Characterization of Physical Properties and Tensile Strength of Chitosan/Fibroin Composite via Freeze Drying Method

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Abstract. Chitosan and silk fibroin composite are natural biopolymers widely used in biomedical engineering applications. This work studied the properties of a chitosan and silk fibroin composite were prepared by freeze drying method. The characterizations were operated by fourier-transform infrared spectroscopy, scanning electron microscopy, and x-ray diffraction. In addition, the tensile strength was obtained for various compositions. The results showed that the freeze-dried composite was of high porosity with 3D interconnected pores that are suitable for tissue engineering. In addition, both Fourier-transform infrared spectroscopy and x-ray diffraction indicated that the composite materials were water soluble.

Keywords: Chitosan, Silk fibroin, Biopolymer, Freeze-drying

1. Introduction
Chitosan (CS) is a polysaccharide consisting of random deacetylated and acetylated units (2-amino-2-deoxy-β-D-glucopyranose, and N-deacetylated, respectively) which are β-(1→4)-linked. Chitosan can be found in various natural products from marine sources [1,2]. Chitosan shows strong biological properties such as being antibacterial, biocompatible with cells, biodegradable, and able to absorb humidity [3]. These properties have led to its wide-spread use in various applications [4]. Silk fibroin (SF) is a linear polypeptide that can be extracted from the Bombyx mori silkworm. Natural silk fibroin is composed of various amino acid types such as glycine, alanine, and serine, which affect the solubility in water [5]. In addition, the properties of silk fibroin depend on the proportion of silk I and silk II, which are both secondary structure molecular conformations. Silk I is an amorphous structure that is water soluble, whereas silk II is more stable with higher crystallinity, which results in the water insolubility property [6,7]. In biomedical engineering, silk fibroin is used in controlled release [8], membranes for regeneration [9], and other high efficiency applications [10]. In this research, we focus on estimation of the physical and mechanical properties of CS/SF composites fabricated via freeze drying for application to various fields of tissue engineering.

2. Materials and Methodology

2.1 Materials
Chitosan was extracted from squid pens and silk fibroin was prepared from silk cocoons produced by Bombyx mori silk worm using the method presented in [11]. Initially, fibroin was dissolved into 99 ml of distilled water overnight then 1 ml of acetic acid (RCI Labscan Ltd., Australia) and chitosan were added to the fibroin solution and shaken gently. For tensile testing, 0.0025 ml of diluted
glutaraldehyde (Loba Chemie Pvt Ltd., India) was added to the solution for comparison of the strength property. A clear, homogeneous solution was obtained after dissolving CS/SF for 24 h. The ratios for blending CS/SF were 75/25, 50/50, and 25/75 by wt% (marked as 75CS/SF, 50CS/SF, and 25CS/SF, respectively) for 1 g of solid loading.

2.2 Freeze drying process
CS/SF solutions measuring 5 ml were drained into 24-well plates for the characterization of physical properties. For tensile testing, 50 ml of solution were transferred into a 70x100 mm acrylic mold. The pre-freeze and freeze stages were conducted at -20 and -80°C, respectively for 24 h. Then all CS/SF solutions were freeze dried at -50°C in a vacuum for 48 h.

2.3 Characterization
Fourier-transform infrared spectroscopy (FTIR) spectra were recorded using a NICOLET 6700 (United States) and collected over the range 4000-400 cm⁻¹ with an accumulation of 64 scans and resolution of 4 cm⁻¹. X-ray diffraction (XRD) patterns were obtained using a Rigaku D/teX 250 (Japan) with CuK radiation in steps of 0.01° from 10 to 60° (2-theta angle). Freeze-dried CS/SF specimens were sectioned with a razor blade in the horizontal plane into 10x10x0.5 mm³ pieces then coated with a gold layer. Scanning electron microscopy (SEM) images were obtained using a JSM 5910 LV (Japan). Porosity and pore sizes were estimated using Image J from various SEM images. For tensile strength testing, a freeze-dried CS/SF sheet was cut into 30x80x2 mm³ pieces and tested by the universal testing machine, Instron 5542 (United Kingdom), with a 500 N load and head cross-speed of 0.05 mm/s at room temperature and humidity lower than 40% RH.

3. Characterization and testing

3.1 General properties of specimen
All freeze-dried CS/SF specimens are shown in Figure. 1. Each of the specimens are white-colored sponges which cannot be identified by the naked eye. Glutaraldehyde is added when forming silk fibroin and was not used in these experiments except for comparison in the tensile strength test. Chitosan acts as a network and binding agent for silk fibroin in the CS/SF composites. Hence, 75CS/SF, and 50CS/SF are formed with smooth surfaces and can be cut into various shapes. However, at low chitosan concentration (25CS/SF), binding of the material was not as strong after freeze drying. Therefore, it collapsed easily and the porosity, pore size, and mechanical strength could not be estimated.

![Figure 1](image-url)
3.2 Fourier-transform infrared spectroscopy

The FTIR spectra of chitosan, silk fibroin, and CS/SF are shown in Figure. 2. The chitosan spectrum Figure. 2(A) shows bands at 3370 and 3236 cm⁻¹ that represent hydrogen bonding, corresponding with the stretching of O-H and N-H respectively. The polysaccharide characteristic is indicated by the C-H bond at 2981 and 2879 cm⁻¹ which correspond with symmetric and asymmetric stretching respectively. The functional group of N-acetyl is found at 1633 cm⁻¹ (C=O stretching of amide I) and 1307 cm⁻¹ (C-N stretching of amide III). However, amide II (N-H) could not be observed at 1150 cm⁻¹ due to overlapping between peaks at close wavelengths. Amine at 1567 cm⁻¹ corresponds with the bending of N-H. Both CH₂ and CH₃ are observed at 1412 and 1373 cm⁻¹ with bending and symmetrical deformations, respectively. The bending vibration of hydroxide is shown at 1226 cm⁻¹. The C=O bond is subject to asymmetric stretching at 1121 cm⁻¹.

For the silk fibroin spectrum shown in Figure. 2(E), bands at 1639 and 1519 cm⁻¹ correspond with amide I (C=O stretching) and amide II (N-H deformation and C–N stretching). The important bands in the range 1500 to 1700 cm⁻¹ are associated with amides in silk fibroin, where 1600 to 1700 cm⁻¹ corresponds with amide I and 1500 to 1600 cm⁻¹ corresponds with amide II. The absorption in these regions is due to the peptide backbones of the amides. Regarding the analysis of the secondary structures of silk fibroin, the difference between silk I and silk II will be indicated by peaks in these bands. Characteristics of silk I with amide I and II peaks are observed at 1640 to 1660 cm⁻¹ and 1535 to 1542 cm⁻¹, respectively. For silk II, there is a small shift from the silk I values. Specifically, amide I shifts from 1610 to 1630 cm⁻¹ and amide II shifts from 1510 to 1520 cm⁻¹ [12]. In this study, both amide peaks are shown at 1639 and 1538 cm⁻¹ and correspond with silk I. All CS/SF specimen spectra have the same peak positions and their intensities are related to the content of pristine chitosan and silk fibroin.

![Figure 2 FTIR spectra: (A) chitosan, (B) 75CS/SF, (C) 50CS/SF, (D) 25CS/SF, and (E) silk fibroin.](image-url)
3.3 X-ray diffraction

Figure 3 shows XRD patterns for all specimens. Both chitosan and silk fibroin show broad diffraction at 21.1° and 23.2° respectively due to their amorphous structure. The XRD pattern of Chitosan was reported in [13]. XRD patterns of silk fibroin correspond to the silk I crystalline peak [14], however, there can be various degrees of broadness in the pattern that depend on the solution for degumming [15], hence, the silk fibroin peak in Figure 3 shows a large, broad diffraction when compared with another solution. XRD patterns of freeze-dried CS/SF are preserved when compared with the pristine diffractogram. Due to the overlap of chitosan and silk fibroin peaks, individual patterns could not be identified. However, a broad diffraction band confirms the large silk fibroin content, whereas more sharp diffraction is associated with chitosan.

![XRD Patterns](image)

**Figure 3** XRD patterns: (A) chitosan, (B) 75CS/SF, (C) 50CS/SF, (D) 25CS/SF, and (E) silk fibroin.

3.4 Scanning electron microscopy

SEM images of the horizontal-section of CS/SF are shown in Figure 4. Three-dimensional interconnected pores are observed with different pore size and porosity. Without added glutaraldehyde for silk fibroin forming, chitosan serves as a network and binding agent in silk fibroin for the CS/SF composites as can be seen in Figure 4 for the 75CS/SF and 50CS/SF. However, in the case of low chitosan content (25CS/SF), it can be seen that the capacity for binding is very low. Consequently, the freeze-dried CS/SF composite was formed with weak bonds as shown in Figure 4. For this composition the porosity, pore size, and mechanical strength could not be estimated. Table 1 presents the pore characteristics of freeze-dried CS/SF. Careful investigation and observation of the pore structure of 75CS/SF in Figure 4(A) shows smooth three-dimensional interconnected pores created by removing crystalline ice from the solution. On the other hand, 50CS/SF in Figure 4(B) shows a pore structure similar to plates that are gently linked to each other due to less strong binding as a consequence of reduced chitosan content. The porosity was seen to decrease from 46.89 (75CS/SF) to 23.57 (50CS/SF) percent (the porosity in the 25CS/SF structure could not be estimated). On the other hand, pore size increased from 21-123 to 113-194 μm for 75CS/SF and 50CS/SF respectively.
3.5 Tensile strength

The mechanical strength of freeze-dried CS/SF is shown in Figure. 5. The results indicate that the tensile strength of CS/SF composites are dependent on chitosan content. Moreover, the mechanical strength increased when glutaraldehyde was added due to a more efficient combination of chitosan and fibroin into the CS/SF structure. Nevertheless, without glutaraldehyde, chitosan is the only substance which affects the strength of CS/SF. Hence, when the chitosan content decreased, the strength of CS/SF also decreased, which is consistent with the results observed in [16]. However, the tensile strength in this study was lower than [17], due to an electrospinning method shows nanofibers and pores in various CS/SF structures. In addition, freeze-dried method shows potential for massive production with appropriate properties.

Figure. 4 SEM images of 3D interconnected pores of freeze-dried CS/SF: (A) 75CS/SF, (B) 50CS/SF, and (C) 25CS/SF.

Figure. 5 tensile strength of freeze-dried CS/SF compared with and without added glutaraldehyde.
Table 1 Pore characteristics of freeze-dried CS/SF

| CS/SF (wt%) | Porosity (%) | Pore size (μm) |
|-------------|--------------|----------------|
| 75/25       | 46.89        | 21-123         |
| 50/50       | 23.57        | 113-194        |
| 25/75       | n/a          | n/a            |

4. Conclusion
Freeze-dried CS/SF comprising of high porosity with 3D interconnected pores that are suitable for tissue engineering were fabricated. The results of FTIR and XRD indicated that the materials were comprised of highly amorphous structures and chemical functional groups that are water soluble. The mechanical strength was positively correlated with the chitosan content. Moreover, glutaraldehyde was seen to increase the tensile strength due to its ability to combine chitosan and fibroin into the CS/SF structure.

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