue, such as tissue stiffness, cell proliferation, and differentiation.

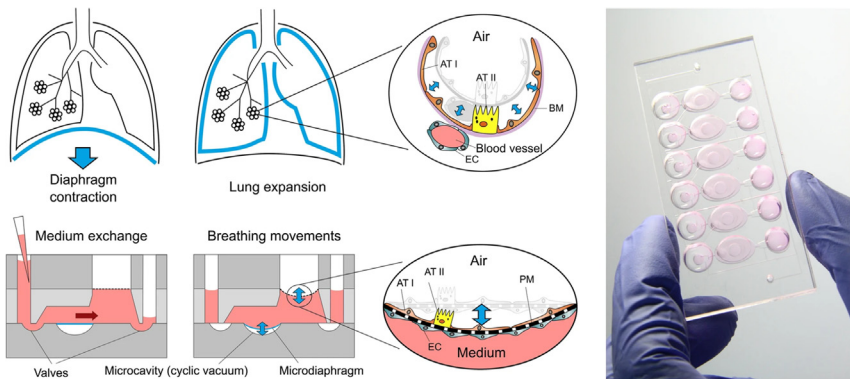


Figure 8.4 The lung-on-chip mimics *in vivo*-like expansion of the alveolar sacs with a thin, flexible, and porous PDMS membrane on which epithelial cells are cultured. The chip is pneumatically connected via the access ports and tubings to an electro-pneumatic setup. The fluidic part comprises two structured plates—the top and middle plates—between which a thin, porous, elastic PDMS membrane is sandwiched. The top plate is made of either PDMS or polycarbonate (PC), whereas the middle plate is made of PDMS. The pneumatic part comprises a structured PDMS base plate. The alveolar barrier consists of a tight alveolar epithelial cell layer—made of type I (AT I) and of type II (AT II) alveolar epithelial cells—and of endothelial cells (EC) between which the basal membrane (BM) is sandwiched. Bottom: Schematic cross-sections of the lung-on-chip with two operation modes: (i) breathing and (ii) medium exchange modes [58]. (From JD. Stucki, N. Hobi, A. Galimov, A.O. Stucki, N. Schneider-Daum, C.M. Lehr, H. Huwer, M. Frick, M. Funke-Chambour, T. Geiser, O.T. Guenat, *Medium throughput breathing human primary cell alveolus-on-chip model*, *Scientific Reports* 8 (1) (2018). <https://doi.org/10.1038/s41598-018-32523-x>.)

8.3.2 Lung-on-a-chip in the pathology of lung disease

Pathological examination, in both malignant and nonmalignant cases, is used for the final diagnosis of respiratory disorders. Organ-on-a-chip technology has contributed to improvement in the pathological diagnosis of these respiratory disorders. Specifically, lung-on-a-chip has been constructed to mimic the pathologic conditions in a physiologic cellular microenvironment, including pulmonary embolism, pulmonary edema, and renal fibrosis [61,62]. Respiratory epithelium or airway epithelium involves regulating immune responses following inhalation of particles, allergens, and pathogens. They are equipped with pattern recognition receptors and rapidly sense and initiate an immune response to microbial threats and cytokine receptors. However, airway epithelial cell dysfunction is strongly associated with the pathogenesis of various airway disorders. On the other hand, epithelial dysfunction can be a driver of numerous chronic diseases affecting the lungs. For example, airway epithelial cells contribute to the pathogenesis of both asthma and obstructive pulmonary disease (COPD) through triggering and inducing immune responses, and their dysfunction is frequently observed. Physiopathology of chronic airway diseases can be better understood by studying the normal and altered epithelial functions. To study airway epithelial cell dysfunction in asthma and COPD, various *in vitro* research models have been described [61]. Nevertheless, lung-on-a-chip can provide novel state-of-the-art alternatives to conventional *in vitro* models. To study smoke-induced pathophysiology, Benam et al. [63,64] developed an airway-on-a-chip device lined by living human bronchiolar epithelium from normal or obstructive pulmonary disease (COPD) patients. With this chip, which was connected to an instrument that “breathes” whole cigarette smoke in and out of the chips, they identified ciliary micropathologies, COPD-specific molecular signatures, and epithelial responses to smoke generated by electronic cigarettes. In a recent study, a 2D hexagonal lung-on-a-chip model was fabricated through replica molding of PDMS. The chip mimics the microenvironment of the air–blood interface to investigate the possible link between cigarette smoke extract (CSE) and associated COPD and lung cancer. The results showed CSE exposure induces inflammation and tumor-like transformation in the coculture of vascular endothelium and bronchial epithelium [65,66].

On the other hand, the lung-on-a-chip is not only helpful in stimulating cells and tissue environment, but it can also be suitable for different

lung disease studies like lung injury, inflammation, and lung cancer. It had better be mentioned that the lung is a dynamic mechanical organ and, as a result, various mechanical forces can affect the alveolar epithelial cells. Consequently, outside forces are continuously the reason for the mechanical injury of cells. To solve this problem, various studies have been done to understand the effect of mechanical forces (shear force model, mechanical stretch model, and a model that includes both forces) on lung injury [67,68]. For instance, Tavana et al. [69,70] could design a microfluidic lung-on-a-chip that showed the ability to simulate the damage of a liquid plug on pulmonary epithelial cells. They fabricated microchannels made of PDMS using soft lithography. In their study, researchers found that considerable cellular injury occurs because of the propagation of liquid plugs devoid of surfactant. Additionally, to reduce cell death, a physiologic concentration of “Survanta” as a clinical surfactant is so important and with this surfactant, researchers could protect the epithelium cells. In another study, Douville et al. [71,72] successfully designed a novel microfluidic device for describing the effects of solid mechanical and fluid mechanical stress on human alveolar cells. For this purpose, they used a three-part microfluidic device. Alveolar epithelial cells were cultured on the upper layer. By changing the liquid load on the alveoli, researchers could simulate physiologic inflation and pathologic edema.

Pulmonary inflammation is another prevalent lung disease all around the world. The biggest reason for this kind of disease is infection by viruses, fungi, bacteria, etc. [73,74]. In recent decades, researchers have been working on a lung-on-a-chip to mimic inflammatory responses. In addition to simulating inflammatory responses, remarkable research has been modeled on some chronic pulmonary inflammatory diseases like asthma and COPD. Benam et al. [75,76], for simulation of the bronchia of the human lungs, designed a human lung small airway-on-a-chip. In this outstanding research, researchers could culture primary human pulmonary microvascular endothelial cells on the lower layer of the collagen-coated porous polyester membrane. In contrast, they cultured primary human airway epithelial cells in the upper layer. With this technique for modeling human lung inflammatory disorders, they could discover different effects of lung epithelium and endothelium on cytokine secretion, measure responses to antiinflammatory compounds that inhibit cytokine-induced recruitment of circulating neutrophils underflow, and detect new biomarkers of disease exacerbation.

8.3.3 Lung-on-a-chip in lung cancer

Tumor and cancer biology, as the second leading cause of death [77,78], are major areas of interest to study. Owing to organ-on-a-chip technology, complex human-relevant model systems have been evolved for the cancer research community. The latest statistics on cancer rates globally have revealed that the leading cause of cancer death worldwide is lung cancer [79,80]. Lung cancer research can provide quality of life for patients and increase the rate of survival by developing effective treatments. Due to the aggressive metastasis of lung cancer cells, developing diagnostic tools to map the molecular mechanisms in patients with lung cancer is a challenge for researchers [81,82]. However, “Cancer on a chip” [83–86] and “metastasis on a chip” [87,88] devices can serve as tools for studying the complex circulation of lung cancer cells and metastasis mechanisms.

To mimic lung cancer cell metastasis to the bone, brain, and liver, Xu et al. [89,90] mimicked the *in vivo* microenvironment of lung cancer metastasis by designing a multiorgan microfluidic chip. The chip was made of a silicon elastomer that contained three microporous PDMA layers coated with basement membrane extract. They proved that this system provides a useful tool to investigate cell–cell interactions during metastasis and mimic the *in vivo* microenvironment of cancer metastasis. They also indicated that lung cancer cells formed a “tumor mass” in this chip. Moreover, the performance of metastasis in the organs-on-chip system was validated by their *in vivo* study in a nude mouse model. In another research, Kong et al. [91,92], to predict the metastatic capabilities of different circulating tumor cells for specific organs, designed a biomimetic microfluidic model to study cancer metastasis and proved that their findings were in agreement with that assessed by the nude mouse model. They used the combination of replicate molding and photolithography to fabricate the microfluidic device with two porous PDMS membranes. They demonstrated that lung metastasis could be effectively inhibited by the metastatic inhibitor AMD3100 in both the microfluidic model and the nude mouse model (Fig. 8.5). On the other hand, a multi-sensor lung cancer-on-chip platform was developed for trans-epithelial electrical impedance-based cytotoxicity evaluation of drug candidates [93,94]. This cancer-on-chip platform, together with its real-time physiological monitoring using integrated biosensors, was used for cytotoxicity evaluation of novel drug compounds, including doxorubicin and docetaxel. The 3D printed microfluidic channel on the glass prepatterned used indium tin oxide

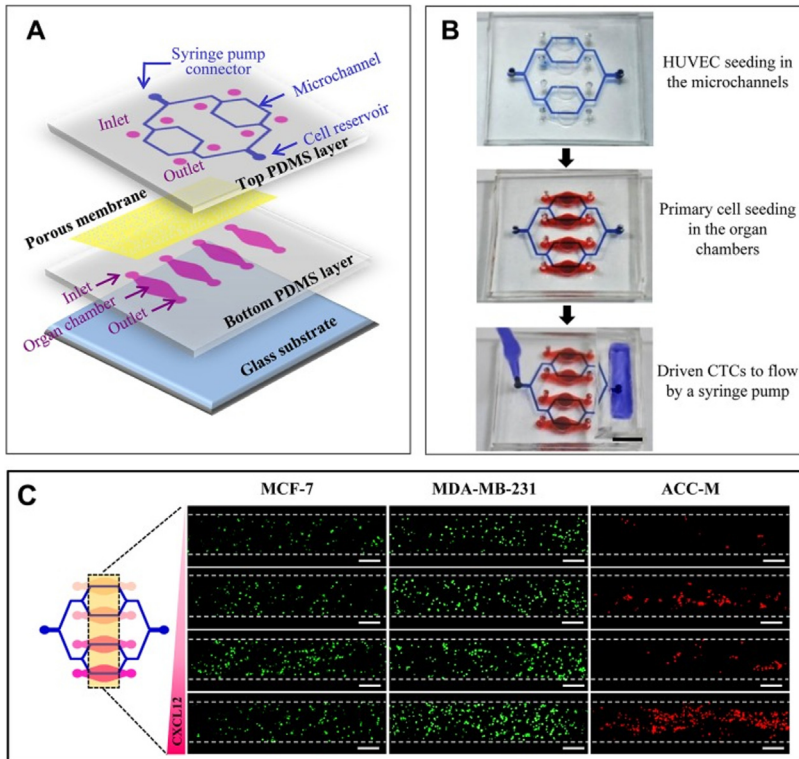


Figure 8.5 Design and assembly of the lung-on-a-chip model for metastatic capabilities of different circulating tumor cells. This model is composed of four layers: one layer of the glass substrate, two layers of a PDMS membrane, and one porous membrane. The porous membrane is sandwiched between two PDMS layers. There are four parallel branched microchannels mimicking vascular microvessels on the top PDMS layer. These microchannels join together at the syringe pump connector at one end and the cell reservoir at the other end. There are four organ chambers on the bottom PDMS layer. After the top PDMS layers and bottom PDMS layers were sealed together, the inlet and outlet of each organ chamber were punched through from the top to the bottom layer. (B) Images of the established microfluidic model. Scale bar = 5 mm. (C) CXCL12-induced CTC metastasis in the microfluidic model. The concentrations of CXCL12 were 0 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL from the upper to the lower chambers. MCF-7 cells (left) and MDA-MB-231 cells (middle) are labeled with CellTracker (green) and ACC-M cells (right) are labeled with CellTracker (red) Scale bar = 200 μm [91]. (From J. Kong, Y. Luo, D. Jin, F. An, W. Zhang, L. Liu, J. Li, S. Fang, X. Li, X. Yang, B. Lin, T. Liu, A novel microfluidic model can mimic organ-specific metastasis of circulating tumor cells, *Oncotarget* 7 (48) (2016), 78421–78432. <https://doi.org/10.18632/oncotarget.9382>.)

electrodes for visual monitoring of cells on the chip. The results indicate that this real-time physiological monitoring system with integrated biosensors could be efficiently used for cancer studies.

Nanoparticles can cause different pulmonary disorders such as pulmonary edema, asthma, COPD, emphysema, and lung cancer. So, analyzing and simulating the effect of nanoparticles and their toxicity on a different part of the body is one of the most attractive and challenging areas for scientists worldwide. The first lung-on-a-chip article was published in 2010. Huh et al. [34,95] developed a bioinspired microdevice that reconstituted the alveolar–capillary interface of a human lung. They used a biomimetic microsystem to analyze the amount of toxicity of silica nanoparticles and find the responses of the lung to these kinds of nanoparticles. With this microdevice, they found mechanical strain not only increases endothelial and epithelial uptake of nanoparticles but also motivates their transport into the underlying microvascular channel. Additionally, because of existing silica nanoparticles, underlying endothelium cells were activated along with the increased amount of Intercellular Adhesion Molecule-1 (ICAM-1) expression. In another study, Zhang et al. [96,97], designed a novel 3D human lung-on-a-chip model to not only mimic the human alveolar–capillary barrier *in vitro* but also examine the pulmonary toxicity of nanoparticles. Their novel microchip had three parallel channels for the coculture of human alveolar epithelial cells, human vascular endothelial cells, and the extracellular matrix, and they have prepared a layer of Matrigel membrane that was located between the endothelial and epithelial cells. Then, they applied ZnO nanoparticles and TiO₂ nanoparticles on the lung-on-a-chip to examine their nanotoxicity on both endothelial cells and epithelial cells. With this novel and useful chip, the researchers could examine expression, junction protein, dose-dependent cytotoxicity, enhanced permeability to macromolecules, apoptosis, etc.

8.3.4 Lung-on-a-chip in drug development

Due to the low predictive value of animal models, drug candidates for respiratory illnesses may fail late in human clinical trials. The development of human “lung-on-a-chips” to mimic the physiological and mechanical microenvironment might be a viable answer to this challenge to assess drug screening and toxicity. These organo-mimetic microdevices might revolutionize drug development by effectively mimicking complex disease processes.

In one of the first attempts, in 2012, researchers to study pulmonary edema disease designed a “lung-on-a-chip” microdevice that could

reconstitute the alveolar–capillary interface of the human lung [98,99]. Simultaneously, this microdevice was exposed to cyclic mechanical strain introduced with interleukin-2 to mimic the rhythmic breathing in the living lung and pulmonary edema. Subsequently, they used a pharmacological agent (GSK2193874) to block certain ion channels to inhibit leakage. They suggested this drug would be a beneficial treatment for pulmonary edema. Xu et al. [100,101] developed an effective lung cancer drug sensitivity test platform to support individualized treatment by mimicking the actual tumor microenvironment in vivo. For this purpose, they designed a three-dimensional (3D) coculture drug sensitivity test platform to culture cells from fresh lung cancer tissues, a mixture of lung cancer and stromal cell lines, mono-lung cancer cell line at the same time under continuous media supplementation, simulating the actual tumor. With this microfluidic chip-based, they could detect the sensitivities of different anticancer drugs for eight patients. All in all, they could fabricate a reliable, simple, and high-throughput microdevice for testing different anticancer sensitivities and this device is an outstanding microfluidic chip to screen suitable chemotherapy schemes.

In another study, in 2018, Yang et al. [102,103] used a lung-on-a-chip model based on poly (lactic-co-glycolic acid) (PLGA) electrospinning nanofiber membrane as cell scaffold and chip substrate. They have used this membrane for simulating the alveolar microenvironment, and they controlled the thickness of the membrane ($\sim 3\ \mu\text{m}$). So, this important membrane was similar to the alveolar membrane. Additionally, the main important properties of this membrane were outstanding biocompatibility, porous structure, and permeability to molecules. In this study, because of the existing PLGA membrane as a 3D cell culture model, they could culture human fetal lung fibroblasts (HFL1), human nonsmall cell lung cancer cells (A549), and human umbilical vein endothelial cells (HUVEC) cell lines (Fig. 8.6). They developed lung tumors-on-a-chip to simulate the alveolar respiratory membrane, aiming to evaluate the effect of gefitinib, an EGFR-targeted antitumor drug. Finally, by mimicking the tumor microenvironment alveolar biochemical factors, they studied the effect of anticancer drugs. As a result, they have understood that A549 cells could cause endothelial cell death or apoptosis and then the occurrence of tumor invasion. In conclusion, they have found this lung-on-a-chip not only is so effective and easy to work, but is also essential in applications to supporting individualized treatment of lung tumors.

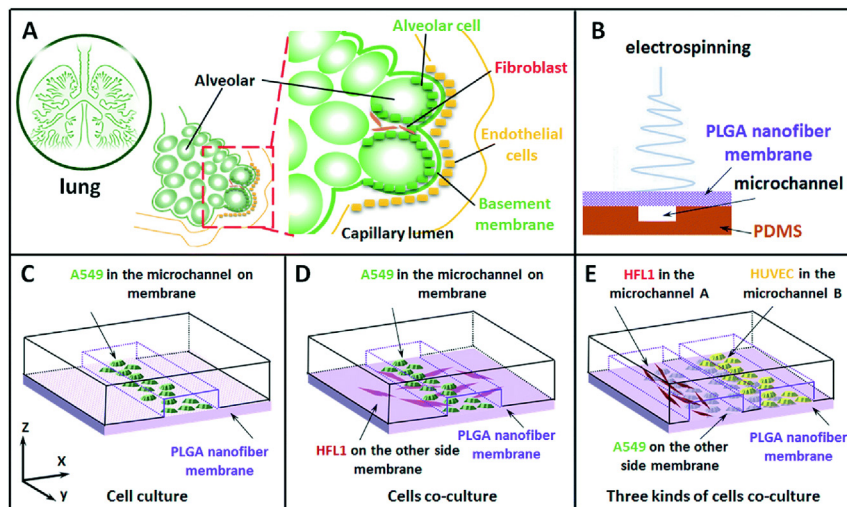


Figure 8.6 (A) Schematic illustration of the alveolar structure. (B) the design and structure of a microfluidic chip with PLGA nanofiber membrane as substrate prepared by electrospinning. (C) Schematic illustration of the culture of A549 cells on the chip. (D) Schematic illustration of the coculture of A549 cells and HFL1 cells. (E) Schematic illustration of the coculture of A549 cells, HFL1 cells, and HUVECs [102]. (From X. Yang, K. Li, X. Zhang, C. Liu, B. Guo, W. Wen, X. Gao, *Nanofiber membrane supported lung-on-a-chip microdevice for anti-cancer drug testing*. *Lab on a Chip*, 18 (3) (2018) 486–495. <https://doi.org/10.1039/c7lc01224a>.)

Drug development is an expensive and time-consuming process; thus, drug repurposing is a significant aid in challenging pandemic situations. The outbreak of severe respiratory diseases like the current pandemic of coronavirus disease 2019 (COVID-19) stressed the necessity for repurposing drugs for effective and rapid treatment [104–107]. In this regard, Sun et al. [108,109] reviewed the potential of the lung microphysiological system to accelerate drug repurposing for COVID-19. Much attention has been paid to the potential of lung-on-a-chip in fighting against COVID-19 [110,111]. Zhang et al. [112,113] fabricated an alveolar chip consisting of two perfused channels sandwiched with an ECM-coated porous PDMS membrane to examine the pathological changes of the epithelium-endothelium interface after virus infection. This work creates a human disease model on a chip that provides new insights into understanding the pathogenesis of SARS-CoV-2.

8.4 Concluding remarks and future perspectives

The complex architecture and dynamic microenvironment of the respiratory system make it difficult to mimic all aspects of its physiology. PDMS is

a common material in microfabrication methods, but its weak absorption of small hydrophobic molecules limits its application in drug screening. Using new materials, although it brings new challenges, leads to new potential [114,115]. In a novel study, recently, Zamprognio et al. [116,117] introduced a second generation of the lung-on-a-chip that mimics the air-blood barrier of the alveoli by using a thin, stretchable membrane made of collagen and elastin. The objective of the lung-on-a-chip second generation is to bring functionality to the designed chip. Huang et al. [118,119] fabricated a three-dimensional porous gelatin methacryloyl hydrogel as a physiologically relevant human alveolar lung-on-a-chip model. The porous hydrogel structure was able to mimic cyclic breathing motions. Their study provides a new sight into the fabrication of full functionality of alveolar units to provide an efficient model for respiratory disease studies. One of the new materials that might be helpful in accelerating the functionality of the chip is off-stoichiometry thiol-ene (OSTE). OSTE showed promising results for lung-on-a-chip fabrication. This new material showed advantages over PDMS, including manufacturability, extensive volume scalability, and negligible molecules' absorption [120,121].

Despite all achievements in the lung-on-a-chip fabrication methods, outcome data are weak to confront the mortality rate of respiratory disease around the world. Scientists provide fresh insight into fabrication methods by combing the conventional and new methods. Hu et al. [122,123] fabricated a microfabricated array device with patient-derived tumor organoids to in vitro tumor model for precision medicine. The super-hydrophobic microwell array chip was fabricated with injection molding then the lung cancer tissue was processing and separated organoids cultured on the chip. They claimed that this platform has a promising potential in screening chemotherapy drugs, especially for personalized cancer treatment. In another study, a polycarbonate microwell array chip was fabricated by injection molding and freezing lung cancer organoids on the chip. Their results showed freeze-thaw process did not affect the viability and growth rate of the organoids. This platform can be used for high-throughput anticancer drug sensitivity analysis [124,125]. Currently, most efforts are related to smart materials, integrating multicellular components, real-time monitoring, tissue-tissue interface, functionality, and fluid flow in the chip [126–131]. These criteria would be valuable in narrowing the gap between in vitro models and organ physiology.

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CHAPTER 9

Kidney-on-a-chip

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9.1 Introduction

Human kidney performs the function of maintaining the constant concentration of different ions and other important substances while removing waste products and other unwanted substances. Structurally, the kidney is composed of functional units, called nephrons, each consisting of glomerulus and tubules. The function of the glomerulus is filtering the blood [1]. The function of renal proximal tubule epithelial cells (PTECs) is active excretion of urea and uremic toxins and reabsorption of essential substances, such as water, salts, glucose, amino acids, and proteins [2]. In addition, renal proximal tubular epithelial cells (RPTECs) are tasked with providing a balance between influx from the kidney interstitial space and efflux to the glomerular filtrate through expressing specialized transmembrane drug transporter proteins while the imbalance between the two events results in susceptibility of RPTEC to drug-induced toxicity [3].

Nephrotoxicity is an important cause of drug failure during preclinical, clinical, and postapproval stages of drug development [4]. Drug toxicity can affect different parts of the kidney; however, the proximal tubule (PT) is the primary site of drug accumulation [5], and it can, therefore, be affected most. Enormous efforts have been dedicated to the investigation of the effect of drug-induced kidney injury using two-dimensional (2D) cell culture and animal models. However, 2D cell culture models are unable to recapitulate the *in vivo* biological functions of the kidney, and the effects of drug toxicity on tight junction, epithelial barrier, and cell polarization [6,7]. Kidney *in vitro* models are important for preclinical studies of drug toxicity; however, these models commonly suffer from partial dedifferentiation [8]. There are also reduced tight junction expression and the function of the epithelial barrier, which limits the replication of cell polarization-dependent drug-induced toxicity [7,9]. Thus, simulating the normal microenvironment of the kidney could prompt kidney histodifferentiation.

Animal models have been used, but they are inappropriate because of the difference in drug pharmacokinetics and pharmacodynamics response between animals and human. Therefore, the development of organ-on-a-

chip (OoC) platforms can help to recapitulate the microenvironment of the kidney and help to determine the transepithelial transport of anionic and cationic organic compounds. In addition, kidney-on-chip (KoC) models with epithelial polarization and localized drug transporters to study drug-induced toxicity have been developed [10–12].

Several research groups have been working on OoC systems and observed that exposure of kidney tubular cells to apical shear stress results in the rearrangement of their actin cytoskeleton, and upregulated tight junction expression as compared to 2D cell culture [13–15]. In this chapter, we focus on reviewing the state-of-the-art of KoC and discuss challenges and future directions.

9.2 Kidney-on-a-chip

9.2.1 Design and fabrication

Establishing 3D models that can recapitulate the anatomical complexity of the kidney will allow for carrying out physiological studies and developing successful therapeutics [16]. The development of KoC provides the capability to reconstruct kidney architecture at multiple scales, for example, tubule–tubule, tubule–interstitial, and tubule–vasculature interactions to investigate biological functionality [17]. KoC platforms comprise mainly three-microfluidic systems, single-layer, multilayer, and tubular microfluidic systems. A single-layer microfluidic system consists commonly of a single monolayer of kidney epithelial cells within microfluidic systems [16]. Moreover, this system can be used for coculturing different cell types to replicate distal nephron structure, and proteinuric nephropathy to investigate the behavior of kidney epithelial cells in coculture [18], and shear stress environment [15]. Microfluidic devices are usually composed of two channels separated by a thin polyester or polycarbonate membrane [19]. This approach imposes fluidic shear stress on cells seeded in the upper channel, while the lower microchannel is exposed to static media [20,21]. Further research to model the local microcurvatures of kidney tubules evolved into the development of circular microchannels, wherein cells could be seeded uniformly on the cylindrical walls, similar to what is seen in vivo. The tubular microfluidic device was fabricated with a single, cylindrical microchannel and coating PDMS microchannels with glass using sol–gel method [22]. This device was further improved by the incorporation of hollow tubular membranes to simulate in vivo transport phenomena through the lumen, and tubule–vasculature interfaces for better modeling of renal physiology (Fig. 9.1) [16].

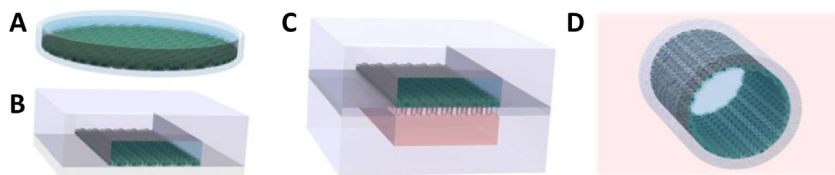


Figure 9.1 Schematic illustration of kidney-on-a-chip. (A) A 2D monolayer in Petri dish under static fluidic conditions. (B) Single layer of kidney epithelial cells in the micro-channel that can be exposed to fluidic shear stress (C) Microfluidic system, where a cell monolayer is cultured in the lower chamber under static conditions while the upper chamber has seeded cells exposed to fluidic shear stress. (D) Cells seeded within a tubular membrane, where they are exposed to fluidic shear stress. Static fluid outside the tube represents the interstitial space. (Reproduced from Sochol et al. [16], with permission from Springer.)

9.2.2 Cells used for kidney-on-a-chip systems

The selection of an appropriate kidney cell source is an important step for the development of microphysiological systems (MPSs). Nephron consists of the functional units of the kidney, which have different epithelial cell types. The generation of these epithelial cells *in vitro* requires complicated efforts [23]. In general, cells used for KoC systems can be classified into three categories, cell lines, primary cells, and stem and progenitor cells.

9.2.2.1 Cell lines

Immortalized cell lines have been used to develop KoC systems as the most common cellular model [24]. They originate from different animals (such as pigs and dogs) or humans. The most common cell lines used for KoC applications are Madin–Darby canine kidney tubular epithelial cells (MDCK cell line) [25–27], Lilly Laboratories porcine kidney tubular epithelial cells (LLC-PK cell line) [28], and opossum kidney (OK) PT epithelial cells (OK cell line) [29,30]. These animal-derived cell lines show interspecies variation, especially in the expression of human-specific transporters [31,32]. Therefore, these cells cannot be recommended for human disease modeling and preclinical drug screening applications.

Human renal cortex cell lines such as human kidney two PTECs (HK-2 cell line) have also been reported for KoC systems [33]. These human cell lines can preserve the PT phenotypes with sensitivity to toxins [34]. Overall, cell lines have some advantages since they are relatively cheap and can easily be maintained. However, none of these immortalized cells completely mimics the primary cell phenotype or functional differentiation [4,34]. Also, continual growth and proliferation of these immortalized cells may obstruct hollow fibers or channels [28].

9.2.2.2 Primary cells

Primary cells can be isolated from kidney tissue, cultured in vitro, and used for the development of MPSs [35]. Primary human cells are better representative of native kidney tissue in comparison to cell lines because they retain their phenotype and functional properties [8,36]. For example, human PTECs are the most common primary cellular models that have been used in KoC systems [24]. Their application has been mainly for developing PT-on-a-chip systems [37], nephrotoxicity studies [4], therapeutic transport evaluation [38], and drug screening [33].

In contrast to their wide range of applications, primary cells lose their function, such as the expression of important genes through passaging, which restricts the number of duplication passages to a maximum of 12 doublings [35] (optimally 2–3 passages in terms of better purity, proliferation, and differentiation [39,40]). Therefore, obtaining an adequate number of primary kidney cells is challenging. The doublings of human PT cells can be enhanced by 3–5 folds via RNA interference or antisense nucleotides. In this regard, the siRNA-mediated lifespan extension has been used to increase the lifespan of the population doublings of human RPTEC by periodic transfection of the cells with small interfering RNA (siRNA) to tumor suppressor (p53) or the cyclin-dependent kinase inhibitor (p16INK4a) (to invalidate messenger RNA of cell cycle-related genes). Hence, a confluent layer of transfected cells can be created, which surrounds the internal surface of fibers and forms detectable microvilli on their apex [35].

In addition to hPTECs, human renal epithelial cells are another primary human cell type explored for kidney MPSs for developing tubular structures [41]. Furthermore, animal-derived cells such as primary rat inner medullary collecting duct cells have been investigated for developing the collecting duct part of the kidney [42].

The probability of contamination by other renal cells and donor-to-donor variability are other limitations of the use of primary kidney cells that should be considered before using them for KoC systems [43].

9.2.2.3 Stem and progenitor cells

Stem cells have been extensively used as cell sources in tissue engineering, microfluidics, and OoC because of two capabilities, including self-renewal and differentiation [44–46]. Their potential to differentiate into different functional cell types varies according to their type. For example, pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent

stem cells (iPSCs) can develop into any cell type apart from extraembryonic cells [47,48]. ESCs are derived from the inner cell mass of the blastocysts and have differentiation ability to almost all of cell types. ESCs can be differentiated into renal cells by using several differentiation protocols [48–53]. However, these pluripotent stem cells have several limitations such as ethical controversies and legal issues due to involvement of destroying embryos to obtain them, rejection by the immune system due to their allogenic nature, and the possibility of developing neoplasms such as teratomas due to their high rate of proliferation [44]. Narayanan et al. showed that human ESCs could be differentiated from human PTECs, providing a favorable cell source for kidney tissue engineering [54]. However, characterization and application of these cells in the kidney MPSs should be carried out.

To overcome these limitations, obtaining pluripotent stem cells from patient's own cells is the best solution. To this end, iPSCs were generated by genetic reprogramming of adult somatic cells [55]. Reprogramming of cells led to pluripotency that can be achieved by inducing four transcription factors (OCT4, SOX2, KLF4, and c-MYC) in adult cells (such as fibroblasts). This method was a revolutionary step in physiology and medicine, resulting in winning the Nobel Prize by Shinya Yamanaka and John Gurdon in 2012 [55]. iPSCs possess many of the characteristics of ESCs in terms of morphology, proliferative capacity, surface markers, gene expression, epigenetic status, etc. Therefore, the use of iPSCs can be an efficient alternative to the use of ESCs as a cell source for OoC [56]. iPSCs maintain the genetic and epigenetic background of their original cells with a lower possibility of immunological rejection as compared to ESCs [57]. There are also established protocols to differentiate iPSCs to renal cells, which can be used for kidney tissue engineering and KoC systems [50,58,59]. For example, Musah et al. successfully differentiated human iPSCs to podocytes to fabricate glomerulus-on-a-chip (GoC) systems [60–62]. In addition, new gene-editing techniques such as CRISPR can be used in kidney organoids and KoC systems for drug discovery and disease modeling applications via pathogenic mutation of iPSCs [63,64].

Progenitor cells indicate the level of potency (self-renewal and differentiation capabilities) between stem cells and adult cells. These cells play a key role in the process of physiological cell turnover and substituting damaged or dead cells *in vivo*. Recent reports have demonstrated that adult kidney consists of renal stem/progenitor cells with high potential for use in autologous therapy [65–68]. In a study by Sciancalepore et al., adult renal

progenitor cells were used in a KoC system for drug testing applications [37]. However, the limited proliferative capacity of these cells is their main disadvantage for use in OoC research.

9.2.3 Structural materials used in organ-on-a-chip systems

The success of an OoC system is heavily dependent on the structural materials used for its fabrication [69–73]. Several criteria should be taken into consideration to select these materials. Biocompatibility, transparency, gas permeability, ease of the process, and cost are just some examples [74]. Since the introduction of OoC, several different materials were exploited to fabricate the chips. Broadly, these materials could be divided into organic and inorganic compounds. In the following paragraphs, we will describe these materials briefly.

The most common material used for OoC fabrication is the elastomer polydimethylsiloxane (PDMS) [59,75]. Elastomers are a family of materials that are defined as polymers with high flexibility, yield strain, and stretchability [76]. Several characteristics of PDMS are favorable for OoC development. PDMS is elastic, transparent, gas permeable, biocompatible, easily processable through several fabrication techniques, and cost-effective [77–79]. The elastic nature of PDMS helps scientists to fabricate OoC devices that are flexible and recapitulate some tissues with motion and mechanical strains [80]. One great example here is the system developed by Ingber and commercialized as Emulate [19,81,82]. The optical transparency of PDMS enables the fabrication of transparent chips, which are suitable for real-time monitoring of cultured organs and for cell imaging or imaging-dependent assessments. Gas permeability is another critical aspect of this elastomer, allowing for proper gas exchange for cell survival over long culturing times. Moreover, PDMS is capable of being shaped through several fabrication methods, including soft-lithography, use of sacrificial templates, hybrid stamp-based approach, razor-printing, and 3D stereolithography techniques [74].

Despite significant advantages, PDMS still has some pitfalls preventing it from being the “best” choice. Most importantly, PDMS absorbs small hydrophobic molecules [83]. PDMS can absorb several compounds available in the medium, including secreted biomolecules from cells or drugs/signaling molecules included in the medium [76,84,85]. Consequently, this can negatively affect the outcome and read-outs from OoC devices designed for drug toxicity evaluation and/or cell response to bioactive

molecules. For instance, in research by van Meer et al., PDMS-coated tissue culture plates (TCPs) were found to be more susceptible to the adsorption of heart-affecting drugs (namely verapamil, nifedipine, and Bepridil) as compared to nontreated TCPs [86]. They also showed that coatings could be a good option for the reduction of drug absorption. Although the gas permeability of PDMS is advantageous for oxygen (O_2) transfer (which is vital for cell survival), it could also play a negative role. Bubble formation due to PDMS high gas permeation and hydrophobicity is a prevalent problem and careful attention is needed to prevent its occurrence [76,87]. On top of these, its incompatibility and swelling within organic solvents, autofluorescence, and relatively time-consuming and expensive fabrication techniques (especially for the soft-lithography as the traditional fabrication technique for PDMS) could be considered as other major drawbacks of PDMS [88,89].

In addition to PDMS other elastomers such as methacrylated polyurethane, tetrafluoroethylene-propylene, poly(polyol sebacate), styrene-(ethylene/butylene)-styrene copolymer, poly(itaconate-co-citrate-co-octanediol), and poly(octamethylene maleate (anhydride) citrate) (POMaC) are used for development of microfluidic and OoC devices [90–92]. POMaC, a citric acid-based polymer, was first developed by Tran et al. through a polycondensation between citric acid, maleic anhydride, and 1, 8-octanediol [93]. They could show that this polymer is highly tunable in terms of mechanical properties, degradation, and other physical properties. In terms of OoC development, Zhang et al. used this elastomer to develop chips they called “AngioChip.” These chips were used to simulate the vessels and were shown to be able to recapitulate several important characteristics of native vasculature and vascularized constructs and could be used as implantable devices [92]. The same group also used POMaC to develop a heart-on-a-chip device. Their new chip, called Biowire II, was capable of recording mechanical forces in developed heart tissue non-invasively and they could be used for disease modeling and investigating cell/drug interactions [94].

Plastics are another family of polymers that are used for the development of OoC systems. Like PDMS and other elastomers, thermoplastic polymers are biocompatible and their use is cost-effective. Plastics are also easily processable and could be made into complex structures with various techniques [95]. In addition, they possess gas permeability properties to allow O_2 to be transferred to cells. Moreover, they are transparent, and their mechanical properties are suitable for developing different tissues and

OoC devices [96,97]. Typical examples of plastic polymers used for OoC fabrication are polymethylmethacrylate (PMMA), polycarbonate, polylactic acid, and thermoplastic polyurethanes [24,98]. In a study using PMMA, a microfluidic chip (UniChip) was developed to enable unidirectional tissue perfusion [99]. PMMA chips were fabricated by laser ablation and solvent-assisted bonding methods with great potential for resembling stress-sensitive tissues. Polycarbonate is also used in the development of OoC devices, mainly as a membrane in OoC systems [70,100]. For instance, in a work by Tian et al., a microporous polycarbonate membrane was sandwiched between two PDMS layers to form a kidney-liver-on-a-chip system [101].

Nevertheless, plastic polymers also show some pitfalls. Several plastic polymers are biodegradable, which could hinder their application for OoC development aimed for long-term screening. Moreover, they might be incompatible with organic solvents and show higher rigidity compared to elastomers, which may limit their application for specific investigations.

Hydrogels are 3D networks that are able to absorb significant amounts of water [102]. Due to their unique properties, and due to the fact that they can resemble the native ECM, hydrogels are widely used as cell culturing and encapsulation materials [103]. Hydrogels could be fabricated from synthetic polymers (e.g., PVA and PEG) or natural polymers (e.g., collagen, gelatin, and fibrin) [104,105]. It is possible to fine-tune hydrogels' physical, mechanical, and biological properties. Several works have shown that the swelling, degradation, stiffness, bioactivity, and cell adhesion properties of hydrogels are adjustable so they could meet required targets [106–109]. Considering OoC platforms, it is possible to use hydrogels and form microchannels inside them. For instance, in one study, Zhang et al. developed a thrombogenesis-on-a-chip platform to study the hemodynamics associated with thrombosis [110]. Using 3D bioprinting technique, they were able to fabricate biomimetic constructs from hydrogels. The constructs were developed from gelatin methacryloyl (GelMA), a well-known biomaterial for hydrogel development. They also implemented sacrificial 3D printing to embed microchannels within GelMA hydrogels [111]. The shortcomings of hydrogels, especially their fragility, relatively low mechanical properties, and possible biodegradation, limit their implementation as the main structural materials for OoC development, especially for long-term studies [112,113]. Therefore, they are primarily used in combination with other materials. In one approach, it is possible to use hydrogels to encapsulate cells within an ECM-relevant environment while

the system is made from another polymer/elastomer such as PDMS [114]. Nevertheless, hydrogels are greatly bioactive and tailorable. For example, they could be manipulated to develop smart structures that are responsive to stimuli such as changes in pH, temperature, and light [115,116]. Hence, they are still considered interesting choices for OoC and microfluidic chip fabrication.

In addition to these organic compounds, inorganic materials, especially silicon and glass, are exploited for the fabrication of OoC devices. Indeed, the application of glasses and silicones as structural materials in microfluidic devices, lab-on-a-chip, or OoC devices was investigated [117]. As a structural material, glass is a good option due to its transparency, low drug or molecule absorption, the possibility of surface modification, and biocompatibility [118]. However, glass is gas impermeable, and glass-based microfluidic devices are relatively expensive. On the other hand, silicon devices are cheap, and they are gas permeable [74]. Therefore, the combination of glass and silicone is a suitable approach for the development of OoC devices. Moreover, it is possible to combine organic and inorganic materials to develop OoC systems.

Paper is another interesting structural material for the development of OoC devices. Paper abundance, low cost, structural similarity to ECM, high porosity, ease of modification and sterilization, lightweight, and biocompatibility are considered as main advantages of paper-based MPSs [119]. However, this material is not commonly considered or investigated for the fabrication of OoC. Low mechanical properties in swollen state and low transparency are considered as the main reasons [76].

9.2.4 Components of kidney-on-a-chip

Nephron, the functional unit of the human kidney consists of different cells and functional parts. An appropriate KoC model should mimic cellular interactions (including interactions between glomerular vascular endothelial cells and podocytes), expression level of transporters, transcellular electrochemical gradients and osmotic pressure differences, architectural organization of renal tubules, cell metabolism and endocrine development, and fluid dynamics [120,121]. Different parts of the nephron have been modeled by researchers via kidney MPSs to recapitulate physiologically pertinent parameters of human KoC [24,122]. These systems include GoC, PT-on-a-chip, and distal tubule/collecting duct-on-a-chip.

9.2.4.1 *Glomerulus-on-a-chip*

Glomerulus is the functional part of the nephron, which is responsible for the filtration of blood and consists of podocytes, epithelial cells, and an array of capillaries [123]. Due to its importance, recapitulation of glomerular function in vitro by using MPSs has attracted the attention of many investigators. On this subject, Zhou et al. fabricated a GoC system having two different microfluidic channels, which mimics the architecture of the glomerulus and can be used for the modeling of hypertensive nephropathy. Immortalized human glomerular endothelial cells and murine podocyte precursor cells were utilized in organized layers lining two compartments. Interestingly, the effect of fluid flow on cytoskeletal reorganization, cell damage, and glomerular leakage was investigated was demonstrated [124]. Increased damage and leakage as a result of glomerular mechanical forces, which recapitulate the disease model, were also seen using this model. In another study, Wang et al. fabricated a GoC device having parallel channels by incorporating rat glomerular endothelial cells, basement membrane, and podocytes to replicate the glomerular filtration barrier and model diabetic nephropathy (a chronic renal disease). Hyperglycemia was indicated to be a cause of proteinuria due to higher barrier permeability to albumin and glomerular disorder. Also, pathological responses related to diabetic nephropathy were observed in this MPS, which can help to investigate disease mechanisms and therapeutic development [125].

To overcome the limitations to developing functional podocytes in vitro, Musah et al. cocultured human iPSCs-derived podocytes and human glomerular endothelial cells in a microfluidic device having distinct channels recapitulating blood and urinary fluids [60,62]. The differentiation protocol was highly efficient (more than 90% terminally differentiated cells) with high expression of maturation markers (such as nephrin, WT1, and podocin). This GoC successfully replicated the native glomerular tissue—tissue interface and filtration function for albumin and inulin clearance with the generation of basement membrane collagen. Also, albuminuria and podocyte damage were detected by using Adriamycin, mimicking in vivo drug nephrotoxicity. Furthermore, Petrosyan et al. cultured human podocytes and glomerular endothelial cells in a GoC device to mimic the structure and function of the glomerular filtration barrier. Retention of cellular morphology, creation of capillaries, upregulation of slit diaphragm proteins, and perm selectivity were observed in long-term cell culture. Interestingly, albuminuria proportional to patients' proteinuria was demonstrated only as a result of exposure to sera from patients with antipodocyte autoantibodies. In

addition, reproducibility was indicated by an assessment of 2000 devices, which confirms the authentication of this chip for high-throughput drug screening. This platform can also be used for modeling diabetic nephropathy and further pathophysiological studies [126].

9.2.4.2 Proximal tubule-on-a-chip

PT is the main part of the nephron responsible for drug elimination and therefore, a place showing renal drug toxicity. Therefore, several PT-on-a-chip systems have been developed for preclinical drug screening. Regulating reabsorption and secretion of compounds is an essential feature of PT-on-a-chip systems to be able to mimic native PT function. Researchers used various strategies for this development such as culturing PT cells in hollow fibers used as tubular scaffolds for cell culture, which can also immunoprotect them [127]. In this regard, a confluent monolayer of hPTECs was seeded on the inner surface of fibrin-coated hollow fibers showing great transport ability for creatinine and glucose [38]. Another model of biofunctionalized renal hollow fibers showed clearance of albumin-bound toxins and albumin reabsorption [128]. In addition, modeling of different renal diseases was demonstrated by the change in glomerular filtration rate, nephrolithiasis, hyperglycemia, and nephrotoxicity caused by different drugs such as cyclosporine and cisplatin [12,129].

Aiming to fabricate a cost-effective PT-on-a-chip model, Jang et al. seeded hPTECs on collagen-type IV-coated polyester membranes that separate microchannels into luminal and interstitial channels. Exposure of cells to fluid shear stress exhibited normal columnar shape, polarity, transporters, and primary cilia. Cellular uptake of albumin and recovery from cisplatin-induced damage were also enhanced [4]. Furthermore, Sciancalepore et al. developed a PDMS microfluidic device that had a fibronectin, laminin, or matrigel-coated porous polycarbonate membrane. Cultured human renal progenitor cells were polarized with a reduction in permeability of urea and creatinine under fluid shear stress, and fibronectin-coated surfaces exhibited the greatest metabolically active cells [37]. In addition, Weber et al. used a microfluidic device comprising an ECM-coated chamber with tubular architectures for hPTECs culture. Development of tubular structures, collagen matrix production, renal epithelial differentiation, cell polarization, and morphology with high cell viability (more than 95%) was confirmed for four weeks of cell culture. Interestingly, basolateral transport and apical uptake with intracellular enzymatic function were indicated as by ammonia genesis and activation of vitamin D. Therefore,

physiological conditions of PT cells can be recapitulated in vitro with higher differentiation and maturation [130].

In another study, Vriend et al. investigated renal drug-transporter interactions by using a predesigned microfluidic OrganoPlate cultured by immortalized PTECs upregulating organic anion transporter 1 (OAT1) [131]. As a result, proper cell polarization with gene expression of drug transporters, function of efflux transporters, and localization of F-actin were demonstrated. Recently, Vormann et al. fabricated a 3D high throughput microfluidic device for drug-induced kidney injury (DIKI) pharmaceutical applications [132]. Four drugs with reported nephrotoxicity effects were used including cisplatin, tenofovir, tobramycin, and cyclosporin A with readouts of cell viability, release of lactate dehydrogenase (LDH), expression of toxicity markers, and specific miRNAs. Accordingly, robust nephro-screening could be conducted for DIKI with high usability and reproducibility.

Emergence of 3D bioprinting allowed for the development of PT-on-a-chip structures with higher complexity [16]. Homan et al. printed a perfusive tubular structure using pluronic ink on a gelatin-fibrinogen ECM hydrogel [133]. The internal tubules were seeded with hPTECs and showed a more organized morphology with higher albumin uptake under perfusion in comparison to 2D culture. Also, dose-dependent exposure to cyclosporin A was observed with stability for more than two months. Moreover, King et al. developed a printed PT-on-a-chip device to mimic renal fibrosis by culturing renal fibroblasts, endothelial cells, and epithelial cells [134]. The stability of this system was more than 30 days.

9.2.4.3 Distal tubule/collecting duct on-a-chip

Distal tubule/collecting duct-on-a-chip has not been widely investigated as compared to other nephron component-on-a-chip models, and there are only a few studies on this subject. As an early study in this field, Baudoin et al. fabricated a PDMS microchip, which consisted of a fibronectin-coated cell microchamber connected to microchannels for nutrient supply and waste product elimination [26]. Jang and Suh developed a multilayer microfluidic PDMS chip with a fibronectin-coated polyester porous membrane for primary rat renal collecting duct cell culture [21]. A fluidic shear stress of 1 dyn/cm^2 was used by controlling flow rate, viscosity, and channel size to mimic in vivo renal tubules. This maintained stress resulted in higher cell polarity, improved cytoskeletal reorganization, increased cell-cell junctions, and enhanced molecular transportation. Furthermore,

collagen hollow fibers were developed as a model of renal tubular structures using sacrificial calcium-crosslinked alginate core and carbodiimide-crosslinked collagen shell. Renal tubular cells formed denser monolayers inside the hollow fibers with organized 3D morphology in comparison to less dense cells in 2D flat outer surfaces. Renal tubules also demonstrated higher activity, enhanced sodium and water transport, and overexpression of brush border enzymes of alkaline phosphatase and γ -glutamyl transferase [29,30]. Also, spatial and architectural cues, especially fiber diameter and surface curvature can significantly influence the functionality of distal tubular cells. It was reported that using a fiber diameter of 50 μm can recapitulate the native distal tubules and optimize cell function [135,136].

9.2.5 Multicomponent kidney-on-a-chip

KoC systems, with on-chip models ranging from simple to advanced, are continuously being developed to better elucidate renal physiology, the pathophysiology of kidney diseases, and drug-induced nephrotoxicity in vitro [24]. In particular, advanced on-chip models are at the stage of resembling structures and functions of individual nephron components including glomerulus, PT, and distal tubule/collecting duct [137]. However, considering that kidney is a highly structured organ, of which efficient function is dependent on a finely balanced interaction between different nephron components, research efforts aimed at developing multicomponent KoC systems comprising multiple parts of the nephron. Although the developments toward achieving this goal hold promise, multicomponent KoC systems reported in the literature are still in their infancy due to the difficulty in replicating the high structural complexity of the nephron [24,137].

One design of multicomponent nephron is the example suggested by Weinberg et al. to replicate the filtration by the glomerular unit, reabsorption by PT, and a large increase in urea concentration within the loop of Henle [122]. Accordingly, the device was composed of two layers and a dialysis membrane sandwiched between them and contained the three functional nephron components, namely the glomerulus, the PT, and the loop of Henle. In the device, a connector was used as an additional component to connect nephron parts with each other and with the blood and waste streams. Considering albumin, urea, Na^+ , and Cl^- solutes, numerical calculations for the function of the glomerulus, the PT, and the loop of Henle within the device were reported, synchronized with the flow and transport properties of normal nephron. Another example is the multichannel microfluidic device (Fig. 9.2A) developed by Sakolish and Mahler to mimic the combined

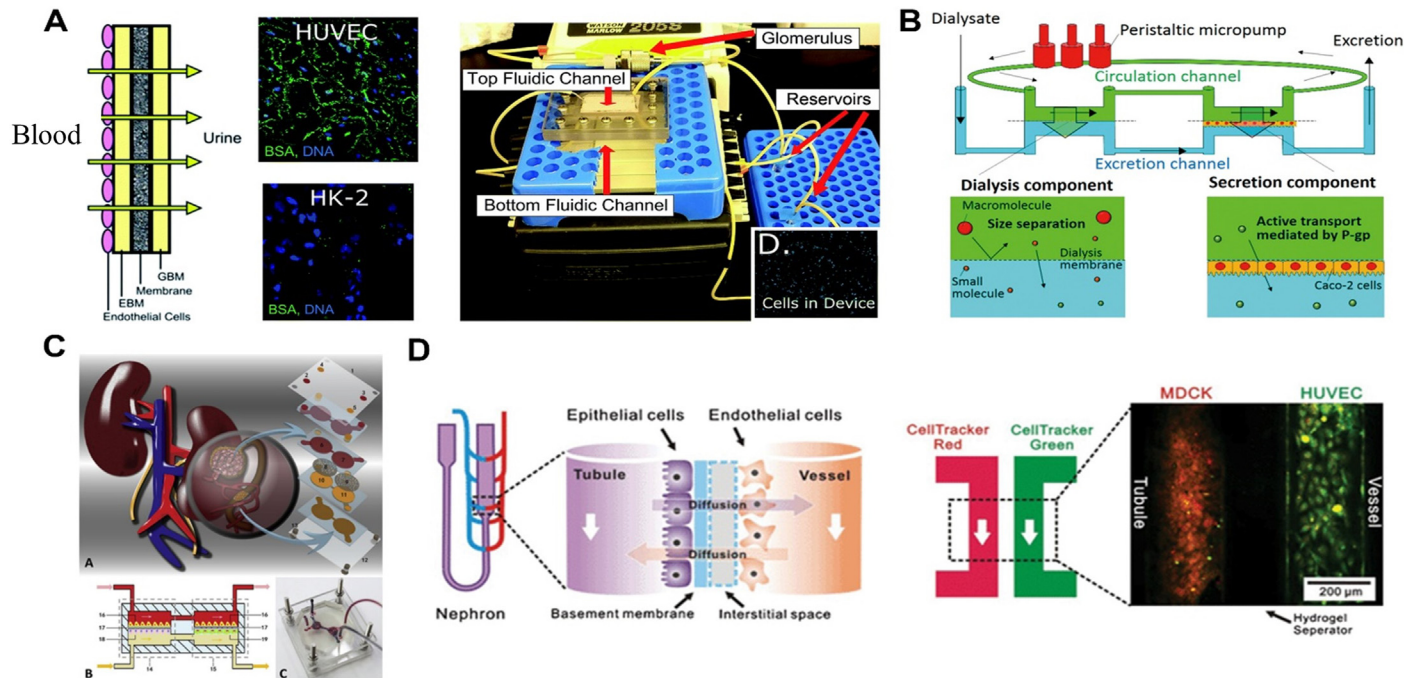


Figure 9.2 Example multicomponent kidney-on-a-chip systems developed to recapitulate the structure and functionalities of combined nephron components. (A) Left panel: Schematic showing the model used to recapitulate the structure of the glomerular barrier using endothelial cells along with epithelial basement membrane (EBM) and glomerular basement membrane (GBM). Middle panel: Bovine serum albumin (BSA) protein was used to show the passive filtration of the glomerulus barrier and the active transport of the proximal tubule (PT) using endothelial cell-seeded glomerular filter cells (HUVEC) and PT epithelial cells (HK-2), respectively. Right panel: Micrograph shows the final assembly of the device, where subpanel D reveals the cell-seeded membrane. (B) Schematic of the microfluidic device

comprised of dialysis and secretion components to mimic combined functionalities of glomerulus and PT of the kidney, respectively. **(C)** Subpanel A: Schematics showing the on-chip nephron model (right part) mimicking the native nephron (left part). 1, top polycarbonate (PC) plate; 2, inlet of “glomerulus”; 3, outlet of “peritubular capillary”; 4, inlet of “Bowman’s capsule”; 5, outlet of “tubular lumen”; 6, “glomerulus”; 7, “peritubular capillary”; 8, interface in the “renal corpuscle”; 9, interface in the “PT”; 10, “Bowman’s capsule”; 11, “tubular lumen”; 12, bottom PC plate; and 13, screw. Subpanel B: The longitudinal-section view of on-chip nephron model. the direction of “renal blood flow” and “glomerular filtrate drainage” is indicated by the pink and yellow arrows, respectively. 14, “renal corpuscle”; 15, “PT”; 16, primary renal endothelial cells; 17, porous membrane with basement membrane matrix extractant on; 18, primary podocytes; and 19, primary renal tubular epithelial cells. Subpanel C: Micrograph shows the final assembly of the device with red (“renal blood flow”) and blue (“glomerular filtrate drainage”) fluids. **(D)** Left panel: Schematic shows structure and passive diffusion of a physiological nephron. Right panel: The vascular network of a nephron was mimicked with independent hydrogel channels (analogous to tubules and vessels) using renal epithelial (MDCK) and endothelial (HUVEC) cells. *(A) Reproduced from Sakolish et al. [138], with permission from the Royal Society of Chemistry. (B) Reproduced from Sakuta et al. [139], which is freely available via the Creative Commons Attribution License (CC BY 4.0). (C) Reproduced from Qu et al. [140], with permission from Elsevier. (D) Reproduced from Naughton et al. [141], with permission from the Royal Society of Chemistry.*

functions of glomerulus and renal tubule [138]. Through the application of physiologically relevant shear stress, a glomerulus-modeling filter attachment, and two independent fluidic channels, the device could provide an improved growth environment for renal endothelial cells, podocytes, and tubular cells compared to traditional methods. The device proved also effective both for the passive filtration barrier function of the human glomerulus, and, for the subsequent reuptake of serum proteins in the PT. More recently, another multicomponent KoC system was developed by Sakuta et al. to mimic the combined functionalities of glomerulus and PT of the kidney [139]. The system consisted of a peristaltic micropump to mimic the heart, a dialysis component (glomerulus and Bowman's capsule) to mimic glomerular filtration, and a secretion component (PT) to mimic renal clearance (Fig. 9.2B). In the device, a dialysis membrane was used to separate analytes by size in the dialysis component, where renal tubular cells, cultured in the excretion channel of the secretion component, demonstrated functions of filtration and secretion of the kidney.

Importantly, the study by Qu et al. demonstrated a multilayer microfluidic device (Fig. 9.2C) to generate the structure and function of the glomerulus, the Bowman's capsule, PT lumen, and peritubular capillary in a single chip [140]. Artificial renal blood flow and glomerular filtrate flow were used to mimic the function of the physiological nephron. As a result, developed nephron-on-a-chip recapitulated renal physiology, including the glomerular basement membrane charge-selective barrier, glomerular size-selective barrier, para-aminohippuric acid secretion, and glucose reabsorption. A developed device was also used to study drug-induced acute renal injury and nephrotoxicity using cisplatin and doxorubicin as model drugs. Another noteworthy study is the one conducted by Mu et al., where hydrogel microchannels were developed in a microfluidic device for mimicking the highly vascular network of the nephron (Fig. 9.2D) [42]. The study demonstrated that generated vascular network is mechanically stable for perfusing solutions and biocompatible for cell adhesion and coverage.

Clearly, combined functions of critically important nephron parts, such as glomerulus and PT, could successfully be mimicked in the reported multicomponent KoC systems. However, in view of the complex character of the nephron, constructing a nephron-on-a-chip that can completely recapitulate the filtration and reabsorption functions of the native kidney is challenging. This is due to fact that recapitulating all specific structures and functions of the nephron in a single KoC system would require developing on-chip models of different essential nephron parts along with

reconstituting tissue–tissue interfaces, recreating relevant chemical micro-environment, and recapitulating the physical microenvironment of the native kidney [13]. Although publications on KoC systems that involve building a multicomponent KoC is scarce, with advancements made in microfluidic technologies and cellular biology, the development of more structurally and physiologically relevant KoC systems is on the horizon.

9.2.6 Multi-organ-on-a-chip systems that include kidney-on-a-chip

Toxic effects of drugs can affect different sites in the kidney through various mechanisms [141]. They can be metabolized and absorbed by the small intestine and the liver and excreted by the kidney, suggesting key determined efficiency for therapeutic application. Thus, detection of these systemic effects of drugs requires the development of a reliable MPS that integrates different organ mimicking modules [4]. A four-organ-chip (4C) platform has been fabricated by Maschmeyer et al. to combine intestine, liver, skin biopsy, and PT. 4C comprised two PDMS layers that contained channels, micropumps, membranes, and openings for culture compartments (Fig. 9.3). In this system, skin biopsy could be used as an absorption route for assessing the toxicity of drugs and their metabolites. It was used for in vitro analysis of absorption, distribution, metabolism, and excretion,

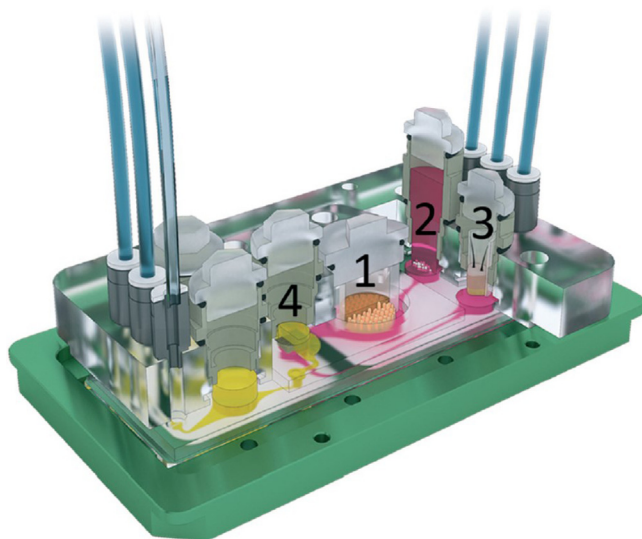


Figure 9.3 3D view of a four-organ-chip consisting of two polycarbonate cover plates, the PDMS-glass chip to accommodate a device with surrogate blood circulation (pink color), and an excretory flow circuit (yellow). It comprises intestine (1), liver (2), skin (3), and kidney (4) representing units. (Reproduced from Maschmeyer et al. [142], with permission from the Royal Society of Chemistry.)

along with repeated dose toxicity testing of drugs through predicting the interaction of metabolites with organs equivalents. A discrete medium reservoir at the apical side of the intestinal barrier was used to represent oral route for ingested drugs, which could be absorbed and get into circulation. Accordingly, drug will reach liver compartment of the system and undergo the first metabolism path, and the kidney for the second metabolism and excretion path [142].

9.3 Applications of kidney-on-a-chip systems

9.3.1 Physiological studies

KoC systems not only provide on-chip models to recapitulate the structure of a highly complex native kidney, but also resemble its physiology [143]. Indeed, assessing renal function *in vivo* using on-chip models could help in understanding mechanisms of kidney function at cellular and tissue levels, such as blood filtration in the glomerulus and secretion/reabsorption in renal tubules. For example, with a two-channel microfluidic chip, one representing the PT lumen and the other peritubular capillary lumen, Vedula et al. were able to replicate the reabsorptive barrier of PT *in vitro* by separating the channels of the chip with a porous membrane [144]. Within the device, renal epithelial and endothelial cells showed enhanced viability, metabolic activity, and compactness of the epithelial layer. The resulting tissue expressed kidney-specific transport proteins. Moreover, cells in the device showed inhibition and recovery of active reabsorption of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), which is a fluorescent glucose analog in response to the drug ouabain. In another study by Ferrell et al., single- and multichamber microfluidic bioreactors were developed to evaluate albumin handling by PT cells (Fig. 9.4A) [29]. In the single-chamber system, cells were perfused with physiologically relevant shear stress to evaluate the effects of mechanical stress on protein uptake. In the multichamber system, apical and basolateral compartments were separated by a porous membrane to evaluate the fate of protein following cellular metabolism. It was found that in cells exposed to flow, cellular uptake and/or degradation was significantly increased as compared to cells under static conditions.

With regard to the formation of tubular structures, Wei et al. developed a microfluidic chip where a monolayer of polarized epithelial cells was formed resembling the structure and function of native epithelia [146]. It

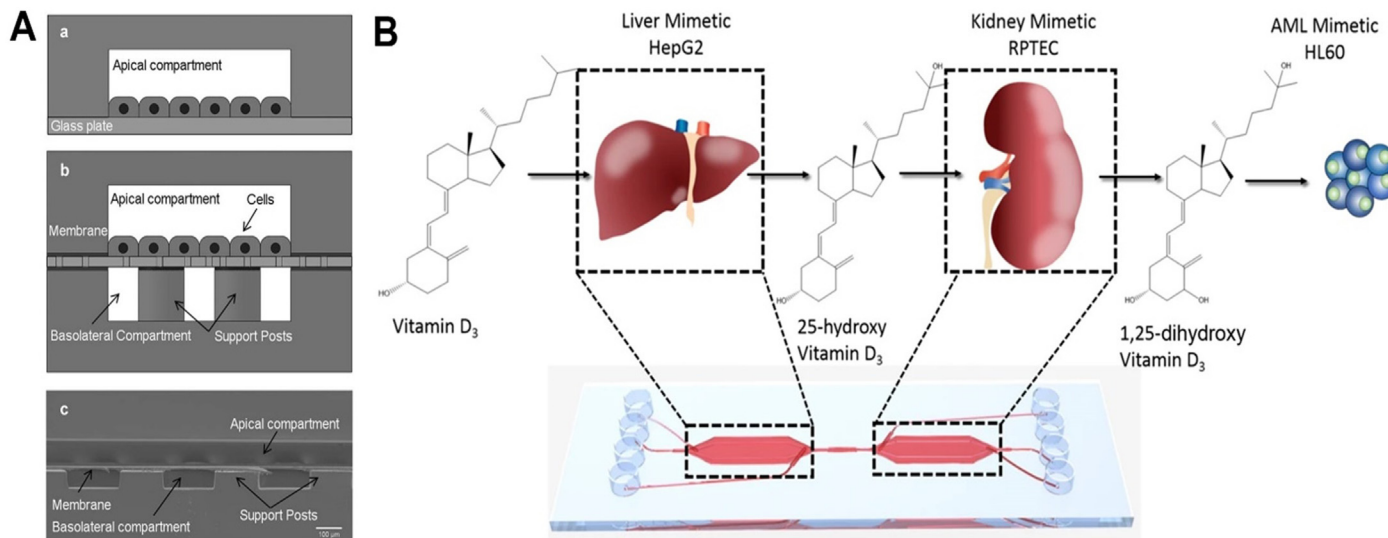


Figure 9.4 Example kidney-on-a-chip systems providing on-chip models to recapitulate the renal physiology. (A) Schematics showing the single- (subpanel a) and multichamber (subpanel b) microfluidic bioreactors developed to evaluate the albumin handling by proximal tubule cells. The electron micrograph in subpanel c shows the cross-section of the multi-chamber microfluidic bioreactor. In the bioreactors, a polycarbonate membrane was used to separate the apical and basolateral compartment, and cells are cultured on the membrane in the apical compartment. (B) Schematic showing an on-chip vitamin D₃ metabolism and bioactivation using a microfluidic device, where the liver chamber is used to metabolize vitamin D₃ to 25-hydroxy vitamin D₃ and the kidney chamber is used to further metabolize 25-hydroxy vitamin D₃ to 1,25-dihydroxy vitamin D₃. The prodifferentiation effects of vitamin D₃ were then investigated using myeloid leukemia cells (HL60) for vitamin D₃ anticancer activity. (A) Reproduced from Ferrell et al. [29], with permission from Wiley. (B) Reproduced from Jourde-Chiche et al. [145], with permission from Nature Publishing Group.)

was possible to assess the overexpression of specific proteins inside tubular structures, the functional kinetic information on Ca^{2+} in cells, in real time with Ca^{2+} transporting epithelia. Moreover, in a millifluidic chip developed by Homan et al., vascularized kidney organoids cultured under flow conditions had more mature podocyte and tubular compartments with enhanced cellular polarity and expression of adult genes as compared to those cultured under static conditions [147]. Further, a multicompartment microfluidic chip developed by Theobald et al. recapitulated hepatic vitamin D metabolism in humans (Fig. 9.4B) [148]. They showed that cultivation of HepG2 and RPTEC cells, which were used to mimic liver and kidney, respectively, in interconnected chambers of the chip led to enhanced expression of vitamin D metabolizing enzymes as compared to cultivation using conventional tissue culture.

Altogether, the aforementioned findings contribute to improving our understanding of the function of the kidney. However, more complex KoC systems need to be established to recapitulate complete renal physiology, which would require providing a physiologically relevant micro-environment to complex on-chip models.

9.3.2 Disease models and pathophysiological studies

Over a few decades, several advances have been made in developing in vitro on-chip models that can recapitulate normal physiology and pathophysiology, as well as cellular and molecular functions of the native kidney [24,142]. In this regard, KoC systems give a great opportunity for replicating key structures and integrated functions of the native kidney in lab settings, where more relevant disease models can be generated to assess pathophysiological mechanisms underlying kidney dysfunction mostly caused by acute kidney injury, chronic kidney failure, and viral infection.

9.3.2.1 Acute kidney injury and chronic kidney failure

Acute kidney injury occurs mainly when the blood flow to the kidneys is reduced or when the urinary tract is obstructed. In particular, in patients with renal hypoperfusion or urinary stone disease, acute injury leads to a reduced glomerular filtration rate [149]. Acute renal injury is also caused by toxic agents, such as drugs, metals, chemicals, and fungal toxins, which damage nephrons leading to nephrotoxicity [145]. With further worsening, acute injury leads to chronic kidney failure, which is associated with increased morbidity and mortality [150].

To study the effect of toxicities produced by drugs, a reusable microfluidic device containing on-chip models of PT and glomerulus was developed using human kidney epithelial cells (Fig. 9.5A) [4]. A chemotherapeutic drug, cisplatin, was administered to the PT cells under static and dynamic conditions, and cell injury was investigated. Cells cultured under flow conditions were less damaged than cells cultured under static conditions, as indicated by lactate dehydrogenase release. Moreover, cisplatin-damaged cells cultured under flow conditions recovered to a significantly greater extent than the cells cultured under static conditions as indicated either by suppression of apoptosis or the number of viable cells remaining attached to the extracellular matrix-coated substrates. These findings coincided with observations made in kidney failure patients, most of whom have recovered after cisplatin toxicity.

In another study, glomerulus was recapitulated, using human endothelial cells and rat podocytes, in a microfluidic device developed as a diabetic neuropathy on-chip model (Fig. 9.5B) [125]. The device, designed to reproduce high glucose-induced critical pathological responses in diabetic nephropathy, showed an increase in barrier permeability to albumin corresponding to high glucose concentrations on podocytes. The findings of this study were verified with a commercialized GoC, lined with human podocytes (primary, immortalized, or amniotic-fluid derived). Immortalized podocytes showed less filtration of albumin than primary and amniotic-fluid derived podocytes [126].

Chemotherapeutic or antiviral drugs might also injure the kidney leading to chronic kidney failure. To evaluate this, another microfluidic device was developed by culturing Madin–Darby canine kidney cells on a fibronectin-coated porous membrane to study the kinetics of gentamicin nephrotoxicity (Fig. 9.5C) [25]. Results of the study revealed that a higher concentration of gentamycin increases membrane permeability, reduces cell viability, and destroys tight junctions of the kidney, indicating that the drug toxicity may lead to chronic kidney failure.

9.3.2.2 *Virus-induced disease models*

OoC can be used to study viral diseases and their therapeutics [151,152,154,155] and can be applied to investigate kidney dysfunction caused by viral infections, for example, using distal tubule-on-a-chip [156]. In this study, an in vivo-like three-layered device (Fig. 9.5D) was fabricated using distal tubule cells, which were then infected with pseudorabies virus. Before infection, the on-chip model was tested for tight reabsorption

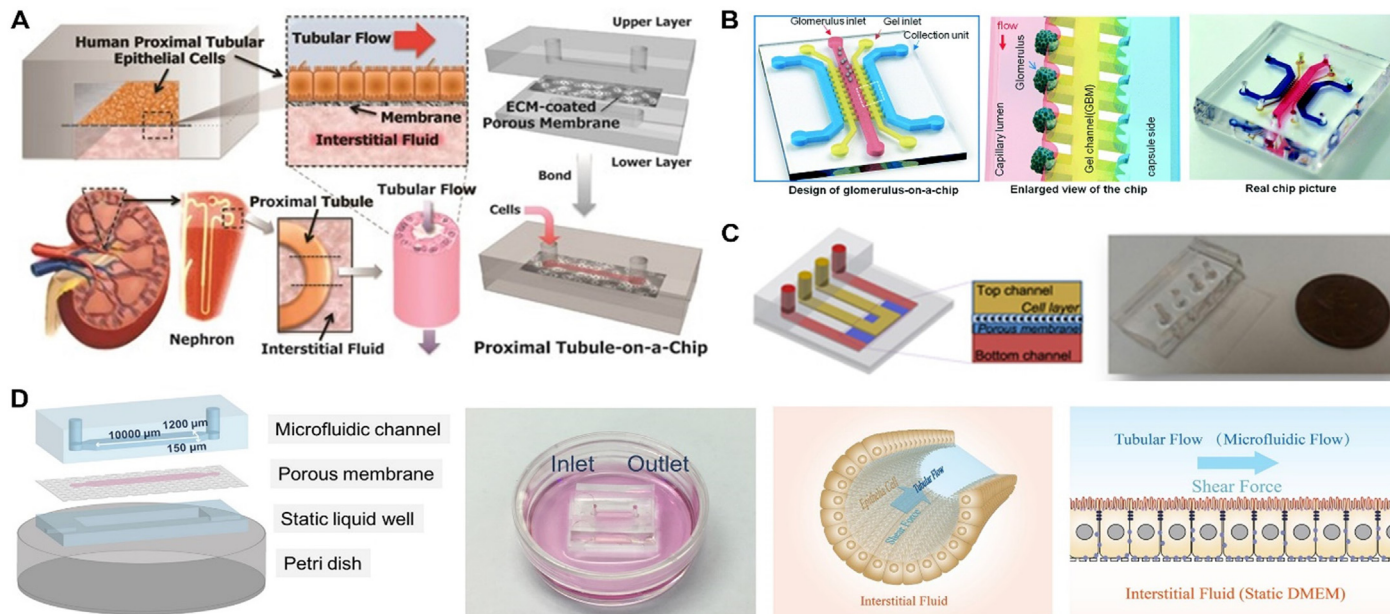


Figure 9.5 Example kidney-on-a-chip systems generating disease models for pathophysiological studies of the kidney. (A) Schematic showing the design of a microfluidic device consisting of an apical (intraluminal) channel and a bottom reservoir (interstitial space), where the compartments are separated by an extracellular matrix (ECM)-coated porous membrane. In the device, primary human proximal tubular epithelial cells are cultured in the presence of physiologically relevant apical fluid shear stress. The basolateral compartment is readily accessible for fluid sampling and addition of test compounds to study active and passive epithelial transport, and drug-induced cell injury. (B) Schematic design and micrograph showing a glomerulus-on-a-chip developed to study diabetic neuropathy. The device consists of capillary, gel, and collection channels, representing the capillary lumen, the glomerular basement membrane, and Bowman's capsule, respectively. (C) Schematic design and micrograph showing another kidney-on-a-chip developed to study the kinetics of cell injury due to gentamicin nephrotoxicity, caused by exposing kidney epithelial cells to various pharmacokinetic profiles of drug concentrations in the bloodstream in humans. (D) A 3-layered microfluidic chip developed to study virus-induced kidney dysfunctions. Illustrations depict the extracellular environment of epithelial cells in distal renal tubules and physiologically relevant fluidic flow within the tubule that generates a shear force to epithelial cells. (A) is reproduced from Ref. [4], with permission from the Royal Society of Chemistry. (B) is reproduced from Ref. [125], with permission from the Royal Society of Chemistry. (C) is reproduced from Ref. [25], with permission from IOP publishing. (D) is reproduced from Ref. [156], with permission from ELSEVIER.

barrier, apical microvilli, and sodium reabsorption. After 24 h postinfection, pathogenesis of the virus affected renal dysfunction in electrolytes regulation, decrease in sodium reabsorption, apical microvilli transformation, and disruption of polarized distribution of functional proteins. Although on-chip models other than KoCs have been applied for in vitro investigation of various viral infections, this study was the first one to investigate physiological and functional changes occurring in the kidney following viral infection.

9.3.3 Drug development and testing

While developing new drugs, there are various steps to perform for assessing drug toxicity. In general, the toxicity of drugs is tested using animal models, but these are expensive, and the results are species-specific. To overcome this limitation, on-chip cell culture models are necessary to recapitulate the structure of the kidney in vitro. Among them, the generation of PT-on-chip models attracts great attention for the creation of physiologic models of the PT, the primary site in the nephron for drug clearance and a primary site for drug-induced nephrotoxicity, because of at least in part due to accumulation of drugs in the PT [5,157]. When PT is exposed to drugs for prolonged periods, chronic kidney failure may ensue. Therefore, it is important that in preclinical trials newly developed drugs are assessed using appropriate and reliable models.

9.3.3.1 Drug screening using cellular models

For drug screening using cellular models, several studies aimed to generate KoC models. For example, in one study podocytes were cultured in a microfluidic device to form glomerulus for nephrotoxicity and drug assessment (Fig. 9.6A) [161]. In another study, the toxicity of two drugs, cisplatin and cyclosporine, were then assessed, showing more sensitivity with integrated glomerulus cells. In another study, PTECs were cultured in a single-channel Nortis microfluidic chip to assess nephrotoxicity caused by a model drug (polymyxin B). The results of the study showed a significant rise of kidney injury molecule-1 (KIM-1) and a panel of injury-associated miRNAs (Fig. 9.6B) [162].

Further studies reported microfluidic devices with more complex microenvironments. For example, in one study a microfluidic device was developed, and it comprised glomerulus, Bowman's capsule, PT lumen, and capillary tubules [140]. Adding drugs, such as cisplatin and doxorubicin, to the renal blood flow showed significantly increased biomarker levels such

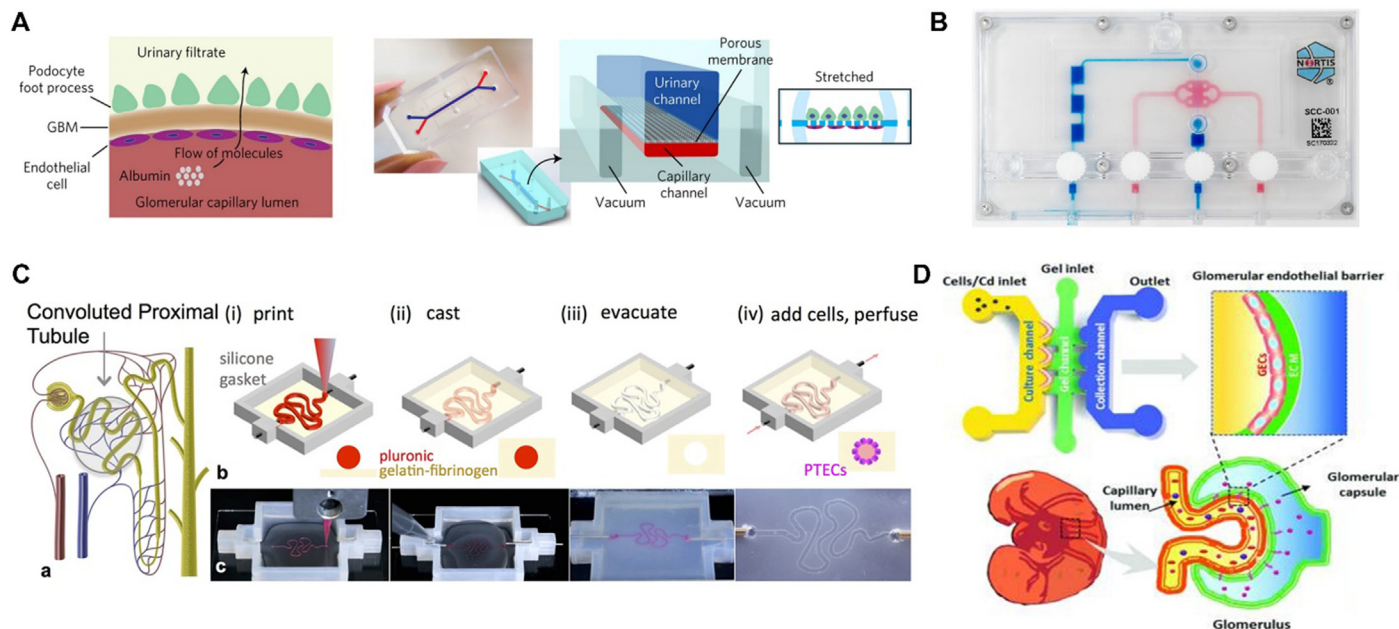


Figure 9.6 Example kidney-on-a-chip systems for nephrotoxicity and drug assessment. (A) From left to right: Schematic representation of the structure and function of a glomerular capillary lumen, where podocytes and endothelial cells are separated by a glomerular basement membrane (GBM). Micrograph and schematic design of the developed glomerulus-on-a-chip to mimic Adriamycin-induced albuminuria and podocyte injury, where cell layers were exposed to cyclic mechanical strain by stretching the flexible PDMS membrane using vacuum. (B) Micrograph showing a single-channel Nortis microfluidic chip developed for safety testing of polymyxin antibiotic nephrotoxicity on human kidney proximal tubule cells. (C) Schematic on the left panel showing a nephron where the convoluted proximal tubule is highlighted, whereas schematics and micrographs on the right panel show the bioprinting steps of 3D convoluted renal proximal tubules on perfusable chips to study the nephrotoxicity of cyclosporine A on the epithelial barrier. (D) Schematics showing a microfluidic device developed to test for cadmium toxicity on glomerular endothelial cells. (A) Reproduced from Lentini et al. [158], with permission from Nature Publishing Group. (B) Reproduced from Lepist et al. [159], which is freely available via the Creative Commons Attribution License (CC BY 4.0). (C) Reproduced from Homan et al. [133], under a Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>). (D) Reproduced from Li et al. [160], with permission from The Royal Society of Chemistry.)

VEGF and E-cadherin, resulting in injury of kidney cells. In another chip, renal tubular epithelial cells and endothelial cells were cocultured on a porous membrane integrated within a microfluidic chip, and model drugs were passed to generate concentration gradients. In two other studies, two-chamber microfluidic devices were developed to study the toxicity of drugs by cocultured kidney and liver cells. Developed systems metabolized the toxins and drugs that were administered and provided toxicity read-out of the metabolites that could be used for drug screening [163,164].

9.3.3.2 Metal-induced nephrotoxicity

Nephrotoxicity is not limited to drugs as exposure to heavy metals such as arsenic, cadmium (Cd), cobalt, copper, and mercury can also be toxic [158]. Although small amounts of these metals are beneficiary for human health as they represent important components of cellular pathways, large amounts contribute to organ damage [158].

To investigate nephrotoxicity induced by Cd [162], which severely damages internal layers of the kidney, KoC with three discrete chambers was fabricated to determine Cd-induced nephrotoxicity. In this device, the capillary and the glomerular capsule sides of the glomerular filtration barrier were fabricated (Fig. 9.6D). Primary rat glomerular endothelial cells were cultured in the middle gel channel demonstrating selective permeability of the renal barrier for investigating the nephrotoxicity induced by exposure to Cd at different concentrations. The results of the study indicated that Cd-induced cytotoxicity led to the disrupted expression of tight junction protein ZO-1 in a dose-dependent manner, which enhanced the permeability of the endothelial layer to large molecules such as immunoglobulin G and albumin.

9.3.3.3 Drug transporter interaction

In drug development research, apart from drug toxicity, evaluation of drug transporter interaction is also important to evaluate, to avoid possible drug withdrawals at a later stage of clinical trials. Regulatory agencies such as the US Food and Drug Administration and European Medicines Agency endorsed the study of the drug–drug interaction using drug transporters [159]. Transporters such as P-glycoprotein are present in the PTECs and the transport of drugs from the blood takes place through organic solute carriers, namely organic cation transporter 2 and organic anion transporter 1 and 3 [165].

To study drug transporter interactions, conditioned PTECs and human primary PT cells have been used. For example, in one study a microfluidic device was developed using cultured human PT cells to study various functionalities of the kidney including drug efflux [4]. Cisplatin was applied, and the P-glycoprotein transporter efflux activity was assessed using calcein-acetoxymethyl-ester. The specificity of P-glycoprotein activity was confirmed by adding the inhibitor verapamil and measuring the fluorescence intensity. In another study, a 3D microfluidic PTEC model was developed for high-throughput drug-transporter interactions [166]. Using the chip, 3D drug efflux assays were analyzed using P-glycoprotein and multidrug resistance-associated protein transporters. Chip was further used in the detection of microRNAs released from kidney cells in response to toxic microenvironment.

Overall, KoC systems have been used to investigate biological acute kidney injury and chronic kidney failure occurring at cellular, molecular, and functional levels. Noteworthy, KoC models can also be applied to study molecular mechanisms of renal injuries caused by viruses, and potentially, renal pathology caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [151–153]. This way, the developed disease models could help in assessing the chemotherapeutic drugs against virus-induced kidney dysfunctions *in vitro*.

9.4 Current challenges and future outlook

OoC is a technologically advanced medical device, which aims to investigate organ function, test drugs, and develop personalized therapeutics [72]. In addition, there is a rising demand for the drug toxicity assessment in the preclinical investigation, among which KoC represents a valuable model [12]. KoC has some benefits of exposing cells to physiological shear stress, which makes cells more sensitive to drugs in the kidney MPS. KoC could provide a platform for better testing of renal toxicity [167]. In this regard, PT epithelium is most prone to drug-induced toxicity, and it is a predominant focus of many studies to reconstitute physiologically relevant microenvironment to provide insights into drug discovery, efficacy, and toxicology.

Kidney MPS models with accurate functions offer the opportunity to investigate kidney physiology, pathophysiology, and drug toxicities. Having a reliable model of kidney biology, researchers have recommended the characterization of three different features of the mechanical environment, transport/metabolic function, and kidney-specific biochemical markers

[168]. In addition, it is critical to incorporate chemical, physical, and biological variables into the MPS models of the kidney. At the organ level, the kidney is composed of various, specialized cells, and different parts. Thus, specific kidney cell types are required for the development of disease models, and chemical screening [169]. Recent advances in iPSCs derived kidney organoids, and CRISPR gene-editing technology enable the development of functional experiments and establishing gene-edited organoids derived from pluripotent stem cells to gain insight into the cellular mechanisms of kidney disease [170].

To realize a more human-relevant in vitro model, it is critical to provide physiological flow to help the physiological function of cells and maintain cells in a highly differentiated state. With the advent of CRISPR/Cas9 technology, the functional consequence of genetic mutations can be investigated, thus new biomarkers for the detection of genes involved in drug-responsive phenotype.

9.5 Conclusions

Various KoC systems have been developed to recapitulate kidney function and model disease or study drug toxicity, and they represent potential tools for use in conducting preclinical studies in the future. To assess also secondary and systemic toxicity, multi-organ-on-a-chip systems that can have kidney as one component are required. With well-developed multi-organ-on-a-chip systems, the use of animal models can largely be reduced, and cost-effective drug development can be anticipated. To accomplish this, a multidisciplinary approach and sustained funding are required.

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CHAPTER 10

Eye-on-a-chip

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10.1 Introduction

The eyes are one of the most essential organs of the body, so loss of vision or low vision (due to diseases, chemical/physical injury, etc.) is known as one of the most frightening situations created for the human that can affect the quality of life of patients. Hence, the research on this organ, its diseases, and therapeutic methods has always been the focus of researchers and scientists.

Recently, novel therapeutic methods for treating ocular diseases/disorders have been developed; tissue engineering is one of these methods. This method includes expansion and proliferation of cells (ex vivo), the cultivation on a suitable substrate, and then in a bioreactor for healing of eyes injury [1,2]. However, the main limitation of this method is the formation of a necrotic core due to an oxygen diffusion length of more than $\sim 100 \mu\text{m}$ for the clinical trial of large tissues, fabrication of the imitating substrate of extracellular matrix (ECM) of tissues, and clinical validation [3,4].

The microfluidic technique is another method to treat eye disease, which is known as a promising tool for delivering drugs. However, low bioavailability of drugs or the entry of hydrophobic or hydrophilic agents into the eye can be challenges in this field due to dynamic motions and the existence of biological barriers of ocular such as the blood-retinal barrier, corneal epithelial layer, and tear flow [5–7]. Although the improvement in drug bioavailability can be provided by some of the polymers (only 5% of the total dose, with the average residence time of less than 5 min), nevertheless, in these formulas, it must be ensured that the drug or nanoparticle possesses the desired PK/PD¹ effect [8,9].

Hence, the concerns about the reliability of drugs, applicability of methods, and ethicality of research led to the development of the biomimetic organ models. These models, which are known as “organ-on-a-chip” and commonly designed by the soft-lithography method, are able to solve the mentioned limitation and problems and understand the biological, pharmacological, and toxicological mechanisms via mathematical and experimental methods (microengineered systems) and creation of a preclinical model (without ethical concerns) [8,10–16].

Eye chips or eye-on-the-chips are one of these platforms that can assess PK/PD of drugs (without animal model) by mimicking anatomy, physiology, and microenvironmental conditions of the eye [3,17–19]. Some mathematical models are not providing relevant parameters on drug PK/PD and can only focus on the profile of tear flow and residence time of the drug [8]. Moreover, in some of the mentioned models, tear-outflux and eyelid or eyeball motions have not been well assessed. However, the MEMS² technology-based eye-on-a-chip models can culture cell or tissue (ex vivo) on predesigned microchips and analyze chemical/biological reactions [20]. Notably, the novel microengineering models can eliminate the problems of current models and lead to successful eye chips with considering the ocular microenvironment.

In this chapter, we have focused on the eye-on-chips and their advantages, limitations, and applications in the treatment of ocular diseases. Moreover, manufacturing technologies and parameters required have been studied for designing the ideal eye chips. Given that knowing eye anatomy and its diseases can play a crucial impact in understanding the physiological

¹ Pharmacokinetics/Pharmacodynamics.

² Microelectromechanical systems.

and anatomical microenvironment of the eye to achieve more accurate eye chips, in the first part, the anatomy of the eye is examined.

10.2 Anatomical structure of the eye, diseases, and treatments

The study of eye anatomy shows that the eyes consist of two segments (anterior and posterior) that each one provides different functions (Fig. 10.1 and Table 10.1). Notably, ocular diseases and disorders often lead to vision loss and impose a socioeconomic burden on the individual and society. Hence, understanding the anatomical structure of the eye and study of its diseases and disorders can be effective in creating new therapeutic methods. Some of the common eye diseases are summarized in Table 10.2.

10.3 Engineered models

Nowadays, various mathematical/physical models have been provided to assess ocular phenomena that mostly focused on the tear film. However, some of these models have been developed for quantifying drug dynamics

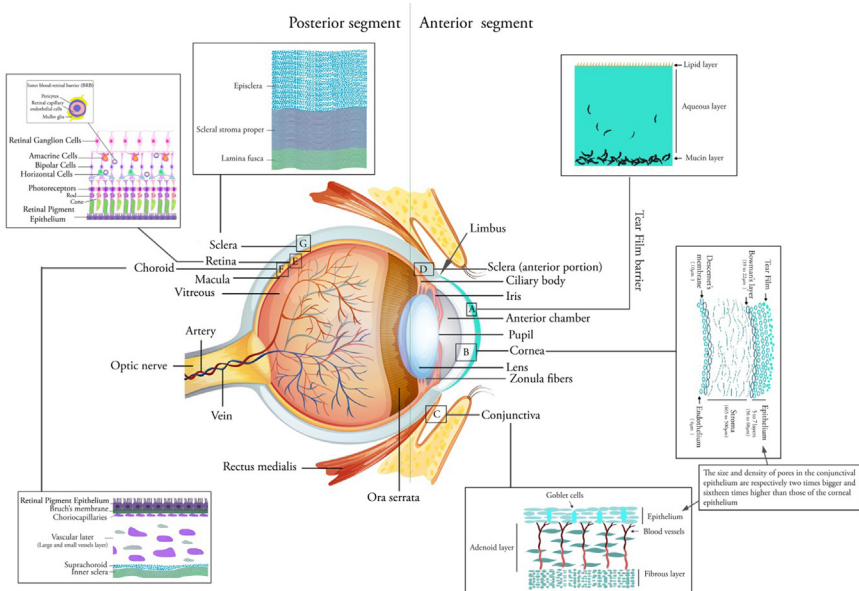


Figure 10.1 Anatomy of the eye along with layers of the retina, sclera, choroid (on the left) and cornea, conjunctiva, and tear film barrier (on the right).

Table 10.1 The anatomical components of the eye.

Eye structure	Ocular segments	Characteristics and function	References
Anterior	Tear film	The tear film that coats the cornea is the first protective barrier for the entry of topical drugs into the cornea and conjunctiva (Fig. 10.1A). Notably, to protect and lubricate the eye, lacrimal glands also secrete minor quantities of tears	[21,22]
	Cornea	The outermost component of the anterior segment that nourished via lacrimal fluid. This part possesses a transparent and refractive structure and includes five transport-limiting layers with different thicknesses (Fig. 10.1B). The cornea is the main route for the absorption of ocular drugs; however, physicochemical properties of the drug such as degree of ionization, hydrophilicity, hydrophobicity, and molecular weight play an essential role in the permeability of a drug into the cornea. The tight junctions of the epithelium and stroma layers of the cornea (as the barriers of adsorption rate-limiting) lead to a reduction in the penetration of hydrophilic and lipophilic drugs, respectively.	[23–29]
	Conjunctiva	The conjunctiva part is a mucous membrane that covers the anterior globe and inner surface of the eyelids. This part includes three layers that possess more permeability than the cornea, and its cells as transport agents of ions and water spread tear film via producing mucin ^a (Fig. 10.1C). Moreover, the conjunctiva provides drugs-systematic circulation due to the existence of microblood vessels.	[8,30,31]

Table 10.1 The anatomical components of the eye.—cont'd

Eye structure	Ocular segments	Characteristics and function	References
	Iris	The iris plays an important role in the control of light entering the eye via opening and closing the pupil. Indeed, the dilation and the constriction of iris muscles lead to such a function.	[32]
	Ciliary body	The ciliary body is a part of the middle layer of the wall of the eye that can change the pupil size and the lens shape by the ring-shaped muscle.	[32]
	Lens	The lens is the second light refracting agent (after the cornea) and is held in place by the ciliary body-originated zonular fibers. Some disorders can impair the transparency of the lens such as aging, genetic factors, metabolic disorders, trauma, and cataracts.	[33–36]
	Anterior chamber	The anterior chamber is located between the cornea and iris, and the posterior chamber is formed by the lens, iris, and zonula fibers. Both chambers have filled with aqueous humor produced in the posterior chamber. Variation in the circulation of aqueous humor (indirectly/ directly) leads to variation in intraocular pressure and subsequent outbreaks of diseases such as glaucoma.	[33,37]
	Sclera (anterior portion)	The anterior sclera is visible through the conjunctiva, possesses a white, opaque appearance, and comes to an end anteriorly where it attaches to the limbus of the cornea. This part may appear blue or yellow, due to connective tissue diseases and scleral thinning and fatty deposits caused by aging and liver disease, respectively.	[38,39]

Continued

Table 10.1 The anatomical components of the eye.—cont'd

Eye structure	Ocular segments	Characteristics and function	References
Posterior	Vitreous humor	The innervation to this part is provided by the ophthalmic nerve-derived long posterior ciliary nerves (Fig. 10.1D). The vitreous humor is a large part of the eyeball that can help with visual clarity and maintain the round shape of the eye. This gel-like substance has occupied space behind the lens and front of the retina (between lens and retina)	[32]
	Retina	The retina is the part of the central nervous system (CNS) and forms a thin part of the posterior globe (0.5 mm) (Fig. 10.1E). This part consists of retinal pigment epithelial cells (reduction of backscattering of light entering the eye and maintaining the integrity of photo-receptors) and the five types of neurons stacked in alternating layers and its neural circuitries convert the electric activity of the photo-receptor into action potential that transfer to the occipital lobe of the cortex via axons. The blood-retinal barrier (BRB) also creates a constant milieu and limits nonspecific transport between the circulating blood and the retina.	[20,40–43]
	Choroid	The choroid is the vascularized layer at the posterior segment and plays a main role in providing nutrients for the retina (Fig. 10.1F). This part of the eye is highly responsible for the drugs transport and can be effective in the clearance mechanism of drug, due to having microblood vessels. The sclera as the main protective wall of the eye plays an important	[8,28,32,44] [30,38]

Table 10.1 The anatomical components of the eye.—cont'd

Eye structure	Ocular segments	Characteristics and function	References
	Sclera (posterior portion)	role in the protection of intraocular structures and pressure (Fig. 10.1G). This part of the eye possesses collagen fibers, elastin, and proteoglycan that are arranged irregularly and lead to scattering light. The sclera has a white and opaque appearance and posteriorly fuses with the sheath of the optic nerve. It also possesses about 10 and 5 times higher permeability than the cornea and conjunctiva, respectively.	
	Optic nerve	The optic nerve or cranial nerve II is part of the vision network located in the back of the eye, and its role is the transformation of visual information from the retina to the brain vision center by the electrical impulse.	[32]

^aMucin possesses important physiological functions in eye lubrication, as well as can help the adherence of tear film to the eyeball. Mucin also plays a crucial impact in the ocular/systemic absorption of drugs.

and pharmacological assessments. Moreover, in vivo, ex vivo, and in vitro models can be effective in designing the eye chips platform.

10.3.1 Theoretical models

Some theoretical models have been created for changing from a one-dimension (1D) form of the tear film to a 2D eye-shaped domain and further study of the flow of tear and tear film, respectively, in the meniscus and from the lacrimal glands to the nasal canthus. Such models can correctly imitate the film hydraulic connectivity. Other models have also involved contact lenses for studying the changes in drug permeability and delivering the ocular drug. These models introduce the permeable layer into the considered domain during problem formulation. Examples of theoretical models along with lubrication theory have been listed in Table 10.3.

Table 10.2 Ocular diseases and therapeutic methods.

Components of eye	Diseases/disorders	Causes of disease	Main symptom	Treatments	References
Corneal	Corneal infections or keratitis	Injury (contact lens wear) or bacteria that cause painful inflammation and infection.	Reduce in clarity of vision, creation of corneal discharges, erode of corneal, and corneal scarring.	Antimicrobial eye drops (minor corneal infections), strong antibiotic or antifungal or antiviral treatments (severe infections), and topical steroid treatments (for decrease of corneal scarring).	[45–49]
	Corneal dystrophies	Corneal dystrophies are inherited	Severe vision problems (in some cases such as Fuchs’ dystrophy, keratoconus, granular dystrophy, lattice dystrophy), no vision problems, severe pain (without permanent vision loss), sensitivity to light, excessive tearing, and foreign body sensation in the eye (map-dot-fingerprint dystrophy)	Specially fitted contact lenses, corneal collagen crosslinking (to stop the progression of keratoconus), corneal transplant (severe scarring, lack of tolerating contact lens), eye drops and ointments (to decrease friction and pain), eye patch (to keep the eyelids still), creation of the anterior corneal punctures (for	[50–53]

	Dry eye	Fewer or lower quality tears, the use of certain medications (antidepressants, decongestants, antihistamines, tranquilizers, etc.), patients with connective tissue diseases (rheumatoid arthritis) are prone to this disease.	Scratchy or sandy feeling (as if something is in the eye), stinging or burning of the eye, excess tearing and dryness, stringy discharge, pain and redness of the eye, feel heaviness in eyelids, vision problems	attachment of cells to the epithelium), corneal scraping (for removal of the damaged area and growth of healthy epithelial tissue), corneal transplant, excimer laser (phototherapeutic keratectomy: PTK). Artificial tears (as eye drops), ointments (to prevent the eye from drying at night), using humidifiers, topical cyclosporine (restasis), and wearing wrap-around glasses when outside, temporarily or permanently blocking the eyelid puncta (severe cases of dry eye)	[54–59] [60–67]

Continued

Table 10.2 Ocular diseases and therapeutic methods.—cont'd

Components of eye	Diseases/disorders	Causes of disease	Main symptom	Treatments	References
	Graft versus host disease (GVHD)	GVHD resulting from GVHD due to donation of bone marrow or transplant of blood stem cell	Pain, burning, inflammation, and irritation of the eye along with foreign body sensation, watering eyes and dry eye, reduction in night vision, blurred vision, sensitivity to light and rubbing of the eye, decreased tear production, chronic conjunctivitis, and redness, as well as corneal thinning, infection, perforation, scarring, and neovascularization	Eye drops, aggressive lubrication, ocular ointments, steroids, punctal occlusion, various autologous serums and antibiotics, immunosuppressants, and scleral lenses, and promotion of tear production with using fish oil	
	Herpes zoster (shingles)	Varicella-zoster virus	Inflammation and scarring in the cornea, decrease in corneal sensitivity	Oral antiviral medication	[68–72] [73–77]

Iridocorneal endothelial syndrome (ICE)	Iris nevus syndrome; Chandler's syndrome; and iris atrophy	The changes in the iris, swelling of the cornea, and the development of glaucoma	There are no treatments; however, medications are given for the treatment of glaucoma associated with ICE. Also, the corneal transplant can be effective in the treatment of corneal swelling	
Limbal stem cell deficiency	Failure in limbal stem cells for repairing and regenerating the surface of the cornea. Notably, radiation, trauma, toxins, chemical/thermal burns, etc., cause limbal stem cell deficiency	Reduced vision, eye irritation, and sensitivity to light, uncontrollable eyelid movement or a twitch (blepharospasm), pain and intolerance to contact lenses, as well as thinning, ulceration, and opacity of the cornea, and eventually possible blindness	Corneal transplant/limbal graft for promoting the regrowth of corneal tissue. Bandages, scleral lenses, autologous serum drops, lubricating agents such as drop and ointment, the use of warm compresses, stem cell therapy, punctal occlusion, steroids (to reduce inflammation)	[78–85] [86–93]

Continued

Table 10.2 Ocular diseases and therapeutic methods.—cont'd

Components of eye	Diseases/disorders	Causes of disease	Main symptom	Treatments	References
	Ocular cicatrice pemphigoid/ mucous membrane pemphigoid	The rare systemic autoimmune disorder (primarily attacks the conjunctiva) Common causes: myopia neurotrophic keratopathy, thermal or chemical burns, splash injuries from hot liquids (oil, water, etc.), contact injuries from flame, radiation or alkali chemicals (bleach, ammonia), industrial and laboratory chemicals, etc.	Conjunctivitis, irritation, burning, tearing along with mucus discharge, and trichiasis ^a (common symptoms), as well as, blistering and scarring of the conjunctiva, symblepharon ^b , damage to structures that lubricate the eye, and scarring of the cornea (advanced symptoms)	Topical medicines along with systemic therapy such as antibiotics and corticosteroids, as well as immuno-suppressant, eye drops, and punctal plugs (to provide more lubrication in the eye)	
	Ocular herpes	Herpes simplex virus	Painful sore on eyelid or surface of the eye as well as inflammation of the cornea	Antiviral medications	[94–101]
	Pterygium	The pinkish, triangular-shaped	The redness and chronic irritation	Surgery to remove a pterygium is not	[102–110]

Retina	Neovascularization	growth of tissue on the cornea. Pterygia causes are unknown however ultraviolet (UV) light may be one of its causes		recommended due to it might grow back. To make growth back less likely, conjunctival autografts, amniotic membrane, and mitomycin-C are suggested. Lubricants (to reduce the redness and irritation), eye drops	[20,111–120]
	Retinal tear	Creation of dysfunctional blood vessels due to unregulated processes that led to hemorrhage or retinal detachment Shrinking of the gel-like substance in the vitreous, and its tugging on the retina	Development of new and abnormal capillaries that break and bleed Seeing floating specks or cobwebs, blurred or distorted vision (straight lines look wavy), the blind spot in the center of the visual field, defects in the side vision, loss of night vision	Using a laser, shrinking abnormal blood vessels, injecting medicine into the eye, freezing or cryopexy (KRY-o-pek-see), injecting air or gas into the eye, indenting the surface of the eye, evacuating and replacing the fluid in the eye, implanting a retinal prosthesis	
	Retinal detachment.	The passing of fluid due to a retinal tear, and lifting from underlying tissue layers			

Continued

Table 10.2 Ocular diseases and therapeutic methods.—cont'd

Components of eye	Diseases/disorders	Causes of disease	Main symptom	Treatments	References
	Diabetic retinopathy	Diabetic retinopathy. The deterioration of capillaries in the back of the eye and leaking fluid into and under the retina			
	Epiretinal membrane	A delicate tissue-like scar or membrane (like crinkled cellophane lying on top of the retina)			
	Macular degeneration	Deterioration of the center of the retina			
	Retinitis pigmentosa	An inherited degenerative disease			
	Macular hole	A small defect in the center of the retina (macula) that is caused by abnormal traction between the retina and the vitreous or an injury to the eye.			

Conjunctiva	Conjunctivitis	The growth of bacteria, viruses, and other organisms lead to inflammation of the conjunctiva	Feeling of grittiness and discomfort (bacterial conjunctivitis), redness, and thick discharge (particularly after sleep), swelling, irritation, and watering of the eye and eyelids Sensitivity to external irritants	Antibiotic drops, artificial tears drops, and cool compresses	[121–126]
	Vernal conjunctivitis (or spring catarrh) Chronic conjunctivitis	An allergic condition (in the spring and early summer) Chronic inflammation of the lid margin (like blepharitis), allergic sensitivity (to cosmetics or drugs), deficiency of tear secretion, foreign bodies	A gritty feeling along with redness of the eyes and a slight mucoid discharge	Antiallergy or antiinflammatory medication	
	Degenerative conditions	Exposure to wind and dust frequently	The creation of a yellow nodule (pinguecula, initially on the nasal side of the cornea and later on the opposite side)	Degenerative is unnoticed until an incidental infection (such as conjunctivitis) occurs simultaneously	
Sclera	Inflammation	A relatively mild nodular inflammation (episcleritis), inflammation of the deeper sclera (scleritis)	Inflammation along with pain (for scleritis)	Usually, without treatment (episcleritis), the use of antiinflammatory medication (in more severe cases)	[127,128]

Continued

Table 10.2 Ocular diseases and therapeutic methods.—cont'd

Components of eye	Diseases/disorders	Causes of disease	Main symptom	Treatments	References
Choroid	Choroidal detachment and hemorrhage	The accumulation of serum-like fluid or blood leads to detached choroid from the underlying sclera. This detachment can be because of the low intraocular pressure due to trauma, inflammatory diseases, ocular surgery, intraocular tumors, etc.	Photopsia and floaters, a visual field defect, pain, metamorphopsia, or impaired vision over time	Topical steroids and intraocular pressure-lowering drugs, as well as, cycloplegics, mydriatics, surgery	[129–131]
	Choroidal folds	Orbital disease, choroidal tumors, ocular hypotony (due to surgery), as well as, idiopathic, chronic papilloedema, and posterior scleritis		Asymptomatic folds do not require treatment	[132]
	Choroidal rupture	Indirect/direct blunt trauma	Acute preretinal, retinal, or subretinal hemorrhaging, decreased vision or a paracentral scotoma	Laser treatment	[133]

^aEyelashes that turn inward.

^bAdhesion of the conjunctiva to the eye.

Table 10.3 Examples of the mathematical models for understanding challenges of ocular drug delivery and prediction of the usability of the drugs.

Parameters/mathematical models	Description	References
Nondimensional parameters: surface tension, gravity, and evaporation. $H_t + E + Q_x = 0$ (the resting and during blinking action) $\eta = \frac{x}{L(t)}$ $Q = \frac{H^3}{12} \times (H_{xxx} + G)$ $H_t + \frac{E}{K+H} [1 - \delta(H_{xx} + \Pi)] = - \left[\frac{H^3}{12} (H_{xxx} + \Pi_x + G) \right]_x$ (partial differential equation) $\Pi = \frac{1}{h^3} [A + B(2HH_x^2 H_{xx} - H_x^4)]$ A and B: related to Van der Waals force. $u = \frac{\gamma(\gamma - H)(-H_{xxx} - 1)}{2}$ $v = \frac{\gamma^2}{4} H_x (-H_{xxx} - 1) - \frac{1}{2} \left(\frac{\gamma^3}{3} - \frac{H\gamma^2}{2} \right)$ $\frac{dv_m}{dt} = Q_{\text{meniscus}} - A_{\text{meniscus}} \times E_{\text{rate}} - Q_{\text{drainage}} + Q_{\text{fornical}}$ The mass balance equation using the tear film (as a control volume).	The boundary conditions consist of fixation of the boundary flux and pinnation of the film height at the domain edge For the change in coordinates of the eye Assuming the effects of gravitational and evaporation (no-slip in the surface of the eyeball), a fixed contact angle for the tear film, varying boundary flux at the edge of the tear film. The influence of initial tear film height, blinking, evaporation, and gravity, on the stability of the tear film. This model is related to the effects of both evaporation and Van der Waals forces on an inter-blink domain and can show similar dry spot formation to that seen in vivo. To study the velocity field for the tear film that applied with the convection-diffusion equation, for modeling the drug diffusion into the tear film, via the corneal epithelial cells. Using a control volume method by taking the idea of the tear film state and solutes introduced to the film; due to the more straightforward solution of control volume equations than those that fully model the tear film state.	[134–152]

A, the surface area; E, δ , and K, the evaporation parameter; G, the gravitational parameter; H_t , the tear film height; $L(t)$, the length of the opening of the eye at time t; Q, the flux (from each respective location); Q_x , the volume flux term (based on different physics such as the surface of the cornea and sclera, etc.); η , the equivalent x coordinate in a fixed domain; Π , the dimensionless conjoining pressure.

Generally, mathematical models can play an important role in the understanding of the processes of drug delivery, although experimental data can help to develop pharmacokinetic models [8]. It seems that modification of these models along with the use of empirical data can lead to new mathematical models that help to select drugs or therapeutic methods.

10.3.2 In vivo models

Since 1964, the use of ocular animal models has been approved by regulatory boards [8]. Nowadays, rabbit and dog eye models are used to study treatment efficacy [21,153]. In this field, we can mention the rabbit model for differentiation and degeneration of the vitreous matrix to study the structures (in more detail) and their effective strategies [154]. The mouse strains have also been used to study the process of diseases such as glaucoma and retinal degeneration [22,155].

Moreover, the development of the rabbit dry eye models by topical medication is a further example from these animal models that assess the preservative-induced dry eye syndrome [156]. Based on the reports, the rabbit models are also applied for studying corneal endothelium by engineered methods; due to the similarity of its characteristics with human corneal endothelium [157–162]. Evaluation of the conjunctiva function by rabbit dry eye model is also one of the other studies that can help to understand the physiology of conjunctival epithelium for better treatment [163]. Likewise, pigs and monkey models have been used in some pharmacokinetic studies, which can be due to the similarity of their eye size to the human eye [21,22].

Notably, the use of animal models, as human therapeutic models to assess ocular drugs, is rugged due to the lack of control of anatomical and physiological parameters [22,164,165]. Moreover, in some cases, in vitro models cannot fully cover the main properties of the human eye, such as mice models (no macula) [22]. One of the other problems of in vitro models is their high cost and the ethical issues that lead to the limitation of using such a model [21,166].

10.3.3 Ex vivo models

In ex vivo ophthalmic studies, animals or human donors-derived retinal and corneal tissues are usually applied as explant cultures [167–171] to study the modeling of diseases, the degeneration of cells [172,173], and functions of drugs. [171]. However, based on the studies, the ocular clinical

characteristics of animal models sometimes differ from those in humans, which can be due to differences in pharmacological/physiological responses of the animal eye and the human eye. Likewise, cell viability and its functions (in long-term culture) are the main challenges in human tissues [8,174,175].

Commonly, the study of ex vivo corneal models relies on porcine and bovine; however, it cannot be controlled. It can be related to the significant variations in the cornea of porcine and bovine and differences in the human cornea with the bovine and porcine cornea (in terms of size, structure, thickness, anatomical properties, blink interval, and tear flow dynamics) [8]. The evaluation of retinal diseases on the zebrafish model has also shown that such models cannot provide a suitable approach due to the visual acuity much less than humans [176,177]. Moreover, performed studies on the human eye models indicated that there are inconsistent results in tear production/evaporation that can be due to the low sensitivity of the mentioned models and tear production responses [8,178].

10.3.4 In vitro models

Given the limitations and challenges of in vivo and ex vivo models (ethical concerns, high cost, poor standardization, etc.), in vitro models as animal and human cell culture models can be a suitable alternative for the production of ocular tissue equivalents [179–182]. These cells can be used to study differentiation, toxicity, drug transport/release, and its permeability, ocular bioavailability of drugs, and biological interactions of materials (Table 10.4).

Based on the studies, to study the toxicity and barriers permeable of the drug, ocular equivalents of monolayer such as corneal epithelial monolayer is still applied. However, reports indicate that 2D in vitro models are not correlated with in vivo models. It can be due to ocular limitations and physiological barriers of the eyes. Nowadays, the 3D bioengineered ocular constructs can solve the drawbacks of 2D in vitro models (2D cell culture) via mimicking the structure and function of the eye. There are various materials in this field that are known as promising tools and engineered substitutes due to the creation of a more relevant microenvironment (physiologically) [199–201].

Moreover, a coculture of ocular cells such as conjunctival and retinal can play an important role in studying drug uptake/permeation, cell transport, biology, and cytotoxicity. Such models as a multilayered 3D culture include

Table 10.4 Examples of the studied in vitro models along with origin and type of cell.

Cell types	Cell origin	Assessment	References
Conjunctiva epithelial cell culture	Bovine	Toxicity tests	[183]
H.Ep.2 laryngeal, liver, conjunctival, lung, skin, and muscle cells	Human	Relative toxicity of six local anesthetics (procaine, lignocaine, piperocaine, amylocaine, amethocaine, and cinchocaine)	[184]
Conjunctival epithelial cells	Rabbit	Permeability of cell to low molecular weight drugs	[185]
Bulbar conjunctival epithelial cells	Human	Growth and differentiation	[186]
Corneal cell	Human	Permeation behavior of drugs and prediction of drug absorption into the human eye (transcorneal permeation of six drugs of pilocarpine hydrochloride, befunolol hydrochloride, hydrocortisone, diclofenac sodium, clindamycin hydrochloride, and timolol maleate	[187]
Corneal epithelium cells	Rabbit	The effects of ophthalmic solution (isopropyl unoprostone) on the barrier function of corneal epithelium cells (permeable support)	[188]
Cornea cell	Human	Determination of ocular drug absorption	[189]
Conjunctival epithelial cells	Rabbit	Permeable support for drug transport studies	[190]
SIRC corneal cells	Rabbit	Growth pattern	[191]
	Rabbit	Growth and differentiation	[192]

Table 10.4 Examples of the studied in vitro models along with origin and type of cell.—cont'd

Cell types	Cell origin	Assessment	References
Bulbar, fornix, and palpebral conjunctival epithelia			
Corneal epithelial cells	Human	Mechanism of cytotoxicity of the various preservatives (benzalkonium chloride, chlorobutanol)	[193]
Corneal endothelial cells	Rabbit	Culture approach, biological interactions, and functional characteristics	[194]
Cell-culture model of the cornea (The cultivated human cornea on permeable polycarbonate filters)	Human	Transcorneal drug absorption/permeation studies (the influence of cultivation parameters on barrier characteristics)	[195]
Immortalized corneal cells and cultivated in serum-free conditions (3D model of the human cornea)	Human	Drug absorption studies	[196]
Cornea reconstruction by using endothelial, stromal, and endothelial cells on the 3D-collagen gel matrix.	Bovine	The biological interactions of materials	[197]
Development of an organotypic cornea equivalent consisting of different cell types of a porcine cornea (epithelial, stromal, and endothelial cells)	Porcine	The permeation study of drug	[198]

several extracellular factors to differentiate the ocular cells, such as corneal epithelium. These models have also been developed to study the corneal wound healing process and its transplantation, as well as the assessment of corneal viability, transfection, and corneal perfusion models [202–204].

However, one of the important disadvantages of these systems is their opacity, which is known as the main challenge in acquiring analytical images. Moreover, all 3D cell culture models cannot be sufficient for considering *in vivo* models (such as aqueous humor and tears) [205]. The spatial conditions of ocular cell culture are also one of the crucial factors to study permeability. In this field, studies have shown that serum-free conditions can be effective in the achievement of high cell integrity, enhancement of cellular barrier integrity, and long-term cell growth [206–208]. However, serum-free media led to conjunctival cells that are unable to produce mucin [208]. Furthermore, the studies showed that none of the 3D *in vitro* models could fully imitate the anatomy and physiology of the human eye. However, the advantages of the 3D cell culture models (such as the flexibility in device design and experimental model, control of system into cellular microenvironment, and the ability of single-cell analysis) have led to their application in microfluidic systems and this system direct integration with other components or on-chip models [209–213]. In this field, Ali et al. showed that *in vitro* models could apply in studies of tear flow and drug release from the contact lenses consists of microfluidic channels [8], although this system cannot reflect the tear volume and flow conditions. These parameters play an essential role in mimicking ocular function/structure and modeling the transport of ocular drugs. It seems that microengineered chips can solve the disadvantages of the mentioned models.

10.3.5 Microengineered human eye-on-chips

In recent years, the combination of sciences of biology and tissue engineering along with microfabrication techniques have led to new platforms called organ-on-chips [214]. These systems, as advanced *in vitro* models are able to develop microscale human tissues into a microfluidic system for incorporating physiological stimuli [215,216]. The advantages of organ chips such as complex structures same to tissue, the effects of systemic and multicellular, as well as the interactions of cell/cell and cell/matrix, etc. lead to the development of this system in therapeutic approaches, toxicity tests, disease modeling, etc. [217–219].

These systems have also been created in ophthalmic research to assess pathology and drug function and imitate physiological responses of tissue [16], although there are relatively few reports about these chips [8,220]. Hence, the study and control of ocular anatomical/physiological parameters

play a crucial impact in the design of microengineered eye chips to mimic the physiological environment of the eye. These parameters include the assessment of tear flow or lacrimal system (effective in the diffusion of drugs), blinking dynamics, corneal epithelium, and the microstructures of the eye, as well as shape and size of the eye (due to the ethnic/genetic differences) [8]. Moreover, attention to the multiple-layered structure of the eye and rate-limiting barriers are important factors in the design of eye-on-a-chip and improvement of its function to mimic the anatomical characteristics of the eye. The movements of the eyeball are one of the other parameters in this field that can be effective in tear flow and drug diffusion. Although such dynamic systems' design is complicated, it can lead to ideal eye chips and alternative models for *in vivo* studies. Notably, the fabrication techniques of chips can be effective in the achievement of the complex architectural structures of eye natural tissue.

10.4 Fabrication technique of tissue models

Based on the reports, the techniques of 3D fabrication/microfabrication led to the imitation of physiological microenvironments of the eye and promotion of chips function *in vitro*. However, chip fabrication processes are usually sophistication and labor-intensive due to the use of multistep lithographic processes, integration, alignment, high expenses, and time-consuming processes. Hence, more automatic and cheaper methods can eliminate industrial barriers in fabricating these tissue models [221].

3D printing is one of these methods that led to a reduction in processing time. The technique of extrusion-assisted 3D printing (the dispensation of material via an extruder or nozzle along with the movement of the nozzle by the computer-controlled arm) also creates 3D shapes [222]. Nozzle-based 3D printing methods include inkjet and microextrusion each of which possesses its advantages and disadvantages in terms of prototyping, processing time, handling, printing speed, resolution, cost, 3D stability, and biocompatibility [223–227] (Fig. 10.2).

Moreover, the use of light reactions (light exposure) is a new approach to solidify the structure via a photochemical reaction and make microchannels (as stereo-lithography) [229,230]. Notably, digital light processing is usually used to design 2D models onto the liquid starting materials. This method relies on the movements of the laser beam [231].

The optical-based 3D printing approach also involves a laser-induced forward transfer, stereo-lithography, digital-light, projection, and two-

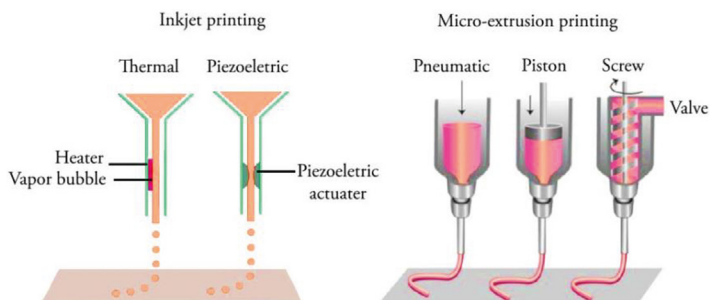


Figure 10.2 Nozzle-based 3D printing methods. (Adopted with permission from Malda et al. [228], copyright (2013), John Wiley & Sons.)

photon absorption methods, with advantages and disadvantages similar to nozzle-based 3D printing methods (Fig. 10.3) [227,233–237]. A comparison of optical-based 3D printing and nozzle-based 3D printing methods indicates that these two approaches possessed differences in terms of printing speed, resolution, 3D instability, and preparation (prototyping).

Given the unique advantages of 3D printing, nowadays, 3D printing technology has developed. The fabrication of micrometer-scale objects by best-in-class 3D printers has led to the creation of microfluidic devices and interface compartments to improve microfluidic chips [238–240]. In this technology, the highest achievable resolution relates to the nanometer scales, and geometric complexities play no role in design. This promising strategy not only controls the spatial distribution but also can lead to the layer-by-layer assembly of ECM, the cell, and biomaterials. Hence, integration of this method with cells to create organ-on-a-chip platforms can facilitate the fabrication of microorgans.

In this field, 3D bioprinting as a combination technique (cell-laden bioink and 3D printing) can provide functional organs with complex

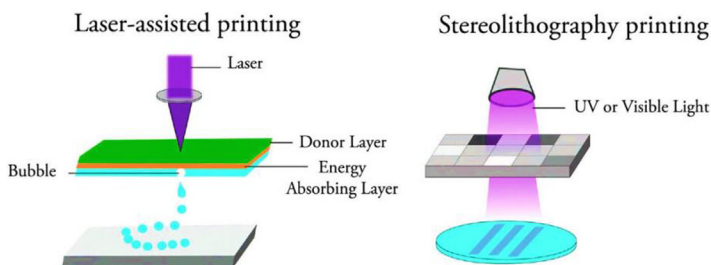


Figure 10.3 Optical-based 3D printing methods. (Adopted with permission from Foyt et al. [232], copyright (2018), WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.)

architectural structures from digital models in the form of organ-on-chip platforms [241–246]. Important factors for using the 3D bioprinting technique (3D bioprinting) to create organs-on-chips involve printing materials/inks and 3D cell-printing methods, which depend on the functions and purposes of the chip (Table 10.5, Fig. 10.4). [256,271].

Although 3D bioprinting technology has widely used in the last decade, its intrinsic limitations for printing hollow constructs such as capillaries and vessels, as well as the proliferation of the cells in thick structures, have led to the development of 4D bioprinting (via integrating time into processes of 3D bioprinting) to better mimic the native tissue along with the higher resolution and most complexity (Fig. 10.4) [270,272].

10.5 Recent studies and advances of eye-on-the-chips

Nowadays, microfluidic-based eye-on-a-chip platforms are widely used for the study of ocular drug permeation and function of the cornea, as well as the development of ocular microphysiological systems.

The corneal multilayered microfluidic devices (containing vitrified collagen) are examples of these systems that can be used to create corneal microtissue patches and act as a substrate for cell growth. Such a multilayered device was initially introduced by Puleo et al. fabricated by fixation of the collagen vitri-gel (under vacuum and on-chip dehydration) and integration with multilayer soft lithographic technique (Fig. 10.5A). This gel-like collagen substrate (PDMS) as the eye-on-chip system provides fluidic connectivity to both sides of the poly(dimethylsiloxane) substrate and results in bilayered culture to achieve corneal microtissue (such as pore size and permeability) [273].

The blinking eye-on-chip is one of the other systems that can recapitulate the 3D eye structure and mimic its dynamic mechanical environment and physiological function. Such microdevices are usually used to study blinking duration, velocity, and frequencies and understand ocular diseases such as eye dryness and the process of wound healing. In this regard, Seo et al. showed that the collagen gel/human keratocytes-impregnated porous scaffolds along with biomimetic hydrogel eyelid could imitate the stroma of the cornea and eye blinking (Fig. 10.5B) [18]. This study also indicated that these dome-shaped polystyrene scaffolds led to the replication of the concentric epithelial pattern in vivo and regeneration of the cornea and conjunctiva; via the depositing corneal epithelial and conjunctival epithelial cells on their surface and blinking motions (by a

Table 10.5 The types of printing materials/inks to create organ-on-a-chip.

Printing ink	Description	Properties	References
Natural materials	Hydrogel-forming materials as bioinks that used for the cell encapsulation in 3D cell-printing: marine algae (alginate, agarose), proteins derived from mammalian tissues (collagen, gelatin, fibrin), plants (gellan gum, cellulose), polysaccharides (chitosan).	The viscoelastic property, high water content, low cost, protection of cell (during the printing process), and exogenous risk factors (mechanical stress, potential contaminating, drying), tunable characteristics (such as gelation kinetics and rheological properties) thermos-reversible gelation kinetics (for agarose and gellan gum), high bioaffinity/activity (for proteins)	[226,228,235,247–255]
Synthetic materials	Polycaprolactone (PCL) (as a thermoplastic polymer), silicone, poly(dimethylsiloxane) (PDMS), Pluronic F127 (triblock copolymer consisting of poly(ethylene oxide) (PEO), and a hydrophobic poly(propylene oxide) (PPO)with viscoelastic property), photo-curable resins (such as Watershed, Visijet SL Clear, PEG-DA, and MED610), and thermoplastic polymers	Biocompatible (low cytotoxicity and bioinert properties), high stiffness/rigidity and fast solidification when extruded from the printing nozzle (for PCL), the flexibility, transparency, and toughness (for PDMS) the support of cells in 3D cell-printing, nondegradable properties, remarkably	[225,235,249,256–269]

Table 10.5 The types of printing materials/inks to create organ-on-a-chip.—cont'd

Printing ink	Description	Properties	References
	(such as acrylonitrile butadiene styrene and cyclic olefin copolymer)	flexible, and easy production (silicone), the use of 3D printing along with laser/visible light-mediated polymerization (for photo-curable resins), adaptable to extrusion-based printing with high clarity (for thermoplastic polymers), suitable for particular purposes such as the construction of organs-on-chips and microfluidic devices.	

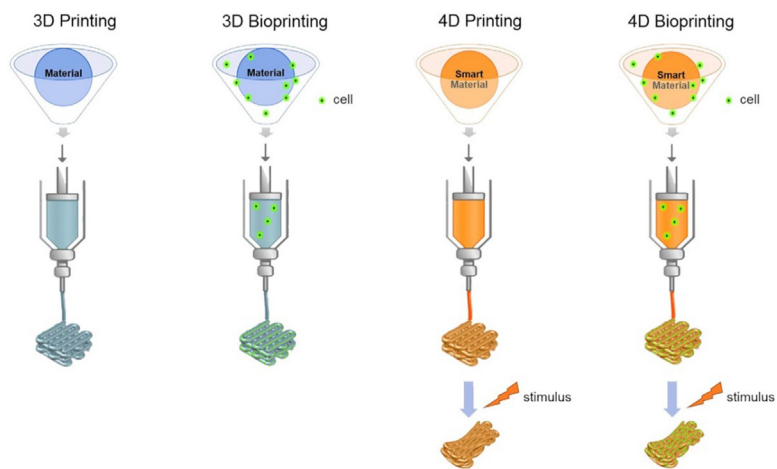


Figure 10.4 Different printing technologies of 3D and 4D printing/bioprinting. (Adopted with permission with Ashammakhi et al. [270], copyright (2018), WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.)

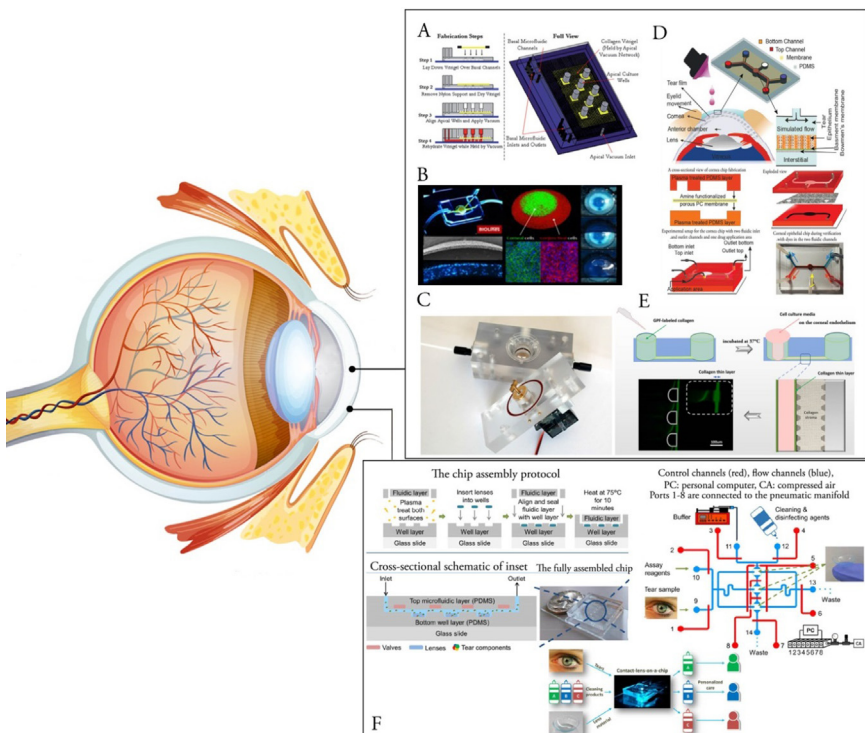


Figure 10.5 (A) The hybrid collagen/PDMS device via assembly and integration of collagen vitrigel [273]. (B) Imitation of the stroma of the cornea by collagen gel/human keratocytes-impregnated porous scaffolds along with biomimetic hydrogel eyelid [18]. (C) The piece of DynaMiTES along with a detached top level and the motor-driven mixer [274]. (D) The cornea chip includes the apical channel separated from a basal channel by a porous membrane coated with ECM [5]. (E) The 3D cornea-on-a-chip by corneal epithelial and endothelial cells (corneal-epithelium-on-a-chip). Adding collagen solution to the microchannel and the formation of the lumen by DMEM, displacing the collagen solution into the center of the channel and leaving the thin layer of condensed collagen (green line), which imitates the Bowman's membrane [275]. (F) Contact-lens-on-a-chip along with the top-view schematic of chip layout and connectivity [19].

small motor). Notably, tear flow, as the main factor for studies of drug permeability and its transport kinetics into the eye, has not been evaluated in this research. Currently, there is no platform in current eye-on-chip systems to assess the effect of blinking and the rate of tear turnover, which can be because of the small volume of tears.

Recently, ocular dynamic microtissue engineering systems, called ocular DynaMiTES, have been developed to achieve a strategy for studying the

absorption of the anterior eye and the introduction of suitable drug candidates. Such systems can provide noninvasive monitoring of the system over time. In this regard, Mattern et al. designed a novel microfluidic from polycarbonate (as an inert material) that can lead to an online analysis of drug permeation via barrier-forming tissues and the optimized electrodes-created transepithelial electrical resistance (TEER) (Fig. 10.5C) [274,276]. They also indicated that this system can be used for drug tests. The comparison of both adsorptions of static and dynamic demonstrated this chip could well mimic the flow pattern and physiological conditions of traditional static-culture systems. Followed by, the 3D-hemicornea structure, along with human keratocytes and epithelial cells, in the designed model, was examined, and its functionality and compatibility on the new chip were confirmed. The authors also concluded downsizing of the compartment could play an essential role in the decrease of the required amounts of tested drugs, especially in the preclinical study.

Corneal epithelium-on-chip systems are also another example of chips, which was introduced by Bennet et al. [5]. The main purpose of designing this system was to create a chip for mimicking precorneal characteristics and its morphology and topography, studying barriers property of the corneal epithelium and the behavior of drugs mass transport, as well as emulating tear flow attributed to eye-blinking, as two parameters influencing residence time of drugs (Fig. 10.5D). This chip was fabricated using epithelial cells cultivated on the synthetic polycarbonate membrane to achieve a 5–7-layered structure of the cornea. To study permeability, the chip surface was evaluated by two topical eye drops and applied three conditions of static, continuous, and pulsatile flow to mimic eye-blink sequences. Authors believe this *in vitro* microengineered model can be applied for imitation of the cornea environment and preclinical study of drug transport for eye allergies and inflammatory diseases.

Afterward, Bai et al. developed a corneal microphysiological model based on the corneal epithelial and endothelial cells that aimed to mimic the *in vivo* cornea function and study the permeation of ocular drugs (Fig. 10.5E) [275]. To this end, a microfluidic device that included two peripheral channels containing collagen matrix and a central channel was fabricated and mouse epithelial/endothelial cells were cultured within all three channels, to create a 3D chip model. The results showed that this 3D cornea chip as a highly accessible platform not only can be used in physiological and pathological studies of the cornea but also can be effective in new studies of the eye-on-chips and facilitate drug delivery tests.

Moreover, as contact lenses may lead to serious eye infections, the contact lens-on-chip systems have developed as valuable tools for studying the influence of disinfection drugs. In this field, Guan et al. introduced poly(dimethylsiloxane)-based chips with the 1 mm diameter of the lens, which led to fluidic control through incorporated valves (Fig. 10.5F) [19]. This lens consisted of channels for tear, disinfection, and cleaning and was able to integrate tear (1 μ L) with contact lens materials and assess the function of lens care solutions. To this end, they incubated the lens with tear samples, and then its surface was cleaned by disinfecting agents to study the influence of cleaning formulations. The authors proposed that these chips can be effective for personalized diagnostic/medicine.

Based on the reports, some systems have also been created to assess the causes of diseases such as retinitis pigmentosa and improve therapeutic methods. The eye-cavity-on-a-chip introduced by Chan et al. is an example of this system that models the posterior eye chamber and assesses the formation of droplets in the eye cavity (Fig. 10.6A). In this model, the silicone oil behavior as a therapeutic method that may result in post-operative complications during vitreoretinal surgery was assessed. The results obtained from this study show that poly (methylmethacrylate)-based chips along with the retinal ganglion cell line can mimic the surface properties of the retina [220]. In this study, the number and size of emulsified silicone oil droplets on-chip, as well as the interface between silicone oil and aqueous, were studied. Chan et al. reported that silicone oil, 5 St, could readily emulsify, and higher droplets form inside the designed chip. Hence, it seems that this system can be a suitable opportunity to assess ocular phenomena. Notably, the previous study of this group was on the emulsification of silicone oil and eye movements. They indicated that the addition of polydimethylsiloxane to silicone oil could provide an easier injection due to the increase of extensional viscosity. However, an increase in shear viscosity leads to a decrease in the formation of emulsified droplets and the oil movement in the eye because shear viscosity is the main factor in the velocity determination between oil and the wall of the vitreous cavity [282].

In a similar study, Lu et al. developed a cell-coated eye-on-chip model to assess the effect of adding high-molecular-weight silicone oil into silicone oil on adhesion and emulsification (Fig. 10.6B) [277]. The samples of silicone oil with/without high molecular weight were included SiOHMW2000, SiOHMW5000, SiO2000, and SiO5000. They proposed a new mechanism in this field: the existence of high molecular weight and

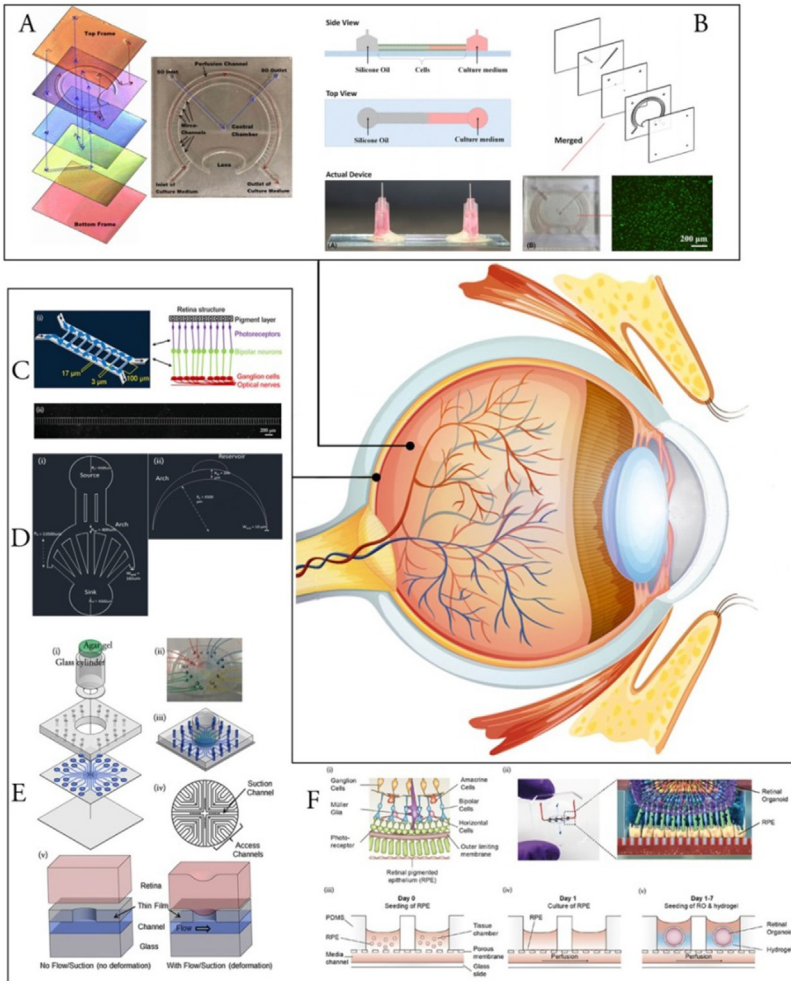


Figure 10.6 (A) Eye-cavity-on-a-chip mimicking the vitreous chamber of the eye. The routes of the culture medium within the chip (red arrows), the route of injection of silicone oil into the chamber (blue arrows) [220]. (B) Eye-cavity-on-a-chip including five layers of poly(methyl methacrylate) along with silicone oil and culture medium to create the silicone oil/aqueous interface. Fluorescence image of retinal ganglion cells, after cell culture on the chip for 12 h [277]. (C) Retinal synaptic regeneration chip. Including two chambers connected by 100 microchannels: (i) design of chip for mimicking retinal structure, (ii) microchannels in the chip [278]. (D) Human and mouse microRetina system including: (i) three compartments that are connected in series by a set of microchannels, (ii) a large compartment along with an arch-shaped gradient compartment [279]. (E) The retina-on-chip microfluidic system. (i) Exploded view with media cylinder broken into the retina well, (ii) top-view with a PDMS disk as a substitute for the retina, (iii) view assembled, (iv) access channels along with the suction

channel on the PDMS layer, (v) Schematics of deflection of the retina into through-holes in PDMS layer [280]. (F) Microfluidic retina-on-chips: (i) composition of the human retinal with the types of cell *in vivo*, (ii) retina-on-chips along with the retinal organoids photoreceptor and retinal pigment epithelium interaction, (iii) the seeded retinal pigment epithelium cells into the chip, (iv) the formation of monolayer after 24 h, and (v) loading retinal organoids and the hyaluronic acid-based hydrogel into the well and onto the retinal pigment epithelium [281].

consequently increase of extensional viscosity may lead to the resistance against the break-up of silicone oil from the substrate for the formation of emulsified droplets. When using the microfluidic device, a high molecular weight in silicone oil leads to a decrease in the number of *in vitro* formed droplets. This study can be effective in understanding how increasing the extensional viscosity of silicone oil can decrease emulsification.

Cell therapy is one of the other themes that play a role in the creation of ophthalmic chips, due to the lower number of migrating cells or lack of maturation of the implanted cells into damaged tissue of the eye (such as the retina). In this field, retina-on-a-chips have been developed for cellular studies after implantation and achievement of models with optical accessibilities [278,279].

Retinal synaptic regeneration chips (RSR chips) are one of these models introduced by Su et al. to regenerate retinal neuronal synapses and imitate the retinal function [278]. They also suggested an image-based method to determine the kinetics of RSR (Fig. 10.6C). In this model, retinal precursor cells were cultured on the designed chip with microfluidic guiding channels. After 3 days, axons emerged, and the formation of retinal synaptic connections was confirmed on the chip. The authors stated that integrating a microfluidic chip with image-based methods is a valuable technique to study factors supporting retinal regeneration and synaptogenesis mechanisms.

Likewise, Mishra et al. developed a microfluidics-based model by the soft lithographic technique called microRetina to study the migratory behavior of retinal cells in humans and mice and create models for retinal transplant therapies (Fig. 10.6D) [279]. In this study, retinal progenitor cells were initially cultured on the designed chips for 48 h, and then cellular behavior was determined by studying the response of cells to concentrations of the stromal cell-derived factor 1 (SDF-1), as a chemoattractant for the central nervous system-derived cells. Notably, the computer simulation along with experimental validation was applied to evaluate the

concentration gradient. The results demonstrated that high concentrations of SDF-1 induce directional cellular movement in the direction of its gradient. However, no cellular movement was observed in low concentrations.

The studies have also demonstrated that ocular geometry plays a vital role in the design of eye chips so that imitation of the surface curvature of the eye can provide a microfluidic platform with the capability of tissue culture and the study of the point access signaling. The research works in this field show that retina-on-chips are usually designed via applying negative pressure and creating curvature in the chip and can be used in the assessment of the tissue behavior, drug functions, and molecular signaling to the cornea [280]. Such chips can provide new approaches for fluidic chemical delivery through micrometer holes embedded in the chip surface as fluidic access points. The designed retina-on-a-chips by Dodson et al. are examples of these platforms (Fig. 10.6E). This chip included 12 channels/holes (100 μm diameter) to deliver drugs and signaling molecules in a controlled and localized manner. The response of the chip to lipopolysaccharide indicated the ability of the chip to imitate inflammation. The authors believe that this chip can create new insights into preclinical studies of various tissues.

In a new study, Achberger et al. introduced technology based on organoid-merged chips that can be effective in the design of human multilayer tissue-based in vitro models. Indeed, they fabricated a retinal microphysiological system that integrated with the types of human induced pluripotent stem cells (iPSCs)-derived retinal cells (Fig. 10.6F) [281]. The designed chip consists of two layers of poly(dimethylsiloxane) and a porous membrane of polyethylene terephthalate between the mentioned two layers, that bonded to a glass slide with a thickness of 170 μm , along with a second wafer to culture retinal organoids and retinal pigment epithelium. Based on the results, this model can create vasculature-like perfusion, increase the formation of the inner/outer segment, and recapitulate the interaction between retinal pigment epithelium and photoreceptor segments. The applicability of the chip for screening the drug was also assessed by gentamicin and chloroquine. The authors envision that human iPSC-based retina chips not only can play an important role in the development of drugs but also create new insight for the pathological understanding of retinal diseases.

As vascularization/neovascularization in the retinal pigment epithelium layer is considered an important factor in the decrease of the barrier

function of the outer blood-retinal barrier and the creation of ocular diseases such as age-related macular degeneration, some researchers have created chips that can model barrier functions and/or imitate the ophthalmic choroid layer. These chips provide a biomimetic model (in terms of phenotypical and functional) for retinal pigment epithelium and choroid layer in vitro that can assess the pathological mechanism and cellular change that occurred during disease [283–285]. In this field, Kaji et al. designed a microfluidic device along with the cell-based model. The designed chip consisted of two independent channels and a separating membrane to culture human retinal pigment epithelial cells and human umbilical vein endothelial cells on both sides of the membrane [285]. The results of this research indicated this coculture chip has developed similar properties to native tissue. This study created a new concept in the design of choroid-on-chips.

Nguyena et al. also developed a biomimetic model of angiogenic sprouting in vitro to screen effective factors on angiogenesis events (Fig. 10.7A) [286]. The results of this study illustrated that angiogenic

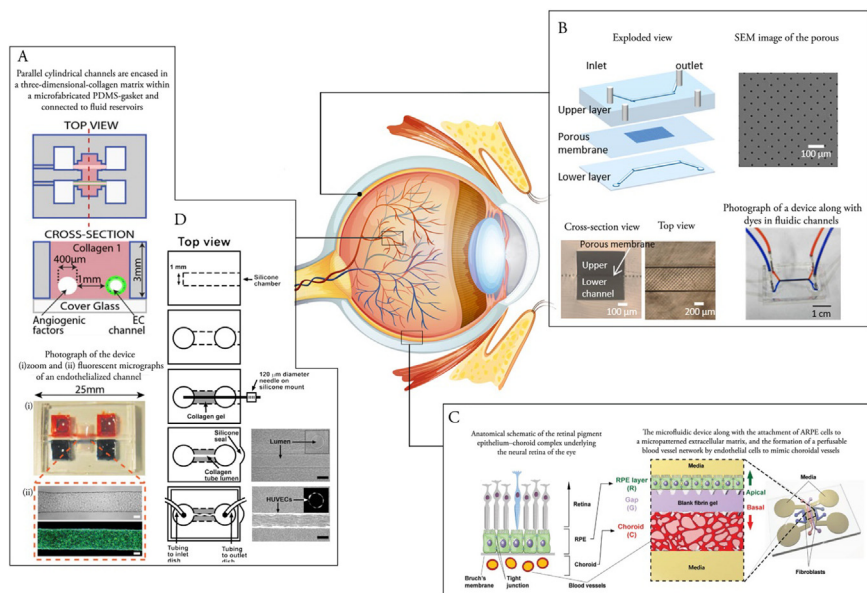


Figure 10.7 (A) The microfluidic system for the 3D formation of endothelial sprouts and neovessels [286]. (B) Microfluidic device to investigate choroidal angiogenesis [287]. (C) Wet-AMD-on-chip to model outer blood-retinal barrier, inhibit angiogenesis on the chip, and mimic 3D retinal pigment epithelium–choroid structure [283]. (D) The in vitro 3D microvascular tubes to mimic venules and capillaries in vivo [288].

sprouts in this model possessed structural similarities (such as lumens and connected branches to the parent vessels, etc.) with angiogenesis *in vivo*. In this study, the effect of angiogenic inhibitors was also assessed via the ability of VEGF receptor inhibition. The authors believe this model not only can reconstitute steps of angiogenic sprouting (morphogenetically) but also elucidates the molecular mechanisms and neovascularization.

Afterward, a coculture system with a more detailed structure to study *in vitro* angiogenesis processes and mimic Bruch's membrane was introduced by Chen et al. (Fig. 10.7B) [287]. In this model, retinal pigment epithelial cells were exposed to cobalt chloride to improve angiogenesis. Based on the results, a low glucose level and alteration in oxygen level (due to chemical hypoxia) are the main factors in increasing secretion of vascular endothelial growth factor in retinal pigment epithelial cells that lead to alteration in the growth of human umbilical vein endothelial cells. Such a system can be an appropriate model for dissecting the angiogenic process.

In a further study, Chung et al. developed a complex model to study angiogenesis-related diseases [283]. This microfluidic consisted of 3D blood vessel network along with an epithelium monolayer that was partitioned into two central and two lateral channels, by the array of microposts (Fig. 10.7C). To form a choroid network, fibrin gel-mixed endothelial cells and fibroblasts were injected into one central and two lateral channels, respectively. To create a gap between the retinal pigment epithelial cells and the choroid layer (as *in vivo* Bruch's membrane) as well as form a monolayer of the mentioned cells, fibrin (cell-free) was injected into the other central channel. Authors find that symmetric injection of fibroblast into lateral channels led to the reconstruction of *in vivo* epithelium monolayer. Moreover, the gap channel provides a stable culture for endothelial cells and retinal pigment epithelial cells. This region is also the main area for quantitative observation of pathological angiogenesis, and adding a vascular endothelial growth factor to the retinal pigment epithelial channel leads to imitation of angiogenesis of the choroid. Finally, to assess the antiangiogenic effects of drugs in the designed chip, bevacizumab was injected into the chip, and the vessel network regression was studied. Given the inhibition of angiogenesis on the chip, the authors stated that the designed chip could also be effective in determining the appropriate dosage of drugs and treating angiogenic ocular diseases.

A further example of eye-on-chip systems is related to the microfluidic device for modeling the blood-retinal barrier. The *in vitro* 3D microvascular tubes introduced by Chrobak et al. are an example of these

biomimetic models that included the culture of endothelial cell monolayer within cylindrical channels (Fig. 10.7D) [288]. To create perfusion as well as assess blood-retinal barrier and microvascular functions, the mentioned channels were created within collagen gels. The results of this study indicated that the barrier functions and adhesiveness of the endothelial layer of this tube mimic venules and capillaries *in vivo* and add inflammatory agonists such as agonists histamine and tumor necrosis factor- α (TNF- α) leading to an increase in adhesiveness and breakdown of barriers.

A further model introduced by Yeste et al. consisted of the arranged cell within parallel compartments along with crisscross microgrooves to facilitate paracrine signaling and heterotypic interactions between cells [289]. In this model, to measure TEER, the electrodes were equipped on the substrate. Then, coculture of human neuroblastoma cells (SH-SY5Y) (in five central compartments) along with human retinal endothelial cells, and human retinal pigment epithelial cells (ARPE-19) (in two outer compartments, separately) was performed to mimic the blood-retinal barrier (Fig. 10.8A). The results showed this designed chip could be a promising *in vitro* tool to monitor the functions of barrier tissues. Such a chip can also extend a new generation of sophisticated organ-on-chip models.

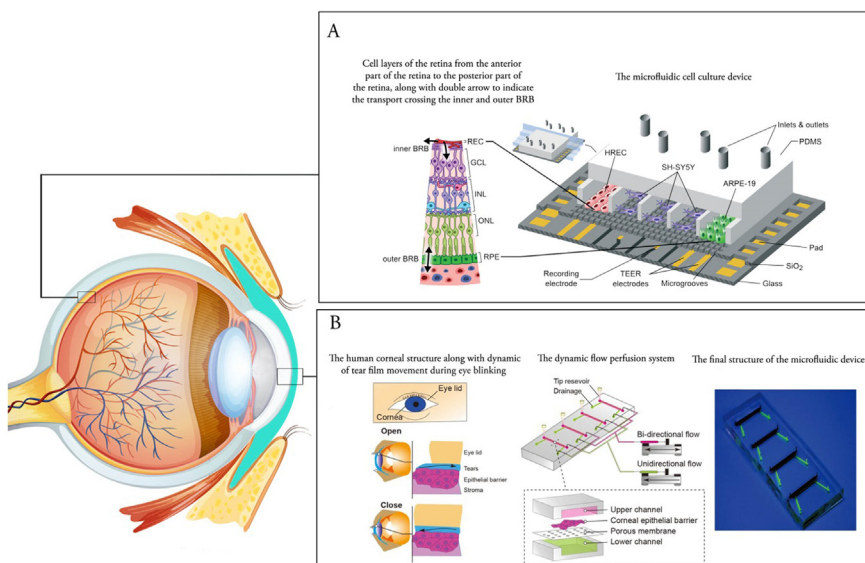


Figure 10.8 (A) The microfluidic chip to model the blood-retinal barrier [289]. (B) A multicorneal barrier-on-a-chip [290].

In recent years, similar models to the blood-retinal barrier, focusing on the blood–brain barrier [11,291,292], have also developed so that learning and using them can be effective in better design of blood-retinal barrier chips.

Based on the reports, shear stress as one of the physiological parameters of blood flow plays an important role in the maturation and stabilization of vessels (vascular homeostasis and pathophysiology). It has been found that shear stress associates with vascular damages caused by inflammation, aging, stroke, Alzheimer, etc. [293–295]. Hence, studies of the vascular response to shear stress and the creation of biomimetic models can be effective in preclinical studies. In this regard, Galie et al. fabricated the microvessels with a diameter of $\sim 400\ \mu\text{m}$, which were encased into glass coverslip-adhered bilayer poly(dimethylsiloxane), by the subtractive method [296]. They studied the threshold of flow-induced shear stress that stimulates angiogenesis by using microfluidic control. The results of their study showed that a shear stress threshold of more than $10\ \text{dyn}/\text{cm}^2$ leads to sprouting (regardless of the transmural or luminal flow). The authors also found that shear consistency throughout the vessels network plays a crucial impact in the achievement of interconnected vascular networks. Moreover, to remove focal leaks, the amount of global shear stress is more than $15\text{--}20\ \text{dyn}/\text{cm}^2$.

In a further study, Abdalkader et al. introduced a multicorneal barriers-on-chip that is able to create a dynamic culture of the human corneal barrier by the recapitulation of eye blinking shear stress forces (Fig. 10.8B) [290]. They fabricated a device of two channels along with a porous membrane for the separation of channels (upper and lower); and cultured human corneal epithelial cells on the upper channel. The result of this culture was zonula occludens-1 expression and consequently the formation of the barrier that was evaluated by translocating fluorescein sodium on the chips. Finally, by creating a bidirectional flow into the upper channel, the cells were stimulated via applying $0.6\ \text{dyn}/\text{cm}^2$ of shear stress. The results revealed that applied shear stress led to stimulation of expression of cytokeratin-19 intermediate and increase of barrier function. The authors believe that such a chip can create new insights into the development of topical ocular drugs and the effect of blinking shear stress.

10.6 Outlook

In this chapter, we have discussed the anatomical structure of the eye, along with its diseases and treatments, as well as the design methods of biomimetic ocular models and eye-on-the-chip systems that to date used in the study of the eye. In this field, the studies have revealed mathematical models play a crucial impact in understanding different ocular phenomena such as eye movements and tear film dynamics and lead to lower cost/time for design and use of eye chips. These models can be a starting point to assess the methods of drug delivery or mechanism of drugs before using drugs on eye chips. However, mathematical models, in terms of the study of drug transport and permeability, need improvement due to the limitations of precorneal and corneal. In this regard, although the eye-on-chips can imitate ocular events and environment, some of them cannot be used to screen drugs due to the lack of study of blinking and how to produce tears. Hence, it seems that the use of membrane deflection theories and their combination with membrane-based micropumps can be effective in the achievement of ideal chips with more accurate flow profiles along with the study of drug permeation.

Further studies showed that cells and organoids-based chips could create a better understanding of in vivo developmental processes, cellular signaling pathways, disease modeling, and the discovery of new drugs. These eye chips can also be effective in the prediction of toxicology and efficacy of ocular drugs and provide a unique window into therapies of eye diseases. In this field, there are various reports that indicate the development of eye-on-a-chip models to deliver drug and model disease, such as blood-retinal barrier, retina-on-a-chip, and corneal epithelium on-a-chip.

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CHAPTER 11

Pancreas-on-a-chip

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11.1 Introduction

The pancreatic islets, or islets of Langerhans, are recognized as the endocrine part of the pancreas and contain different types of hormone-secreting cells. The most important function of the pancreatic islet is to maintain normoglycemia that mostly depends on the secretion of insulin by the β -cells. Meanwhile, secretion of other cells within the islets (glucagon secretion of α -cells) and proper functioning of other organs including the liver and skeletal muscle plays an important role in this function. The inability of the body to maintain the glucose blood level in a normal range is the onset of a metabolic disorder called diabetes mellitus (DM) [1]. The

most common types of DM are type 1 diabetes mellitus (T1DM), which is recognized as an autoimmune disease causing the destruction of β -cells, and type 2 diabetes mellitus (T2DM), which is characterized by insulin resistance and β -cell dysfunction. Also, there are rare types of DM such as the maturity-onset diabetes of the young and neonatal DM that are due to genetic disorders. According to the international diabetes federation, approximately 463 million adults are diagnosed with DM in 2019, and it has been predicted that this number will reach 700 million by 2045. The direct cost of DM (health expenditures) was estimated to be USD 760 billion in 2019, and it will reach USD 845 billion by 2045. Besides, indirect costs of DM, including labor-force dropout, mortality, absenteeism, and presenteeism, increase the economic burden of DM and were estimated to constitute 34.7% of the total cost of DM in 2015 [2].

A better understanding of pancreatic islets and conditions leading to their malfunctioning could improve our knowledge of DM and results in developing treatment methods and medications for prevention, treatment, and possibly reversing the course of the disease. One of the requirements for pancreatic islet research is high-fidelity in vitro models that could reduce the cost and time of animal research. The current standard method for cell culture is based on static culture; however, pancreatic islets are vascularized mini-organs and rapidly lose their functionality in vitro due to the lack of perfusion. Some macroscale cell cultures have been developed to overcome this problem, yet they suffer from problems such as lack of physiological relevancy and limited flow control [1].

The organ-on-a-chip (OOC) technology is the most promising approach to overcome this problem. OOCs are microfluidic-based in vitro models that could mimic the characteristics of the cells' microenvironment including 3D architecture, presence of extracellular matrix (ECM), and cell-cell interaction. Besides, precise fluid flow control allows the proper exchange of nutrients, oxygen, drugs, and waste between the media and cells. It also enables online measurement of cell secretion that is significantly essential in culturing pancreatic islets. Therefore, pancreas-on-a-chip (POC) models have been developed by culturing pancreatic islets in these models that could be used for islet quality assurance and drug development [3,4]. In this chapter, we first briefly describe the physiology of the pancreatic islets. In the next section, we explain what parameters must be taken into account for developing a POC. Then, we describe possible applications of POCs in the field of pancreatic islet and diabetic research. Finally, we briefly reported some of the studies that used multi-organ-on-a-chip (MOC) to investigate the interactions of pancreatic islets with other organs.

11.2 Physiology of pancreatic islets

Pancreatic islets are spherical-shaped clusters of cells that are responsible for the endocrine functions of the pancreas. β -cells are the major component of the islets (about 80%) and secrete insulin that is responsible for reducing the blood glucose level. α -cells constitute about 20% of the cell population in the islets and produce glucagon that increases blood glucose levels by promoting glycogenolysis. δ -cells or D-cells that are about 5% of the number of islet cells release somatostatin that inhibits hormone secretion of other islet cells, anterior pituitary, and gastric parietal cells. ϵ -cells and pancreatic polypeptide cells (PP cells) or F-cells are the rare components of these clusters that are responsible for the secretion of ghrelin and pancreatic polypeptide, respectively [5,6]. In addition, other cell types reside in pancreatic islets including vascular cells, immune cells, neurons, and glial cells [7]. The secretion of these hormones is regulated by factors from the outside of the islets such as blood glucose level and interactions of cells inside the islets [5]. The crucial role of pancreatic islets is to maintain normoglycemia, which is regulated by the secretion of insulin and glucagon by β - and α -cells, respectively.

Insulin secretion by β -cells is a series of cellular events which is triggered by glucose stimulation that leads to the electrical excitation of these cells (Fig. 11.1). Upon stimulation, membrane-bound glucose transporter (GLUT2) will be activated and glucose will be transferred into the β -cell. This will be followed by glucose catabolism through the glycolysis pathway that leads to the production of pyruvate and eventually the generation of ATP in the mitochondria. An increased level of ATP closes the ATP-sensitive potassium channel and prevents K^+ efflux resulting in membrane depolarization. When the membrane potential reaches -30 mV, the voltage-gated Ca^{2+} channel opens and the influx of calcium ions triggers the exocytosis of insulin [7]. Insulin secretion by β -cells is a biphasic process. The first response is due to the release of the stored insulin which lasts about 5 min and shows a sudden increase in insulin secretion, reaches a maximum level, and rapidly decreases to the baseline. The second phase, which is due to the secretion of newly synthesized insulin, sustains while the stimulation is applied. Another important feature of insulin secretion is pulsatile behavior. This property is in synchrony with Ca^{2+} oscillation that implies the direct regulation of insulin secretion by Ca^{2+} oscillation [7,8].

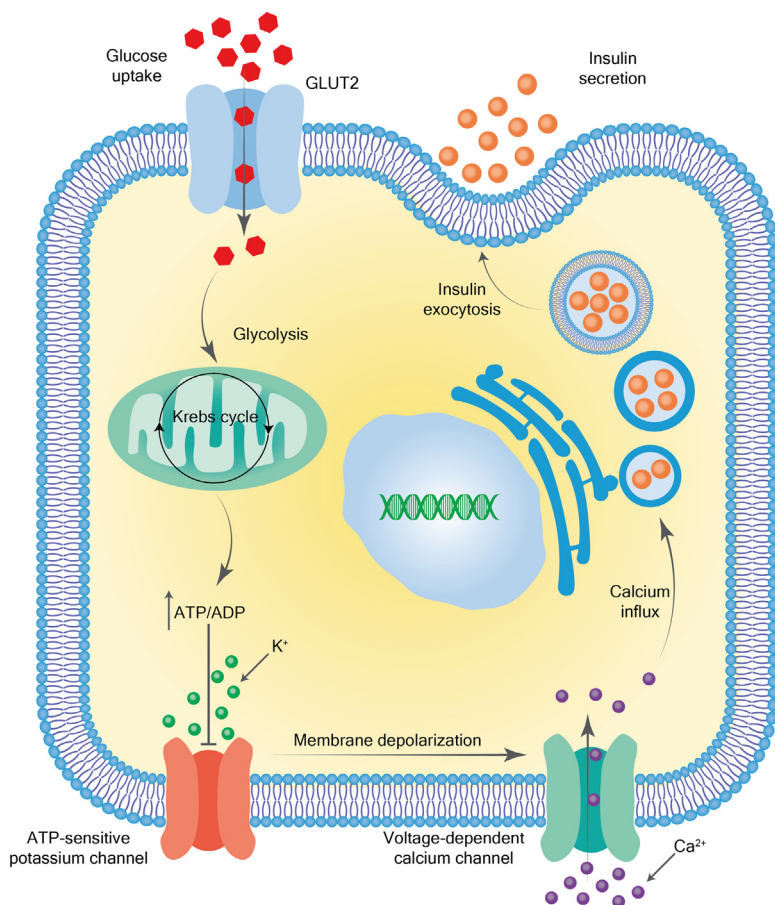


Figure 11.1 Signaling pathway of insulin secretion by β -cells.

11.3 Design considerations of a POC

11.3.1 Cell source

One of the essential components of an *in vitro* model is the cell source as the functionality of the cells affects the accuracy and its availability ensures the possibility of developing the model based on that source. Available sources for islet research include an animal or human pancreatic islets, cell lines, and stem cells (SCs) (Table 11.1 provides a comparison of these sources). Animal and human pancreatic islets are the gold standard cellular sources for pancreas and diabetic research. This is because the presence of the whole islet could resemble the mass transfer between the cells in the

Table 11.1 Comparison of different cell sources for pancreatic islet research.

Source	Advantages	Disadvantages
Human islets	• Presence of a multicellular, 3D structure • Secretion of all hormones of islets • High physiological relevancy	• Availability in small quantity • Time-consuming and expensive isolation method • Loss of functionality in vitro • Donor-to-donor variation
Animal islets	• Presence of a multicellular, 3D structure • Secretion of all hormones of islets • High physiological relevancy • More available compared to the human islet	• Interspecies differences • time-consuming and expensive isolation method • Loss of functionality in vitro • Donor-to-donor variation
Reaggregated Islets	• Presence of a multicellular, 3D structure • secretion of all hormones of islets • high physiological relevancy • Uniform cellular composition • Similar functionality • Optimized size to avoid necrosis • Easy genetic modification	• Time-consuming and expensive isolation method • Additional postisolation process • Donor-to-donor variation
Cell lines	• Availability • Low cost • Reproducibility • Easy genetic modification	• Partial loss of the cell functionality after immortalization • Absence of some islet cell types and their interactions • Lack of some of the hormone secretions • Further process for creating 3D structure
Patient SCs	• Patient specificity • Preferable for studying organogenesis and tissue regeneration	• Lack of standardized protocol for complete differentiation of SCs • Lack of some of the hormone secretions • Further process for creating 3D structure

islets and the surrounding environment. Besides, interactions between different cell types affect the secretion of the hormone by the islets. Therefore, primary islets could incorporate essential molecular and cellular mechanisms into the model. However, using primary islets could face different obstacles. The isolation procedure of islets is expensive and labor-intensive, and it could damage the vasculature of the islets that disrupts mass transfer inside the islets. Also, primary islets rapidly lose their functionality *in vitro* that limits the time span of the experiments. Another problem with using human islets is that they might be available for transplantation, but they are not accessible research. In the case of animal islets, they may not perfectly represent the human islet due to interspecies differences; for instance, different cell composition between human and murine islet results in different homotypic and heterotypic interactions that could lead to different underlying cellular mechanisms and functionality of islets [1,9].

Another problem with using primary islets is their size heterogeneity, ranging from 50 to 400 μm , which could cause several experimental challenges. Due to the disruption of the vasculature, necrosis could happen at the core of larger islets that leads to lower viability and function. In addition, islets of different sizes vary in their response to external stimuli, and due to the variation in the cell number, the interpretation of the results could be difficult. The solution to this problem is the generation of homogenous islets. For this purpose, first islet cells will be dispersed with trypsin digestion. Then, a predefined number of cells will be transferred to a container with low attachment property. The spontaneous reaggregation of cells results in clusters that are similar in size, cell number, and functionality [10,11]. Aside from uniformity, the size of these clusters could be controlled to avoid hypoxia and lack of nutrients at the core. Another advantage of this method is that while the islet cells are dispersed, transduction is easier due to the better viral penetration to the single cells compared to the whole islet. Therefore, more efficiently, adenoviruses that encode a specific gene could be introduced to islet cells to alter their gene expression and study their cellular mechanisms under certain circumstances [12].

As islets are primarily investigated for their glucose-induced insulin secretion, immortalized β -cells are an alternative for primary islets that prevent the time-consuming and expensive isolation procedure. The EndoC- β H1-3 cells are the human-derived [13–15] and MIN6 and INS-1 cells are the rodent immortalized β -cells that have been used to study the function and physiology of β -cells [16,17]. Despite their benefit for β -cells study, they cannot represent pancreatic islets due to the absence of other

cell types and a 3D structure. Accordingly, Lecomte et al. generated aggregation of EndoC- β H3 that resulted in the improved functionality of cells due to homotypic interactions and the 3D structure [18]. Also, due to the proximity of β -cells to endothelial cells in the pancreas, coculturing β -cell lines with human umbilical vein endothelial cells or islet-derived endothelial cells will improve insulin secretion and glucose sensitivity [19,20]. Moreover, other islet cell types could be added to the cell aggregate to resemble different pancreatic islet functionality. For instance, Sokolowska et al. generated aggregation of rodent α - and β -cells that could secrete both insulin and glucagon [21]. Although these cells eliminate the cost and time of islet isolation, the immortalization process changes the phenotype of these cells; therefore, they might behave differently compared to the cells of primary islet under certain circumstances.

The other cell source for developing in vitro models is SCs, which include human adult stem cells (hASCs), human embryonic stem cells (hESCs), and human-induced pluripotent stem cells (hiPSCs). Among these, hESCs and hiPSCs are more exciting candidates due to their higher ability to differentiate into different cell types [9]. In vitro models based on hESCs could help to unravel the underlying mechanisms in organogenesis and β -cell maturation. Such studies could lead to improving in vitro differentiation protocols for β -cell differentiation [11]. hiPSCs bear the genetic information of the donor; therefore, they are useful to develop patient-specific disease models and propose the most efficient treatment method for each individual. This is significantly important in the case of diabetic research as genetic background could affect the pathogenesis and complications of the disease [9]. For instance, Lovino et al. studied hiPSCs with mutations in the insulin receptor. They observed that this genetic disorder could affect proliferation, gene expression, and cellular context of the cell that indicates the potential of hiPSCs for disease modeling [22]. It is worth mentioning that, like cell lines, only β -cell-like cells are not sufficient to model pancreatic islets. Besides, pancreas organogenesis comprises the interaction of different cells with the surrounding environment and different signaling events. Likewise, the maturation of SCs toward insulin-secreting cells could be affected by parameters such as 3D environment and interaction with different cells such as neuronal cells, ECs, and mesenchymal stem cells. One approach to providing such an environment is to generate organoids. In fact, organoids consist of different cells with self-renewal and self-organizing abilities that can differentiate into a 3D structure with organ-level function [23,24]. Accordingly, such 3D models provide niche signaling and

cell–cell interactions that are necessary for the maturation of cells and improve the physiological relevancy of the model [11].

11.4 Microenvironment of the islets

Chemical, biological, and mechanical cues in the niche of the cells impact their viability and functionality. Accordingly, in developing *in vitro* models, mimicking the microenvironment of the target tissue is significantly important. First, a 3D environment is necessary for culturing cells as they experience a 3D environment in the human body. Daud et al. cultured human islets on a 3D scaffold made of poly(lactic-co-glycolic acid) (PLGA). Their result showed that not only did the 3D scaffold improve the viability of islet cells, but also it enhanced the secretion of insulin, glucagon, and somatostatin [25]. Another crucial factor of the cell microenvironment is the ability of the scaffold to prepare anchoring sites for cells. A promising approach to fulfill this purpose is to use the component of the ECM that consists of polysaccharides and proteins including fibronectin, laminin, and collagen [26]. The ECM surrounding the human islet consists of collagen fibers and a matrix called the periinsular basement membrane, and it is mostly composed of collagen type I, IV, fibronectin, and laminin [27]. Daud et al. investigated the effect of these proteins on the survival and functionality of islets *in vitro*. Unlike laminin, collagen I, IV, and fibronectin improved cell attachment to the surface. While viability and gene expression of the islets were higher on collagen type I and IV, morphology and phenotype of the islets were better preserved on fibronectin that was also confirmed by higher stimulation index (SI) and insulin release [28]. Krishnamurthy showed that culturing INS-1 on collagen type I and IV enhanced insulin secretion and expression of *pdx-1* and insulin genes due to the binding of the $\alpha 3 \beta 1$ integrin to these proteins [29]. Accordingly, by providing these binding sites, not only did the ECM components enhance the functionality of cells, but also they improve the viability of peripheral islet cells, which is a common problem for *in vitro* islet culture [30].

Another important feature of the microenvironment of islets is perfusion. Pancreatic islets are heavily vascularized mini-organs that account for 1% of pancreas mass while their capillary network is five times greater than the exocrine part of the pancreas [31,32]. As the pancreas mainly comprises metabolically active β -cells, the lack of proper oxygen and nutrient exchange will lead to necrosis at the islet core. Besides, low concentrations of serum proteins and oxygen adversely impact ECs in the islets, the

functionality of which directly affects β -cells as each β -cell is associated with at least one EC [33,34]. Accordingly, traditional static cell culture models are not a proper choice for culturing islets, and they lead to rapid loss of islet function and viability. The advantage of microfluidic devices is the possibility of a perfused mode of action to precisely control fluid flow and resemble the transport phenomena between cells and the vasculature that involves continuous delivery of oxygen and nutrients and removal of metabolites and wastes. Previous studies by the Rocheleau group showed that a perfused microfluidic device for culturing islets could reduce necrosis, increase length and fractional area of ECs, and result in higher density and vasculature structure throughout the islets. The proposed mechanism was that the perfusion improves the media access to intercellular spaces and the center of the islet. This was proven by imaging islets perfused with fluorescent dextran-supplemented media and improved functionality and density of ECs with serum-supplemented media [34,35]. In addition to improving physiological relevancy, perfusion enables monitoring hormone kinetics in response to external stimuli with high temporal resolution. Moreover, multiple microphysiological systems (MPSs) could be integrated to investigate tissue-tissue cross-talk [4,9].

11.5 Trapping site for islets

Fixation of pancreatic islets is necessary for their *in vitro* survival and functionality as it mimics the presence of periinsular basement [25]. In addition, from an operational viewpoint, a predetermined site for islet trapping allows the imaging process to be performed automatically [36]. Hence, different strategies have been used for trapping islets in the design of a POC. Constriction of the islet pathway in microchannels is a method for trapping islets in a POC. Rocheleau and Piston developed a movable dam-like wall using an actuator (Fig. 11.2a). Pressurizing the actuator moves the dam toward the islet trapping region so that this region could fit the size of the islet that entered from the islet inlet [37]. Sankar et al. also created a dam by dropping the height of the channel from 125 to 25 μm that prevents further movement of the islet in the channel while allowing the perfusion of media through and around the islets (Fig. 11.2b) [34]. The main disadvantage of these designs is that while they hold islets in place, the imposed shear stress on islets could lead to a dampened glucose-stimulated response of periphery β -cells [34,35].

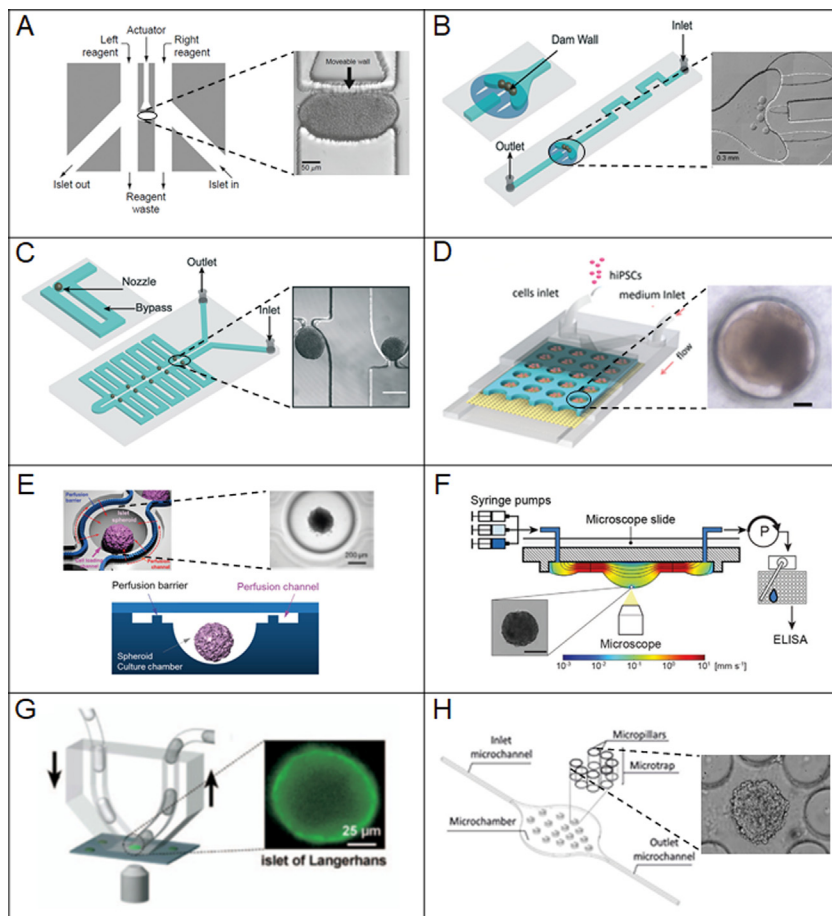


Figure 11.2 Different strategies for trapping pancreatic islets in POCs. (a, b) Dam-like structure, (c) hydrodynamic trapping, (d, e) microwell types, (f, g) open-channel systems, and (h) micropillar type. (A) *reprinted and modified with permission from J.V. Rocheleau, D.W. Piston, Combining microfluidics and quantitative fluorescence microscopy to examine pancreatic islet molecular physiology. in: Methods in Cell Biology [Internet]. 1st ed. Elsevier Inc., 2008, pp. 71–92. Available from: [https://doi.org/10.1016/S0091-679X\(08\)00604-3](https://doi.org/10.1016/S0091-679X(08)00604-3) © 2008 Elsevier, (B) *reprinted and modified with permission from P.N. Silva, B.J. Green, S.M. Altamentova, J.V. Rocheleau, A microfluidic device designed to induce media flow throughout pancreatic islets while limiting shear-induced damage. Lab Chip 13(22) (2013) 4374–4384. © 2013 The Royal Society of Chemistry and K.S. Sankar, B.J. Green, A.R. Crocker, J.E. Verity, S.M. Altamentova, J.V. Rocheleau, Culturing pancreatic islets in microfluidic flow enhances morphology of the associated endothelial cells, in K. Maedler (Ed.), PLoS One [Internet]. September 22 [cited 2019 August 7]; 6(9) (2011) e24904. Available from: <https://dx.plos.org/10.1371/journal.pone.0024904> © 2011 Sankar et al., (C) *reprinted and modified with permission from P.N. Silva, B.J. Green, S.M. Altamentova, J.V. Rocheleau, A microfluidic device designed to induce media flow***

One approach to confine cells in a microchannel while controlling the microenvironment of the cell and reducing shear stress is hydrodynamic trapping [35]. In this approach, the fluidic resistance and eventually the cell movement will be controlled by the microstructures called trapping sites [38,39]. For example, Silva et al. developed a microfluidic device containing 10 cup-shape nozzles as the trapping islets (Fig. 11.2c). When islet suspension is inserted into the system, the islets will be delivered to the trapping site due to the higher fluidic resistance of the bypass channel, which was controlled by changing the length of this channel. However, when a trapping site is occupied, the islet will bypass the site due to its higher resistance and move toward the bypass channel that will deliver the islet to the next empty site and ensure sequential occupation of the trapping sites [35].

Microwell design is another approach to avoid high shear stress imposed on cells. Having developed a multilayer microfluidic device, Tao et al. attached a polycarbonate porous membrane to a through-hole poly(-dimethylsiloxane) (PDMS) membrane to produce microwell structures in

throughout pancreatic islets while limiting shear-induced damage. *Lab Chip* 13(22) (2013) 4374–4384. © 2013 The Royal Society of Chemistry, (D) reprinted and modified with permission from T. Tao, Y. Wang, W. Chen, Z. Li, W. Su, Y. Guo et al. Engineering human islet organoids from iPSCs using an organ-on-chip platform. *Lab Chip* [Internet]. 19(6) (2019) 948–958. Available from: <http://xlink.rsc.org/?DOI=C8LC01298A> © 2019 The Royal Society of Chemistry (E) reprinted and modified with permission from S.H. Lee, S. Hong, J. Song, B. Cho, E.J. Han, S. Kondapavulur et al. Microphysiological analysis platform of pancreatic islet β -cell spheroids. *Adv. Healthc. Mater.* [Internet]. January; 7(2) (2018) 1701111. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/adhm.201701111> © 2017 WILEY-VCH Verlag GmbH and Co. KGaA, Weinheim, (F) reprinted and modified with permission from P.M. Misun, B. Yesildag, F. Forschler, A. Neelakandhan, N. Rousset, A. Biernath et al. In vitro platform for studying human insulin release dynamics of single pancreatic islet microtissues at high resolution. *Adv. Biosyst.* [Internet]. March 29; 4(3) (2020) 1900291. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/adbi.201900291> © 2020 WILEY-VCH Verlag GmbH and Co. KGaA, (G) reprinted and modified with permission from D. Chen, W. Du, Y. Liu, W. Liu, A. Kuznetsov, F.E. Mendez et al. The chemistode: a droplet-based microfluidic device for stimulation and recording with high temporal, spatial, and chemical resolution. *Proc. Natl. Acad. Sci. India.* November; 4 105(44) (2008) 16843–16848. Available from: <http://www.pnas.org/cgi/doi/10.1073/pnas.0807916105> © 2008 The National Academy of Sciences of the USA, and (H) reprinted and modified with permission from P. Sokolowska, K. Zukowski, J. Janikiewicz, E. Jastrzebska, A. Dobrzyn, Z. Brzozka, Islet-on-a-chip: biomimetic micropillar-based microfluidic system for three-dimensional pancreatic islet cell culture. *Biosens. Bioelectron.* [Internet]. July; 183 (2021) 113215. Available from: © 2021 Elsevier.)

the device and culture several islets in parallel (Fig. 11.2d) [40]. Lee et al. developed an SU-8 mold with concave cavities using gray-scale lithography. Next, they replicated the pattern using PDMS, and subsequently, the PDMS layer was replicated on a polyurethane layer to create a master mold. Then, soft lithography was used to develop a PDMS layer with a hemispherical concave network for trapping islets (Fig. 11.2e) [41]. In another approach, Jun et al. developed an array of concave microwells using the surface tension of the PDMS prepolymer. After fabrication of a PDMS layer with flat chambers, a mixture of PDMS precursor and its curing agent was poured onto this layer to fill the chambers, and the unwanted amount of the solution was removed by applying pressure and wiping. Finally, the remaining solution formed a concave surface in the chambers and was polymerized by heating [10].

Open microfluidic systems have also been used for islet immobilization. In hanging drop design, islets and a small liquid volume will be loaded into the droplet compartment and a standing drop will form. Then, the chip will be flipped to a hanging drop configuration and the islet will sediment at the bottom of the drop (Fig. 11.2f). Such design benefits from easy loading and retrieval of islets, which is ideal for further analysis. Besides, compared to closed microfluidic systems, open microfluidic systems provide sufficient oxygen supply due to the liquid–air interface and reduce the possibility of analyte adsorption by decreasing the surface area of the PDMS layer. However, it must be mentioned that controlling the fluid flow and precise volume of the hanging drop is more difficult in this design. Moreover, hanging drops might evaporate, which affects the concentration of the analyte in the outlet [42,43]. Another open-channel device was also designed by Chen et al. In this study, an islet was cultured on a glass substrate, and the rest of the device was placed on top of the islet and fixed on it by surface tension (Fig. 11.2g) [44]. This device benefits from a plug-based microfluidic device to deliver arrays of aqueous plugs to the device and record the response of the islets to the stimuli with high temporal resolution. The other advantage is that this method does not require islet loading to the system [1].

Micropillars have been used to trap cells in a perfused system for the in situ formation of spheroids and organoids. Sokolowska et al. developed a microfluidic device containing 15 microtraps for culturing cell aggregate (Fig. 11.2h). Each of these microtraps was created using seven micropillars that enabled cell aggregation by reducing shear stress and the growth surface. Introducing a cell suspension containing α and β cell lines, they were

able to generate pseudo islets within the first 24 h that secreted both insulin and glucagon. When they compared it to a microwell design, they observed that the microwell design led to necrotic cores and a random distribution of the two cell types. However, the micropillar design resulted in 40% higher cell viability and a microarchitecture that is more relevant to native islets, which is composed of most of the β -cells in the core and α -cells in the peripheral regions [21]. Using another strategy, Wang et al. developed a micropillar-based OOC to generate hepatic organoids. The device consisted of micropillar arrays, and the hiPSCs were inserted into the system utilizing a perfusion system. The gaps between these micropillars provided a microenvironment for the formation of hepatic embryonic buds and eventually the generation of hepatic organoids [45]. Such design could be used for the in situ generation of pancreas organoids to develop patient-specific POCs.

11.6 Integration of analytical tools

Conventional methods to assess the viability and functionality of cells in vitro are invasive, labor-intensive, and have a low temporal resolution. Besides, online monitoring of cellular activity could help to find the optimum and reproducible conditions for cell culture. Therefore, integration of analytical methods to OOCs is critical to monitor the mechanical, (bio) chemical, and electrical activities of the cells. In particular, an in-line (bio) sensor is an essential feature of a POC to capture the activity and glucose-stimulated response of β -cells. The most important features of these sensors include high spatiotemporal resolution, high selectivity for the analyte so that other cosecreted metabolites do not disturb the signal of the analyte, being minimally invasive to cells, and being compatible with the microfluidic device [7,46].

Peptide hormones secreted by pancreatic islets play important roles in glucose metabolism rendering them target analytes that must be measured to ensure the functionality of islets. Traditional methods for monitoring these peptides are based on radioimmunoassay and enzyme-linked immunosorbent assay (ELISA); however, offline processing and low temporal resolution make these methods inefficient for studying the kinetic of hormone secretion. Capillary electrophoresis immunoassay is the most commonly used method for online measurement of insulin [47], glucagon [48], and amyloid polypeptide [49]. In this method, the effluent of the cultured islet, antibody, and fluorescent

antigen will be transferred to a reaction channel for their online mixing before entering the electrophoresis channel. Then, the mixture will be injected into the electrophoresis channel wherein an applied voltage separates the free and bound antigen. Finally, the ratio of the free and bound antigen will be used to quantify the antigen in the cell culture effluent [47,48].

Localized surface plasmon resonance (LSPR) is another technique that has been used for insulin quantitative measurement [50]. This phenomenon results from the interaction between a beam of light and conductive nanoparticles that are smaller than the wavelength of the incident light. Upon LSPR excitation, both adsorption and scattering properties of the particle are enhanced; consequently, LSPR could be detected by optical spectroscopy [51,52]. One exciting property of LSPR is that it occurs in the proximity of the nanoparticle that localized the detection, consequently, reducing the required sensing area. Besides, a simple optical device could be used for the detection of this phenomenon. Therefore, it has a high compatibility for integration with OOCs [53]. Ortega et al. integrated an LSPR sensing module to a POC for the detection of insulin secretion. The sensing compartment was composed of an array of gold nanorods that were functionalized with mercaptoundecanoic acid to immobilize insulin antibodies. The binding of insulin to the antibody will lead to a shift in the extinction spectrum of the nanorod that could be used for the measurement of insulin concentration in the effluent of the POC [50].

Aside from peptide hormones, ionic compounds involved in the cellular activity of β -cells could be monitored for indirect quantification of hormone secretion. Due to the transparency of materials that are commonly being used for microfluidic device fabrication such as PDMS and Borofloat glass, fluorescent microscopy could be used for live-cell imaging. Accordingly, transportation and oscillation of Ca^{2+} , which plays a significant role in the metabolism and insulin secretion of β -cells, have been visualized using calcium-sensitive dyes such as Fluo-4 and Fura-2 [1]. For instance, calcium signaling has been used to determine the effect of shear on the oscillatory response of periphery islets to glucose stimulation [34,35]. GCaMP6m has also been used for the online detection of calcium flux. It is a genetically encoded calcium indicator that will be delivered to cells or islets through viral infection. It contains green fluorescent protein and when

it binds to Ca^{2+} , the conformational change induces a fluorescence signal that could be used for detecting calcium oscillation in islets [12,30]. Other regulatory species for quantitative measurement of islets' performance are zinc ions as they are cosecreted with insulin and regulate insulin secretion through ATP-gated and zinc-modulated P2XR channels. In this regard, combining zinc indicators and confocal laser scanning microscopy could be used to quantify insulin secretion [1,54]. To perform on-chip measurement of Zn^{2+} , Easley et al. continuously perfused a single islet with a FluoZin-3-containing media and implemented a water-in-oil droplet generator downstream of the effluent. This device enables high temporal resolution for zinc measurement that could detect both fast and slow oscillatory zinc secretion [55].

11.7 Applications of POCs

11.7.1 Islet physiology and biology

Due to precise control of islets microenvironment in POCs, they could be used to study the effect of different conditions on the functionality of islets. As islets are heavily vascularized, parameters such as oxygen content and shear stress significantly impact their morphology and function. Sankar et al. studied the effect of CoCl_2 -induced hypoxia on perfused pancreatic islets and compared it to that of the exogenous VEGF-A treatment (Fig. 11.3). Their result showed that CoCl_2 -induced hypoxia was more effective for the survival and functionality of ECs compared to exogenous VEGF-A treatment. This means that hypoxia did not only induce VEGF-A expression and had other effects on ECs; therefore, it could prolong the viability and functionality of islets in vitro [56]. Jun et al. investigated the impact of two different flow rates (8 and 25 $\mu\text{L}/\text{h}$) on reaggregated islets. Compared to the static model, both dynamic models resulted in a higher level of microvilli on the surface and the interior of the islets that are enriched in GLUT2 and play an important role in cell signaling and insulin secretion. Comparing the results on day 14 of the two dynamic models showed that the model with the higher flow rate led to rounded cell and loss of cell–cell contact while the lower flow rate preserved the morphology of islets and microvilli. These results were also confirmed with higher SI in the model with a lower flow rate implying that proper shear stress could maintain the morphology and phenotype of islets [10].

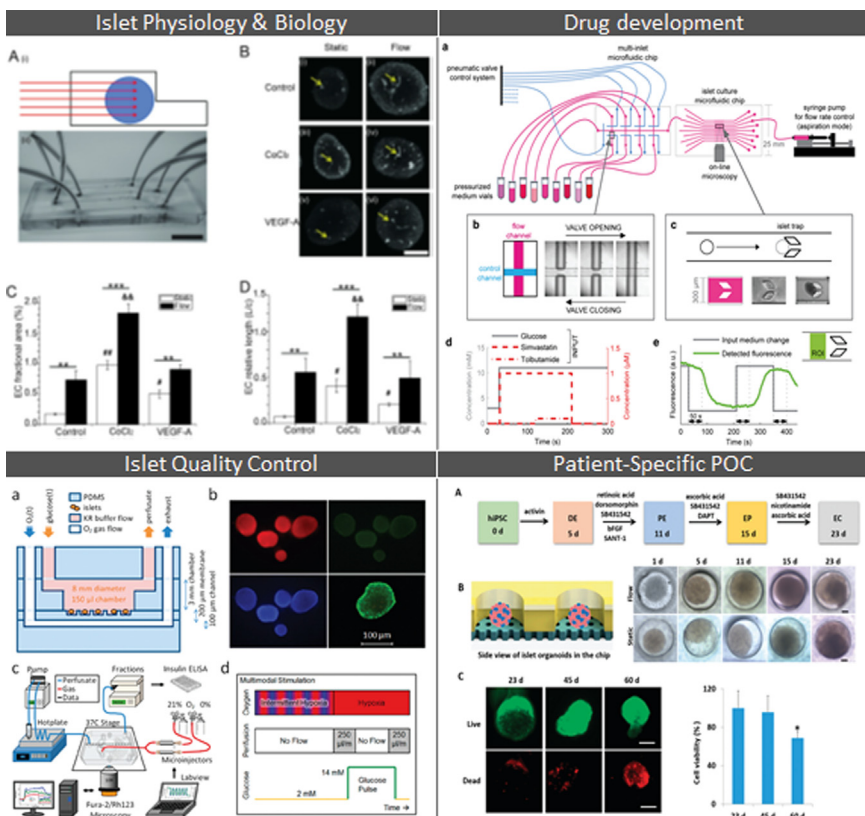


Figure 11.3 Applications of POCs in diabetic research. (Reprinted and modified with permission from K.S. Sankar, S.M. Altamentova, J.V. Rocheleau, Hypoxia induction in cultured pancreatic islets enhances endothelial cell morphology and survival while maintaining beta-cell function. Zhao F, editor. *PLoS One* [Internet]. October 10; 14(10) (2019) e0222424. Available from: <https://doi.org/10.1371/journal.pone.0222424> © 2019, Sankar et al., V. Scattolini, C. Luni, A. Zambon, S. Galvanin, O. Gagliano, C.D. Ciubotaru et al. Simvastatin rapidly and reversibly inhibits insulin secretion in intact single-islet cultures. *Diabetes Ther.* [Internet]. December 9; 7(4) (2016) 679–693. Available from: <http://link.springer.com/10.1007/s13300-016-0210-y>, © 2016, The Authors J.F. Lo, Y. Wang, A. Blake, G. Yu, T.A. Harvat, H. Jeon et al. Islet preconditioning via multimodal microfluidic modulation of intermittent hypoxia. *Anal. Chem.* [Internet]. February 21; 84(4) (2012) 1987–1993. Available from: <https://pubs.acs.org/doi/10.1021/ac2030909>, © 2012, American Chemical Society T. Tao, Y. Wang, W. Chen, Z. Li, W. Su, Y. Guo et al. Engineering human islet organoids from iPSCs using an organ-on-chip platform. *Lab Chip* [Internet]. 19(6) (2019) 948–958. Available from: <http://xlink.rsc.org/?DOI=C8LC01298A>, © 2019, The Royal Society of Chemistry)

Calcium propagation through pancreatic islets is another important phenomenon that impacts insulin exocytosis. Pistons group investigated the parameters that govern Ca^{2+} oscillation [57–60] that are summarized in Table 11.2. For example, they used a microfluidic device to stimulate a single islet from each side. Stimulation from either side led to a gradient in NAD(P)H autofluorescence throughout the islet and a flat calcium concentration from β -cells residing on the opposite side of glucose stimulation.

Table 11.2 Examples of studies in the biology and physiology of pancreatic islets using POCs.

Cell source	Islet trapping method	Purpose	Results	References
Rodent islets	Microwell	Modulating the oxygen content to study the effect of hypoxia on glucose-stimulated islet response	• Loss of magnitude and biphasic characteristic of calcium signaling • Reduced insulin secretion and mitochondrial hyperpolarization • Improved islet responses by intermittent hypoxia preconditioning	[76]
Rodent islets	Dam-like wall	Mimicking hypoxia by CoCl_2 treatment and observing its effect on the islet cells	• 8-fold increase in HIF-1α • Enhanced glycolysis while mitochondrial metabolism being preserved • Enhanced VEGF-A expression by ECs • Increased EC area, number, and vessel length	[56]

Continued

Table 11.2 Examples of studies in the biology and physiology of pancreatic islets using POCs.—cont'd

Cell source	Islet trapping method	Purpose	Results	References
Reaggregated rodent islet spheroids	Microwell	Effect of flow on the islet cells	• Stable size and DNA content of spheroids with increased number and area of ECs in the presence of perfusion • Higher level of F-actin—enriched filopodia • Improved ECM synthesis by ECs • Upregulation of Pdx1, Glut2, and glucagon	[10]
Rodent islets	Dam-like wall	Role of gap junctions in glucose-stimulated response of the islets	• The sufficiency of 30% KATP-present β-cells for glucose-dependent response • Asynchronous Ca^{2+} oscillation due to the treatment of islets with an inhibitor of gap junction • Dependence of glucose response on the gap junction coupling of KATP channel activity	[58]
Rodent islets	Dam-like wall	Effect of electrical coupling on glucose-stimulated calcium response of the islets	• A minimum required local glucose concentration (6.6 μM) for Ca^{2+} oscillation • Inability of β-cells in the	[57]

Table 11.2 Examples of studies in the biology and physiology of pancreatic islets using POCs.—cont'd

Cell source	Islet trapping method	Purpose	Results	References
Rodent islets	Dam-like wall	Studying factors that contribute to Ca^{2+} wave dynamics	transmission of glucose-stimulated calcium response due to insufficient electrical coupling • Increased transmission of Ca^{2+} oscillation by KATP channel antagonist (tolbutamide) • Propagation of Ca^{2+} wave from locations with higher metabolic activity • higher velocity of Ca^{2+} wave caused by uniform excitability • Regulation of Ca^{2+} wave direction by the origin of applied glucose stimulation • Heterogenous calcium concentration within β-cells with lack of electrical coupling • Regulation of the extent of Ca^{2+} wave propagation by gap junctions	[59]

Continued

Table 11.2 Examples of studies in the biology and physiology of pancreatic islets using POCs.—cont'd

Cell source	Islet trapping method	Purpose	Results	References
Reaggregated human islet spheroids	Microwell	The effects of G_i and G_q GPCR pathways on insulin and glucagon secretion	• Attenuation of insulin and glucagon secretion by G_i signaling • A first stimulatory and then continued inhibitory effect of G_q signaling on insulin secretion • Increased secretion of glucagon and somatostatin by α- and δ- cells, respectively, due to activation of G_q signaling	[12]

However, stimulation from both sides led to a uniform NAD(P)H concentration and Ca^{2+} oscillation of all representative β -cells implies that the electrical coupling of β -cells is not sufficient to transmit the glucose-stimulated response to the nonstimulated part of the islet. Incorporating a K_{ATP} channel antagonist resulted in further transmission of Ca^{2+} oscillation through the islet with a lower frequency. This indicates that while Ca^{2+} oscillation depends on the number of closed K_{ATP} channels, the frequency of the oscillation is governed by the glucose concentration [57].

Aside from the physiology of islets, the cellular mechanisms that govern the functionality of the islets could be studied using POCs. One example is the signaling pathway of G protein-coupled receptors (GPCRs), integral membrane signaling proteins that respond to external signals. Particularly in islet cells, these proteins affect hormone secretion. Accordingly, Walker et al. virally manipulated dispersed islet cells and reaggregated them in islet spheroids to study the effects of G_i and G_q GPCR pathways on islet cells. Their results showed that G_i signaling inhibits the secretion of insulin and glucagon. In the case of G_q signaling, higher glucagon secretion was

observed by α -cells. In contrast, a transient stimulation followed by an inhibitory effect was observed for the insulin secretion of β -cells. They also directly activated islet cells using KCl that could not overcome the inhibitory effects suggesting that these effects occur downstream of glucose metabolism [12].

It is worth mentioning that although most of these studies used rodent islets, these are proof of concept for the capability of microfluidic technology for islet research, and integration of human islets with these devices could improve our understanding of the biology and physiology of the human pancreatic islet.

11.8 Drug development

The treatment for T2DM is mainly based on monotherapy or combination therapy of antidiabetic drugs. The most common antidiabetic drugs include sulfonylureas, biguanides, insulin sensitizers, α -glucosidase inhibitors, amylin agonists, incretin mimetics, and SGLT2 inhibitors. The first prescribed drug is metformin (a biguanide antidiabetic drug) unless there is a contraindication of its usage for the patient. In this case, other hypoglycemic agents will be chosen based on the conditions of the patients. Besides, in case monotherapy based on one of these drugs is ineffective, combination therapy could be used to maintain normoglycemia in the patient. Moreover, novel drug delivery systems including microparticles, nanoparticles, liposomes, and niosomes have been proposed to overcome some of the problems with conventional drug delivery systems such as the solubility of the drugs, loss of potency, and lack of specific target [61].

Robust and accurate pancreas models are required to assess the required dosage regimen of existing drugs, combine therapy based on these drugs, and develop new antidiabetic drugs or drug delivery systems. 2D cell culture and animal models are the most common models that are used in the pharmaceutical industry for this purpose. 2D static models cannot mimic the physiological conditions of the cells' environment resulting in loss of functionality in cells and inaccurate prediction of their response to drug candidates. The result of animal studies may not be extrapolated to human studies due to interspecies differences. Besides, animal models are expensive, time consuming, and raise ethical concerns. As a result, the lack of a robust and accurate model for this purpose prolongs the drug development process and makes this industry less profitable [62,63]. As microfluidic devices could provide a perfused model and mimic the niche of the islets, POCs have been proposed for drug testing and screening (summarized in Table 11.3). Lee

Table 11.3 Applications of POCs for drug testing.

Drug category	Drug candidate	Drug concentration (μM)	Cell source	Outcome	References
Antidiabetic drug	Tolbutamide GLP-1	0–200 0–0.1	Reaggregated islet spheroids	• Increased level of insulin secretion in a dose-dependent manner • higher sensitivity of the POC to the drug candidates compared to the conventional models	[10]
	Tolbutamide	10, 25	Rodent islets	• Increase in the depth of calcium propagation across the islet • Capability of the POC for precise determination of islet physiology	[57]
	GLP-1	0.1	Rodent islets	• Upregulation of Glp-1r, Gcgr, Reg3a, Ins1, Ins2, and Stt and downregulation of neurod • Higher effect of GLP-1 on gene expression of islets cultured on a chip compared to that of the islets cultured petri dish	[3]
Immunosuppressive drug	Rapamycin	0–0.4	Reaggregated islet spheroids	• Decrease in cell viability and SI in a dose-dependent manner • Lower toxicity of rapamycin in case of dynamic POC, compared to that of the static POC and intact islets	[10]
Drug for T2DM complications	Simvastatin	1	Rodent islets	• Rapid and reversible decrease in insulin secretion • Observing intact calcium oscillation that is unlike previous studies	[66]

et al. examined the possibility of creating a T2DM model by perfusing β -cell spheroids with media containing high concentrations of glucose or palmitic acid. The results showed a higher level of intracellular reactive oxygen species (ROS) generation due to glucolipotoxicity. Also, higher activity of caspase 3/7 indicated that apoptosis cascade was induced in the β -cell spheroids. Moreover, the study of gene expression showed higher expression of uncoupling protein 1, which negatively impacts insulin secretion, and upregulation of glutathione peroxidases, peroxiredoxins, and superoxide dismutase, which protects cells against oxidative damage, indicating the ROS-mediate damage due to hyperglycemia and hyperlipidemia. Therefore, such a POC could be used to induce a T2DM environment in vitro to assess the efficacy of hypoglycemic agents [41].

Aside from disrupted glucose homeostasis, there are complications such as cardiovascular disorders, kidney dysfunction, and neurodegenerative diseases from which DM patients might suffer [64,65]. As a result, aside from hypoglycemic agents, other drugs must be prescribed to stop the progression of these conditions. Moreover, in the case of T1DM, immunosuppressive drugs must be used posttransplantation to avoid the destruction of transplanted islets by the immune response. One important notion about these drugs is that they could be toxic toward pancreatic islets and exacerbate the problems with maintaining the blood glucose level in a normal range. Therefore, POCs could be used to assess the toxicity of these medications to pancreatic islets. Statins are one of these drugs that are recommended to reduce the risk of cardiovascular morbidity and mortality; meanwhile, they could disrupt normoglycemia in both healthy and diabetic patients. Scattolini et al. developed a POC capable of temporal control of the media that is fed to a single islet to study the effect of simvastatin on pancreatic islets (Fig. 11.3). They showed that while simvastatin inhibited insulin secretion, it did not impact intracellular calcium concentration. Therefore, simvastatin affects insulin exocytosis, downstream of Ca^{2+} regulation. They also showed that the inhibitory effect of simvastatin could be reversed by tolbutamide treatment [66].

While more studies are required in this area, even these limited studies demonstrate the potential of POCs to study the effect of drugs and chemicals on pancreatic islets. For instance, Jun et al. showed that a dynamic model with precise control of the fluid flow could improve the sensitivity of islets to drug candidates [10]. Besides, it prevents the accumulation of the examined toxic agent and waste metabolites in the microenvironment of the cells and could avoid overestimating the toxicity of that compound. Besides, the study of Scattolini et al. showed that these models can provide us with more

accurate information about the effect of drugs on cellular pathways of islet cells [66]. This also implies the application of POCs in toxicological studies. Previous studies have investigated the impact of different chemicals such as benzene [67], styrene [68], and diazinon [69] on pancreatic islets using traditional 2D models or animal models. Substituting these models with POCs will allow a better understanding of the mechanisms by which these chemicals cause toxicity to pancreatic islets. Therefore, not only could POCs accelerate the drug development process and lead to a broad range of drugs and treatment methods for DM and its complications, but also they could be used as a tool for studying the toxicological effects of toxins and chemicals.

11.9 Islet quality assurance for transplantation

As mentioned earlier, T1DM is an autoimmune disease that impairs insulin secretion. The most common treatment for T1DM is insulin therapy, which is not completely successful. Besides, its failure could lead to the progress of the disease and development of diabetic complications such as neuropathy, nephropathy, retinopathy, heart disease, and atherosclerosis. Besides, insulin therapy could cause oppressive episodes of hypoglycemia that will affect the patients' quality of life [70,71]. Islet transplantation has been proposed as a promising treatment for T1DM that has received FDA approval for clinical studies. As organ procurement and islet isolation process could negatively impact the functionality and microvasculature of islets, it is important to ensure the quality of isolated islets before transplantation [72]. Therefore, standard and reliable methods are required to assess the functionality of the islets postisolation.

One approach for the islet assessment is *in vitro* glucose-stimulated insulin secretion. As islets rapidly lose their functionality in traditional *in vitro* models, the glucose-stimulated response may not be satisfactory and cannot predict the posttransplantation *in vivo* response. Diabetic mouse models are another method for this purpose. In these models, islets are grafted to the animals, and the potency of islets will be measured by their ability to reverse the disease. Although useful, this method is time consuming and requires experts to perform the surgical procedure. Besides, rodents are nocturnal animals while experiments are performed during the day, which will affect the reliability of the results [4]. Therefore, POCs could be used to assess islet quality because (1) they can ensure the precise control of the microenvironment that prevents loss of functionality, (2) they need a small amount of tissue that is ideal in islet

research, (3) dynamic mode of action enables detection of temporal dynamics of insulin secretion, and (4) online quantitative measuring techniques could be integrated to these systems to monitor the real-time response of islets to a stimulus [73,74].

As mentioned in section 3.4, Several studies have been proposed POCs to assess the quality of islets based on measurement of Ca^{2+} oscillation [57], Zn^{+2} exocytosis [55], and secretion of hormones [47–49]. As the functionality of islets and secretion of insulin are complex phenomena and depend on different parameters, devices that permit multiparametric assessment of islets could provide us with coherent information about the viability and potency of islets for transplantation [73]. Oberholzer and Eddington team developed POCs for simultaneous measurement of mitochondrial membrane potential change and intracellular Ca^{2+} oscillation. For this purpose, they used Rhodamine 123 (Rh123) to detect mitochondrial hyperpolarization and Fura-2 for Ca^{2+} influx measurement [74,75]. Due to the high metabolic activity of β -cells, the oxygen consumption rate is also an important parameter that affects the cellular activity of β -cells. Accordingly, Schulze et al. simultaneously measured oxygen consumption rate and analyzed NAD(P)H autofluorescence to investigate the relation between the kinetic of these two phenomena and insulin exocytosis [73].

In addition to evaluating the potency of the islets, in vitro modeling of conditions that islets experience during isolation and transplantation could provide us with information to precisely predict the outcome of islet transplantation. The hepatic portal vein is one the most common site for islet transplantation that has a lower oxygen concentration compared to the pancreas; therefore, islets that are transplanted to this region could undergo hypoxia. Lo et al. modulated oxygen concentration by controlling the opening of two microdispensing nozzles that were connected to two inputs of 0% and 21% oxygen concentrations (Fig. 11.3). In this manner, they could impose hypoxia on islets and quantitatively measure their response by monitoring intracellular Ca^{2+} , mitochondrial potential, and insulin secretion [76]. Another challenge in islet transplantation is the immune response that could reduce the viability and functionality of the islet. The solution to this problem is the encapsulation of islets, which protects them from the immune system. One of the most investigated forms of encapsulated islets is alginate microparticles [77]. Accordingly, Nourmohammadzadeh et al. developed a microfluidic device for hydrodynamic trapping of microencapsulated pancreatic islets. They showed

disrupted activity of encapsulated islets under hypoxia by monitoring intracellular calcium concentration, mitochondrial potential, and redox ability of mitochondria [38].

11.10 Patient-specific POC

Personalized medicine, also called precision medicine, is a strategy to diagnose, prevent, and develop new therapeutic interventions for each individual. In this approach, factors such as genetic background, gender, and age of the patient could play a major role in proposing the treatment method. Accordingly, instead of in vitro models based on cell lines, using patient-specific platforms could be a better option to incorporate the above-mentioned factors into drug screening [78]. This is also of high importance in diabetic research as studies showed that genetic information plays a major role in T1DM and T2DM [9]. For example, patient-specific models could be used to investigate the effect of the immunosuppressive drugs on the immune cells or the islets of the patients, which is a common medication after islet transplantation to treat T1DM or propose therapeutic interventions for T2DM. Mun et al. developed a POC based on the pancreatic islets and pancreatic ductal epithelial cells (PDECs) of the patients. For this purpose, they collected the remnant cell pellets following total pancreatectomy with islet autotransplantation, and isolated pancreatic islets and PDECs. Then, they were cultured on two sides of a microfluidic, and the system was used to study the cross-talk between pancreatic islets and PDECs and whether malfunctioning of cystic fibrosis transmembrane conductance regulator (CFTR) proteins could impair the insulin secretion of the islets. Moreover, such models are useful for other purposes such as assessing the endocrine functionality of the patients' islets or investigating the effect of alcohol abuse on the functionality of CFTR proteins [79].

Although patient-derived pancreatic islets could provide the possibility of patient-specific models, scarcity of donors and the invasive, costly, and time-consuming islet isolation procedure render this source inapplicable for this purpose. A promising candidate for this purpose could be hiPSCs. As mentioned earlier, they bear the genetic information of the patient and their somatic origin makes them more accessible [9]. As a result, their differentiation to insulin-secreting β -cells provides an unlimited source for pancreatic islet study. However, as pancreatic islets contain different cell types, only insulin-secreting β -cells could not be sufficient to recapitulate islet physiology and function. One approach to tackle this problem is to

develop hiPSC-derived organoids and create a tissue-like structure on a smaller scale compare to the original size of the tissue that resembles the architecture and function of islets [40].

Despite exciting characteristics of organoids such as multicellularity and organ-like architecture, lack of vasculature may reduce their functionality and viability during in vitro culture. One approach to providing a dynamic system to resemble blood flow and facilitate media exchange is using a perfused OOC [11]. For the in situ generation of hiPSC-derived islet organoids, Tao et al. developed a multilayer microfluidic chip consisting of upper and bottom layers with microchannels for perfusion of media and cell insertion, and a through-hole PDMS bonded to a polycarbonate porous membrane to create parallel 3D chambers for cell culture (Fig. 11.3). To form embryonic buds, a suspension of hiPSCs was infused into the microfluidic device and it was followed by a differentiation protocol. Gene expression analysis of the organoid showed expression of specific markers of both α -cells and β -cells, which proves heterogeneity of the organoid. In addition, the pancreatic organoids showed a dose-dependent insulin secretion in response to glucose and depolarization via KCl increased insulin secretion that can prove mature β cell-like properties of the organoids [40]. Accordingly, such methodology could be used to develop personalized models with applications in diabetic research.

11.11 MOC

In the human body, organs are separated from each other, but they communicate through the blood and lymph circulation. This is of high importance in the case of the endocrine system due to the presence of hormonal feedback loops that control the secretion of homeostasis-regulating hormones [80]. However, traditional in vitro models only rely on culturing cells without considering the effect of other organs that cannot resemble the phenomena that occurs in the human body and negatively impacts the accuracy of the results. Consequently, this could increase the cost and time of the drug development process as these models may predict the efficacy of drugs inaccurately and cannot include phenomena such as toxicity of the drugs to other organs or secondary toxicity. On the other hand, microfluidic technology is capable of integrating different MPSs to develop MOCs and include tissue-tissue cross-talk into the system [81].

Glucose homeostasis is a complex phenomenon in the human body that involves different organs. Upon food intake, insulin is secreted by β -cells of

the pancreas that mediates glucose uptake by the liver and skeletal muscles. Also, when the blood glucose level is low, α -cells of the pancreas release glucagon that stimulates glycogenolysis in the liver to increase the glucose level back to normal [82]. In addition, disruption of normoglycemia is involved in different complications, specifically in the case of T2DM. Aside from lower secretion of insulin and increased glucagon secretion that is related to the pancreas malfunction, these complications include (1) insulin resistance in insulin-sensitive tissues such as liver and skeletal muscle, (2) elevated level of glucose production in the liver, (3) higher level of glucose reabsorption in the kidney leading to sustained hyperglycemia, (4) damage to the neural transmitters that are responsible for insulin-associated appetite-suppressing signaling, (5) enhanced level of lipolysis in adipose tissue, and (6) deficiency of incretin effect in the small intestine [9].

Due to the presence of perfusion, it is possible to integrate different OOC systems to study the interactions of different organs, which is called MOC. Nguyen et al. developed a microfluidic device for integrating the 3D culture of L-cells, which resides in the small intestine, and INS-1 to observe the effect of glucagon-like peptide-1 (GLP-1) secretion by L-cells. GLP-1 changed the dynamic of insulin secretion of β -cells resulting in a faster secretion rate after 30 min and a higher maximum level compared to that of culturing only INS-1 [83]. The intercommunication between the liver and pancreas is significantly important in maintaining normoglycemia, the anabolic activity of the liver, and insulin secretion of islets. Developing a pancreas-liver-on-a-chip system, Essaouiba et al. studied the effect of coculture on each of the two MPSs. The expression levels of CK18 and CYP3A2, which are hepatic differentiation marker, were higher in hepatocytes of the coculture system. Pancreatic islets also showed more stable secretion of insulin and glucagon over a longer period that indicates the positive effect of the MOC system on both the MPSs [63]. Bauer et al. also integrated liver and pancreas MPSs to investigate the effect of the cross-talk on glucose and insulin levels. In the coculture system, glucose level was lower compared to that of the islets monoculture that indicates insulin-stimulated glucose consumption by the liver MPS. In addition, when a high glucose load was inserted into the monoculture system, the level of glucose and insulin increased over time; however, in the coculture system, normoglycemia was obtained within 24 h and insulin secretion reached a steady state once the glucose level was stable, which indicates the feedback loop between the pancreas and liver MPSs [84].

Although more studies are required on this topic, the existing research shows the potential of microfluidic technology for integrating pancreas MPS with other organs to study its interaction with them. Such MOC systems could be used to understand the intercommunication between organs that are involved in maintaining normoglycemia. Also, by resembling T2DM, it is possible to study how high insulin and blood glucose level impacts other organs and how these organs function under these conditions. Finally, by integrating the liver and immune system MPSs, it is possible to study the effect of immunosuppressive drugs and their metabolites, and the immune response on the functionality of pancreatic islets, which are possible scenarios that might happen posttransplantation.

11.12 Conclusions

In conclusion, POCs could be significantly useful for islet research as they can provide precise control of fluid flow and mechanical, biological, and biochemical cues in the microenvironment of the cells. Besides, perfusion and integration of quantitative measurement methods enable high spatio-temporal resolution detection of hormone secretion by the islet cells. These features provide the opportunity to develop a fast, precise, and reliable method for islet quality control before islet transplantation, and avoid traditional *in vitro* and animal models that might lead to inconclusive results. Also, they could be used for prolonged culture of islets *in vitro* to study the underlying mechanisms in the (patho)physiology of pancreatic islets. This could improve our understanding of the biology of human pancreatic islets and create robust and efficient tools for drug screening to develop new antidiabetic drugs or treatment methods. In addition, the possibility of integrating different MPSs could be significantly useful for diabetic research. This is because several tissues and organs are involved in this phenomenon and, its malfunctioning could negatively impact these organs as well.

As mentioned, expensive and labor-intensive islet isolation and altered phenotype of cell lines limit their application in islet and diabetic research and there is still a need for an available cell source of high functionality. One approach to answering this problem is generating mature-like β -cells from hiPSCs; however, current protocols cannot lead to cells with enough maturity and function, and the lack of standard protocols renders them less applicable. To answer this problem, POCs could be used to develop standard protocols for generating mature-like β -cells as they can reduce the cost of such studies by minimizing the required reagents and media per

experiment. Besides, creating OOCs based on hiPSCs allows to better understand the organogenesis of pancreatic islets and to develop personalized disease models for each individual with a specific treatment method.

Nomenclature

CFTR	Cystic fibrosis transmembrane conductance regulator
DM	Diabetes mellitus
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GPCR	G protein-coupled receptor
hASC	Human adult stem cell
hESC	Human embryonic stem cell
hiPSC	Human-induced pluripotent stem cell
HUVEC	Human umbilical vein endothelial cell
LSPR	Localized surface plasmon resonance
MOC	Multiorgans-on-a-chip
MPS	Microphysiological system
OOC	Organ-on-a-chip
PDEC	Pancreatic ductal epithelial cells
PLGA	poly(lactic-co-glycolic acid)
POC	Pancreas-on-a-chip
PP	pancreatic polypeptide
Rh123	Rhodamine 123
ROS	Reactive oxygen species
SC	Stem cell
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

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CHAPTER 12

Musculoskeletal tissue-on-a-chip

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12.1 Introduction

Bone is one of the largest organ systems (approximately 15% of the weight) in the human body [1,2]. This substantially mineralized tissue serves various critical physiological functions in the body, including shape formation, organ protection, locomotion, muscle anchorage, mineral storage, hormone regulation, and hematopoietic niche maintenance [3,4]. To support these essential functions and preserve the skeleton's integrity while lifetime, this dynamic organ consistently undergoes resorption to remove the old or damaged bone by osteoclast cells and subsequent new bone formation osteoblast cells [5,6]. Normal bone remodeling is an essential process for the

human body, and any disruption in this well-coordinated process might result in severe bone disorder [3]. While genetic disorders make up significant components of bone failures, abnormalities can also arise from toxic radiation and drug exposure could result in bone diseases [7]. These diseases affect the ability of hematopoietic cells to proliferate and differentiate within the bone marrow [3,7,8]. The most common bone disease caused by elevated bone resorption is osteoporosis, which affects 1 in 3 women over the age of 50 [3,9], and it is predicted that this disease will annually increase the economic burden to over 25 billion dollars by the year 2025 [3]. Furthermore, recent reports revealed that bone is the third most prevalent site of cancer metastasis for breast, lung, and prostate cancer [3,10]. Also, other cancers including melanoma, thyroid, and kidney cancer can metastasize into bone, posing serious bone problems [3,8]. Therefore, to avoid the mentioned consequences more research on bone regeneration and bone diseases seems necessary.

In recent years, reproducing the peculiarities of the human bone niche *in vitro* has been one of the leading research fields in managing various human bone concerns [8,11]. Although conventional *in vitro* 2D cultures have been the groundwork of many scientific advances, due to different surface stiffness, surface topography, and cell–cell and cell–extracellular matrix (ECM) interactions, these cultures cannot successfully mimic the *in vivo* microenvironment [3,8]. To address these limitations, 3D bioengineered systems based on human patient-derived cells were designed to provide a more precise simulation of cell interactions through *in vivo* environments [3,12]. Recently, the invention of *ex vivo* and *in vitro* on-chip devices has helped to simulate the bone microenvironment to improve the development of therapies and drug diagnosis [13]. Organ on a chip (OOC) can be presented as a microengineered biomimetic platform developed to assist in mimicking the physiological functions of the organs or specific cells in a similar environment to *in vivo* [12]. These devices are minuscule systems with channels varying in size from 10 to 1000 μm that allow the flowing of small quantities of fluid (between 10^{-9} and 10^{-18} L) [14]. OOC devices are capable of simulating the microarchitecture, cell environment, and cell signaling [12]. Also, the more advanced devices could mimic the mechanical, physical, and biochemical characteristics of a living organ [3]. The materials used for this application should be: (1) biocompatible: to support an adequate cellular response in a specific application, (2) long-term durable in culture medium: to support biological experiments such as differentiation of mesenchymal stem cells or ECM secretion, (3) capable of gas

exchange: to provide the required O_2 and CO_2 concentrations and avoid cell damage [12]. As a consequence of nonrepresentative preclinical models and inconsistency between clinical and preclinical trial results, the vast number of therapeutic candidates cannot enter the market [8,15]. Additionally, the incorrect assessment of the adverse effects of such drugs has ended in increasing the hospitalization rate [14,16,17]. An accurate OOC is capable of providing a timely and high-throughput platform that leads to more precise results due to patient-derived tissue samples assist for personalized drug screening or help to have a better simulation of specific diseases [8,15]. This review presents new advances in designing different 3D bone-on-a-chips that mimic the in vivo environment characteristics. We cover the potential of bone-on-a-chip technology in investigating the foundational biology of bone diseases, therapeutics identification, and providing a more precise condition to actual tissue for further investigation.

12.1.1 Bone marrow-on-a-chip: a general overview

The bone cells derive from the human bone marrow (hBM), and significant damage or injury to bone marrow significantly increases health risks and can lead to severe bone diseases or death [7,11]. Until now, there has been no way to study the progression of hematopoietic cell responses in humans without conducting invasive biopsy procedures [7]. Additionally, studies specializing in bone regeneration commonly use 2D cell cultures to test in vitro or animal models to test in vivo, yet are constantly limited from replicating the real characteristics of the microenvironment within the bone [3,8]. Also, to obtain more realistic details such as cell-to-matrix and cell-to-cell interactions, understanding the physiological bone function in a similar niche is unavoidable [3]. Though many OOC studies typically use animal cell lines, a bone marrow-on-a-chip study is capable of using primary human cells at their source [18]. Furthermore, the currently used culture systems and animal models are especially limited at translational value for the development of medications [3,7].

Overall, human bone-marrow-on-a-chip (BM chip) has the capability of supporting hematopoiesis in vitro where the stem cells and the progenitor cells can properly differentiate into red and white blood cells from interactions with stromal cells due to the proper chemical and physical gradients present within the microenvironment [13]. These devices can predict the hematotoxicities during patient exposure to a specific drug and model the bone marrow recovery and cytotoxicity after maturation [7].

The 3D bone tissue modeling through the BM chip has improved mineral deposition, response to stimuli, cell differentiation, and tissue organization, better mimicking a bone microenvironment composed of a mineralized extracellular matrix not seen in 2D models [8]. The BM chip has a huge potential for patients with genetic disorders once deemed incurable, cancer metastases, and osteoporosis, creating a pathway to accurately simulate the human processes and improve the quality of medical care [3,19].

12.1.2 Bone marrow-on-a-chip: utilizing nanotechnology to simulate bone marrow microenvironment

Due to the various factors that play a role in the simulation of a micro-environment, building an in vitro model can bring a more in-depth understanding of a more precise condition of cells and their interactions [20]. For instance, Nelson et al. designed a multi-niche, microfluidic, micro-vascular human BM-on-a-chip to explore the behavior of hematopoietic stem cells (HSC) and progenitor cells in a 96-well plate. This platform included three primary niches on a chip: (1) endosteal niche, (2) stromal cell containing central marrow niche, and (3) interconnected microvasculature network. The device was designed with five channels: two media channels, two outer gel channels, and one gel channel. The array of 5-channel devices was connected into standard well-plates and the polydimethylsiloxane (PDMS) layer to a bottomless 96-well plate (Fig. 12.1A). They could successfully recapitulate both the central perivascular BM niche and the vascularized endosteal BM niche on this device. To assess mineralization, they performed alizarin red and von Kossa staining on seeded mesenchymal stromal cells (MSCs) on the coated central channel and differentiated in osteogenic media. Their results showed a significant increase in mineralization after 21 days (Fig. 12.1B and C). Also, by examining the HSC proliferation and CD34 expression, the capability of simulating the HSC maintenance in the endosteum by the designed hBM-chip was verified (Fig. 12.1D). Additionally, the biologically relevant cytokines secreted from osteoblasts, Interleukin 6 (IL-6), Interleukin 11 (IL-11), macrophage colony-stimulating factor (M-CSF), Stem cell factor, C-X-C Motif Chemokine Ligand 12 (CXCL12), and Leukemia inhibitory factor, confirmed the successful simulation of the physiologically relevant human BM niche on a chip (Fig. 12.1E) [11].

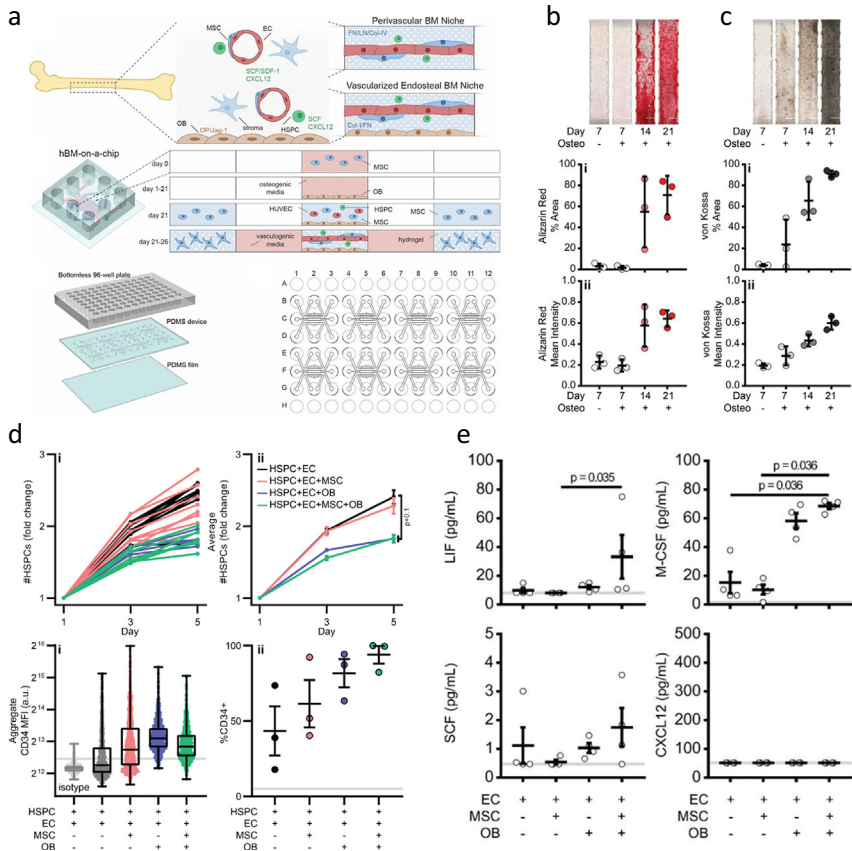


Figure 12.1 (A) A brief schematic of human bone marrow-on-a-chip (5-channel PDMS microfluidic device) and the device potential to recapitulate both the central perivascular BM niche and the vascularized endosteal BM niche. (B) Mineralization assessment of differentiated MSCs on day 21 on the central channel of the hBM-on-a-chip by alizarin red and (C) Von Kossa staining, Quantification of alizarin red and von Kossa staining reported by (i) percent area and (ii) mean intensity. (D) CD34⁺ HSPC behavior in hBM-on-a-chip. (E) Hematopoietic cytokine secretion measured in device supernatant collected on day 5. (Reprinted with permission from Ref. [11].)

12.1.3 Bone marrow-on-a-chip: diseases treatment application

Advanced bone metastasis is associated with a high morbidity rate of patients diagnosed with cancer [21]. The metastasis rate of cancers such as breast, prostate, lung, and melanoma carcinomas is significantly high (~70%) [8,22]. Unfortunately, there are several inherent obstacles that impede the study of cancer metastasis in the bone marrow, including the

physical difficulty of manipulating bone as a tissue, complexity of obtaining antemortem samples of bone metastases from human patients, a limited number of in vivo and ex vivo models that effectively mimic human disease, and inconvenience of identifying or monitoring cancer cells using in vivo or ex vivo models [8,23]. In addition, the existing in vitro models are unable to replicate the pathological condition precisely [19]. The bone marrow needs to be first generated in mice and then explanted whole and maintained in vitro within a microfluidic device. The need for an in vitro growth process prevents its application to large-scale bone study. Other shortcomings of the existing models include high unit cost, the large dead volume of individual bioreactors, and the difficulty to find the same region of interest during long-term cell culture. To address those shortcomings, a microfluidic 3D bone model to study cancer cell extravasation to bone is necessary [8].

Metastasis-on-a-chip models show the metastasis cascade with separate primary and secondary tumor sites to show the live monitoring of the invasion of cancer and tumor cell migration [17,20]. Bersini et al. developed a 3D microfluidic model to investigate the specification of breast cancer metastasis to bone. Their assessment of the extravasation rate and extravasated distance of breast cancer cells in the presence of a desirable matrix (Fig. 12.2A and B) indicated that the extravasated cancer cells could potentially proliferate and form micrometastases within the bone microenvironment. Compared to collagen gel-only matrices, the extravasation of cancer cells in their model was significantly higher in the bone microenvironment (Fig. 12.2C). They could also validate the role of C-X-C chemokine receptor 2 (CXCR2) as a breast cancer cell receptor and the C-X-C Motif Chemokine Ligand 5 (CXCL5) as the bone-secreted chemokine in the extravasation rate and their traveling distance (Fig. 12.2D and E). Their results showed cancer cells could form micrometastases of various sizes comprising 4–60 within the osteo-cell-conditioned microenvironment (Fig. 12.2F and H). This model demonstrated the capabilities of the bone-specific model and could potentially assist researchers in gaining more information about cancer biology to develop more specific and effective anticancer therapeutics [24].

In another study, Aleman et al. fabricated PDMS devices in which the cell-growth chamber and medium reservoir are linked to a tank through an additional tube to facilitate cell seeding and regular daily medium exchanges. They classified the BM into four major niches to investigate the preferred interactions of hematopoietic stem and progenitor cells (HSPCs), leukemic,

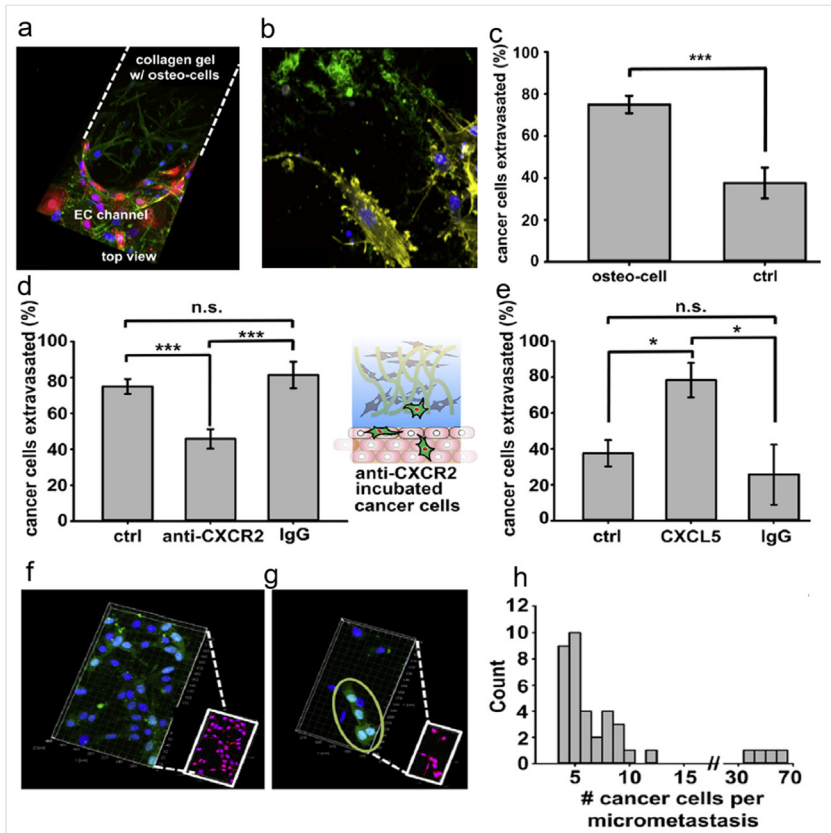


Figure 12.2 (A) Top view of the 3D reconstruction of a confocal stack on day 3, endothelial cells (ECs) are stained in red, cancer cells are stained green and, nuclei were stained blue. (B) Capability of differentiated hBM-MSCs to secrete osteocalcin (green) within the 3D microenvironment. nuclei were stained with DAPI in blue and phalloidin stained by F-actin in yellow. (C) Comparison between average percentage of extravasated cancer cells in the collagen gel with differentiated hBM-MSCs. (D) Effect of CXCR2 in cancer cell extravasation within collagen gel containing osteo-differentiated hBM-MSCs compared to nontreated cancer cells (ctrl) or IgG incubated cancer cells. (E) Effect of CXCL5 in cancer cell extravasation within collagen gel containing osteo-differentiated hBM-MSCs compared to nontreated cancer cells (ctrl) or IgG incubated cancer cells. Generation of large (F) and small. (G) Cancer cell micrometastasis within the osteo-cell-conditioned microenvironment. (H) Cancer cells distribution histogram per micrometastasis. (Reprinted with permission from Ref. [24].)

and lymphoma cells with the perisinusoidal, periaarterial, osteoblastic, or mesenchymal niches. This group used “on-a-chip” technology to examine the interactions of the different niches within the BM microenvironment to

evaluate the differences in normal or malignant HSPC within the niches mentioned earlier. All four niches of cell populations are encapsulated in a hyaluronic acid–gelatin-based hydrogel. In this closed system, the medium is pumped through a battery-powered peristaltic micropump to eliminate the manual medium exchange over time. To ensure the even distribution of the cells to four niche constructs, the consistent fluid flow in each of the four arms of the device is required. In this study, they observed the flow consistency by infusing dyed phosphate-buffered saline (PBS). Moreover, they created a fluid dynamics computational model of the device using Flow3D software to simulate the infusing cells into the device. The obtained heatmap of the fluid flow rates through the device model confirmed the consistent fluid flow in each region of the device. On day 5, the results showed CD34+HSPC lodged within the mesenchymal and sinusoidal niches, lymphoma cells homed in the vicinity of the osteoblastic niche as well as mesenchymal and sinusoidal niches, and the leukemic cells localized mainly in the area of the osteoblastic niche and had less tendency to the mesenchymal in sinusoidal niches. These results could potentially assist in developing more novel and accurate therapies by targeting the abnormal cells or signaling in the malignant niche [13].

As mentioned previously, bone diseases, which arise from an imbalance in bone cells [25,26], can be studied in a simulated environment using osteoblasts embedded into collagen and cultured among tumor cells [8]. With this microfluidic platform, it is capable of determining the tumor cells, morphological changes, cell death, and visualizing mechanisms in pathology [26]. A crucial component that a bone chip model further emphasizes is the extravasation of tumor cells during colonization of bone [27]. The presence of biophysical stimuli adds to the accuracy of the human internal environment, which is otherwise limited in nonchip models [19]. Myeloma and hematological malignancies are also among the bone diseases where bone chips can be applied [26]. To accomplish this, immortalized cell lines of hematological diseases are cultured with mesenchymal stromal cells from bone marrow as well as osteoblasts [28–30]. Models can further be improved with pumpless microfluidic platforms and syringe pumps to promote interstitial perfusion [28,30]. Also, it is possible to propagate primary cells and maintain unique patient marker expression and heterogeneity within a bone-on-a-chip model [26,31].

In summary, bone chip models increase the potential for personalized medicine wherein patient-derived bone marrow cells can be used to obtain a better understanding of drug responses and opens the possibility of

tailoring the highest effective treatment to patient care. The bone chip models also pave a path for studying microenvironment-induced drug resistance and cell adhesion-mediated drug resistance [25]. As drug resistance increasingly hinders medical treatment, the simulated environment provided by the bone chip model allows for the study of the mechanisms under which drugs develop resistance [32].

12.1.4 Bone marrow-on-a-chip: testing new drugs and therapeutics

Many hematopoietic drug studies currently being conducted depend upon animal studies *in vivo* to test for efficacy [33]. Due to the high sensitivity of the hematopoietic system to damage from radiation, bone marrow failure among other risks of lethal health is concerning. Therefore, it has become increasingly important to accurately study drug responses as countermeasures to radiation toxicity [33,34]. As animal studies have many drawbacks with high expenses and an inaccurate assessment of relevance to humans in general, a BM chip can better predict and highlight drug efficacy by mimicking bone marrow structures and functions [33,35]. With the development of an *in vitro* model, bone marrow toxicity screens can be performed, the hematopoiesis mechanism can be studied, and drug testing can reveal new input about the formation of blood cells [35].

In this manner, Torisawa et al. have developed a BM chip microfluidic device where the living bone marrow can flourish as an essential niche for hematopoiesis while simultaneously recreating radiation injury response seen in bone marrow *in vivo*. In this study, the formation of bone marrow was induced through surgical removal of the bone and placed into the BM chip microfluidic device, where it was submerged in the medium. From this model, the dynamics of continuous blood cell production and pharmacological countermeasures can be tested on the repair of radiation-damaged bone marrow. Also, this BM chip showed great potential in screening for drugs that are capable of reinitiating the production of red blood cells as the addition of erythropoietin, an erythrocyte stimulating agent [35,36]. In addition, the BM chip could function as a platform for closely examining the effect pharmacological countermeasures provide against radiation exposure seen through the study's administration of granulocyte colony-stimulating factor (G-CSF) and bactericidal/permeability-increasing protein (BPI) [37–39]. Both G-CSF and BPI are known to advance recovery of hematopoiesis after radiation damage that causes bone marrow failure and the similarity between the result of BM

chip in vitro model and in vivo systems was confirmed [37–39]. In comparison, conventional static bone marrow cultures could not detect the effects of these radiation countermeasure drugs, whereas the BM chip microfluidic device could detect the applied drugs successfully [35]. The results of the study above were further echoed by the BM chip developed by Wyss Institute [40]. This chip eliminated the need for animal studies and replaced them with an engineered bone marrow with results showing the effectiveness of preventive drugs against radiation exposure successfully left the bone marrow intact [40]. The BM chip, therefore, provides a successful and novel method to test the effects of harmful agents and drugs in a reproduced bone marrow microenvironment [35,40].

12.1.5 Cartilage-on-a-chip: design and manufacturing techniques

Approaches to developing cartilage-on-a-chip platforms are necessary for the advancement of disease modeling and drug testing platforms [41]. Generally, there are two main approaches to develop cartilage-on-chips including harvesting the tissue explants and the use of three-dimensional bioprinting to achieve the proper 3D environment that not only represents the mechanical niche of the tissue being analyzed but also retains the functionality of the whole unit being studied. These two general techniques have also been further specialized by incorporating gene modification of host tissue before explantation and 3D print culturing in bioreactors to promote endothelial cell alignment in structures needing vascularization [42]. These systems could be obtained either by adding nonbiological materials to act as scaffolds for cell cultures or including the direct additive manufacturing of biological materials into their intended three-dimensional shape [42,43]. Additionally, these platforms are highly representative of the specific organ or tissue environments and can even be adjusted to add the stresses induced by fluid (blood) flow into the system [3]. Design and prototyping of lab-on-a-chip models in the past have been long and costly, with the requirement of system culturing in bioreactors being a significant contributor. With the introduction of bio-3D printing, rapid prototyping and testing of high-resolution systems have become a reality. Live cells and biocompatible structures can be printed simultaneously in an incredibly timely manner and are highly reproducible [44].

A critical component of designing cartilage-on-a-chip systems is that they could represent in vivo conditions. While the techniques are representative of the stationary tissue units, they are not necessarily mimic the

actively mechanically stimulated tissues. Due to the different locations in which cartilage is located in the body, it is crucial to include the design method of cartilage-on-a-chip systems that are representative of cartilage that endures loading and mechanical stress for each specific organ. The technique of creating 3D environments using cell cultures in highly flexible hydrogel matrices allows for the implementation of real-world mechanical stresses on tissues such as athletic compression [45]. These matrices are usually either produced with soft lithography or are microfabricated and allow for integrating multiple cellular components into a single tissue [26].

12.1.6 Cartilage-on-a-chip: disease treatment application

One of the most challenging issues for developing a treatment for cartilage diseases and injuries is the lack of blood supply. Regeneration in cartilage tissue in the body does not occur because of this lack of blood flow [46], and this fact also makes drug delivery to the site extremely difficult. This issue also impacts testing techniques on cartilage tissue because it makes both traditional in vitro and animal trials less capable to accurately predict the results of human trials. Currently, osteoarthritis (OA) is the most common musculoskeletal disease in the elderly population. It is classified as a multifactorial polygenic disease with genetic and environmental factors that contribute to its development and expression [47]. This issue causes the disease to be extremely difficult to exactly model in vitro, and up to now, the treatment options for those with OA have been extremely limited due to the lack of entirely relevant and representative models of the disease [47,48]. With the emerging technology of cartilage-on-a-chip (COC) models, the possibility of designing more accurate models of OA treatment has become closer to reality. Paola Occhetta et al. could successfully create a physical COC model of OA by hyper physiological compression of cartilage tissue. Their device includes two chambers surrounded by two side channels for culture medium supplementation and divided by a flexible membrane (Fig. 12.3A and B). An elastic recoil was used to help to support the membrane and 3D microconstruct to relax after the pressure was released. This microscale system was able to tailor either physiological compression (10%) or hyperphysiological compression (30%) by adjusting the gap underneath the hanging posts (Fig. 12.3C and D). Due to the importance of providing a dense cartilage-like model, the expression of collagen type I and collagen type II was assessed by immunofluorescence. The results proved a dense cartilage-like ECM deposition after 14 d of

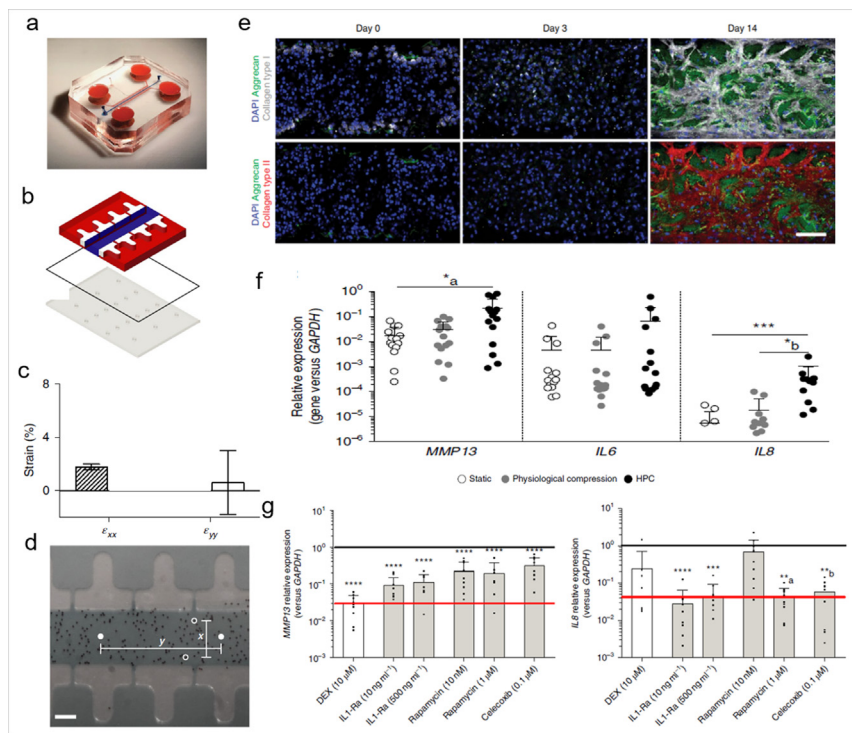


Figure 12.3 (A, B) Design of microfluidic device including two chambers, two side channels, and a flexible membrane. (C) Strain deformation of fabricated COC. (D) Deformed state of fabricated COC. (E) Evaluation of aggrecan, collagen type I, and collagen type II expression by immunofluorescence, after 14 days of differentiation. (F) The expression of genes responsible for matrix degradation and inflammation (*MMP13*, *IL6*, and *IL8*) induced by mechanical compression on fabricated COC. (G) Drug screening using the OA COC model by measuring the levels of *MMP13* and *IL8* expression to specific antiinflammatory compounds. (Reprinted with permission from Ref. [48].)

differentiation (Fig. 12.3E). Additionally, by inducing the physical factors required to induce the phenotype of osteoarthritis, they were able to induce the same gene activation that is expressed by OA patients (Fig. 12.3F). Finally, by providing an accurate model of OA, the effects of antiinflammatory/anticatabolic drugs, were examined and the results revealed high consistency with existing preclinical and clinical reports (Fig. 12.3G). Therefore, Three-dimensional COC models may also serve to be excellent platforms for fabricating small-scale in vitro models closely simulating a degenerative joint pathology such as OA [48].

On the other hand, cartilage inherently has a low capability to repair itself after damage or regular wearing over time [49]. As load-bearing cartilage is such an integrated tissue type in most living beings, isolating the system is extremely hard, yet the importance of repair attempts at such an integral tissue is high. Considering this, new COC technologies offer remarkable observation and testing tools for strategies to induce self-healing in cartilage tissue. Aside from the benefits as testing platforms for diseases such as OA, COCs are also helpful for testing treatments of injuries such as lesions and general trauma over time. As injuries to load-bearing cartilage tissues are often a combination of physical damage and chemical environment changes. As a result, an efficient COC could be promising for mechanical and chemical analysis of cartilage injury treatment [50].

12.1.7 Cartilage-on-a-chip: potential application for implantation

Aside from the possibilities that COC's pose for accurate models of diseases and drug testing, they also show the promising capability of enhancing engineered prosthetics and transplanted tissues. In joints highly susceptible to injury and disease like the knees and ankles, perfect healing can be impossible [51,52]. With the advancements in tissue engineering, the possibility of cultured cartilage tissue in engineered scaffolds as tested implants is extremely promising. Additionally, advances in additive manufacturing have an increasing capability to create personalized articulating joints [53]. Combining these technologies has improved the treatment of joint and injuries, such as osteochondral defects and lower limb osteoarthritis to decrease the socio-economic impact on the individuals [54].

12.1.8 Cartilage-on-a-chip: testing new drugs and therapeutics

Unfortunately, as testing practices have increased in technological advancement, the lack of efficacy of past models for drug testing has become more apparent. Even in drug testing on mice, despite the fact that humans share nearly 92% of our DNA [16], toxicity for humans is often undetected. Looking at statistics from current drug trials, approximately 89% of new drugs fail human clinical trials, and about half of those are due to unexpected toxicity that had been unrepresented in preclinical animal trials [55]. With debilitating cartilage diseases such as osteoarthritis being so prevalent, these numbers have sparked innovation to change the way drugs are tested [26,48].

Many earlier models rely on the introduction of inflammatory cytokines to attempt to induce the conditions of OA in cartilage tissues; however, these models fall short in relevance compared to the multi-factored comprehensive models. The new generation of cartilage-on-a-chip models has allowed for accurate testing of drugs such as anakinra, rapamycin, and celecoxib, which all yielded the same outcomes in vivo studies [32]. The advances in models mimicking joint microenvironments are also projected to further improvements in drug delivery systems for cartilage disease therapies. As OA and a few other cartilage diseases mainly tend to affect the tissues and joints as closed structures, many systemic drugs and treatments are not highly efficient. Relevant models of OA will allow for the possibility of increased testing of intra-articular drug delivery systems to target the closed system of the joints [56]. Moreover, the in vitro cartilage testing models are vastly improved by COCs not only with regard to drug testing but also through physical stress testing. It is critical to know in load-bearing cartilage locations that the tissue can withstand any stresses and loading applied to it after any chemical or physical alterations had been done to it, assuming that these tests would be done on a patient as a treatment [57].

12.1.9 Skeletal muscle on-a-chip: design and manufacturing techniques

PDMS is the most widely used substrate for the fabrication of organ-on-chip devices [58]. PDMS is a silicone-based synthetic polymer that is widely favored for microchip construction because of the low cost, low cytotoxicity, and ease of processing [59]. The materials used in this application must follow the following general characteristics: adequate mechanical strength to support the cells, appropriate porosity, compatibility with cells, and they must remain intact long enough for the cells to organize but not long enough to where it disturbs the coupling of cells required for the formation of a functional tissue [59]. The use of appropriate biomaterials allows scientists and researchers to select the most suitable design for their targeted tissue functionality, as well as what they intend to test with the device. In terms of skeletal muscle tissue-on-chip, skeletal myoblasts, and myotubes are placed with nanofibers, nanowhiskers, and microgrooves within PDMS substrates.

12.1.10 Skeletal muscle-on-a-chip: disease treatment application

Development of skeletal-muscle-on-a-chip offers a biologically relevant platform to most accurately portray disease modeling for drug discovery and toxicological testing [60,61]. Research utilizing organ-on-chip allows scientists to further investigate the various diseases that affect skeletal muscle [61]. Skeletal muscle is such an essential organ because its primary function is to generate contractile forces that enable respiration, posture, and locomotion [62]. Skeletal muscle is highly productive and functional due to its regenerative ability that is possible with the aid of myogenic satellite cells. Upon aging, or diseases such as cancer or large traumatic muscle loss injury, the function of these cells can degenerate, causing the naturally occurring reparation and healing within the skeletal tissues to be diminished [62]. Defects of these cells lead to a defect in function, in turn causing various other diseases including but not limited to diabetes, muscle atrophy, and adiposity [63]. Additionally, skeletal muscle can be impaired upon infection from other diseases, as seen in rheumatoid arthritis (RA) [64]. RA causes chronic inflammation, medication toxicities, and physical inactivity that further impair skeletal muscle tissue [64]. Another disease that threatens the state of skeletal tissue is a neuromuscular disease. Neuromuscular diseases impair the function of muscles as a result of altercations within the nerves and muscles of the body. The neuromuscular disease can result from dysfunction of muscular tissue, further causing potentially fatal or life-altering repercussions. The severity and specificity of these diseases call for a physiologically relevant model of the neuromuscular system [65]. Fortunately, an neuromuscular junction (NMJ) chip has been developed by Osaki et al. based on the serious need to construct and capture physiological and pathophysiological characteristics of the human skeletal muscle system. The NMJ chip is a microfluidic device that consists of motor neurons coupled to skeletal muscles that interact at the neuromuscular junction. The design of the NMJ chip includes compartments for the neurons and the muscle, and spaces to separate cell types to mimic their condition in the body. The NMJ chip was fabricated by conventional photolithography including three culture compartments to culture both skeletal muscle cells (compartment 1) injected from port 1 and MN spheroids (compartment 2) injected from port 2, and to observe axonal outgrowth (compartment 3) separately. The stimulation of neural activity and muscle contraction was performed using 488-nm light. Then, the differentiated MN spheroids and skeletal myoblasts were injected into the NMJ chip in a collagen type I and

Matrigel mixture to support the successful skeletal muscle formation. This chip could accurately aid to predict the capability of the drugs to cross the blood—spinal cord barrier and, reach the neurons in the spinal cord. Also, this model helped to provide more specific information about the interaction between an MN and a skeletal muscle throughout the process of NMJ formation, for example, synapse connectivity and signaling via various cytokines or myokines and exosomes [66].

12.2 Bone-on-a-chip devices: challenges and future direction

Although the bone chip models have a wide range of advantages from a more precise microenvironment to a drug investigation, under conditions that are specific to the bone microenvironment and relevant for various diseases, several challenges are continually being assessed [26]. Due to the importance of mechanical loading as an essential component of bone tissue function and physiology, many efforts have been made to recreate different types of loads distributed across bone units to regenerate similar mechanical properties [67,68]. For instance, conventional models use precalcified polymers or ceramics [3]. These materials are commonly used due to their similarity to the composition of bone and their ability to enhance bone growth and cellular activities [69]. Despite these benefits, models with these components do not replicate the complex extracellular matrix and bone microenvironment structurally [3]. However, as some studies have done, it is possible to overcome this limitation using hydroxyapatite nanoparticles that provide a more similar bone microenvironment and recreates the mechanical properties seen in the matrix [70]. Another alternative is to grow decellularized bone cells with mesenchymal and endothelial cells, which will create a capillary network to produce interstitial perfusion [21]. Yet, a third method is the use of cell-laden hydrogels [3]. Natural polymers and synthetic polymers are among the hydrogel biomaterials implemented in bone modeling [71]. However, even with cell-laden hydrogels, caution must be used as these are not mineralized, and many current methods simply use exogenous supplements that are not natural to bone tissue, creating an inaccurate model with slowly coordinated mineral nodules [3]. To address this, tissues can be engineered to control the mineralization at a nano level to match the mechanical properties, structure, and chemical composition of the bone [3,72]. Recently, the incorporation of controllable and cyclic mechanical stresses has attracted considerable attention in this

field. However, developed bone-on-chip models that combine multiple cell types, complex loading, and vascular capillaries have not been developed. Therefore, it is likely that scientists will try to replicate the bone tissue dynamics precisely [3].

Additionally, studying bone remodeling has also proved to be challenging as osteoclasts are often not present in the model [26]. This issue can also be overcome with the differentiation of osteoclast precursor cells into osteoclasts and coculturing them into the model. Moreover, the present models do not include a mineralized matrix or osteoclasts, preventing the study of bone lesions lysing as seen in hematological tumors, yet with the alternatives described above, this may now be possible to be explored in future models [73]. Also, due to the high integration of cartilage tissues with other tissues for its function, modeling it outside the body on a small scale is extremely challenging. Multiple factors such as rigidity, force application, and multitissue integration must be considered for an accurate COC model for each specific cartilage tissue in each organ. Another issue with COC models is keeping up with the consistent real-time monitoring and assessment that they require while maintaining minimal invasiveness and disruption to the environment. A large percentage of assessments done on tissue cultures require off-chip testing, such as ELISA. Therefore, the model disruption or possible invalidation of the system may be occurred [74,75].

In addition, despite the widespread use of PDMS as a substrate to create the basis of the chip, its hydrophobic nature, hinders the integration of certain biomaterials, and limits cell attachment without further surface modification [3,59]. To solve this issue, new alternative substrates should be used to decrease the hydrophobicity nature of the surface. Another problem with bone-on-chip platforms lies within the maturation stage of the cells; ensuring that the cells produce an adult phenotype that is replicable of an adult human organ can be challenging. Usually, the product of maturation of the stem cells more closely represents an organ of a fetus than that of an adult organ [76]. To solve this issue, scientists are trying to use electrical or mechanical stimulation, allowing for the alteration of the electrophysiological features of these cells [61,76].

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CHAPTER 13

Tumour-on-a-chip

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13.1 Introduction

Cancer is intrinsically complex and involves heterogeneous cellular compositions and microenvironmental cues. During the various stages of cancer initiation, development, and metastasis, cell–cell interactions (involving vascular and immune cells in addition to cancerous cells) and cell–extracellular matrix (ECM) interactions (e.g., alteration in stiffness and composition of the surrounding matrix) play major roles. Cancer cells gradually expand in multiple stages [1]. First, the mutated cells proliferate uncontrollably, showing metabolic heterogeneity, such as the Warburg effect. Increasing the preference for aerobic glycolysis accelerates the transformation and growth of malignant cells [2,3]. As a result of gross enlargement of cancer, severe depletion of oxygen and nutrients triggers angiogenesis and the process of new blood vessel formation from preexisting vasculature [4]. Second, cancer cells invade the adjacent tissue and blood vessels. Owing to their changing their morphological and phenotypical plasticity via epithelial–mesenchymal transition (EMT), cancer cells have migratory ability [5]. Downregulation of adherence junctions and rearrangement of the cytoskeleton facilitate translocation of the cancer cells along with surrounding ECM fibers [6]. Once the carcinogenic cells encounter the vasculature, transendothelial migration, called intravasation, occurs. Then, the metastatic cancer cells spread into the blood circulation and disseminate at a secondary site [7]. Conventional cancer models, both two- and three-dimensional (2D and 3D), have numerous limitations as they lack good vascularization and cannot mimic the complexity of tumors, thereby restricting their use as biomimetic models in drug screening and fundamental cancer biology studies. The development of 3D bioprinting technology has overcome the limitations of standard preclinical models [8]. The technology for precise and versatile deposition of bioinks enables spatiotemporal patterning to reconstruct the complex geometry of the tumor microenvironment in a layer-by-layer manner. In particular, on cancer-on-chips, multiple materials can be applied to closely mimic physiological implementation, including cell–cell and/or cell–substrate

interactions. The effects of biochemical and physical cues, such as cytokine gradient, oxygen gradient, extracellular matrix stiffness, and shear stress, have also been studied using this platform [9]. Recently, multiorgan-on-chip with several tissue models have been established to show consecutive metastatic progression [10]. The systems have helped to reveal hidden rate-limiting steps of cancer progression and also provided systemic insights for new anticancer drug discovery.

13.2 Features of tumor microenvironment

The tumor microenvironment (TME) is a complex ecosystem, where tumor cells acquire malignancy via cellular, biochemical, and mechanical cues and transform their genetic and phenotypic features to survive [11]. In response to external stimuli by ligands from the TME, cancer cell receptors are activated and initiate signaling cascades. Stromal cells (e.g., fibroblasts, immune cells, endothelial cells) and ECM components [12] are key regulators and mediators of cancer progression in the TME (Fig. 13.1). The

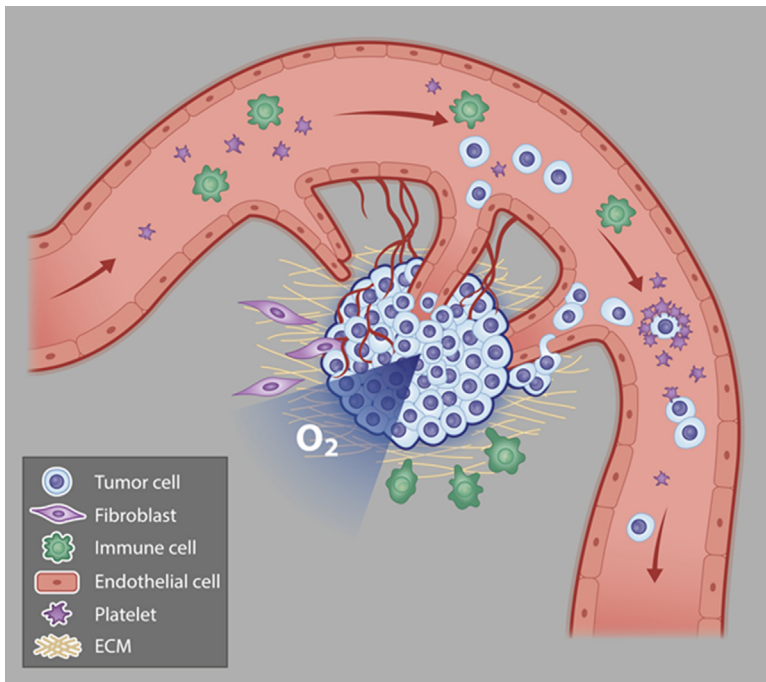


Figure 13.1 Tumor microenvironment consists of stromal cells and extracellular matrix. Cellular, Biochemical, and physical constraints govern cancer progression. Only the malignant cells that adapt to hostile conditions by changing genetic, epigenetic, and phenotypic characteristics can survive, metastasize, and destroy the whole body system.

dynamic interactions between cancer cells and the TME lead to the evolution of cancer cells, resulting in metastatic outgrowth and enhanced multidrug resistance [13]. Therefore, understanding the underlying mechanisms governing this interplay is essential for identifying a novel anticancer drug approach.

13.2.1 Fibroblast

Cancer-associated fibroblasts (CAFs) are a group of fibroblasts whose function is manipulated by nearby cancer cells and redirected toward carcinogenesis. CAFs usually originate from normal fibroblasts in the stroma but can be derived from endothelial cells, mesenchymal stem cells, immune cells, or pericytes [14,15]. The crosstalk between cancer cells and CAFs is an important contributor to cancer proliferation, migration, and invasion, leading to cancer metastasis development. During progression, cancer cells can break down the basement membrane and migrate to the surrounding tissues, and CAFs induce EMT of cancer cells, upregulating their mobility and metastatic potential. Transforming growth factor-beta (TGF- β) is a well-known prometastatic factor produced by CAFs. Many studies have reported that TGF- β produced by CAFs stimulates mesenchymal marker expression in various types of cancer cells and enhances the migratory behavior, showing escalated invasion into the surrounding matrix [16–18]. Several markers are used to identify CAFs, including fibroblast activation protein, alpha-smooth muscle actin, vimentin, and platelet-derived growth factor receptor-alpha.

13.2.2 Immune cells

In the TME, immune cells also play an important role in tumorigenesis. These tumor-associated immune cells are known to possess tumor-promoting functions. In the early stages of cancer, immune cells are tumor-antagonized and tend to target and kill cancer cells. However, in the TME, cancer cells tend to escape immune surveillance and inhibit tumor-antagonizing immune cells through a variety of mechanisms. Among the various immune cells, tumor-associated macrophages (TAMs) play a significant role in inflammation and cancer progression. Similar to CAFs, TAMs produce various factors that stimulate cancer cell migration. Several studies suggest that TAMs support tumor angiogenesis by secreting vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and nitric oxide synthase [19], in addition to proinflammatory molecules secreted by TAMs, such as TGF- β , tumor necrosis factor-alpha (TNF- α), interleukin 10 (IL-10), and chemokine (C-X-C motif) ligand

10 (CXCL10). Moreover, TAMs produce ECM remodeling molecules, such as matrix metalloproteinase 9 (MMP9), MMP2, and MT1-MMP, to degrade the surrounding matrix and make a path for cancer cell migration [20]. Therefore, an increased number of TAMs in the tumor region is strongly correlated with a worse prognosis.

13.2.3 Hypoxia

During cancer development and progression, the TME is often hypoxic. As the tumor mass increases, cancer cells have restricted access to nutrients and oxygen. The central region of the solid tumor is normally necrotic owing to the absence of blood supply. Therefore, tumor cells develop new blood vessels to obtain nutrients and oxygen. However, newly formed blood vessels are structurally unstable and leaky. Hence, tumor hypoxia causes leakage of the surrounding vasculature and increases interstitial fluid pressure [21]. The hypoxic environment leads to the upregulation of hypoxia-inducible factors (HIFs), which promote angiogenesis and adaptation of hypoxic cancer cells. Among the various factors, HIF-1 α is a hallmark of tumor hypoxia. In a hypoxic TME, activation of HIF-1 α increases Snail or Twist, which are key transcriptional factors that reduce E-cadherin expression and promote cancer migration [22]. These factors also promote angiogenesis, cancer invasion, inflammation, and resistance to chemotherapy. HIF-1 α is also related to the expression of ECM remodeling molecules to align collagen fibers and facilitate cancer migration.

13.2.4 Extracellular matrix

The ECM is a three-dimensional network consisting of macromolecular components, such as glycosaminoglycan, polysaccharides, and fibrous proteins, and provides biochemical and biomechanical cues essential for cell proliferation, differentiation, and homeostasis [23]. Because the molecules are generated and remodeled by the residing cellular components during tissue development, each tissue cannot help acquire unique compositions and heterogeneity. As a biological shelter, the ECM supports the cells to adhere and mature to be functional [24]. The adhesions are mediated by ECM receptors, such as integrin receptors and discoidin domain receptors [25]. Furthermore, ECM restores signaling molecules, such as growth factors and cytokines, and mediates interactions between cells by delivering molecules. Several studies have demonstrated that the stiffness and elasticity of the ECM also determine cell fate [26]. ECM homeostasis is a fundamental factor in tissue development and function.

During cancer progression, the ECM is compositionally changed and develops unique characteristics that accelerate tumor growth and survival [27]. This change is influenced by all cellular components in the TME, including fibroblasts, immune cells, and endothelial cells. For example, collagen is produced by CAFs and TAMs, and the increase in collagen reinforces ECM rigidity and induces EMT in cancer cells. Meanwhile, glycoproteins, such as laminin, fibronectin, and fibulin, are overexpressed and support the self-renewal of cancer cells [28,29]. By stretching their pseudopodia and secreting enzymes, such as MMPs, cancer cells detect and track the gradient of ECM fibers to proliferate and migrate out from the harsh microenvironment [30,31]. However, the exact role of each component remains unclear, and further research is required.

13.2.5 Vasculature

The tumor vasculature is critical for cancer growth and survival. The essential nutrients and oxygen are delivered, and metabolites are eliminated through blood vessels. Hence, cancer cells take over surrounding blood vessels and entice angiogenic sprouting from existing vessels [32]. Some cancer cells with cancer stem cell features mimic the vessel networks to actively secure blood supply independent of endothelial cells. However, owing to the distinctive morphologies of tumor blood vessels with irregular diameters and wall thicknesses as well as leaky vessel networks resulting from the lack of essential cell components including pericytes, a high local interstitial pressure is generated, which leads to impairment of material transfer [33]. Furthermore, although a tendency to accumulate drugs in these locations is observed because of these phenomena, therapeutic trials are still unsuccessful owing to increased chemoresistance of cancer cells [34]. Therefore, tumor vasculature is regarded as one of the most important therapeutic targets.

Along with cancer development regarding metastasis, the importance of blood vessels has risen. Cancer cells are transferred throughout the body through the nutrient supply path. To induce transendothelial migration, malignant cancer cells secrete VEGF. In response, angiocrine factors, including interleukin and basic fibroblast growth factor (bFGF) from endothelial cells, make cancer cells more invasive and chemoresistant [35]. Cancer cells are squeezed on the penetration of the endothelial barrier because of its tight junction. Furthermore, by traveling across the body through capillaries, veins, and arteries, cancer cells suffer anoikis owing to the absence of adhesion proteins and are exposed to various stimuli from

blood cells and hemodynamic forces in the bloodstream. This generates large genetic alterations and dramatic chromosomal rearrangements, and only a few adapted cancer cells survive and reach distal organs [36]. Therefore, systemic interaction between tumor blood vessels and cancer cells in the pathophysiological microenvironment is required to discover novel therapeutics for cancer treatment.

13.3 Cancer progression

Dynamic interactions between cancer cells and the microenvironment can promote cancer progression (Fig. 13.2) [37]. In response to the soluble factors secreted by the stromal cells or tumor cells themselves, intracellular signaling pathways are activated and genetic and phenotypic features of cancer cells are altered to adapt and create an environment conducive to their development [38]. However, cancer cells impair themselves by exposing them to harsh environments with low oxygen and pH levels caused by their abnormal proliferation. Nevertheless, cancer cells not only reprogram the metabolic cascade but also facilitate angiogenesis [39]. Malignant cells invade the surrounding ECM and penetrate the blood vessels. Less than 0.01% of circulatory cancer cells can reach secondary tissue enduring physical and biochemical stresses caused by blood flow and

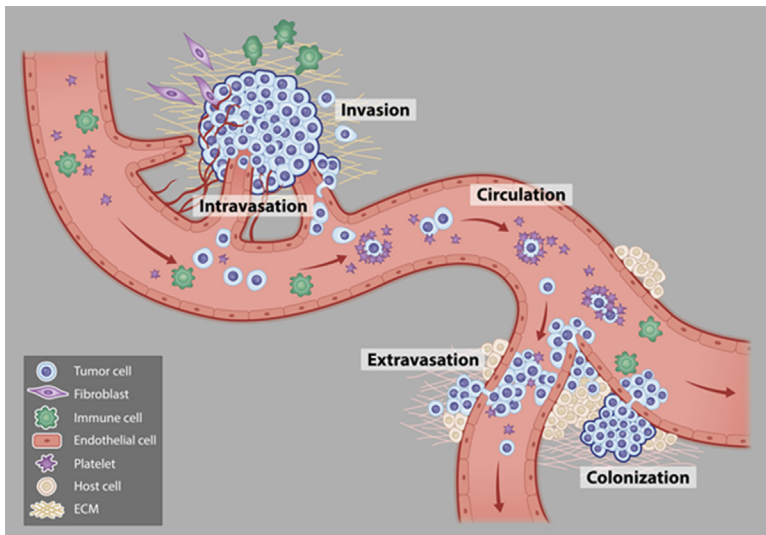


Figure 13.2 Cancer progression occurs in multiple steps: local invasion, intravasation, circulation, entrapment, extravasation, and colonization.

various cellular constraints [40]. Even in secondary tissue, malignant cells constantly adapt to their surroundings, undergo genetic, epigenetic, and phenotypic changes, and finally succeed in metastasis [7].

13.3.1 Tumor development and angiogenesis

13.3.1.1 Angiogenic switch

Tumors require sustained delivery of oxygen and nutrients from the blood vessels. Therefore, for survival and growth, malignant cancer cells need to reside near the surrounding blood vessels. Because of abnormal proliferation of cancer cells, the central region of the solid tumor is devoid of blood vessels, resulting in the formation of the hypoxic region or even necrosis. Thus, the early stage of cancer progression is usually accompanied by angiogenesis, a process that involves the formation of new capillaries from the growth of preexisting blood vessels [41]. In healthy tissues, vascular homeostasis is controlled by a balance between the pro- and antiangiogenic factors. When these are in balance, endothelial cells are quiescent and the vasculature is inactive. However, tumors lose the balance between pro- and antiangiogenic signals. Once a tumor size exceeds a few millimeters in diameter, hypoxia triggers an “angiogenic switch” to allow proangiogenic factor secretion and blood vessel formation. The angiogenic switch relieves cancer cells from nutrient deprivation and flares malignant cancer progression by enabling nutrient supply from the newly formed blood vessel.

13.3.1.2 Tumor-induced angiogenesis

Sprouting angiogenesis is the most common method of blood vessel formation in the TME. It is a multistep process through which new capillaries sprout from preexisting blood vessels. The basic steps of sprouting angiogenesis include (a) tip and stalk cell selection: migratory leading cells are selected from preexisting blood vessels, (b) migration of endothelial cells: the tip cell migrates along the chemoattractant signal, and stalk cells follow behind a tip cell, (c) lumen formation: connection of the lumen of the sprouting capillary to the parent vessel, and (d) anastomosis: integration of the developed sprouts with other vessels [41].

Endothelial cells are normally quiescent, but in the TME, they can be induced to initiate angiogenesis by proangiogenic factors including VEGF-family members, placental growth factor, and angiopoietin-1 (ANG1). The endothelial tip cells and stalk cells are selected through VEGF and DLL4/Notch signaling [42]. Tip cells are characterized by their elongated filopodia, which secrete large amounts of proteolytic enzymes to digest the

surrounding ECM and create a pathway for the development of sprouts. In response to VEGF, the filopodia of tip cells are endowed with VEGF receptor-2 (VEGFR-2), VEGFR-3, platelet-derived growth factor-B (PDGF-B), and Delta-like-4 (DLL4) ligands, allowing them to sense and align with the VEGF gradient. In the neighboring stalk endothelial cells, DLL4 secreted by tip cells activates Notch signaling to suppress VEGFR-2 and VEGFR-3 expression. Therefore, stalk cells proliferate and follow tip cells, forming the vascular lumen and junction. Next, vascular anastomosis occurs in two forms: (1) a junction between the newly formed sprouts and (2) a junction between the developing sprouts and the existing blood vessel. Evidence suggests that stromal cells in the TME play a critical role in angiogenesis.

13.3.2 Cancer invasion and intravasation

The development of metastasis requires cancer cells to invade the surrounding matrix and leave the primary tumor site. Cancer cells invade other tissues either by migrating as single-cell dissemination or collective migration as a cell cluster. EMT is a process in which epithelial cancer cells transform their plasticity and acquire the ability to migrate, invade, and disseminate from the primary tumor site [43]. Under normal conditions, epithelial cells are immotile and adhere to neighboring ECM. In the last few decades, EMT has been recognized as an important process in cancer. One of the main hallmarks of EMT is the downregulation or even loss of E-cadherin, which is a key molecule for cell-to-cell adhesion [44]. E-cadherin helps epithelial cells assemble and maintain quiescence. The loss of E-cadherin is a fundamental event in EMT. EMT can be induced by transcription factors that repress E-cadherin expression. Transcription factors, such as SNAI1/Snail 1, SNAI2/Snail 2, and ZEB1/2 bind to the promoter of E-cadherin and repress its transcription. In the TME, hypoxia and several signaling pathways (TGF- β , FGF, EGF, Notch, and Wnt/ β -catenin) induce EMT [45]. For example, snail and slug regulate the expression of p63 isoforms that play a significant role in epithelial structure development. When p63 isoforms are repressed, cell-to-cell adhesion is reduced, and cancer cells exhibit increased migratory properties. Activation of the Wnt pathway induces SNAI1 and upregulates vimentin, which is a well-known mesenchymal marker. Intravasation is the following step of cancer metastasis, in which cancer cells invade through the basement membrane to enter the blood or lymphatic vessels. Under normal conditions, intravasation is an inefficient process in which most cancer cells may

be killed by the shear stress of blood flow. However, compared to the normal vasculature, the newly formed blood or lymphatic vessels in the TME are often leaky. Tumor-induced angiogenesis leads to a network of leaky vasculature that facilitates access to cancer cells.

13.3.3 Circulation

Circulating tumor cells (CTCs) are cancer cells that migrate away from a primary site and are swept away by blood circulation toward the distal site. Only a fraction of CTCs succeed in surviving against mechanical and physiological obstacles. Mostly, just before intravasation, the cancer cells detached from the ECM suffer anoikis, a programmed cell death, owing to the absence of adhesion signals [46]. Furthermore, immediately after facing the bloodstream, hemodynamic shear stress causes physical collision, induces apoptotic signaling, destroying CTCs, and prevents metastasis. To survive and metastasize successfully, cancer cells acquire mechano-resistance by activating signal transduction such as ras homolog family member A (RHOA)-dependent mechanism [47]. However, CTCs form coagulant platelets that serve as physical and biochemical shields during circulation [48]. Platelets protect vulnerable CTCs from immune cells by secreting cytokines such as TGF- β and providing platelet MHC-I to cancer cells [49,50]. Some CTCs exist as circulating tumor microemboli composed of more than two CTCs, which reinforce the potential of metastasis by protecting the innermost cancer cells from physical stress and immune attack [51]. However, although the aggression of CTCs has consistently raised the urgency for related research, it is still in its infancy stage due to experimental challenges in monitoring rare CTC populations ranging from 1 to 10 CTCs per mL of blood in metastatic cancer [47]. Therefore, an accurate bioengineered tumor model should be developed to improve our understanding and clinical applications.

13.3.4 Extravasation and colonization

Cancer cells arrive in secondary tissue secured spots through extravasation and colonization. Extravasation is a process in which invasive cancer cells disrupt the endothelial barrier and penetrate the secondary tissue. The physical entrapment of CTCs due to size restriction and tortuosity of the microvasculature activates the adhesion process between the cancer cells and the endothelium [52]. At the complementary sites, cancer cells and endothelial cells express adhesion ligands and receptors, such as selectins, integrins, cadherins, and immunoglobulin receptors [53]. As soon as tumor

cells interact, cancer extravasation is directly accelerated by various factors, such as angiopoietin-like-4 (ANGPTL4), epiregulin, and matrix metalloproteinase [54]. Migrating through the stroma, the disseminated cancer cells form a supportive metastatic niche. The niche comprises metastatic cancer cells and other supportive stromal cells, which play a crucial role in evolutionary adaptation for cancer colonization. The niche provides not only physical shelters but also biochemical stimulatory mediators, such as Wnt, Stat3, TGF- β , and MAPK, to support self-renewal, cell metabolism, proliferation, and survival pathways [55]. Eventually, the cancer cells adapt to the distinctive microenvironment and form overt secondary tissue metastases.

13.4 Standard preclinical models for engineering cancer in vitro

Preclinical human cancer models have been investigated to understand pathophysiological heterogeneity and discover novel therapeutic strategies [56]. In particular, mouse models have been frequently used for preclinical testing of therapeutics because of their decisive advantages, including genetic and physiological similarities to humans ($\sim 80\%$) and cost-efficiency resulting in high-throughput studies. Despite the wide spectrum of engineered animal models, many issues, including species differences, genetic and immunological variation, ethical problems, have been raised [57]. To overcome the limited availability and feasibility, various in vitro models, such as 2D culture, transwell-based culture, spheroid culture, and hydrogel embedded culture have been developed. In this section, we introduce standard preclinical in vitro models for engineering in vitro cancer platforms and their current limitations.

13.4.1 2D monoculture models and transwell-based models

In vitro human cancer models have been developed to overcome the drawbacks of animal models. Since the early 1900s, the 2D monoculture system, the easiest in vitro culture model has been used. On the flat surface of culture dishes or flasks, cancer cells grow in culture medium and anti-cancer drugs are introduced for simple anticancer drug screening [58]. Conversely, Transwell-based models have been widely used to investigate the cancer migration and invasion depending on the biochemical and biomechanical gradients. Utilizing different substances contained in the two distinct spaces, behavioral changes in cancer cells can be observed. For

example, cancer cells seeded on top of a porous membrane can move toward the bottom well containing chemoattractant molecules [59]. In fact, over 80% of recent cancer studies rely on 2D culture techniques for convenience [60].

These conventional *in vitro* culture systems have limitations considering the significant gap from real tissue. The structural and cellular dynamics cannot be satisfied resulting in a change in the artificially polarized cytoskeleton of cancer cells. Hence, the specific functions of cells deteriorate [61]. Researchers have been trying to create a physiological environment suitable for cell growth with biological materials such as collagen, elastin, fibronectin, and chemical coatings such as poly-L-ornithine and poly-L-lysine. However, unfortunately, the biological environment of an actual cell is much more complex and three-dimensional culture systems are required to provide an environment similar to that in the natural state by securing an appropriate space [62]. In the next section, we introduce stereoscopic culture methods for cancer research.

13.4.2 Spheroidal culture

Two-dimensional *in vitro* tumor models have been useful tools for screening compounds and for the identification of the pathophysiology of cancer cells [59]. Although they show considerable efficacy and can recapitulate genetic pathways *in vivo*, 2D tumor models have limitations. Cancer cells on 2D culture substrates cannot accurately represent solid tumors because they lack ECM, structural architecture, and nutrient gradients that play significant roles in cell growth and tumor progression [63]. Consequently, the absence of the key features of the solid tumor leads to loss of the natural phenotype of cancer cells (e.g., adhesion, migration, invasiveness, and metastasis) and different growth behaviors from those observed in solid tumors. Therefore, translation of the results obtained from 2D tumor models to patients is still challenging. Therefore, the need for more predictive reproduction of tumor characteristics has prompted the development of three-dimensional tumor models for better recapitulation of the actual TME.

Cancer spheroids are the most common 3D tumor model. Cancer spheroids, which are aggregates of cancer cells grown in suspension or embedded in hydrogels, have been considered as the gold standard to stimulate *in vivo* solid tumors [64]. Owing to their ability to recapitulate the structural and physiological features of solid tumors, they have been widely used to investigate basic tumor biology. Indeed, cancer spheroids

Table 13.1 Advantages and disadvantages of various techniques for cancer spheroid formation.

Methods	Description	Advantages	Disadvantages
Liquid overlay culture	Spheroid formation on a substrate-coated cell culture plate	Production of large number of spheroids applicable for high-throughput testing	Extra step is required for coating Labor intensive
Hanging drop	Spheroid formation by reversing the lid	Uniform spheroid size applicable for numerous cell lines	Labor intensive difficulty in long term culture
Bioreactor	Spheroid formation by continuous agitation	Allow uniform distribution of nutrient and oxygen applicable for large production	Large variation in spheroid formation shear-induced cell damage or cell death

can reproduce cell–cell and cell–ECM interactions, nutrient gradients, formation of necrotic cores by hypoxia, and local invasion, which cannot be recapitulated in 2D culture. Moreover, many researchers have suggested that these differences can affect drug response; cancer cells cultured in 3D environments are less susceptible to anticancer drugs compared to cells cultured in a monolayer. Several methods have been developed to fabricate cancer spheroids, such as liquid overlay culture, hanging drop, or bioreactor techniques (Table 13.1).

13.4.2.1 Liquid overlay culture

Liquid overlay culture is a simple method for generating cancer spheroids. The surface of the cell culture plates was coated with a substrate that prevented cell adhesion and promoted cell aggregation. Due to the relatively low adhesive force between the cells and substrates compared to that between the cells, cancer cells form spheroids on the substrate [65]. Various substrates, such as agarose, poly-2-hydroxyethyl methacrylate, or Matrigel, are used to coat the plate [66,67]. Using this approach, researchers have developed a method for producing 3D cancer spheroids in 96-well plates using various breast cancer cell lines [68]. Liquid overlay culture has many advantages. With the incorporation of multiwell plates (e.g., 384- or 96-well plates), a large number of cancer spheroids can be easily reproduced with low variation. Therefore, it can be applied to high-throughput efficacy testing of drugs or biochemical assays. However, the main drawback of

liquid overlay culture is that it requires an extra step to precoat the cell culture plates before cell seeding.

13.4.2.2 Hanging drop

The hanging drop method, introduced by Johannes Holtfreter in 1994, is the most commonly used technique to promote cancer spheroid formation through the natural aggregation of cells. To obtain the cell spheroids, a small volume of cell suspension (usually 20–50 μL) was pipetted onto the inside of the lid of a cell culture plate. After reversing the lid, gravity concentrates the cells at the liquid–air interface of the droplet to form a spheroid. The hanging drop method is relatively simple, cheap, highly reproducible, and does not require specialized equipment. The size of the spheroid can be adjusted by varying the cell density of the cell suspension. Owing to its versatility, the hanging drop method was applied to produce 3D spheroids using numerous cell lines. It has been reported that cancer spheroids produced by the hanging drop method are physiologically relevant to solid tumors. However, the hanging drop method is not appropriate for long-term cultures and is labor-intensive and requires a multistep process, and requires constant monitoring of the cell culture medium to avoid evaporation of the media.

13.4.2.3 Bioreactor

Spinner flasks or rotating flasks are commonly used bioreactors for the large-scale production of cancer spheroids [69]. In a bioreactor-based 3D culture system, cell suspensions with the desired cell density were filled into the cell culture chamber. Continuous agitation during cell culture, either by rotating, stirring, or perfusing, inhibits flowing cells from adhesion to the surface, but instead leads to cell spheroid formation. In addition, bioreactor-based systems can ensure the expulsion of metabolic waste from the spheroids and homogeneous distribution of nutrients, growth factors, and oxygen to the cells [69]. Therefore, long-term culture of 3D cancer spheroids can be accomplished. However, the main drawbacks of using bioreactors are shear stress induced by repetitive agitation can adversely affect cell physiology or even cause cell death. Furthermore, batches of 3D spheroids formed in spinner flasks typically have a broad range of sizes, which might result in physiological variation between spheroids. To avoid this variation, a postprocess of manual selection is necessary to obtain 3D spheroids of similar sizes.

13.5 Bioprinting technologies

Recently, bioprinting technology has gained attention owing to its ability to overcome existing limitations in traditional tissue engineering. The main component of bioprinting is bioink, which is a printable hydrogel containing various types of cells, biomaterials, and/or biomolecules. Due to its ability to precisely position the cells, bioprinting has emerged as an effective technology for fabricating complex 3D structures of human tissues and organs. To build a 3D functional tissue/organ construct, bioink is deposited from the printing module in a layer-by-layer manner. Bioprinting technologies can be classified into four types [1]: inkjet-based bioprinting [2], orifice-free bioprinting, and [3] extrusion-based bioprinting (Fig. 13.3) [70].

13.5.1 Bioprinting techniques

13.5.1.1 Inkjet-based bioprinting

The inkjet-based bioprinting system is a noncontact technique that dispenses picoliter (1–100 pL) bioink droplets (10–100 μm in diameter) to a

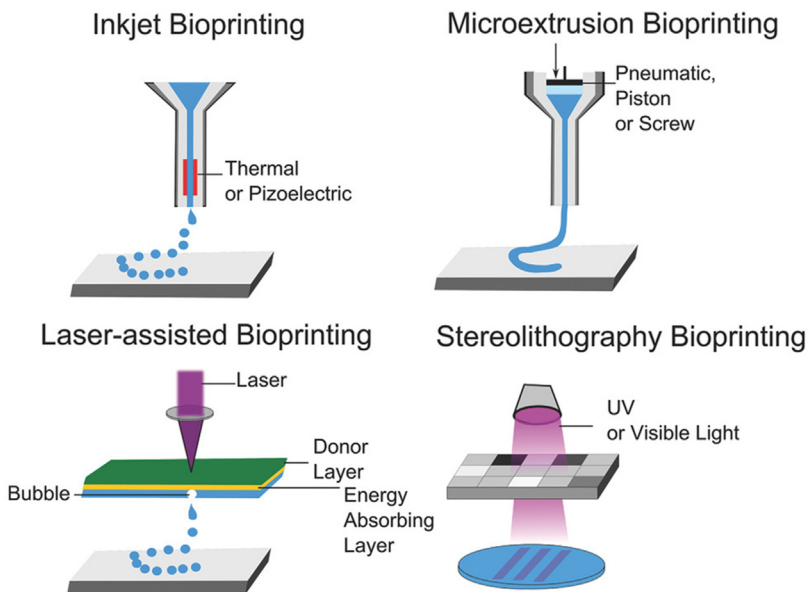


Figure 13.3 The most common 3D bioprinting techniques. Inkjet bioprinting deposits droplets either by heat or piezoelectric actuation. Extrusion bioprinting utilizes pneumatic or piston or screw-driven pressure to extrude bioink. Laser-assisted bioprinting uses a laser source to generate a vapor bubble and propel bioink droplets on a substrate. Stereolithography bioprinting uses UV or visible light to selectively crosslink bioink. (Adapted with permission from Ref. [71].)

predefined location of the substrate with high fabrication resolution. Thermal and piezoelectric inkjet printing are commonly used to print bioink droplets [70]. The thermal inkjet printing system uses a heat generator to increase the temperature within the bioink chamber. Then, the localized heating produces pressure pulses by generating bubbles around the heating element, yielding droplets through a micron-sized nozzle. The piezoelectric printing system uses an acoustic wave generated by a voltage-mediated actuation to produce a droplet. Using the inkjet-based bioprinting system, direct printing of cells with complex patterns at a high resolution can be achieved. However, the disadvantages include the inability to fabricate 3D volumetric constructs owing to insufficient mechanical strength. Moreover, bioinks with high viscosity or high cell density cannot be applied to inkjet printing systems owing to nozzle clogging.

13.5.1.2 Extrusion-based bioprinting

The extrusion-based bioprinting system dispenses cell-laden bioinks through a micro-sized nozzle in a continuous manner. To extrude the bioink, the extrusion-based bioprinting system uses either pneumatic pressure or piston, and the amount of dispensed bioink can be controlled by the printing pressure or displacement of the piston. The piston-driven system can offer more direct manipulation of the flow of the bioink and provide more spatial control, which is appropriate for dispensing viscous bioinks [72]. However, the shear force generated by the piston can damage the encapsulated cells or even result in cell death. Meanwhile, the pneumatic system is controlled by the air pressure to dispense a wider range of bioinks with varying types and viscosities, which might cause less damage to the suspended cells. However, the pneumatic system is accompanied by a time delay associated with compressed gas volume. The extrusion bioprinting system offers advantages in a wider selection of bioink materials, and the producible construct size is scalable. The drawbacks are its low resolution (200–400 μm), potential nozzle clogging, and cell death due to the increased shear stress during extrusion through the narrow nozzle.

13.5.1.3 Light-assisted bioprinting

Light-assisted bioprinting uses a light source to adjust the properties of the bioink to either facilitate the deposition of the bioink or polymerize photocrosslinkable bioink. Light-assisted bioprinting technologies include laser-induced forward transfer (LIFT), stereolithography (SLA) bioprinting, and digital micromirror devices (DMDs).

LIFT printing is a noncontact, nozzle-free, laser-associated printing process developed for the fabrication of high-resolution patterns. LIFT includes a laser that produces a pulsed laser beam, an absorbing layer (e.g., gold or titanium) where a layer of bioink is coated, and a collecting platform on which bioink is deposited [73]. The pulsed laser energy is deposited in an absorbing layer and causes focal evaporation of the material, thereby creating vapor bubbles or mechanical waves that transfer bioink droplets to a collecting platform. The dimensions of the bioink droplet can be controlled by manipulating the properties of the laser (e.g., wavelength and power). Although LIFT is helpful for the fabrication of 3D patterns with a high spatial resolution (20–30 μm), cell viability, the complexity of the laser source, and control of the laser make the process labor-intensive and expensive.

SLA bioprinting uses a light source to polymerize photocrosslinkable bioink. In SLA bioprinting, the fabrication of a 3D construct is achieved by light-initiated polymerization. The light source polymerized a bath filled with liquid resin onto a moving chamber. After polymerization, the chamber descends a certain distance from the upper layer to be polymerized. Compared to LIFT, SLA bioprinting is fast; however, it has low biocompatibility and resolution.

While SLA bioprinting uses an intensive light source to polymerize the photocurable resin, DMD or digital light processing (DLP)-based bioprinting is a method for constructing a tissue construct with a computer-controlled array of micromirrors to simultaneously polymerize the resin. DLP-based bioprinting utilizes a DMD chip, motorized stage, and printing head. In DLP-based bioprinting, UV light is illuminated, and each layer of the liquid resin is polymerized through the pattern of the DMD chip. After patterning, the liquid substrate is lowered by the motorized stage to cure the next layer. The resolution of DLP-based bioprinting is approximately 5 μm .

13.5.2 Bioinks

Bioink, a mixture of biomaterials and living cells, is one of the most essential components of 3D bioprinting. The material should possess not only printability to fabricate tissue-mimetic complexes while protecting the cells from external stresses, but also biocompatibility to support the functionality of engineered constructs, providing signals for cell proliferation and differentiation [70]. Furthermore, depending on target bioprinting modalities, distinct characteristics, such as resolution, physical integrity, and

degradability, should also be equipped for the proper performance of bioink. Selecting adequate bioinks is one of the most important steps in generating reliable tumor models [74]. Hydrogels are the most commonly used bioinks in tissue engineering because they can provide a highly hydrated and permeable 3D microenvironment and enhance the metabolic activity of encapsulated cells. In general, hydrogels can be classified into two groups: natural polymers (e.g., collagen, gelatin, alginate, fibrin, decellularized extracellular matrix (dECM)) and synthetic polymers (e.g., gelatin methacrylate (GelMA) and pluronic) (Table 13.2) [97,98]. Natural

Table 13.2 Natural and synthetic bioinks and their properties regarding applicability for 3D bioprinting.

Bioinks	Type	Gelation method	Advantages (A) and disadvantages (DA)	Refs.
Collagen	Natural	Thermal and pH	A: Promotes cell attachment, good printability DA: Poor mechanical stability, slow gelation	[75–77]
Gelatin	Natural	Thermal	A: Reversible, promotes cell attachment DA: Poor mechanical stability, low printability	[78–81]
Alginate	Natural	Ionic	A: Rapid gelation, high shape integrity DA: Poor cell attachment, easily clogs	[82,83]
Fibrin	Natural	Fibrinogen-thrombin	A: Promotes angiogenesis, rapid gelation DA: Poor mechanical stability, easily clogs	[84–86]
dECM	Natural	Thermal and pH	A: Promotes cell attachment, good printability DA: Poor mechanical stability, slow gelation	[87–89]
GelMA	Synthetic	Photo	A: Easy degeneration, high mechanical stability, good printability DA: Slow gelation, ultraviolet light causing cell damage	[90–92]
Pluronic	Synthetic	Photo	A: Reversible (good sacrificial material) DA: Poor mechanical stability, rapid degradation	[93–96]

polymers are present in plants, animals, and microorganisms. The solubilized ECM molecules are cell-friendly and can support cellular activities. However, owing to their unstable bonding forces, such as hydrogen bonds and ionic bonds, these materials have a weak mechanical strength [99]. On the other hand, synthetic polymers are artificially synthesized polymers having tunable rheological and mechanical properties. However, these materials usually have no appropriate cell-binding sites that guarantee cellular functionality, leading to limited medical applications. Recently, to strengthen and compensate for the weakness, hybrid polymers have been highlighted as complementary strategies [100].

13.5.3 3D bioprinted in vitro tumor models

13.5.3.1 3D bioprinted cancer models

3D heterogeneity of the TME promotes cancer development. 3D culture platforms have been highlighted because of the higher similarity in structural integrity with native than 2D cell culture models [101]. Integration of the cancer spheroid with the ECM-like hydrogels is a promising approach because the aggregate can present cellular heterogeneity and pathophysiological features of solid tumors. Hribar et al. fabricated concave hydrogel microstructures using photocrosslinkable poly(ethylene glycol) diacrylate for long-term multicellular spheroid culture [102]. Furthermore, Ling et al. suggested a high-throughput bioprinting fabrication strategy for in situ breast cancer spheroid formation by utilizing cell-embedded gelatin arrays. The cancer droplets with the desired size and shape could be produced in a controllable manner, while concave wells were produced as a result of the dissolution of gelatin (Fig. 13.4) [103]. Representative features, including hypoxic and necrotic cores, were reproduced and their applicability to embryogenesis study and patient-derived disease modeling were also shown. The scaffold-based 3D cancer model is another promising strategy that has been widely used to investigate the chemotherapeutic response of cancer cells. Wang et al. developed a 3D glioma cell-laden gelatin-alginate-fibrinogen hydrogel scaffold. Under 3D culture conditions, gene expression levels related to cancer stemness and EMT increased. Moreover, higher tumorigenicity of the 3D cultured cancer cells was verified, as well as higher chemoresistance against Temozolomide (TMZ). In addition to solid tumors, metastatic cancer models utilizing 3D printed scaffolds have also been developed. Holmes et al. fabricated a nanohydroxyapatite-coated poly lactic acid scaffold with microstructures varying from 150 to 250 μm square and hexagonal pores. Cancer cell adhesion and proliferation increased in a

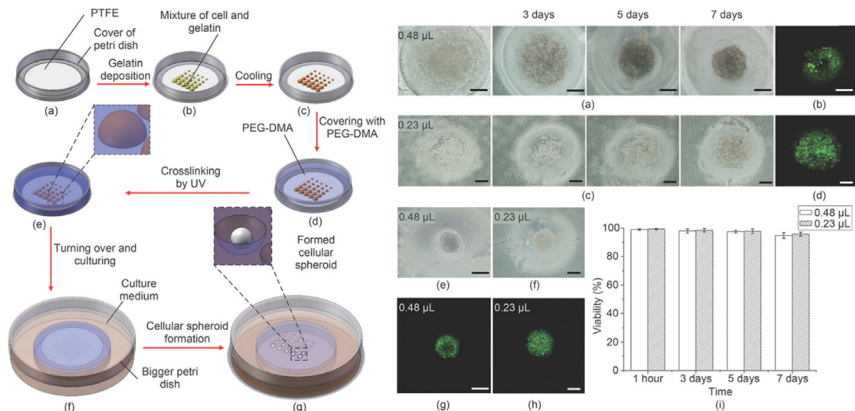


Figure 13.4 Bioprinting of cancer spheroids. Bioprinting technology was applied for the fabrication of sacrificial gelatin arrays and concave wells in a high-throughput manner. By using the gelatin arrays, cancer spheroids with the desired dimension could be produced in a controllable manner. (Adapted with permission from Ref. [103].)

small hexagonal pore model [104]. However, the absence of stromal cells might lead to modeling failure in most 3D cancer models because of the lack of reproducibility of actual cancer tissue. Various types of tumor-associated stromal cells exhibit substantial crosstalk and significantly contribute to tumorigenic progression.

13.5.3.2 3D bioprinted multicellular cancer models

To recapitulate the pathological features of cancer *in vitro*, cellular crosstalk between cancer and stroma has attracted the attention of many researchers. The effects of soluble mediators secreted from stromal cells, including fibroblasts, endothelial cells, and immune cells, on behavioral changes in cancer cells have been investigated [105–108]. For instance, Jiang et al. established a new strategy using 3D bioprinting technology to generate self-assembled multicellular tumor spheroids as a result from a crosstalk between breast cancer cells and cancer-associated fibroblasts (CAFs) [109]. MDA-MB-231 triple-negative breast cancer cells were deposited at central hub departed from outer segment with IMR-90 fibroblasts within the heterogeneous tumor model (Fig. 13.5). During culture, CAFs migrated toward the central hubs and MDA-MB-231/IMR-90 multicellular spheroids were spontaneously formed without any biochemical and physical stimuli. It demonstrates that carcinogenic progression is promoted by the interaction between cancer cells and stromal cells, as well as it can be reproduced *in vitro* by constructing the biomimetic structure using 3D bioprinting

effects on chemoresistance were also investigated [112]. To study multicellular communications for cancer progression, Langer et al. integrated four cell types, that is, cancer cells, endothelial cells, fibroblasts, and bone marrow-derived mesenchymal stem cells, and showed self-organization and neo-extracellular matrix deposition resulting from cancer tissue maturation. Extrinsic signals toward cancer cells accelerate cancer proliferation and migration, mimicking the *in vivo* morphogenetic features of primary patient tumors [113]. In addition to the pathological changes in solid cancer, metastatic development has also been recapitulated. To study breast cancer bone metastasis, *in vitro* bone matrices using nanocrystalline hydroxyapatite composite hydrogels were 3D printed [114,115]. Due to the presence of osteoblasts, the cancer cell proliferation rate and cytokine secretion level, such as VEGF, were stimulated, indicating the interactive effects on post-metastatic breast cancer progression in bone tissue. However, evolutionary changes in cancer cells depending on metastatic-developmental stages could not be recapitulated in previous models. To overcome this issue, an approach that can unveil the genetic and phenotypic changes in metastatic cancer should be presented.

13.5.3.3 3D bioprinting of vascularized cancer models

The spread of cancer cells usually occurs through blood and/or lymphatic vessels. In perfusable vessels, metastatic cancer cells move toward secondary organs. To develop a reliable anticancer drug testing platform, Han et al. constructed a vascularized microenvironment using endothelial cells, fibroblasts, and gelatin/alginate/fibrinogen hydrogel and seeded multicellular tumor spheroids, which were cultured in concave microwells, to show tumorigenic progression (Fig. 13.6). Recruitment of blood vessels and generation of angiogenic sprouts were also observed. Furthermore, the feasibility of the model was verified by testing the effects of the anticancer drugs temozolomide and sunitinib [116]. Kim et al. combined *in situ* cell printing technology and multicellular composition for the one-step fabrication of vascularized metastatic melanoma-on-a-chip. In this study, a high density of metastatic melanoma cell-encapsulated dECM bioink was printed to fabricate cancer spheroids. Printed cancer spheroids recapitulate tumor-like properties (local invasion, hypoxia, and secretion of angiogenic factors). Meng et al. printed programmable release capsules containing growth factors, including VEGF and EGF. In response to the slope of the growth factors generated when the capsules burst, cancer cells migrated and angiogenic sprouts were formed. Key initial steps of cancer metastasis,

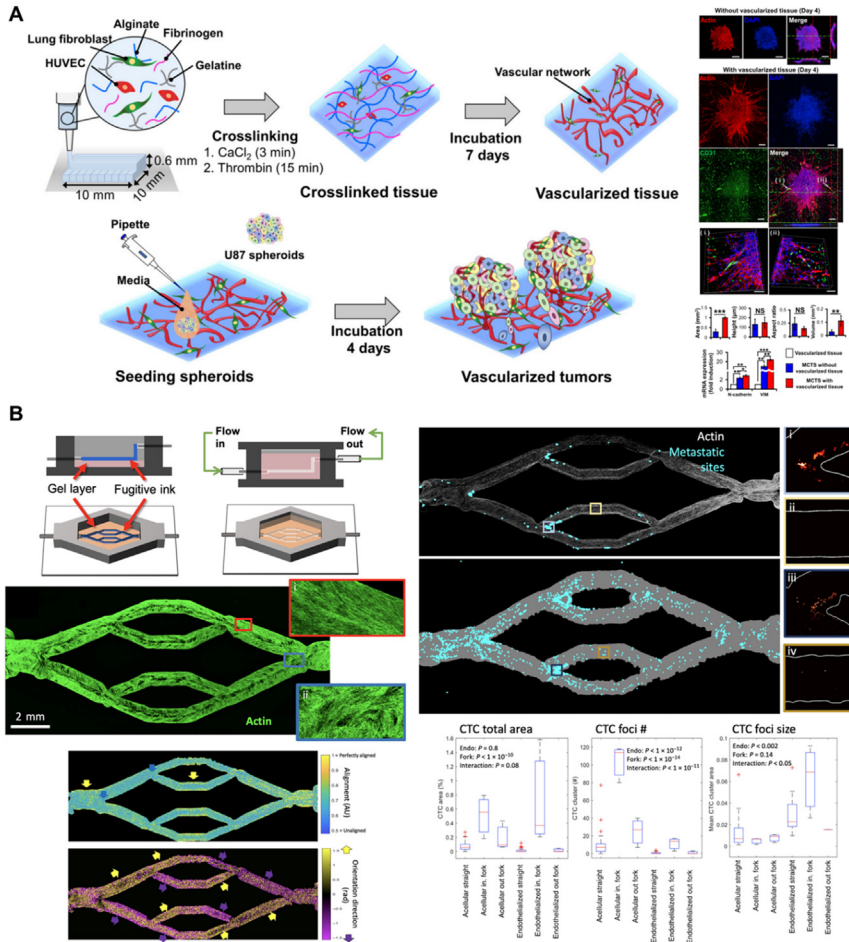


Figure 13.6 Bioprinting of vascularized cancer models. Construction of vascularized cancer models: (A) The vascularized TME was constructed by printing a vascular bed consisting of fibroblast and endothelial cells. Cancer spheroids of glioblastoma cells (U87 MG) were seeded onto the blood vessel layer; (B) Blood vessels were bioprinted and perfused with metastatic mammary gland cells under physiological flow rates. Therefore, the dynamics of circulating tumor cell behavior within the vasculature were studied. (Adapted with permission from Refs. [116,119].)

including invasion, intravasation, and angiogenesis, could be created. Furthermore, immunotoxins were treated, and the utility of the model as a drug screening platform was evaluated [117]. Recently, the importance of lymphatic vessels in cancer dissemination has also emerged. Cao et al. presented a tumor-on-a-chip possessing a tumor mass with a blood and

lymph vessel pair and compared the therapeutic effects of one- and two-channel systems. Through the lymph drainage system, the drug molecules were eliminated in a timely manner and significantly higher viability was observed, which demonstrated the efficacy of simulating the transport mechanisms of anticancer drugs in the TME [118]. Interestingly, studies on adhered cancer, CTCs, and their metastatic behavior have been examined. Hynes et al. fabricated bifurcated vessels and evaluated the effects of flow dynamics on cancer cell attachment. Depending on the flow pattern, preferential attachment was detected in the branched lesion [119]. From the 3D printed cancer-on-chips, insights into cancer progression and ideas for novel therapeutic approaches can be provided.

13.6 Conclusion and future perspectives

Cancer is a systemic disease that occurs throughout the body [120]. Advances in bioprinting technologies have enabled the fabrication of complex TME of the disease by integrating various types of bioinks and cells [121]. Despite many recent advances in the field of cancer research, the overall developmental mechanism of cancer on multiorgan-on-chips has not been accomplished. In particular, the accumulation of genetic alterations, including chromosomal abnormalities resulting from internal and external stimuli, has not been reproduced in recent *in vitro* models. Only the changes in gene and protein expression levels have been verified, which is insufficient to represent the pathophysiological characteristics of the disease. Therefore, the integrity and functionality of the generated artificial cancer tissue on multiorgan-on-chips should be elaborated, considering the biological and physiological properties of real tissue [10]. This technology is expected to stimulate human physiology, which is seldom limited to a single tissue or organ. Recently, a combination of 3D bioprinting and other manufacturing technologies is expected to integrate a multitissue/organ model for a more reliable study of cancer metastasis and *in vitro* drug testing. The development of microfluidic technologies provides a possible solution for manufacturing complex vascular structures at the micrometer scale and for controlling parameters such as the chemical gradient, flow of the culture medium, and spatial distribution of cells [122]. This technology can be integrated with an imaging system, which then enables real-time imaging of cancer cells to study the multiple steps of tumorigenesis and observe drug efficacy. Using microfluidic technology, Lee et al. developed a heart-breast cancer-on-a-chip platform to study cardiotoxicity induced by

cancer chemotherapy [123]. This model reproduced chemotherapy-induced cardiotoxicity in patients with breast cancer. Moreover, integration of the electrochemical immune-aptasensors enabled the detection of biomarkers with high sensitivity and a low detection limit. Recently, the development of hybrid bioprinters has enabled the printing of chips and tissues [124]. These platforms include SLA printing for chip fabrication as well as extrusion-based bioprinting for microtissue simultaneously to overcome the limitations of conventional bioprinting techniques. Integration of bioprinting and organ-on-a-chip platforms adds another level of complexity to tumor models and presents a promising direction for testing as an alternative to animal and human models.

Another limitation of the current *in vitro* cancer model is the need to generate a patient-specific model that can reflect each cancer patient's unique characteristics and complexity. Extreme cancer heterogeneity and variation in cancer molecular characteristics from individual patients make treatment difficult. Because each patient has a different composition of cancer clones with dozens of mutations, a treatment method that considers these variations must be considered. Research on patient-specific treatment has been actively conducted based on technological advancements in the genomic analysis [125]. However, it seems that it is still difficult to find the ideal combination of anticancer drugs for personal medicine due to the lack of basic studies and proper models that can reproduce the pathophysiology of cancer that differs from patient to patient [126]. Recently, patient-derived organoids (PDOs) have emerged as a suitable model to recapitulate the complexity and morphological features of the original solid tumor. The advantage of PDOs is that organoids can also be established from tumor-adjacent healthy tissues, providing normal organoids that can serve as experimental controls [127]. Moreover, PDOs can be easily cocultured with a variety of cell types and can be utilized to study the role of TME components in tumorigenesis. Owing to their versatility, PDOs derived from a wide range of cancer types are incorporated with bioprinting technology to mimic the dynamic *in vivo* environment more closely [128,129]. It is anticipated that the synergetic applications of bioprinting to PDOs present a breakthrough to achieve a more comprehensive understanding of tumorigenesis and the mechanisms of metastasis. Overall, these emerging advances in *in vitro* cancer models will lead to new advances in cancer research and therapeutics.

To conclude, the innovative progression of tumor-on-chip models has been reported, but further studies to understand the pathophysiological

features of cancer and fundamental technological developments with high resolution are urgently required. These systemic developments may accelerate the discovery of new therapeutic medications for advancing patient care.

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