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Synthesis and characterization of gelatin-based quaternizable hydrogels

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Abstract

Gelatin, a water-soluble natural polymer with excellent film-forming properties, exhibits high biocompatibility due to its amino acid composition, which closely resembles that of proteins. However, gelatin has poor mechanical properties and poses a risk of bacterial infection when films are composed solely of gelatin. In this study, gelatin-based crosslinked polymers with quaternary amine groups, exhibiting potential antibacterial properties, were developed. To achieve this, gelatin was first modified with methacrylate via an isocyanate-amine reaction, and the resulting material was characterized using Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR). Hydrogels were successfully synthesized by photopolymerization of gelatin methacryloyl, with a tertiary amine-containing monomer and a four-arm crosslinker, and characterized using FTIR, scanning electron microscope (SEM), and thermogravimetric analysis (TGA). Subsequently, the hydrogel was prepared as a film on a glass surface, and quaternization of the tertiary amine groups imparted polycationic properties to the hydrogel coatings, enabling further investigation into their antibacterial potential.

Keywords: *Gelatin, photopolymerization, hydrogels, quaternization, isocyanate-amine.*

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Jelatin bazlı kuaternize edilebilir hidrojellerin sentezi ve karakterizasyonu

Öz

Üstün film oluşturma özelliklerine sahip, suda çözünebilen doğal bir polimer olan jelatin, proteinlere benzer amino asit bileşimi sayesinde yüksek biyouyumluluk sergilemektedir. Ancak jelatinin mekanik özellikleri zayıftır ve yalnızca jelatinden oluşan filmler, bakteriyel enfeksiyon riski taşımaktadır. Bu çalışmada, potansiyel antibakteriyel özelliklere sahip kuaterner amin grupları içeren jelatin bazlı çapraz bağlı polimerlerin geliştirilmesi amaçlanmıştır. Bunun için önce jelatin izosiyanat-amin reaksiyonu kullanarak metakrilat ile modifiye edilmiş ve ardından fourier dönüşümlü kızılötesi spektroskopisi (FTIR) ve nükleer manyetik rezonans spektroskopisi HNMR ile yapısı aydınlatılmıştır. Jelatin metakriloilin tersiyer amin içeren monomer ve dört kollu bir çapraz bağlayıcı monomer ile fotopolimerleşmesi sonucu hidrojeller elde edilmiş ve FTIR, taramalı elektron mikroskobu (SEM) ve Termogravimetrik Analiz (TGA) teknikleri ile karakterize edilmiştir. Ardından, hidrojeller cam yüzeyde bir film olarak da hazırlanmış ve üçüncül amin gruplarının kuaternizasyonu ile hidrojel kaplamalara polikatyonik özellikler kazandırılarak, antibakteriyel potansiyel barındıran yeni malzemeler elde edilmiştir.

Anahtar kelimeler: *Jelatin, fotopolimerleşme, hidrojeller, kuaternizasyon, izosiyanat-amin.*

1. Introduction

Gelatin, a water-soluble collagen, is a biopolymer with a typical repeating amino acid sequence of Gly-X-Y, where X and Y are mostly proline and hydroxyproline. It is a linear polymer with excellent film-forming properties [1]. Due to its biocompatibility, gelatin has attracted significant attention in the biomedical field [2,3]. However, its poor mechanical properties limit its practical applications [4,5]. Additionally, gelatin serves as a good nitrogen source for microorganisms [6], which may lead to bacterial infection in gelatin-based films [7]. As a result, efforts have been made to improve its mechanical properties and reduce its susceptibility to microbial attraction.

One widely used approach to enhance the mechanical properties of gelatin is crosslinking [8]. The utilization of bifunctional crosslinkers has been demonstrated to produce crosslinked gelatin hydrogels [9-11]. Alternatively, methacrylation facilitates further crosslinking through photopolymerization, thermal gelation, or redox reactions [12,13]. Methacrylation allows control of the crosslinking density [14] and incorporation of different monomers [15] to tune the chemical and physical properties of the resulting product. Initial studies demonstrated gelatin methacrylation using methacrylic anhydride through amino groups present on the lysine side chains of gelatin. Notably, the resulting hydrogels retained their ability to support cell behavior due to the preservation of Arg-Gly-Asp (RGD) peptides and matrix metalloproteinase (MMP)-responsive sites, ensuring that gelatin's biocompatibility and degradation properties remain unaffected [16]. More recent studies have explored alternative methods for gelatin methacrylation, such as using epoxy-amine [17,18], N-hydroxy succinimide-amine [19], or isocyanate-amine reactions

instead of methacrylic anhydride-based esterification [20]. Among those approaches, the rapid isocyanate-amine reaction [21-23], makes 2-Isocyanatoethyl methacrylate an excellent choice for reducing reaction times in gelatin modification.

Polycationic structures are known to bind negatively charged macromolecules on microbial cell surfaces, thereby achieving bacterial stasis [24]. Poly(2-(dimethylaminoethyl)methacrylate) (pDMAEMA) is a mucoadhesive polymer that becomes cationic upon quaternization with an alkylating agent [25,26]. pDMAEMA has been used to modify various surfaces, including polystyrene [27], polypropylene [28], polyethylene/polypropylene nonwoven fabric [29], filter paper [30,31], glass [32,33], and nanoparticles [34], serving as an antimicrobial coating to inhibit the growth of *Escherichia coli* and/or *Bacillus subtilis* after quaternization in acidic media or in the presence of methyl iodide.

The modification of gelatin with methacrylate, followed by crosslinking in the presence of DMAEMA (which contains post-quaternizable groups) and pentaerythritol tetraacrylate (PETA), a crosslinker with four reactive units, has the potential to enhance the mechanical properties and impart antibacterial activity after quaternization. This hydrogel can be considered a novel material due to the combination of these three components, which provide biocompatible, quaternizable, and highly crosslinked properties. In this work, we modified gelatin with methacrylate using an isocyanate-amine reaction, a method that is not widely reported in the literature, and introduced a cost-effective precipitation method for purification. Methacrylate conjugation was confirmed via FTIR and HNMR, and the degree of substitution was calculated. By employing a tertiary amine-containing monomer and a four-arm crosslinker along with methacrylated gelatin, we obtained bulk hydrogels, which were characterized using FTIR, SEM, and TGA. Additionally, one of the hydrogels was covalently coated onto a glass surface. The tertiary amine groups were then quaternized to impart polycationic properties to the hydrogel coatings, providing antibacterial potential to the gelatin-based film (Fig. 1).

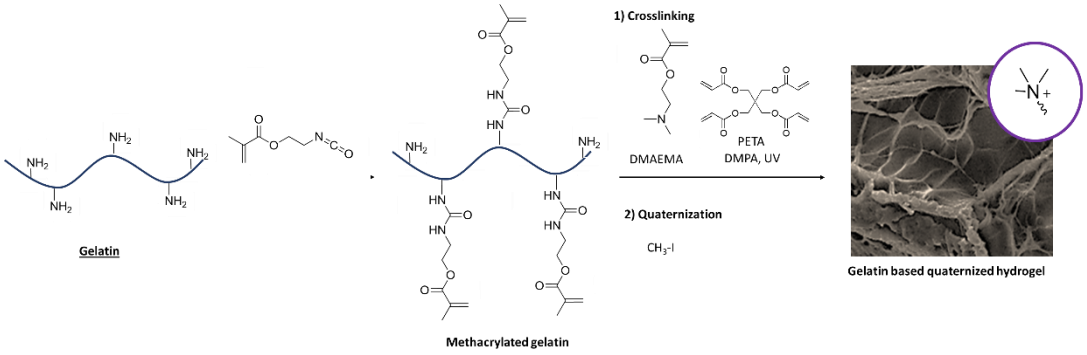

Figure 1. General scheme of the study.

2. Material and method

2.1. Material

Gelatine (Pharmaceutical limed bone Gelatine, 200 Bloom, 8 mesh) was purchased from PB Gelatins, GmbH. 2-isocyanatoethyl methacrylate (ICM) (98%) pentaerythritol tetraacrylate (PETA), 2,2-dimethoxy-2-phenylacetophenone (DMPA) were purchased

from Chem Cruz. 2-(dimethylamino ethyl)methacrylate was obtained from Merck. Dimethyl sulfoxide (DMSO), ethyl acetate (EtOAC), hexane, Tetrahydrofuran (THF) and toluene were purchased from Merck, and used as received. Methacrylate modified glass surface was prepared as our previous study [35].

2.2. Instrumentation

Polymerization was performed under UVP Blak-Ray™ B-100A UV Lamps365 nm ultraviolet light (365 nm, 100 watts). FTIR spectra were measured using a Perkin Elmer Paragon 100 ATR-IR instrument in the range of 4000–650 cm^{-1} was used for chemical characterization of gelatin derivatives and hydrogels. ^{1}H NMR spectra were recorded with a Varian 500 MHz instrument at room temperature. The thermal analysis of the hydrogels was investigated by Mettler Toledo model TGA/851 equipped with Mettler Toledo Star^{e} software at a heating rate of 10°C/min under N_2 gas flow. The morphology of the hydrogels was observed using a Jeol Neoscope JCM-5000 scanning electron microscopy.

2.3. Method

Synthesis of pendant methacrylated gelatin: GelMA-1 and GelMA-2 with different degree of substitution was prepared. For GelMA-1, Gelatin (1g) was taken in a 25 mL beaker dissolved in 10 mL of DMSO. The mixture was stirred in an oil bath at 50°C. After complete dissolution of gelatin, 3 mL of solution was taken reaction vessel. 15 µL of 2-Isocyanatoethyl methacrylate (ICM) was placed in the vessel and mixed for half an hour. Purification was performed by precipitating the product in ethyl acetate and keep stirring in ethyl acetate for 1 week by regular changing the solvent. Removal of unbound components were confirmed by TLC analysis in 50:50 ethyl acetate: hexane. GelMA-1 was dried under vacuum, FTIR and HNMR analyses were performed. Same protocol was used for GelMA-2 by using 30 µL of ICM. HNMR analysis in D_2O was used to calculate the degree of substitutions (DS) of free amine groups in gelatin samples. For the quantification of the DS, phenylalanine peaks between 7.0-7.5 ppm was used as they depict the concentration of amines [19,36]. Then, the lysine methylene signals between 2.9-3.0 ppm were integrated to obtain the areas [A (lysine methylene of non-modified gelatin) and A (lysine methylene of GelMA)]. The DS of the GelMA-1 and GelMA-2 was calculated as equation (1):

$$DS(\%) = 1 - \left(\frac{A(lysine\ methylene\ of\ GELMA))}{A(lysine\ methylene\ of\ gelatin)}\right) x100 \qquad (1)$$

Synthesis of bulk hydrogels: PETA (210 µL, 0.71 mmol) was dissolved in 1.5 mL of DMSO. DMPA (29 mg, 0.11 mmol) was dissolved in 500 µL of DMSO. Those stock solutions were kept in freezer. 200 µL of 50 mg/mL GelMA-1 was taken in a vial. DMAEMA (5.37 µL, 36.3 mmol), 22 µL of PETA (0.47M), and 20 µL of DMPA (0.22 M) were added. After mixing well, the solution was transferred to a 5 mL syringe and placed under UV light for 40 minutes. After curing, the gel was washed with THF and distilled water. It was then left to swell in 10 mL of distilled water for 2 hours in a Falcon tube, placed in the freezer, and dried in a lyophilizer.

Hydrogel coating on glass surfaces and quaternization: 20 µL of GelMA-2 hydrogel precursor, prepared in the same way as the bulk gelatin solution, was placed on a methacrylate-modified glass slide and covered with a coverslip. The sample was exposed to UV irradiation for 40 minutes. After gelation, the surfaces were washed with DMSO and THF, dried with a stream of argon gas, and placed under vacuum. The same procedure

was followed for the coating of GEL-30. For quaternization of the hydrogels on the glass surface, 60 µL of a methyl iodide mixture (50:50, v/v) was applied to the HG-2 film. It was covered with a coverslip, left in a beaker at room temperature for 18 hours, then washed with THF and dried under vacuum.

Water uptake study of hydrogels: The dry hydrogel was placed in a beaker containing 10 mL of distilled water at room temperature. At predetermined intervals, the hydrogel sample was removed from the beaker, carefully drained of excess water using tissue paper, and then weighed. Water uptake % (Wup) was determined using the weights of the swollen (*Wwet*) and dried (*Wdry*) hydrogel samples according to the equation (2). For each sample, water uptake tests were carried out three times.

$$Wup = \frac{Wwet - Wdry}{Wdry}\ x\ 100 \qquad (2)$$

3. Results and discussion

Gelatin methacryloyl was synthesized via isocyanate-amin reaction and an alternative purification method for the obtained product was proposed. Since DMSO was the only organic solvent that could dissolve both ICM and gelatin, DMSO was used as the medium, although it is not an ideal solvent for isocyanate-based reactions due to its water content. At first gelatin was dissolved in a closed system to reduce the possibility of moisture absorption and then ICM was directly added via a syringe and the mixture was mixed for 30 minutes with a stirrer bar. The reaction mixture was then precipitated in ethyl acetate to remove DMSO and the unreacted monomer, and the ethyl acetate was refreshed three times a day by pouring out the used solvent. The solution phase was checked by TLC by using ethyl acetate: hexane (50:50, v/v) and stained in $KMnO_4$. Washing continued until the unreacted monomer no longer appeared on the TLC plate. Although the purification of gelatin is usually performed using a dialysis membrane, precipitation in ethyl acetate offers a more economical alternative.

Due to high reactivity of isocyanates, the conjugation of ICM to gelatin competes with its conversion into carbamic acid. However, since the isocyanate-amine reaction is also quite fast,[2] we were able to attach a portion of methacrylate via urea bonding. The conjugation of ICM to the gelatin was first confirmed using FTIR spectroscopy. In the spectra of GelMA-1 and GelMA-2, strong peaks at 1631 cm^{-1} and 1527 cm^{-1}, observed in gelatin, correspond to the presence of amide I and amide II, respectively. The observation of these peaks in GelMA-1 and GelMA-2 indicates that no chemical changes occurred in the amide bonds. More importantly, small bands appeared at 1720 cm^{-1} in the spectra of GelMA-1 and GelMA-2, attributed to the incorporation of ester groups into the gelatin molecule during the methacrylation process [37] (Fig. 2). Although this peak is also seen in some methacrylated gelatin studies [38], other research articles attribute the peak in a similar region to hydrogen bonding of urethane moieties, [20, 39, 40]. Therefore, we believe that both ester and hydrogen bonding contribute to this absorption peak, and either or both confirm the conjugation of ICM to gelatin.

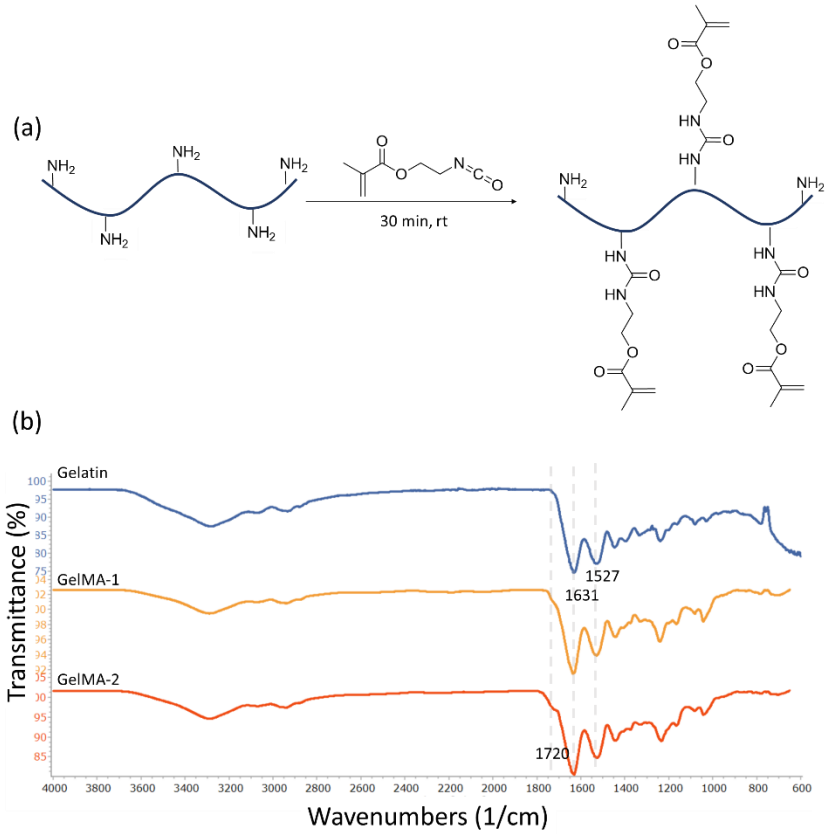

Figure 2. (a) Methacrylation of gelatin with ICM. (b) FTIR spectra of Gelatin, GelMA-1 and GelMA-2.

Further verification of the conjugation of isocyanates to gelatin was performed using ^{1}H NMR in D_2O, comparing GelMA-1 and GelMA-2 with unmodified gelatin. Peaks observed at $\delta = 6.0$ and $\delta = 5.6$ ppm after reaction with isocyanate indicate the conjugation of methacrylate units to gelatin. Additionally, the decrease in the area of the lysine methylene signal at $\delta = 2.9$ ppm in GelMA-1 and GelMA-2 provided further evidence the substitution of ICM onto gelatin. The spectra were normalized to the phenylalanine signal (δ = 7.0-7.5 ppm) as the aromatic ring does not react with ICM (Fig. 3). Using the decrease in the integrated area of lysine methylene signals, the degree of substitution (DS), which is defined as the ratio of functionalized to originally available amino groups, was calculated. According to Equation (1) in the methods section, the degree of modification of gelatin was calculated to be 11.8% for GelMA-1 and 22.9% for GelMA-2. The higher integrated area of methacrylate-related signals in GelMA-2 compared to GelMA-1 further demonstrated that DS can be controlled by adjusting the amount of ICM used. These results confirm the successful modification of gelatin and highlight the ability to precisely control the degree of substitution through the amount of ICM added.

Methacrylated gelatins were crosslinked under UV light using the tertiary amine-containing monomer DMAEMA and the 4-arm crosslinker PETA for increasing crosslinking density. The reaction was carried out in the presence of the photoinitiator DMPA at room temperature (Fig. 4). A 5 mL syringe, cut to serve as a mold, was used for gelation. After 30 minutes of UV irradiation, the hydrogels were removed from the mold and washed thoroughly with THF and water. The gel precursor was transparent and

clear prior to gelation. Following UV irradiation, the resulting hydrogel appeared slightly yellow but remained clear (Fig. 4).

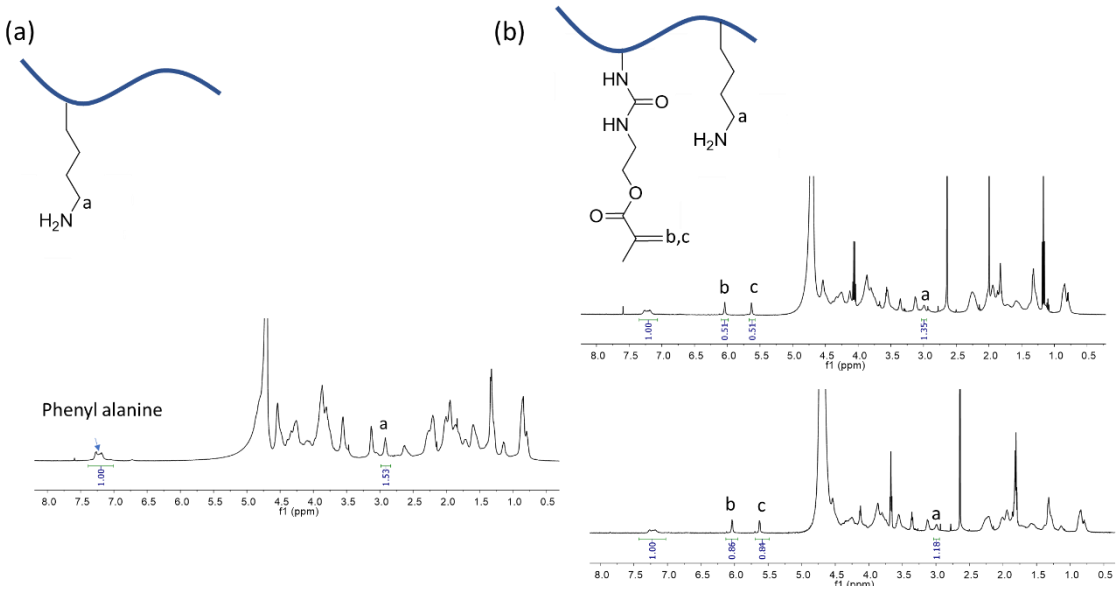

Figure 3. (a) ^{1}HNMR spectrum of bare Gelatin. (b) ^{1}HNMR spectra of GelMA-1 (top) and GelMA-2 (bottom).

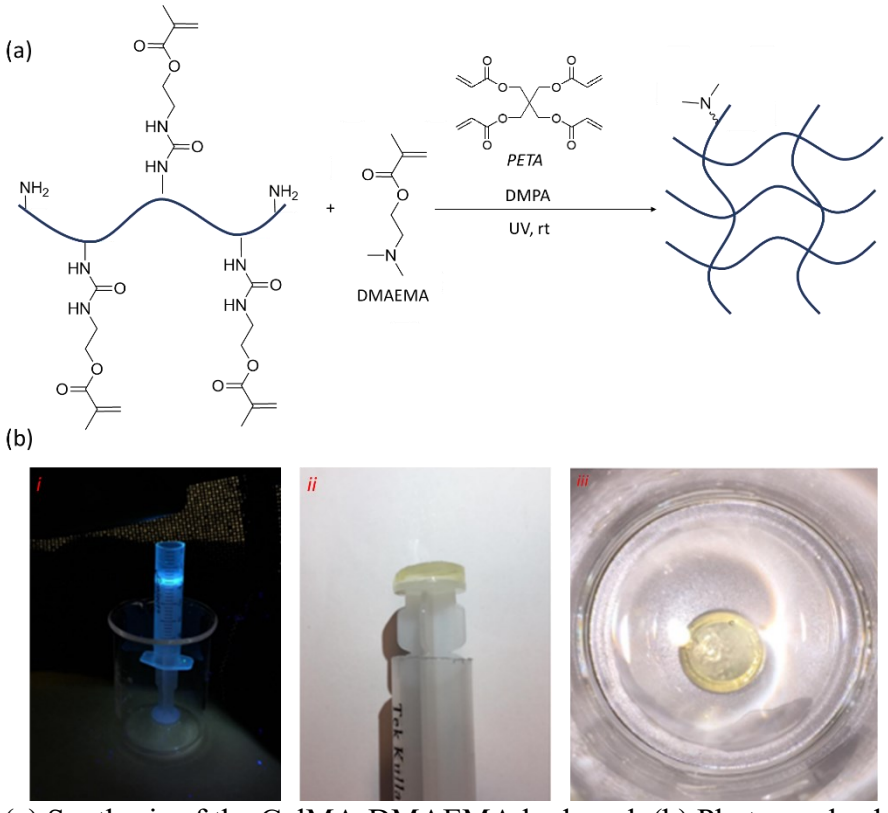

Figure 4. (a) Synthesis of the GelMA-DMAEMA hydrogel. (b) Photographs depicting: the crosslinking stage under UV irradiation inside a syringe (i), the hydrogel (HG-1) immediately after the reaction (ii), and the hydrogel after being removed from the syringe (iii).

The obtained hydrogels were initially characterized using FTIR spectroscopy (Fig. 5). A significant increase in the absorption at 1725 cm^{-1}, attributed to ester units, was observed in the hydrogels. This increase was due to the incorporation of methacrylate and acrylate-containing components, specifically DMAEMA and PETA. Peaks at 2821 cm^{-1} and 2776 cm^{-1} were assigned to the symmetric and asymmetric stretching vibrations of the $(CH_3)_2$–N group in DMAEMA [34, 41–43], confirming the integration of this monomer into the polymer network. Peaks associated with the gelatin backbone were still visible at 1645 cm^{-1} and 1544 cm^{-1}, albeit with a slight shift.

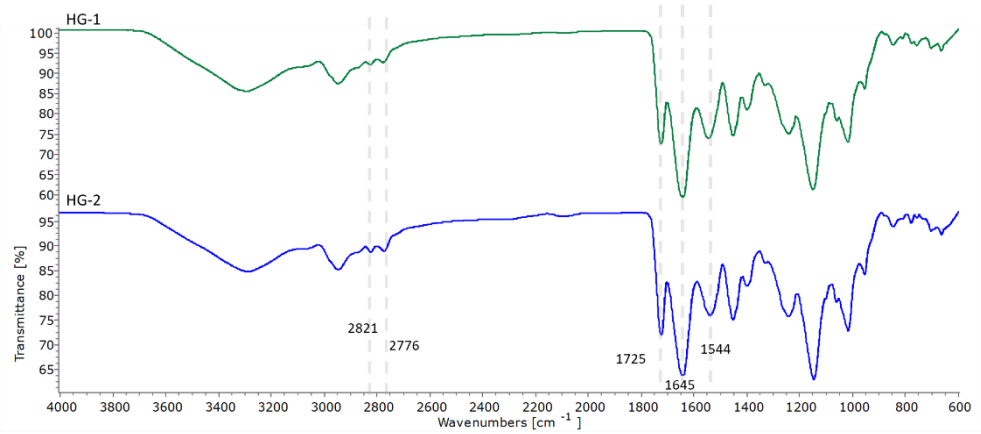

Figure 5. FTIR spectra of hydrogels HG-1 and HG-2.

Two hydrogels were prepared using gelatin with different degrees of substitution (DS), leading to variations in physical properties such as porosity and swelling capacity. To examine these differences, the morphologies of the hydrogels were visualized through SEM. SEM analysis revealed that both hydrogels exhibited a porous structure, which is advantageous for applications such as tissue engineering and drug delivery. As anticipated, HG-2 displayed larger pore sizes compared to HG-1, attributed to its higher crosslinking density of this sample (Fig. 6a). The water absorption capacities of the hydrogels were assessed gravimetrically. Dry gels were weighed, immersed in distilled water, and their weights were recorded at intervals over 90 minutes at room temperature. HG-1 demonstrated a slightly higher water absorption capacity than HG-2, consistent with its lower crosslinking density and the observations from SEM images (Fig. 6b). Thermal stability was evaluated using TGA. The thermograms revealed three distinct degradation stages, with the first occurring below 100 ºC, attributed to water evaporation. Both hydrogels showed stability up to approximately 125 ºC. While HG-1 and HG-2 exhibited similar weight loss behaviors, HG-2 was marginally more thermally stable, likely due to its higher crosslinking density (Fig. 6c).

It is well known that surfaces with quaternary ammonium units can inhibit or minimize bacterial growth. In this study, we aimed to demonstrate the preparation of a gelatin-based hydrogel film with potential antibacterial properties due to its positive charges. To achieve this, one of the hydrogels (HG-2) was prepared as a coating on a glass surface to explore the quaternization of tertiary amino groups, which impart positive charges to the hydrogel layer. The hydrogel precursor solution was spread onto the TMSMA modified glass, covered with a clean coverslip to ensure uniformity, and subsequently washed. Quaternization was performed by incubating the hydrogel-coated surface in a solution of methyl iodide (MeI) in tetrahydrofuran (THF) for 24 hours (Fig. 7a). After incubation,

the surface was washed with THF and dried under vacuum. The thickness of the hydrogel film on the glass surface was measured from cross-sectional SEM images, which indicated a thickness of 2.4 µm (Fig. 7b). The film initially appeared clear with a slight yellow tint but turned a darker yellow following treatment with MeI (Fig. 7b). Quaternization was confirmed via FTIR analysis, as the characteristic peaks at 2776 and 2821 cm^{-1}, associated with tertiary amines, disappeared after methyl iodide treatment, indicating near-complete quaternization (Fig. 7c).

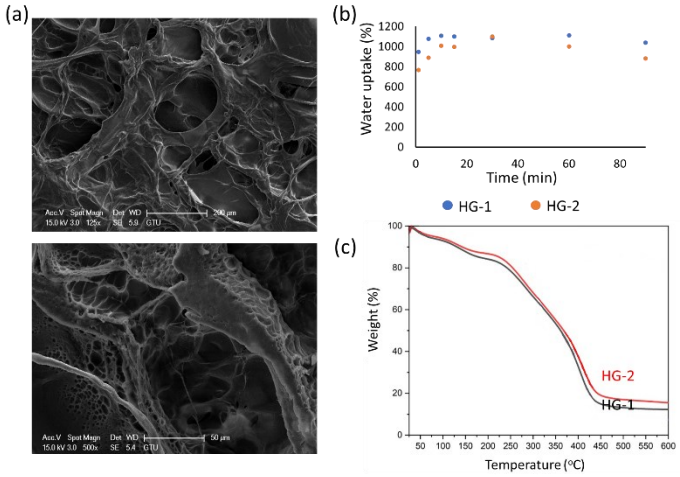

Figure 6. (a) SEM images of the lyophilized hydrogels: HG-1 (top) and HG-2 (bottom). (b) Water absorption plots of the hydrogels at room temperature. (c) Thermogravimetric analysis (TGA) curves of HG-1 and HG-2.

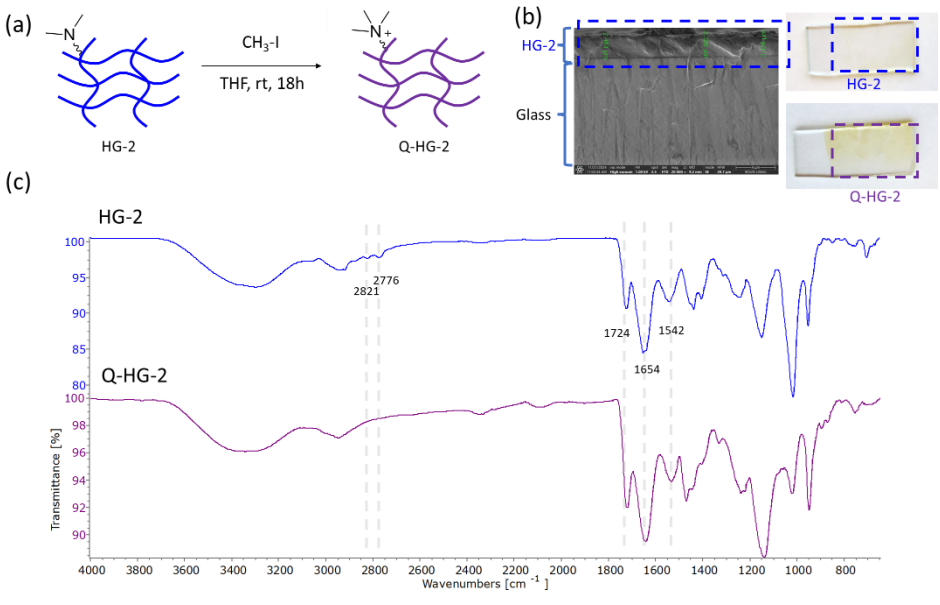

Figure 7. (a) Schematic representation of the quaternization process of the hydrogels. (b) SEM image of the cross section of the HG-2 film on a glass surface (right) Photographs of HG-2 (top-left) and Q-HG-2 (bottom-left) coatings on a 2 × 1 cm area

4. Conclusion

In this study, we demonstrated the rapid modification of gelatin with methacrylate using a room temperature isocyanate-amine reaction, along with inexpensive and straightforward purification without dialysis membranes. Successful preparation of gelatin methacryloyl was confirmed by FTIR and NMR analyses and degree of substitutions were calculated. The modified polymers were then used to synthesize hydrogels with a tertiary amine-containing monomer and four-arm crosslinker via photopolymerization. One of the hydrogels was also synthesized on a methacrylated glass surface as a covalently-attached film. The tertiary amine units on the surface were subsequently quaternized using methyl iodide. We believe that the resulting material has potential antibacterial properties and biocompatibility due to its quaternary amine and gelatin components, and it can find applications in the biomedical field from antibacterial coatings to drug release.

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