Preparation and Characterization of Collagen-Based Composite Conduit for Peripheral Nerve Regeneration

Wang Xiangmei,[1] Zhang Jing,[2] Chen Hao,[2] Wang Qingrui[3]

[1]*Chemical Industry and Ecology Institute, North University of China, Shanxisheng-Taiyuan 030051, People's Republic of China*
[2]*College of Basic Science, Donghua University, Shanghai 200051, People's Republic of China*
[3]*The State Key Lab of Modification for Chemical Fibers and Polymer Materials, Donghua University, Shanghai 200051, People's Republic of China*

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ABSTRACT: Collagen-based composite nerve conduit scaffold was prepared by freeze-drying steam-extrusion method and modified chemically with glutaraldehyde (GTA) by adding chitosan into collagen. Fourier transform infrared spectroscopy showed that the collagen and chitosan are certainly crosslinked through GTA. It was observed under scanning electron microscope that the modified nerve conduit material is a porous three-dimensional crosslinked structure and the quantity ratio of the collagen to chitosan has influence on the morphology. The cell proliferation experiment results showed that the collagen-based composite scaffold prompts the adhesion and proliferation of cells, but as the chitosan increasing, the cell proliferation decreased slightly. The swelling property, the collagenase degradation, and the mechanical property of the scaffold are tested at the quantity ratios of collagen to chitosan 4 : 3, 3 : 1, and 4 : 1 and crosslinking time 0.5 and 1.0 h. The experiments show that the stability of the scaffold is enhanced with decreasing the quantity ratio of collagen to chitosan and increasing crosslinking time. Through the experimental investigations, the modifying technique parameters are discussed and the scaffold exhibits better physical and chemical properties at the quantity ratio of collagen to chitosan 3 : 1 and crosslinking time 0.5 h. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 3652–3662, 2009

Key words: collagen-based composite scaffold; biostability; crosslink; peripheral nerve regeneration; conduit

INTRODUCTION

The nerve autograft is the mostly used method for repair of peripheral nerve injuries. However, because of donor site morbidity, such as loss of sensory function and formation of neuromas, lack of donor tissues, and potential differences in tissue structure and size,[1,2] which severely limited its application in clinical surgeries, the use of nerve guidance conduit for peripheral nerve regeneration is introduced. With the rapid development of tissue engineering, more and more studies have been attracted to this field.[3–5] In general, the suitable material for peripheral nerve regeneration should have the following properties[6–8]: (1) it must allow diffusion transport of nutrients while preventing external cells from entering the conduit, (2) it must become revascularized fast enough to overcome nutrient transport limitations into the graft, (3) it must be able to degrade slowly enough to maintain a stable support structure for the entire regeneration process, but should not remain in the body much longer than needed to prevent later compression of the nerve, (4) the material must be immunologically compatible with the host, (5) it must be surgically, and (6) it must be able to support cell adhesion and cell spreading on its surface.

Naturally derived polymers, for example, collagen, have the advantages of biologic properties, such as cell proliferation, biocompatibility, and maintaining its biologic functions. Therefore, natural polymer becomes main materials for artificial nerve conduit. However, natural polymers have some disadvantages as nerve conduit materials. They have poor mechanical strength, fast degrade, and too much uptake of water, which can result in slump or deform of the nerve conduit during the bridging process. Therefore, these materials could not be used for repair of damaged peripheral nerves. Many researchers have been tried to use the various molding methods and modification methods to modify these natural polymers.[9–14]

For the molding, many efforts were dedicated to improve the molding method (solution casting and freeze-drying) of the collagen conduit to obtain the high porosity and required mechanical property of the nerve conduit. Li and Qakland[9] used the dip-

Correspondence to: W. Xiangmei (wangxiangmei@nuc.edu.cn).

coating–freeze-drying, and Ahmed et al.[10–12]used the laminar evaporation of collagen solution, and after crosslinking the multilayered semipermeable conduit achieved. Brendan et al.[13] fabricated tubular scaffolds with a radial pore size gradient by a spinning technique. All their works aimed to obtain the pore scaffolds which facilitate cell adhesion and cell spreading.

For the modification of the material, Itoh et al.[14] evaluated the different crosslinking methods of ultraviolet (UV) irradiation, heating, and immersing in glutaraldehyde (GTA or GA). Itoh et al. studied the effective crosslinking of collagen with UV irradiation for peripheral nerve repair. Ahmed et al.[10–12] gave a discussion of mechanical properties, cell proliferation of the collagen conduit irradiated by microwave and crosslinked with GTA. The microwave-irradiated collagen conduits result in ample myelinated axons compared with GTA group. They concluded that physical crosslinking (UV irradiation, microwave irradiation, or heat treatment) makes the collagen conduit better cellular activity and good nerve regeneration, but poor mechanical properties. Otherwise, the chemical crosslinking effectively prompts the crosslinking degree, and the physical characteristics of the collagen conduit are improved, but make the collagen conduit less cellular activity and poor nerve regeneration.

In this study, chitosan, which has a better mechanical strength, hemostatic activity, and wound-healing functions and can react with collagen, was added into collagen solution during the preparation of artificial nerve conduit to improve the biostability of collagen. The addition of the chitosan also reduces the amount of GTA and thus lowers the potential cytotoxicity brought from GTA crosslinking.[15–17] First, the freeze-drying steam-extrusion method was designed to prepare nerve conduit of high porosity. Then, the effects of quantity ratio of collagen to chitosan and crosslinking time on the properties of the conduit were studied. Through the experimental investigations, the optimum technical parameters for preparation of the nerve conduit are chosen, and the way to improve the physical property and to not depress the biocompatibility was discussed.

EXPERIMENTS

Materials

Pepsins were purchased from Shanghai Yuanju Biology Technology Co., China. Hydroxyprolines were purchased from Shanghai Chemical Reagent Co, China. Chitosans (96.7% deacetylated, $[\overline{M}_n] = 1.5 \times 10^5$) were purchased from Shandong Weifang-Kehai, Co., China. Collagenases were purchased from Sigma. Glutaralaldehyde (GTA), 25% water solution were purchased from Shanghai Pharm, Co., China. All other reagents and solvents are of analytical grade and used as received.

Collagen was isolated from fresh bovine tendon by pepsin digestion and citric acid dissolution method. Its purity was confirmed by UV spectroscopy, Fourier transform infrared spectroscopy (FTIR), and amino acid analysis. The natural triple helixes of collagen were preserved during the extraction process. All of the collagen scaffolds were made of the same batch to avoid influence of the raw material differences in this study.

TABLE I
Feed Composition and Crosslinking Condition for Collagen-Based Composite

Sample code	Collagen/chitosan of the quantity ratio	TGA crosslinking times (h)
CC-4	4 : 3	0
CC-7	3 : 1	0
CC-8	4 : 1	0
CC-4h05	4 : 3	0.5
CC-7h05	3 : 1	0.5
CC-8h05	4 : 1	0.5
CC-4h10	4 : 3	1.0
CC-7h10	3 : 1	1.0
CC-8h10	4 : 1	1.0

Preparation and modification of collagen-based composite materials

A total of 1.2% (w/w) collagen solution (1.00 mol/L citric acid solution pH = 2–3) was completely mixed with 4% chitosan solution (1% of aqueous acetic solution) in certain proportions at 37°C with stirring. The resulting mixture of a quantity ratio of collagen to chitosan, after defoamed in a centrifuge defoaming device, was introduced into a mold with a central axis (polytetrafluoroethylene) of an inner diameter 3.2 mm. The mold was placed in a freeze-drying machine for prefreezing for 4 h at −50°C, and then was kept under vacuum condition at the same temperature for 24 h. The porous collagen-based composite conduits were formed when solid water was vaporized under vacuum. The conduits were processed by compressing in flow of steam until their wall thickness is 2.0 ± 0.2 mm. Then, the conduits were compressed into ribbed ones with pitch of 1.0 mm. The resulting conduits of collagen-based composite scaffolds were dipped in a solution of 0.25% of GTA at 25°C for the crosslinking times 0.5 and 1.0 h, and then washed repeatedly with deionized water for 3 h. After the conduits were dried, they were treated with ethylene oxide at 37°C for sterilization. Feed composition and crosslinking condition for collagen-based composites are listed in Table I.

Fourier transform infrared spectroscopy of collagen composite film

Specimens of collagen-based composite thin film, uncrosslinked and GTA crosslinked, were used for FTIR measurements with Nicolet-20sx-B.

Scanning electron microscopic analysis of collagen-based composite scaffolds

The morphology of the surface, the radial cross sections of the scaffolds were observed by scanning electron microscopy (SEM, JSM-5600LV JEOL Japan). Specimens were placed on a Cu mount and coated using a gold-coating apparatus.

Porosity of collagen-based composite scaffolds

The porosity of collagen-based composite scaffolds was measured by volume mensuration at temperature 25°C. A pycnometer filled with ethanol was weighted as W_1. The sample of collagen composite scaffolds, weighted W_s, was immerged into the ethanol in the pycnometer. After elimination of gases in the ethanol and waiting enough time for the ethanol entering the scaffolds completely, the pycnometer was weighted again as W_2. Removing the scaffolds soaked with ethanol, the pycnometer was weighted as W_3. Consequently, the porosity of collagen-based composite scaffolds is calculated as follows.

$$\text{Volume of the scaffolds} : V_s = (W_1 + W_s - W_2)/\rho$$

$$\text{Volume of apertures in the scaffolds} : V_p = (W_2 - W_3 - W_s)/\rho$$

where ρ is the mass density of ethanol.

$$\text{Porosity of the scaffolds} : \varepsilon = V_p/(V_p + V_s) = (W_2 - W_3 - W_s)/(W_1 - W_3).$$

Equilibrium swelling studies

The influence of alkaline pH on swelling behavior of the scaffold was determined by using phosphate buffer (PBS) (pH 7.4, 25°C), until equilibrium was obtained. Samples used for determining the equilibrium swelling characteristics were made by cutting a scaffold conduit into small pieces. The swollen weights of various scaffolds were accurately determined after removing the water adsorbed by them using filter paper. The weights of the scaffolds were recorded every hour until equilibrium stage was reached.

Percentage swelling of scaffold at equilibrium was calculated as[18]:

$$E_{\text{sw}} = \left[\frac{W_e - W_0}{W_e}\right] \times 100,$$

where W_e is weight of the scaffold at equilibrium swelling, W_o is weight of the dry scaffold, and E_{sw} is the percent swelling at equilibrium (four samples were tested).

Mechanical tests of collagen-based composite conduits

Radial compressive measurements

Because of very small diameter of the conduits, conventional testing devices are not suitable for testing their compressive properties in the radial direction. A custom-built compression device, manufactured by Laizhou Electron Instrument Co., was used to test the properties of the conduits. The testing parameters were chosen as follows.

The testing conditions: diameter of the punch, 5 mm; length of samples, 12 mm; compression–decompression cycle number, 5; maximal displacement, 50% of the initial diameter of sample; compression rate, 6 mm/min.

$$\text{Elastic recovery rate } (\%) = \frac{L_1^1 - L_1}{L_0 - L_1} \times 100\%$$

where L_0 is the initial diameter of samples, mm; L_1 is the diameter of sample after compression, mm; L_1^1 is the diameter of sample after recovery, mm.

Wet condition: specimen is soaked to the equilibrium state at temperature 25°C.

In the experiment of radial compressive properties, three characteristics, maximum strength of compression and rebound, compression–decompression cycle number, and elastic recovery rate, which denoted the resisting dent capability of scaffolds, were tested.

Tensile strength measurements

Besides the compressive strength, the tensile strength of the collagen-based composite conduits is also an important factor to ensure that the conduits could withstand suturing and remain intact after surgery. The characteristics denoted the tension properties of scaffolds are elastic modulus, tensile strength, yield point, and percentage elongation at fracture point.

Samples of length 50 mm were tested in wet condition by universal testing machine (AGS-500ND) of Shimadzu Corp. of Japan to examine strength properties. A stress–strain plot was made for each specimen at a speed of 10 mm/min. Tensile strength was calculated by the breaking load and area of cross

section, and the percentage elongation was calculated by the ratio of increase in length to original length.

In vitro collagenase degradation

The biological stability of the crosslinked collagen-based composite scaffolds was evaluated by *in vitro* collagenase biodegradation test. Each kind of scaffolds was incubated in PBS (pH 7.4) containing a given concentration of type I collagenase at 37°C for 24 h in atmosphere of 5% CO_2. The degradation was stopped at a given time interval by incubating the assay mixture in an ice bath immediately. Centrifuging the assay mixture at 1000 rpm for 10 min, the clear supernatant was hydrolyzed with 6*M* HCl at 120°C for 24 h. The content of hydroxyproline released from the collagen molecules in the scaffold was measured with ultraviolet spectroscopy.[19,20] The biodegradation degree is defined as the percentage of the released hydroxyproline from the scaffolds to that from the completely degraded collagen material with the same composition and weight.

In vitro culture studies

Resuscitated and incubated for a while, the retina cells were cultured over the various sterilized materials. The cells were cultured for 24 h and measured by MTT. All cells were from the same batch of resuscitated retina cells.

As the absorbency is proportional to the cell proliferation, the absorbency of the solution containing the cells denotes the cell number.

RESULTS AND DISCUSSIONS

Fourier transform infrared studies

The FTIR spectra obtained from UCC-7 and CC-7h10 are shown in Figure 1.

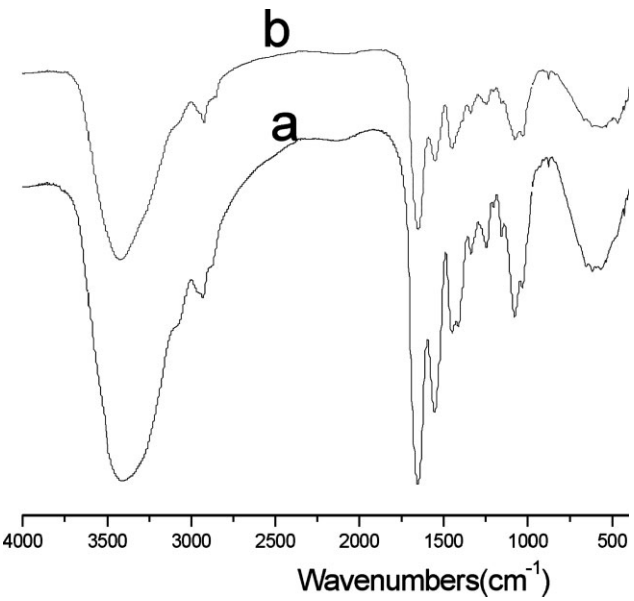

Figure 1 FTIR spectra of (a) uncrosslinked and (b) GTA crosslinked collagen-based composite scaffolds.

It is well known that the characteristic absorption bands of pure collagen[21] appear at the frequencies of 3324, 1650, and 1560 cm^{-1}. Generally, amide I bands (1650 cm^{-1}) originate from C=O stretching vibrations coupled to N—H bending vibration. The amide II bands (1560 cm^{-1}) arise from the N—H bending vibrations coupled to C—N stretching vibrations. The other amine, arising from the stretching vibrations of N—H group, of a medium to weak intensity, appear at 3324 cm^{-1}. Moreover, it is well known that the characteristic absorption bands of chitosan appear at 1655 (C=O stretching), 1585 (—NH_2 bending), and 1380 cm^{-1} (—CH_2 bending). And, the absorption bands at 1152 (antisymmetric stretching of the C—O—C bridge), 1084, and 1040 cm^{-1} (skeletal vibrations involving the C—O stretching) are characteristics of saccharide structure of chitosan.[22]

The spectra of uncrosslinked collagen-based composite (UCC-7) and GTA crosslinked collagen-based composite (CC-7h10) show that the amide II bands (1560 cm^{-1}) and peak at 3439 cm^{-1} corresponding to NH_2 group bands of CC-7h10 decreased greatly. The change of amide II bands means that the free —NH_2 groups in collagen molecules and the —NH_2 (1585 cm^{-1}) groups of chitosan were changed to N—H groups (i.e., intermolecular crosslinkages between chitosan and collagen or within collagen molecules formed through GTA), as shown in Figure 2.[23] The vital observation from this study is that the collagen and chitosan are certainly crosslinked through GTA without any significant change in the chemical property. Hence, they can exert their characteristics individually during *in vitro* culture studies.

Scanning electron microscopy analysis

A rough and porous surface of the uncrosslinked collagen-based composite conduit (UCC-7) was observed under SEM (Fig. 3). The size of the pores was not uniform. The overall surface had a streaky appearance, and there were large gaps between streaks. The surface of the GTA crosslinked collagen-based composite conduit (CC-7h05) (Fig. 3) was netlike and looked dense. The average size of pores was about a few micrometers and appeared even. The surface of crosslinked collagen-based composite conduit was smoother than that of uncrosslinked collagen-based composite conduit. This makes cells

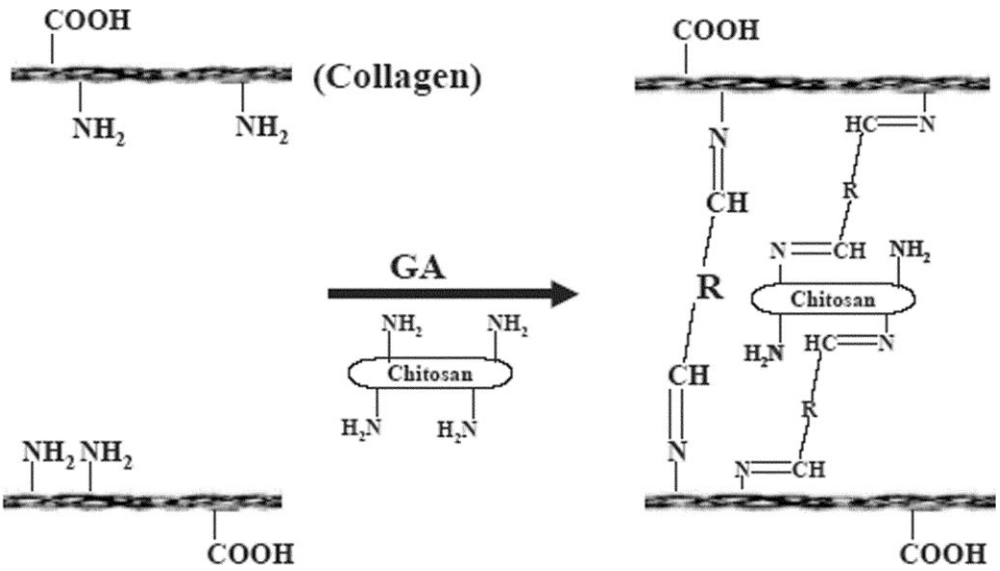

Figure 2 Schematic presentation of collagen crosslinked with GTA in the presence of chitosan.

more easily move on it. With the crosslinking of GTA, the structure of the conduit changed from steaks to nets, which led to an increase in mechanical strength of the conduit.

The radial cross sections of collagen-based composite scaffolds, uncrosslinked and crosslinked, were examined by SEM (Fig. 4). After crosslinking, the pore size of the composite scaffold became smaller and a three-dimensional network structure was formed in it. And, with the chitosan increasing, the microstructure density of scaffolds becomes a little higher.

Effects of the modifying techniques on the porosity of collagen composite scaffolds

For migration of the cells and exchange of metabolized substance, the porosity of collagen-based composite scaffolds should be selected carefully. Some patents indicated that the optimal porosity of the nerve conduit is 70–98%.[9]

The different collagen-based composite scaffolds with various quantity ratios of collagen to chitosan and several crosslinking times with GTA were investigated through experiments. The results are shown in Figure 5.

It is showed that the crosslinking time with GTA has an obvious effect on the porosity of collagen-based composite scaffolds. Although the porosity of uncrosslinked scaffolds is about 90%, the porosity of crosslinked scaffolds with GTA for 0.5 h is about 80% and that of crosslinked scaffolds with GTA for 1.0 h is about 50–60%. That is because of dense fabric of the scaffolds crosslinked with GTA, which makes the volume of the scaffolds shrink and the porosity reduce.

It is also displayed that the porosity of collagen-based composite scaffolds with various quantity

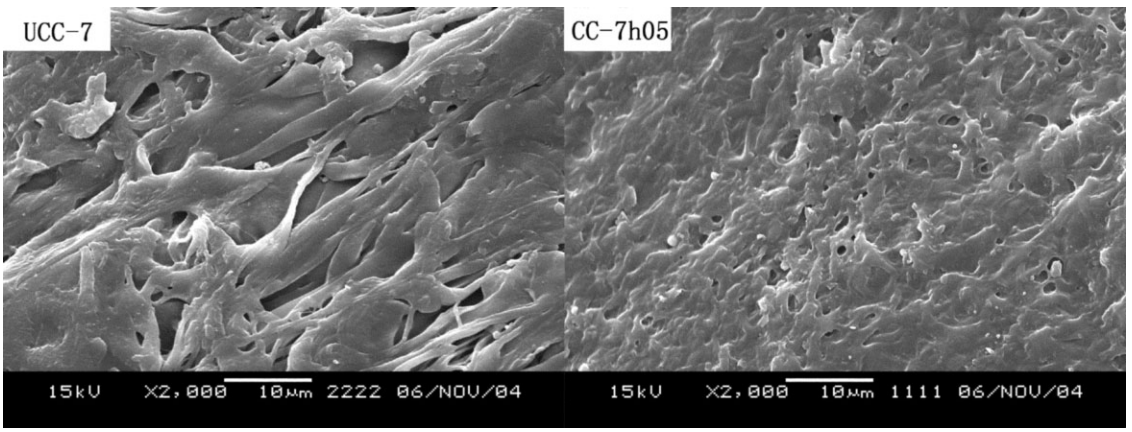

Figure 3 Scanning electron micrograph of surface of uncrosslinked and GTA crosslinked scaffolds, original magnification ×2000; scale bar:10 μm.

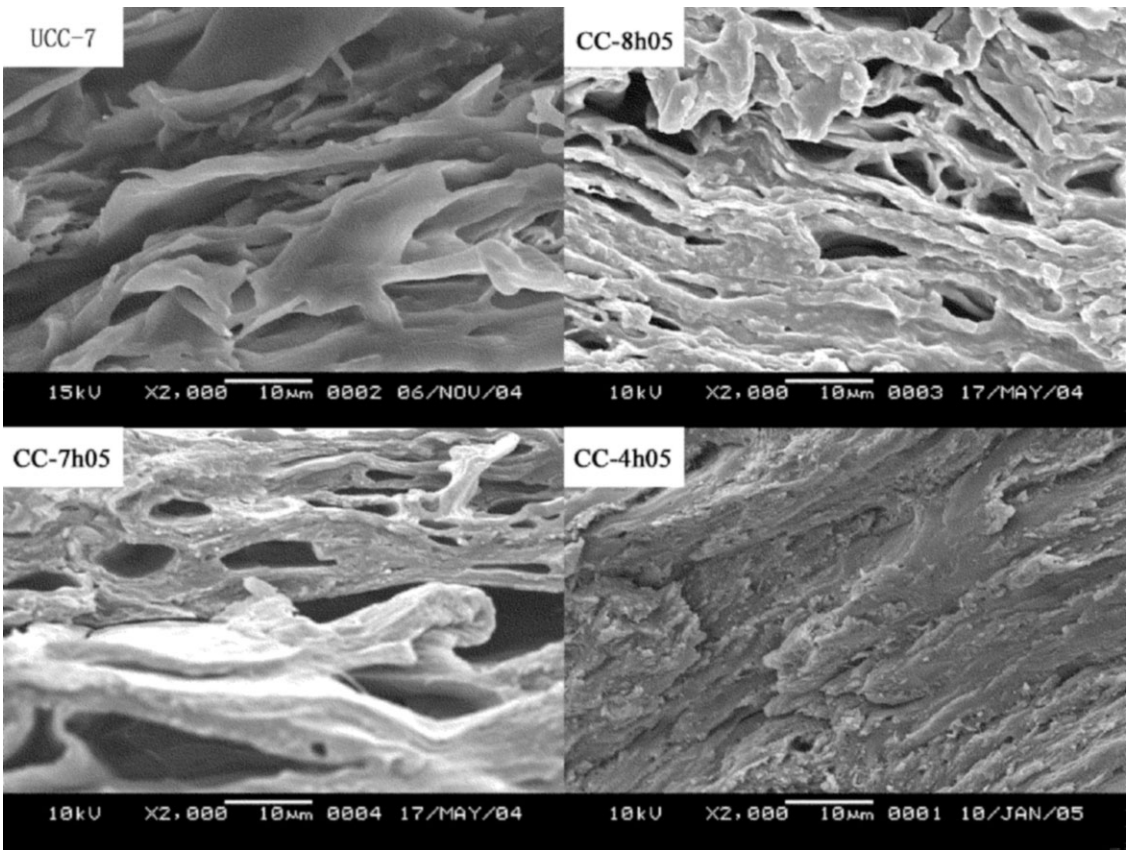

Figure 4 Scanning electron micrograph of radial cross section of crosslinked scaffolds, original magnification ×2000, scale bar:10 μm.

ratios of collagen to chitosan has a little difference. The porosity of collagen-based composite scaffolds tends to be declined with chitosan increasing. Here, the chitosan, like a crosslinking reagent, makes the fabric of the scaffolds dense. That is verified by SEM.

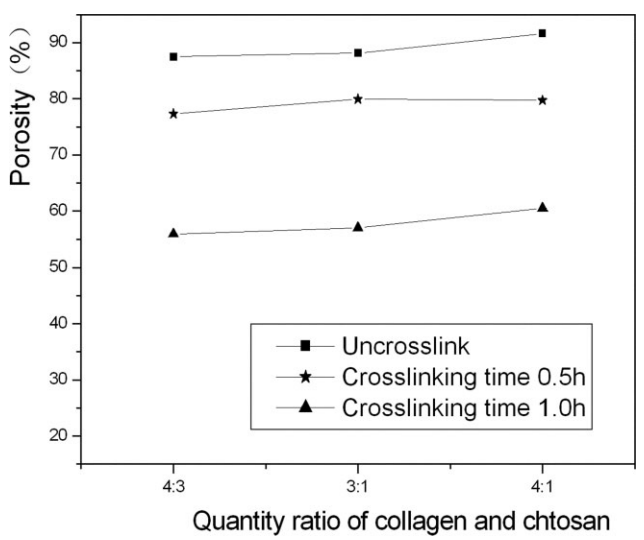

Figure 5 Effects of technological parameters on porosity of collagen-based composite scaffolds.

Equilibrium swelling studies

The water uptake of the composite scaffolds is a very important factor *in vitro* or *in vivo* experiment. If its water uptake is too low, the collagen-based composite conduit will have a poor biocompatibility and poor bioactivity. On the other hand, if the uptake is too high, the lumen of the conduit will be distorted. Den Dunnetal et al.'s[24] studies revealed that the deformation of the nerve conduit would hinder nerve regeneration. In addition, after the conduit takes up water, its wall gets thicker, that will hinder the exchange of nutrients between inside and outside of the conduit.

Percentage swelling of the collagen-based composite scaffolds was shown in Figure 6.

From comparison of the swelling properties of various scaffolds, the scaffold prepared from crosslinking time 0.5 h and the quantity ratio of collagen to chitosan 4 : 1 showed maximum swelling of 132.06%, at the 3rd hour in PBS and the equilibrium stage reached there after, and the scaffold prepared

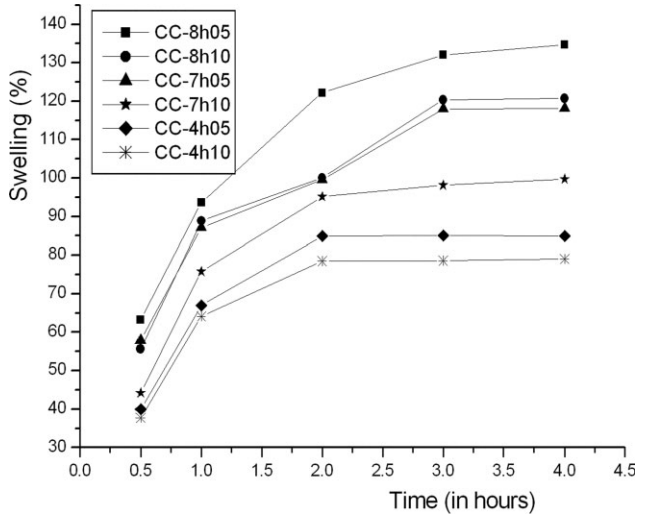

Figure 6 The swelling curves of various collagen-based composite scaffolds in phosphate buffer (pH 7.4) at temperature $T = 25°C$.

from crosslinking time 1.0 h and the quantity ratio of collagen to chitosan 4 : 3 showed minimum swelling of 78.46%, at the 2nd hour in PBS and the equilibrium stage reached there after. PBS of pH 7.4 was selected as the swelling agent to simulate the condition *in vivo*.

The results of equilibrium swelling of experiments indicate that the swelling ratio is decreased as the quantity of chitosan and the crosslinking degree increasing.

Water uptake of the scaffolds is different from the porosity. Although the porosity of the scaffolds depends on the apertures, water uptake of the scaffolds depends not only on the hydrophilicity but also on the microstructure of the scaffolds. The hydrophilicity of the scaffolds is affected by crosslinking degree and hydrophilic groups.

To explain the aforementioned results, the measurements of contact angle between deionized water and the collagen-based composite membrane were carried out. The feed composition and crosslinking condition is as same as that in section "preparation and modification of collagen-based composite materials," except the method of the membrane drying. The collagen-based composite solutions were laid on the fluoride glass plate and then were spread using glass stick to even membranes. They were dried under vacuum condition at temperature 37°C. The thickness of the dried membranes is 0.25 mm. The contact angles were tested after 10, 60, and 120 s of the contact time, respectively. The results, averaged over seven times for each sample, were listed in Table II. It is read in Table II that the water contact angle is enlarged and the hydrophilicity of the collagen-based composite is lower, as the quantity of chitosan and the crosslinking time increasing.

It is proved that the prolonged crosslinking time and increasing quantity of chitosan affect the water uptake of the collagen-based composite scaffolds. But the effects of them are different. Increase of crosslinking time enhances the crosslink degree greatly (Fig. 2), lowers the hydrophilicity and causes the fabric of the scaffolds dense, which brings on the water molecule permeating into the composite difficultly. Therefore, the time of equilibrium swelling becomes shorter and water uptake declines. Moreover, increase of quantity of chitosan accelerates the reaction of the —OH groups and —COOH groups in collagen molecules with $—NH_2$ groups in the chitosan molecules, which lead to slightly enhancing the crosslink degree and the poor hydrophilic property.

Based on the Ahmed et al.'s and the aforementioned results, the optimal range for water uptake for collagen-based composite conduit for peripheral nerve regeneration is about 90–120% at 25°C.

Results of the radial compressive test

The typical curves of force versus displacement of specimen CC-7h10 are shown in Figure 7.

Radial compressing behaviors with different quantity ratios of collagen to chitosan and different crosslinking times are listed in Table III.

It was shown in the experiments that the maximum compressive force and elastic recovery rate, which denoted the compressive property, are inversely proportional to the quantity ratio of collagen to chitosan for same crosslinking time. For example, for 1 h of crosslinking time, both of the maximum compressive force and the elastic recovery rate increase with decreasing the quantity ratio of collagen to chitosan, the former from 13.3 ± 1.0 cN to 40.8 ± 0.6 cN, the latter from 87.5 to 93.7%, in the first cycle, as the quantity ratio of collagen to chitosan changes from 4 : 1 to 4 : 3. With the increase in the

TABLE II
Contact Angle of Various Collagen-Based Composite Membrane

Quantity ratio of collagen to chitosan	Crosslinking times	Contact angle (°)		
		Contact time		
		10 s	60 s	120 s
4 : 1	0	34.0	28.0	20.0
	0.5	63.0	56.0	42.0
	1.0	67.0	60.0	52.0
3 : 1	0	45.5	37.0	27.0
	0.5	73.0	63.0	59.0
	1.0	79.0	65.0	60.0
4 : 3	0	60.0	44.0	33.0
	0.5	86.0	81.0	78.5
	1.0	90.0	88.5	80.0

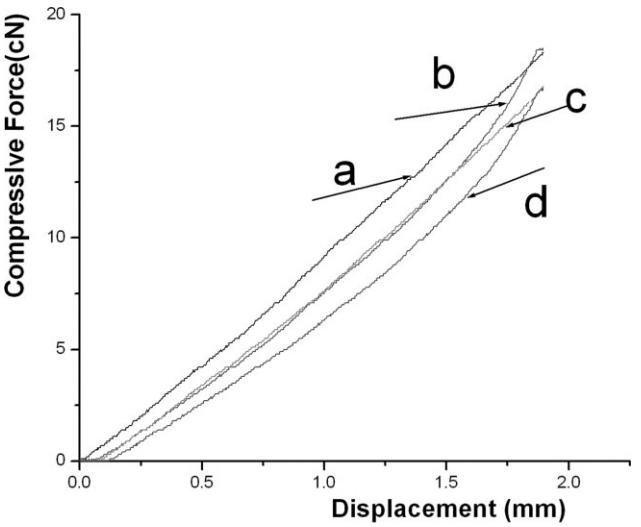

Figure 7 Representative diagram of force versus displacement of crosslinked collagen-based composite conduit. a,b: curves of first cycle; c,d: curves of last cycle.

crosslinking time, the compressive property is also enhanced. For example, at the quantity ratio of collagen to chitosan 3 : 1, both of the maximum compressive force and the elastic recovery rate increase when crosslinking time increases, the former from 16.5 ± 0.3 to 18.4 ± 0.5 cN, the latter from 87.0 to 88.3%, in the first cycle. In addition, the times of cycle have an effect on the compressive property and the maximum compressive force exhibits descending trend with increase of cycles.

It is concluded that the resisting dent capability and fatigue resistance of the scaffolds can be improved by varying the quantity ratio of collagen to chitosan and the crosslinking time.

Results of the tension test

The results data are listed in Table IV.

It is shown, through comparison of values of the ultimate stress and elastic modulus in the Table IV, that the collagen-based composite scaffolds with higher quantity of chitosan and longer crosslinked time are the more rigid and strong than that of lower quantity of chitosan and shorter crosslinked time. The rigid and strong scaffold is necessary to resist the deformation, but a brittle rupture will happen if the scaffold is over rigid.

To determine the effects of the modifying conditions on the toughness of the scaffolds in detail, the fracture energy is to be calculated. It is revealed from the Table IV that when the crosslinking time with GTA is 0.5 h, the fracture energy of the collagen-based composite scaffolds with large quantity ratio of collagen to chitosan (4 : 1–3 : 1) alters more rapidly than that of the scaffolds with low quantity ratio of collagen to chitosan (3 : 1–4 : 3). That is due to the low crosslinking degree resulted in the short crosslinking time, at which the effect of the quantity ratio of collagen to chitosan on the toughness is significant. The more quantity of collagen in the scaffolds is, the better the toughness is. But when the crosslinking time with GTA is 1.0 h, the fracture energy of the scaffolds is slowly increased as the quantity of collagen increasing. This is due to the high crosslinking degree resulted in the long crosslinking time. If the crosslinking degree is over high, the fracture energy will decrease and the scaffold is easy to take place brittle fracture.

In vitro collagenase degradation

Figure 8 compares the biodegradation degree of the pure collagen scaffold and the various collagen-based composite scaffolds before and after GTA crosslinking. After incubated in collagenase solution for 24 h, the pure collagen scaffold (col) had been thoroughly biodegraded. The addition of chitosan (UCC) can somewhat increase the biostability, and the biodegradation degree was slightly lower. The biostability of GTA crosslinked collagen-based composite scaffolds were greatly enhanced, where only 2.51% (CC-4h05), 6.42% (CC-7h05), and 7.00%(CC-8h05) were degraded in 24 h. These results reveal that addition of chitosan and GTA crosslinking is indispensable for improving the scaffold biostability.

TABLE III
Radial Compressive Properties of the Collagen-Based Composite Conduits

Quantity ratio of collagen to chitosan	Crosslinking time 0.5 h			Crosslinking time 1.0 h		
	Max. compressive force (cN)		Elastic recovery rate (%)	Max. compressive force (cN)		Elastic recovery rate (%)
	First cycle	Last cycle	Last cycle	First cycle	Last cycle	Last cycle
4 : 1	9.7 ± 0.5	8.7 ± 0.9	83.7 ± 1.5	13.3 ± 1.0	11.3 ± 0.8	87.5 ± 2.0
3 : 1	16.5 ± 0.3	15.5 ± 0.4	87.0 ± 1.7	18.4 ± 0.5	16.8 ± 0.6	88.3 ± 0.9
4 : 3	32.2 ± 0.2	30.7 ± 0.6	90.3 ± 0.8	40.8 ± 0.6	38.7 ± 0.2	93.7 ± 0.7

The values indicated represent mean ± SD, where $n = 5$.

TABLE IV
Tensile Properties of the Collagen-Based Composite Conduits

Quantity ratio of collagen to chitosan	Crosslinking time 0.5 h					Crosslinking time 1.0 h				
	Ultimate stress (MPa)	Yield point	Percentage elongation (%)	Elastic modulus (kPa)	Fracture energy (MJ/m^3)	Ultimate stress (MPa)	Yield point	Percentage elongation (%)	Elastic modulus (kPa)	Fracture energy (MJ/m^3)
4 : 1	9.10 ± 0.14	Yes	52.1 ± 0.97	98 ± 9	232 ± 10	10.04 ± 0.10	Yes	39.3 ± 0.80	87 ± 7	127 ± 8
3 : 1	12.96 ± 0.12	Yes	24.9 ± 0.10	249 ± 7	135 ± 6	13.79 ± 0.15	No	23.8 ± 0.50	326 ± 11	121 ± 5
4 : 3	15.26 ± 0.10	No	17.5 ± 0.23	880 ± 7	133 ± 9	20.75 ± 0.11	No	16.5 ± 0.40	886 ± 3	119 ± 8

The ability to resist collagenase degradation was enhanced for the collagen-based composite scaffolds, because of addition of chitosan. Furthermore, the presence of chitosan can obviously improve the biostability of the collagen-based composite scaffolds under the GTA treatment, where chitosan functions as a bridge to increase the crosslinking efficiency of GTA (Fig. 2).

In vitro culture studies

The high-porosity collagen-based composite scaffolds were molded by freeze-drying steam-extrusion method, and the collagen-based composite membranes were compared to explore the effects of the quantity ratio of collagen to chitosan and their microstructure on the adhesion and growth of the cells. The collagen-based composite membranes are prepared as follows. The collagen-based composite solutions prepared in the way in the section "preparation and modification of collagen-based composite materials" were laid on the fluoride glass plate and then were spread using glass stick to even membranes. They were dried under vacuum condition at temperature 37°C. The thickness of the dried membranes is 2.0 ± 0.2 mm.

Effects of the quantity ratio of collagen to chitosan of the membrane on the growth of cells

The cell proliferation experiments were carried out using collagen composite membranes, which were not crosslinked with GTA, of different quantity ratios of collagen to chitosan. The results are shown in Figure 9.

It is shown in the Figure 9 that the cell proliferation of collagen composite membranes is higher than that of the control group. That indicated the collagen

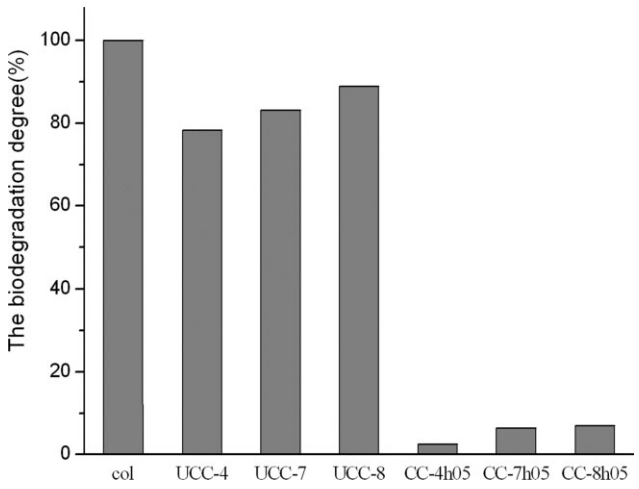

Figure 8 The biodegradation degree of the pure collagen scaffolds and the collagen-based composite scaffolds.

composite membranes promote the adhesion and proliferation of the RPE cells. It is also shown that the effects of the various quantity ratios of collagen to chitosan on the cell proliferation have a little difference.

Effects of the quantity ratio of collagen to chitosan of the collagen-based composite scaffolds on the growth of cells

The cell proliferation results of the collagen-based composite scaffolds were shown in Figure 10. The cell growths on glass plates (control), pure collagen (Col), UCC-7, UCC-4, UCC-7h05, and UCC-4h05 were denoted by their absorbencies. Experiments showed that the absorbencies for all the collagen-based scaffolds were significantly higher than absorbency (0.485) for the control or absorbency (0.034) for the blank. It is proved that the collagen-based composite scaffolds prompt the adhesion and proliferation of cells, but the absorbency varied with the different modifying technique parameters. It is summed up that: (1) the absorbencies for the scaffolds are deceased a little after GTA crosslinking; (2) whether the scaffolds crosslinked or uncrosslinked, the absorbencies for the scaffolds of quantity ratio of collagen to chitosan 3 : 1 are higher than that for the scaffolds of quantity ratio of collagen to chitosan 4 : 3. That indicates that both GTA crosslinking and addition of chitosan are not of advantage to the adhesion and proliferation of cells.

The effects of the quantity ratio of collagen to chitosan on the cell proliferation exhibited different in the sections "effects of the quantity ratio of collagen to chitosan of the membrane on the growth of cells" and "effects of the quantity ratio of collagen to chitosan of the collagen-based composite scaffolds on the growth of cells." That is due to the microstructure of the materials molded by different preparation methods. The collagen-based composite scaffold, molded by freeze-drying steam-extrusion method, is a high porosity structure. The collagen-based composite membrane has a little aperture. Measurement results proved that the high porosity structure is of advantage to the cell proliferation. Otherwise, as the quantity of chitosan increasing in the porous scaffold, the microstructure of scaffold becomes dense and causes the adhesion slightly less and proliferation of cells slowly.

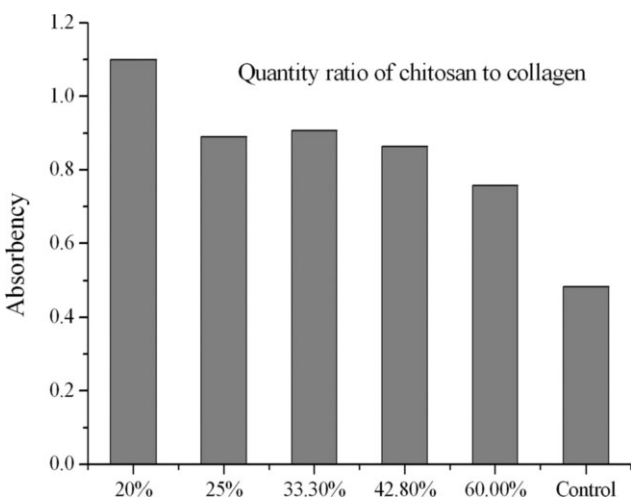

Figure 9 Effects of chitosan contents on RPE growth from the collagen-based composite membranes.

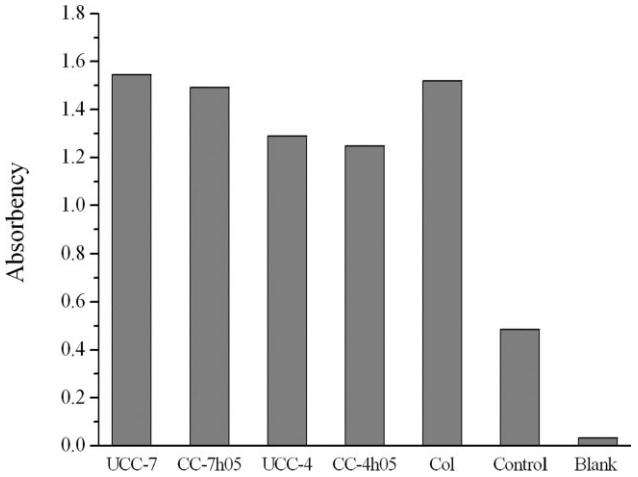

Figure 10 Effect of quality ratio of collagen to chitosan on RPE growth on the collagen-based composite scaffolds.

CONCLUSIONS

In this study, the molding technology and the modification method of collagen-based nerve conduit scaffold were researched. A freeze-drying steam-extrusion method was proposed for preparing the collagen-based composite nerve conduit scaffold to obtain porosity structure. To improve the biostability of collagen material, chitosan was added into collagen solution during the preparation of artificial nerve conduit scaffold, and the scaffold was further crosslinked with GTA. Investigations on the properties of the scaffold were made through experiments and the conclusions are as follows:

1. FTIR spectroscopy showed that the chitosan is certainly crosslinked through GTA without any significant change in the chemical property of the collagen.
2. SEM observation indicated that the crosslinked nerve conduit material is a porous three-dimensional network structure.
3. The water uptake of the collagen-based composite material is related to the hydrophilic property and the microstructure of the material. It is proved that the prolonged crosslinking

time and increasing quantity of chitosan affect the water uptake of the collagen-based composite scaffolds. But the effects of them are different. Increase of crosslinking time enhances the crosslink degree greatly. Increase of quantity of chitosan leads to slightly enhancing the crosslink degree.

4. Increasing the quantity of chitosan in the collagen-based composite material causes the adhesion slightly less and proliferation of cells slowly.
5. Decreasing the quantity ratio of collagen to chitosan and increasing crosslinking time with GTA enhance the mechanical properties and collagenase degradation of the scaffold.

The results showed that the quantity ratio of collagen to chitosan, crosslinking time with GTA, and the microstructure are the important factors to improve the physical property and to not depress the biocompatibility for preparation of collagen-based composite conduit. Through the swelling property, collagenase degradation and mechanical property measurements, and the cell proliferation experiments, the optimum modifying technique parameters are the quantity ratio of collagen to chitosan 3 : 1 and crosslinking time 0.5 h.

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