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Fish Skin-Isolated Collagen Cryogels for Tissue Engineering Applications: Purification, Synthesis, and Characterization

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Abstract: Tissue engineering aims regenerating damaged tissues by using porous scaffolds, cells, and bioactive agents. The scaffolds are produced from a variety of natural and synthetic polymers. Collagen is a natural polymer widely used for scaffold production because of its being the most important component of the connective tissue and biocompatibility. Cryogelation is a relatively simple technique compared to other scaffold production methods, which enables to produce interconnected porous matrices from the frozen reaction mixtures of polymers or monomeric precursors. Considering these, in this study, collagen was isolated from fish skin which is a non-commercial waste material, and scaffolds were produced from this collagen by cryogelation method. Pore morphology and protein structure of collagen was proven by SEM and UV-Vis analysis, respectively. Iso-electrical point of the protein was determined by zeta potential analysis. Amide A, Amide B, Amide I, Amide II and Amide III characteristic peaks of collagen was demonstrated by FTIR analysis. The yield of isolated protein was 14.53% and 2.42% for acid-soluble and pepsin-soluble collagen, respectively. Scaffolds were produced by crosslinking isolated acid soluble collagen with glutaraldehyde at cryogenic conditions. With FTIR analysis, C=N bond which belongs to the reaction between glutaraldehyde and collagen was found to be at 1655 cm^{-1}. It was demonstrated by SEM analysis that collagen and glutaraldeyhde concentration had significant effects on the pore morphology, diameter, and wall thickness of the cryogels, which in turn changed the swelling ratio and degradation profiles of the scaffolds. In this study, synthesis and characterization results of a fish skin isolated collagen cryogel scaffold that may be potentially used in the regeneration of damaged tissues are presented.

Keywords: Tissue engineering, collagen, isolation of collagen, scaffold, cryogel.

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INTRODUCTION

Tissue engineering is a discipline which aims to regenerate damaged tissues by using porous scaffolds and cells. Porous scaffolds that are produced from various polymers are used in regeneration of various organs and tissues. Interconnected pore structure of the scaffolds supports cell attachment, homogeneous cell distribution, differentiation, and proliferation [1]. Many natural polymers such as collagen have shown potential to be used in the fabrication of tissue engineering scaffolds [2]. Collagen is a natural polymer which is present in the extracellular matrix. Because of its being the most important component of extracellular matrix, and its biocompatibility and biodegradability, collagen is widely used in the preparation of tissue engineering scaffolds [3]. In recent years, researchers are searching for alternative natural sources of collagen and improved technologies for collagen isolation. Fish skin is a potential source of collagen. Therefore, skin collagen from several fish species have been extracted and characterized recently. Examples of those species are Baltic cod (*Gadus morhua*) [4], silvertip shark (*Carcharhinus albimarginatus*) [5], deep-sea redfish (*Sebastes mentella*) [6], striped catfish (*Pangasianodon hypophthalmus*) [7] and carp (*Catla catla* and *Cirrhinus mrigala*) [8]. Cryogelation is a rather simple scaffold production technique compared to other techniques. Cryogels produced by this technique have interconnected macropores that allow diffusion of nutrients and wastes as well as mass transport of nano- and microparticles, microorganisms and cells [9].

The aim of this study is extraction and characterization of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from the skin of shark fish and fabrication and characterization of collagen cryogel scaffolds for tissue engineering applications. Extracted collagens (ASC and PSC) were characterized by fourier transform infrared spectroscopy (FTIR), ultraviolet visible spectroscopy (UV-Vis), scanning electron microscopy (SEM) and Zeta potential analysis. Cryogels were synthesized by using ASC as the natural polymer and glutaraldehyde as the crosslinker. The chemical structure, swelling ratio, degradation profile, pore morphology, and pore size of collagen cryogels were demonstrated.

MATERIALS AND METHODS

Chemicals

KOH, NaOH, acetic acid (Glacial), and pepsin (from porcine gastric stomach mucosa) were purchased from Sigma Aldrich, USA. NaCl powder was obtained from Emsure, Germany. Butyl alcohol was obtained from Laba Chemie, India. Spectra/Por (12-14.000 Da, Cellulose) dialysis membrane was purchased from Spectrum Laboratories, USA. A cheese cloth used in filtration process was obtained from a local market in Gaziantep, Turkey. Distilled water was used in all experimental steps.

Isolation of collagen

ASC and PSC were isolated by using the method demonstrated by Benjakul *et al.*, with some modifications [10].

Fish skin preparation

Shark skin was a gift from a local fish market in Mersin, Turkey. The skin was washed with cold distilled water and then cut into small pieces (approximately 0.5 x 0.5 cm^2) by a pair of scissors. 50 grams of shark skin was treated with 0.1 M NaOH solution at 4 °C for 6 hours with continously stirring at 250 rpm in order to remove the non-collagenous compounds. The solution was replaced every two hours.

Isolation of ASC

After separating the non-collagenous proteins, the skin samples were mixed with 10% (v/v) butyl alcohol solution at a ratio of 1:10 (w/v) and continiously stirred for 48 hours at 4 °C to remove fat. The solution was replaced every 12 hours. After washing with cold water, fat-free skin was treated with 0.5 M acetic acid solution (skin/solution ratio was 1:15, w/v) for 24 hours and the extract was filtered by using the cheese cloth. The residue was re-extracted with 0.5 M acetic acid solution. Filtrates, which were very viscous, were combined. The filtrate was stirred for 24 hours, after addition of NaCl

powder to a final concentration of 2.6 M. The resultant precipitate was collected by centrifuging at 6000 rpm for 30 minutes and dissolved in a minimum volume of 0.5 M acetic acid solution, and dialyzed against 50 volumes of 0.1 M acetic acid solution for 24 hours at 4°C. It was then dialyzed against 50 volumes of distilled water for 24 hours. The dialyzate was freeze-dried by using a lyophilizer. The ASC was obtained.

Isolation of PSC

The remaining skin after ASC filtration was mixed with 0.5 M acetic acid solution [ratio of solid/solution 1:15 (w/v)] at 250 rpm for 24 hours after adding pepsin from porcine gastric stomach mucosa (20 units/g residue). The PSC was obtained followed by the same steps (filtration, centrifugation, dialysis and lyophilization) which were used in the isolation of ASC.

Characterization of isolated collagen

FTIR analysis

The obtained ASC and PSC samples were analyzed by FTIR. FTIR spectra in the range of 400- 4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1}.

UV-Vis Analysis

The ultraviolet spectra were recorded by using a UV-Vis (Specord 210 Plus, Analytik Jena, Germany). 1 mg of collagen (ASC or PSC) was dissolved in 2 mL of 0.5 M acetic acid solution and centrifuged at 5000 rpm for 10 minutes. The measurement was done in the wavelength range of 200-420 nm.

ζ - Potential Analysis

The zeta potential of ASC and PSC solutions was measured by zeta potential analyzer (Zeta sizer, Malvern, UK) [10]. The samples were mixed with 0.5 M acetic acid solution to a final concentration of 0.05% (w/v) and stirred at 4 °C until they were completely

dissolved. The ζ-potential of ASC and PSC solutions at different pH values (2, 3, 4, 5, 6) were recorded.

SEM Analysis

The surface morphology of platinium coated ASC, PSC and cryogel samples was examined by SEM (Supro 55, Zeiss, Germany). The avarage pore sizes of the samples were calculated after measuring the size of ten pores.

Cryogel synthesis

Cryogels were prepared by using ASC as the polymer and glutaraldehyde as the crosslinker. The calculated amount of collagen was dissolved in distilled water at three different concentrations (3.0 - 6.0 - 8.0; w/v). The prepared collagen solutions were mixed with varying concentrations (0.5 - 1.0 - 2.0% ; v/v) of aqeous glutaraldehyde solution (25%; v/v) and then poured into plastic syringes (2 mL). The solution was immediately immersed in cryostat, incubated at -12 °C for 2 hours and then placed in the freezer (at -16°C) for 24 hours. After the crosslinking reaction was completed, the frozen samples were thawed at room temperature and washed with distilled water until the unreacted ingredients were removed. The prepeared cryogels were then dried overnight at room temperature. Schematic illustration of collagen isolation and cryogel production is demonstrated in Figure 1. The effect of collagen and glutaraldehyde concentration on the properties of the cryogels was evaluated. When the collagen concentration changed, the glutaraldehyde concentration was kept constant at 0.5% (Table 1). And when the glutaraldehyde concentration changed the collagen ratio was kept constant at 8% (Table 2).

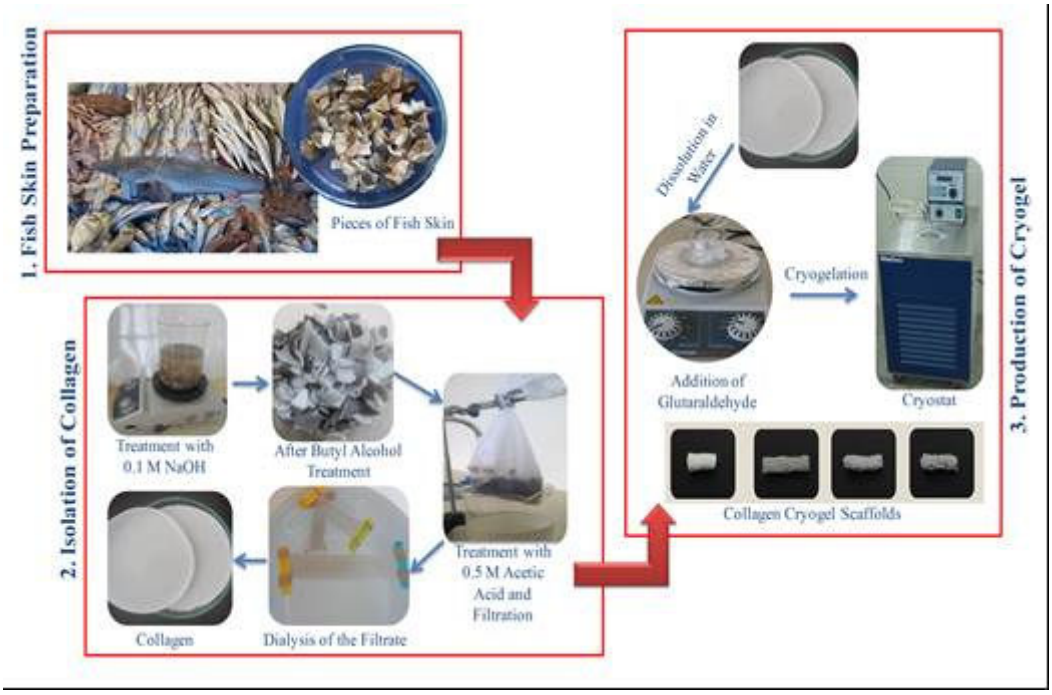

Figure 1. Schematic illustration of collagen isolation and cryogel production.

Table 1. Cryogels synthesized with different amounts of collagen.

Concentration of Collagen (%, w/v)	Concentration of Glutaraldehyde (%, v/v)
3.0	0.5
6.0	0.5
8.0	0.5

Table 2. Cryogels synthesized with different amounts of glutaraldehyde.

Concentration of Collagen (%, w/v)	Concentration of Glutaraldehyde (%, v/v)
8.0	0.5
8.0	1.0
8.0	2.0

Swelling ratio

The swelling behavior of collagen cryogels were determined according to a previously demonstrated method [11]. The dried cryogels were weighed (W_d). Then dried cryogels were kept in 50 mL distilled water at room temperature for 360 minutes. The excess

water on the surface of the cryogels was removed with a filter paper and the cryogels were then weighed (W_w). The ratio of swelling (SR%) was calculated using equation (1).

$$SR\% = [(W_w-W_d)/W_d]*100 \quad \text{(Eq.1)}$$

Degradation studies

Collagen cryogels were incubated at 37°C in 10 mL of distilled water at static conditions to measure their degradation rate. The samples were taken out from incubation after 42 days, dried at room temparature and weighed. The weight loss of samples were obtained according to equation (2):

$$\text{Weight Loss}\% = [(W_0-W_t)/W_0]*100 \quad \text{(Eq.2)}$$

where W_0 and W_t are the weights of cryogels' initial dry weight and dry weight after degradation, respectively.

RESULTS AND DISCUSSION

Yield of ASC and PSC

The yields of ASC and PSC calculated directly based on the initial weight of skin were 14.53% and 2.42%, respectively. The following analyses were conducted in order to characterize the properties of the collagen.

FTIR analysis of collagen

The FTIR spectras of ASC and PSC were shown in Figure 2 and depicted in Table 3. According to Wang et al. [12] the Amide A band of ASC and PSC occurs at wavenumbers of 3314.82 cm^{-1} and 3315.81 cm^{-1}, respectively, were associated with NH stretch coupled with hydrogen bond. Amide B band of those collagens at 2940.72 cm^{-1} and 2940.80 cm^{-1}, respectively, corresponds to asymmetrical stretch vibration of CH_2 [10]. Amide I, Amide II, and Amide III bands of ASC were found at 1652.10, 1550.21 and 1238.64 cm^{-1}, respectively. Those bands of PSC were found at 1658.30, 1552.90 and 1239.16 cm^{-1}. Amide I band that occurs in the range of 1600 - 1700 cm^{-1} shows the existence of stretching vibration of C=O or hydrogen bond coupled with COO- group. The result of

Amide II and Amide III bands were presumed that there were stretching N-H bending vibrations and C - H stretching.

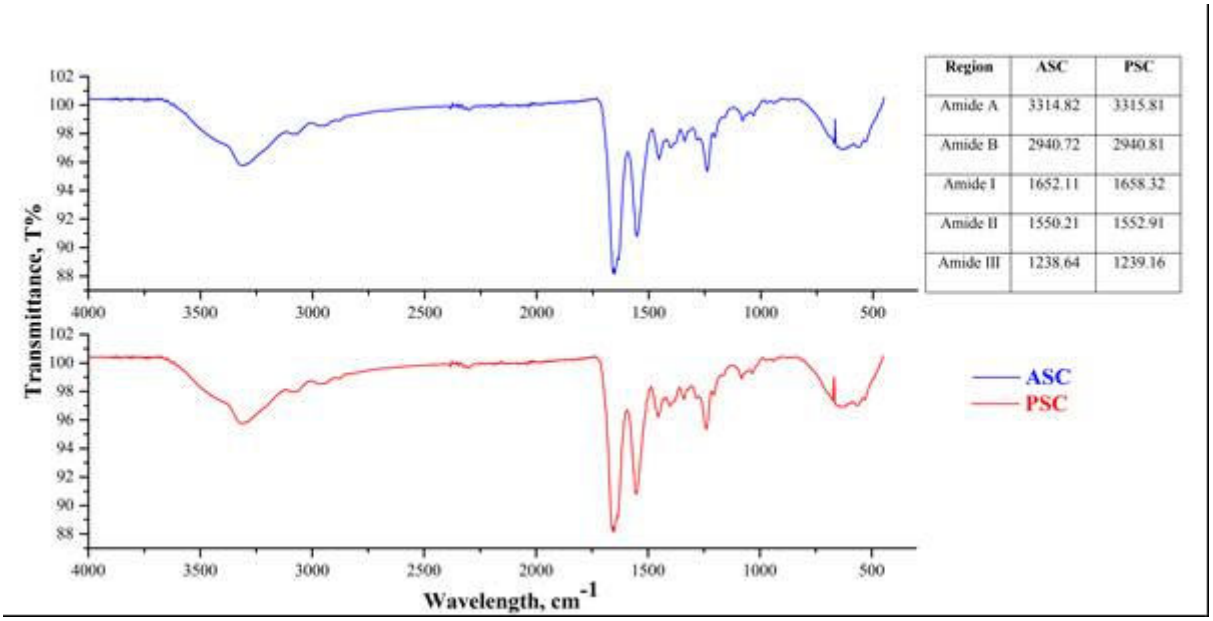

Figure 2. FTIR spectra of ASC and PSC.

Table 3. FTIR spectras peak wave numbers and assigments for ASC and PSC.

Region	Peak Wave Number (cm^{-1})		Assigment	References
	ASC	PSC		
Amide A	3314.82	3315.81	NH stretch coupled with hydrogen bond	Wang et al. [13]
Amide B	2940.72	2940.80	Asymmetrical stretch of CH_2	Benjakul et al. [10]
Amide I	1652.10	1658.30	Stretching vibration of C=O or hydrogen coupled with COO^-	Zhang et al. [14]
Amide II	1550.21	1552.90	Vibration of NH bending	Benjakul et al. [10]
Amide III	1238.64	1239.16	C-H stretching	Kittiphattanabawon et al. [15]

UV-Vis analysis

The maximum absorptions of ASC and PSC, were determined at the wavelengths of 230 - 240 nm region, as shown in Figure 3. This result is in agreement with the result of the

study presented by Huang *et al.* Moreover the absorbances at near 280 nm is related to the tyrosine in collagen [16].

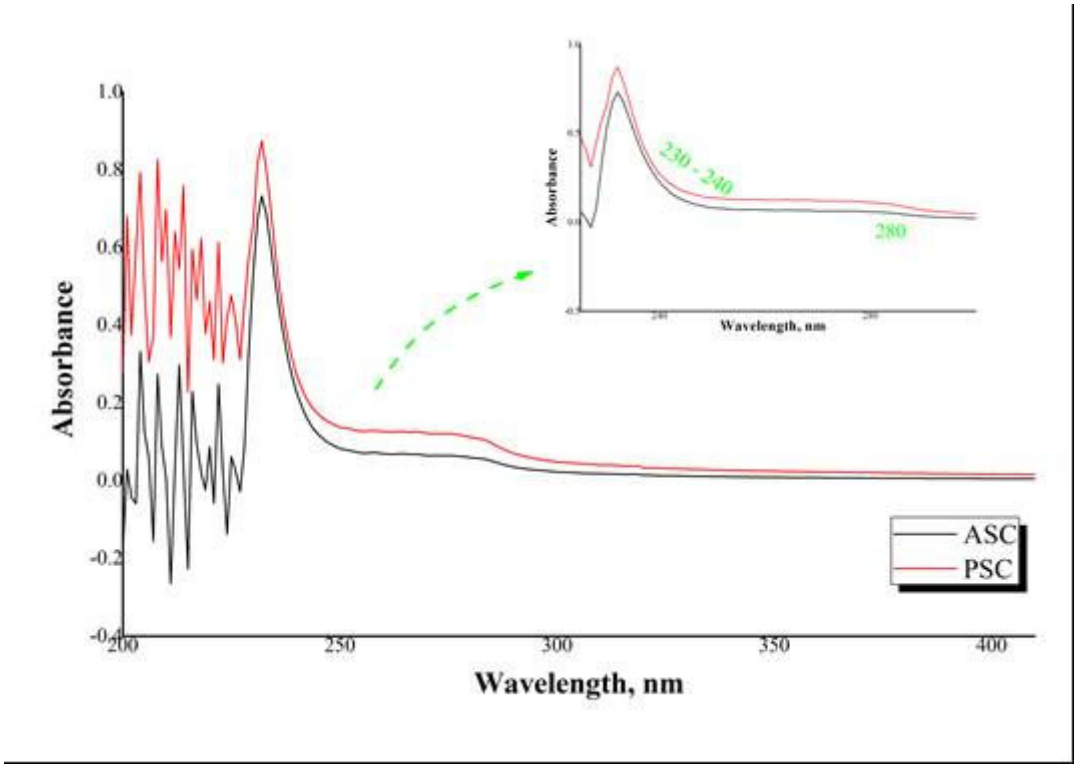

Figure 3. UV - Vis spectra of ASC and PSC.

ζ - Potential analysis

The zeta potential values of ASC and PSC solutions at different pH values (2, 3, 4, 5 and 6) were determined as shown in Figure 4. The zeta potential was zero at pH 4.8 for ASC and 5.9 for PSC. This result can be explained by the balance between positive and negative charges as reported by Bonner [17]. According to Vojdani, a protein in an aqueous system (like collagen in acetic acid solution) has a zero net charge at its isoelectric point [18]. However, the differences of surface charges of ASC and PSC might be explained by their various amino acid compositions.

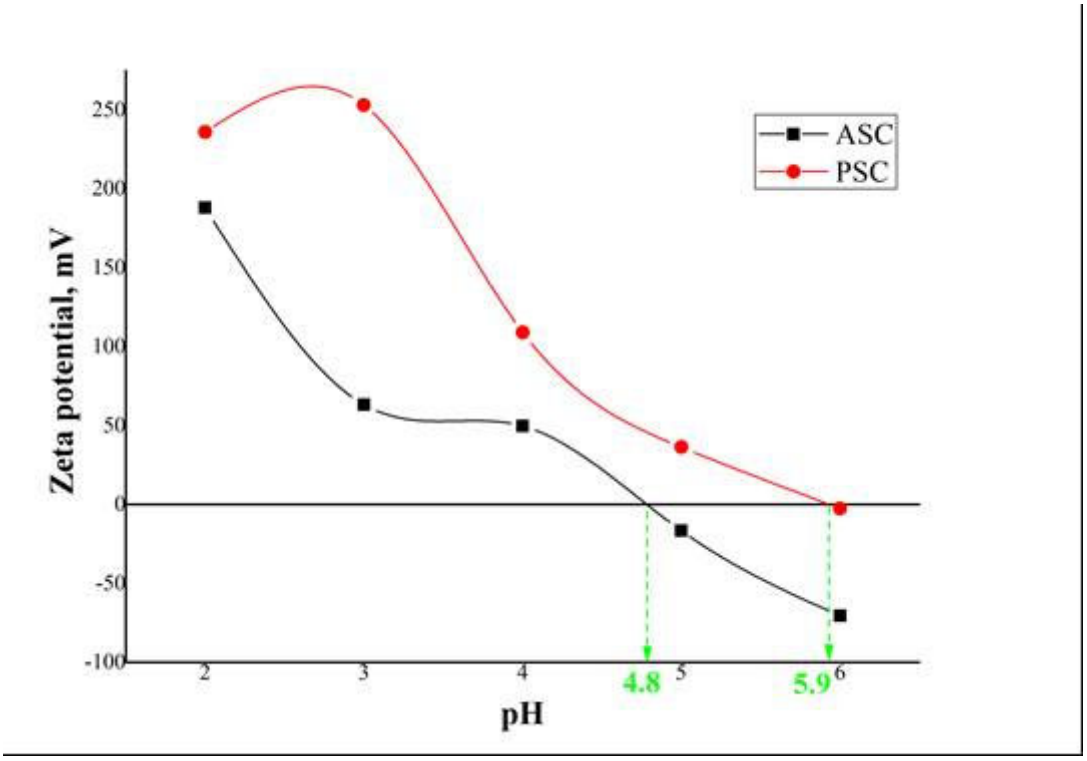

Figure 4. The zeta potential of ASC and PSC solutions at different pH values.

SEM analysis of collagen

The pore morphology of the lyophilized collagens (ASC and PSC) were quite similar when compared to each other. The average pore sizes of ASC and PSC samples were 61.071 ± 27.51 and 50.308 ± 22.89 μm (Figure 5).

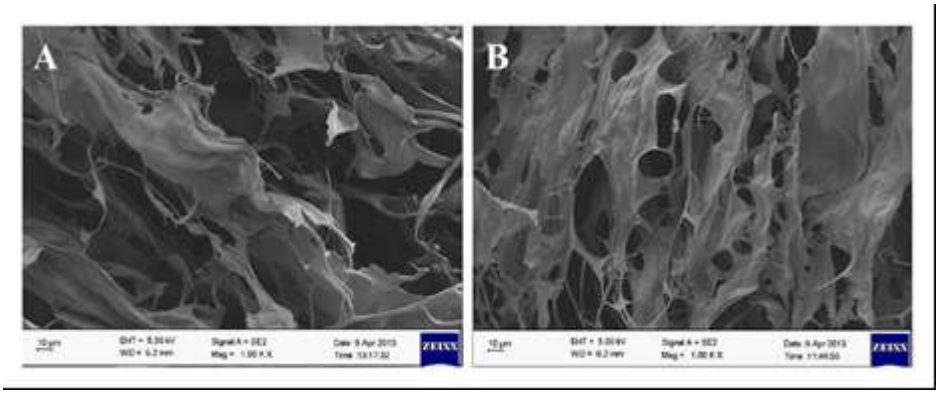

Figure 5. SEM images of ASC (A) and PSC (B) (at 1000x magnification).

Synthesis and characterization of cryogels

FTIR analysis

The chemical structure of the cryogels were analyzed by FTIR and the resulting spectra were demonstrated in Figures 6 and 7. The Amide B bands which belong to the asymmetrical stretch of CH_2 at near by 2400 cm^{-1} disappeared in FTIR of crosslinked collagen cryogels, compared to uncrosslinked collagen which may be due to the hindrance of the peak by glutaraldehyde crosslinking of collagen. The appearance of the peak at 1630 - 1648 cm^{-1} represents stretching vibrations of C=N in Schiff's base formed by the crosslinking reaction between the amide group of collagen and the aldehyde group of glutaraldehyde [7,19]. In addition, the peak at 2929 cm^{-1} of C-H streching are related to the aldehyde group of glutaraldeyde.

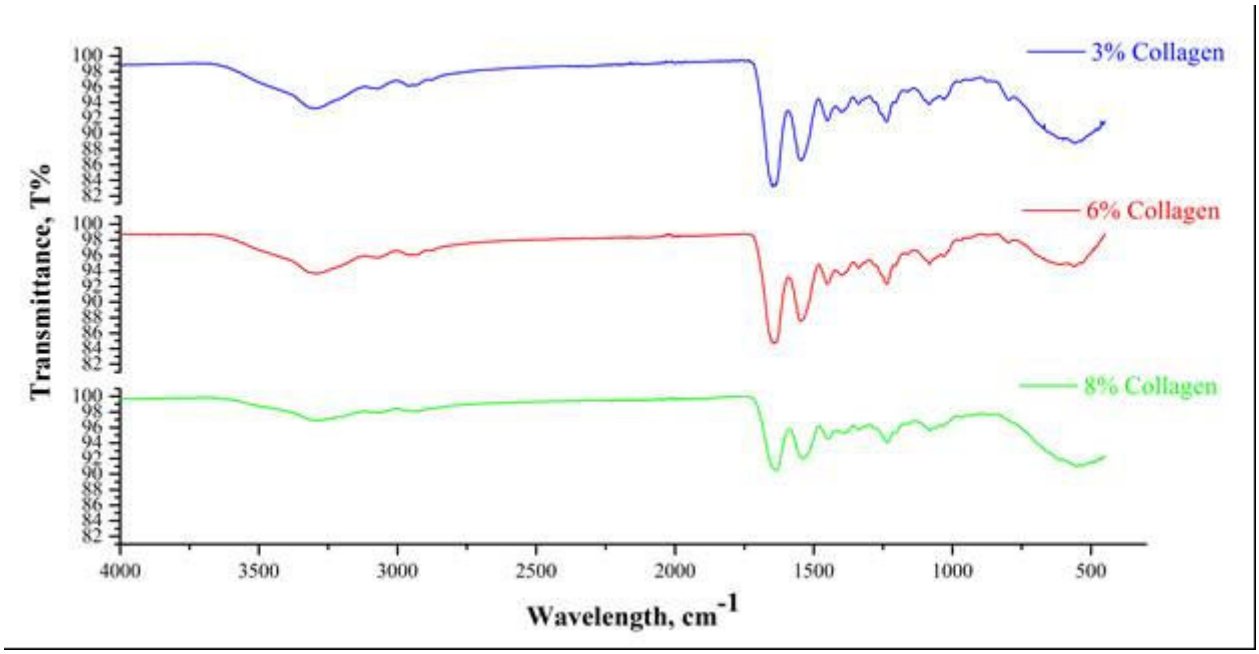

Figure 6. FTIR spectrum of cryogels prepared at different collagen concentrations while the glutaraldehyde concentration was kept constant at 0.5% (v/v).

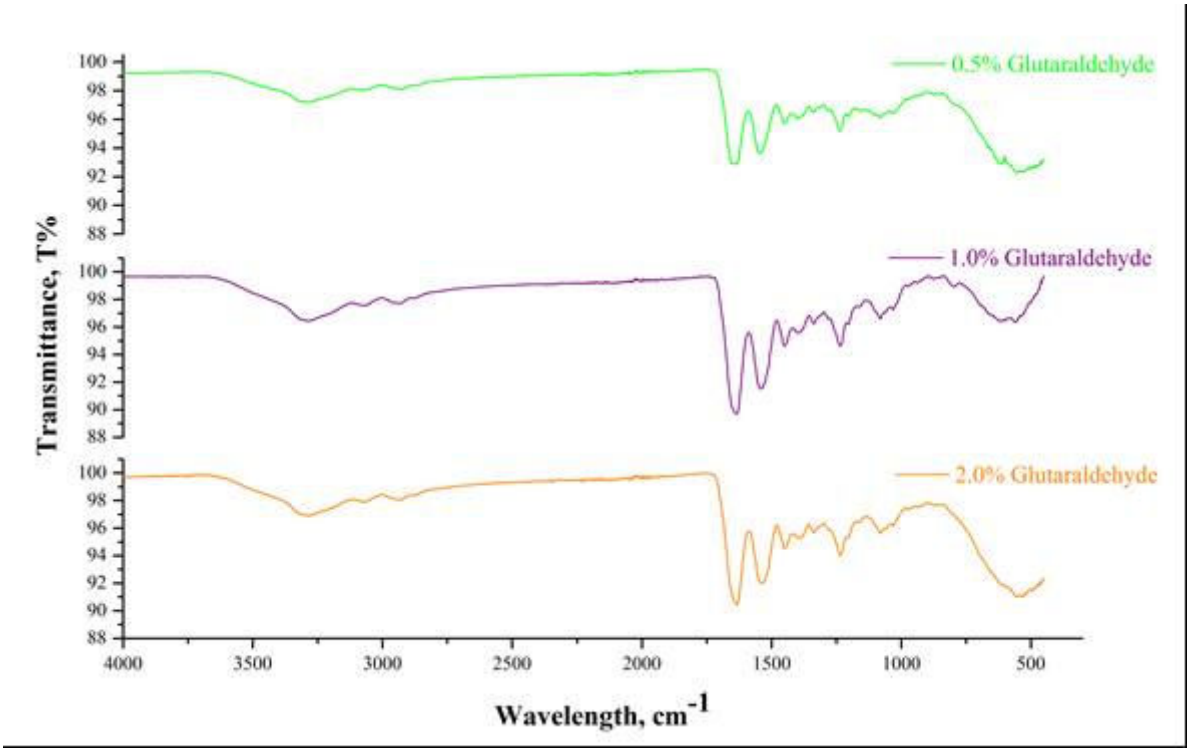

Figure 7. FTIR spectrum of cryogels prepared at different glutaraldehyde concentrations while the collagen concentration was kept constant at 8.0% (w/v).

SEM analysis

Cryogels were prepared with different ratios of collagen and glutaraldehyde, and the effect of those parameters (polymer and crosslinker concentration) on the final properties of the cryogel scaffolds were evaluated. Porosity and pore sizes are considered as important parameters in the design of scaffolds for tissue engineering applications. The adequate pore structure and pore size allow adhesion, migration, and differentation of cells and new tissue formation [20]. In this study, the morphological structure of collagen cryogels were examined by SEM analysis to assess the effects of polymer and crosslinking agent on the morphology. The results demonstrated that the cryogels exhibited interconnected porous morphology as shown in Figures 8 and 9. The pore sizes of the cryogels prepared with 3.0, 6.0 and 8.0% (w/v) concentration of collagen were 123.302 ± 35.08, 145.79 ± 49.37 ve 217.4 ± 63.96 μm, respectively. It was observed that the pore size increased with increasing the collagen concentration. In addition to this, increasing the amount of collagen from 3.0 to 6.0 and to 8.0% (w/v) increased the wall thickness of prepared cryogels from 0.96 ± 0.06 to 1.38 ± 0.09 and to 1.71 ± 0.09 μm, respectively.

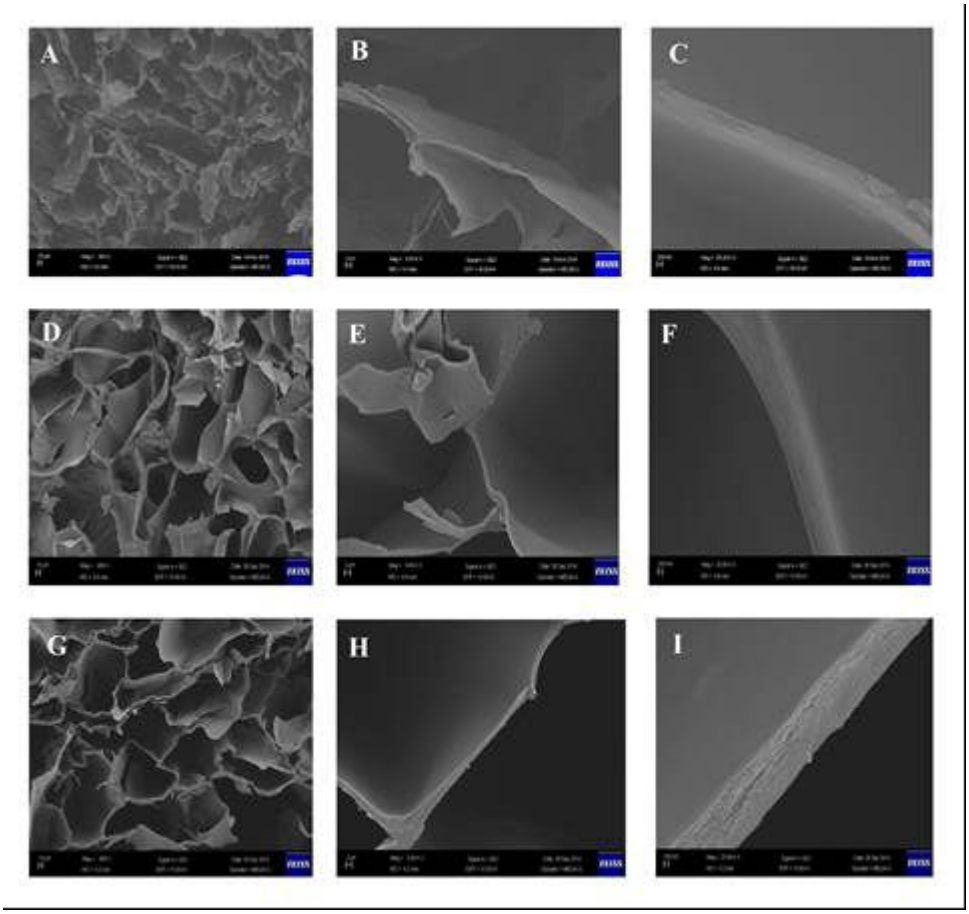

Figure 8. SEM images of the cryogels' porous structure and pore walls. The collagen concentration was changed while the crosslinker ratio was kept constant at 0.5% (v/v). Collagen concentration (w/v): (A) 3.0 %, (D) 6.0%, (G) 8.0%. Magnification: x400 in A,D,G ; x3000 in B,E,H ; x25000 in C,F,I.

The pore sizes of the cryogels prepared with 0.5, 1.0 and 2.0% (v/v) concentration of glutaraldehyde were 217.4 ± 63.96, 180.88 ± 73.31, and 157.21 ± 32.05 μm, respectively. It was observed that the pore size decreased with increasing the crosslinker concentration. Increasing the amount of crosslinker from 0.5 to 1.0 and to 2.0% (v/v), the wall thickness of prepared cryogels changed from 1.72 ± 0.09 to 0.07 ± 0.03 and to 1.61 ± 0.04 μm, respectively.

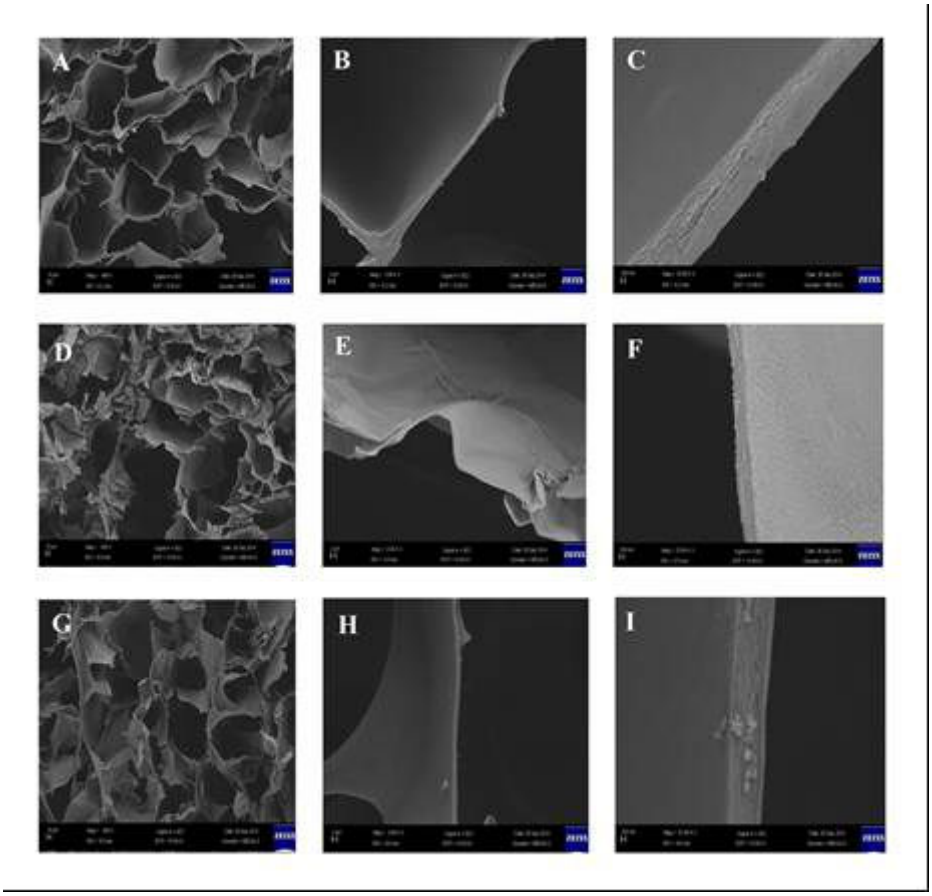

Figure 9. SEM images of the cryogels' porous structure and pore walls. The glutaraldehyde concentration was changed while the collagen ratio was kept constant at 8.0% (w/v). Glutaraldehyde concentration (v/v): (A) 0.5%, (D) 1.0%, (G) 2.0%. Magnification: x400 in A,D,G ; x3000 in B,E,H ; x25000 in C,F,I.

Swelling ratio analysis

The swelling ratios of cryogels are presented in Figure 10. All samples exhibited a swelling behavior and have the ability to retain more water than its own dry weight. It is observed that when the concentration of collagen increased from 3.0 to 6.0 and to 8.0% (w/v) swelling ratio of cryogels decreased from 4292.38 ± 446.68 to 2664.89 ± 227.85 and to 2036.27 ± 51.95%, respectively, at 360 min (Figure 10A). A more rigid structure was formed as collagen concentration increased and higher wall thickness caused less water uptake, therefore the swelling ratio was decreased. As seen in Figure 10B, at 360. minute as the crosslinker ratio was increased the swelling ratio was decreased.

Figure 10C demonstrates the cryogel's flexible behavior after swelling (a representative image). It was possible to squeeze and relax the scaffold easily after it swells the water, which showed the mechanical stability and elasticity of the cryogel.

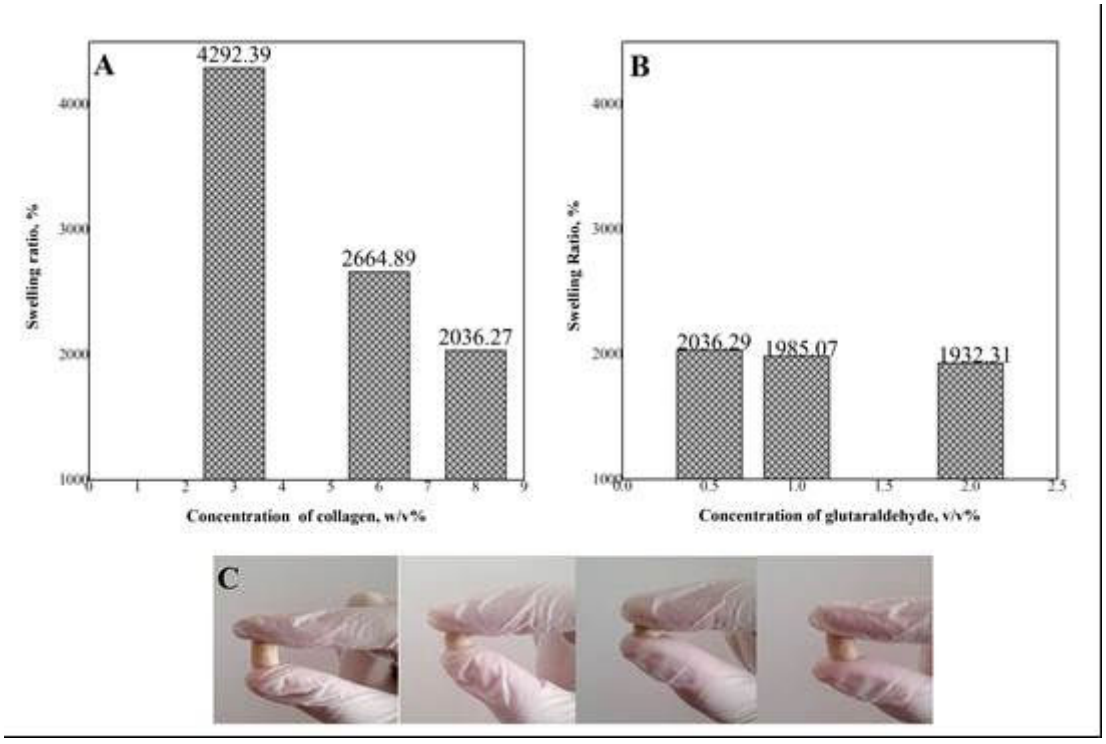

Figure 6. Swelling behavior of collagen cryogels: A) prepared at different collagen concentrations, B) prepared at different glutaraldehyde concentrations, C) cryogel's flexible structure after swelling.

In vitro degradation studies

The degradation of a scaffold is an important point in the design of a tissue engineering application [21]. For determination of how far collagen cryogel scaffolds can withstand to support tissue formation or restoration of damaged or diseased tissues, the weight loss of the collagen cryogels was demonstrated (Figure 11A and B) by the *in vitro* degradation test during 42 days and the images of cryogels before and after degradation process are shown in Figure 11C.

At the end of 42 days, as the collagen and glutaraldehyde concentration decreased, the degradation rate increased. The results were related to the results of swelling ratio studies. As the swelling ratio increased more water was uptaken by the scaffolds, which resulted in a faster degradation rate of the scaffold.

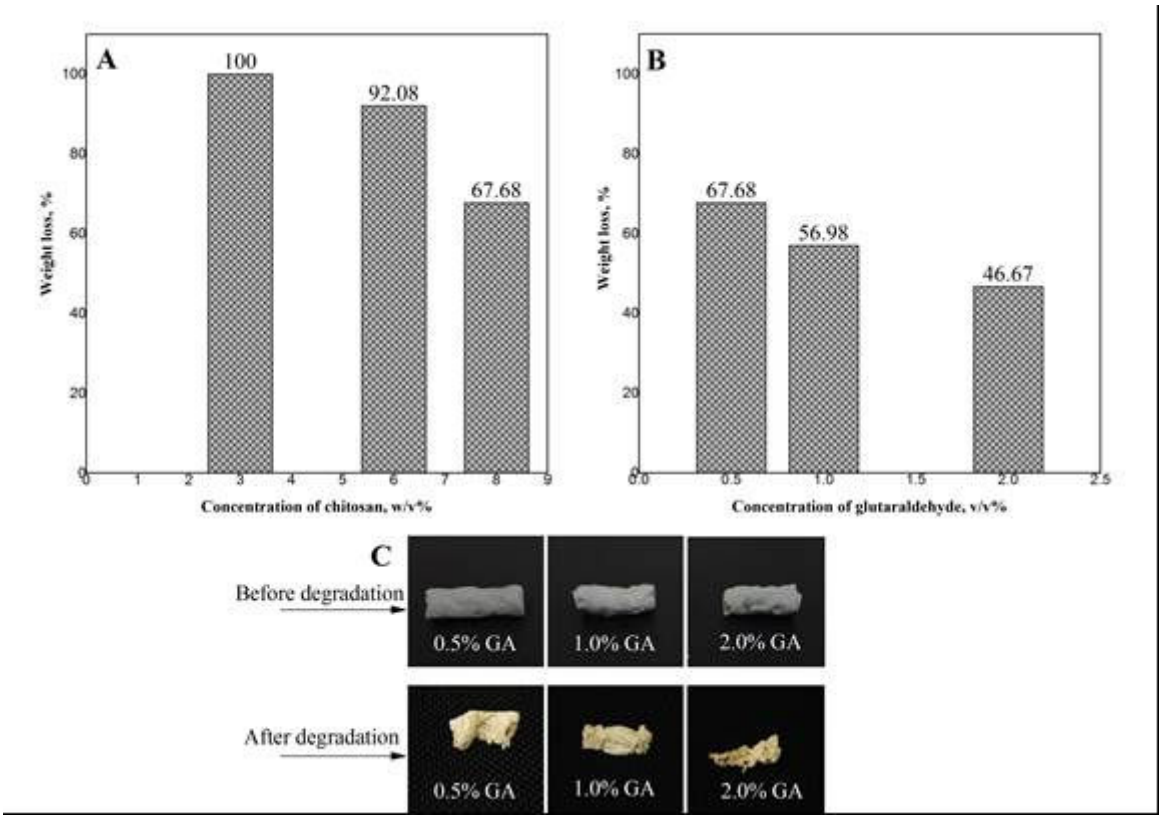

Figure 7. Degradation profiles of collagen cryogels: A) prepared at different collagen concentrations, B) prepared at different glutaraldehyde concentrations, C) cryogel images before and after degradation process.

CONCLUSION

In this study, acid-soluble collagen (ASC) with a yield of 14.53% and pepsin-soluble collagen (PSC) with a yield of 2.42% were successfully isolated from fish skin which is a waste material of the fish industry and characterized on the basis of SEM, UV-Vis, Zeta potentital and FTIR analysis. The cryogel scaffolds were produced from isolated collagen by applying the cryogelation technique which is a very unique technique in the design of a qualified scaffold with an interconnected porous structure that allows the three dimensional tissue growth and the diffusion of nutrients and wastes during tissue regeneration. Using different amounts of collagen and glutaraldehyde in the recipes resulted in properties of the scaffold such as having interconnected porosity, mechanical stability, spongy and elastic structure. Tailoring these properties of the cryogels with their swelling ability and degradation behavior would enable to design novel scaffolds for use in various applications in tissue engineering field. This study demonstrated extraction

of collagen and synthesizing cryogels from this collagen. For future tissue engineering applications, *in vitro* and *in vivo* biocompatiblity experiments would be performed.

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Türkçe Öz ve Anahtar Kelimeler

Doku Mühendisliği Uygulamaları için Balık Derisinden İzole Edilmiş Kolajenden Üretilen Kriyojeller: Saflaştırma, Sentez ve Karakterizasyon

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Öz: Doku mühendisliği gözenekli doku iskeleleri, hücreler ve biyoaktif araçlar kullanmak suretiyle zarar görmüş dokuları yenilemeyi hedefler. Doku iskeleleri bir çok doğal ve sentetik polimerden üretilir. Kolajen, bağ dokunun en önemli bileşeni olması ve biyouyumluluğu sebebiyle doku iskelesi üretiminde geniş bir kullanım alanına sahip olan bir doğal polimerdir. Kriyojelleşme, diğer yapı iskelesi üretimi yöntemlerine göre nispeten daha basit bir teknik olup polimer karışımı veya monomerik öncül maddeleri içeren donmuş tepkime karışımlarından birbiri ile bağlantılı gözenekli matrisler üretme olanağı sunar. Bunlar dikkate alındığında, bu çalışmada, kolajen balık derisi gibi ticari olmayan bir atık malzemeden izole edilmiş ve bu kolajenden kriyojelleşme yöntemiyle doku iskeleleri üretilmiştir. Kolajenin gözenek morfolojisi ve protein yapısı sırasıyla SEM ve UV-Vis analizi ile doğrulanmıştır. Proteinin izoelektrik noktası zeta potansiyel analizi ile belirlenmiştir. Kolajenin Amid A, Amid B, Amid I, Amid II ve Amid III karakteristik pikleri, FTIR analizi ile gösterilmiştir. İzole edilmiş proteinin verimi asitte çözünen ve pepsinde çözünen kolajen için sırasıyla %14,53 ve %2,42 olarak bulunmuştur. Doku iskeleleri izole edilmiş asitte çözünür kolajenin, kriyojenik koşullar altında, glutaraldehitle çapraz bağlanması ile üretilmiştir. FTIR analizi ile, glutaraldehit ile kolajen arasındaki tepkimeden oluşan C=N bağının konumu 1655 cm^{-1} olarak bulunmuştur. SEM analizi ile, kolajen ve glutaraldehit derişiminin kriyojellerin gözeneklerinin morfolojisi, çapı ve duvar kalınlığı üzerine belirgin etkide bulunduğu gösterilmiştir, bu etkiler doku iskelelerinin şişme oranını ve bozunma profillerini değiştirmiştir. Bu çalışmada, hasar görmüş dokuların yenilenmesinde potansiyel olarak kullanılabilecek, balık derisinden izole edilmiş kolajen kriyojel doku iskelesinin sentezi ve karakterizasyon sonuçları sunulmuştur.

Anahtar kelimeler: Doku mühendisliği, kolajen, kolajenin izolasyonu, doku iskelesi, kriyojel.

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