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Preparation, Characterization, and Potential Biomedical Application of Composite Sponges Based on Collagen from Silver Carp Skin

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ABSTRACT: To reuse fish processing waste for biomedical materials, collagen (Col) was extracted from silver carp skin, and Col–chitosan (Ch) composite sponges were prepared by a freeze-drying method. The atomic force microscopy and electrophoresis results suggest the Col might have been type I. To obtain the optimum conditions for the manufacture of the Col–Ch sponges, the characteristics of sponges composed of different ratios of Col to Ch with different crosslinkers were evaluated. Scanning electron microscopy showed that the sponges had an interconnected network structure with porosity. Infrared spectroscopy demonstrated that intermolecular crosslinkages between Col and Ch occurred. The swelling measurements implied that all of the sponges could bind an 18- to 36-fold amount of distilled water and still maintain their form and stability. When the ratio of Col to Ch was higher than 1:0.25, the swelling and degradation rate decreased with increasing Ch. Cell proliferation, hemolysis, and hemostasis assay indicated that the sponges exhibited noncytotoxicity, biocompatibility, nonhemolysis, and hemostatic efficacy. Overall, we concluded that the optimal ratio of Col and Ch for the sponges was 1:0.25, and glutaraldehyde crosslinking was more suitable than 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. These results demonstrate the potential application of silver carp skin Col–Ch sponges for tissue engineering and wound dressing in non-weight-bearing tissue. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40998.

KEYWORDS: biomaterials; chitosan; collagen; composites; silver carp skin

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INTRODUCTION

Much attention has been paid to collagen (Col) as a biomedical material for tissue engineering and wound dressings because of its excellent biocompatibility, nontoxicity, low antigenicity, biodegradability, good hemostasis, and good cell adhesion properties. However, its fast biodegradation rate and poor mechanical strength limit the use of Col. Crosslinking and the creation of mixed systems with other materials are effective methods for modifying the biodegradation rate and other physicochemical properties.[1]

Chitosan (Ch), a natural cationic polysaccharide, has a variety of outstanding biological properties, including biocompatibility, biodegradability, nontoxicity, antimicrobial activity, and hemostasis.[2] It is used in broad biomedical fields such as surgical thread, tissue engineering, drug delivery, and wound healing.[3] Col–Ch composite sponges have been reported to enhance the healing of dermal excision wounds to a higher extent compared with individual biopolymers.[4] It can also promote cell proliferation and tissue growth.[5,6] However, the sources of Col are exclusively limited to bovine and porcine skins and bones; these carry a high risk of diseases, such as bovine spongiform encephalopathy, transmissible spongiform encephalopathy, and foot and mouth disease.[7–9] In addition, the Col extracted from pigs cannot be used in some regions because of religious barriers. Therefore, a variety of alternative sources for Col have been proposed, including fresh water and marine fishes, and different marine animals, such as jumbo squid and jellyfish.[10,11]

Fish skin and scales, major byproducts of the fish processing industry that lead to waste and pollution, could provide a large amount of Col. Fish Col has similar amino acids to that of mammal Col, and it is more available and much cheaper and does not have the previously discussed infectious diseases and religion problem.[12,13] On the other hand, if we can reuse fish

Table I. Design of the Col–Ch Complex Sponges

	Composition	
Col–Ch sponge	Col (g)	Ch (g)
Col1Ch0	1	0
Col1Ch0.125	1	0.125
Col1Ch0.25	1	0.25
Col1Ch1	1	1

processing wastes for biomaterials, such as pharmaceutical microencapsulation, light-sensitive coatings, and food packaging films, this may increase the economic value of the fish. Recently, Cols from balloon fish, grass carp, catfish, and more, have been isolated and characterized.[14–16] The extraction methods and physicochemical properties of fish Col have been investigated. Nevertheless, their application is mainly in the food field, whereas its biocompatibility and use for tissue engineering and wound dressings have seldom been examined so far.

Silver carp (hypophthalmichthys molitrix) is one of the most abundant species of freshwater fish in China. Previously, our group fabricated an edible active packaging film with silver carp skin gelatin.[17] However, the biomedical applications of silver carp Col has not been examined. To reuse silver carp processing wastes for biomedical materials, in this study, Col was extracted from silver carp skin, and Col–Ch composite sponges of different ratios of Col and Ch with different crosslinkers were prepared by freeze-drying. The physicochemical characteristics of the sponges were investigated to obtain the optimum conditions for Col–Ch sponge fabrication. Furthermore, cell proliferation, hemolysis, and hemostasis assay of the sponges were evaluated to further demonstrate their potential for biomedical application.

EXPERIMENTAL

Materials

Cultured silver carp was supplied by a fish market of Fuzhou, China. Ch (viscosity-average molecular weight = 5×10^5 Da, 85% deacetylation degree) was purchased from Shanghai Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). Type I Col from bovine tendon was obtained from BMT Biotech Co., Ltd. (Fuzhou, China). Glutaraldehyde (GA; 25% water solution) was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Collagenase was obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium was obtained from Hyclone (Logan, UT). All other chemical reagents were analytical grade and were obtained from commercial sources.

Isolation of Col from Silver Carp Skin

The isolation of the Col was carried out as previously described.[14,17] Briefly, silver carp skins were cut into small pieces and mixed with 1% H_2O_2 in 0.01*M* NaOH at a solid/solution ratio of 1:20 w/v with stirring for 24 h. The previous alkali-treated skins were washed with distilled water until they reached neutrality and were then defatted with 10% isopropyl alcohol at a solid/solvent ratio of 1:10 w/v for 4 h and washed with distilled water again. After it was immersed in 2.5% NaCl for 10 h, the obtained raw Col was extracted with 2% pepsin at pH 2.5 for 20 h and centrifuged at 11,000 rpm for 20 min. The viscous liquid was collected, dissolved in 2% acetic acid (HAc), and dialyzed against distilled water for 24 h. Then, the Col solution was obtained. All of the previous procedures were performed at 4°C. The microstructure of the Col fibrils was observed by atomic force microscopy (AFM; Veeco Nanoscope Multimode IIIa). Finally, the Col was freeze-dried.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis (PAGE)

SDS–PAGE was performed according to the method of Laemmli with 8% separating gel and 4% stacking gel.[18] The Col sample was dissolved in 2% HAc. The solubilized sample was mixed with a fourfold-concentrated loading buffer (200 m*M* Tris HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue, and 40% glycerol). A 10-μL sample was loaded per well. Type I Col from bovine tendon was prepared with the same method used for the sample preparation. A high-molecular-weight marker (protein-molecular-weight marker, molecular weight range = 14,400–116,000 Da) was loaded alongside the calf Col. Electrophoresis was performed with the MiniProtean II unit (Bio-Rad Laboratories, Hercules, CA) at 15 mA/gel. The gels were stained with 0.025% Coomassie Brilliant Blue R 250 dissolved in distilled water/HAc/methanol (85/10/5% v/v/v) and destained with a solution containing methanol/distilled water/HAc (50/40/10% v/v/v).

Preparation of the Col–Ch Composite Sponges

Different ratios of Col and Ch were dissolved in 2% HAc and homogenized to obtain a Col–Ch blend solution (Col1Ch0, Col1Ch0.125, Col1Ch0.25, and Col1Ch1; Table I). After treating by freeze lyophilization, the sponges were immersed in a 0.25% GA or 50 m*M* EDC crosslinking solution at 4°C for 8 h. After the reaction, the sponges were washed repeatedly with deionized water for 1 day to remove the excess crosslinking agent.[19,20] Finally, the sponges were freeze-dried again to obtain GA-crosslinked Col–Ch sponges (S1) and EDC-crosslinked Col–Ch sponges (S2).

The morphology of the Col–Ch sponges was observed by scanning electron microscopy (SEM; Nova NanoSEM 230). The mean pore size of the sponges was determined by cross-sectional views of the SEM images. In each view, 10 apparent pores were measured with a ruler, and the average value was taken as the mean pore size. The porosity of the Col–Ch sponges was calculated by an ethanol infiltration method. Briefly, the weighed sponges (W_0) were immersed in ethanol at room temperature. The system was maintained for 20 min in a desiccator under reduced pressure to remove air bubbles. The sponges were taken out and wiped superficially with a filter paper to remove the surface ethanol and then weighed immediately (W_e). The pore volume (V_p) was defined as follows:

$$V_p = (W_e - W_0)/\rho_e$$

where ρ_e (0.789 mg/mL) is the ethanol density at room temperature. The volume of the sponges (V_s) was measured from the

sponge geometry (length, width, and height). The porosity (P) was calculated according to the following equation:

$$P = V_p / V_s \times 100\%$$

The chemical structures of the sponges were determined by Fourier transform infrared (FTIR) spectroscopy (FTIR Spectrum 2000, PerkinElmer).

Swelling Measurements

The dry sponges were weighed (W_1) and placed in distilled water at 37°C for 24 h. After we poured out the unabsorbed solution, the weight of the wet sponge was recorded as W_2. The swelling ratio of the sponges was calculated as follows:

$$\text{Swelling ratio } (\%) = \frac{W_2 - W_1}{W_1} \times 100$$

In Vitro Collagenase Degradation

The biodegradation test of the sponges was performed in phosphate-buffered saline (pH 7.4) with collagenase (200 μg/mL) at 37°C. The sponges were incubated at different time points, and then, the reaction was stopped by the addition of ice. After centrifugation at 1500 rpm for 15 min, the pellucid supernatant was hydrolyzed with 6*M* HCl at 37°C for 10 h. The degradation percentage of the sponges ($n = 3$) was determined by the measurement of the hydroxyproline content of the hydrolysate with UV spectroscopy (UV-1600, China).[21]

Cell Proliferation Assay

The cell proliferation assay was measured on the basis of the ISO10993-5 standard test method.[22] After they were sterilized by γ rays, the sponges were incubated in an RPMI 1640 containing 15% fetal bovine serum at 37°C for 24 h with an extraction ratio of 5 mg/mL. The extraction solution was stored at 4°C for further experiments.

An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the cell viability. Normal human liver cells (L02) were seeded on 96-well plates at 5 × 10^3 cells per well and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Then, the medium was removed and replaced with 100 μL of extraction solution. After culturing periods of 1, 3, and 5 days, 100 μL of 1 mg/mL MTT in culture medium was added to each well, and the cells were further incubated at 37°C for 4 h. To dissolve the formazan precipitate, the medium was removed, and 100 μL of dimethyl sulfoxide was added to each well. Subsequently, the UV absorbance at 570 nm was measured with a microplate reader (DNM-9602, China). Each sample was tested in six replicates per plate, and the results are expressed as a percentage of the absorbance of the nontreated cells.

In Vitro Hemolysis Assay

The hemolysis test was carried out according to a method reported previously.[23] Col–Ch sponge pieces (0.2 g) were immersed into 20 mL of physiological saline at 37°C for 24 h. A volume of 10 mL of the previous extraction solution was added to each sample tube. A positive control group (deionized water) and negative control group (sodium chloride injection) were used for comparison. Three parallel samples were used in each group. A volume of 5 mL of rabbit blood containing sodium citrate (0.5 mL, 2.5%) was diluted by 5 mL of sodium chloride injection (0.9%). Then, fresh anticoagulant-diluted rabbit blood was obtained. A volume of 0.2 mL of diluted blood was added to each tube, and the tubes were incubated at 37°C within a shaking water bath for 60 min. Subsequently, the solution was centrifuged at 2000 rpm for 5 min, and the supernatant was collected. The absorbance (A) value was measured with UV spectroscopy at 545 nm. The hemolysis ratio (HR) was obtained by the following equation:

$$\text{HR } (\%) = \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100$$

Hemostasis Test[24]

All animals used in this research were cared for according to the policies and principles established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty-four New Zealand rabbits were randomly assigned to six groups: S1–Col1Ch0.25, S1–Col1Ch0, S2–Col1Ch0.25, S2–Col1Ch0, gauze, and blank groups ($n = 6$ per group). Rabbits were anesthetized with 20% ethyl carbamate (2 mL/kg) via intravenous injection. A 2 × 2 cm^2 excision was created on the hepatic anterior lobe. In the experimental group, the sponges were immediately applied to the bleeding site until hemostasis was achieved. The same procedure was repeated in the gauze group, whereas free bleeding was allowed without any treatment in the blank group. The amount of blood loss and the bleeding time were recorded to evaluate the hemostatic efficacy of the sponges.

Statistical Analysis

The experimental data were analyzed with a one-way analysis of variance test of variance with a Student's t test. The results were reported as means and standard deviations. The significance level was set as $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of Col from Silver Carp Skin

To reuse fish processing wastes as a novel biomedical material for tissue engineering and wound dressings, in this study, Col was isolated from silver carp skin with pepsin first. It is known that Col has a unique structure and size, which are related to its outstanding physicochemical and biological properties.[25,26] A large amount of type I Col is present in silver carp skins. As we all known, type I Col fibrils typically display a banding pattern of 67-nm spacing called the *D-periodicity* when they are observed by electron microscopy or AFM.[27,28] As shown in Figure 1, the Col fiber was about 100 nm in diameter, and the average width of periodic banding with elevated and depressed zones was 66 nm; this suggested that the material extracted from the silver carp skin might have been type I Col.

SDS–PAGE was performed to further confirm the type of Col. As shown in Figure 2, the Col from silver carp skin had a similar electrophoretic profile to that of type I Col from bovine tendon; it contained at least two α chains (α_1 and α_2) with different mobilities. Although pepsin removed the crosslink-containing telopeptide, a β chain considered to be a dimer of the α chains was observed. These results indicate that the Col from silver carp skin might have been type I; this was supported by the AFM results. On the other hand, the subunit molecular

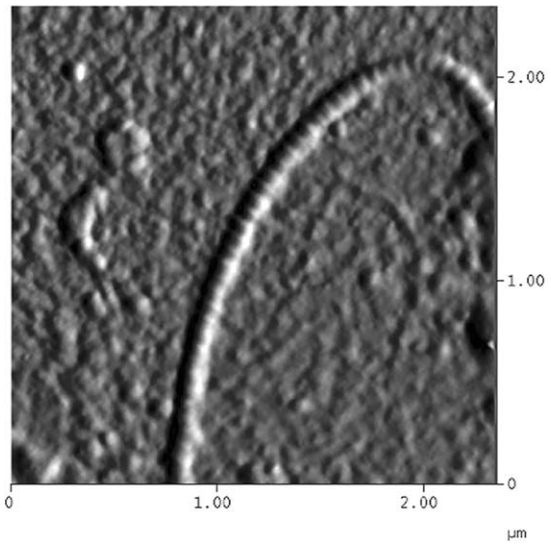

Figure 1. AFM image of Col fiber from silver carp skin. It presents the typical D-periodicity of type I Col fibrils.

weights (of the α_1, α_2, and β chains) of the silver carp Cols were lower than those of bovine Col; this was similar to another report.[29] This was due to different sources of Col, namely, mammalian animals versus aquatic animals.

Preparation and Characterization of the Col–Ch Sponges

The Col–Ch sponges were fabricated by lyophilization, and their porous structures were visually confirmed by SEM (Figure 3). The sponges had an interconnected network structure with porosity, the mean pore size was 54 ± 13 μm, and the porosity was more than 90% (Figure 4). The rapid uncontrolled quench freezing rate resulted in space- and time-variable heat transfer through the suspension; this caused nucleation, growth of the ice crystals, and sponge heterogeneity. Hence, the Col–Ch sponges showed a soft and flexible structure with interconnected porosity, which could have had good fluid absorption capability. In addition, all of the sponges (S1 and S2) showed a similar pore size, regardless of the ratio of Col to Ch or the type of crosslinking agent. With increasing Ch, the porosity of the

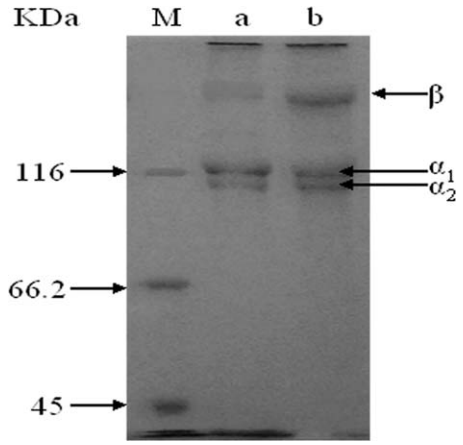

Figure 2. SDS–PAGE analysis of (M) a protein molecular weight marker, (a) type I Col from bovine tendon, and (b) Col from silver carp skin.

sponges was decreased, and that of Col1Ch0.25 was the lowest. This implied that the addition of Ch changed the porosity and that Col1Ch0.25 had the highest extent of crosslinking. On the other hand, the porosity of S1 was less than that of S2; this indicated that the crosslinking degree of GA was higher than that of EDC.

Figure 5 shows the FTIR spectra of Ch, Col, S1–Col1Ch0.25, and S2–Col1Ch0.25. In Ch, characteristic absorption bands appeared at 1659 cm^{-1} (C═O stretching), 1593 cm^{-1} (—NH_2 bending), and 1374 cm^{-1} (—CH_2 bending). The absorption bands at 1160 cm^{-1} (antisymmetric stretching of the C—O—C bridge) and 1040–1090 cm^{-1} (skeletal vibrations involving the C—O stretching) were characteristic of the saccharide structure of Ch. In Col, three amide bands at frequencies of 1659 cm^{-1} (amide I bands), 1551 cm^{-1} (amide II bands), and 1239 cm^{-1} (amide III bands) were observed; this further proved the triple helical structure of Col isolated from the silver carp skin.[30] Compared to pure Col, absorption bands at 1157, 1052, or 1092 cm^{-1} appeared in S1 and S2; this confirmed the saccharide structure of Ch.[31] In addition, the amide II bands of Col decreased greatly; this implied that a part of —NH_2 groups were changed into N—H groups because of the formation of intermolecular crosslinks between Col and Ch or within Col molecules. The position of the broad absorption bands at 3335 cm^{-1} (O—H stretch overlapped with N—H stretch) was shifted to lower frequencies; this resulted from the formation of hydrogen bonds. These results suggest that intermolecular crosslinks between the Col and Ch occurred.

Swelling Measurements

The ability of a sponge to retain water is an important property for evaluating its efficacy for biomedical materials. The swelling ratios of S1 and S2 are shown in Figure 6. All of the sponges could bind 18- to 36-fold of distilled water and still keep their form and stability. With increasing Ch, the swelling ratios of the sponges decreased, except in Col1Ch1. The water-binding ability of the Col–Ch sponges could be ascribed to their hydrophilicity and the maintenance of their three-dimensional structure. Col and Ch had a good water-binding ability; however, chemical crosslinking by GA or EDC treatment and carboxyl activation for the coupling of amine groups led to an increase in the hydrophobicity. Moreover, the hydrophilic amino acid and aldehyde were bonded after crosslinking with GA or EDC. Generally, a higher extent of crosslinking results in a lower porosity and water uptake because of the decrease of the hydrophilic groups.[32] This might explain why the swelling ratios of the sponges decreased with the addition of Ch. Nevertheless, the swelling amount did not decrease when the ratio of Col and Ch was less than 1:0.25. The reason might have been that the presence of Ch provided more amino groups than was required for crosslinking; this caused a decrease in the degree of relative crosslinking.[33] These suggested that the optimum ratio of Col and Ch was 1:0.25. On the other hand, the swelling amount of S1 was lower than that of S2 at the same ratio of Col to Ch ($p < 0.05$); this implied that the effect of GA crosslinking on the reduction of the hydrophilic groups and water-binding capacity of the sponges was more obvious than that of the EDC treatment. Therefore, in this study, the crosslinking degree of GA

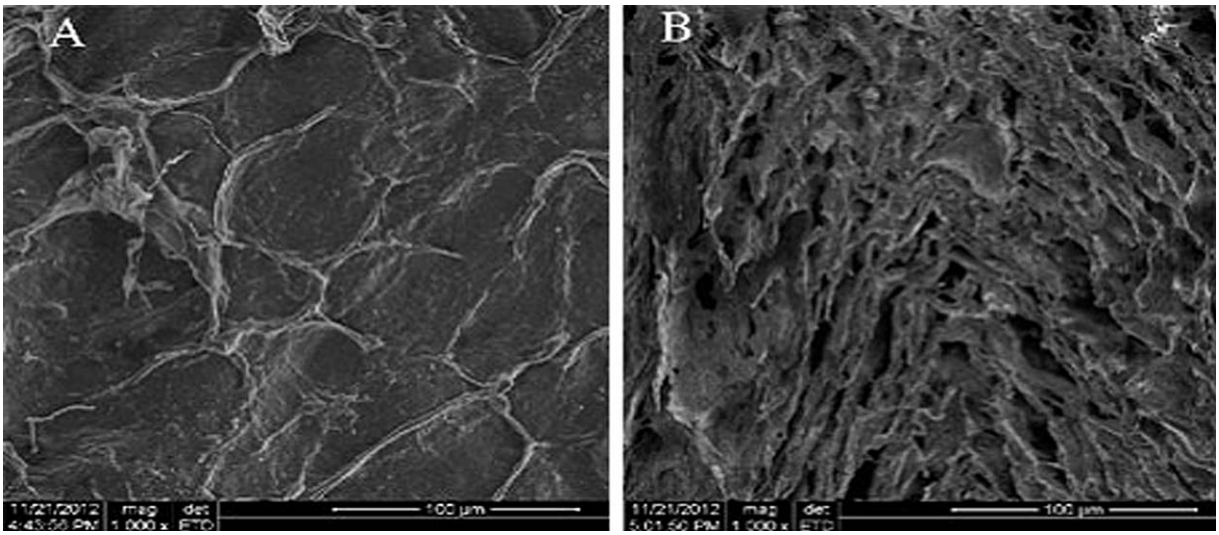

Figure 3. SEM images of a Col–Ch sponge (S1–Col1Ch0.25): (A) a surface view and (B) a cross-sectional view.

was higher than that of EDC, and the optimum ratio of Col to Ch was 1:0.25.

In Vitro Collagenase Degradation

The biostability of the Col–Ch sponges was investigated by an *in vitro* collagenase degradation test. As shown in Figure 7, most of the Col (Col1Ch0) was biodegraded after hydrolysis by collagenase for 1 day, and the degradation rate decreased with increasing Ch, except in Col1Ch1. This suggested that the resistance to collagenase increased along with the dose of Ch; this implied that the presence of Ch improved the biostability of the sponges. The degradation rate of the Col–Ch sponges might have been related to the swelling ratio, crosslinking degree, and safeguarding by Ch. When the sponge swelling ratio was higher, the collagenase solution penetrated the sponge more easily, and the contact area was much larger;[34,35] as a result, the sponges degraded more quickly. The higher degree of crosslinking led to more resistance to degradation. Furthermore, hydrogen bonding and ionic interactions acted as a mechanism for increased resistance to enzymatic hydrolysis. In this study, the degradation rate of Col1Ch0.25 was lower than those of the other groups; this might have been due to Col1Ch0.25 having the smallest swelling ratio and a larger Ch content. When the ratio of Col to Ch was less than 1:0.25, the swelling ratio, crosslinking degree, and interactions did not increase again. This indicated that the optimum ratio of Col to Ch was 1:0.25, and the degradation rate of the Col–Ch sponges could be controlled for clinical requirements by a variation in the ratio of Col to Ch. In addition, the degradation rate of S1 was lower than that of S2 at the same ratio of Col to Ch; this suggested that enzyme resistance of the sponge with GA crosslinking was more clear than that of the sponge with EDC.

On the basis of the results of the porosity, swelling ratio, and degradation *in vitro,* 1:0.25 was the optimum ratio of Col to Ch

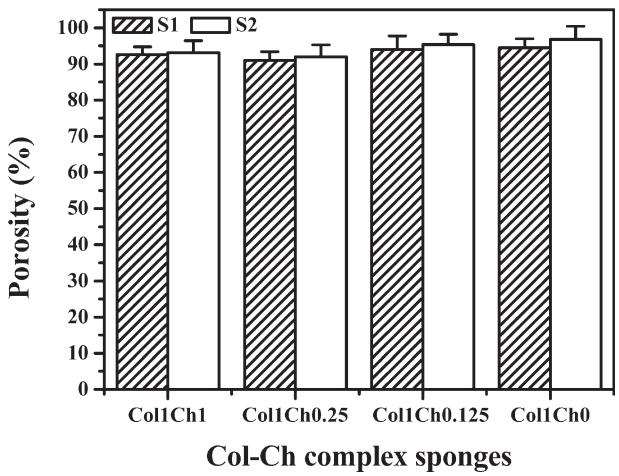

Figure 4. Porosity of S1 and S2 with different ratios of Col to Ch. The values are the means and standard deviations ($n = 6$). The data indicate no significant difference ($p > 0.1$) in comparison with S2 with the same ratio of Col to Ch.

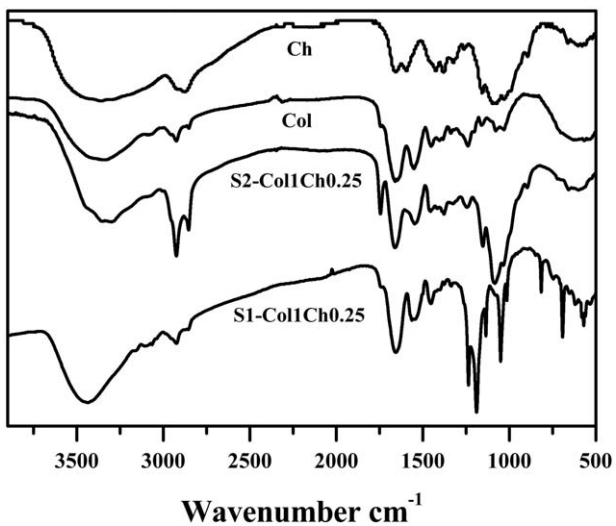

Figure 5. FTIR spectra of Ch, Col, S1–Col1Ch0.25, and S2–Col1Ch0.25.

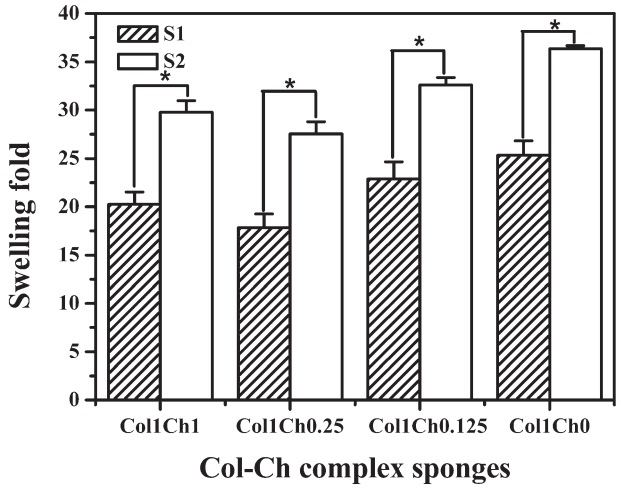

Figure 6. Effects of different ratios of Col to Ch on the swelling ratios of S1 and S2. The error bars represent the means and standard deviations ($n = 3$). The asterisks indicate statistically significant differences ($p < 0.05$).

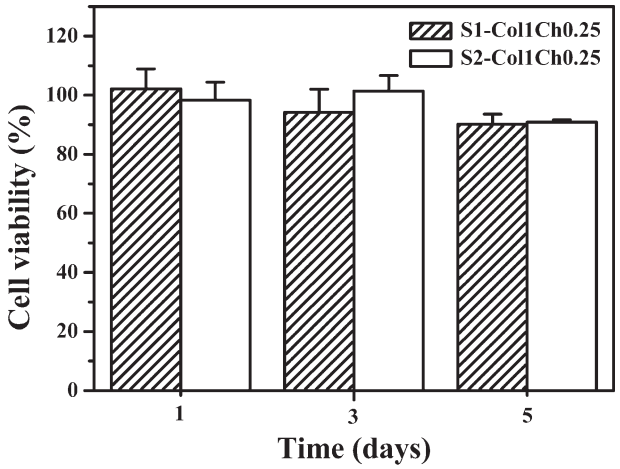

Figure 8. Cell viability of S1–Col1Ch0.25 and S2–Col1Ch0.25 on the L02 cells. The error bars represent the means and standard deviations ($n = 6$). The data indicate no significant difference ($p > 0.1$) in comparison with S2–Col1Ch0.25.

for preparing the Col–Ch composite sponges. Thus, Col1Ch0.25 was chosen for further experiments.

Cell Proliferation Assay

To further demonstrate the biocompatibility of the sponges, the cell viability of the sponges on L02 cells was evaluated by the MTT assay. As shown in Figure 8, the cell viabilities of S1–Col1Ch0.25 and S2–Col1Ch0.25 were greater than 90% after 5 days of culturing, and there was no significant difference in the cell viability between S1–Col1Ch0.25 and S2–Col1Ch0.25. The results reveal that the cytotoxicity of the GA or EDC residue was not evident; this implied that the sponges had noncytotoxicity against the L02 cells and biocompatibility.

In Vitro Hemolysis Assay

According to ISO 10993-4,[36] medical biomaterials that will be applied to the body and biological tissue should be evaluated by a hemolysis test. In this study, the HRs of S1–Col1Ch0.25 and S2–Col1Ch0.25 were 2.8 ± 0.2 and $2.2 \pm 0.3\%$, respectively, less than 5% HR. This suggested that S1–Col1Ch0.25 and S2–Col1Ch0.25 had nonhemolysis and blood compatibility, and this implied that the sponges would be compatible with erythrocytes when they were circulating in human blood.

Hemostasis Test

To evaluate the hemostatic efficacy of the Col–Ch sponges, a hepatic hemorrhage model was chosen, with the consideration that the liver is the most representative and important organ with its extremely abundant blood supply, and it is sensitive to severely traumatic hemorrhaging. The hemostatic efficacy of the Col–Ch sponges is shown in Table II. Compared to the control, medical gauze, and pure Col with the same crosslinking agent, the S1–Col1Ch0.25 and S2–Col1Ch0.25 sponges had less bleeding time and less blood loss. The hemostatic mechanism of the sponges might have consisted of three aspects. First, Col induced platelet aggregation, the coagulation factor, and thrombin generation.[37] Second, Ch quickly bonded to the red blood cells, and this resulted in a cellular hemostatic plug and the

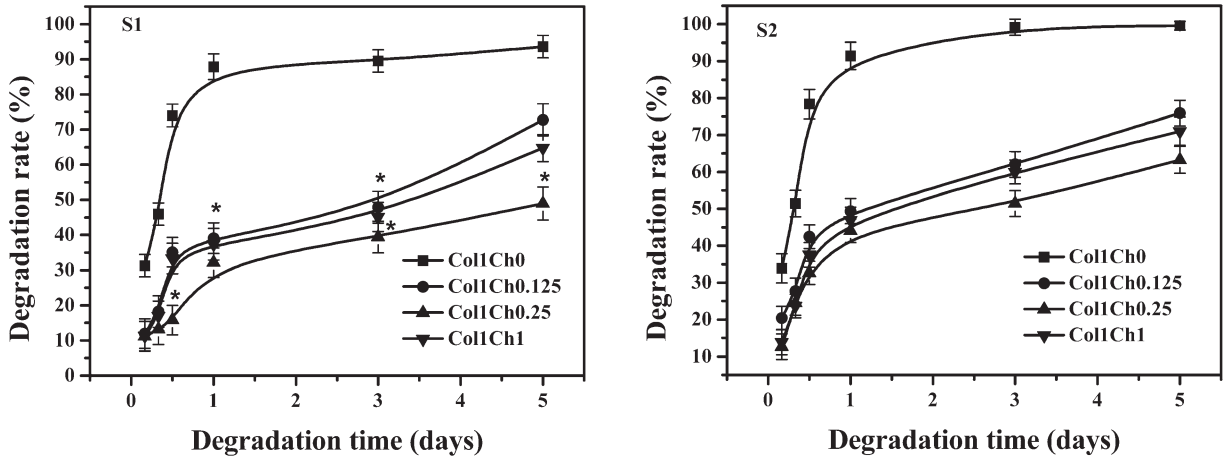

Figure 7. *In vitro* degradation of S1 and S2 with different ratios of Col to Ch. The values are the means and standard deviations ($n = 3$). The asterisks indicate a statistically significant difference in comparison with S2 with the same ratio of Col to Ch ($p < 0.05$).

Table II. Bleeding Time and Blood Loss for Different Materials

Material	Control	Medical gauze	S1-Col1Ch0	S2-Col1Ch0	S1-Col1Ch0.25	S2-Col1Ch0.25
Bleeding time (s)	413 ± 3	382 ± 2^{a}	$329 \pm 3^{a,b,d}$	$353 \pm 4^{a,b}$	$275 \pm 3^{a-d}$	$318 \pm 2^{a,b,c}$
Blood loss (g)	3.92 ± 0.12	1.66 ± 0.21^{a}	$1.16 \pm 0.17^{a,b}$	1.48 ± 0.23^{a}	$0.98 \pm 0.11^{a,b}$	$1.05 \pm 0.20^{a,b,c}$

The values are means and standard deviations ($n = 6$). Different superscript letters in the same row indicate significantly different ($p < 0.05$).
[a] Statistically significant difference in comparison with the control.
[b] Difference in comparison with medical gauze.
[c] Difference in comparison with pure Col with the same crosslinking agent.
[d] Difference in comparison with S2 with the same ratios of Col to Ch.

acceleration of local clot formation. At the same time, Ch significantly activated platelets and promoted thrombin formation.[38] Third, the three-dimensional structure of the sponges supported red blood cell aggregation and clot organization, assisted in the solidification of the growing plug, and led to a stable clot that stopped the bleeding. In view of the hemostatic mechanism, the sponges showed superior hemostatic efficacy compared to the control, gauze, and pure Col groups. Among all of the materials, S1–Col1Ch0.25 had the least bleeding time and blood loss; this indicated that GA crosslinking was more suitable than that of EDC. Therefore, we concluded that S1–Col1Ch0.25 and S2–Col1Ch0.25, especially S1–Col1Ch0.25, have the potential to be used as biomedical materials for wound dressing and tissue engineering in non-weight-bearing tissues.

CONCLUSIONS

Col was extracted from silver carp skin, and Col–Ch composite sponges composed of different ratios of Col and Ch were fabricated by freeze-drying. The sponges had an interconnected network structure with porosity and maintained their form and stability after swelling. In addition, the sponges exhibited noncytotoxicity, biocompatibility, and nonhemolysis and superior hemostatic efficacy. On the basis of the properties of the sponges, we concluded that the optimal ratio of Col to Ch for the Col–Ch sponges was 1:0.25, and GA crosslinking was more suitable than that of EDC. Therefore, the Col–Ch sponges, especially S1–Col1Ch0.25, have potential applications as biomedical materials for tissue engineering and wound dressings in non-weight-bearing tissues.

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