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Research article

# Temperature-dependent formulation of a hydrogel based on Hyaluronic acid-polydimethylsiloxane for biomedical applications

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ABSTRACT

Hyaluronic acid (HA), as a safe biomaterial with minimal immunogenicity, is being employed in a broad range of medical applications. Since unmodified HA has a high potential for biodegradation in the physiological condition, herein, an HA-based cross-linked hydrogel was formulated using polydimethylsiloxane-diglycidyl ether terminated (PDMS-DG) via epoxide-OH reaction. The formation of HA-PDMS hydrogel was confirmed using FTIR, NMR, and FESEM. Temperature demonstrated a critical role in the physicochemical properties of the final products. Gel-37, which formed at 37 °C, had a higher modification degree (MD) and more stability against hyaluronidase and oxidative stress than the hydrogel formulated at 25 °C (Gel-25). In addition, the swelling ratio, roughness, and porous network topology of Gel-25 and Gel-37 were different. The rheology measurement indicated that HA-PDMS hydrogel had a stable viscoelastic character. The hydrogel was also biocompatible, non-cytotoxic, and considerably stable during 7-months storage. Overall, various determined parameters confirmed that HA-PDMS hydrogel is worth using in different medical applications. Keywords: Hyaluronic acid; Polydimethylsiloxane-diglycidyl ether terminated; Hydrogels; Long-term stability; Viscoelastic behavior; Biocompatibility.

## 1. Introduction

Hyaluronic acid (HA) is a high molecular weight anionic polysaccharide composed of repeating disaccharide units (N-acetyl glucosamine and D-glucuronic acid). In physiological condition and commercially available samples, HA is in its sodium salt form (Na-HA), as shown in Figure 1A [1-4]. HA naturally exists in many human tissues such as skin (with 50% of total HA), connective tissue, cartilage, synovial fluid of joints, and vitreous body of the eye [5, 6, 7]. Due to the highly negative charge and hydrophilic property, the water absorption capacity (WAC) is high in HA. Owing to the physicochemical properties such as high WAC, high molecular weight, high flexibility with non-immunotoxicity, HA is applied in biomedical usages e.g. in dermal fillers [8, 9], cell and tissue culture scaffolds [10], cartilage tissue engineering [11], drug delivery systems [12], and wound healing [13].

Although HA has many advantages, its rapid degradation in tissues limits its application. Two main mechanisms for HA degradation include the enzymatic (by hyaluronidase) and chemical (by oxidative stress) degradations [14, 15]. Degradation process decreases the half-life of injected HA in the skin and joints, about one day or less [14, 16], which causes to need many injections during the treatment period. Developing methods to increase the HA longevity at injection site even for several months, is an essential step in the processing of HA-based medical products [1, 17, 18].

HA modification not only helps to overcome the enzymatic/chemical digestion but also gives it some new intriguing features. HA has two functional groups, hydroxyl and carboxyl groups, that are highly susceptible to react with different materials and provide various HA modifications (Figure 1A). One of the most useful modification strategies is the co-polymerization of HA with other polymers and creating hydrogels. Hydrogels are cross-linked polymeric networks that swell quickly without dissolved in water. Despite, hydrogels are resistant to dissolve in aqueous solutions, the hydrophilicity character of the resulted porous structure drives the hydrogel to capture lots of water molecules [19, 20].

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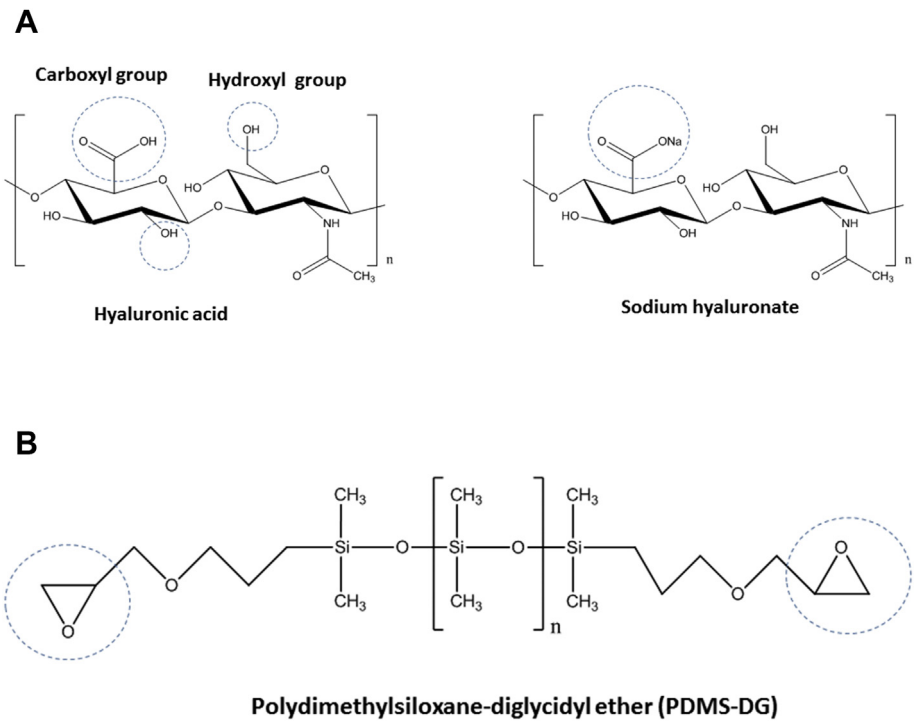


**Figure 1.** Chemical structure of HA and Sodium hyaluronate (A) and PDMS-DG (B). Two functional groups, hydroxyl and carboxyl, are shown by blue circles. Sodium hyaluronate is dominant form of HA at physiological conditions. Polydimethylsiloxane has two methyl groups attached to its silicon structure. PDMS-DG has two epoxy groups in its ends (blue circles). The chemical structures present here have been drawn with ChemBioDraw Uitra 12.0.

It is believed that HA is also more stable against the enzymatic and oxidation degradation in the three-dimensional cross-linked hydrogel structures than its natural, linear form [21].

So far, many methods for cross-linking HA molecules to each other (self-cross-linking) or other polymers have been examined to increase the half-life of HA in the body as well as create new biomaterials with novel properties [2, 17]. In this study, PDMS-DG was used as a cross-linker polymer with its functional bis-epoxide (diglycidyl) part, which depending on the pH condition, could make ether bonds with the hydroxyl groups (high pH) or ester bonds with the carboxyl groups of HA (low pH) [22]. Formation of the ether bonds using epoxide was performed by Laurent et al., in 1964. They used 1,2,3,4-diepoxybutane as a cross-linker at basic pH [23]. Butanediol-diglycidyl ether can also be referred to as a non-cytotoxic cross-linker that has been used in many commercial hydrogels [24]. Some studies have shown that epoxide mediated cross-linked HA hydrogels are more stable than the hydrogels produced from other cross-linking agents such as carbodiimide and can last for long time [25, 26]. It has also been identified that the cross-linked HA hydrogels produced by ether bonds are more stable than those produced by ester bonds [27]. Some of the special polymers which can crosslink with HA are silicon-based synthetic polymers such as PDMS. Although pure silicon is a popular filler with considerable properties such as high flexibility, high gas permeability, non-toxicity, and biocompatibility, it may cause undesirable symptoms [28]; for instance, the filler makes up of PDMS has been associated with migration, overcorrection, and deformation in the treatment place [29]. Nevertheless, a study on 95 patients who received silicon oil emulsified with HA (without any chemical bond formation between them) during the 2-years medical following-up, showed very slight complications such as some small and temporary nodules, with no observation of granulomas or filler migration [30].

In this study, for obtaining a multi-functional biomaterial, we fabricated HA-PDMS hydrogel, which has not been reported so far. A modified form of PDMS containing two epoxy groups (bis-epoxide PDMS) called PDMS-diglycidyl ether (PDMS-DG) was employed for appropriate cross-linking reactions (Figure 1B). PDMS-DG reacted with HA via epoxy groups at its ends, in the alkaline conditions. The effect of the reaction temperature on the features of the hydrogel was evaluated at 25 °C and 37 °C. Besides, the hydrogel's swelling capacity, rheological behavior, bio-compatibility, bio-stability, and cytotoxicity were measured.

## 2. Experimental section

### 2.1. Materials

Dry powder of sodium hyaluronate (HA) (average molecular weight = 50000 Da) was purchased from BulkActives (Taiwan). Poly dimethylsiloxane-diglycidyl ether terminated (epoxy terminated PDMS or PDMS-DG, 480282-50 ML, average Mn- 800, Japan), hyaluronidase (EC 3.2.1.35, 400–1000 U/mg), 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltertazolium bromide (MTT) and 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (USA). The lactate dehydrogenase (LDH) measurement kit was from Pishtazteb Co. (Iran). All other chemicals were analytical grade and obtained from Merck (Germany). The mouse fibroblast cell line (L929) was from the Pasteur Institute of Iran. Cell culture media (DMEM) and antibiotics were from GibcoBRL (Life Technologies, paisley, Scotland), and fetal bovine serum (FBS) was from Biosera (Tehran, Iran). Commercial hydrogel (Perfectha, France) was donated by Dr. Afshar Ramezanpour from Zanjan University of Medical Science.

### 2.2. Hydrogel preparation

100 mg (0.128 mmol) of HA powder was dissolved in 0.5 mL of NaOH (1N, pH > 10) and then left at 4 °C for 24 h. In another container, 200 μL of DMSO was added to 1 mL (1.23 mmol) of PDMS and mixed thoroughly

by mechanical agitation for 60 s. Then, the PDMS solution was added to the HA-NaOH solution, stirred for 20 s, and left at 25 °C or 37 °C for 2 h. In this step, the excess solution was removed from the synthesized hydrogel in the di-phasic sample. The resulted hydrogels produced at 25 °C and 37 °C were regarded as "Gel-25" and "Gel-37", respectively, and kept at 4 °C or 25 °C.

### 2.3. Characterization of the hydrogel

#### 2.3.1. Fourier-transformed infrared (FTIR) analysis

FTIR spectra were recorded for native HA, PDMS-DG, and dried hydrogels (Gel-25 and Gel-37). Samples were prepared using the KBr pellet method, and all spectra were recorded in the range of 400–4000 $cm^{-1}$, using a Nicolet iS10 FTIR spectrometer.

#### 2.3.2. Nuclear magnetic resonance spectroscopy (NMR)

Hydrogels were dialyzed for 24 h against distilled water and then were lyophilized. The lyophilized hydrogels were dissolved in $D_2O$ and used as NMR samples on a Bruker Avance 250 MHZ spectrometer. NMR data processing was performed using MestReNova software.

### 2.4. Swelling ratio (SR) measurement

Equivalent amounts (0.1 gr) of the room temperature dried hydrogels (Gel-25 and Gel-37) were weighed (Wd). Samples were then incubated in 1 mL distilled water at 37 °C. At selected time intervals, the swollen hydrogels were reweighed after removing excess water using tissue paper (Ws). After that, the hydrogels were reloaded with 1 mL of fresh water. The measurement was continuing until the swelling rate did not change. SR was also measured in phosphate-buffered saline (PBS). SR was calculated by Eq. (1):

$$\text{Swelling ratio (SR)} = \frac{Ws}{Wd} \quad (1)$$

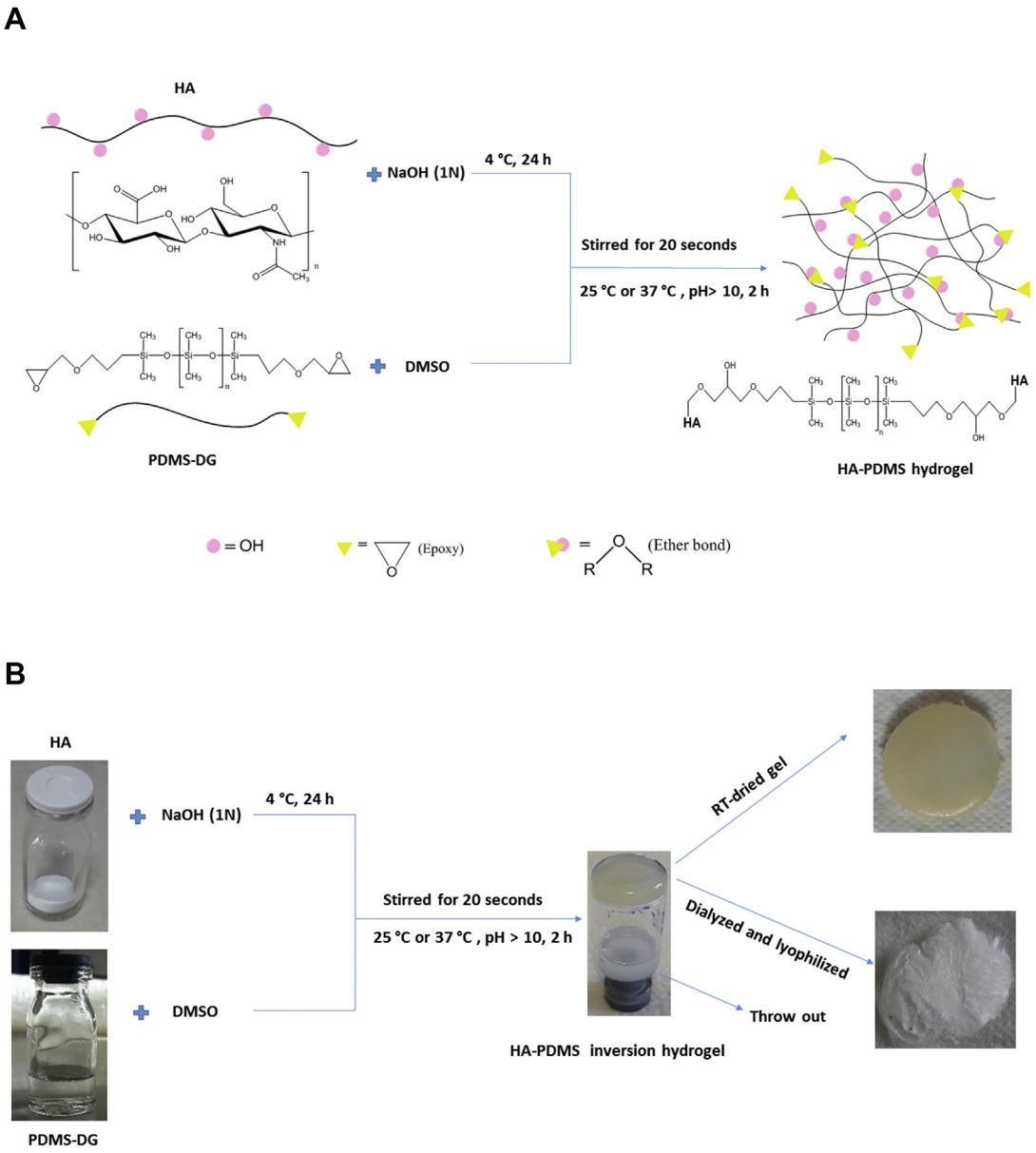


**Figure 2.** HA-PDMS hydrogel synthesis at different temperatures. (A) Schematic diagram and (B) real materials representation. HA was incubated at basic pH, and interaction of the epoxide groups with the HA hydroxyl groups leads to ether bond formation. This cross-linking reaction created HA-PDMS 3D hydrogel network.

### 2.5. Resistance of the hydrogels against enzymatic and chemical degradations

#### 2.5.1. Enzymatic degradation assay

The method of Nimmo et al. with slight modification was used to determine the stability of the hydrogels against hyaluronidase [31]. Firstly, the dried hydrogels (Gel-25 and Gel-37, 0.05 gr) allowed swelling in PBS at 37 °C. After removing the non-reacted solution, the swelled hydrogels were weighed ($W_i$). Then, the hyaluronidase with activity of 7 U/mL (17.5 μg/mL) was added to the swelled hydrogels, and the mixtures were incubated at 37 °C using a water bath. At different time points, the supernatants were removed, and the remaining hydrogels were weighed ($W_t$). The percentage of weight-losing was calculated by Eq. (2).

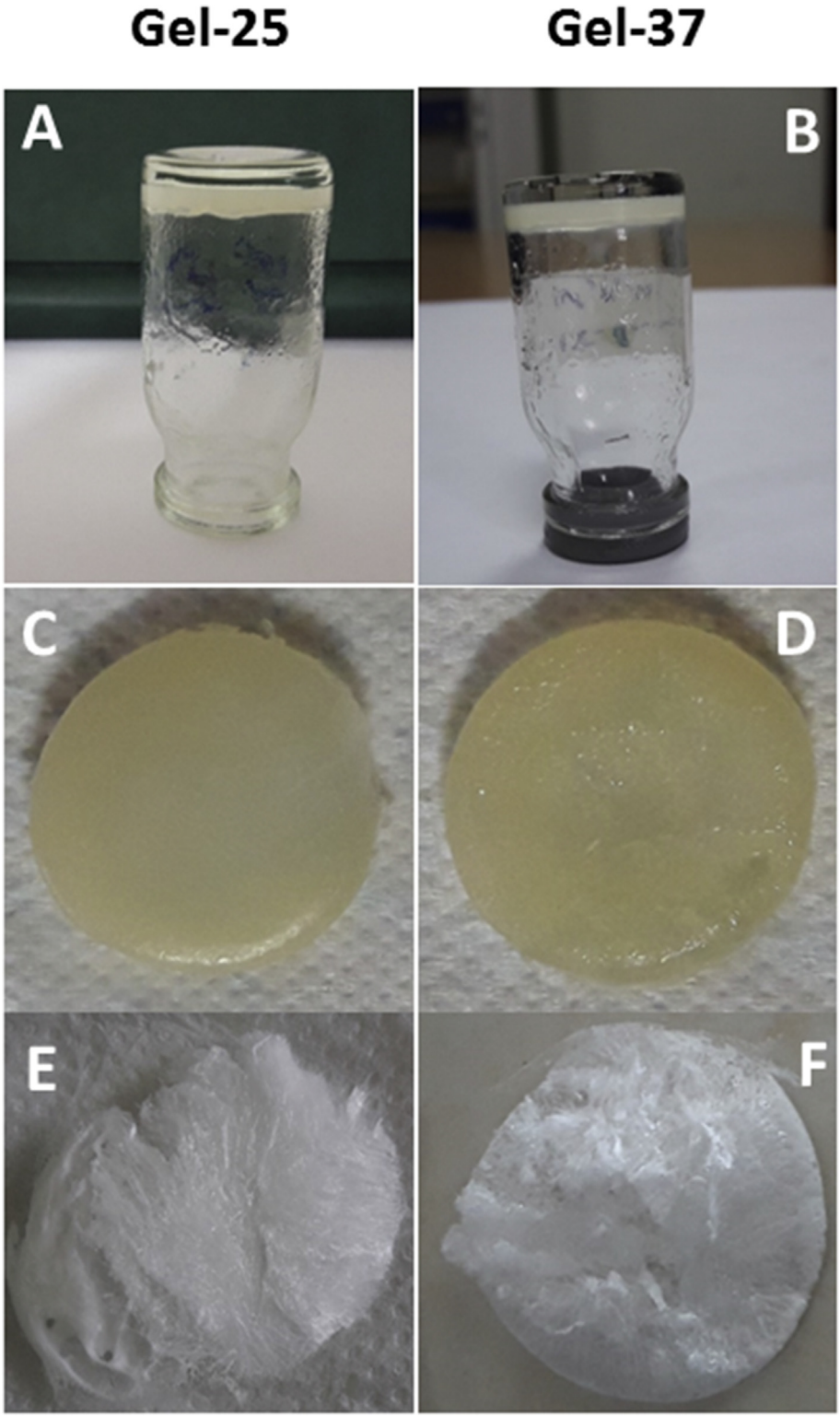


**Figure 3.** The hydrogels producing at 25 °C (A, C, E) and 37 °C (B, D, F). Inversion property of the gels was tested by inverting the vessels after removing the non-reacted materials (A, B). The dried gels at room temperature, or the dialyzed/lyophilized gels are represented in (C, D) and (E, F), respectively.

In the control samples, equivalent hydrogels incubated in similar conditions without the enzyme.

$$\text{Weight loss } (\%) = \frac{(Wi - Wt)}{Wi} \times 100 \quad (2)$$

### 2.5.2. Chemical degradation assay

To determine the stability of the hydrogels against free radicals, hydrogen peroxide ($H_2O_2$) at a final concentration of 2.5% (v/v) of the samples was used. After adding $H_2O_2$ to the swelled hydrogels, at different time points, the supernatants were removed, and the remaining hydrogels were weighted ($W_t$). The percentage of weight loss was calculated according to Eq. (2). In the control samples, the equivalent hydrogels incubated at similar conditions without $H_2O_2$. The reaction was performed at 37 °C.

## 2.6. Field emission scanning electron microscopy (FESEM)

The microstructure, energy dispersive x-ray spectroscopy (EDS), and elemental map (EM) of the gels were examined using a TESCAN mira2 (the Czech Republic) FESEM, after coating the lyophilized hydrogels with gold.

## 2.7. Rheological study

The rheological test was performed using an Anton-Paar oscillatory rheometer (Physica, MCR300, Austria) with parallel-plate geometry. For this end, HA and dried Gel-37 powders were soaked in distilled water for about 3 h. The storage module (G′), elasticity index, and loss module (G″), viscosity index, were measured as functions of frequency at room temperature.

## 2.8. Cytotoxicity assays of the synthesized hydrogels

### 2.8.1. Assessment the cellular metabolic activity by MTT assay

The colorimetric method using 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltertazolium bromide (MTT) was employed to evaluate the effect of the hydrogels on the metabolic activity of the L929 cell line. MTT is a tetrazolium dye that reduces to insoluble formazan by the activity of the dehydrogenase in the mitochondria of the live cells. L929 cells were seeded in 96-well plates at densities of $3\times10^4$ cell/mL and incubated in the incubator with 5% $CO_2$ and 90% humidity for 24 h. The cells were then incubated for more than 24 h after treated with different concentrations (5–200 μg/mL) of Gel-25, Gel-37, or Perfectha (as a control hydrogel) dissolved in DMEM medium. Then, the wells were washed with PBS, and the medium was replaced with a fresh medium containing 10% (v/v) MTT (5 mg/mL in PBS) followed by incubation for 4 h in the dark. After that, the formazan crystals were dissolved in DMSO and read at 570 nm in a plate reader (Microplate Spectrophotometer, Epoch 2, BioTek Company, Gen5 Software, USA), and the viability percentage was calculated using Eq. (3):

$$\text{Viability } (\%) = \frac{\text{Absorbance 570 nm (treated cells)}}{\text{Absorbance 570 nm (untreated cells)}} \times 100 \quad (3)$$

### 2.8.2. Lactate dehydrogenase (LDH) assay

The release of the cytoplasmic enzyme LDH reflects the loss of membrane integrity and inference cell death [32]. The cells were incubated for more than 24 h after treatment with different concentrations (5–200 μg/mL dissolved in DMEM medium) of Gel-25, Gel-37, or Perfectha. Then, a 100 μL of growth media was added to 1 mL of the kit substrate and absorbance at 340 nm was measured in four consecutive minutes with an interval of one minute at 37 °C. The LDH (U/L) was then measured according to the Pishtazteb manufacture kit instruction.

### 2.8.3. Measurement of the intracellular ROS

To verify the effect of the synthesized hydrogels on the hemostasis of reactive oxygen species (ROS) in the cells, the intracellular ROS was monitored by the DCFH-DA probe, a cell-permeable reagent. DCFH-DA reacts with ROS, where fluorescent 2′7′- dichlorodihydrofluorescein (DCF) is generated [32]. Accordingly, $6\times10^4$ cells/mL of L929 cells were seeded and incubated for 24 h in the 96-well plates. The cells were then treated with different concentrations of Gel-25, Gel-37 or Perfectha (5–1000 μg/mL dissolved in DMEM medium),

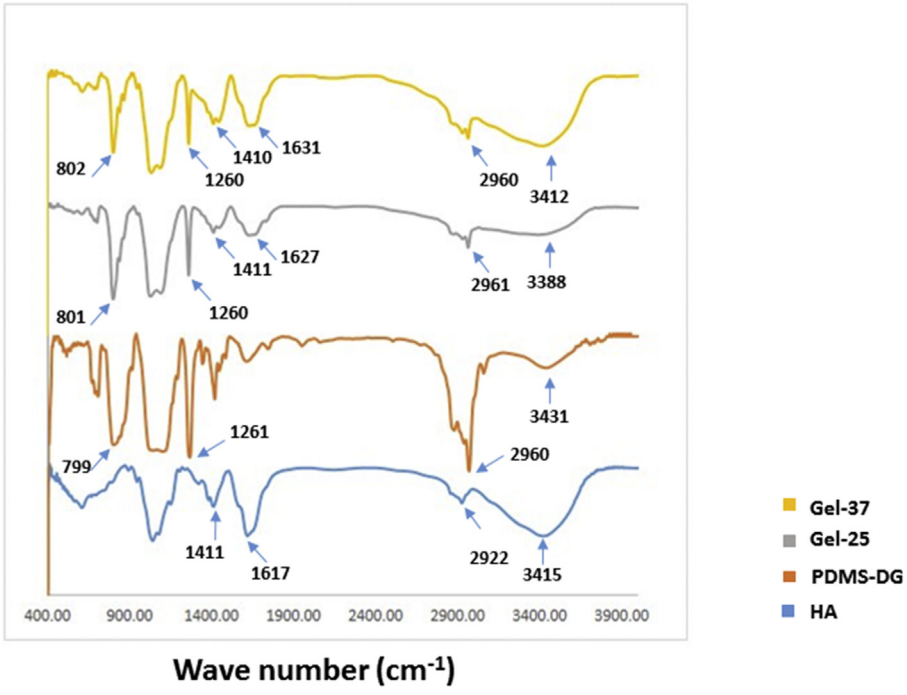


**Figure 4.** FTIR spectra of HA, PDMS-DG, Gel-25, and Gel-37. The characteristic peaks of HA and PDMS-DG were indicated by blue arrows, which also presented in the constructed hydrogels.

and incubated for more than 6 h. After washing the cells with PBS, the culture mediums were replaced by PBS containing 15 μM DCFH-DA. The plate was incubated for 45 min in the dark, and then the cells were washed with PBS, and the fluorescence emission intensity of DCF was recorded at excitation and emission of 495 and 528 nm, respectively.

#### 2.8.4. Hemolysis assay

The standard blood agar plates were obtained from Sarinalab (Tehran, Iran). A total of 100 μL of 20 mg/mL Gel-25, Gel-37, or Perfectha (dissolved in PBS) were spread on the surface of blood agar plates, and then hemolysis was analyzed after incubation for 24 h at 37 °C. As a control, an equivalent of 0.5 McFarland of *Bacillus Cereus* was spread on the blood agar plates in the same condition.

### 2.9. Statistical analysis

The experiments were done in triplicate, and results are indicated as means ± SD. The statistical significance within the groups was assessed using One-way ANOVA. The significance outcome between the groups was also compute using unpaired Student's t-test (p value $<0.05$ was considered significant).

## 3. Results and discussion

### 3.1. The hydrogels of HA and PDMS-DG

PDMS-DG was employed to create linking between HA and PDMS. As the epoxide rings were opened in PDMS-DG, they formed ether bonds

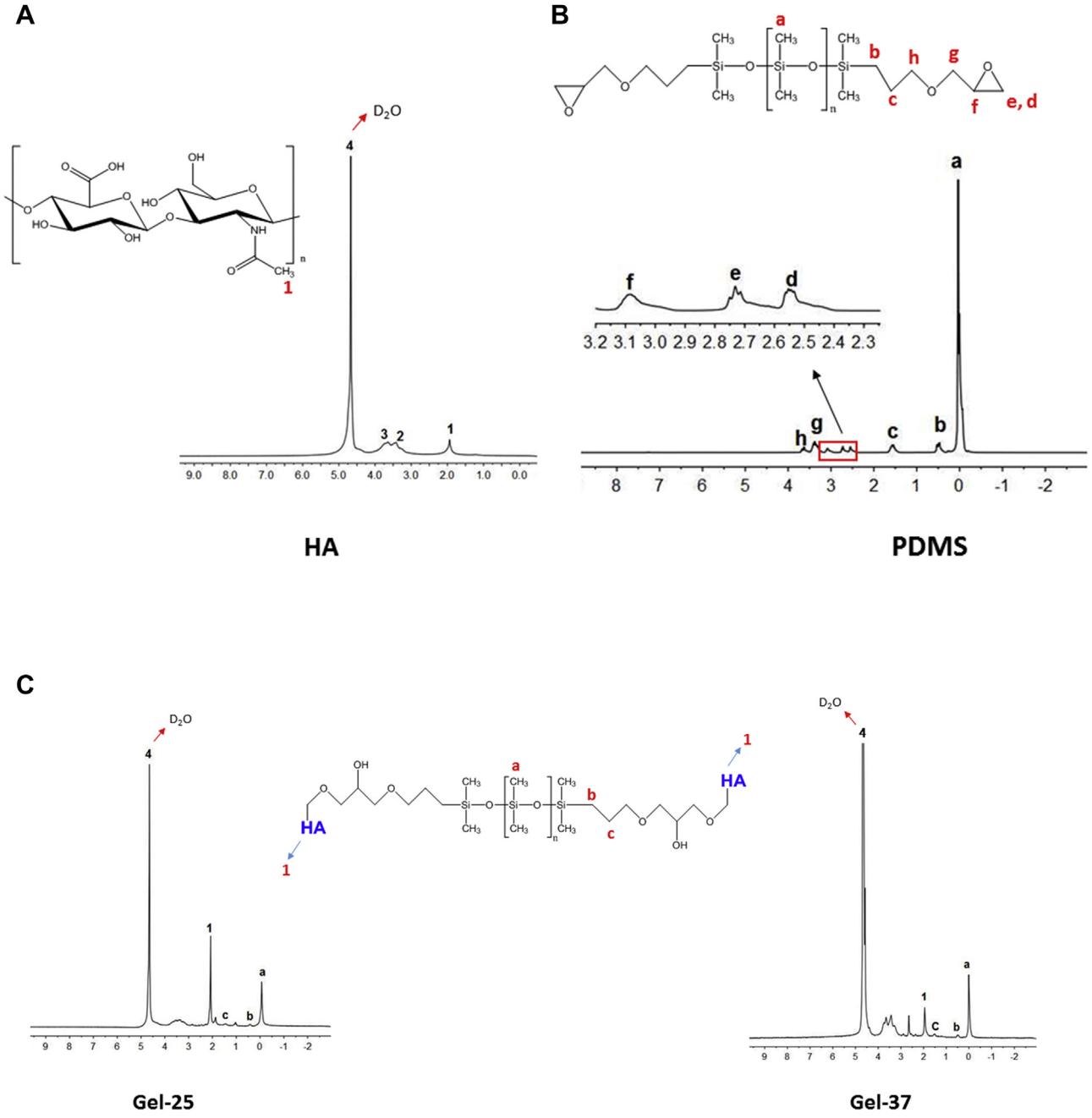


**Figure 5.** $^{1}$H NMR spectra of HA, PDMS-DG, Gel-25, and Gel-37. (A) The HA spectrum in $D_2O$ with specific peaks for the N-acetyl group (1) and sugar rings (2, 3). (B) The PDMS-DG spectrum in chloroform with specific peaks for the methyl (a), epoxy (d, e, f), and $CH_2$ (b, c, g, h) groups. (C) The Gel-25 and the Gel-37 spectra in $D_2O$ with the specific peaks of HA and PDMS-DG. The red number or letters in the chemical structures represented the chemical groups detected by NMR.

with the hydroxyl groups of HA. It was assumed that in the alkaline condition (pH > 10), the deprotonated hydroxyl groups of HA are more nucleophilic than the carboxyl groups and prefer to react with the PDMS epoxide groups and form ether bonds (Figure 2) [3]. Due to the insolubility of Na-HA in organic solvents, $Na^+$ ions are commonly exchanged with TBA (tetrabutylammonium) through ion-exchange chromatography, however; adding DMSO to the PDMS solution also helped us to eliminate the chromatography step [33, 34]. While the gelation process was applied at different temperatures, in our experiment the stable gels were only produced at 25 °C and 37 °C after 2 h (Figure 3). Because the temperature might have an influential role in the gelation processes, more details of the produced hydrogels at different temperatures were explored.

### 3.2. Temperature affected the cross-linking reaction

FTIR and NMR analyses were performed to obtain information about the structure of Gel-25 and Gel-37. FTIR spectroscopy showed that the characteristic peaks of the HA spectrum, such as 1411 $cm^{-1}$ (carboxylic acid: O–H bend) and 1617 $cm^{-1}$ (amid: C=O stretch), are present in the spectra of the hydrogels but not in the PDMS spectrum. Also, characteristic peaks of the PDMS spectrum, such as peaks around 1261 $cm^{-1}$ and 799 $cm^{-1}$ (Si–C), were observed in the spectra of the hydrogels but not in the spectrum of HA (Figure 4). This data indicates that both HA and PDMS are present in the final hydrogel products.

The NMR analysis of the hydrogels was also performed and shown in Figure 5A-C. The characteristic peak of HA appeared at 1.9 ppm ($CH_3$ of N-acetyl glucosamine) (Figure 5A) as indicated in the NMR spectra of Gel-25 and Gel-37 (Figure 5C). The characteristic peak of PDMS-DG between -0.1, and 0.1 ppm, which related to Si–$CH_3$, also appeared in the NMR spectra of the hydrogels (Figure 5C). However, the peaks of the epoxy ring at about 2.5, 2.7, and 3.1 ppm in the NMR spectrum of PDMS-DG (Figure 5B) were disappeared or weakened in the NMR spectra of the hydrogels (Figure 5C). This data implies the opening of epoxy rings during the reaction.

Moreover, the modification level of HA in Gel-25 and Gel-37 was quantified by the integration of the signal at about 0.5 ppm (-$CH_2$- from PDMS-DG) and the signal at 1.9 ppm (-$CH_3$ of N-acetyl glucosamine from HA). The modification degree (MD) of HA in Gel-25 and Gel-37 was about 5.11% and 6.60%, respectively. According to these data, it can be deduced that temperature is another factor that influences the characteristics of the produced hydrogel.

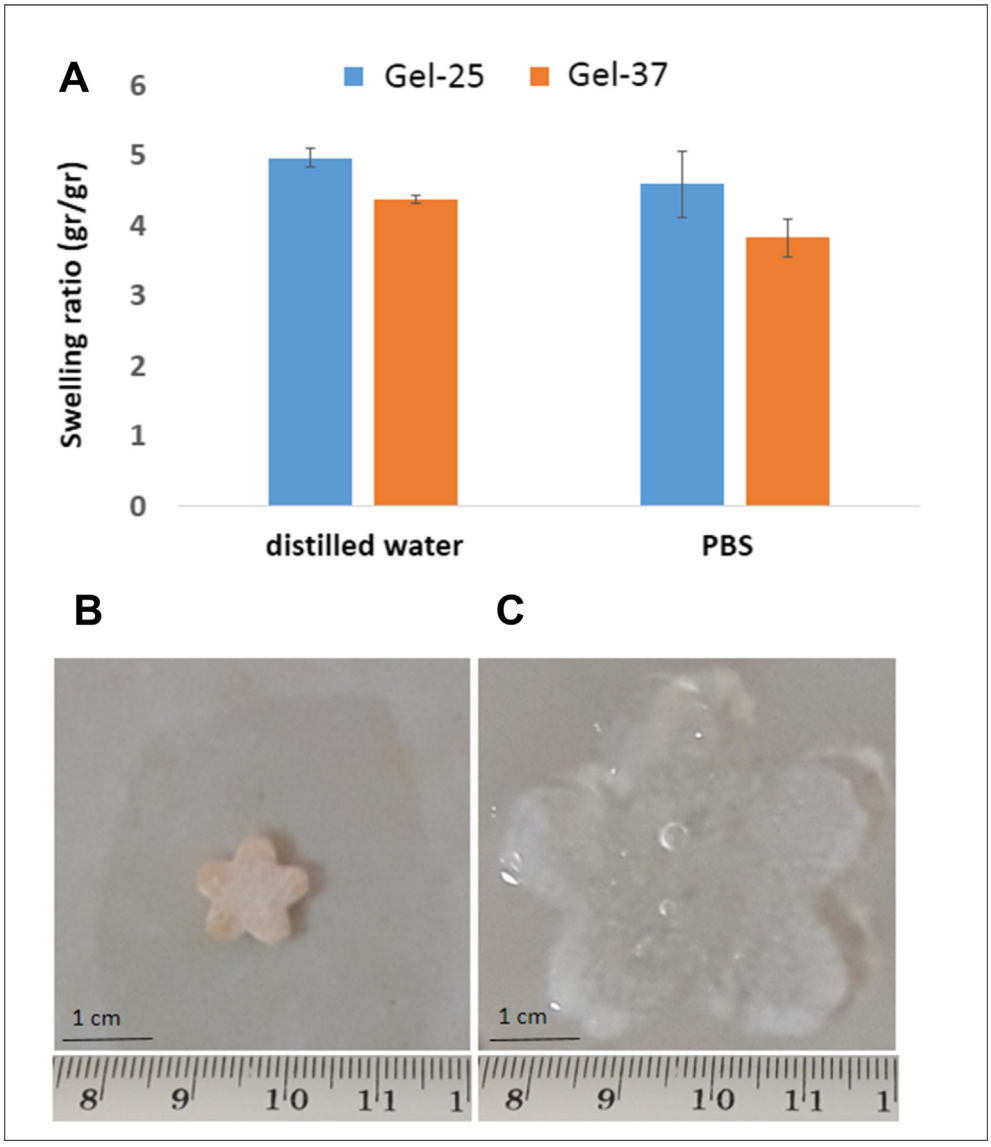


**Figure 6.** Assessment of the swelling properties of the hydrogels. (A) The swelling ratio of Gel-25 and Gel-37 in distilled water and PBS at 37 °C. (B) A designed pluck of Gel-37 after dried in room temperature and (C) after re-hydration.

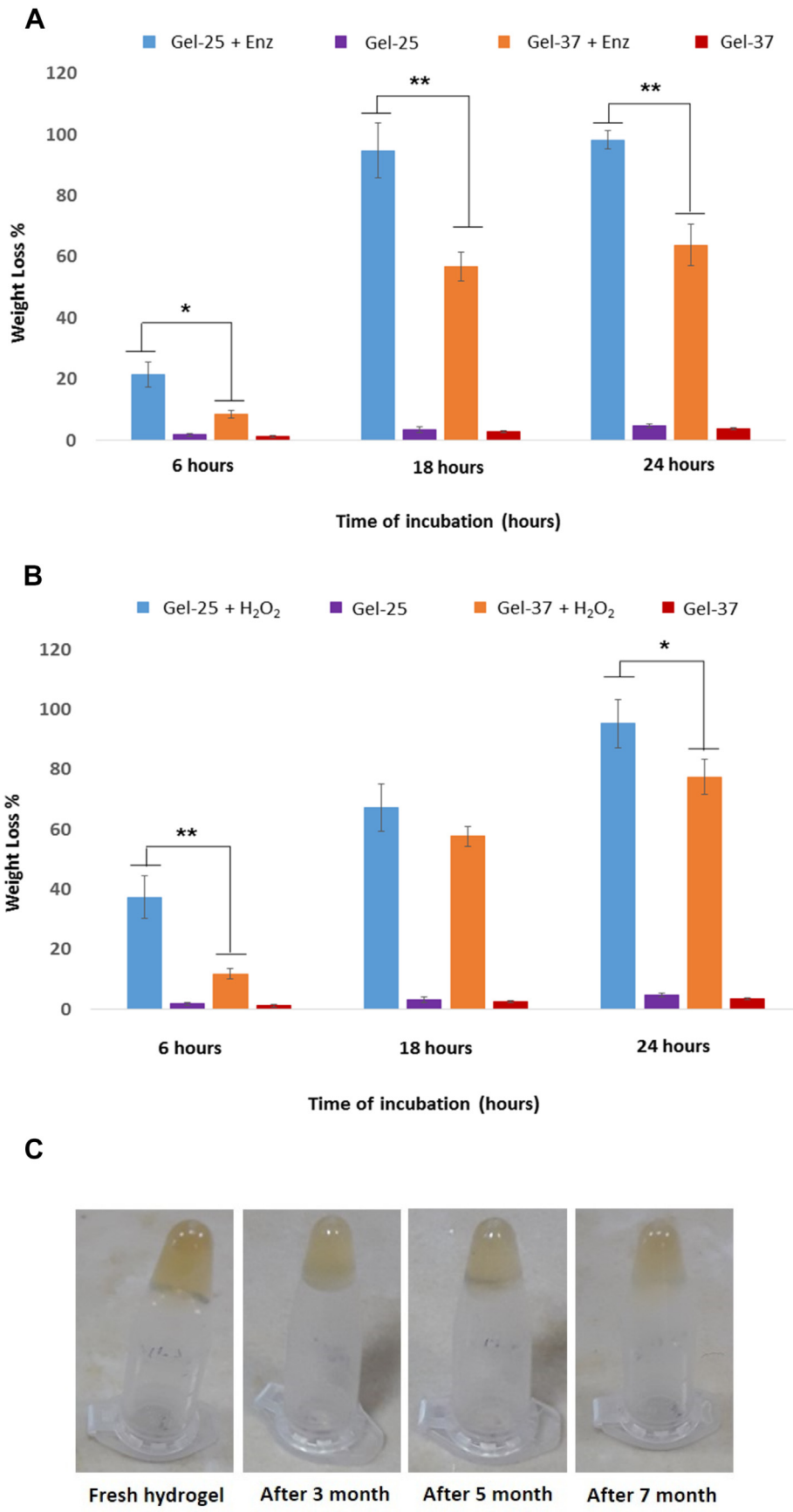


**Figure 7.** Assessing the stability of the hydrogels against degradation. (A) Enzymatic degradation of Gel-25 and Gel-37 using 7 U/mL hyaluronidase at 37 °C. (B) Chemical degradation of the Gel-25 and Gel-37 using 2.5 % (v/v) $H_2O_2$ at 37 °C. The control samples were incubated in PBS without any enzyme or $H_2O_2$ (N = 3, mean ± SD, *P < 0.05, **P < 0.01). (C) Follow up the inversion property of Gel-37 after seven months staying at 25 °C.

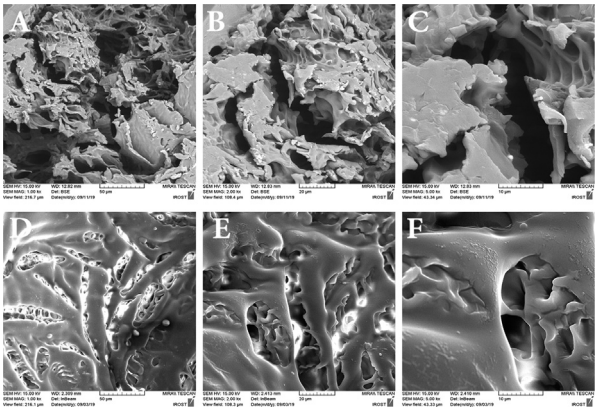


**Figure 8.** FESEM micrographs of the hydrogels. A, B, and C are for Gel-25 and D, E, and F are for Gel-37. Imaging was performed after coating the lyophilized hydrogels with gold. The detail of magnification and scale bars are demonstrated under each image.

Commercial products of injectable HA with different modification ratio (3-25%) are used for inclusive applications [4]. High HA-modified hydrogels such as Hylaform, Hylaform plus and Prevell (with 23% MD) are applied in regions that need to resist against dynamic forces such as nasolabial fold and marionette line, while hydrogels with low MD like Restylane and Perlane (with 3% MD) usually used for dynamic wrinkles such as tear troughs or lips [4].

### 3.3. The hydrogels had a controllable swelling ratio

The swelling ratio (SR) of the hydrogels was measured after soaking the dried Gel-25 and Gel-37 in distilled water or PBS at 37 °C. The SR of Gel-25 and Gel-37 in distilled water were 4.97 and 4.38 g/g and decreased in PBS to 4.6 and 3.83 g/g, respectively (Figure 6A). The presence of large quantities of PDMS as a hydrophobic polymer may cause limitation to the absorption capacity of water in the gels [35]. In support of this claim, the SR in Gel-37 (with MD 6.6%) was less than Gel-25 (with MD 5.11%). Besides, several studies have suggested that the denser the hydrogel network, the lower its water uptake capacity and vice versa [1, 36]. Therefore, the lower swelling ratio of the Gel-37 than the Gel-25 can reflect the denser structure of the Gel-37 in comparison with Gel-25. SR is a critical parameter in the hydrogel materials, and various SR indexes can donate different applications for fabricated hydrogels. Some of the HA-based hydrogels have high SR; for instance, SR of HA-BDDE cross-linked hydrogel is 135 gr/gr [1]. The high SR can cause undesirable results, especially for medical and cosmetic applications. This is because of an uncontrollable increase in volume and conformation changes of the hydrogel. To test the ability of the hydrogel to maintain its shape and integrity during the swelling, the dried Gel-37 in a designed shape was soaked in distilled water. The hydrogel kept its shape, while its size increased several times (Figure 6B and C).

### 3.4. The resistance of hydrogels against bio-degradation

The *in vivo* degradation of HA occurs through two main ways: enzymatic and chemical processes [4], which limit its applications, e.g., for injection, local usage, or the cell culture scaffold. The resistance of the synthetic hydrogels was studied by the *in vitro* enzymatic degradation assay, in which the hyaluronidase activity was more than ten times of its real activity in physiological conditions [37]. As shown in Figure 7A, Gel-37 was significantly more resistant against hyaluronidase than Gel-25, which might be related to its higher MD, the result was consistent with NMR and swelling outcomes. In addition, by increases the MD, the hydrogel network becomes denser with smaller pore size. Therefore, the penetration of the degradation agents into the network is reduced. Bulpitt et al., have synthesized hydrogels with different cross-linking agents, and suggested that by decreasing the porosity, the effect of the enzyme may be limited to only the hydrogel surface [21]. Kablik et al. by comparing some commercial hydrogels, have emphasized that the degree of modification can play an essential role in determining the characteristics of a hydrogel [4]. Even though the stability of HA improved by the formation of HA-PDMS hydrogels, these hydrogels were biodegradable and able to correct after injection by local hyaluronidase. In addition, to investigate the resistance of hydrogels to the oxidative stress conditions, the hydrogen peroxide ($H_2O_2$) containing media was used. As Figure 7B shows, Gel-25 was more vulnerable to degradation in the presence of oxidative reagents than Gel-37. It seems that the presence of PDMS was an advantage for the hydrogels to increase their stability against both enzymatic and chemical degradation. It is intriguing that by following the swelled Gel-37, its inversion character was persistent over seven months, as shown in Figure 7C.

### 3.5. Gel-25 and Gel-37 had different topologies

The FESEM micrographs of the hydrogels are shown in Figure 8. Both hydrogels had rough, non-uniform, and relatively porous surfaces; however, pore networks were more regularly and shapely in Gel-37 than

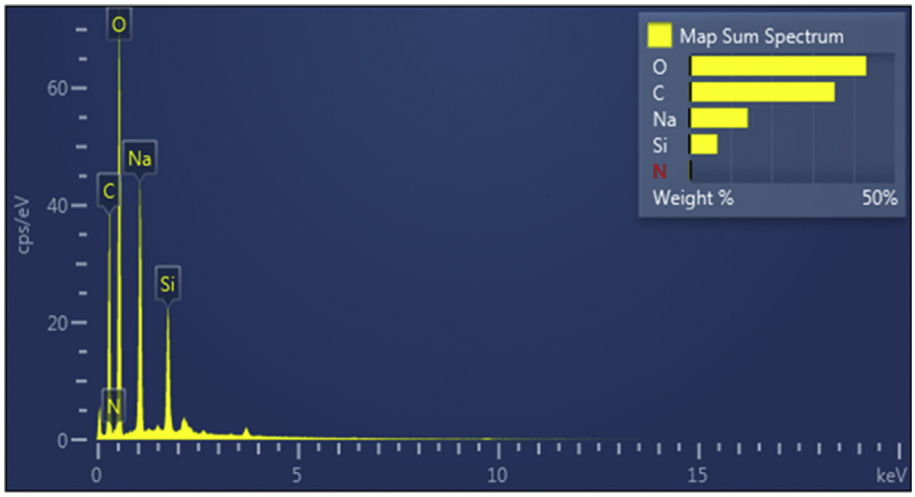


**Figure 9.** Distribution of the elements in the hydrogel, EDS curve of Gel-37. The inset indicated the percentage of each element, including O, C, N, Na, and Si.

**Table 1.** Elemental analysis of Gel-37.

| Element | C | O | Na | Si | N |
|---|---|---|---|---|---|
| Atomic% | 45.07 | 41.19 | 9.48 | 3.74 | 0.15 |

spectrogram of Gel-37 is also shown in Figure 9. C and O are common elements of HA and PDMS. N and Na are specific for HA, while Si is specific for PDMS. The results indicated that the characteristic elements of HA and PDMS, including C, O, N, Na, and Si (except H), were present

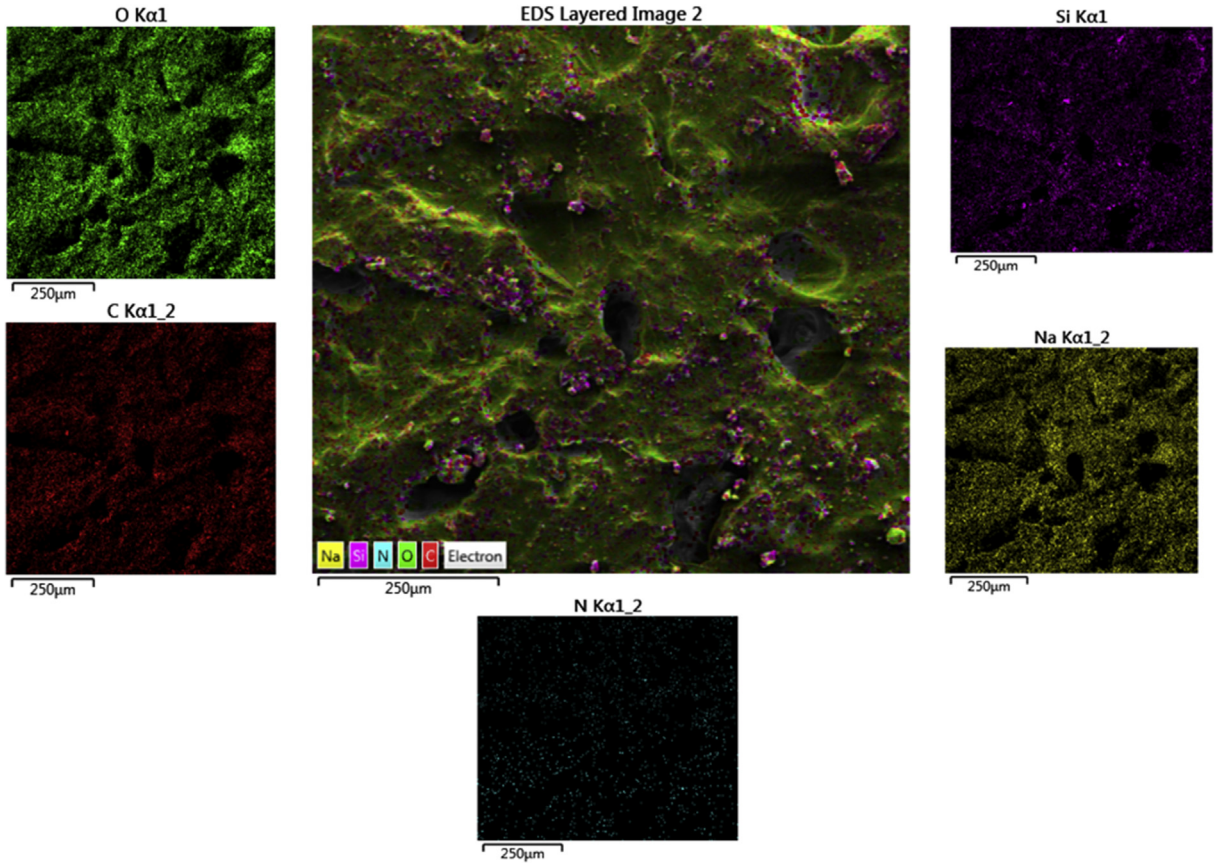


**Figure 10.** Distribution of the elements in the hydrogel, Elemental mapping (EM) profile of Gel-37. All of the Na-HA and PDMS-DG chemical elements (C, O, N, Na, and Si) except H are visible in the hydrogel.

Gel-25, which perhaps related to its high cross-linking rate. According to differences between porosity, roughness, and water absorption level, it seems that by small modification in the process of the synthesis, the hydrogels with different properties would be achieved. The EDS with different contribution C > O > Na > Si > N in the hydrogels (Table 1). Elemental mapping (EM) indicated that both HA and PDMS homogeneously distributed in the hydrogel with a similar contribution of N (as an element of HA) and Si (as an element of PDMS) (Figure 10).

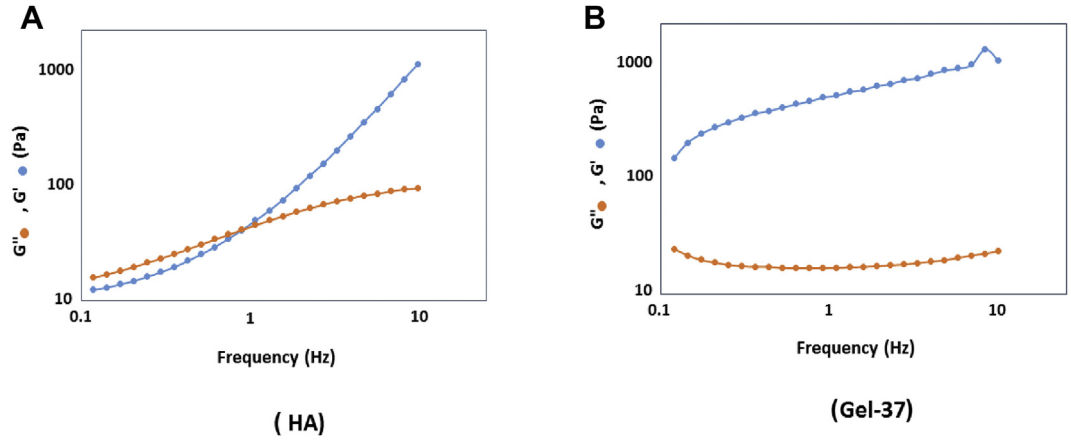


**Figure 11.** Rheological evaluating of the hydrogel. Logarithmic variations of the storage module, G′, and the loss module, G″, of (A) pure HA and (B) Gel-37 based on the loading frequency.

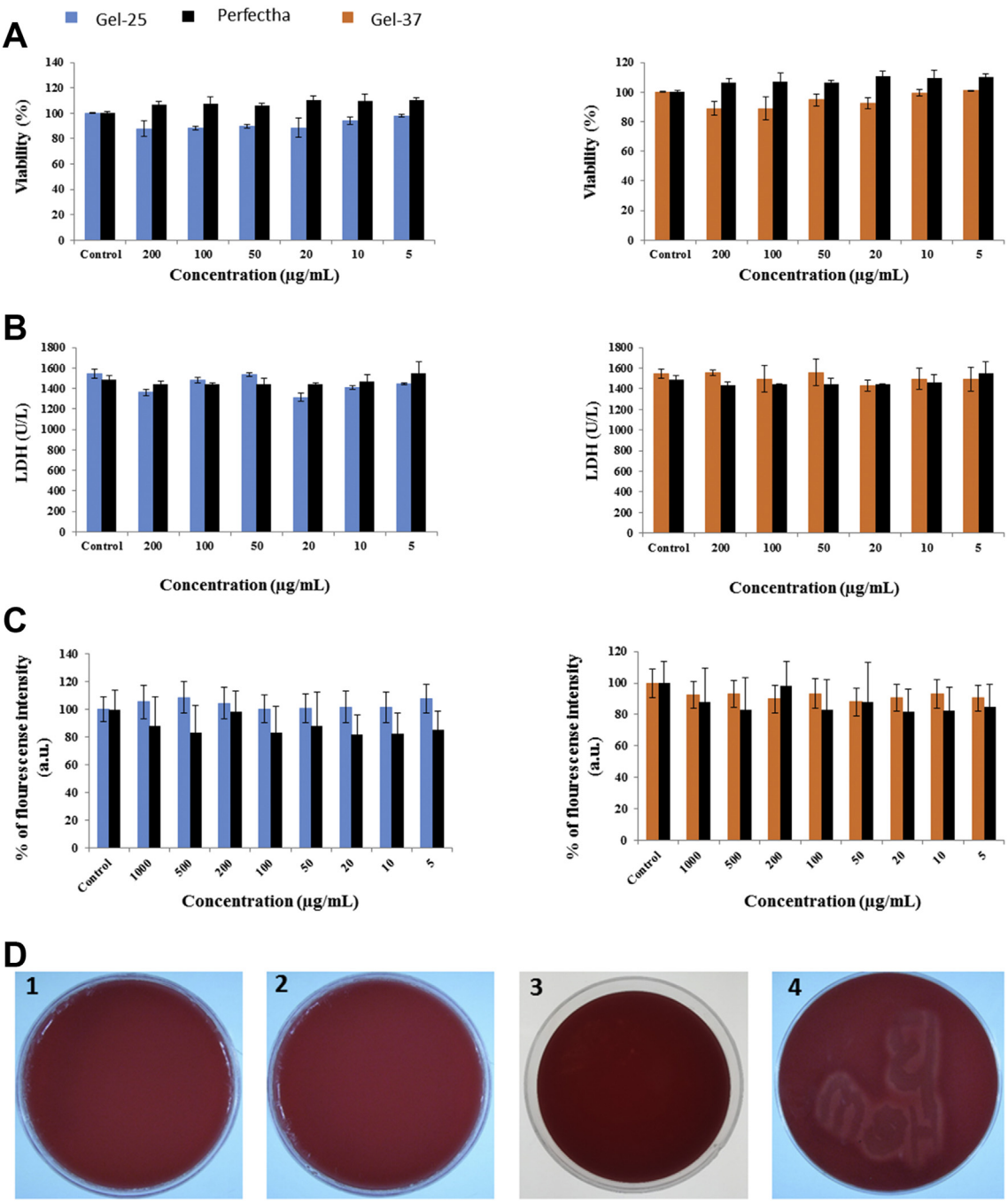


**Figure 12.** Cytotoxicity and biocompatibility measurements of Gel-25 and Gel-37. The viability of L929 fibroblastic cell line treated with different concentrations of the hydrogels for 24 h, were analyzed using (A) MTT and (B) LDH assays. (C) Evaluation of the ROS generation in the cells by measuring the DCF fluorescence intensity in the presence of different concentrations of Gel-25, Gel-37, or Perfectha. (D) Biocompatibility assay using the hemolysis test, Gel-25 (1), Gel-37 (2) or Perfectha (3), and *Bacillus Cereus* as a positive control (4).

### *3.6. HA-PDMS hydrogel showed the viscoelastic property*

The rheological behavior of HA and Gel-37 was obviously different (Figure 11). HA solution as a concentrated polymer solution had a frequency-dependent mechanical behavior, and by increasing the frequency, a cross-over point was seen in the rheological graph (Figure 11A). In this case, viscosity ($G'' > G'$) and elasticity ($G' > G''$) was dominant at low and high frequencies, respectively. It seems that at low frequencies the HA chains were capable to relieve the stress by Brownian motions but by increasing the frequency and consequently reducing the oscillation period, the polymer chains became impotent to disentanglement and therefore temporarily behaved like a cross-linked network [38, 39]. While in the case of Gel-37, $G'$ and $G''$ curves not only exhibited a frequency-independent behavior and were relatively parallel to each other in all ranges of the frequency, but also $G'$ was always higher than $G''$ (Figure 11B). This assessment may support that the fabricated hydrogel has enough strength [38].

Rheological behavior of cross-linked HA hydrogel has also been investigated by others. They have discussed that in the rheological profile of a cross-linked hydrogel, moduli (both G′ and G″) are independent of frequency and G′ is always greater than the G" [39, 40].

### 3.7. Biocompatibility and non-cytotoxicity of the hydrogels

Biomaterials are replaced with whole or part of organs after their safety and biocompatibility have been tested. According to ISO 10993-5:2009, the viability rate of treated standard cells with the consider material should be more than 70% [41]. In this regard, the viability of fibroblast cells in the presence of different concentrations of the hydrogel was examined. MTT assay (Figure 12A) indicated that there was no significant cytotoxicity in the levels between 5-200 μg/mL of the hydrogels. The LDH assay also evaluated the cytotoxicity as an alternative cytotoxicity measurement method. As shown in Figure 12B, both synthesized hydrogels were not harming the integrity of the plasma membrane. The antioxidant activity of HA is observed in some studies [6, 42]. Here the issue of whether Gel-25 or Gel-37 can affect the intracellular ROS content of the treated cells was investigated in comparison with a commercial hydrogel, Perfectha. All examined concentrations of either Gel-25 or Gel-37 did not induce the generation of intracellular ROS and were comparable to the results of Perfectha (Figure 12C). Besides, in the hemolysis test, lacking any transparency on the treated blood agar plates confirmed the biocompatibility of the hydrogels (Figure 12D). All findings in this section indicated the non-toxicity and biocompatibility of the hydrogels consisting of HA and PDMS-DG. These results followed previous evidence concerning the biosafety of HA and PDMS [4, 43, 44, 45].

## 4. Conclusion

HA, as a natural material, is used in many different medical applications. However, HA has a high tendency to degradation; therefore, its usage in natural form is limited and needs to modify. In this study, by using properly cross-linkable polymer, PDMS-DG, and simple methodology, the new hydrogel was formulated. HA-PDMS hydrogel depicted a controllable swelling ratio and also stable viscoelasticity character. The results determined that by applying a proper temperature during the hydrogel fabrication, the determinative parameters of the produced hydrogel, such as morphology as well as resistance to enzymatic and chemical degradation, significantly improved. This study suggests that different physicochemical factors can be employed during the fabrication of HA-PDMS hydrogel to achieve various properties. Furthermore, cytotoxicity assessment indicated that the presentation of PDMS-DG did not mostly induce toxicity in the produced hydrogel. Taken together, HA-PDMS hydrogel can be an excellent candidate for many biomedical purposes.

## Data availability

In the present work, the datasets generated are demonstrated in the manuscript.

## Declarations

### Author contribution statement

Maryam Khaleghi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ebrahim Ahmadi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Mahvash K. Shahraki: Contributed reagents, materials, analysis tools or data.

Farhang Aliakbari: Performed the experiments; Analyzed and interpreted the data.

Dina Morshedi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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