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Optimization Studies of Cryogel Scaffolds Prepared Using Different Chitosan and Polyvinyl Alcohol Ratios

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ABSTRACT

Cryogels are scaffolds structured with interconnected porous matrices produced from frozen solutions of monomeric or polymeric initiators. These scaffolds are seen as unique candidates with desirable properties for the different biomedical fields including tissue engineering, wound dressing, and drug delivery systems. In this study, polyvinyl alcohol (PVA) and chitosan (CS) were used to fabricate PVA:CS composite cryogels. Cryogels prepared at different polymer ratios were evaluated in terms of chemical structure, morphology, porosity, and swelling ratio. The chemical structure of composite cryogels was determined using Fourier-Transform Infrared Spectroscopy (FTIR). The interconnected pore morphology was observed using Scanning Electron Microscopy (SEM). Porosity and swelling ratio values were determined based on the weight change of the cryogels. All samples retained their shape during swelling experiments and exhibited swelling ratios in the range of about 3000-6000%. In general, all samples exhibited a porous structure, and it was revealed that porosity and other properties differ according to the ratio of each polymer in the scaffolds.

Farklı Kitosan ve Polivinil Alkol Oranları Kullanılarak Hazırlanan Kriyojel Doku İskelelerinin Optimizasyon Çalışmaları

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ÖZ

Kriyojeller, monomerik veya polimerik başlatıcıların donmuş çözeltilerinden üretilen birbirine bağlı gözenekli matrislerle yapılandırılmış yapı iskeleleridir. Bu yapı iskeleleri, doku mühendisliği, yara örtü ve ilaç salım sistemleri dahil olmak üzere farklı biyomedikal alanlar için arzu edilen özelliklere sahip benzersiz adaylar olarak görülmektedir. Bu çalışmada, polivinil alkol (PVA) ve kitosan (CS) polimerleri kullanılarak PVA:CS kompozit kriyojelleri üretilmiştir. Farklı polimer oranlarında hazırlanan kriyojeller kimyasal yapı, morfoloji, gözeneklilik ve şişme oranı açısından değerlendirilmiştir. Kompozit kriyojellerin kimyasal yapısı Fourier Dönüşümlü Kızılötesi Spektroskopisi (Fourier-Transform Infrared Spectroscopy, FTIR) kullanılarak belirlenmiştir. Birbirine bağlı gözenek morfolojisi, Taramalı Elektron Mikroskobu (Scanning Electron Microscopy, SEM) kullanılarak gözlemlenmiştir. Porozite ve şişme oranı değerleri, kriyojellerin ağırlık değişimi esas alınarak belirlenmiştir. Tüm örnekler şişme deneyleri esnasında şeklillerini muhafaza etmiş ve yaklaşık %3000-%6000 aralığında şişme oranları sergilemişlerdir. Genel olarak tüm numuneler gözenekli bir yapı sergilemiş ve gözeneklilik dahil diğer özelliklerin doku iskelesi yapısındaki her bir polimerin oranına göre farklılık gösterdiği ortaya

konmuştur.

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1. Introduction

Hydrogels are three-dimensional (3D) network structures which are able to retaining large amounts of water without dissolving. They can be prepared by different polymerization methods using both chemical and physical crosslinking routes. Owing to their excellent features and ability to imitate the biological environment, they are widely used in tissue engineering applications such as regenerative medicine, wound healing and drug delivery (Savina et al., 2021). Hydrogel scaffolds for tissue engineering applications need to mimic the extracellular matrix (ECM) in a way that can promote cell attachment and growth (El-Sherbiny and Yacoub, 2013). This requires a porous scaffold structure with a high degree of pore connectivity that can support cell migration, proliferation and metabolic activity and provide adequate mechanical strength and stability. Hydrogel matrices can be either non-porous (typically having only small pores in the tens of nm range for the gel network) or macropores (typically in the range of 10–500 μm) (Sergeeva et al., 2019). Traditional techniques for making 3D porous hydrogel scaffolds include solvent casting/particle leaching, fiber bonding, gas foaming, phase separation, reswelling and freeze drying (Annabi et al., 2010; Sornkamnerd et al., 2017). In addition to these methods, another popular method especially in the last quarter is the cryogelation method, which is based on freezing the solvent to create pores in the hydrogel material. Hydrogels with an inherent interconnected macro-porous structure prepared by this method are called cryogels or cryotropic hydrogels (Henderson et al., 2013). The details of the cryogelation method can be listed as follows: First, a homogeneous mixture of monomers/small molecule precursors or polymers in a suitable solvent (water and other solvents with a suitable melting/freezing temperature) is prepared. Afterwards, the prepared solution is cooled below the freezing point of the solvent, thereby forming ice crystals. Here, the solvent freezes to solidify and forms porogens within the structure, allowing the polymer network to form around these ice crystals, template the porous structure of the final cryogel. The last frozen structure is thawed at room temperature, the ice crystals melt and cryogel material is formed with an interconnected, macroporous network surrounded by a highly dense polymer wall (Henderson et al., 2013; Rogers and Bencherif, 2019). This structure, unlike conventional nanoporous hydrogels, allows unhindered diffusion of solutes of almost any size, survival of cells, and facilitates cell migration and tissue ingrowth (Razavi et al., 2019).

Many synthetic and natural polymers can be used to fabricate cryogels with different properties. For example, chitosan (CS), collagen, gelatin, silk, starch, cellulose and pectin have been used as natural polymers while poly(vinyl alcohol) (PVA), poly(ethylene glycol), poly(ε-caprolactone), poly(glycolic acid) and poly(lactic acid) have been used as synthetic polymers. Both polymer groups have significant advantages. Naturally derived polymers are of great importance because of their easy

availability, safe use, biocompatibility, biodegradability, regulating cellular interactions with minimal immune response, and low toxicity (Akgönüllü et al., 2020). Synthetic polymers are also preferred because of their ability to be prepared with the desired molecular weight and properties, their easy processing and their mechanical properties. The ideal is the production of functional scaffolds in composite structure by combining the advantages of both polymer classes. In this way, important features coming separately from natural and synthetic polymers will be combined, deficiencies will be eliminated, and cryogel scaffolds with different properties will be produced for different application areas by changing polymer ratios and types.

Based on this approach, it is aimed to produce composite cryogel scaffolds for different tissue engineering applications by combining CS and PVA. CS is a natural polymer derived from chitin, known as one of the most abundant amino-polysaccharide in nature. It is frequently preferred because it is antibacterial, biocompatible and biodegradable (Demir et al., 2016). PVA is a water-soluble synthetic polymer that possesses excellent mechanical properties. It is approved by Food and Drug Administration (FDA) due to its biocompatibility and biodegradability (Ceylan et al., 2017).

In this study, the variability of the morphological structure and physical properties of the final cryogels formed by using different ratios of CS and PVA has been demonstrated. In tissue engineering applications, it is expected that the properties of the produced scaffolds according to the type of region to be implanted (such as bone, soft tissue or cartilage tissue) will be different. Therefore, it is important to diversify the features of scaffolds by changing the polymer ratios. To observe this, the swelling ratio, porosity, pore size and chemical properties of composite scaffolds prepared at different ratios were characterized using a series of physicochemical analyzes. Composite cryogel scaffolds prepared using biocompatible and biodegradable polymers are assumed to be a suitable scaffold for potential tissue engineering applications based on their interconnected porous structure, chemical structure, high water holding capacity and physical strength.

2. Material and Method

2.1. Materials

Polyvinyl alcohol (99+% hydrolyzed, average mol wt 89.000-98.000 g/mol) and chitosan (low molecular weight, 50.000-190.000 Da based on viscosity) polymers were obtained from Sigma Aldrich, USA. Glutaraldehyde (25% in H_2O) as the crosslinking agent, acetic acid (glacial) as the solvent and ethanol (absolute) were purchased from Merck, Germany. During experiments, preparing solutions, dilutions and washing steps were carried out using distilled water.

2.2. Production of Cryogel Scaffolds

Different ratios of chitosan natural polymer and PVA synthetic polymer were used in the production of scaffolds. The cryogels were prepared with a total polymer content of 2% by weight and

PVA:CS ratios of 70:30, 40:60, 30:70, 20:80, 10:90 (v:v), respectively. The PVA solution was prepared by dissolving the calculated amount of polymer in distilled water at 90°C, while the CS solution was prepared by dissolving the calculated amount of polymer in 6% (v/v) acetic acid solution. The separately prepared PVA and CS solutions were brought together in the volume ratios specified above and mixed until the solutions became homogeneous. After homogenization, 2 mL of glutaraldehyde solution (3%, v/v) was added to the 10 mL of each composite polymer solution. The mixture was quickly filled into 2,5 mL plastic syringes and incubated for 2 hours in a cooling thermostat (Wisd Lab. Inst., WiseCircu WCR–P6 CFC 404 A, Germany) set at two different temperatures (-8°C) for freezing to occur. Then, the frozen samples were allowed to stand in refrigerator at same temperatures for 24 hours. Finally, the frozen samples in the syringe molds were thawed to room temperature. The obtained samples were washed 3 times with distilled water in 1000 mL beakers to remove the unreacted ingredients and then were completely dried by using a freeze-dryer (Labconco, FreeZone Benchtop Freeze DrySystem-7670531, USA). The dried cylindrical samples of 3 mm height were cut and stored for further analysis.

In order to distinguish the samples easily in the continuation of the study, the names given to the samples and their contents are summarized in Table 1.

Table 1. The sample names and their polymer contents

Sample name (PVA:CS)	PVA amount, g	CS amount, g
70:30	0,14	0,06
40:60	0,08	0,12
30:70	0,06	0,14
20:80	0,04	0,16
10:90	0,02	0,18

*PVA: polyvinyl alcohol, CS: chitosan

2.3. Physicochemical Characterization

The physicochemical properties of the gel scaffolds produced in different polymer ratios were determined by chemical and morphological analyzes. Crosslinking of polymers in the presence of a crosslinking agent at subzero temperature was primarily evaluated using FTIR spectrophotometer (PerkinElmer, FT-IR/FIR/NIR Frontier-ATR, USA). The spectrum of samples were recorded within the wavelength range of 400 to 4500 cm^{-1}. Morphologically, the produced cryogels were evaluated by determining their microscopic and porosity properties. Images were obtained at different magnifications by using SEM (FE-SEM Zeiss/Supra55, Quanta 400F Field Emission, USA) operated at 5 kV to visualize the internal structures of the samples after coating with platinum. In addition, the mean pore diameter was calculated by taking the average of the pore sizes measured as a result of processing SEM images with the Image-J Software.

The porosity (PR) values of the cryogels were calculated using ethanol penetration method (Choudhury et al., 2015; Demir et al., 2020). According to method, pre-weigthed dry cryogel speciemens (W_{dry}) were immersed in ethanol ($\rho_{ethanol}$ = 0,987 g/mL) filled falcon tubes and incubated for 120 min. The sample was then removed from the tube, the excess ethanol on the surface of the cryogel was blotted using filter paper and the wet cryogel mass (W_{wet}) was recorded. Diameter and height of the cylindirical scaffold were used to calculate the sample volume (Vol_c). The percent porosity was calculated as:

$$PR, \% = [(W_{wet}-W_{dry})/\rho_{ethanol})/(Vol_c)] \times 100 \quad (1)$$

2.4. Swelling Measurements

The swelling measurements of the cryogels were performed using gravimetric difference after immersing the cryogels in distilled water. To do this, first, weight of dry samples was recorded (W_0) and then scaffolds were immersed in distilled water for a certain time. The swollen cryogels were removed from the water at pre-determined time intervals, excess water on their surfaces was removed with filter paper, and then their weights were recorded (W_i). The swelling ratio of cryogels was calculated with respect to time by the following equation:

$$\text{Swelling ratio}, \% = [(W_i - W_0)/W_0] * 100 \quad (2)$$

3. Results and Discussion

3.1. Physical Evaluation of Cryogels Produced at Different PVA:CS Ratio

In this study, the polymer-based scaffolds prepared under cryotropic conditions were planned to be used for potential tissue engineering applications. In the light of characterization analysis, different polymer ratios that may cause changes in the final material were investigated in terms of pore diameter, porosity, morphology, chemical structure, swelling and degradation rates. As the first step, the cryogels produced by changing the ratios of PVA and CS polymers were evaluated physically as presented in the Table 2. The gels were produced successfully using different polymer ratios. As the chitosan ratio increases, the color of the cryogels changes from light yellow to dark yellow. This is due to the yellowish color formed by the crosslinking of chitosan with glutaraldehyde (Demir et al., 2016; Pavoni et al., 2021).

Chitosan is a remarkable polymer in tissue engineering applications due to its high biocompatibility and antimicrobial properties and its most important disadvantage is low mechanical properties. PVA is ideal for bone and cartilage tissue engineering applications as it is a biocompatible polymer with high mechanical strength. Therefore, it is aimed to obtain composite scaffolds with stronger properties by using these two polymers together.

As seen in Table 2, the cryogels exhibited a harder and brittle structure at high PVA ratios, while they became more elastic and spongy with the addition of chitosan. The elastic and spongy structure of cryogel scaffolds is largely the result of porosity. The cryogelation technique used in scaffold

fabrication is known to exhibit an open interconnected 3D porosity and this morphology is essential for cell nutrition, proliferation, and migration for tissue vascularization and new tissue formation (Loh and Choong, 2013; Tripathi et al., 2009). In our study, the porosity values were determined using ethanol penetration method and results are presented in Table 2. It was observed that the % porosity value of the cryogels increased approximately 2 times with the addition of chitosan (from 38,25±2,20 to 77,02±4,60). Similarly, Kanimozhi et al. found the porosity of CS/PVA scaffolds was about 81% (Kanimozhi et al., 2016).

Table 2. Evaluation of cryogels based on their physical appearance and their porosity (%) values

Sample	Basic Properties	Pysical Appearance	Porosity, %
70:30	Light yellow, homogeneous, brittle		38,25±2,20
40:60	Yellow, homogeneous, brittle		46,56±2,61
30:70	Yellow, homogeneous, brittle		55,24±3,25
20:80	Dark Yellow, homogeneous, elastic		63,55±5,12
10:90	Dark Yellow, homogeneous, spongy		77,02±4,60

3.2. Chemical Analysis of Cryogels

The characteristic peaks of CS and PVA polymers used in the production of scaffolds and the chemical changes in their structures as a result of cross-linking of these polymers in the presence of glutaraldehyde were revealed by FTIR analysis. Figure 1 shows the FTIR spectrum of cryogel scaffolds prepared at different polymer ratios. Since the scaffold content is the same in all samples, only the amount changes, in general, the same peaks are located at different intensities in all samples. There is an absorption peak at 3328 cm^{-1} and a vibrational band at 2930 cm^{-1} that can be associated with C-H stretching of alkyl groups, expressing the intermolecular hydrogen bonding and –OH stretching vibration due to the presence of PVA. The presence of CS is seen as a broad band at 3320 cm^{-1} caused by -OH stretching and a band at 1560 cm^{-1} due to NH bending (Amide II). In the spectra, the sharp and wide dense peaks at 3437 cm^{-1} shows the –NH and -OH symmetrical stretching frequency, while the more intense peaks at 1610 and 1010 cm^{-1} show the –C=N and -C-O stretching

frequencies, the formation of the imine group. also explains the crosslinking between CHI and PVA with glutaraldehyde.

When we examine the effect of the polymer ratio change on the FTIR spectra, it is seen that there are changes in the peak intensities, although there is no change in the locations of the peaks, since the same composition (PVA, CS and glutaraldehyde) is used in all samples. There is an increase of intensities from the main absorption bands related to chitosan, for instance amine region (around 1560 cm^{-1}), as its content was increased from 30% (70:30, PVA:CS) to 90% (10:90, PVA:CS). In addition, a slight shift was observed in the band associated with the bending vibration of the C=H group with the increase in the amount of chitosan. This can be explained by the fact that covalent chemical bonds preferentially react with the amine groups of chitosan and less interact with the hydroxyl groups of PVA, since the blend crosslinking reaction is carried out at acidic pH. Similar results were obtained for glutaraldehyde crosslinked chitosan-PVA blends studied by Parida and others (Parida et al., 2011).

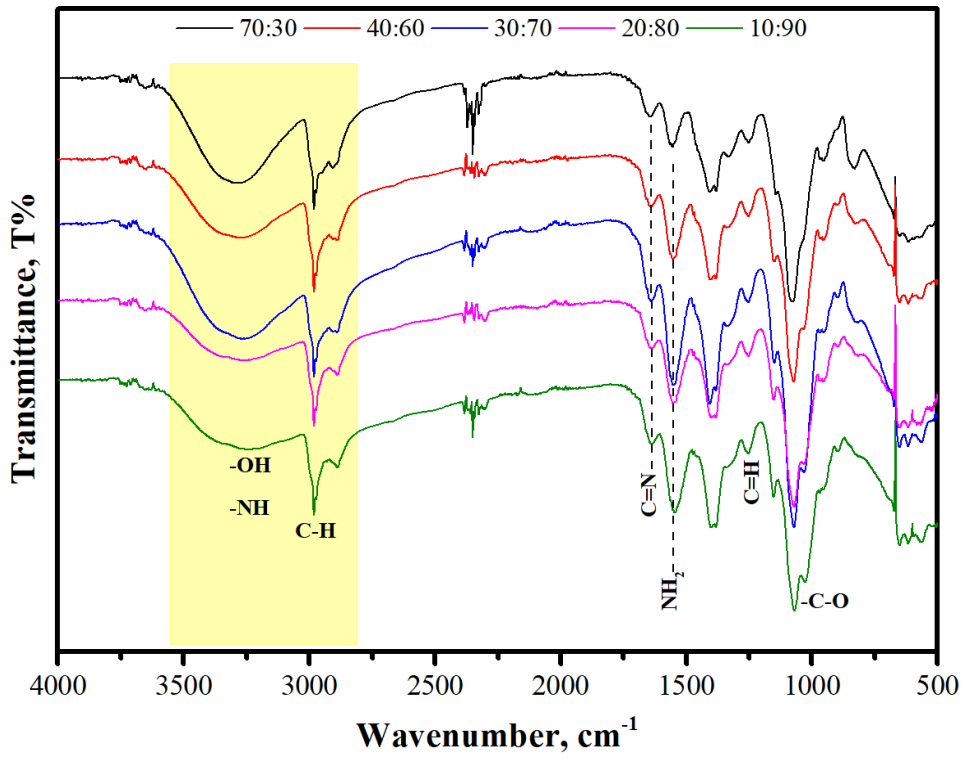

Figure 1. FTIR spectra of PVA:CS cryogels prepared at different ratios

3.3. Morphological Evaluation

In tissue engineering applications, porosity of scaffolds is an important feature for cells to provide nutrients, remove waste, adhere to the inner parts of the scaffold, spread, reproduce, and vascularization (Kumar, 2016). For this reason, scaffolds should have a structure with interconnected, controllable, high porosity and appropriate pore sizes in order to provide an environment for cell growth and formation of new tissue matrix.

Cryogels are candidate biomaterials for scaffolds with desired morphology with their interconnected porous matrix structures. In the cryogelation process, the matrix structures formed by freezing the

aqueous polymer solution and crosslinker mixture at subzero temperatures, when brought to room temperature and melted, the voids remaining as a result of the melting of the ice crystals form the pores, while the polymers connected with the crosslinker form the pore walls (Bölgen et al., 2020; Ceylan et al., 2017).

When the SEM images presented in Figure 2 are examined, it can be observed that the dark areas represent the pore spaces, while the light gray colored surfaces represent the polymer walls. All samples exhibited a heterogeneous morphology with pores at both macro and micro scales. Although it is a heterogeneous structure, it can be said that the pore size of the polymers increases with the increase in the amount of chitosan. An average pore size was calculated for each cryogel by averaging different pore measurements from at least 20 points on SEM images at low magnifications. The pore sizes are 20,82±2,87 µm for 70:30, 31,40±4,30 µm for 40:60, 35,55±5,51 µm for 30:70, 44,23±7,19 µm for 20:80 and 61,07±16,74 µm for 10:90 cryogel sample.

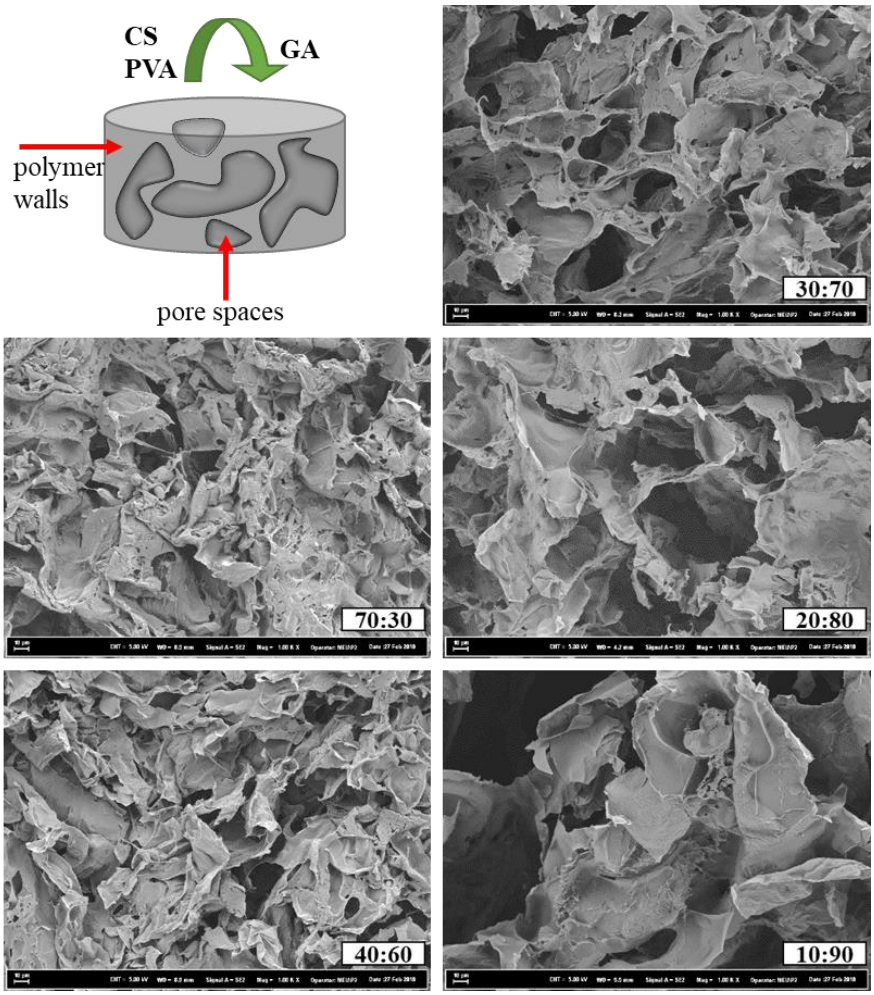

Figure 2. Representative drawing of the morphological structure of the cryogels and SEM images of PVA:CS cryogels prepared at different polymer ratios

3.4. Swelling Results of Cryogels

One of the characteristic features of cryogels is that they absorb high volumes of water due to their porous structure, release water without deteriorating their physical structure when a mechanical force is applied, and swell by reabsorbing water when placed in an aqueous environment. Repeating this process several times will not cause the structure of the cryogel to deteriorate. Similarly, the cryogels obtained in this study exhibited high water holding capacity without any degradation in their physical structure. These features allow the cryogel scaffolds to fill the space in the damaged area by quickly absorbing body fluids after implantation into living organism. The swelling behavior of PVA:CS composite cryogels produced at -8°C cryogelation temperature with different polymer ratios is presented in Figure 3. During the first 5 minutes, very rapid water uptake was observed by all the samples, then it slowed down and reached equilibrium at the end of approximately 120 minutes. In addition, it is seen that the swelling ratio of the cryogels increases as the chitosan ratio in the total polymer amount increases. The water uptake capacity of PVA:CS composite cryogels can be related to the chemical structure of PVA and CS and morphology of cryogels. When examined in terms of chemical structure, the hydroxyl (-OH) groups of PVA and the amino ($-NH_2$) groups of CS can interact with water molecules and form hydrogen bonds. Morphologically, composite cryogels which exhibit high pore structure may have facilitated the diffusion of water. Scaffolds with high PVA additives may have exhibited less swelling, especially as a result of the formation of a tighter structure with PVA additive and low pore diameter and porosity values. Similar results were obtained in the study performed by Tang et al. They investigated the structural characterization of thermosensitive PVA/Chitosan hydrogels. In the swelling experiments, they have seen that if the amount of chitosan is the same, the swelling rate decreases when the amount of PVA is increased. They explained the reason for this as the increase in PVA content causes a denser gel structure, which has a negative effect on the swelling ratio (Tang et al., 2007).

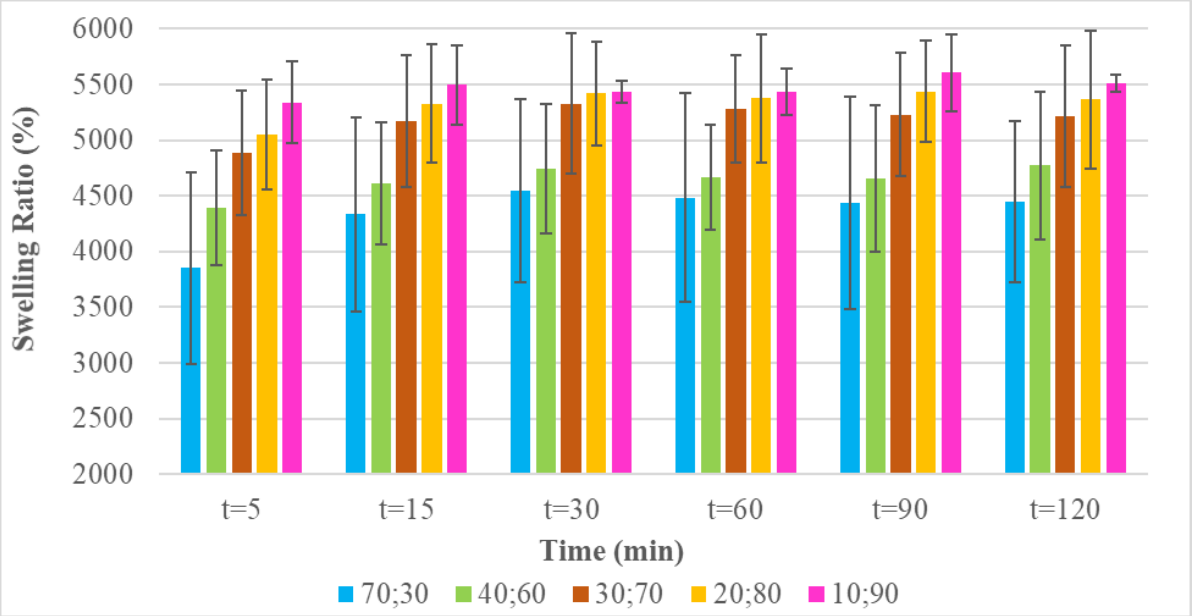

Figure 3. Swelling ratio of PVA:CS cryogels prepared at different polymer ratios

4. Conclusions

In our previous studies, we fabricated different types of scaffolds such as film, hydrogel, cryogel, electrospun nanofiber and microspheres using the composition of different syntetic and natural polymers (chitosan, collagen, gelatin, starch, pectin, PVA, PCL) for tissue engineering applications. In the current study, we aimed to perform preliminary optimization studies of cryogels with optimum properties according to the application area, in a composite scaffold to be prepared using a selected natural and synthetic polymer. For this purpose, a series of cryogel scaffolds were produced by using different ratios of CS and PVA. The produced scaffolds were successfully chemically crosslinked in the presence of glutaraldehyde. Gels prepared in monolith structure showed different porosity, pore size and physical stability depending on the variation of polymer ratios. In addition, all gels exhibited high swelling ability due to to their interconnected pore structure. The results obtained will be used as a preliminary step in determining the optimum scaffold to be selected for future drug release, microbial and biological analyzes.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Authors' Contributions

The authors contributed equally to this study.

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