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REVIEW

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Methods for fabricating oxygen releasing biomaterials

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ABSTRACT

Sustained external supply of oxygen (O_2) to engineered tissue constructs is important for their survival in the body while angiogenesis is taking place. In the recent years, the trend towards the fabrication of various O_2-generating materials that can provide prolonged and controlled O_2 source to the large volume tissue constructs resulted in preventing necrosis associated with the lack of O_2 supply. In this review, we explain different methods employed in the fabrication of O_2-generating materials such as emulsion, microfluidics, solvent casting, freeze drying, electrospraying, gelation, microfluidic and three-dimensional (3D) bioprinting methods. After discussing pros and cons of each method, we review physical, chemical, and biological characterisation techniques used to analyse the resulting product. Finally, the challenges and future directions in the field are discussed.

ARTICLE HISTORY

KEYWORDS

3D bioprinting; oxygen generating; biomaterial; tissue engineering

Introduction

Tissue engineering aims to develop products for the repair and regeneration of diseased or lost tissues [1]. Although substantial progress has been made in this area, difficulties still face the survival and unction of engineered constructs [2]. One of the biggest challenges is supplying the necessary oxygen (O_2) to the newly formed tissue constructs, following their implantation in the body [3,4]. Because vascularisation takes some time [5], tissue constructs rely on diffusion with limited access to receiving sufficient O_2 [6]. O_2-generating materials have thus, been developed to overcome this problem by providing the O_2 to implanted constructs [7]. There are various materials that can be used as a source for the generation of O_2, among which most commonly used ones comprise calcium peroxide [8], magnesium peroxide [9] and hydrogen peroxide [10]. However, the common problem with the O_2 delivery systems is the sudden or burst release of O_2. Fast O_2 generation of O_2 results in the release of hydroxyl radicals which lead to the formation of hyperoxide conditions and cell injury [11]. One strategy to prevent this is to encapsulate O_2 source into a polymeric material, such as poly(lactide-*co*-glycolide) (PLGA) [12], polycaprolactone (PCL) [13], and polyvinylpyrrolidone (PVP) [12] or into ceramics/polymer carrier materials [3,4] with varying degrees of success. When an O_2 generating material is encapsulated in a carrier polymer, the polymer decomposes in aqueous environment leading to the exposure of O_2 source material which reacts with water to produce H_2O_2, which dissociates then to water and O_2 [5]. To have appropriate control over O_2 release important factors include the use of appropriate method for the fabrication of O_2-releasing products. These methods include emulsion [10], solvent casting [14], freeze-drying [15], electrospraying [12], gelation [16] and microfluidic [17] fabrication techniques. Each method has its advantages and limitations [7]. Therefore, we explain procedures in each of these methods, and compare and contrast them. The characterisation methods used to define the properties of resulting O_2 releasing materials such as chemical, physical and biological investigations are also explained in this review.

Materials

O_2 source

For the oxygenation of tissue engineered constructs, an O_2 source either in a liquid or a solid form can be used. The former includes hydrogen peroxide (H_2O_2) [10] and the latter includes calcium peroxide (CPO or CaO_2) [8], magnesium peroxide (MPO or MgO_2) [9], sodium percarbonate (SPO or $(Na_2CO_3)_2.3H_2O_2$) [18], and zinc peroxide (ZnO_2) [19] as the most commonly used O_2 solid materials.

In general, solid peroxides, in the presence of water, dissociate into their corresponding metal hydroxides and H_2O_2, which further decompose to release O_2 and water. Hydrogen peroxide is the only liquid peroxide that can be used for oxygen generation. As this is a slowly decomposing source, it produces highly-reactive radicals that can cause cellular damage [20]. Therefore, catalase is used for the acceleration of H_2O_2 decomposition into water and oxygen with high turnover efficiency in the O_2 production process [21]. Encapsulation of catalase also helps to control the O_2 release from the materials [22]. Release kinetics of O_2 from peroxides are influenced by several factors such as temperature and pH [23–25]. Additionally, the purity and solubility of the peroxides can also affect the rate of O_2 release from the material. Like H_2O_2, SPO also readily dissolves in water and decomposes rapidly. As a result,

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Table 1. O_2 generating materials.

O_2 generating source	Pros	Cons
H_2O_2	It can bind to high molecular weight polymer for encapsulation.	Difficulty in controlling O_2 release rate.
CPO	High purity More sustained release	Less solubility
MPO	Slow O_2 formation	Less purity Less solubility
SPO	High solubility Biocompatible byproducts	Fast decomposition rate Rapid O_2 release
ZnO_2 [19]	Stable in high pH aqueous solutions. Good anti-bacterial and antimicrobial activity	Insoluble in water
Benzoyl peroxide (BPO) [26]	Prolonged O_2 release Biocompatibility	Hydrophobicity No release during the first two days

It is adapted from [7] with modification and permission from the American Chemical Society.

SPO releases O_2 at faster rate. Thus, sustained O_2 release over a prolonged period of time can be a challenge. In these peroxides, H_2O_2 is; however, dissolved in water, and it is difficult to encapsulate using conventional methods. Instead, it can be complexed with polyvinyl pyrolidone (PVP) for encapsulation [12]. On the contrary, MPO and CPO are insoluble in water (Table 1). Therefore, the material has a low decomposition rate, which leads to the slow release of O_2 [27]. The purity of the MPO is 15–25%, while CPO has a purity of 60–80% [28]. Thus, among all, CPO is considered as the most preferred O_2-generating material for delivering sustainable and controllable O_2 for a prolonged time. Unlike peroxides, perfluoro-based hydrocarbons, endoperoxides, and microtanks have been utilised for O_2 delivery [29–31] and not generation, which are discussed elsewhere and are out of the scope of this review.

Carrier materials

Materials that are used to carry an O_2-generating source need to be biocompatible, biodegradable, capable of controlling the release of O_2, and minimise the risk of sudden release of a large amount of O_2 that can be toxic to cells. There are various polymers, including synthetic, natural, and the combination of both, which have been used for this purpose and were previously discussed in details [32]. Thus far, polymers have been utilised as carrier materials for O_2 sources include PLGA, PCL, PVP, polydimethyl siloxane (PDMS), polyurethane and *N*-isopropylacrylamide (NIPAAm) [12,13,33]. Carrier materials are usually mixed with O_2 generating sources, and the physical combination of these materials is further formulated to produce various forms of O_2 generating materials.

In the form of hydrogels such as gelatine methacryloyl (GelMA) [16] gellan gum [34] and alginate [26], polymers have also been utilised not only as carriers for O_2 generating sources but also to encapsulate cells, adsorb growth factors and drugs, and as a printable constructs that can mimic the three dimensional (3D) environment of the body and used for tissue engineering applications [16,35].

The selection of the polymer for a carrier is based on its physical properties, which play a key role in controlling the release of O_2 from the source. Particularly, the hydrophilicity and degradation profile of the material governs the kinetics of the O_2 release and determines the level of toxicity. For example, less hydrophilic polymers such as PCL and PLGA have less interaction with water molecules, thereby control the degree of degradation and provide sustainable release of O_2 for an extended period of time; while water can easily reach peroxide in the more hydrophilic polymers such as gelatine and alginate, and lead to the release oxygen that lasts for a short period of time. Interestingly, nondegradable polymers such as PDMS and ceramics or ceramic polymer composites have also been explored for O_2 releasing and considering them for unique applications where the stability of the device and implants are favoured [36]. The type of the polymer plays an important role in determining material morphology that can be used to control the release of O_2, for example dimethyl sulfoxide (DMSO) soluble gelatine derivatives and 3D printable PCL were used to make porous scaffolds. Furthermore, stimuli-responsive materials have opened the doors for controlling O_2 release by using local and external triggers such as temperature, pH, electric field, magnetic field, and light [37–41].

Fabrication methods

After selecting the O_2 source and the encapsulation material, the next step is to choose the right method to fabricate the O_2 releasing product. Encapsulation method should be simple, rapid, highly reproducible, and allow maximum loading capacity. Finally, the last step is to identify an appropriate method for storage that prevents O_2 loss when O_2 generating material is not in use [42]. In this section, we explain different fabrication methods that are used to produce O_2 releasing materials.

Emulsion method

Emulsion solvent evaporation method is a useful method that can be used to fabricate O_2 releasing microparticles. The choice of solvent and surfactant, as well as the rate of solvent evaporation, can directly influence microparticle size, morphology, and porosity, thus control O_2 release time. Single emulsion and double emulsion methods, alternatively known as multiple emulsion methods are common methods employed for the fabrication of O_2 generating materials [43].

Single emulsion method

For example, single emulsion of water in oil can be used to fabricate H_2O_2 loaded microcapsules [9]. Poly(methyl methacrylate) (PMMA) polymer is dissolved in water solution of H_2O_2 in a mixture of acetone and acetonitrile, and the solution is emulsified further in mineral oil containing a surfactant (Figure 1). After solvent evaporation, microcapsules are separated using centrifugation, washed, and dried at ambient temperature, and stored in refrigerator until use. Although the resultant microcapsules (5–30 μm) can release O_2 for over 24 h, direct contact of H_2O_2 with water can trigger the decomposition of peroxide [9]. To avoid this drawback, an alternative method of oil-in oil emulsion can be used. For

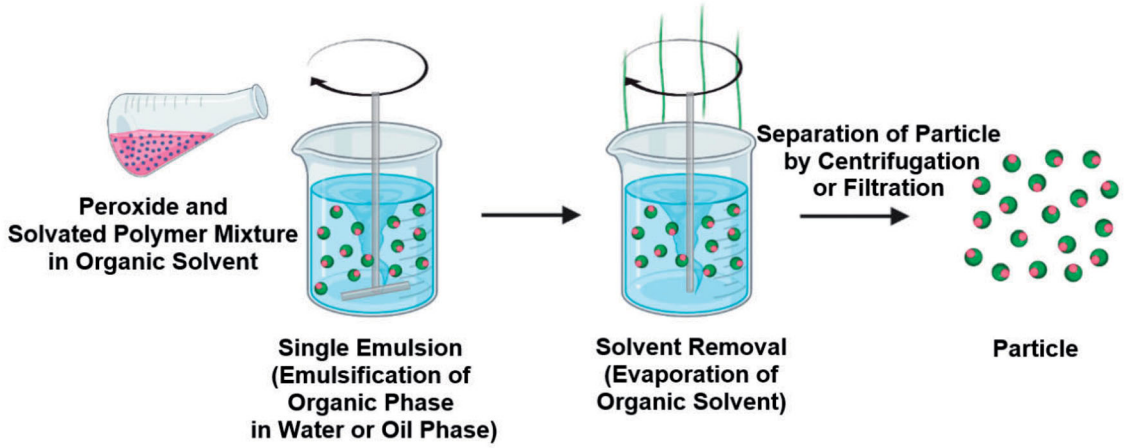

Figure 1. Illustration showing the process of fabrication of O_2-generating microcapsules using single emulsion method. Adapted from [7], with permission from the American Chemical Society.

example, composite microspheres comprising of CPO and poly(trimethylene carbonate) (PTMC) can be produced using an oil-in-oil emulsion method. For this purpose, CPO is dispersed in a solution of PTMC in acetonitrile, and the dispersion is pipetted into a mineral oil supplemented with Span 80 surfactant. The resultant microspheres (~200 μm) are washed with n-hexane, vacuum dried, and stored at 20 °C until use. Since during this procedure, CPO is never exposed to water, O_2 releasing time can be extended for 20 days [44]. Suvarnapathaki et al. prepared CPO-loaded PCL microparticles (average diameter 100 mm) with oil-in-water solvent evaporation method. In this method, they used PVA as a surfactant that can be separated by washing with water several times. These particles were used to fabricate an O_2 generating scaffold by the gelation method inside ultraviolet (UV) curable GelMA. The obtained gel based scaffold sustained O_2 release for up to 35 days.

Double emulsion method

Single emulsion method is preferred for the encapsulation of poorly water-soluble and lipophilic compounds. It is usually not suitable for water-soluble compounds; otherwise their encapsulation requires a high dose of compounds to be loaded. To overcome this limitation, a double emulsion method to encapsulate water-soluble drugs or proteins can be used [43]. There are various types of double emulsion methods such as water/oil/water, oil/water/oil, water/oil/oil, solvent/oil/water, and solvent/oil/oil that can be used to engineer stable formulations [45].

One of the double emulsion methods that has been frequently used for encapsulation of H_2O_2 is water/oil/water (w/o/w) double emulsion solvent evaporation method, in which external and internal water phases containing dissolved H_2O_2 are separated by an oil layer. The w/o/w emulsion is an excellent system for the development of a product with sustained release of H_2O_2 due to the presence of the intermediate oil layer acting as a liquid membrane. Based on this criterion, a w/o/w emulsion solvent evaporation method can be used to encapsulate a large amount of H_2O_2 in PLGA, for prolonged O_2 release. To achieve primary w_1/o emulsion, PLGA was dissolved in dichloromethane (DCM) as a volatile organic phase (o), and H_2O_2 as an aqueous phase (w_1) was then emulsified under high speed shaking. Synthesised emulsion is then added to H_2O_2 containing PVA (w_2) to form a second emulsion layer. Subsequently, DCM is evaporated under continuous stirring, and the resultant microparticles (in the size range of 25–250 μm) are filter-separated, surfactant (PVA) is washed off, and the microparticles are freeze-dried, and stored at low temperature to prevent H_2O_2 decomposition [10]. Because H_2O_2 is toxic to the cells, a second layer of alginate shell around the microparticles [46] can be used to prevent direct cell contact with H_2O_2 with the cells [10], which can also extend the O_2 release time [35,46].

Although the conventional emulsion-based method is used to produce microparticles, preparation always ends the production of particles having different diameters. Despite utilising these microparticles for various applications such as drug delivery, the absence of size homogeneity limits the use of microparticles for drug release studies.

Microfluidic fabrication method

Microfluidic technology enables the production of microparticles with homogeneous controlled particle size, shape, and shell thickness [47]. To do this, hydrodynamic flow focussing, T-shape flow focussing, or their combination with a spiral mixer can be utilised [48]. Most of the device preparation methodologies have similar principle; however, they vary in their channel configurations. For example, T-flow focussing devices can be fabricated from PDMS, using standard soft lithography method. The PDMS slab is then fixed to a silica glass plate using vacuum and O_2 plasma treatment. To produce O_2-releasing microparticles, a continuous phase of dissolved 1% PVA (to function as a surfactant) and dispersed (organic) phase of dissolved 5% w/v PLGA in dichloromethane (DCM) are used (Figure 2). CPO is added to the dispersed phase. The flow rates of the solution for the PLGA-CPO are set at 1 ml/h and for the PVA solution at 5 ml/h. Microparticles are then collected from the device, purified by applying vacuum pressure to evaporate DCM, and subsequently, the solution is centrifuged for 3 min to obtain PLGA encapsulated CPO microparticles. By utilising this method, CPO of different concentrations can be loaded into microparticles. Produced particles can release O_2 for up to two weeks.

Solvent casting method

Solvent casting method is a useful method for the preparation of film-based O_2 generating materials. This method is based on the principle of dissolving the polymer and O_2 source in an organic solvent and then evaporating the solvent from the solution after

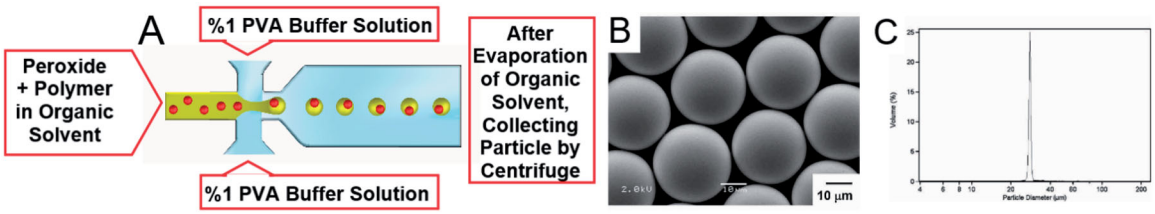

Figure 2. (A) Illustration of the fabrication process of O_2-generating microcapsules using microfluidic method. (B) SEM images of monodisperse PLGA microparticles (diameter ≈ 28 µm). (C) Size distribution of the microparticles measured using a Coulter counter. Adapted from Xu et al. [17], with the permission from Wiley.

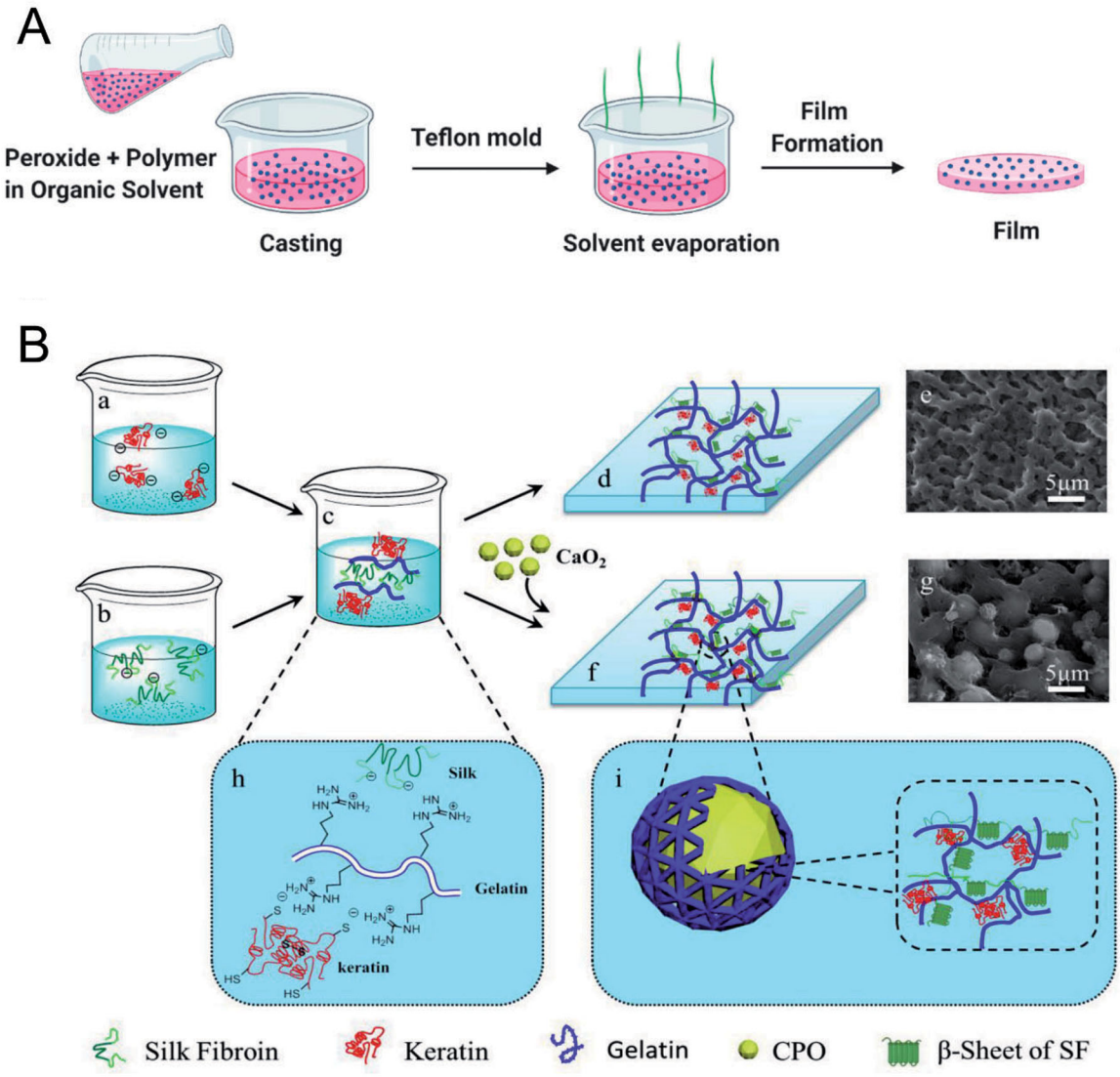

Figure 3. (A) Schematic illustration of the fabrication process of O_2-generating film using solvent casting method. Adapted from [7], with the permission from the American Chemical Society. (B) Schematic illustration of O_2-generating materials composed of mixture of gelatine, keratin and silk fibroin fabricated using solvent casting method. Adapted from Lv et al. [49], with permission from Elsevier.

it is poured into a mould (Figure 3). In this method, organic solvents with a low boiling point along with water-insoluble polymers are often used. For example, SPO can be dispersed in methylene chloride solution containing 5% PLGA, and then evaporating methylene chloride for 5 days to produce a film. Produced film can release O_2 for up to 70 h [14]. Chloroform solution containing 10% (w/v) PLGA or polylactide (PLA) can also be used to produce films containing 5% (w/w) CPO in ree days. In these films, catalase is also included in the films to reduce the risk of accumulation of free radicals. The encapsulation of CPO prolongs the release of O_2 as compared to non-capsulated CPO [8].

Using this method, multi-layered films can also be produced, where one layer releases O_2, while other layers enhance product properties such as flexibility and gas permeability of the film. O2 releasing layer is prepared by using a 4.44% (w/v) CPO and 3.33% (w/v) SPO in 9.5% (w/v) PCL that are dissolved in hexafluoro-2-propanol. A blend of gelatine (in concentration of 1%, 2% or 3% w/v) and CPO (5%, 10%, 15%, 20%, and 25%, wt%) with different

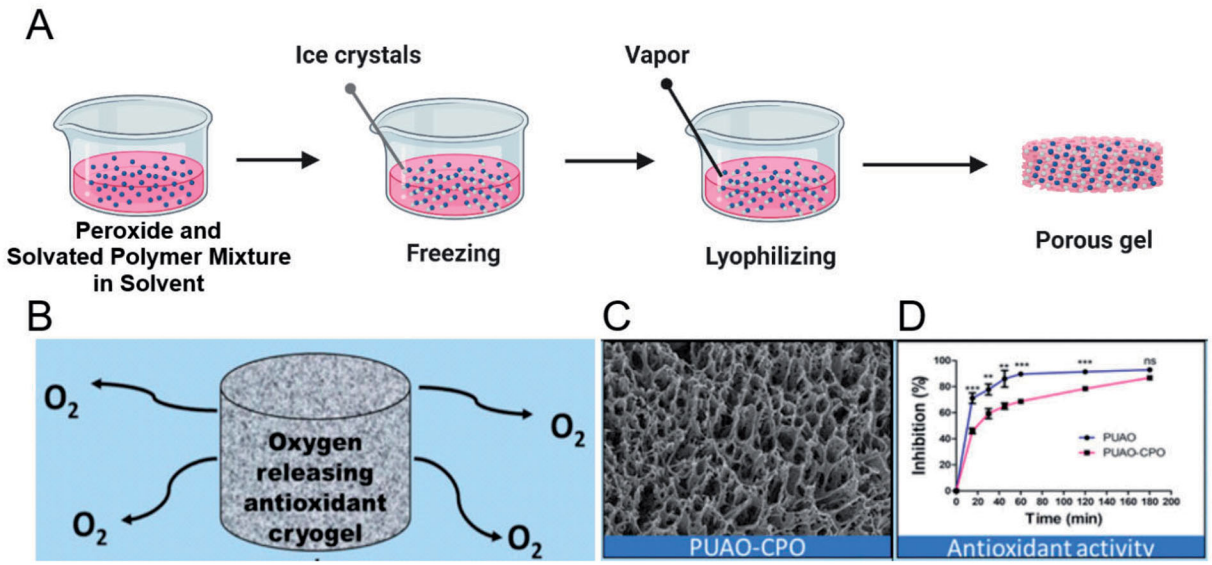

Figure 4. (A) Fabrication process of O_2-generating macroporous film using freeze-drying method. Adopted from [7], with the permission from the American Chemical Society. (B) Representative image of O_2 releasing antioxidant PUAO-CPO cryogel. (C) SEM micrographs of PUAO-CPO cryogels and (D) DPPH assay showing the antioxidant properties of 1% PUAO-CPO cryogels after the incorporation of CPO. Adapted from Shiekh et al. [15], with permission from the American Chemical Society.

ratios of silk/keratin (30/70, 40/60, 50/50, 60/40, and 70/30) dissolved in water at pH 7.0 can also be used (Figure 3). Films are obtained by casting the blend in plastic dishes and drying at room temperature for 24 h, then incubating with 100% ethanol for 1 h. Produced films can release O_2 for more than two weeks [49].

Freeze-drying method

The freeze-drying method is a simple method for fabricating O_2 releasing materials. An O_2 source can either be dissolved or dispersed in a solution of a carrier material and frozen to a low temperature to eliminate the solvent from the carrier material by sublimation process at low pressure. For example, an O_2-generating anti-oxidant polymeric cryogel scaffold can be produced by dispersing CPO in 5 w/v% of anti-oxidant polyurethane (PUAO) in DMSO at 60 °C. The mixture is stirred overnight until a homogenous distribution of CPO is achieved. Cryogel is formed by freezing the mixture at −20 °C and thawing in absolute ethanol/cold water, followed by lyophilisation (Figure 4). Resulting cryogel showed release O_2 for more than a week [15]. Otherolymers that can be freeze-dried, can also be used as alternative materials in the future.

Electrospinning and electrospraying methods

Electrospinning and electrospraying techniques are based on applying an electric field to a polymer solution, which is ejected from a syringe. These techniques are useful for producing submicron materials. The yield of electrospraying is particles, which are obtained by using a low polymer concentration, while the yield of electrospinning process is fibres, which are obtained by using high polymer concentrations [50]. For example, PCL [51] or poly(-glycerol sebacate) (PGS) and PCL [52] can be used to encapsulate CPO and produce O_2-releasing nanofibers (Figure 5). The concentration of CPO can be varied (1%, 5% and 10% w/w) [51]. For preparing precursor solution, polymer, e.g. PGS (10% w/v) and PCL (10% w/v) are dissolved in chloroform: ethanol mixture (9:1) by 1 h sonication and then mixed with CPO (In ratios of 0–10% w/v). Then, the precursor solution is electrospun at a distance of 15 cm with 1.2 ml/h of feed rate, and using an 18 G needle, cleaned every 2 min. This can result in the production of O2 releasing nanofibrous of ∼600 nm in diameter that can release O_2 for up to seven days [52].

O2 releasing microparticles can be produced by using electrospraying of a PLGA solution (5 wt% in DCM) and H_2O_2/PVP complex containing different molar ratios of H_2O_2 and PVP (6/1, 4.5/1, and 3/1). A coaxial device can be utilised to produce core-shell O_2 releasing microparticles by including H_2O_2/PVP in the core and using PLGA for the shell. An infusion rate of 0.2 for H_2O_2/PVP complex and 1 ml/h for PLGA solution through the coaxial device, are used. Produced microspheres can release O_2 for up to 14 days [12].

PDMS curing

PDMS is a synthetic polymer, however its O_2 permeability feature provides a great advantage. PDMS curing method is based on mixing an O_2 source with a PDMS pre-polymer solution, then removing air bubbles from the mixture with vacuum, then, curing this mixture with heat (Figure 6). For example, discs of CPO containing PDMS can be produced by curing the mixture of the two at 40 °C for 24 h. Produced PDMS-CPO discs can release O_2 for more than seven weeks [36]. CPO containing PDMS ring scaffolds can also be produced by using different ratios of CPO (in concentration of 25%, 50%, and 75%) and curing at 50 °C for 6 h. Produced scaffolds can release O2 for 24 h [53]. A double-layer O_2 releasing films can also be produced by curing a SPO containing PDMS. In this process, PDMS is poured on top of silicone and cured to make the first layer, and then covered by a second layer of SPO particles followed by curing at room temperature for 48 h [54]. Using this method, burst release of O2 form SPO can be avoided and O_2 release can be sustained for more than four days.

Gelation method

For the gelation method, a synthesised pre-polymer containing O_2 generating agent is solidified physically or by chemical reactions or photocrosslinking. For the UV-based crosslinking, a

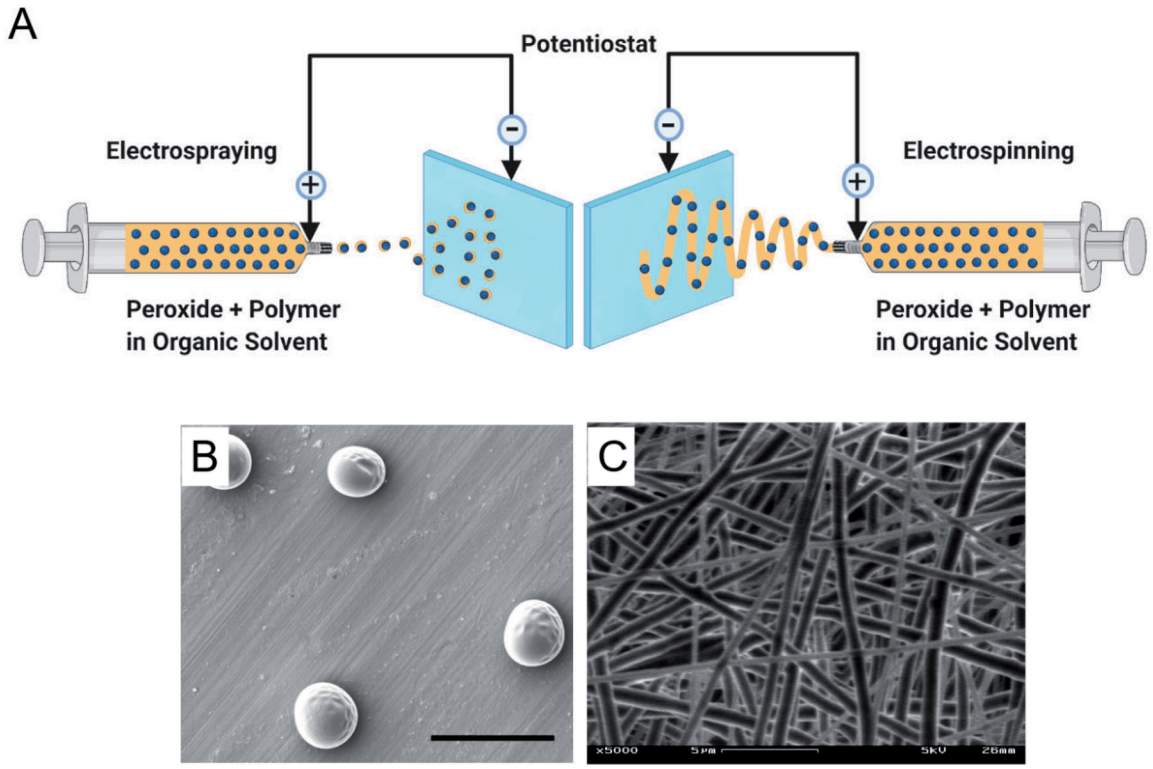

Figure 5. (A) Schematic illustration of the fabrication process of O_2-generating materials using electrospraying and electrospinning methods. Adapted from [7], with the permission from the American Chemical Society. (B) SEM image of core–shell H_2O_2-releasing PLGA microspheres. Adapted from Li et al. [12], with permission from Elsevier. (C) SEM micrographs of electrospun nanofibers with compositions of PCL/Calcium peroxide/AC 10%. Adapted from Wang et al. [51], with permission from the American Chemical Society.

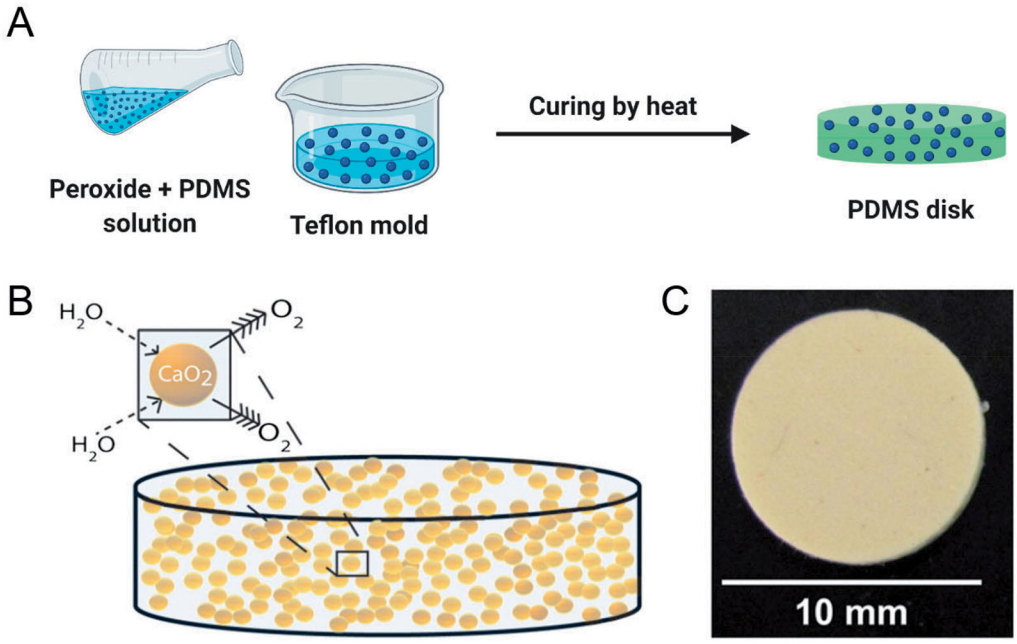

Figure 6. (A) Fabrication process of O_2-generating films using PDMS curing method. Adapted from [7], with the permission from the American Chemical Society. (B) Schematic of O_2-releasing biomaterial, fabricated by using PDMS and CaO_2. Water diffusion is hindered by the hydrophobicity of the PDMS, whereas O_2, generated *via* hydrolytic reaction with CaO_2, quickly diffuses out of the PDMS material. (C) Photograph of PDMS-CaO_2 disc (10-mm diameter; 1-mm height). Adapted from Pedraza et al. [36], with permission from the National Academy of Sciences.

photoinitiator (PI) such as 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) or lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) can be used. For visible light-based crosslinking, vinyl caprolactam, triethanolamine (TEA) in combination with Eosin Y can be used [55–58]. For gelation, a polymer as GelMA can be used. CPO is mixed with GelMA pre-polymer solution, and 0.1% Irgacure 2959 is used as a PI to crosslink the mixture with UV light and fabricate a hydrogel (Figure 7). Using gelation method, O_2 can be released for five days [56]. Gellan gum pre-polymer can also be used and physically crosslinked

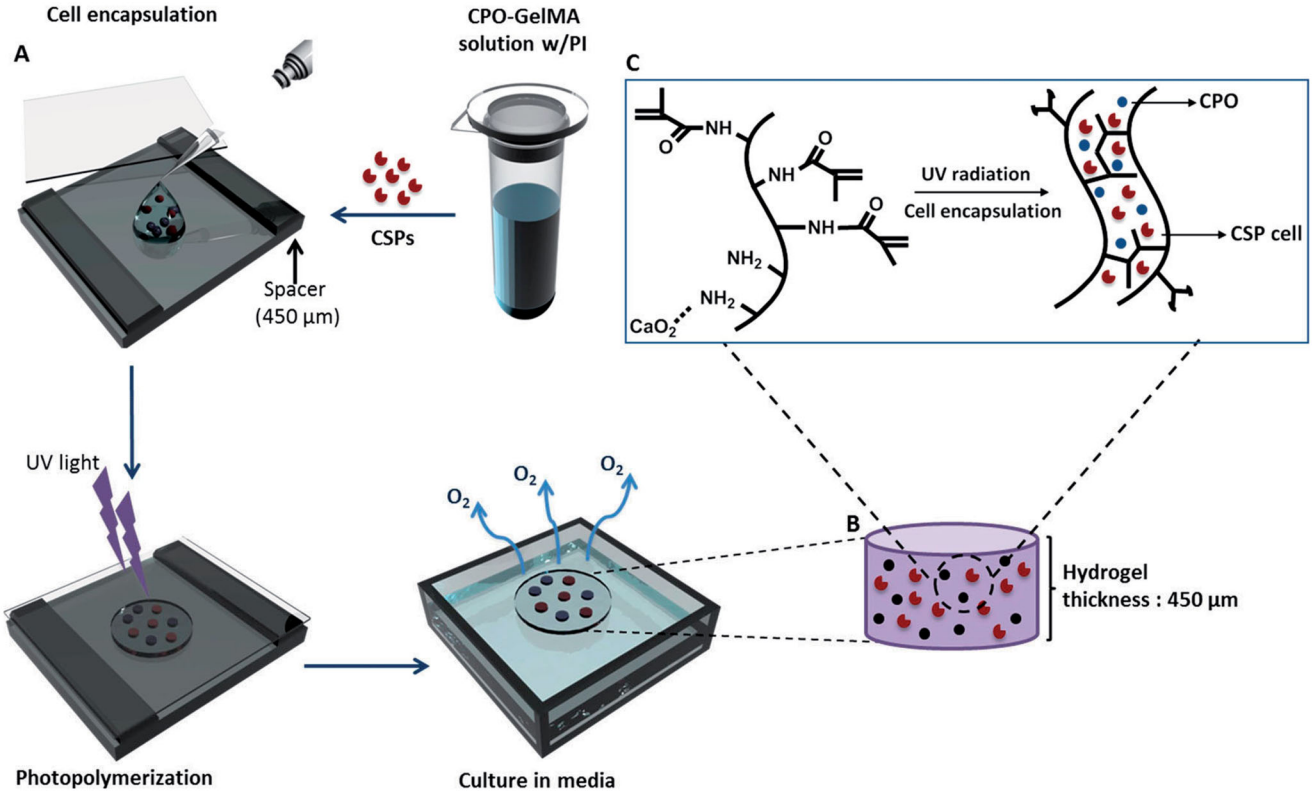

Figure 7. Schematic illustration of gelation method used for production of 450 µm thick-CPO encapsulated GelMA hydrogel *via* UV irradiation photopolymerization. Adapted from Alemdar et al. [56], with permission from the American Chemical Society.

using calcium ions which are released from contained CPO to form a solidified hydrogel [34]. A catalase enzyme can also be included in the mixture to enhance the conversion of H_2O_2 to O_2. O_2 release from the gelly gum is CPO-concentration dependent. Because the release of O_2 from materials produced by gelation is rapid, a combination of gelation and encapsulated O_2 releasing particles can be used. For example, a hydrogel can also be prepared, and used to encapsulate PVP H_2O_2/PLGA core/shell microspheres to develop an O_2 releasing system with extended O_2 release for up to 4 weeks [33]. In addition to UV and physical cross-linking, gelation can be performed with many various methods. Thanks to this diversity, the gelation method can be used in the preparation of different O_2-releasing materials in the future.

Three-dimensional (3D) bioprinting method

Three-dimensional (3D) bioprinting can be used to develop oxygenated 3D constructs, which can address the challenge of cell survival in engineered tissues. To 3D print an O_2 releasing material, an O_2 source material is added to the bioink while in solution. A source such as CPO can be added to the solution of, e.g. an adipose tissue-derived stem cell-containing alginate bioink [59]. In the preparation, CPO is washed with buffer solution on a rotating shaker at 37 °C overnight. Then CPO is added in different concentrations (0.1, 03 and 0.6 mg/mL) to the acellular alginate solution that has a concentration of 4%, 6%, 8%, 10% or 12%, and stir it for 3 h. The mixture is then kept at room temperature for 1 h. Cells are then added to the mixture solution, and extruded using a bioprinter into culture plates containing ionic cross-linker solution (100 mM $CaCl_2$) to obtain a cell-laden O_2 releasing constructs (Figure 8). O_2 releasing bioinks can also be produced using cardiomyocyte-laden GelMA (10%) bioink solution, which can be prepared in HEPES buffer, 0.1% photo initiator (Irgacure 2959) and different ratios of CPO (0.1, 0.5 and 1.0%) can be added [16]. Cell-laden bioinks are then printed using extrusion method and cross-linked using UV.

Comparison of fabrication methods

Each of the mentioned methods has its advantages and limitations and the selection of the method should be carefully made (Table 2). The duration of O_2 release from fabricated constructs depends on the location of the O_2 source in the construct and the properties of the containing polymer. For example, PDMS curing results in very slow O_2 release kinetics because PDMS is highly hydrophobic, which can limit the O_2 release. Using microfluidic techniques, O_2 releasing materials can be embedded deep into the carrier polymers, which can result in slow O_2 release. However, microfluidic approaches are much more complex compared to other methods because they require a flow focussing microchip device, and related multiple steps of design and fabrication processes. The time required to produce O_2 releasing materials also varies between different approaches where gelation requiring the least time (minutes to an hour) due to the quick solidification of the gelating polymers, and used crosslinking and photoinitiator agents. On the other hand, electrospraying can take days because of the drying process is lengthy. Yield is another parameter that needs to be taken into consideration when producing considerable amounts of O_2 releasing materials is required. While gelation produces the highest yield, microfluidic systems and PDMS curing methods suffer from producing samples in low

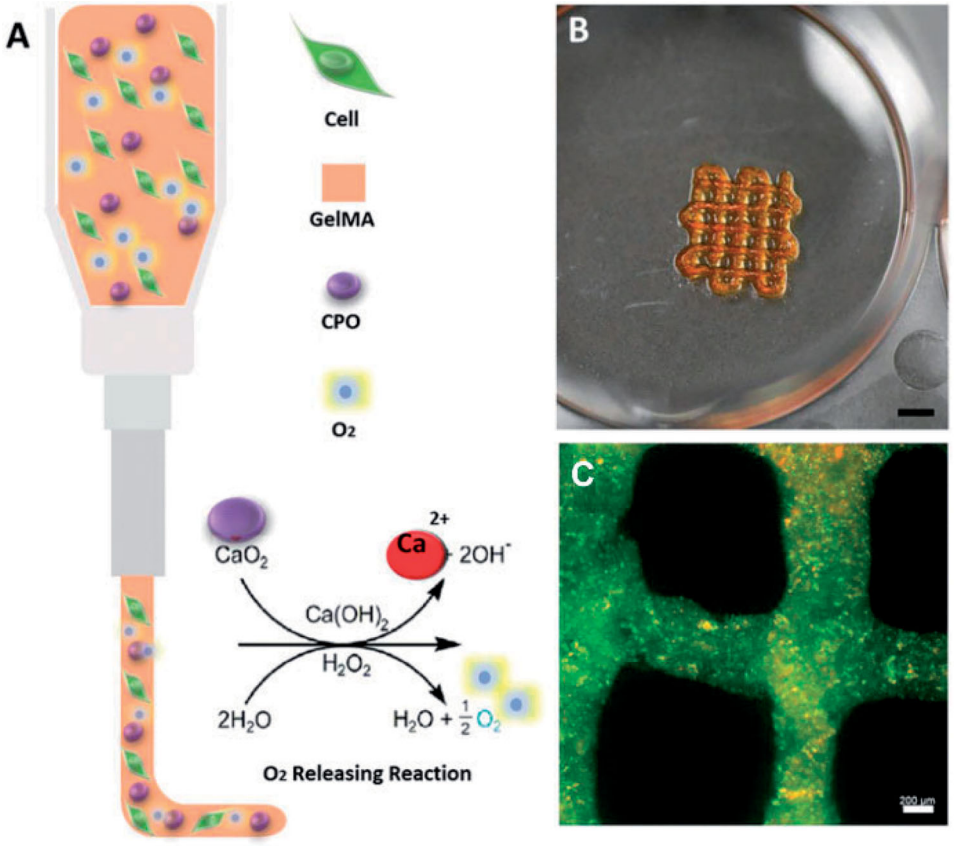

Figure 8. Illustration showing the procedure of 3D bioprinting of oxygenated cardiomyocytes-laden bioink (A). (B) Produced 3D bioprinted construct. (C) Live (green) cardiomyocytes seen in the construct 7 days after printing (scale bars for (B) is 2 mm, and (C) is 200 µm). Reproduced from [16], with permission from Wiley.

quantities. Quality in terms of homogeneity and the ideal release kinetics, is best obtained with the use of microfluidic approaches.

Characterisation techniques

Depending on the purpose of the application, O_2 releasing materials can be fabricated in the form of microspheres [54], nanofibers [51], scaffolds [15,60], devices [59], films [13,14], and coatings [61] for tissue engineering [60], 3D bioprinting constructs [59], preservation of organs [61], and regenerative therapy [14]. The products can in general be characterised using methods outlined below.

Methods for the characterisations of physical and morphological properties

Particles structure, porosity, pore size, surface charge and viscosity are the most common physical and morphological characteristics of the O_2 generating materials that should be determined. Scanning electron microscopy (SEM) is frequently used to investigate O_2 releasing material structure [54], morphology [59], porosity [62], pore size [29] and particle size by using image processing software [63]. However, particle size analyser is comparatively a more accurate instrument to measure the size and analyse size distribution of the microparticles [59]. SEM can also be coupled with a field emission electron source and energy dispersive X-ray spectroscopy (EDS) to analyse morphology of fabricated O_2 generating microparticles [64]. EDS analysis is used to confirm the presence of the CPO in the O_2 releasing materials [64].

Another physical characteristic of the O_2 generating materials, which affects directly their applications in biomedical research, is viscoelasticity. A rheometer can be used for the determination of the viscoelasticity of O_2 releasing materials [65]. O_2 releasing hydrogels having different viscoelasticity properties can be used to suit different biomedical applications.

Methods for the analyses of degradation rate

The degradation rate of the O_2 releasing materials is an important parameter for determining their biomedical applications. Especially for tissue engineering and regenerative medicine applications, O_2 generating materials should be biodegradable so that no further surgery required to remove them. The O_2 generating materials should be engineered in such a way that they undergo slow degradation by time while also be stable until cells regenerate desired tissues [26]. The degradation of O_2 generating materials is also important to study the release kinetics of loaded O_2. It is worth noting that the incorporation of hydrophobic O_2 generating peroxide salts decreases the degradation rate by reducing the rate of water diffusion into the polymer network [26].

The common method to assess degradation rate of the O_2 generating materials is by keeping them in the Dulbecco's phosphate-buffered saline (DPBS), or culture media in the presence or absence of the relevant enzyme and incubate the materials at 37 °C. At predetermined incubation time points, samples are taken out, dried and weighed. Subsequently, the degradation rate can be estimated by assessing dry weight of the samples at given

Table 2. Comparison of different O_2 generating biomaterial fabrication methods.

No.	Parameter	Microfluidic	Solvent casting	3D Printing	Gelation	Freeze- drying	PDMS curing	Emulsion	Electro-spraying /electrospinning
1	O_2 release	Slow (week)	Moderate (4–14 days)	Moderate (7–10 days)	Moderate (one week)	Moderate (9 days)	Very slow (weeks)	Fast (hours days)	Fast (hours–1 day)
2	Complexity	Very complex	Simple	Moderately simple	Simple	Simple	Simple	Moderately simple	Complex
3	Time consumption	Long (min-hours) for quantity	(24–72 h-)	4–6h	Fast (min.-1h)	Long (>2d)	4–6h	Moderate-Long (6 h)	Long (1 day) because of drying
4	Cost	High	Low	High	Moderate	Low	Low	Moderate	Low to moderate
5	Operator training	Highly trained	No specific training required	Specific training required	No specific training required	No specific training required	No specific training required	Specific training required	specific training required
6	Additives	Surfactant	None	Change according to composition	Photoinitiator	None	Crosslinker	Surfactant	None
7	Suitability for the range of the materials	Complicated	Acceptable	Acceptable	Minor problems	Only for limited number of materials	Limited	Robust	Robust
8	Efficacy and productivity	Low	Medium	Medium	High	High	low	high	Medium
9	Homogeneity	Very high	Moderate	Moderate	Moderate	Moderate	Moderate	Very low	Very low
10	Quality of the product	Very high	Moderate	High	Moderate	Moderate	Moderate	Very Low	Moderate
11	Product	Microparticles	Particles	3D Construct	Hydrogel	Particles	PDMS device	Microparticles	Droplets and fibres

time point and subtract this from their initial dry weight per dry weight at given time point.

Methods for the assessment of O_2 release kinetics

The first and easy step for the evaluation of the release of O_2 from produced constructs is the observation of O_2 bubbles by naked eye or optical microscopy. Different O_2 bubble sizes can be observed on a glass slide by using optical microscopy [66]. An inverted laser scan confocal microscope can be used to monitor real time growth and detachment of O_2 bubbles from H_2O_2 decomposition catalysed by gold [67]. The radius of O_2 bubbles can be measured by using thin dual layer gold – titanium coated coverslips. Therefore, confocal microscope can be a better tool for studying O_2 bubbles as compared to the optical microscope. Although the immediate observation of O_2 bubbles in the construct is a direct evidence proving the release of the O_2, this method is qualitative at the best.

In order to quantify the amount of the O_2 released in the DPBS or cell media, an O_2 probe/microsensor is found to be the gold standard tool. To do this, different methods can be used. For example, an O_2-sensitive dye can be used to assess O_2 release form core/shell microspheres. In this method, DPBS supplemented with 1 mg/mL catalase is used in a hypoxic environment (1% O_2). Two different dyes, luminophore $Ru(Ph_2phen_3)Cl_2$ -an O_2-sensitive dye are used to determine the O_2 level and rhodamine B-an O_2-insensitive fluorophore to serve as a reference. PDMS membranes, which allow dyes to interact with the O_2 are placed in tissue culture well plates, and DPBs containing catalase added to each well and incubated with 50 mg O_2-contaning microspheres at 37 °C. To estimate the O_2 level, fluorescence intensities at emission wavelength of 610 nm for $Ru(Ph_2phen_3)Cl_2$ and wavelength of 576 nm for rhodamine-B using a fluorescent plate reader are measured [33]. Blood gas analyser can also be used to monitor O_2 release kinetics from CPO included in 3D-printed constructs, which are kept in DPBS containing tissue culture plates in a hypoxic incubator (1% O_2) [59]. Dissolved O_2 is measured in 1 ml of DPBS solutions taken from each well at defined time points.

Methods for biological characterisation

Since the ultimate goal of fabricating O_2 generating materials is to utilise them in biological systems, it is necessary to undertake biological characterisation. In addition to O_2 release, these materials should also be biocompatible, and their biocompatibility determined using various methods. Commonly, the initial biological method to evaluate safety of O_2 releasing materials before using them for any *in vivo* applications is cell culture-based cytocompatibility test [68]. Broad range of biological assays are performed to assess cell viability, metabolic activity, proliferation, and immune responses. The cytocompatibility of O_2 generating materials in contact with different cell types can be evaluated by using calcein/ethidium homodimer-1 live/dead cell viability assay [56,65], prestoblue metabolic activity assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay [56,59,63] Resazurin assay [61], lactate dehydrogenase (LDH) release assay [56], cell proliferation WST-1 assay [65], and cell proliferation 5-ethynyl-20-deoxyuridine (EdU) Clik-it assay [59]. Usually the principle of these assays relies on the ability of the cells to maintain their viability and allow colorimetric evaluation.

Future directions and conclusions

Several organic and metal peroxides such as H_2O_2, SPO, MgO_2 CPO and BPO have been utilised as O_2 generating sources and biomaterials to fabricate various O_2 releasing products. Various methods have been discovered for successful synthesis O_2 generating sources and encapsulation of these sources in the carrier materials, which were explained in this review. However, several challenges remain to be addressed, before these O2 releasing products can be used for clinical applications. One the main concerns associated with the fabrication of the O_2 generating materials, is the size of the particles which are quite big and in the range of several micrometres. Large size microparticles can affect the physical and mechanical properties, e.g. of the resulting scaffolds intended for use in tissue engineering [42]. Using current techniques, it is difficult to create a nanosize range of particles. Future generation of the sophisticated microfluidics system might be able to revolutionise the fabrication methods by producing size-homogenous particles with diameter much smaller than the currently available microparticles. The other concern in selecting the O_2-generating source for their *in vivo* applications is the toxicity. Generation of H_2O_2, increasing O_2 free radicals and change in pH could be bottleneck for the researchers to use the O_2 sources in biomedical applications. Encapsulation of the O_2 source in the carrier materials could effectively reduce burst release of the O_2 and enhance controllable O_2 release at the target location, resulting in elimination of potential toxicity. Using carrier materials with tuneable degradation rates, or stimuli-responsive/smart materials, which can release O_2 when triggered by environmental changes such as temperature or pH are some of the strategies that can be applied to control/prolong O_2 release. Although, the release of O_2 can be quantified with different characterisation methods *in vitro,* one of the challenges is to measure the released O_2 *in vivo*. Coupling sensors with O_2-generating systems to continuously monitor the release of the O_2 and byproducts can be one of the future directions that can be persued to monitor *in vivo* safety of O_2-generating materials [69]. Furthermore, non-invasive imaging and computational modelling along with machine learning techniques may also provide comprehensive information about the release kinetics of the O_2 *in vivo*. Altogether, the development of new class of O_2 releasing biomaterials, and O_2 release monitoring methods will open the doors to utilise O_2-generating materials for application in future tissue engineering and for other medical indications such as the treatment of myocardial infarction and chronic wounds.

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