

**Research Article**

## Influence of fabrication temperature on the structural features of chitosan gels for tissue engineering applications

Nimet Bölgen <sup>a,\*</sup> , Seda Ceylan <sup>b</sup>  and Didem Demir <sup>a</sup> 

<sup>a</sup>Department of Chemical Engineering, Mersin University, Mersin 33110, Turkey

<sup>b</sup>Department of Bioengineering, Adana Alparslan Türkeş Science and Technology University, Adana 01250, Turkey

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**ABSTRACT**

Chitosan is a natural polymer synthesized from the chitin of crab, lobster shells, fungal mycelia and shrimp. It has been used for biomedical applications in many different structures including thin film, nanofibrous membrane, sponge, microsphere, hydrogel and cryogel because of its non-toxicity, biodegradability, biocompatibility and antibacterial properties. Cryogelation technique is based on the crosslinking of polymers or crosslinking polymerization of monomers in the presence of crosslinking agents at temperatures below zero. On the other hand, hydrogels are mainly prepared at room temperature. In this study, chitosan gels were prepared at different reaction temperatures (-25, 0 and +25°C). Swelling profiles revealed that with decreasing reaction temperature swelling ratio increased. In addition, the degradation rate of chitosan gels prepared at -25 and +25°C was measured 50.60 and 30.88%, respectively. Results indicate that reaction temperature affects the architecture and characterization results of the gels.

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**1. Introduction**

Tissue engineering is a scientific discipline that includes medical, engineering and biological sciences and works to preserve and improve tissue structure and quality of life. The aim of tissue engineering is to regenerate damaged tissues by combining scaffolds with cells and biomolecules.

Tissues made up of cells and extracellular matrix (ECM). A well-designed scaffold for tissue engineering applications should have similar mechanical and biophysical properties to ECM. Scaffolds are considered as an artificial ECM [1]. The ECM provides a three-dimensional microenvironment for the cells. In addition to providing physical support for cells, it organizes cell-cell interactions and provides various biochemical and biophysical cues for cell adhesion, cell-cell communication, migration, growth, proliferation, differentiation and ECM accumulation [2]. The use of polymeric materials to mimic the target tissue and produce

scaffolds with different properties is a common practice in tissue engineering applications. The biomaterials used for this purpose can be formed in gel form. Hydrogels are obtained by gelation of the polymers at room temperature. The gels obtained by freezing the solution in the cooler is called cryogel.

**Hydrogels:** Polymeric hydrogels are biomaterials that have water absorption capacity and are water-insoluble due to the chemical or physical crosslinking of the polymer chains [3],[4]. Hydrogels may be prepared from natural and synthetic polymers. Examples of natural polymers are proteins (collagen) and polysaccharides (chitosan, dextran and alginate). Polymers from natural origin support cellular activities such as proliferation, differentiation and migration. They are biocompatible and biodegradable. On the other hand, they have low mechanical strength and batch variation. Hydrogels are commonly used in tissue engineering studies such as ECM, drug release systems and biosensors. Vinyl acetate, acrylamide, ethylene glycol monomers can be used to obtain synthetic polymers.

\* Corresponding author. Tel.: +90 324 361 00 01; Fax: +90 324 361 00 32

E-mail addresses: [nimet@mersin.edu.tr](mailto:nimet@mersin.edu.tr) (N. Bölgen), [sceylan@adanabtu.edu.tr](mailto:sceylan@adanabtu.edu.tr) (S. Ceylan), [didemdemir@mersin.edu.tr](mailto:didemdemir@mersin.edu.tr) (D. Demir)

ORCID: 0000-0003-3162-0803 (N. Bölgen), 0000-0002-1088-7886 (S. Ceylan), 0000-0002-2977-2077 (D. Demir)

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Characterization properties of synthetic polymers can be precisely controlled and tailored. Hydrogels produced from synthetic polymers can have different degradability and functionality due to their modifiable properties. Hydrogels show a great resemblance to natural soft or hard tissues because of the biocompatibility, biodegradability and water absorption capacity. The gel cross-linking grade, the chemical structure of the hydrogel-forming monomers and environmental stimulants such as temperature, pH and ionic strength are important factors affecting the hydrogel bond structure [5]. Hydrogels are inert in normal biological reactions and resistant to degradation. In addition, hydrogels can be sterilized easily by heat, ethanol or UV radiation [6].

**Cryogels:** Cryogels are super macroporous three-dimensional polymeric gel matrices prepared from partially frozen solutions of monomeric or polymeric initiators. In the cryogel preparation process, the initiators are rapidly dissolved in an appropriate solvent. The steps of cryogelation process can be sorted as phase separation with ice-crystal formation, secondly crosslinking-polymerization and finally thawing of ice crystals to obtain cryogels with interconnected pores [7]. The interconnected macropores of the cryogels provide mass transfer of nanoparticles and nutrients. Unique pore structures, mechanical, osmotic and chemical stability makes the cryogels interesting biomaterials for the chromatographic applications of biological nanoparticles such as viruses and plasmids and cell organelles. Characteristic properties of cryogels can be changed by optimizing steps of the process for targeted tissue.

Effects of the different factors of cryogelation process can be listed as follows:

**Freezing process:** The macropores of cryogels can be hundreds of micrometers. The pore size varies depending on the cryogelation regime and temperature. As the freezing temperature drops, smaller ice crystals will be obtained. In order to optimize pore diameter, cryogelation temperature can be adjusted for targeted tissue [8],[9].

**Crosslinker:** Crosslinking amount is important in terms of the properties of cryogels. The higher amount of crosslinker is used, the lower water swelling rate and absorption capacity are obtained. Cryogels can also be produced by freezing/thawing techniques without crosslinkers. In this case, no additional treatment is required to remove crosslinking agents remaining in the tissue scaffold [8],[9].

**Polymer concentration:** Elasticity and fragility of cryogels depend on the initial polymer concentration. The higher polymer concentration of the initial reaction mixture is prepared, the denser and fragile matrices are obtained, and as the initial concentration is reduced, the matrices begin to exhibit a more elastic and spongy structure [7],[8].

In this study, scaffolds were produced in hydrogel and cryogel forms at different reaction temperatures using chitosan natural polymer. Chitosan is obtained from deacetylation of chitin by alkaline hydrolysis or enzymatic methods [10]. Chitosan is a biocompatible, biodegradable, non-toxic and antimicrobial polymer and these properties make it a suitable source for use in the biomedical field.

The chemical structure, surface morphology, swelling and degradation behaviors of the produced gels were investigated and the effect of the reaction temperature on the characterization of the material was compared for tissue engineering applications.

## 2. Materials and Methods

### 2.1 Materials

Chitosan (low molecular weight) was obtained from Sigma Aldrich (USA). Glutaraldehyde (25%, v/v, GA) and acetic acid (100%, glacial) were purchased from Merck (Germany).

### 2.2 Production of Chitosan Cryogels and Hydrogels

All gels were prepared at 1% (w/v) constant chitosan concentration. After the calculated amount was weighed, 6% (v/v) acetic acid solution was added and the solution was mixed until a homogeneous solvent was obtained. At room temperature, magnetic stirrer was used to mix solution. The structural differences of the gels were examined by changing gelation temperature as -25, 0 and +25°C.

GA was added as a crosslinking agent (5% weight of total polymer concentration) to chitosan solutions. The prepared solution was quickly put into a plastic syringe and placed into the cryostat for crosslinking reaction at specified temperatures (-25 and 0°C). Gelling at +25°C was carried out at room temperature. The structure formed at -25 and 0°C is called cryogel and the structure formed at +25°C is called hydrogel.

The cryogels formed at -25 and 0°C were kept at the specified temperatures for 4 hours while the hydrogel formed at +25°C was kept at room temperature for 4 hours. Then all the gels were left in the freezer for 20 hours. The frozen materials were thawed at room temperature. All gels were washed several times with distilled water to remove the unreacted chitosan and GA. The produced gels were freeze-dried by lyophilization.

### 2.3 Characterization of Gels

Functional groups of the produced chitosan gels were analyzed by Fourier Transform Infrared Spectrometer (FTIR) (Perkin Elmer, USA) between 500 and 4000  $\text{cm}^{-1}$  wavelengths. Scanning electron microscopy (SEM) (Zeiss, Supra55, USA) was used to determine the morphology (pore structure, size, wall thicknesses and homogeneity) of the gels after coating with platinum at 5 kV for 1000x magnification ratios. The swelling capacity of the chitosan gels was calculated according to the method demonstrated in our

previous study [11]. Firstly, all samples were dried and weighed ( $W_i$ ). At specified time intervals, filter paper was used to remove the excess water from the surface of cryogels and the cryogels were then weighed again ( $W_f$ ). The swelling ratio (SR%) was determined using Equation (1):

$$SR\% = \frac{W_f - W_i}{W_i} \times 100 \quad (1)$$

Degradation behavior of the chitosan gels was determined by gravimetric analysis. The samples were incubated in distilled water at 37°C in a shaking waterbath (DaihanScientific, WiseBath WB-22, Korea) at 60 rpm for 21 days. After 21 days the gels were dried and weighed ( $W_d$ ). The degradation rate (DR%) was calculated according to Equation (2):

$$DR\% = \frac{W_i - W_d}{W_i} \times 100 \quad (2)$$

Statistical analysis of degradation ratio data was performed with one-way analysis of ANOVA. Statistical significance was defined as  $p < 0.05$ .

### 3. Results and Discussion

In the present study, we aimed to investigate the effect of reaction (between chitosan and glutaraldehyde) temperature on the structure and physicochemical properties of the produced gels. In our preliminary studies, all gels were held at determined reaction temperatures (-25, 0 and +25°C) for 24 hours, respectively. Then, all gels were washed with large volume of distilled water to eliminate the uncrosslinked ingredients especially glutaraldehyde which is highly toxic for cells. However, while doing washing, the surface of the gel prepared at +25°C called hydrogel began to crack with rapid swelling behavior of hydrogel (Figure 1A). The high amount of water in the resulting structure could not be sublimated within 2 days in a lyophilizer. To overcome this, the crosslinking reaction time was limited to 4 hours (by considering that the reaction will be completed in a shorter time) and left in the freezer for 20 hours for mechanical stability. There are many studies in the literature where low reaction times are used to produce scaffolds from different polymers [12],[13]. It can be seen that the gels produced by this procedure are more stable and smoother as shown in Figure 1B.

#### 3.1 FTIR

The chemical structure of the chitosan and crosslinked chitosan gels at different temperatures was analyzed using FTIR. The FTIR spectra of the samples are demonstrated in Figure 2. The spectra show the typical bands of chitosan at 2925 and 3445  $\text{cm}^{-1}$  ( $-\text{NH}_2$ ) and carbonyl group band at 1477  $\text{cm}^{-1}$ .

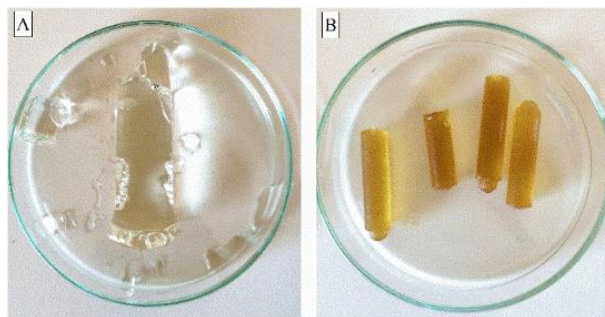


Figure 1. Gels with different crosslinking reaction times (A: 24 hours at room temperature, B: 20 hours at refrigerator after 4 hours at room temperature)

The crosslinking reaction between the aldehyde groups of glutaraldehyde and free amino groups of chitosan was proven by the existence of the peak at 1646  $\text{cm}^{-1}$  from the imine bonds  $\text{N}=\text{C}$  [14]. This peak was observed for all the crosslinked chitosan gels.

The FTIR spectrum of pure chitosan and gels revealed that the C-OH stretching vibration at 1073  $\text{cm}^{-1}$  for pure chitosan shift to 1037  $\text{cm}^{-1}$  after crosslinking reaction with GA. The intensity of the peaks located at 1037 and 1646  $\text{cm}^{-1}$  increase with increasing reaction temperature. The increasing band intensity for high temperatures may be related to the crosslinking degree of chitosan gels.

Furthermore, the  $\text{CH}_3$  bending vibration at 1363  $\text{cm}^{-1}$  and C-O-C bending vibration at 1155  $\text{cm}^{-1}$  were observed in the spectrum of chitosan, but not in the spectrum of crosslinked chitosan [15]. In contrast, the band at 1559  $\text{cm}^{-1}$  (amide II) was found in the spectra of GA crosslinked chitosan, but not in the spectra of pure chitosan.

Also, the formation of cross-links between chitosan and GA causes a color change of the chitosan gels from transparent to yellows [16].

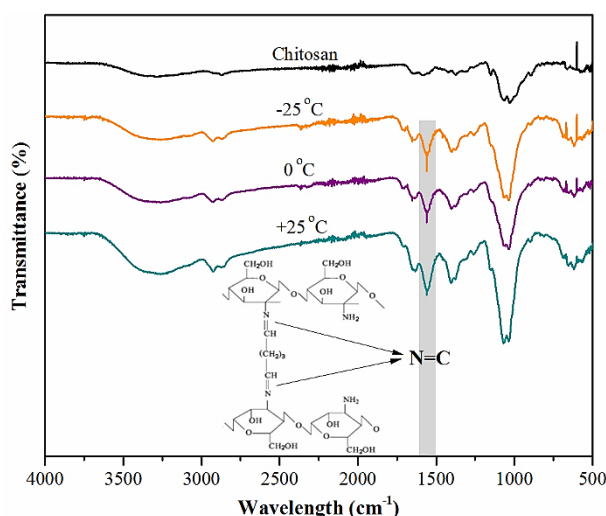


Figure 2. FTIR spectra of chitosan and crosslinked chitosan gels

### 3.2 SEM

Pore size and porosity design of scaffolds are considered as important parameters for tissue engineering applications. Figure 3 shows a comparison of the pore morphology and pore size distribution of the produced chitosan cryogels and hydrogel. The images demonstrated that the cryogels exhibited an interconnected pore morphology as seen in Figure 3A and B. The steps of the cryogelation process can be sorted as phase separation with ice-crystal formation, crosslinking and polymerization followed by thawing of ice-crystals to form an interconnected porous cryogel network [7]. The microenvironment is important for cell behavior because cell attachment, infiltration, proliferation and differentiation were affected interconnected pore structure of the cryogels [17].

The pore size distribution of cryogels prepared at  $-25$  and  $0^{\circ}\text{C}$  was shown in Figure 3A and B, where the mean pore size was calculated as  $21.57$  and  $24.22\ \mu\text{m}$ , respectively. Although the average pore size of the cryogels was not directly affected by the cooling temperature, the pore size distribution diagram showed that the cryogels produced at  $-25^{\circ}\text{C}$  were relatively regular (Figure 3). This is due to the formation of ice crystals at  $-25^{\circ}\text{C}$ . The ice crystals formed acted as pore-forming agents during the crosslinking process. Another reason is that it may contribute to the formation of more regular pores by stabilizing the polymer network [18].

The inner matrix of the hydrogel was also explored by SEM as seen in Figure 3C. The hydrogel surface displayed thin polymer walls with irregular pores. The mean pore size diameter of hydrogel was  $38.95\ \mu\text{m}$  with a wide distribution of pore size.

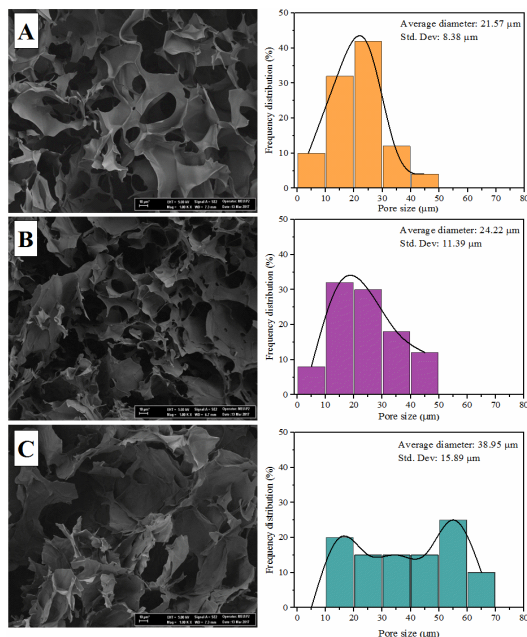


Figure 3. SEM images and pore size distribution diagram of chitosan gels (A:  $-25^{\circ}\text{C}$ , B:  $0^{\circ}\text{C}$  and C:  $+25^{\circ}\text{C}$  at  $1000\times$  magnification)

### 3.3 Swelling and Degradation Behaviors of Gels

Swelling behavior is an important parameter which influences the physical and chemical properties of the scaffolds after and before implantation and supplies of nutrients and oxygen to the interior regions [19].

The swelling ratios of the produced gels are presented in Figure 4. The samples were monitored and the duration of the equilibrium was determined as approximately 20 min. All gels have the ability to retain more water than their dry weight due to the porous and hydrophilic structures [20].

The degradation of polymeric network is another significant parameter for determination of how far a scaffold can withstand to support tissue regeneration or formation [20]. The weight loss of the gels was demonstrated in Figure 5 by the *in vitro* degradation test for 21 days. The results were related to the water uptake capacity of gels as determined in swelling ratio analysis. As the swelling ratio increased more water was absorbed by the gels, which resulted in a faster degradation rate.

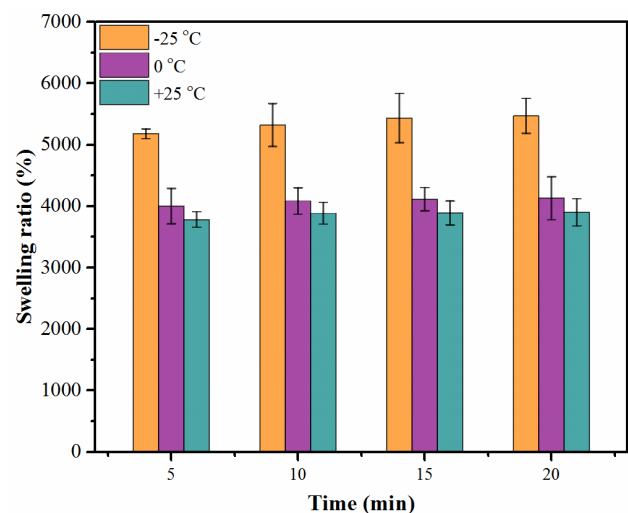


Figure 4. Swelling ratio of chitosan gels

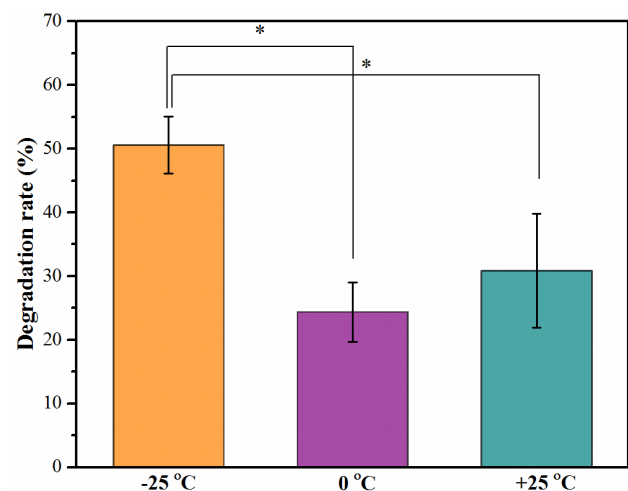


Figure 5. Degradation rate of chitosan gels [Asterisk (\*) indicates significant statistical difference ( $p \leq 0.05$ , compared between gels)]

#### 4. Conclusions

Chitosan gels were fabricated by crosslinking of chitosan in the presence of a crosslinker at different reaction temperatures. The synthesis and characterization of the gels were successfully shown. FTIR spectrum of scaffolds proves crosslinking reaction between the free amino groups of chitosan and aldehyde groups of glutaraldehyde. Characterization studies have revealed that the surface morphology (pore size and pore size distribution), swelling and degradation profiles of produced cryogels and hydrogel are different. The synthesized cryogels exhibit an interconnected and homogeneous pore structure, while the hydrogel is an irregular pore structure with thin polymer walls. Comparing the swelling and degradation behavior of cryogels, it was observed that gel prepared at  $-25^{\circ}\text{C}$  showed a higher water uptake and weight loss rate than gels prepared at  $0^{\circ}\text{C}$  and  $+25^{\circ}\text{C}$ . This distinction is due to the basic difference in their pore morphology and wall thickness. Furthermore, the desired properties of chitosan gels such as swelling, degradation and morphology can be adjustable by changing the reaction temperature. The high-water uptake capacity and porous structure of the produced gels would enable to design novel scaffolds for use in potential applications in tissue engineering and biomedical field.

#### Nomenclature

<i>DR</i>	: Degradation rate
<i>ECM</i>	: Extracellular matrix
<i>FTIR</i>	: Fourier Transform Infrared Spectrometer
<i>min</i>	: minute
<i>SEM</i>	: Scanning electron microscopy
<i>SR</i>	: Swelling ratio
<i>Std. dev</i>	: Standard deviation
<i>Wd</i>	: Degradation weight
<i>Wf</i>	: Final weight
<i>Wi</i>	: Initial weight

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