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Extraction and Characterization of Chitin and Chitosan from Blue Crab and Synthesis of Chitosan Cryogel Scaffolds

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Abstract: Polymeric scaffolds produced by cryogelation technique have attracted increasing attention for tissue engineering applications. Cryogelation is a technique which enables to produce interconnected porous matrices from the frozen reaction mixtures of polymers or monomeric precursors. Chitosan is a biocompatible, biodegradable, nontoxic, antibacterial, antioxidant, and antifungal natural polymer that is obtained by deacetylation of chitin, which is mostly found in the exoskeleton of many crustaceans. In this study, chitin was chemically isolated from the exoskeleton of blue crab (*Callinectes sapidus*). *Callinectes sapidus* samples were collected from a market, as a waste material after it has been consumed as food. Demineralization, deproteinization, and decolorization steps were applied to the samples to obtain chitin. Chitosan was prepared from isolated chitin by deacetylation at high temperatures. The chemical composition of crab shell, extracted chitin and chitosan were characterized with FTIR analysis. Moreover, in order to determine the physicochemical and functional properties of the produced chitosan, solubility, water uptake, and oil uptake analysis were performed. Chitosan cryogel scaffolds were prepared by crosslinking reaction at cryogenic conditions at constant amount of chitosan (1%, w/v) with different ratios of glutaraldehyde (1, 3, and 6%, v/v) as crosslinker. The chemical structure of the scaffolds were examined by FTIR. Also, the water uptake capacity of scaffolds have been determined. Collectively, the results suggested that the characterized chitosan cryogels can be potential scaffolds to be used in tissue engineering applications.

Keywords: Tissue engineering, crab shells, chitin, chitosan, scaffold, cryogel.

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INTRODUCTION

Tissue engineering is one of the topics in biomedical engineering that can provide many alternatives for the repair of damaged tissues [1]. By applying the principles of tissue engineering, characteristic properties of the original tissues can be mimicked by scaffolds. An ideal scaffold should be biocompatible, biodegradable and does not induce an immune reaction or inflammation. These scaffolds can be obtained from natural or synthetic polymers [2-5].

Chitin is a a naturally abundant mucopolysaccharide which can be obtained from crab shells [6,7]. It is a nitrogenous polysaccharide that is white, rigid, with inelastic structure. Chitin contains 2-acetamido-2-deoxy-b-D-glucose groups by (1→4) linkage which named as *N*-acetylglucosamine [7, 8]. After the cellulose, chitin is the second most abundant biopolymer over the world. Invertebrates, insects, marine diatoms, algae, fungi and crustaceans like crabs, shrimps, and lobsters are the source of chitin [9].

Chitosan is one of the chitin derivatived products that can be obtained by N-deacetylation [6,10]. Moreover, it has also biological properties including biocompatibility, biodegradability and non-toxicity for living cells [10].

Blue crab, called *Callinectes sapidus,* contains chitin in its shell, lives in North America, Mersin-Silifke area lagoons, İskenderun coasts and Adana-Yumurtalık lagoon in Turkey [11].

In this study, we aimed to extract chitin from shells of blue crab and produce chitosan from extracted chitin by deacetylation at high temperatures. The chemical compositon of extracted chitin and chitosan were characterized by FTIR analyses. In addition to this, solubility, water uptake and oil uptake analysis were performed to determine the physicochemical and functional properties of the produced chitosan. Chitosan-based cyrogels were produced by using gluteraldehyde as a crosslinking agent. Characteristic properties of the produced cryogels were demonstrated in order to be used in tissue engineering applications.

MATERIALS AND METHODS

Materials

Blue crab (*Callinectes sapidus*) shell wastes were collected from a local market in Mersin, Turkey after it has been consumed as food. The samples were transferred to the laboratory as soon as possible and stored in a freezer at -16°C, until starting the extraction procedure. Hydrochloric acid was obtained from Merck, Germany, sodium hydroxide and acetone were purchased from Emir Kimya, Turkey for use in experimental steps of extracting chitin and chitosan. Glutaraldeyde (GA) solution 25% in water as a crosslinking agent and 100% (v/v) glacial acetic acid as a solvent were both obtained from Merck for the preparation of cryogel scaffolds.

Chitin and chitosan extraction from crab shells

Extraction of chitin from crab shells: Crab shells were washed several times with distilled water and then dried at 40 °C in an oven. Dried shells were powdered by a grinder. First step of extraction of chitin was demineralization. 40 g of powdered sample was treated with 2 M HCl solution for 24 h at 80 °C to remove all minerals from the sample. The second step of extraction was deproteinization in which the sample was treated in 2 M NaOH solution at 110 ºC for 20 h at a solid to solution ratio of 1:10 (w/v) to remove all proteins of the sample. After deproteinization, sample was treated with acetone for decolorization of extracted chitin. Chitin was obtained after applying these three steps respectively. At the end of each step, the sample was filtered, washed several times with distilled water, and dried in an oven at 40 °C.

Production of chitosan from extracted chitin

Extracted chitin was treated with 50% concentrated NaOH (w/v) solution at 150 °C for 4 h at a solid to solution ratio of 1:10 (w/v) to remove the acetyl groups of chitin. This process is called as deacetylation. After deacetylation, the sample was filtered and washed several times with distilled water until pH was neutral. The obtained chitosan was then dried before using for production of chitosan cryogel scaffolds. Schematic illustration of extraction steps of chitin and production of chitosan is demonstrated in Figure 1.

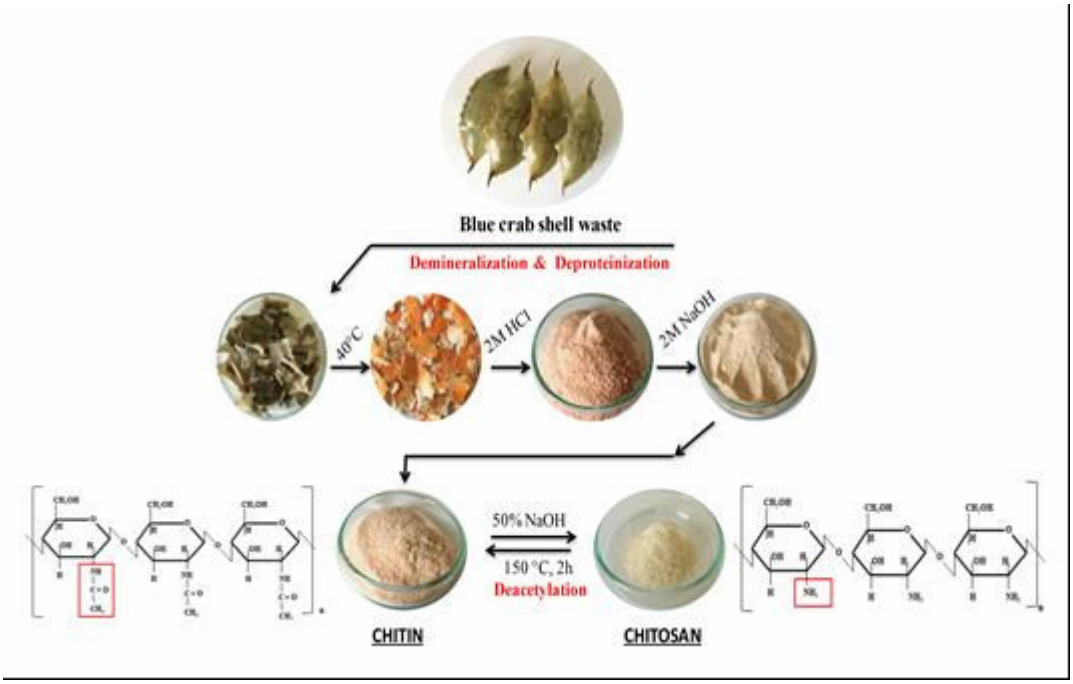

Figure 1. Schematic illustration of extraction steps of chitin and production of chitosan.

Production of chitosan cryogels

The chitosan cryogels were synthesized at three different concentrations of GA. 2 mL of chitosan solution was prepared in (6%, v/v) acetic acid solution and mixed until the solution was homogenous and clear. 1 mL of GA solution (1, 3 and 6%, v/v) was poured to the prepared chitosan solutions. The polymer and crosslinker mixture was immediately poured into a plastic syringe and placed into the cryostat. The mixture was incubated in the cryostat at -16 °C for 2 h and stored in the freezer at the same temperature for 24 h. The reaction mixture was thawed to room temperature and the formed blocks were washed in distilled water until the unreacted polymer and crosslinker was removed.

Characterization of chitin and chitosan

Yield of chitin and chitosan: The percantage of the yield of chitin was calculated by dividing the weight of extracted chitin to initial dry crab shell weight and the percentage of the yield of chitosan was calculated by dividing the weight of produced chitosan to dry chitin weight before deacetylation. Yields were calculated as follows:

$$\text{Yield of chitin (\%)} = [\text{Extracted chitin (g)}/\text{Crab shells (g)}] \times 100 \qquad \text{(Eq. 1)}$$

$$\text{Yield of chitosan (\%)} = [\text{Produced chitosan (g)}/\text{Chitin (g)}] \times 100 \qquad \text{(Eq. 2)}$$

FTIR analysis of chitin and chitosan

The infrared spectral analysis of the crab shell, extracted chitin, and produced chitosan samples was measured by Fourier Transform Infrared Spectrometry, FTIR (Frontier Spectrometer, Perkin Elmer, USA) in the wavelength range of 450 - 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Solubility

10 mL of 1% acetic acid solution was put in a centrifuge tube containing 0.1 g of produced chitosan. The sample was centrifuged at 10,000 rpm for 30 min. After the supernatant was poured away, the undissolved part of chitosan was washed with 25 mL of distilled water and then centrifuged at 6,000 rpm. The supernatant liquid was poured away and the undissolved solid was dried at 60 °C for 24 h in an oven. Lastly, the amount of the dried solid was weighed and the percentage of solubility was determined [12].

Water uptake capacity (WUC)

10 mL of distilled water was put in a centrifuge tube containing 0.5 g of produced chitosan. The sample was mixed on a vortex about 5 min until the sample was dispersed. Then, the dispersed sample was vortexed for 5 s every 10 min (for a total of 30 min) and centrifuged at 3500 rpm for 30 min. After centrifugation, supernatant was poured off and the sample was weighed. WUC was calculated as follows [12]:

$$\text{WUC (\%)} = [\text{Bound water (g)}/\text{Initial chitosan weight (g)}]\text{x}100 \qquad \text{(Eq. 3)}$$

Oil uptake capacity (OUC)

10 mL of sunflower oil was put in a centrifuge tube containing 0.5 g of produced chitosan. The sample was mixed on a vortex about 5 min until the sample was dispersed. Then, the dispersed sample was vortexed for 5 s every 10 min (for a total of 30 min) and centrifuged at 3500 rpm for 30 min. After centrifugation, supernatant was poured away and the sample was weighed. OUC was calculated as follows [12]:

$$\text{OUC (\%)} = [\text{Bound oil (g)}/\text{Initial chitosan weight (g)}]\text{x}100 \qquad \text{(Eq. 4)}$$

Characterization of Chitosan Cryogels

FTIR analysis of cryogels: Chemical surface analysis of the produced chitosan cryogels was performed by Fourier Transform Infrared Spectrometry, FTIR (Perkin Elmer, FT-IR/FIR/NIR Spectrometer Frontier, ATR, USA) with the range of 450 - 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Water uptake capacity: Water uptake capacity of chitosan cryogels was measured by a gravimetric analysis. The chitosan cryogels of about 50 mg weight (the surface bound water was removed by filter paper) were incubated in 10 mL of distilled water at room temperature. After specified time intervals (5, 15 30, 60 and 90 min) the wet weight of cryogels was determined and the water uptake capacity calculated according to the following equation:

$$WUC\ (\%) = [(Ws - W_w) / Ww]*100 \quad \text{(Eq. 5)}$$

Where WUC is water uptake capacity, Ws is weight of cryogel and Wd is weight of swollen cryogel.

RESULTS AND DISCUSSION

Chitin extraction and chitosan production

Yield of extracted chitin and produced chitosan: The yields have been calculated for extracted chitin and produced chitosan. The yield of chitin extraction from dry crab shells was 11.73%. The yield of chitosan produced from extracted chitin was 77.78%, which was similar to the results that was reported in the literature by Kaya *et al.* (76% yield of chitosan from *Callinectes sapidus* from İskenderun, Turkey) and Odote *et al.* (74.6% yield of chitosan of *Sylla cerrata* from Mombasa, Kenya) [13, 14]. The yields were above average in these studies and our study indicated that crabs are one of the major resources of chitin and chitosan among the other crustacean group of organisms.

FTIR spectra of chitin and chitosan

Figure 2 demonstrates the FTIR spectra of the crab shell, chitin, and chitosan. The FTIR spectra shows the characteristic bands of $-NH_2$ at 3447 cm^{-1} and carbonyl group band at 1477 cm^{-1} [15]. The band at 3448 cm^{-1} could be assigned to (N-H), (O-H) and (NH_2) groups. The band at 3267 cm^{-1} is associated with (N-H) in secondary amides only with trans-configuration and usually is due to the formation of linear associates [16]. Additionally, the lower intensity

band at 3110 cm^{-1} confirmed trans-configuration of NH-CO group in chitin. The presence of methine group in pyranose ring and methyl group in methylene group was proved by the corresponding stretching vibrations of these groups in the range of 2892-2961 cm^{-1} [17]. The only one intense peak at 626 cm^{-1} indicate crystalline state of chitin [18]. Furthermore, the spectra of the samples indicated the presence of two bands, one at 1626 cm^{-1} and another at 1657 cm^{-1}, probably indicating an amorphous state. The peak at 1626 cm^{-1} could be based on to the stretching of C–N vibration, linked to OH group by bonding [19,20]. The characteristic bands for chitosan can be observed in Figure 2. The spectra showing the amine peak at 2923.88 cm^{-1} indicates the presence of CH stretch and the peak at 3400 cm^{-1} indicates symmetric stretching vibration of OH. In addition to this, the peak at 1650.95 cm^{-1} was due to C=O stretching (amide I) the peaks at 1095.49 cm^{-1} and 1033.77 cm^{-1} show C-O stretching [15]. The wavelength at 894.91 cm^{-1} represent a ring stretching, a characteristic bond for β-1-4 glycosidic linkage.

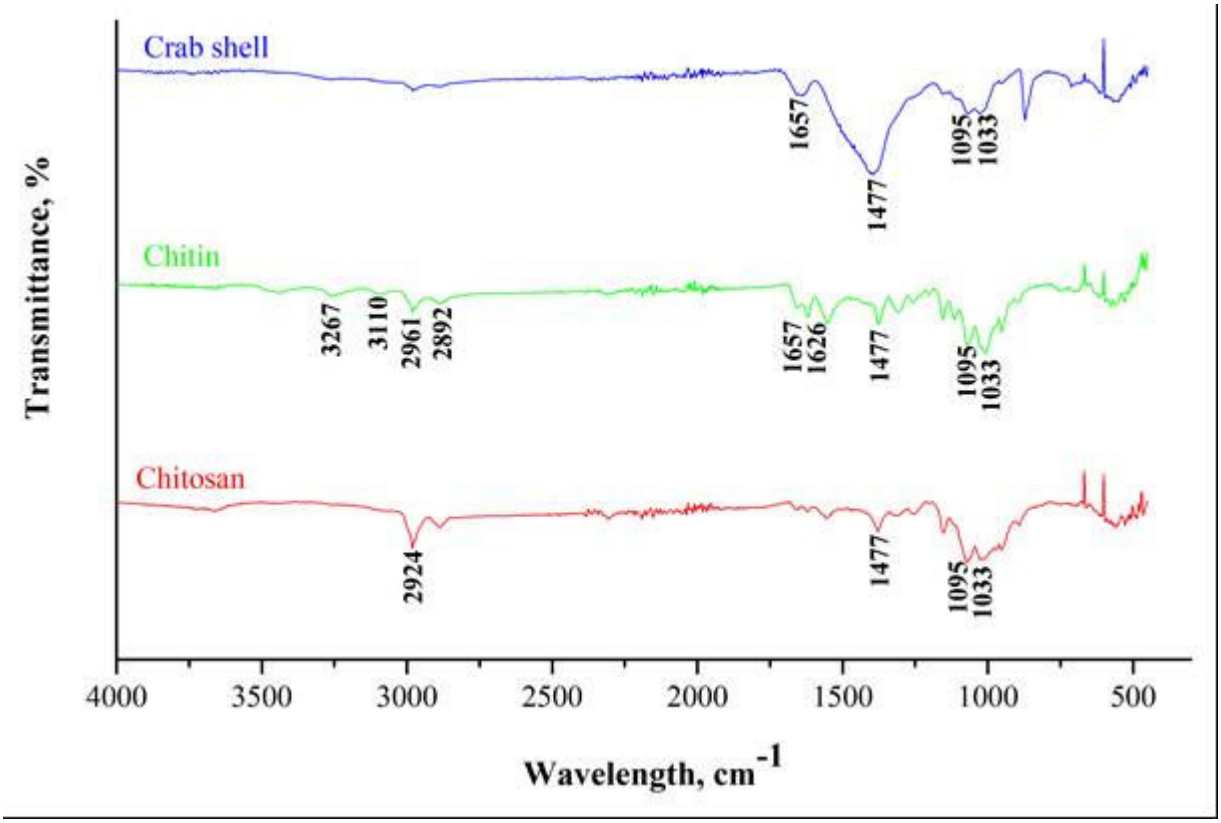

Figure 2. FTIR spectrum of crab shell, extracted chitin and produced chitosan.

Solubility

Chitosan had an excellent solubility of 99.29% ± 0.001 in 1% acetic acid solution. The high solubility of produced chitosan was due to the process conditions in deacetylation step. The temperature was 150 °C, period of deacetylation was 2 h, and alkaline concentration was 50% NaOH. The high solubility of chitosan in acetic acid indicates that the deacetylation degree is at least 85% [21].

Water uptake capacity

Water uptake capacity of produced chitosan was 582.59% ± 58.67. The chitosan showed similar WUC compared with the results of Özbay *et al.* (650.51% ±18.55 WUC, blue crab) [22].

Oil uptake capacity

Oil uptake capacity of produced chitosan was 372.21% ± 9.29. The percentage OUC of chitosan is in agreement with the result (437.82 % ± 21.48 OUC, blue crab) reported by Özbay *et al.* [22].

Production of chitosan cryogels

Cryogelation process: The concentration of GA as a crosslinking agent was varied in this study. The concentration of GA affects the chemical, physical, mechanical, morphological, and porous structure of cryogels. Cryogelation technique was used in the synthesis of chitosan cryogels. Figure 3 shows the image of GA crosslinked chitosan cryogels after cryogelation reaction is completed (Figure 3A) and behavior of cryogels before, under, and after force (Figure 3B). The chitosan cryogels showed a yellowish color with increasing the GA ratio, which can be due to the double bonds resulting after GA crosslinking [23]. Chitosan cryogels which were crosslinked with 1% and 3% GA did not show a significant deformation after applying force. However, although the cryogels prepared with 6% GA were elastic, when the applied force was increased they were deformed.

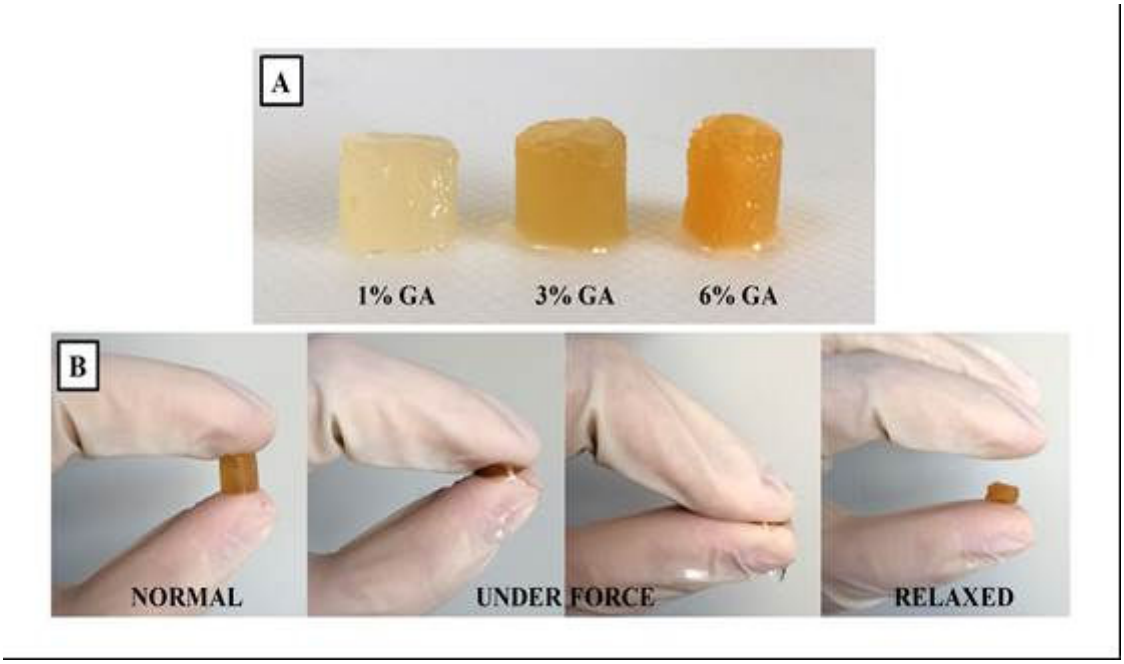

Figure 3. The image of prepared chitosan cryogels: (A) cryogels after cryogelation reaction is completed, (B) behavior of cryogels (crosslinked with 3% GA) before, under, and after applying force.

FTIR spectra of cryogels

Figure 4 demonstrates the FTIR spectra of crosslinked cyrogels with GA (1, 3, and 6%, v/v). As a result of the imine bonds N=C, the crosslinking of the scaffolds with GA shows the main absorption peak at 1646 cm^{-1} [24]. Shoulders at 1587 cm^{-1} appeared due to the ethylenic bonds [24, 25]. Increasing gluteraldehyde (crosslinker) concentration, caused increase in the intensity of ethylenic bond frequency at 1562 cm^{-1}. For the C-H stretching vibration frequency at 2936 cm^{-1} was observed. In addition, the peak at 1100 cm^{-1} demonstrated the aliphatic amino groups [25,26].

Water uptake capacity

WUC is releated with the highly porous and spongy structure of the cryogels. The WUC results of the synthesized cryogels are demonstrated in Figure 5. WUC of all chitosan cryogels was higher than 120% in the first 15 min. It was observed that the WUC of cryogels decreased as the concentration of crosslinker (GA) increased. Mirzaei *et al.* reported a similar trend related to the effect of different glutaraldehyde concentrations on the swelling behavior of freeze-dried chitosan hydrogels [25]. Cryogels crosslinked with 1% GA showed the highest WUC (514.51% ± 31.91) compared to the ones prepared with 3% and 6% GA which showed a WUC of 257.14% ± 29.28 and 154.18% ± 24.17, respectively.

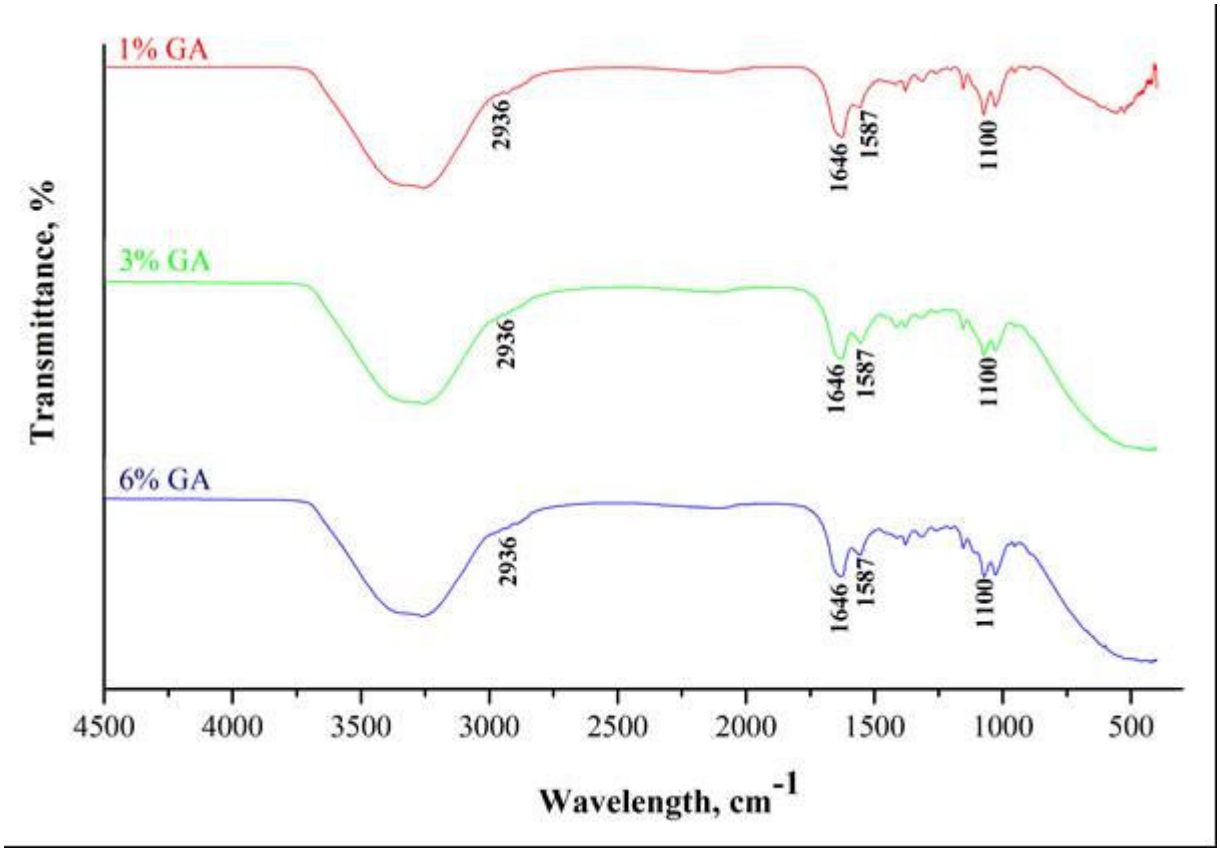

Figure 4. FTIR spectrum of synthesized chitosan cryogels.

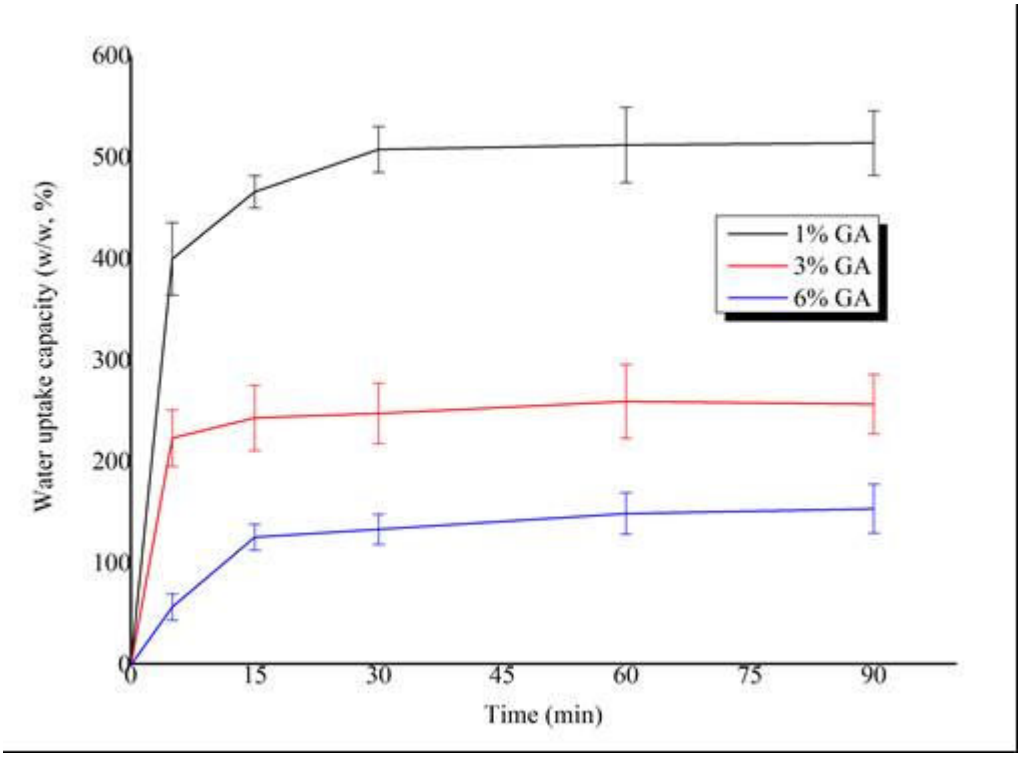

Figure 5. Water uptake profiles of the synthesized cryogels.

CONCLUSION

Chitin was extracted from the waste of blue crab shells (*Callinectes sapidus*) and chitosan was produced from extracted chitin by deacetylation. The chemical composition of crab shell, extracted chitin, and chitosan were characterized with FTIR. In addition to this, physicochemical properties of obtained chitosan was determined by solubility, water uptake and oil uptake capacity analysis. According to these results, obtained chitosan can be used as a biomaterial in tissue enginnering applications. For this purpose, chitosan scaffolds were synthesized by cryogelation technique and characterized by FTIR and water uptake capacity tests. Increasing the crosslinker (GA) concentration affected the properties of cryogels. The cryogel scaffolds were mechanicaly stable and had water uptake ability. The cryogels synthesized in this study have potential to be used as tissue engineering scaffolds in biomedical applications.

CONFLICT OF INTEREST

The authors declare that no conflict of interests exist in relation to the writing of this article.

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Türkçe Öz ve Anahtar Kelimeler

Mavi Yengeçten Kitin ve Kitosanın Ekstraksiyonu ve Karakterizasyonu, ve Kitosan Kriyojel Doku İskelelerinin Sentezi

Didem Demir, Fatma Öfkeli, Seda Ceylan, Nimet Bölgen Karagülle*

Öz: Kriyojelleşme tekniği ile elde edilen polimerik doku iskeleleri, doku mühendisliği uygulamaları için artan ilgi konusu olmaktadır. Kriyojelleşme, polimerler veya monomerik öncül maddelerin donmuş tepkime karışımlarından birbiri ile bağlantılı gözenekli matrisler elde edilmesine imkân tanıyan bir tekniktir. Kitosan, biyo-uyumlu, biyo-bozunur, toksik olmayan, antibakteriyel, antioksidan ve antifungal olan bir doğal polimerdir ve pek çok kabuklu hayvanın dış iskeletinde bulunan kitinin deasetilasyonu ile elde edilir. Bu çalışmada, kitin mavi yengeç (*Callinectes sapidus*) dış iskeletinden kimyasal olarak izole edilmiştir. *Callinectes sapidus* örnekleri bir marketten gıda olarak tüketilen yerleri bitirildikten sonra çöpe atılan alandan toplanmıştır. Kitini elde etmek için örneklere demineralizasyon, deproteinizasyon ve renksizleştirme adımları uygulanmıştır. Kitosan, yüksek sıcaklıklarda kitinin deasetilasyonu ile hazırlanmıştır. Yengecin kabuğu, ekstrakte edilen kitin ve kitosana ait kimyasal bileşim FTIR analizi ile karakterize edilmiştir. Bunun dışında, üretilen kitosanın fizikokimyasal ve işlevsel özelliklerini belirlemek için, çözünürlük, su alımı ve yağ alımı analizleri gerçekleştirilmiştir. Kitosan kriyojel doku iskeleleri, kriyojenik koşullarda sabit miktarda kitosan (%1, w/v) ile çapraz bağlayıcı olarak farklı oranlarda glutaraldehit (%1, 3 ve 6) karışımının çapraz bağlanma tepkimesinden elde edilmiştir. Doku iskelelerinin kimyasal yapıları FTIR ile incelenmiştir. Ayrıca, doku iskelelerinin su alma kapasiteleri de ölçülmüştür. Sonuç olarak, elde edilen neticeler karakterize edilmiş kitosan kriyojellerinin doku mühendisliği uygulamalarında potansiyel doku iskeleleri olarak kullanılabileceğini göstermiştir.

Anahtar kelimeler: Doku mühendisliği, yengeç kabukları, kitin, kitosan, doku iskelesi, kriyojel.

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