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Chitosan-*graft*-Pomegranate Extract Hydrogel: A Dual-Functional Pad for Antibacterial and Antioxidant Enhancement for Shelf Life Extension in Food Packaging

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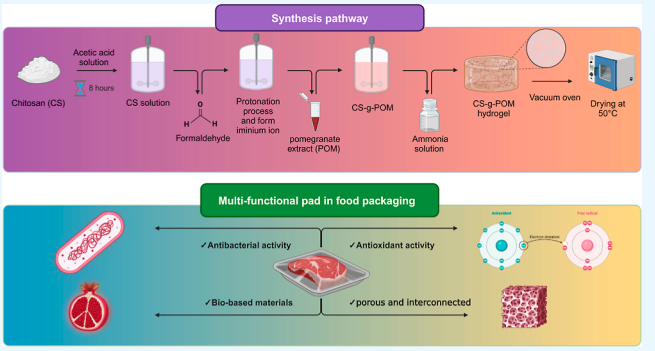
ABSTRACT: In the preservation of water-rich foods, especially meat, spoilage can occur due to bacterial contamination and oxidative degradation. This study aimed to determine the structural characteristics and synthesis of the chitosan-*graft*-pomegranate extract (CS-*g*-POM) hydrogel using a cost-effective and biobased method to develop antibacterial and antioxidant pads. Pomegranate extract is used to fabricate covalently and noncovalently interconnected chitosan hydrogel networks. Several characterization methods, such as Fourier transform infrared spectroscopy, thermogravimetric analysis, field emission scanning electron microscopy, atomic force microscopy, and rheological analysis, confirmed the successful design of the produced hydrogel. The freeze-dried CS-*g*-POM hydrogel had a swelling ratio of 373%, 614%, and 508.5% at pH 5, 7.4, and 10, respectively. FE-SEM images showed the porous hydrogel, which confirmed the cross-linking process. The addition of pomegranate extract increased the phenolic content, resulting in a DPPH radical scavenging activity of 50.20%. The hydrogel also had good antibacterial properties, with the inhibition zones against *Escherichia coli* and *Staphylococcus aureus* measuring 18 ± 1 mm and 12 ± 1 mm. The CS-*g*-POM hydrogel plays a crucial role in moisture absorption in water-rich foods such as meat due to its high degree of swelling and could be used as an antibacterial absorption pad.



KEYWORDS: *hydrogel, chitosan, active food packaging, antibacterial pad, antioxidant enhancement, pomegranate extract*

1. INTRODUCTION

Extending the shelf life of fresh products and enhancing their quality is a challenging subject in the field of market economy and health policy.[1] Fresh foods, including meat, fish, and poultry, naturally deteriorate due to high moisture content within a few days after being packed.[2] Therefore, in order to avoid the growth of microorganisms that can cause rotting, it is crucial to regulate the moisture content of the packaging. Absorbent sachets and pads that contain moisture absorbers have been recently used in packages. Traditionally, cellulosic pads have been utilized for the purpose of absorbing liquids at the bottom of trays in packages containing chicken, fresh meat, and fish. However, complete absorption of moisture is not possible with this approach, which may lead to food rotting.[3] One of the main objectives of this research is to develop versatile sachets or pads that possess antioxidant and antibacterial qualities as well as the ability to reduce water activity.

Hydrogels are 3D networks composed of hydrophilic polymers that can absorb significant amounts of water, often several times their dry weight, with varying capacities depending on their composition, making them suitable carriers for bioactive components.[4,5] Although hydrogels and other polymer-based materials have found broad applications in food packaging, they do have some limitations, such as biodegradability issues, possible toxicity of cross-linking agents, and inadequate mechanical strength. Moreover, in maintaining fresh perishable commodities for a more extended period, both antibacterial and antioxidant properties are fundamental, but most traditional packaging materials provide only one. It is possible to form hydrogels from synthetic or natural polymers.[6] However, natural polymers are more appropriate to be employed in food packaging due to their degradability and safety.[7] Proteins such as soy, collagen, gelatin, and fish proteins, along with

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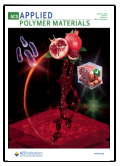

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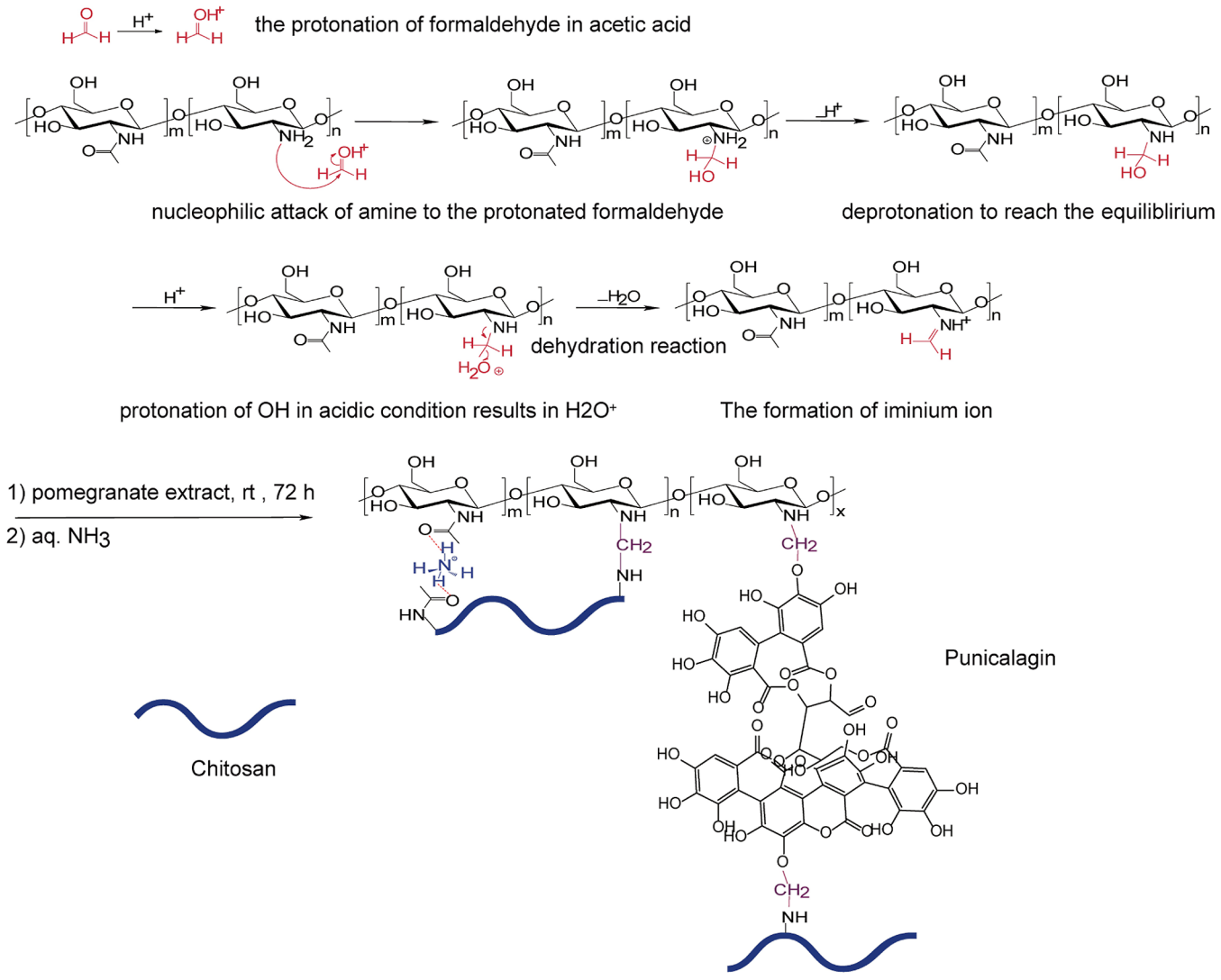

Figure 1. Synthesis pathway of the CS-*g*-POM hydrogel.

polysaccharides like cellulose, starch, gums, and chitosan, can be used as natural polymers to create a hydrogel system.[8] Chitosan (CS) is a naturally occurring polysaccharide with a positive charge that may dissolve in acidic liquids. It is derived from chitin by a process called deacetylation. Chitin biomass is the second most prevalent polysaccharide in nature, following cellulose.[9,10] CS has gained extensive utilization in many applications owing to its nontoxic nature, low cost, biocompatibility, capacity to break down naturally, and antibacterial activity.[11−13] CS-based hydrogels have been widely investigated in the field of tissue engineering and drug delivery systems.[14,15] Recently, there have been efforts to develop chitosan-based hydrogels for food packaging applications due to their significant potential and properties.[16]

By incorporating bioactive materials into the packaging film, the shelf life and safety of food can be increased.[17−19] Prior research has demonstrated the capacity of phenolic compounds, a type of natural antioxidant, to effectively postpone rotting.[20,21] Recent investigations have demonstrated that pomegranate peel extract (PPE) has significant antibacterial properties against foodborne microorganisms.[22,23] Pomegranate (*Punica granatum* L.) is among the oldest fruits that are abundant in most subtropical and tropical countries. Therefore, it was found that pomegranate peel, as a byproduct of home uses and breweries, contains the largest level of antioxidants.[24,25] PPE includes polyphenolic compounds that exhibit considerable levels of antimicrobial and antioxidant activities.[26,27] Biopolymer-based films and hydrogels have been utilized for food packaging so that humidity inside a package can be controlled.[28−30] Chitosan/dialdehyde guar gum hydrogels loaded with PPE exhibited both antioxidant and antimicrobial activities, making them suitable for use as antibacterial pads.[28] Elsewhere, *P. granatum* L. peel powder was added to a fish gelatin film-forming solution to create an active packaging film.[29] The cross-linkers used in many commercial hydrogels are often toxic or not cost-effective.[31] Therefore, it is important to develop and use environmentally friendly and biobased cross-linkers. Antimicrobial and antioxidant properties are prominent in polyphenolic compounds contained in PPE. Its nontoxicity, biodegradability, and biocompatibility make it a promising cross-linker.

By grafting chitosan with pomegranate extract, the goal is to create food packaging solutions that are sustainable, effective at extending the shelf life of food products, and capable of improving food safety. The originality of the chitosan-*graft*-pomegranate extract (CS-*g*-POM) hydrogel lies in its composition and structure. In this regard, the CS-*g*-POM

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hydrogel has been designed to overcome the setbacks of hydrogels intended for food packaging by combining the intrinsic antibacterial properties of chitosan with the effective antioxidant properties of pomegranate extract. Such a hydrogel will benefit food safety and preservation by mechanisms that prevent bacterial growth while stopping oxidation degradation. This paper presents a breakthrough approach to the synthesis of eco-friendly and biobased hydrogels as sustainable food packaging materials, where covalent and noncovalent interactions result in high stability and the enhancement of functional properties.

The formation of the hydrogel occurs by a unique reaction that involves the covalent interaction of CS and formaldehyde as well as formaldehyde with pomegranate extract. The covalent bonds in the hydrogel matrix contribute to its stability and durability. In addition, CS and the ammonium ion create hydrogen bonds, which enhance the structure through noncovalent physical interactions.[32] The demand for the CS-*g*-POM hydrogel stems from the necessity to address bacterial growth and oxidative degradation in water-rich food such as meat.[28] Food deterioration is primarily caused by bacterial infection and oxidation. The antibacterial efficacy of CS, in conjunction with the antioxidant potential of pomegranate extract, effectively tackles these concerns.[33] CS is highly acknowledged for its antibacterial capabilities as it may efficiently inhibit the growth and proliferation of bacteria.[33] Pomegranate extract, abundant in polyphenols, possesses antioxidant activity that can scavenge free radicals and prevent the oxidative degradation of food.[28] This ensures that the hydrogel can effectively serve as a protective barrier against bacterial contamination and oxidative damage.

To the best of our knowledge, there is no published report on the development of CS-*g*-POM hydrogels for the application of antibacterial moisture-absorbent pads. The objective of this study was to explore the formation of hydrogels by POM and CS, as well as to analyze their swelling properties under varying pH conditions. In order to ascertain the interactions among the components, ^{1}H NMR, Fourier transform infrared (FTIR) spectroscopy, and thermogravimetric analysis (TGA) measurements were conducted. The morphology of the freeze-dried CS hydrogel cross-linked with POM was investigated using field emission scanning electron microscopy (FE-SEM) and atomic force microscopy (AFM) analyses. Subsequently, the synthesized hydrogel was tested for its antibacterial activity, ability to scavenge DPPH free radicals, and total phenolic content (TPC). The CS-*g*-POM hydrogel can effectively prolong the shelf life of food by absorbing moisture and generating desired antioxidant and antibacterial properties.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Materials. Chitosan with medium molecular weight (MW = 200,000 and 98% deacetylation) was obtained from Sigma-Aldrich. Pomegranate fleshy seed coat extract was obtained from a local market, where the extract contains pomegranate juice concentrated from pomegranate fruit by reducing the pressure and without cooking. Glacial acetic acid, formaldehyde (37% in water), ammonia (25% in water), hydrochloric acid (37%), and ethanol (99.8%) were obtained from Neutron, Iran. Gallic acid, Folin−Ciocalteu, sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric chloride ethylenediamine tetraacetic acid (EDTA), ascorbate, hydrogen peroxide (H_2O_2), thiobarbituric acid, and hydrochloric acid (HCl) were acquired from Merck Chemical Co. (Darmstadt, Germany).

2.2. Synthesis Method. The preparation of the CS-*g*-POM hydrogel was based on a modified version of the procedure initially established by Farasati Far et al.[9] In this process, 1200 mg of CS was dissolved in 1% acetic acid solution (v/v) and mixed thoroughly for 8 h using a magnetic stirrer to achieve a transparent chitosan solution. Subsequently, 5 mL of a 37% formaldehyde solution was gradually added to initiate the protonation process and form an iminium ion for 2 h as illustrated in Figure 1. For cross-linking, 500 mg of pomegranate extract was incorporated into the chitosan solution. This mixture was then stirred for another 48 h using a magnetic stirrer. To induce gelation, a 25% ammonia solution was introduced, resulting in immediate hydrogel formation. To purify the hydrogel, it was washed with ethanol and methanol to eliminate any unbound or unreacted substances. This was followed by vacuum filtration and drying in a vacuum oven at 50 °C.

2.3. Characterization of Hydrogels. ^{1}H NMR spectra were measured on an AVANCE Bruker DRX-500 spectrometer. Deuterium (D_2O) was used as the solvent, and the solvent signal was used for internal calibration (D_2O): δ (1H) = 4.79 ppm. Without further sample preparation, FTIR spectra were obtained in transmission mode on an FTIR spectrometer (Agilent Cary 630). Field emission scanning electron microscopy (FE-SEM, Topcon) was used to study the superficial morphologies of the CS-*g*-POM. The samples were coated with a thin layer (16 nm) of gold film (Bal-tec). TGA was performed on a calorimeter (STA 449F3, Netzsch). This calorimetric experiment was performed at a heating rate of 10 °C/min under a nitrogen atmosphere over the temperature range of 25−300 °C. Contact mode AFM was performed using a NanoScope E system (Digital Instruments).

2.4. Swelling Behavior of Hydrogels. The CS-*g*-POM hydrogel was prepared into 250 mg tablets to determine the swelling rate and the percentage of water absorbed by the hydrogel. To assess the swelling behavior of the CS-*g*-POM hydrogel at various pH values (pH 5 and 7.4), each CS-*g*-POM hydrogel was stored in a buffer solution for 24 h at room temperature. This was followed by the removal of all excess water not incorporated into the hydrogel structure and weighing of each interval of excess water. Dry hydrogels were immersed in a buffer solution and weighed using eq 1, where W_s and W_d are the swollen and dried weights, respectively (n = 3 for each data point). The equilibrium water content (EWC %) was calculated using eq 2.

$$\text{Swelling ratio(SR)} = (W_s - W_d)/W_d \quad (1)$$

$$\text{equilibrium water content(EWC \%)} = (W_s - W_d)/W_s \times 100 \quad (2)$$

2.5. Total Phenolic Assay and Antioxidant Activity of Hydrogels. *2.5.1. Total Phenolic Content.* The Folin−Ciocalteu method was used to calculate the TPC (milligrams of gallic acid equivalents, GAE/g) with slight modification.[28] To obtain the supernatant, sample parts were immersed in distilled water for 24 h at 25 °C, followed by centrifugation for 10 min at 3500 rpm. After preparation of the hydrogel extract solution, 2.5 mL of Folin−Ciocalteu reagent and 2 mL of sodium carbonate (7.5%) were added to it. A further 30 min of incubation at 25 °C was performed on the samples. A spectrophotometer was used to measure the absorbance at a wavelength of 765 nm (Spectrum SP-UV500DB). The standard for this experiment was gallic acid.

2.5.2. DPPH Free Radical Scavenging Activity. Based on the previously reported method, the radical scavenging activity of the hydrogel was calculated using DPPH, with a slight modification.[28] In brief, 1.5 mL of hydrogel extract was vortexed with 2 mL of ethanolic DPPH solution (0.1 mM) and the solution was incubated in darkness for 45 min at 25 °C. Using a UV−visible spectrophotometer (Spectrum SPUV500DB), absorbance was determined at 517 nm. In order to measure the DPPH radical activity, the following equation was used, where each sample was tested in triplicate.

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{DPPH}}} \times 100 \quad (3)$$

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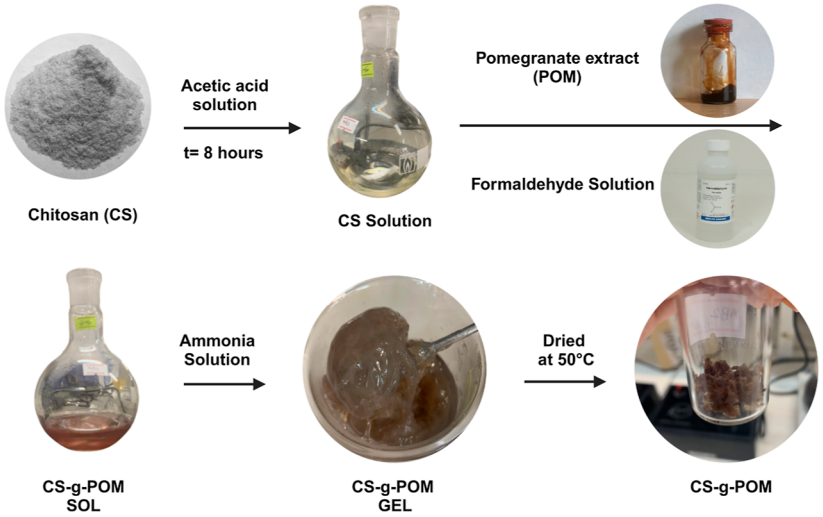

Figure 2. Schematic illustration of the formation of the CS-*g*-POM hydrogel.

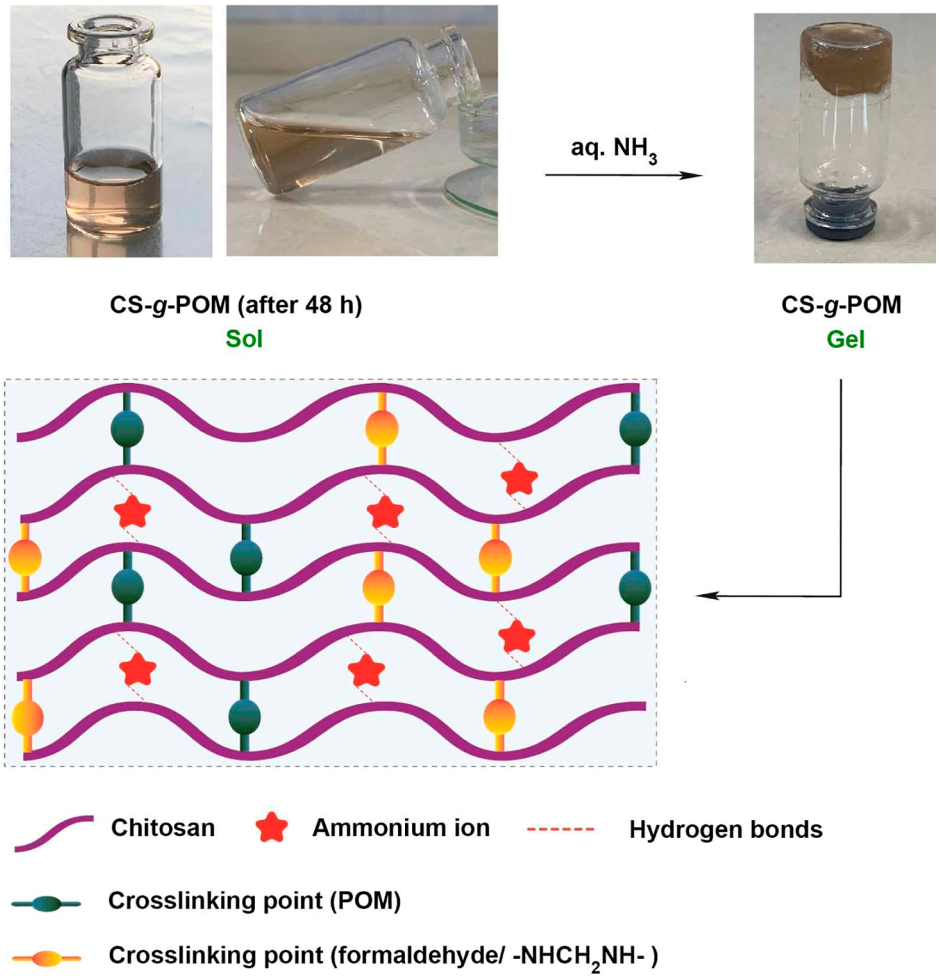

Figure 3. Design strategy for the CS-*g*-POM hydrogel: gelling of the CS-*g*-POM hydrogel with an NH_4^+ binder after 48 h.

2.5.3. Antioxidant Characterization by the Deoxyribose Assay. Briefly, the reaction mixture was prepared to contain 100 μM $FeCl_3$, 100 μM EDTA, 2 μM ascorbate, and 8 mM H_2O_2 in phosphate buffer, at pH 7.4. To complete the Fenton reaction, 2.8 mM deoxyribose was added to the reaction mixture, and it was put into a water bath that had been warmed to 37 °C for an hour. This was followed by incubation and the addition of 1 mL of 2.8% thiobarbituric acid and 1 mL of 10% TCA into the reaction mixture. The solution was then transferred to a boiling water bath for 15 min to develop a dark-pink chromogen. The activity of the formed dark-pink chromogen was then determined at 532 nm for the hydroxyl radical-scavenging activity.

2.6. Assessment of Antibacterial Activity. The antibacterial activity of the prepared hydrogel against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) was evaluated

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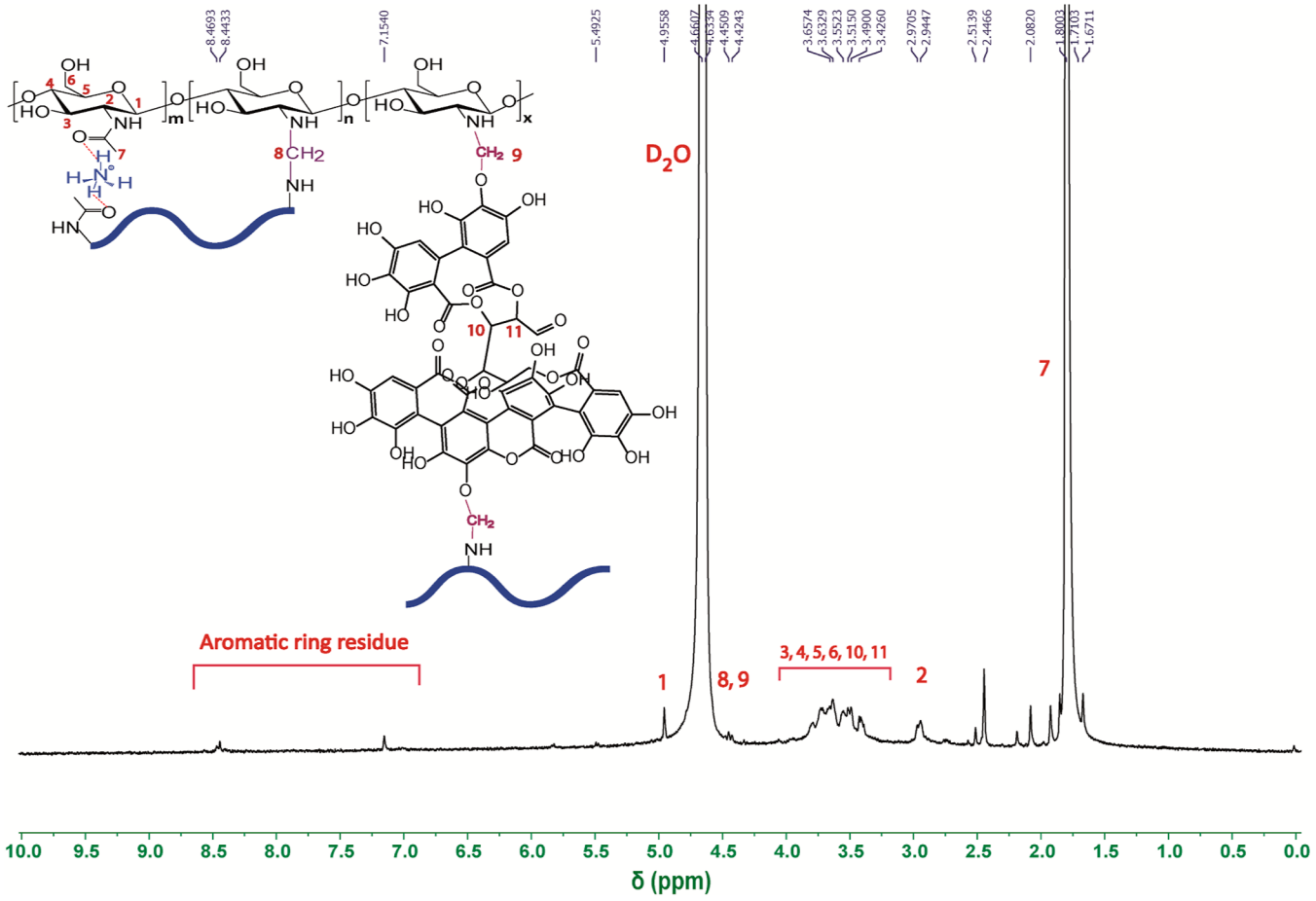

Figure 4. ^{1}HNMR spectrum of CS-*g*-POM (500 MHz, D_2O, 25 °C).

using the agar disk diffusion method (ADDM).[34] Fresh cultures of the target bacteria were prepared, and a bacterial suspension with a concentration of 0.5 McFarland (equivalent to 1.5×10^8 CFU/mL) was created using a diluent solution, such as a physiological serum. Dilution of the bacterial suspension was carried out to achieve the desired cell count. The bacterial suspension was then applied to the surface of the agar plates using a sterile cotton swab. In the ADDM, sterile discs were utilized. 15 mL of hydrogel samples were loaded onto the surface of the discs. The plates were incubated in a temperature-controlled incubator at 30 ± 5 °C for 48 h. After incubation, the diameter of the growth inhibition zone surrounding the discs was measured using a ruler or vernier caliper and recorded in millimeters. Each bacterial strain was analyzed three times, and the mean values were calculated.

2.7. Statistical Analysis. The statistical analysis was conducted using GraphPad Prism software version 10. The means and standard deviations (SD) of experimental results are reported. The one-way ANOVA test with the post-hoc Tukey method was utilized to compare conditions. A value of $p < 0.05$ was considered statistically significant by SPSS (v.17). Statistical significance is indicated on the graphs with asterisks referring to: * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, and **** for $p \leq 0.0001$. GraphPad Prism was also utilized for graphical representation, generating bar charts and line graphs to visually convey trends and group differences.

3. RESULTS AND DISCUSSION

3.1. Synthesis Mechanism of CS-Based Hydrogels. Chitosan-based hydrogels can be fabricated by using various methods, including physical and chemical cross-linking techniques. Physical cross-linking relies on noncovalent interactions between polymer chains, such as hydrogen bonds and hydrophobic interactions, to form a three-dimensional network, whereas chemical cross-linking involves the formation of covalent bonds between polymer chains to provide structural stability.[35] In this study, chemical bonds (covalent bonds between CS and formaldehyde and formaldehyde with POM) and noncovalent physical interactions (hydrogen bonds between CS and ammonium ion) are the mechanisms by which the CS-*g*-POM hydrogel was formed. The synthesis mechanism of the hydrogel is illustrated in Figure 1.

The schematic illustration and design strategy for the CS-*g*-POM hydrogel are shown in Figures 2 and 3. The process begins by preparing a chitosan solution, which undergoes protonation under acidic conditions. This is achieved by exposing the chitosan's amine groups to an acid, typically acetic acid, resulting in the amine groups gaining a positive charge. Next, formaldehyde is introduced to the protonated chitosan. The formaldehyde reacts with the protonated amine groups to form iminium ions, a type of stabilized cation. This reaction involves the loss of a water molecule and is commonly referred to as the formation of a Schiff base. Subsequently, pomegranate extract, which contains reactive phenolic compounds, is added to the mixture. A significant metabolite of ellagitannins, ellagic acid, also exhibits strong antioxidant properties. Researchers have indicated that ellagitannins, particularly punicalagin, are primarily responsible for many of the health benefits associated with pomegranate extract.[36] Punicalagin (2,3-hexahydroxydiphenoyl-gallagyl-D-glucose) is the major ellagitannin isolated from pomegranate fruit and is the main component of pomegranate polyphenols and is found abundantly in pomegranate. Punicalagin exhibits strong antioxidative, anti-inflammatory, and antineoplastic properties.[37] The quantity of punicalagin aril pomegranate juice ranges from 4100 to 233,000 μg/L, and in whole fruit, pomegranate juice ranges from 166,000 to 800,000 μg/L.[38] The phenolic compounds from the pomegranate extract react with the iminium ions on the chitosan backbone. This step is crucial as it leads to the cross-linking of the chitosan chains, effectively transforming the solution into a gel-like substance. The cross-linking reaction is facilitated over a period of time

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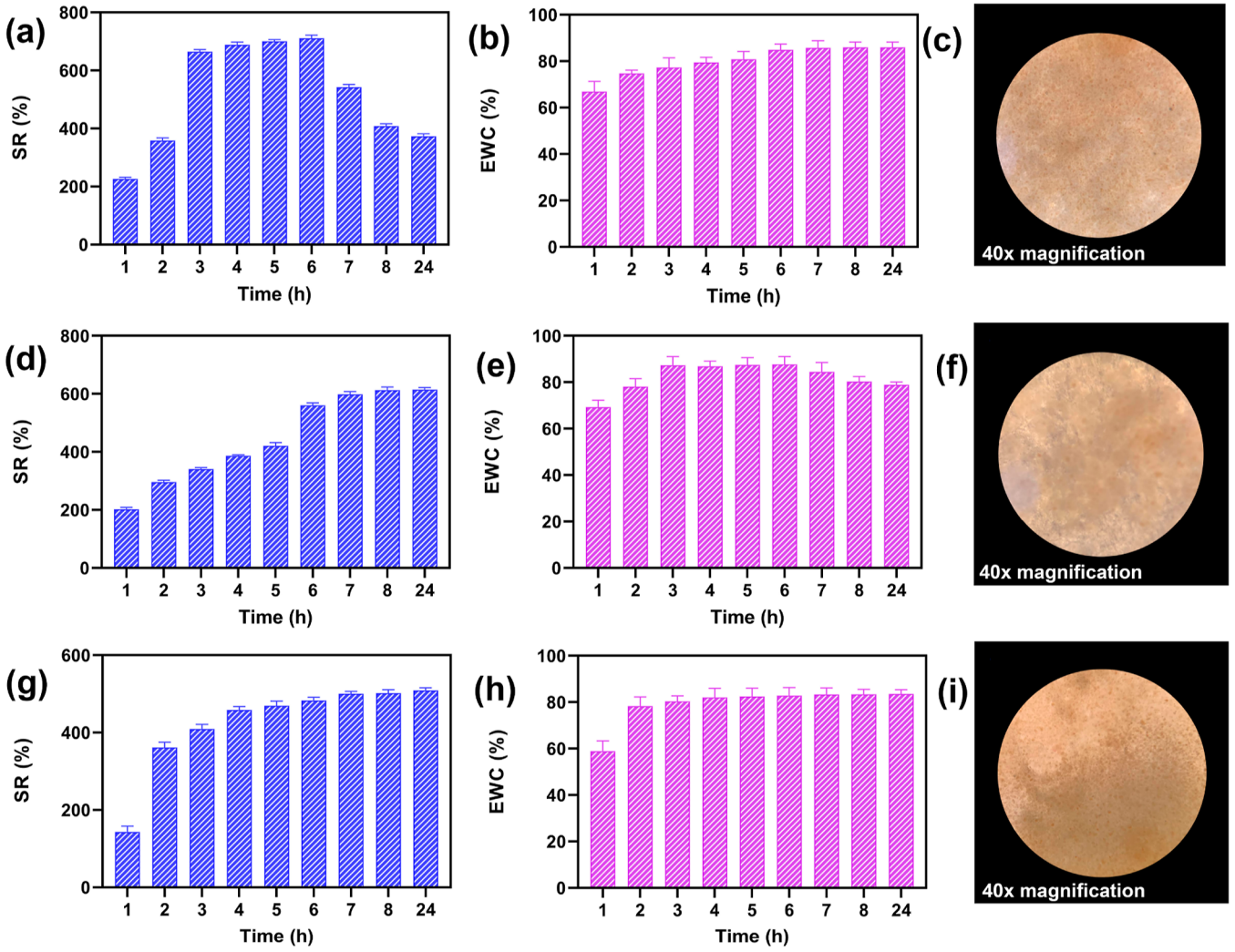

Figure 5. Swelling ratios (%) of the CS-*g*-POM hydrogel at (a) pH = 5, (d) pH = 7.4, and (g) pH = 10, equilibrium water contents (%) of the CS-*g*-POM hydrogel at (b) pH = 5, (e) pH = 7.4, and (h) pH = 10, and optical microscopic images of swelling of the CS-*g*-POM hydrogel at (c) pH = 5, (f) pH = 7.4, and (i) pH = 10.

at room temperature. Finally, to complete the hydrogel formation, an ammonia solution is added. The ammonia likely acts to neutralize the excess hydrogen ions and stabilize the hydrogel matrix. The resulting material is a hydrogel with pomegranate extract molecules grafted to a chitosan structure. This hydrogel has the potential to possess the biological properties of both chitosan and pomegranate extract, which include antibacterial and antioxidant activities. It should be noted that hydrogen bonds, electrostatic attraction, and hydrophobic interactions play a significant role in the gelation process, and these mechanisms may have played a role in gelation. The CS-*g*-POM hydrogel demonstrates potential applications in various fields due to its antibacterial and antioxidant properties. One notable application is in the preservation of water-rich foods, particularly meat, where bacterial contamination and oxidative degradation can lead to spoilage. The antibacterial property of the hydrogel inhibits the growth of pathogenic bacteria, extending the shelf life of the food product. Additionally, the antioxidant activity of pomegranate extract contributes to scavenging free radicals, thereby preventing oxidative degradation and preserving the quality and nutritional value of the food.[28]

3.2. Characterization of Hydrogels. *3.2.1. ^1H NMR.* The ^1H NMR spectrum of CS-*g*-POM in D_2O is shown in Figure 4. The peak at 1.80 ppm can be attributed to the acetyl group ($-CH_3$) while that at 2.94 ppm represents the H2 proton of CS. The chemical shifts between 3.42 and 3.65 ppm are assigned to H3−H6 and protons of CS. The signal at 4.42−4.45 ppm can be attributed to the methylene groups formed by cross-linking POM and CS (H8, H9). The hydrogen bonded to the anomeric carbon (H1) gives rise to the signal in the range of 4.95 ppm. In ^{1}HNMR results, peaks at 7.15−8.4 ppm (denoted with a bracket) in the spectra correspond to aromatic protons on POM. The two signals at 3.4−3.6 ppm assigned to H10 and H11, respectively, have a meta coupling to each other (4JHH = 1.8 Hz). Peaks around 6.5 to 8.5 ppm indicate the presence of aromatic protons, which are characteristic of the phenolic compounds present in pomegranate extract. The presence of observed peaks corresponding to different functional groups (peaks corresponding to the aromatic and aliphatic protons of the pomegranate extract) provides strong evidence supporting the successful grafting of POM into CS. These peaks confirm the occurrence of the grafting process and validate its effectiveness.

3.2.2. Swelling Properties. Moisture absorption and swelling properties are important aspects of preventing food decay or deterioration. Hydrogels must possess swelling properties that are suitable for applications in food packaging.[39] After immersing the CS-*g*-POM hydrogel at pH 7.4 and buffers at pH 5 and pH 10, the swelling ratio [SR (%)] and equilibrium water content [EWC (%)] were determined. As illustrated in Figure 5a,d,g, pH levels have an influence on the swelling ratios of the CS-*g*-POM hydrogel over time. This study shows that the CS-*g*-POM hydrogel is formed by covalently cross-linked mechanisms and physical interactions in the presence of POM. The cross-linking process results in a structure that contains a large number of pores that are filled with water. At different pH levels,

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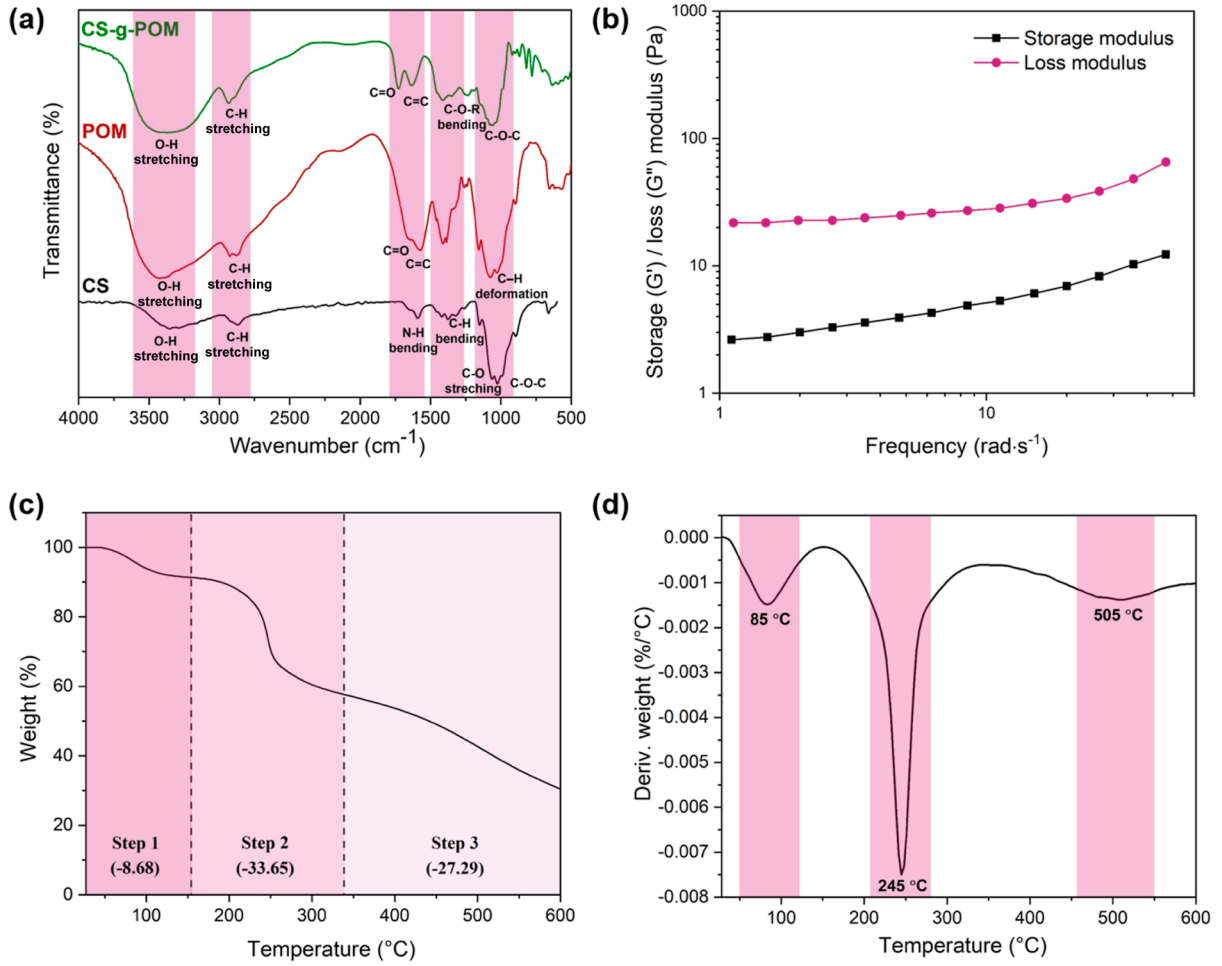

Figure 6. (a) FTIR spectra of CS, POM, and CS-*g*-POM hydrogel, (b) frequency sweeps of CS-*g*-POM hydrogel, (c) TGA, and (d) DTG analyses of CS-*g*-POM hydrogel.

hydrogels typically change their swelling ratio. CS-*g*-POM hydrogel swelling ratios were measured at various pH values. The swelling ratios of 373%, 614%, and 508.5% were obtained for the CS-*g*-POM hydrogel at pH = 5, 7.4, and 10, respectively. Protonation and deprotonation of functional groups can affect swelling ratios as pH changes.[40] The primary amine in chitosan has a pK_a of 6.5, so when the pH of the solution is less than 6.5, the amines in the polymer chain become positively charged (NH_3^+).[41] The equilibrium water contents of 85.99%, 78.85%, and 83.56% were obtained for the CS-*g*-POM hydrogel at pH 5, 7.4, and pH = 10, respectively. The swelling behavior at pH 10 is represented by the repulsion forces between the negatively charged oxygen ions (O^-) and carboxylate ions (COO^-) since at this pH, these groups can be deprotonated.

In an acidic environment, unreacted amino groups of CS become protonated, forming ammonium ions, particularly noticable at pH = 5 and below. Hydrogels would be attached to ammonium ions through ionic bonds. Thus, in an acidic buffer, the hydrogel weight increased in comparison to a neutral buffer. The amino group of CS, however, occurs as $-NH_2$ at high pH, leading to a lower EWC than it shows at lower pH (Figure 5b,e,h). Significant amounts of moisture can be absorbed and retained by the CS-*g*-POM hydrogel due to its high swelling capacity and high equilibrium water content. This is essential for managing the humidity within the packaging and stopping the growth of microorganisms and spoiling. In a study conducted by Omrani et al., the swelling ratio (SR) of a CS-diethyl malonate hydrogel was investigated, where the hydrogel exhibited a swelling ratio of around 600% at pH = 7.4 and a swelling ratio of 900% at pH = 6.8 after 24 h, highlighting its capacity to absorb water and expand under these specific pH conditions.[42] Additionally, Farasati Far et al. conducted a study on the equilibrium water content of CS-*g*-glycerol and carboxymethyl chitosan-*g*-glycerol hydrogels. The CS-*g*-glycerol hydrogel exhibited EWC values of 98% and 97% at pH 5 and 7.4, respectively, after 24 h. In contrast, the carboxymethyl chitosan-*g*-glycerol hydrogel displayed EWC values of 59% and 75% at pH 5 and pH 7.4, respectively, after 24 h.[9] The optical microscopy images in Figure 5 are useful in observing and comprehending the structural and morphological features of the CS-*g*-POM hydrogel at various pH levels. These images allow observing the direct structure of the hydrogel that is crucial for assessing swelling and the functionality of the material. The microstructures of the hydrogel in buffer solutions are shown in Figure 5b,e,h.

3.2.3. FTIR Characterization of the CS-g-POM Hydrogel. Figure 6a shows the FTIR analysis of the CS, POM, and CS-*g*-POM hydrogel. The CS spectrum shows specific absorption at 975 cm^{-1} (C−O stretching), 1023 cm^{-1} (C−O−C stretching), 1070 cm^{-1}, and 1150 cm^{-1} (C−O stretching).[43] Furthermore, amides I and III showed absorption peaks at 1641 and 1364 cm^{-1}, respectively. A broad peak at 3350 cm^{-1} was also related to amino group NH symmetric vibrations.[44] For the POM, the maximum absorption band at 1014 cm^{-1} corresponds to the aromatic ring C−H deformation, and specific absorptions at 1650 and 1440 cm^{-1} are assigned to the stretching vibration of the C═C aromatic ring. A maximum absorption band with a wavelength of 1249 cm^{-1} can be attributed to pyran ring stretching, which is typical of flavonoid compounds. Some bands can be observed between 1300 and 1380 cm^{-1}, which can be attributed to phenols' C−O angular deformations. In the FTIR spectrum of the CS-*g*-POM, the appearance of a shift in the O−H stretching region may indicate hydrogen bonding or interactions between CS and POM. A change in

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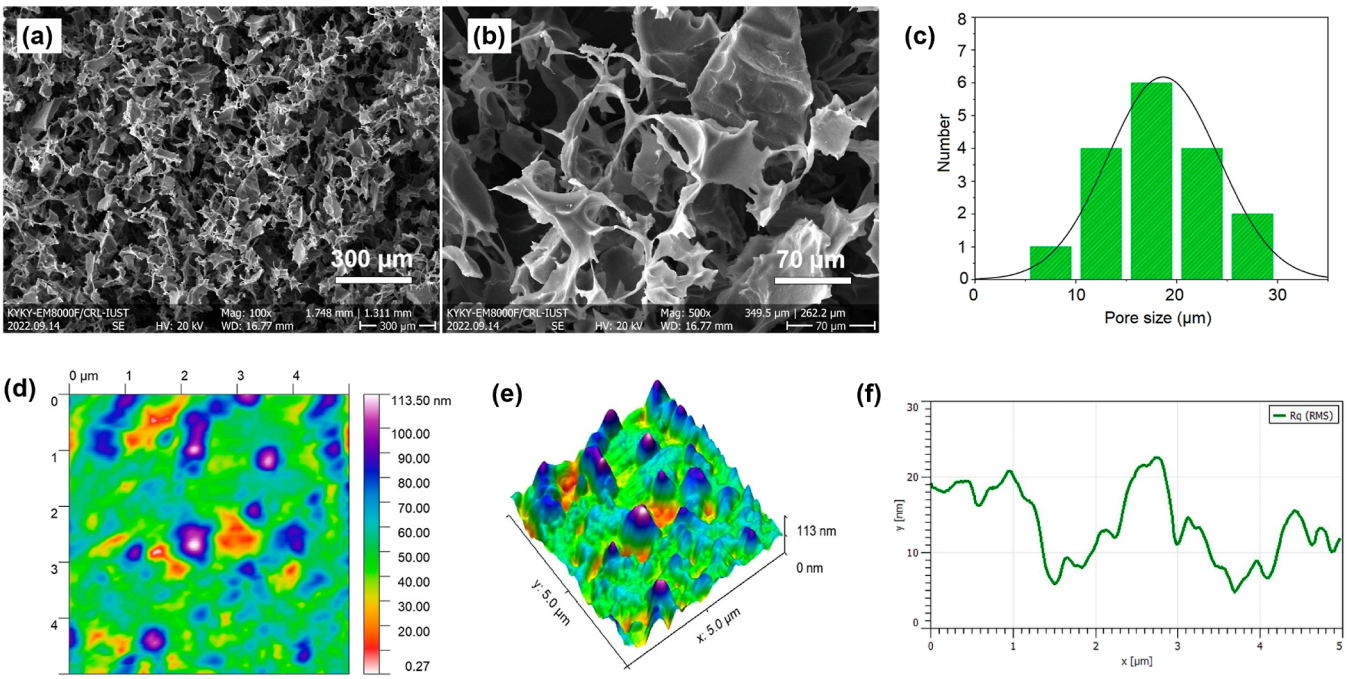

Figure 7. (a,b) FE-SEM images of the cross-sectional CS-*g*-POM hydrogel at two different magnifications, (c) pore size of the CS-*g*-POM hydrogel, (d,e) 2D and 3D AFM images of the CS-*g*-POM hydrogel, and (f) the quantitative roughness of the surface of the CS-*g*-POM hydrogel.

the intensity or position of the amide I band could suggest an interaction between the carbonyl group of CS and the phenolic hydroxyl groups of POM. Moreover, the presence of additional bands in the fingerprint region (below 1500 cm^{-1}) might be attributed to new C−O−C, C−N, or C−C linkages, suggesting successful grafting of POM onto CS. The peak at around 1402 cm^{-1} could be attributed to the cross-linking of POM to CS (C−O−R). Since CS-*g*-POM exhibited characteristics of C═C stretching from aromatic rings, the POM has been successfully grafted into the CS. In addition, the FTIR spectrum of the CS-*g*-POM indicates the presence of aromatic rings with ortho substitution, indicating the presence of aromatic rings with ortho substitution in the CS-*g*-POM hydrogel.

3.2.4. Rheological Properties of the CS-g-POM Hydrogel. A key factor in the CS-*g*-POM hydrogel's efficiency as a food packaging material is its viscoelastic and swelling characteristics. Because of its viscoelasticity, the hydrogel is able to maintain its structural integrity and conform to the shape of the food product, offering reliable protection. Furthermore, the hydrogel can absorb excess moisture from foods high in water, thanks to its swelling ability, which lowers the chance of microbial growth and spoiling. The CS-*g*-POM hydrogel extends the shelf life and safety of packaged foods by regulating moisture levels and releasing bioactive compounds in a controlled manner. Hydrogels are viscoelastic solids characterized by their softness, which enables them to retain and release energy. The assessment of the viscoelastic properties of a hydrogel in dynamic rheology is conducted via loss (G'') and storage (G') moduli. These moduli correspond to the stress in phase and out of phase, respectively, induced by an applied oscillatory deformation. As shown in Figure 6b, the CS-*g*-POM hydrogel exhibited exceptional mechanical strength. The finding that the storage modulus (G') of the CS-*g*-POM hydrogel consistently exceeded the loss modulus (G'') suggests that this hydrogel possesses the characteristics of elastic soft solids. In the "elastic" area, a substance may undergo deformation under stress and then regain its initial state after the tension is released, provided the storage modulus is larger than the loss modulus. This phenomenon could be more readily understood if it is considered how the unsubstituted groups of POM and CS and water produce intermolecular hydrogen bonds that promote the strength of the gel network. These links may establish connections between nearby polymer strands.[45]

3.2.5. TGA and DTG Analyses of the CS-g-POM Hydrogel. TGA was performed on the CS-*g*-POM hydrogel to determine its thermal stability. The CS-*g*-POM hydrogel exhibited a three-step weight loss mechanism. The first weight loss can be attributed to the physical absorption of water by the hydrogel at 28 to 154 °C. Based on the results, the decomposition of organic components and the POM grafted into the polymer structure, along with its interaction with the polymer matrix, may be responsible for the second step, which accounts for approximately 33.65% of the weight loss. The high degree of bonding produced and the high degree of cross-linking are responsible for the high thermal stability of the hydrogel at this stage. Thermal decomposition and gradual degradation of the polymer structure are responsible for 27.29% of the weight loss between 338 and 600 °C (Figure 6c). Furthermore, differential thermogravimetry (DTG) results were investigated for the CS-*g*-POM hydrogel (Figure 6d). CS-*g*-POM was also confirmed to be degrading in three steps between 27 and 600 °C. The physical absorption of water is responsible for the maximum band at around 90 °C. For the CS-*g*-POM hydrogel, the maximum degradation at around 245 °C can be due to the degradation of the grafted pomegranate extract in the hydrogel. Also, the broad degradation peak between 400 and 550 °C may be responsible for the chitosan backbone or the remaining organic structure of the hydrogel.

3.2.6. Morphological Properties. The morphology of the freeze-dried CS hydrogel cross-linked with POM is illustrated in Figure 7a,b. FE-SEM images show high porosities and a cross-linked 3D structure of the hydrogel, which is similar to previous findings.[4,28] The CS-*g*-POM hydrogel may be interconnected due to cross-linked networks. Furthermore, after freeze-drying, hydrogels become porous due to the water that is absorbed by their hydrophilic sites. Due to its porous structure, the hydrogel can swell, therefore reducing resistance resulting from water flow. These porous structures are responsible for the hydrogel's high water absorption capacity, allowing it to swell upon contact with food materials. The CS-*g*-POM hydrogel had a porous structure with pores of 18.64 μm diameter (Figure 7c). A study by Sekine et al. explored the freeze cross-linking method to obtain physically cross-linked hydrogels from carboxymethyl cellulose nanofibers and citric acid. The SEM images of different types of freeze-dried hydrogels showed a porous structure with a pore diameter in the range of 22−80 μm.[46] Butylina et al. conducted a comprehensive study on

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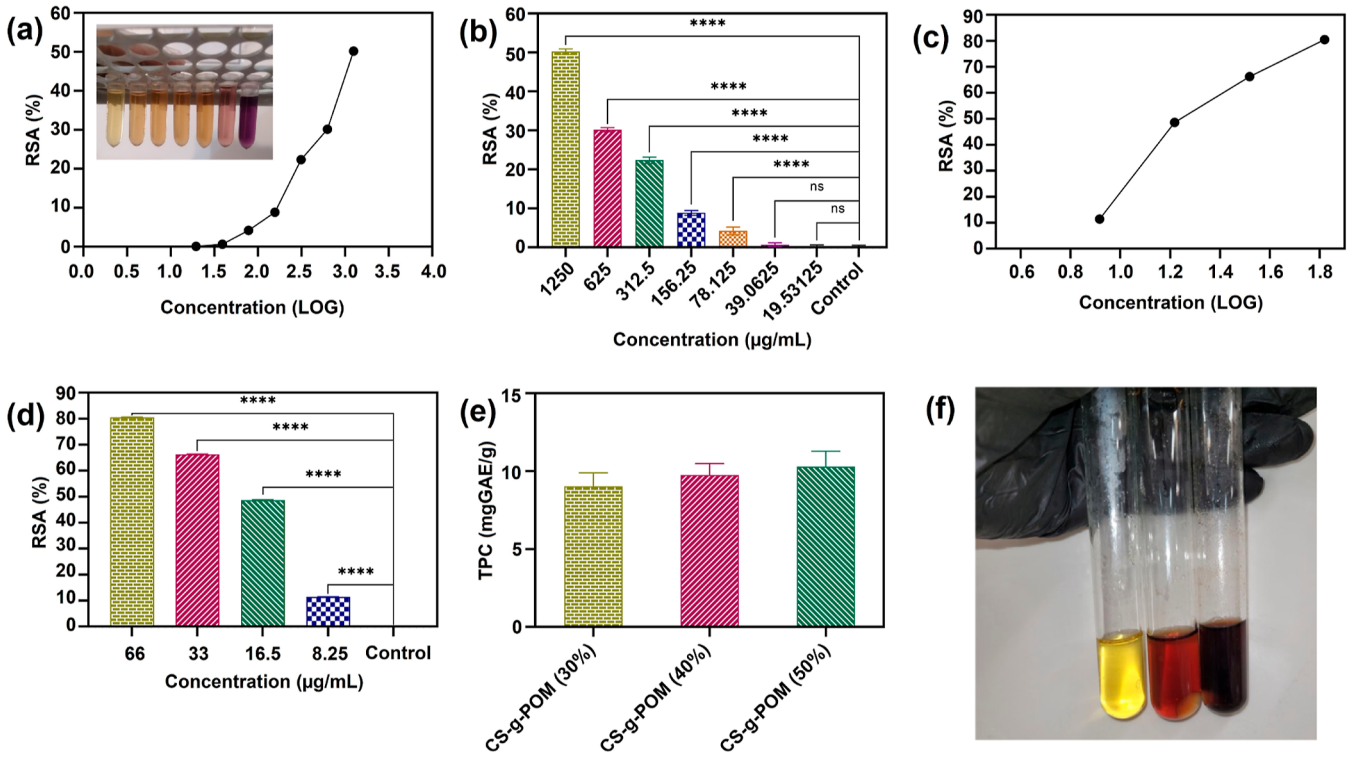

Figure 8. Percentage DPPH radical scavenging activity (% RSA) of (a,b) CS-*g*-POM hydrogel, (c,d) VitC as a control sample, (e) the total phenolic content (TPC) of the CS-*g*-POM hydrogel, and (f) photograph from the deoxyribose assay.

hydrogels composed of poly(vinyl alcohol) and cellulose nanocrystals. They examined how variations in poly(vinyl alcohol) concentration, cellulose nanocrystal addition, the number of freeze−thaw cycles, and the freeze-drying process affected the hydrogels' properties and morphology. The results showed that freeze drying resulted in hydrogels with a highly porous and interconnected structure, which may enhance their potential applications in areas requiring enhanced mechanical strength and bioactivity such as drug delivery and tissue engineering.[47] Figure 7d,e shows two- and three-dimensional AFM images of selected hydrogel samples, where the peaks are both quite large and very small in dimension on the surfaces of hydrogels due to their nodular structure. AFM was also used to measure the quantitative roughness of the surface (Figure 7f). The values of R_{ms} = 18.64 ± 7.68 nm and R_a = 13.84 ± 5.96 nm were obtained, indicating the hydrogel surface was smooth.[48] The results are consistent with those reported in the literature.[49]

3.3. Total Phenolic Assay and Antioxidant Activity of Hydrogels. Figure 8 demonstrates the total phenolic component and DPPH radical scavenging activity of the CS-*g*-POM hydrogel.

The results show that the addition of POM increases the total phenol content of the hydrogel. Polyphenols are powerful antioxidant compounds, and by increasing their number, hydrogels become more capable of inhibiting free radicals by donating electrons or hydrogen atoms.[28] Additionally, the CS-*g*-POM hydrogel displayed an excellent amount of DPPH radical scavenging activity (50.20%) (Figure 8a,b). The data in Figure 8a,b indicate that there is a concentration-dependent relationship in the scavenging ability. The scavenging activity of the CS-*g*-POM hydrogel increased from 30.18% to 50.20% as the concentration increased from 625 to 1250 μg/mL. Figure 8c,d demonstrates the DPPH radical scavenging activity of VitC as a control sample, where a concentration-dependent relationship in the scavenging ability of VitC was observed. As the concentration of VitC rose from 16.5 to 33 μg/mL, the scavenging activity increased from 48.66% to 66.27%. When POM was added in the range of 30−50% compared to the amount of CS as a cross-linking agent, the total phenolic component increased. The CS-*g*-POM hydrogel (50%) had a higher TPC than CS-*g*-POM (30%) and CS-*g*-POM (40%) (Figure 8e).

Through the presence of the polyphenol compounds, gallic acid, hydroxybenzoic acids, hydroxycinnamic acids, tannins from pomegranates—ellagitannins and punicalagin—and anthocyanins, POM has been shown to have potency as an antioxidant. Accordingly, oxidation is responsible for food rancidity and loss of nutritional value in certain foods that contain a high percentage of fat. The hydrogel, through the scavenging of free radicals, avoids the loss of freshness and quality in the food.

The results of previous studies indicate that pomegranate extract could be used in the food industry as a preservative and stabilizing agent, as well as to scavenge free radicals, combat pathogenic bacteria, and prevent atheroma.[28,50] Licciardello et al. proved that the edible coatings using pomegranate peel extract help to preserve the quality of the white shrimp as it gives an antioxidant shield. This corroborates our conclusion that when applied to perishable foods, the antioxidant qualities of the hydrogel can help extend the products' shelf life.[22] The presence of anthocyanin in PPE could also be used as a color change indicator, which provide valuable information on the product's pH as well as the possibility of spoilage.[51] Active food packaging requires antioxidant activity because free radicals are known as a leading cause of meat product spoilage.[52]

3.4. Hydroxyl Radical Scavenging Capacity Assay. One of the most popular methods to estimate hydroxyl radical-scavenging capacity is the "deoxyribose assay". In this assay, a set of reactions occur in which ferric chloride and ethylene diamine tetraacetic acid complexed with ascorbate are converted to iron II-EDTA and oxidized ascorbate when the ascorbate reacts in the presence of hydrogen peroxide. Afterward, the iron II-EDTA reacts itself with H_2O_2 through the Fenton reaction to yield iron III-EDTA, hydroxide ions, and hydroxyl radicals

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

Ultimately, these hydroxyl radicals formed in this reaction will attack deoxyribose, forming fragments. At low pH, with heating in thiobarbituric acid, these fragments react to form a pink chromogen. This chromogen can be quantitated, and the extent of its formation is used to indicate the presence of hydroxyl radicals.

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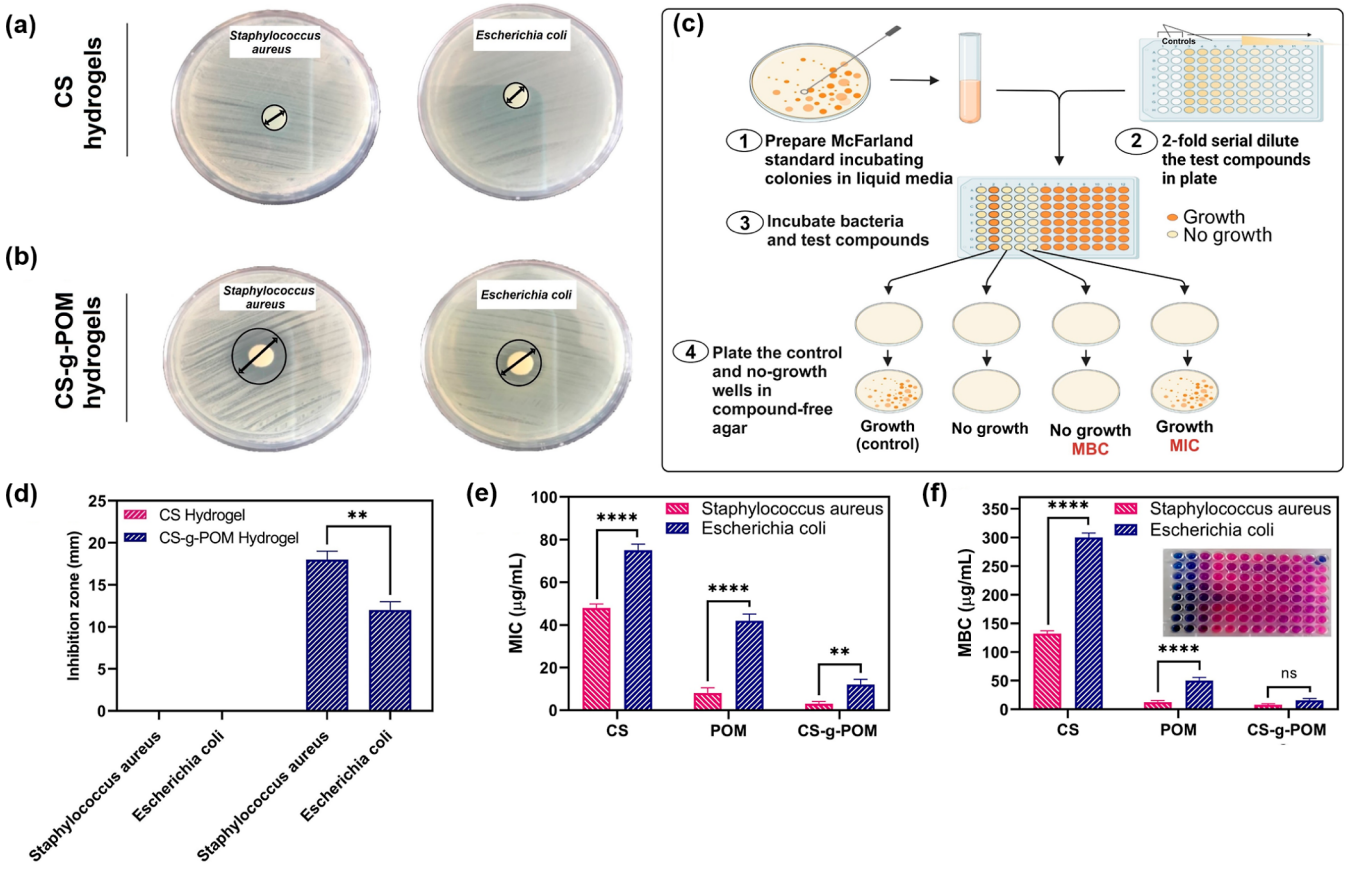

Figure 9. Antibacterial inhibition zone for the CS hydrogel (a) and CS-*g*-POM hydrogel (b) against *S. aureus* (ATCC 12600) and *E. coli* (ATCC 11775) bacteria respectively, schematic illustration of MIC and MBC test created by Biorender.com (c), comparison of the antibacterial inhibition zone for the CS hydrogel and CS-*g*-POM hydrogel (d), and MIC and MBC values of CS, pomegranate extract, and CS-*g*-POM hydrogel, respectively (e,f).

The assays on deoxyribose showed that the CS-*g*-POM hydrogel possesses a relatively high scavenging activity toward hydroxyl radicals (Figure 8f). It may be due to the phenolic compounds from pomegranate extract, punicalagin, ellagic acid, and gallic acid. These compounds either provide the hydrogen atom or electrons to neutralize the hydroxyl radicals and free biological molecules. The first chemical mechanism includes the reaction of phenolic compounds. The hydroxyl groups present in the phenolic compounds are capable of donating a hydrogen atom, like in the case of neutralizing the hydroxyl radicals

$$\mathrm{Ph-OH + HO^{\bullet} \rightarrow Ph-O^{\bullet} + H_2O}$$

By a similar mechanism, the amino groups in chitosan can also scavenge free radicals

$$\mathrm{R-NH_2 + HO^{\bullet} \rightarrow R-NH^{\bullet} + H_2O}$$

These additional tests provide further support for the broad-spectrum antioxidant capacity of the hydrogel and increase its potential as a protective agent in food packaging. This hydroxyl radical scavenging activity provides complementary information to the previously reported DPPH radical scavenging activity, thus enabling the complete evaluation of the antioxidant properties exhibited by the hydrogel.

These findings were based on and agreed with earlier studies on similar materials, which used corresponding methods to determine the antioxidant capacity of assorted materials.[53,54] Therefore, our results indicate that the CS-*g*-POM hydrogel can neutralize different types of reactive oxygen species, offering higher protection against oxidative damage in food products.

3.5. Antibacterial Activity. The agar disk diffusion method was used to assess the antibacterial activity of CS and the CS-*g*-POM hydrogel. The CS hydrogel exhibited no antibacterial activity against *E. coli* (ATCC 11775) and *S. aureus* bacteria (Figure 9a), whereas the CS-*g*-POM hydrogel, due to the presence of POM, had considerable antibacterial activity with more effectiveness against *S. aureus* compared to *E. coli* (Figure 9b). While a low dose of chitosan hydrogel was used to be compelling enough for antibacterial correspondence, several factors could influence the antibacterial outcome of chitosan as a hydrogel. In this regard, deacetylation and molecular weight will directly influence how well chitosan performs. Lower degrees of deacetylation or higher molecular weights imply fewer cationic sites and, therefore, less interaction with the bacterial cell membrane.

Also, during its immobilization into the hydrogel, chitosan could interact with its network structure or water content, modifying its antibacterial activity. The hydrogel matrix may decrease the mobility and accessibility of cationic groups for effective interaction of chitosan with bacterial cells. In addition, chitosan with an aminopolysaccharide backbone, which negatively poses antibacterial activity, implies a very high pH sensitivity. At pH below 6, where chitosan is positively charged, it has a higher degradation efficiency. Nonetheless, in a neutral or slightly basic environment, which is generally the case within the body, chitosan solubility is decreased and hence has low antibacterial efficiency.

The most critical factor is microbial contamination, which may be considered the limitation of this work due to the antibacterial properties of the CS-*g*-POM hydrogel. In the present work, extended zones of inhibition for *E. coli* of 12 ± 1 mm and for *S. aureus* of 18 ± 1 mm were observed, proving that it has a potent antibacterial effect. This can be explained by the synergistic interaction of chitosan and pomegranate extract, both possessing antimicrobial activity.[55,56] In this regard, Maroufi et al. showed how hydrogels containing peal extract of pomegranate demonstrated high antibacterial activity against food-borne pathogens, confirming our findings and showing just how

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Table 1. Hydrogels Are Derived from Natural Polymers for Food Packaging Purposes

polymer	food system	key benefits and applications	refs
chitosan, Melissa officinalis essences, and pomegranate	cream cheese	minimizing water loss while enhancing antioxidant effects, with films serving as indicators for bacterial spoilage in cream cheeses	4,61
gelatin, chitosan, and 3-phenyllactic acid	chicken meat	enhances antibacterial properties and extends shelf life	62
citric acid cross-linked chitosan/poly(vinyl alcohol) films	cherry	presents a promising option for materials used in food packaging and preservation	63
carboxylated nanocellulose	chicken breast	serves as a smart indicator of freshness by changing colors	4
nanocellulose/nisin hybrid film	ham	offers antimicrobial protection, excellent light transmission, and strong barrier qualities against moisture and air	64
cellulose films	various applications	noted for superior optical, thermal, and structural characteristics	65
carboxymethylcellulose	banana	absorbs humidity and ethylene, enhancing shelf life	66
bacterial cellulose, guar gum	blueberries	increases biodegradability and provides better barrier and hydrophobic properties	67
gelatin and modified cellulose	various foods	strengthens tensile and thermal stability while offering clear UV barrier properties	68
chitosan	chicken meat	creates a highly flexible and biodegradable film	69

excellent the enhancing capabilities of pomegranate extract are for its antibacterial properties with polyphenolic compounds (Figure 9d).[28] This observation can be related to POMs that are rich in polyphenols. POM has been shown to inhibit the growth of Gram-positive and Gram-negative bacteria in other studies.[23,57]

There may be an association between this capability and the presence of polyphenols such as punicalagin, ellagic acid, and gallic acid.[29,58] Abu-Dalo et al. reported the synthesis of a TiO_2 NPs/pristine pomegranate peel extract nanocomposite, where a maximum zone of inhibition of 22 mm was observed against *S. aureus*.[58] The study conducted by Dahham et al. provided evidence that pomegranate demonstrated the strongest antimicrobial activity against *S. aureus*.[59] Hanani et al. conducted a study revealing that the antimicrobial efficacy of pomegranate peel was more pronounced against Gram-positive bacteria, specifically *S. aureus* and L. monocytes, compared to Gram-negative bacteria such as *E. coli*.[29] The findings align with the research by Hamed et al., who observed that phenolic compounds exhibit greater efficacy against Gram-positive organisms.[60]

Figure 9c shows a schematic illustration of the MIC and MBC tests. Figure 9e displays the minimum inhibitory concentration (MIC) values, which are the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC results show that CS exhibited a higher inhibitory effect on *S. aureus* (48 μg/mL) compared to *E. coli* (75 μg/mL). POM also demonstrates antibacterial activity, but it is more effective against *S. aureus* than against *E. coli*, as indicated by the lower MIC value for *S. aureus* (8 μg/mL). The CS-*g*-POM hydrogel showed an enhanced effect against both bacteria, with significantly lower MIC values for both strains, indicating that the grafting of POM onto CS amplifies the antibacterial properties of the hydrogel. Figure 9f presents the minimum bactericidal concentration (MBC) values, which indicate the lowest concentration of antibacterial agent required to kill a particular bacterium. Similar to the MIC values, the MBC for CS was lower for *S. aureus* than for *E. coli*, implying that it is more effective at killing *S. aureus*. POM, on its own, has a higher MBC for both bacterial strains than that of CS. However, the CS-*g*-POM hydrogel exhibited a marked decrease in MBC values for both bacterial strains, with 8 μg/mL against *S. aureus* and 16 μg/mL against *E. coli*, indicating that the combination of CS and POM results in a significantly more potent bactericidal hydrogel.[45] These results suggest that the combination of chitosan and pomegranate extract in the form of the CS-*g*-POM hydrogel offers a synergistic effect that enhances both the inhibitory and bactericidal properties of the individual components. The significant reduction in both MIC and MBC values for CS-*g*-POM compared to CS and POM alone indicates that this hydrogel could be an effective material for antibacterial applications, such as the development of antibacterial pads. Table 1 shows the key benefits and applications of hydrogels derived from natural polymers for food packaging purposes.

3.6. Effect of the CS-*g*-POM Hydrogel on Preventing Food Decay or Deterioration. The CS-*g*-POM hydrogel pad possesses antibacterial activity, antioxidant activity, and moisture absorption capabilities, which can be considered a multifunctional foodstuff preservation method. This multifaceted functionality is especially valuable for preserving the shelf life of water-intrusive products such as meat, poultry, and fish products. The first use of these hydrogels is to reduce microbial growth. The hydrogel thereby inhibits the growth of pathogenic bacteria and, consequently, the risk of foodborne infections and spoiling. Previous research has shown that antimicrobial agents in food packaging materials are highly effective in decreasing microbial presence and increasing shelf life.[70]

The CS-*g*-POM hydrogel also offers significant protection against oxidative damage, marking it as a key benefit. Antioxidant properties help prevent food from undergoing oxidation reactions that lead to rancidity and nutrient degradation. Components used in the packing of food items have also been confirmed to extend the oxidation period, thus helping in the conservation of food nutritional value and taste.[71] Another crucial feature in stopping food deterioration is moisture control. Because of the hydrogel's capacity to absorb excess moisture, there is no buildup of water, which could otherwise foster the growth of microbes. In addition to lowering the risk of microbial deterioration, efficient moisture control in packaging preserves the texture and appearance of food.[72]

4. CONCLUSIONS

The objective of this study was to develop a nontoxic and biobased method to synthesize chitosan-*g*-pomegranate POM extract hydrogel. Chitosan hydrogel networks contain covalent and noncovalent bond connections made from pomegranate extract. FTIR, ^{1}HNMR, FE-SEM, AFM, and TGA measurements were used to determine the interactions among hydrogel components. For the freeze-dried powder of the CS-*g*-POM hydrogel, swelling ratios of 614%, 373%, and 508.5% and equilibrium water contents of 78.85%, 85.99%, and 83.56% were obtained for the CS-*g*-POM hydrogel at pH = 7.4, pH = 5, and pH = 10, respectively. The FTIR analysis confirmed the formation of a covalent bond between the amide groups in CS and the functional groups in POM. The incorporation of POM into the CS-*g*-POM hydrogel at varying proportions (30% to 50%) relative to the amount of CS used for cross-linking resulted in an elevation in the overall phenolic content. Additionally, the CS-g-POM hydrogel exhibited impressive DPPH radical scavenging activity, with a substantial percentage of 50.20%. This hydrogel also demonstrated significant antibacterial properties in addition to its antioxidant activity. The agar disk diffusion method was used to determine the inhibition zones of the CS-g-POM hydrogel against *E. coli* and *S. aureus*, which were 12 ± 1 and 18 ± 1 mm, respectively. The results indicate that the CS-*g*-POM hydrogel is an interesting material with potential applications as antibacterial pads that can inhibit food spoilage

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and degradation by targeting crucial elements, such as microbial contamination, oxidative degradation, and moisture imbalance. Its integration into food packaging materials offers a promising solution for extending the shelf life of perishable food products, ensuring their safety and quality over extended storage periods.

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B.F.F. conceptualized the study, collected data, generated the figures, and contributed to manuscript writing and editing. M.J. conceptualized the study, generated the figures, and contributed to manuscript writing and editing. L.J. and P.T. contributed to data collection and manuscript writing. F.Z. contributed to data collection, figure generation, and manuscript writing. Y.N.E. conceptualized the study, generated the figures, and contributed to manuscript writing, reviewing, and editing. All authors reviewed and approved the final version of the manuscript.

Notes

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ABBREVIATIONS

ADDM	agar disk diffusion method
AFM	atomic force microscopy
EWC	equilibrium water content
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
PPE	pomegranate peel extract
SD	standard deviations
SR	swelling ratio
TPC	total phenolic content

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