Research Article

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Preparation and characterization of a novel composite membrane of natural silk fiber/nano-hydroxyapatite/chitosan for guided bone tissue regeneration

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Abstract: Natural silk fiber (SF) was introduced into the chitosan/nano-hydroxyapatite (CS/n-HA) system to fabricate a novel guided bone tissue regeneration (GBR) membrane. The effect of different treatment methods (degummed, un-degummed, or dissolved SF) and different contents of SF on the properties of the CS/n-HA composite membrane was investigated. Results demonstrated that the degummed SF/CS/n-HA composite membrane with a weight ratio of 2:6:2 possessed the highest mechanical strength, where SF supported the composite membrane as a skeleton frame in the form of primeval state, while the un-degummed SF and dissolved SF had weaker reinforce effect due to the poor interface or poor interaction between SF and CS, and the dissolved SF/CS/n-HA composite membrane displayed the fastest degradation. However, the three SF could all improve the cell biocompatibility of the CS/n-HA composite membrane. Conclusively, the study revealed that degummed SF could *in situ* reinforce the CS/n-HA composite membrane with a simple and green processing method, which would provide an important guidance significant to develop a novel GBR membrane.

Keywords: natural polymer, silk fiber, biomaterials, degradation, guided bone tissue regeneration membrane

1 Introduction

Guided bone tissue regeneration (GBR) is an effective method for regeneration of many bony defects, where an ideal membrane was placed on the defect acting as a mechanical barrier membrane, aimed to prevent epithelial cells and fibroblasts from growing into the defect; thus, a singular space was created under the membrane, so as to permit the essential time for the proliferation of osteoblasts and new bone formation. Generally, the GBR membranes should have high mechanical strength, suitable biodegradation, and excellent biocompatibility (1–3).

Among the selection of membrane, the nano-hydroxyapatite/chitosan (n-HA/CS) composite membrane has been usually regarded as a candidate for the GBR membrane (4,5). However, it displayed relatively poor mechanical strength and fast degradation. To solve these problems, glutaraldehyde was added acting as chemical crosslinking. Unluckily, the remaining glutaraldehyde would inevitably bring toxicity, which was not conducive to its biocompatibility of the n-HA/CS composite (6). Therefore, it was expected to introduce other polymers to improve the mechanical strength and slow down the degradation of the n-HA/CS composite membrane.

With the research of natural fibers, natural hemp, kenaf and silk were known to reinforce polymers (7–10). Especially, silk fiber (SF), a natural macromolecule polypeptide produced by *Bombyx mori*, is mainly composed of silk fibroin and sericin (11). SF has good mechanical properties, blood compatibility, water permeability, and antibacterial property, which is known as the “queen of fiber”, owing to its good arrangement structure and component. Therefore, SF was used to reinforce polymers (12–15). However, most of researches focused on the addition of silk protein, not natural SF, which required a variety of complicated steps, such as degumming, dissolution, dialysis, and filtration, then the silk protein solution was combined with

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other polymers to be manufactured into membrane or other scaffolds (16–19). Inevitably, the tedious procession would be adverse for production, and the fiber's strengthening effect might be significantly reduced after the silk was dissolved.

Additionally, based on its excellent biological and mechanical properties, many studies have demonstrated the importance of the development of SF-based bone bio-materials, in particular GBR composite membranes by addition of SF (20–22). However, SF has not been used for reinforcing the n-HA/CS membrane. Therefore, it is worth exploring whether natural SF would *in situ* reinforce the CS/n-HA composite membrane by a simple and green processing method.

Based on the above considerations, in this work, natural SF was used to prepare the SF/CS/n-HA composite membrane, in which natural SF would support the composite as a skeleton frame in the form of primeval state, whose weight ratios of the SF/CS/n-HA were 1:6:2, 2:6:2, and 6:6:2, respectively, compared with the CS/n-HA, un-degummed SF/CS/n-HA, and dissolved SF/CS/n-HA composite membranes. Moreover, Fourier transformation infrared (FTIR), scanning electron microscope (SEM), and mechanical analyses were carried out. Furthermore, *in vitro* degradation behavior of the SF/CS/n-HA composite membranes soaked in simulated body fluid (SBF) was investigated by weight loss, water absorption rate, tensile strength reduction, and SEM morphology observation. Finally, the preliminary cell culture experiment was carried out, including fluorescence microscope observation and cell proliferation. The main aim of the study is to explore a simple and green processing method by adding the primeval state natural SF into the CS/n-HA composite membrane as a skeleton frame, so as to obtain an SF/CS/n-HA composite membrane for the GBR membrane.

2 Experimental

2.1 Materials

Chitosan (CS) was purchased from Jinan haidebei marine Bioengineering Co. Ltd., whose degree of deacetylation was 90% and molecular weight was 3×10^5 . n-HA was prepared in our laboratory by the solution precipitation method according to the relative literature (23), whose size was 100–200 nm in length and 30–70 nm in width. Silkworm silks were obtained by home feeding in Xian Zi

Jiao Zhen, Dao County, Yongzhou City, Hunan Province. All other agents were of analytical grade.

2.2 Preparation of SF/CS/n-HA composite membranes

A total of 5.0 g silkworm silks were boiled in 300 mL of 0.02 mol/L Na_2CO_3 aqueous solution for 30 min, rinsed, and dried, noted as degummed SF. A 10 mg degummed SF was dissolved in 25 mL of mixture solution composed of 11.1 g CaCl_2 , 9.2 g ethanol, and 14.4 g deionized water, named as dissolved SF. Some of the silkworm silks were rinsed with distilled water and dried, noted as un-degummed SF.

CS solution (3 wt%) was obtained with acetic acid 2%, and n-HA slurry was added with the weight ratio of CS/n-HA of 6:2. Half of the CS/n-HA mixture solution was poured into a clean and dry glass plate, degummed SF was spread out on it, and then the remaining half of the CS/n-HA mixture solution was casted on the degummed SF, whose weight ratio was 1:6:2, 2:6:2, and 6:6:2, respectively. Similarly, the un-degummed SF or dissolved SF was introduced according to the same procedure with a weight ratio of 1:6:2, and the CS/n-HA composite membrane (6:2) was also prepared by the solution cast method as controls.

2.3 Material characterization

2.3.1 Fourier transformation infrared (FTIR)

The FTIR analysis of samples was performed with a Thermo Niclet 670 spectrometer, and the spectra were collected between 600 and $4,000 \text{ cm}^{-1}$.

2.3.2 Surface morphological analysis

The surface morphology of samples was observed by SEM (S-520, Hitachi, Japan) after being sprayed with gold for 60 s.

2.3.3 Tensile strength measurement

The tensile strength of dry samples was measured using a universal testing machine (CMT6000, Sans, China) with a speed of 50 mm/min at 25°C. Three parallel samples of

each specimen were tested and the mean values were given.

2.3.4 *In vitro* degradation investigation

The samples were immersed in the SBF for 8 weeks according to related reference (24). After soaking for 2, 4, and 8 weeks, the samples were taken out from SBF, washed with deionized water, and the surface water was adsorbed with filter paper for about 5 s. The weight loss and water absorption rate were given as follows:

$$\text{Weight loss (\%)} = \frac{W_1 - W_3}{W_1} \times 100\% \quad (1)$$

$$\text{Water absorption (\%)} = \frac{W_2 - W_3}{W_3} \times 100\% \quad (2)$$

where W_1 was the original weight of the sample, and W_2 and W_3 were the wet weight and the dry weight after being entirely dried, respectively.

Moreover, the tensile strength reduction was measured after soaking. The surface morphologies of samples after being soaked for 2 and 8 weeks were observed with SEM according to the previous procedure.

2.3.5 *In vitro* cell experiment

To primarily evaluate *in vitro* cell biocompatibility, bone mesenchymal stem cells (BMSCs) were isolated from a week-old SD (Sprague Dawley) rat neonatal by the sequential enzymatic digestive process (25,26). Briefly, the femur and tibia of the hind legs of SD rats were taken out and cut off, DMEM/F-12 medium containing 10% fetal bovine serum (FBS) was added with a 1 mL syringe, and washed until the bone wall turns white. The mixture was centrifuged at 800 rpm for 3 min, and the supernatant was resuspended with 1 mL of DMEM/F-12 culture solution containing 10% FBS, then 9 mL of culture medium was added into the resulting mixture, gently shook to fully mix with the cells, finally, the supernatant was placed in the incubator at 37°C, 5% CO₂, and 90% relative humidity. After five passages, hematopoietic stem cells, red blood cells, and other hybrid cells were removed to obtain pure BMSC. The samples with the size of $\Phi 6.1 \text{ mm} \times 0.2 \text{ mm}$ were soaked with 75% ethanol solution and sterilized with an ultraviolet lamp. The treated samples were placed in a 96-well plate with the density of 8,000 cells per well, and incubated without being disturbed for 3 h, followed by an additional 1 mL culture medium into each well.

Figure 1: FT-IR spectra of the composite membranes: (a) CS/n-HA (6:2), (b) SF/CS/n-HA (1:6:2), (c) SF/CS/n-HA (2:6:2), (d) SF/CS/n-HA (6:6:2), (e) un-degummed SF/CS/n-HA (1:6:2), and (f) dissolved SF/CS/n-HA (1:6:2).

Fluorescent pictures were used to intuitively evaluate the adhesion and proliferation of BMSCs on the surface of composite membranes. After incubation for 1, 2, and 3 days, the samples were taken out and rinsed with phosphate buffer solution (PBS) twice, then dyed for 10 min with 1 mL of acridine orange (AO) solution (0.1 mg/mL), and rinsed with PBS twice again. Finally, the fluorescence microscope (Axioskop 2, ZEISS, Germany) was used to observe the morphology and growth of cells.

MTT (Cell Proliferation and Cytotoxicity Assay Kit) assay was performed to evaluate the cell proliferation (27,28). After 1, 2, and 3 days, the medium in the cell-seeded materials was discarded, and 1 mL of MTT solution of 3 mg/mL was added, followed by incubation for over 4 h at 37°C in an air atmosphere containing 5% CO₂. Then, the formazan crystals were dissolved with 1 mL of DMSO, and 100 µL of purple solution was transferred into a new 96-well plate, whose optical density values were measured with a microplate reader (Synergy HTX, BIOTEK, USA) at 492 nm.

3 Results and discussion

3.1 Physical–chemical properties of SF/CS/n-HA composite membrane

3.1.1 FT-IR analysis

Figure 1 shows FT-IR spectra of samples. It can be found that there was an obvious wide peak of 3,500 cm⁻¹ attributed

to -OH or -NH₂, when different contents of degummed SF were introduced, which showed that there was no chemical bonding between degummed SF and CS/n-HA matrix, meaning they were physical adsorption among various components, that is to say, degummed SF only acted as a skeleton frame to support the CS/n-HA composite. However, the wide peak disappeared when dissolved or un-degummed SF was added. Especially, when the peaks of 800–1,800 cm⁻¹ were partially enlarged, it could be seen that the peak of 1,645 cm⁻¹ (Amid I and II of CS) also disappeared, suggesting that there existed chemical bonding, which was formed between amino acid of sericin and some functional groups of CS (29). The reason was that the dissolved SF contained amino acids, and un-degummed SF remained sericin, as we know, sericin was composed of i, ii, iii, and iv proteins from outside to inside, and it accounted for 20–30% of silk.

3.1.2 Surface morphology observation

Figure 2 gives the surface morphology of samples. The un-degummed SF displayed some jelly on the SF surface (noted in red arrow in Figure 2g), while the degummed SF seemed smoother (shown in Figure 2h). For the CS/n-HA composite membrane, some agglomerate n-HA nanoparticles were present in the CS matrix. For the un-degummed SF or dissolved SF, there were still a few agglomerated n-HA nanoparticles. While the degummed SF membrane was embedded between the two CS/n-HA layers, SF was covered with CS and n-HA nanoparticles without agglomeration, and even the SF amount was increased to 43 wt% (namely a weight ratio of SF/CS/n-HA

Figure 2: SEM micrographs of the composite membranes: (a) CS/n-HA (6:2), (b) SF/CS/n-HA (1:6:2), (c) SF/CS/n-HA (2:6:2), (d) SF/CS/n-HA (6:6:2), (e) un-degummed SF/CS/n-HA (1:6:2), (f) dissolved SF/CS/n-HA (1:6:2), (g) un-degummed SF, and (h) degummed SF.

Figure 3: Tensile strengths of the composite membranes: (a) CS/n-HA (6:2), (b) SF/CS/n-HA (1:6:2), (c) SF/CS/n-HA (2:6:2), (d) SF/CS/n-HA (6:6:2), (e) un-degummed SF/CS/n-HA (1:6:2), and (f) dissolved SF/CS/n-HA (1:6:2).

of 6:6:2). The reason was that n-HA nanoparticles were adhered on the SF, showing that the degummed SF could prevent the agglomeration of n-HA nanoparticles.

3.1.3 Tensile strength analysis

Figure 3 shows the tensile strength of the membranes, when the degummed SF was introduced to the CS/n-HA composite membrane, whose tensile strength was enhanced with the increasing of degummed SF, and the reinforce effect was by far better than that of the un-degummed SF and dissolved SF. The reason was that sericin was removed for the degummed SF, which was more propitious to uniformly attach n-HA nanoparticles, and SF would act as a membrane

Figure 4: Weight loss of the composite membranes during the soaking.

Figure 5: Water absorption of the composite membranes during the soaking.

skeleton, so as to endow the composite membrane with excellent mechanical property, which was consistent with the SEM observation. While the dissolved SF displayed worst reinforce effect, which was caused by the poor interaction between SF and CS.

3.2 Degradation of SF/CS/n-HA composite membrane

3.2.1 Weight loss and water absorption rate analysis

The weight loss and water absorption rate of samples are shown in Figures 4 and 5, respectively. The data indicated that the weight loss rate of all composite membranes

Figure 6: Tensile strength reduction of the composite membranes during the soaking.

gradually decreased, and the dissolved SF/CS/n-HA composite membrane reflected the greatest weight loss rate among all the samples, and other samples showed little difference. Meanwhile, it could be seen that the water absorption of the dissolved SF/CS/n-HA composite membrane was the highest, further suggesting that the dissolved SF/CS/n-HA composite membrane displayed the fastest degradation rate, which were all originated from the better hydrophilicity of the dissolved SF (30).

3.2.2 Tensile strength reduction

Figure 6 shows the mechanical strength reduction during the soaking period. It could be found that the tensile strength of samples gradually reduced with the prolonging degradation time. The SF/CS/n-HA composite membranes possessed higher tensile strength than the un-degummed SF and dissolved SF composite membrane at 8 weeks, which could keep over 17 MPa, while the

Figure 7: SEM micrographs of composite scaffolds with different weight ratios after soaking in SBF: (a and A) CS/n-HA (6:2), (b and B) SF/CS/n-HA (1:6:2), (c and C) SF/CS/n-HA (2:6:2), (d and D) SF/CS/n-HA (6:6:2), (e and E) dissolved SF/CS/n-HA (1:6:2), and (f and F) un-degummed SF/CS/n-HA (1:6:2). (a–f) Soaked for 2 weeks, (A–F) soaked for 8 weeks.

Figure 8: Fluorescence staining micrographs on sample surface: (a) CS/n-HA (6:2), (b) SF/CS/n-HA (1:6:2), (c) SF/CS/n-HA (2:6:2), (d) SF/CS/n-HA (6:6:2), (e) dissolved SF/CS/n-HA (1:6:2), and (f) un-degummed SF/CS/n-HA (1:6:2). The magnification was 40 times.

dissolved SF/CS/n-HA composite membrane and CS/n-HA composite membrane only maintained 5 MPa or so.

The reason was contributed to the faster degradation of dissolved SF. Obviously, the degummed SF/CS/n-HA

composite membranes would keep mechanical support longer, and the mechanical support strength was higher than the relative literature (31).

3.2.3 Surface morphology change

To investigate the bioactivity, Figure 7 shows the SEM photograph of samples after being soaked for 2 and 8 weeks. As expected, it could be clearly found that the surface of all SF/CS/n-HA composite membranes got whiter, compared with the CS/n-HA composite membrane, which showed a large amount of apatite deposition on the surface of the SF/CS/n-HA composite membrane, and there was no difference for different treated SF, suggesting that SF was adverse for apatite deposition (32).

3.3 Cell biocompatibility of SF/CS/n-HA composite membrane

3.3.1 Fluorescence photograph observation

Figure 8 presents the cell fluorescence photographs of the samples. It could be seen that all of the SF/CS/n-HA composite membranes exhibited better cell attachment, compared with the CS/n-HA composite membrane, which demonstrated that the introduction of SF was profitable for cell biocompatibility. As we know, both chemical and topographical features of a surface could influence cell adhesion. Many relative literature indicated that SF possessed good biocompatibility (33), and the agglomerated n-HA nanoparticles in the matrix were not conducive to cell adhesion. So, we think that the reason was contributed to the good biocompatibility of SF and the better dispersion of n-HA in the matrix of SF/CS/n-HA composite membranes, compared with the CS/n-HA composite membrane.

3.3.2 Cell proliferation analysis

Figure 9 shows the cell proliferation, and it was expected to find the cells had distinct proliferation tendency after being cultivated for 1, 2, and 3 days, indicating that all samples were nontoxic. Comparing the subtle difference, the SF/CS/n-HA composite membranes possessed more remarkable proliferation than the CS/n-HA composite membrane, and the SF/CS/n-HA composite membrane

Figure 9: MTT assays of cell culture on sample surface.

with higher SF content displayed better cell proliferation. However, there was no significant difference for different SFs, which was consistent with the results of apatite deposition and fluorescence photographs.

4 Conclusion

In this study, SF was introduced into the CS/n-HA composite membrane by different treatment methods and different contents. Results showed that the degummed SF supported the composite membrane as a skeleton frame in the form of primeval state, which produced the highest mechanical strength and appropriate degradation of the composite membrane, compared with the membrane with un-degummed SF or dissolved SF, although the three different SFs were all profitable for cell biocompatibility without significant difference. The study revealed that the degummed SF could be used to *in situ* reinforce a CS/n-HA composite membrane by a simple and green processing method, which would have a great potential for the GBR membrane.

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