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Preparation and Characterization of Collagen–Chitosan–Chondroitin Sulfate Composite Membranes

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Abstract Collagen (Col)–chitosan (Chi) membrane was modified by a hot dehydrogenation cross-linking method. Carbodiimide was added for further crossing modification. Chondroitin sulfate (CS) was added so that Col–Chi sulfate composite membranes were prepared. The structure of the composite membranes was characterized by Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy, and its mechanical properties, degradation, and cytotoxicity were characterized. The composite membrane was applied to a full-thickness skin injury in animal experiments performed in rabbits. Strong interactions and good compatibility among Col, Chi, and CS in the composite membrane were present. The good mechanical properties, biocompatibility, digestion resistance, and wound healing promotion of the composite membrane make it a potential wound dressing or skin scaffold for tissue engineering.

Keywords Chitosan · Chondroitin sulfate · Collagen · Medical composite membrane · Wound repair

Biomedical membrane is an important kind of biomedical material for tissue-engineering scaffolds, wound dressings, and hemostatic products, and it has been a high-priority research topic for the past few decades (Wang et al. 2011).

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Wound healing comprises a complicated sequence of cellular and biochemical events involving inflammation, migration, and proliferation of a variety of cell types; production of extracellular matrix (ECM) and proteins; and neovascularization (Chen et al. 2006).

It has been demonstrated that the ECM, including such elements as collagen (Col) and glycosaminoglycans (GAGs), plays an important role in the process of injury repair. In particular, Col has been demonstrated to be a good ECM substitute in injury repair (Bissell 2001; Midwood 2004). Col can identify the specific molecular signal and mediate cell adhesion and migration. It also has low immunogenicity and good histocompatibility. In addition, its small-chain polypeptide of protein and amino acid in the catabolite could be used as the nutrient component of tissue-engineered cells. However, Col has several negative effects when extracted, including overall structure change, diminished intensity, and degradation of collagenase in vivo. Wound repair materials that use Col as a membrane have been extensively studied during the past few years (Braga-Vilela et al. 2008; Rho et al. 2006). Various technologies and methods have been applied to increase the intensity of Col and to control its degradation in vivo. In particular, Col may be mixed with chitosan (Chi) and GAGs.

Chi is a natural polysaccharide that is structurally similar to GAGs. It consists of β-(1-4) linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups. It has been reported to be nontoxic and biocompatible, and it promotes wound healing (Francis and Howard 2000). Furthermore, the incorporation of Chi into a Col scaffold is known to increase its mechanical strength, as it forms an ionic complex between the positively charged Chi and the negatively charged Col (Taravel and Domard 1996). In addition, the biodegradable

Chi itself provides bacteriostatic and fungistatic activities (Tomihata and Ikada 1997). For these reasons, Chi has been an important biomaterial for wound management. A novel asymmetric Chi membrane has been prepared by an immersion–precipitation phase-inversion method and evaluated as a wound covering (Mi et al. 2001).

GAGs, including hyaluronan and chondroitin sulfate (CS), are amino-sugar-containing polysaccharides in the ECM of all vertebrates. CS is composed of alternating units of β-1,3-linked glucuronic acid and (β-1,4)-*N*-acetyl-galactosamine (GalNAc), and it is sulfated in either the 4 or 6 position of the GalNAc residues (Kirker et al. 2002). Furthermore, CS has biocharacteristics that include the binding and modulation of growth factors and cytokines, and CS is involved in the adhesion, migration, proliferation, and differentiation of cells (Pieper et al. 2000; Lee et al. 2004).

By dehydrothermal treatment (DHT) modification and an EDC (1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide) cross-linking method (Ma et al. 2004), following the Col–Chi preparation technology, CS was introduced to combine with the composite membrane in order to provide the biological activity of polysaccharides and to further improve its stability and biocompatibility (Ye et al. 2007a, b). The purpose of this study was to develop a composite membrane for tissue-engineered skin scaffolding or wound dressing.

Experimental Materials

Materials and Reagents

The following chemicals, all of analytical grade and used as received without any further purification, were purchased: 2-*N*-morpholino ethanesulfonic acid (Amresco, Dallas, TX); carbodiimide (EDC, Yanchang Biochemistry Company, Shang Hai, China); *N*-hydroxysuccinimide (NHS; Yanchang Biochemistry Company); Chi (degree of deacetylation >87 %, Cheng Du Kelong Chemical Reagents, Chengdu, China); cartilage (CS, Sigma, St. Louis, MO); ninhydrin (Merck, Darmstadt, Germany); lysozyme (Cheng Du Tian Tai Life Sciences, Chengdu, China); glycine (Amresco); and recombinant human basic fibroblast growth factor (bFGF; Sigma). Col was obtained in our laboratory (molecular weight 300,000 Da).

The following experimental apparatuses were used: lyophilizer (Freeze 6; Labconco, Kansas City, MO); CO_2 cell incubator (Sanyo, Tokyo, Japan); inverted phase contrast microscope (Olympus IX70; Olympus, Tokyo, Japan); centrifuge (Varifuge 3.0RS; Thermo Scientific Heraeus, Hanau, Germany); and scanning electron microscope (SEM; JSM-5900LV; JEOL, Tokyo, Japan).

Methods

Preparation of DHT-EDC-Modified Col–Chi–CS Membrane

The membrane was prepared by blending 0.5 mol/L aqueous HAc at a given ratio (Col/Chi = 1:1). After freeze-drying, the unmodified membrane was vacuumed at room temperature, then treated for 2 h after increasing the temperature to 110 °C. The resulting membrane was soaked for 30 min in 40 ml 40 % ethanol solution (pH 5.0–6.0) containing 50 mM 2-*N*-morpholino ethanesulfonic acid, then soaked for 4 h at room temperature in 40 % ethanol solution that contained 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC, 20 mM *N*-hydroxysuccinimide, NHS, and 1 % CS. Next 0.1 M Na_2HPO_4 solution (pH 9.1) was introduced to wash the composite scaffold for 4 h. The material was washed overnight in different concentrations of NaCl (aq) and neutralized to pH 7.0 via freeze-drying. This process completed the preparation of the DHT-EDC composite modified membrane.

After EDC modification, the Col–Chi–CS membrane was washed thoroughly with 0.1–1.0 M Na_2HPO_4 (aq) (pH 8.0–9.1), 1–2 M NaCl (aq), 5–50 mM glycine, and double-distilled water. The self-made bFGF controlled microsphere dispersed with phosphate-buffered saline (PBS) was evenly sprayed onto the surface of the membrane. After drying, the wound healing membrane based on Col was obtained. It was stored it in a medical package that was irradiation-sterilized by a ^{60}Co irradiation apparatus with 25 kGy before further evaluation.

Structure Characterization of Modified Col–Chi–CS Membrane

X-ray Photoelectron Spectroscopy X-ray photoelectron spectroscopy (XPS) was performed regulated with a standard sample by the XSAM800 ESCA system Au (Au4f = 84.0 eV) and Ag (Ag3d = 386.3 eV).

Determination of Free Amino Groups and Modification Index We drew standard curves with the ninhydrin colorimetry method. We then calculated the modification index by the equation $[(M_0 - M_1)/M_0]$, where M_0 is the free amino group amount before modification and M_1 is the free amino group amount after modification.

Fourier Transform Infrared Spectroscopy The modified Col–Chi–CS membrane was analyzed by Fourier transform infrared spectroscopy (FTIR) with the KBr pellet pressing method. The accompanying scanning wave number was

450 ~ 4,000 cm^{-1} and the resolving power was 2 cm^{-1}. Each scan was repeated 15 times.

Properties of Modified Col–Chi–CS Membrane

Tensile Strength Dumbbell-shaped membrane was prepared and put in the condition (temperature 20 ± 2 °C, relative humidity 65 ± 2 %) for 48 h. The tensile strength was determined on a tensile testing machine with a velocity of 10 mm/min; each sample was tested five times and the average value calculated.

Degradation Test Vacuum-dried membrane samples (10 × 25 mm) were stored in test tubes after being precisely weighed. The samples were then soaked with 5 ml lysozyme degradation solution (1.0 mg/ml PBS). After putting the sealed test tubes in a bath rotator (fixed rotation speed 80 rpm), samples were withdrawn for testing at 1, 2, 3, 5, 8, 12, and 15 days. The degradation solution was replaced every 72 h to maintain enzyme activity. Samples were washed with deionized water and dried to a constant weight. The degree of degradation was characterized by the decrease in weight.

Cytotoxic Test The membrane samples were cut into small samples of 1 cm^2. Extracted fluid was withdrawn daily after soaking the samples in 4 ml of culture medium at 37 °C for 3 days. Fibroblast cells were cultured in extracted fluid for 3 days, then tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and observed via optical microscope.

Cell Culture The Col–Chi–CS membrane was cut and placed into a 24-well culture plate. Fibroblast suspension (5 × 10^4 cells/ml) was then placed on the membrane and cultured under 37 °C 5 % CO_2 for 2 h. Three milliliters of culture medium was added, and it was allowed to sit for another 3 days. The results were observed by SEM.

Wound Healing Test Twenty New Zealand rabbits were selected, 10 each male and female, weighing 1.5–2.0 kg. General anesthetized rabbits were dehaired and sterilized on two sides of their backs to permit full-layer wounds 3 × 4 cm in size. In the experimental group, the wounds on the left side were covered with Col–Chi–CS membrane; in the control group, they were not. Then all the wounds were swabbed with oil and stitched. After 1 week, the dressing was removed. Each rabbit was injected with veterinary-quality ampicillin trihydrate 0.25 g for 3 days. We then recorded the amount of bleeding, the duration of the healing period, and the appearance of the wounds.

Results and Discussion

XPS Analysis

The composite membrane was prepared by introducing CS during EDC modification. To find out whether the CS had combined with the membrane or whether it was easily washed away during the follow-up processes (to ensure that the Col–Chi–CS membrane was firmly formed), XPS was performed to analyze elements of the membrane surface (Fig. 1). There was an S-element peak at 167.8 eV in the Col–Chi–CS membrane, while there was only a signal peak in the Col–Chi membrane (Fig. 2). We speculate that CS bonded with the Col–Chi membrane. The element content of the two different membranes is listed in Table 1. Although there was 0.2 % S in the Col–Chi–CS membrane, there was none detected in the Col–Chi membrane; in addition, an increase in O and a decrease in N and C were also measured. CS belongs to the polysaccharides, which contain large amount of SO_4 and –OH, leading to the appearance of S and the increase in O detected in the Col–Chi–CS membrane. This element change further clarified the existence of CS.

The C1s peak was fitted and separated into several peaks: C–H, C–OH, C–NH_2, and C=O (Fig. 3; Table 2). The Col–Chi–CS membrane has more C–OH and C–NH_2 polar groups and fewer C–H nonpolar groups than the Col–Chi membrane. The change in the content of the polar and nonpolar groups indicated a change in the hydrophilic and hydrophobic abilities of the membrane surface. Compared with the Col–Chi membrane, the Col–Chi–CS membrane maintained a higher hydrophilic but a lower hydrophobic performance, which simultaneously affected the cell adhesion and biocompatibility, resulting in a marked application difference.

Free Amino and Modification Index Analysis

CS contains large amounts of hydroxide radicals, carboxyl groups, sulfate radicals, and so on. During EDC modification, an amino link or ester bond was generated by the reaction of the carboxyl group from CS with a hydroxide radical or an amino group from the composite membrane, respectively. A hydrogen or ester bond was generated by the reaction of the hydroxide radical of CS with the amino group or the carboxyl group from the composite membrane, respectively. As a bridge, not only were the active groups of the binary membrane (Col–Chi) cross-linked with CS, but also the distant nonactive groups, thus improving its performance. The ternary membrane (Col–Chi–CS) cross-link reaction degree was determined by the analysis of free amino content. At the same condition of

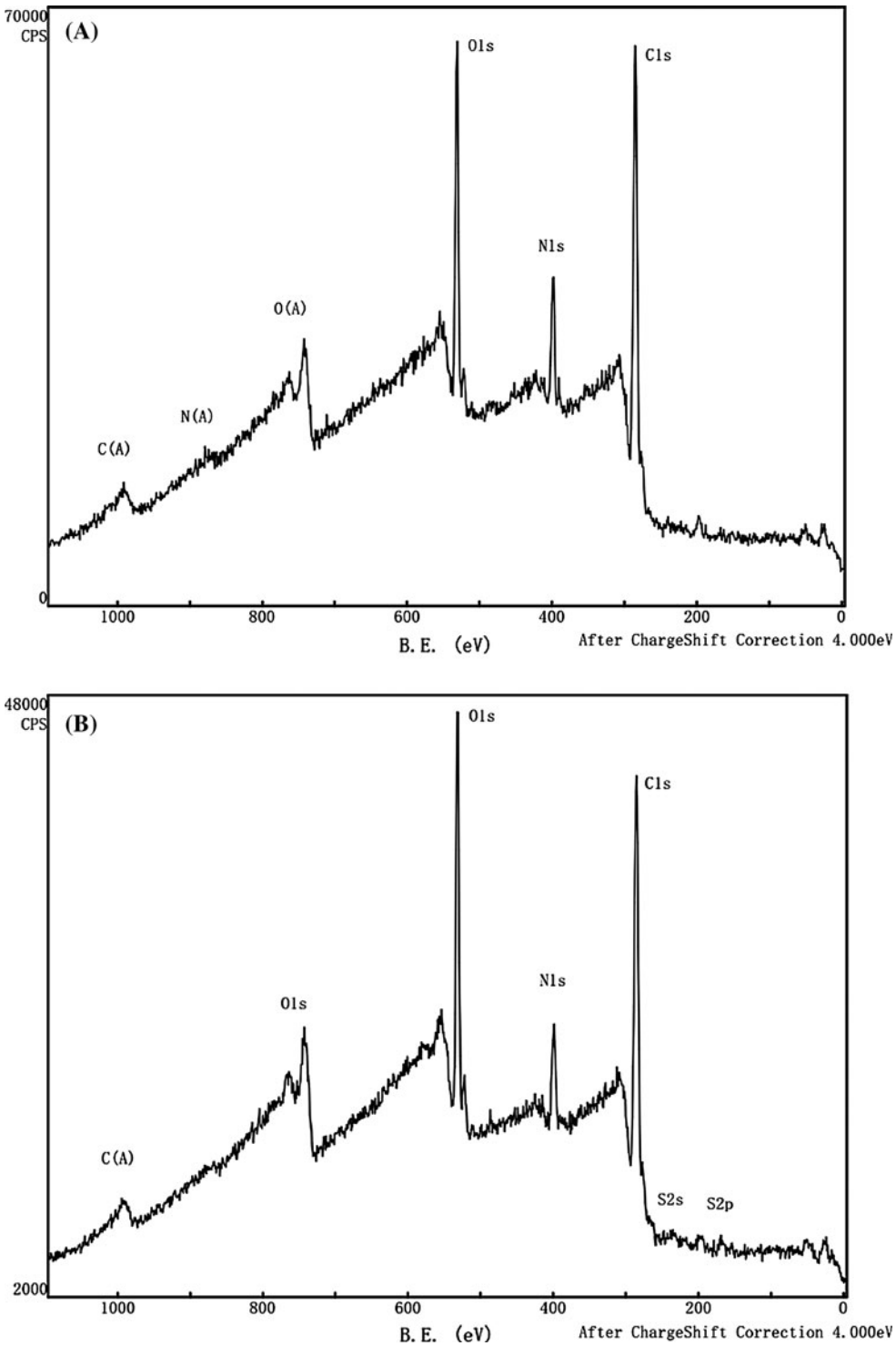

Fig. 1 XPS of composite membrane. **a** Col–Chi membrane. **b** Col–Chi–CS membrane

EDC modification compared with two-component membrane, the cross-linking index of the three-component membrane was larger, which, as a result of the bridge function of CS combined the distant active groups, accordingly improved the degree of the cross-link reaction (Fig. 4).

FTIR Analysis

Figure 5 shows the FTIR curves of the Col–Chi and Col–Chi–CS membranes. A blueshift occurred in the Col–Chi–CS membrane (amide 1,658.80 cm^{-1}) compared to that of Col–Chi membrane (1,658.62 cm^{-1}). The peak at around

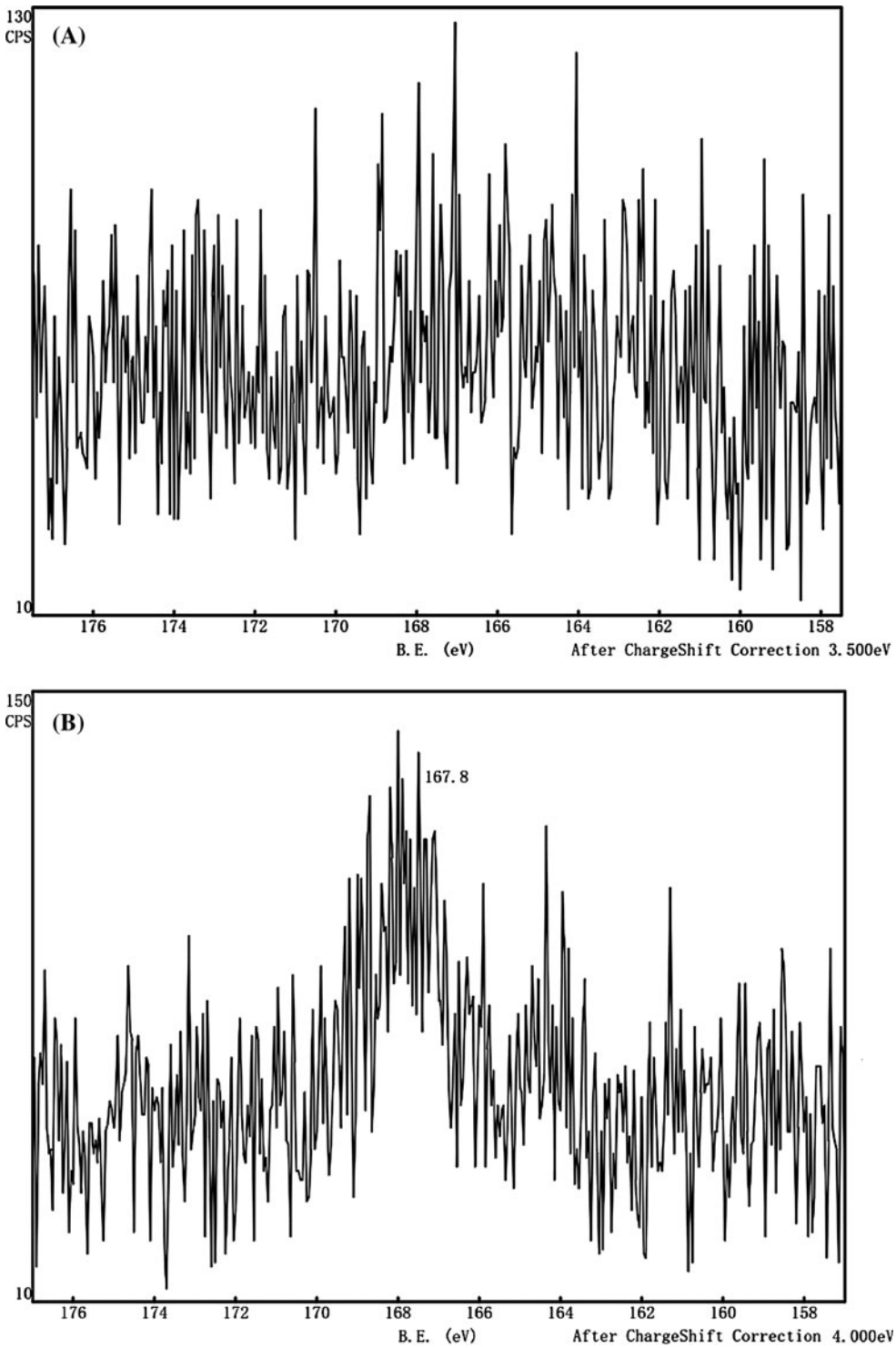

Fig. 2 S energy spectra of XPS. **a** Col–Chi membrane. **b** Col–Chi–CS membrane

Table 1 Content of surface element of Col-based membrane

	C	O	N	S
Col–Chi	66.9	22.8	10.3	0
Col–Chi–CS	65.2	25.9	8.7	0.2

620 cm^{-1} was attributed to an out-of-plane deformation vibration of the primary amine. The *D* value between intensities of deformation vibration and amide to some extent reflects the content of the primary amine in these materials. According to our calculations, the *D* values of

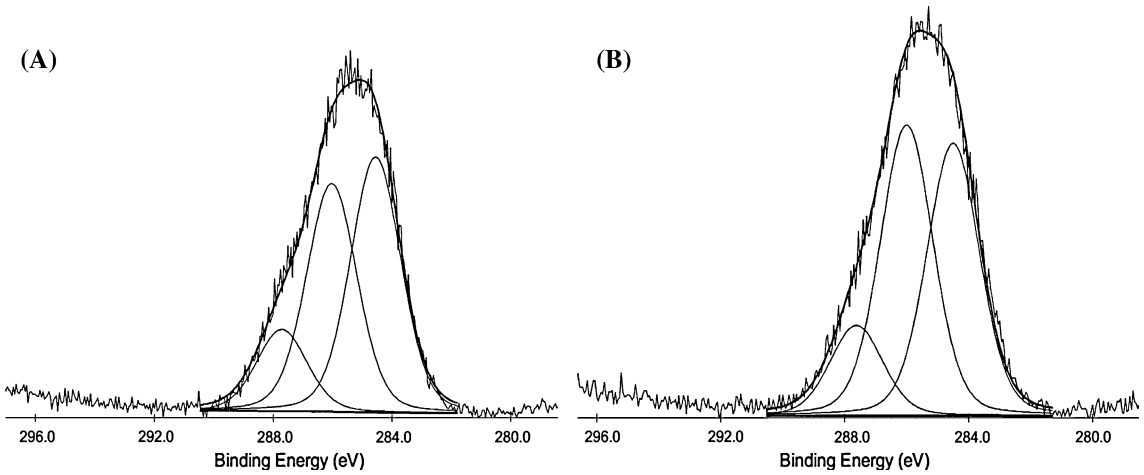

Fig. 3 Fitting *curves* of C1s energy spectrum of blended membrane surfaces. **a** Col–Chi membrane. **b** Col–Chi–CS membrane

Table 2 Content of C1s energy spectra (%)

	C–H	C–OH, C–NH_2	C=O
Col–Chi	45.20	40.36	14.44
Col–Chi–CS	41.68	44.51	13.81

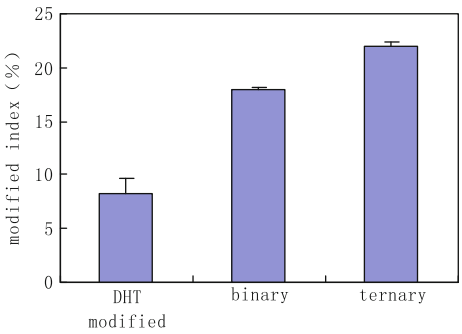

Fig. 4 Contrast of cross-linking index of modified Col-based membrane

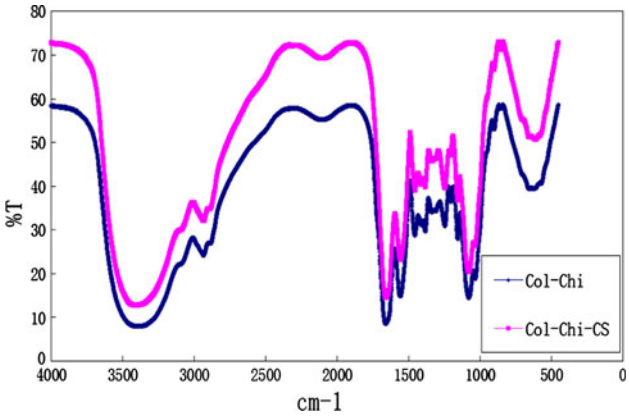

Fig. 5 FTIR spectra of Col-based membrane

the Col–Chi and Col–Chi–CS membranes were 30.89 and 36.60, respectively. Compared to the Col–Chi membrane, the higher *D* value of the Col–Chi–CS membrane indicated a lower content of primary amine. It was thus clear that the number of unreacted amino groups of the Col–Chi–CS membrane was less than that of the Col–Chi membrane, which suggested that the degree of cross-linking of the former was much higher than that of the latter. This finding was in agreement with the measured results of the free amino group.

Tensile Strength

The tensile strength of EDC modified Col–Chi membrane was 1.06 MPa, while that of the EDC modified Col–Chi–CS membrane was 1.19 MPa, which can account for the higher mechanical strength of three-component membrane. This result was related to the modification index.

Degradation Test

Figure 6 shows the degradation of modified membrane in lysozyme solution. When modified and unmodified membrane are compared, the modified one performed better in degradation. After soaking in the solution for 15 days, the remaining quality of EDC-modified two- and three-component membrane was 89.74 and 93.02 %, respectively, of the initial weight, while it was less than 60 % of that of the unmodified one. These indicate the capability of improving the cross-link degree and the antizymohydrolysis ability of the EDC modification technology. EDC-modified membrane has a superior performance. By introducing CS, the antizymohydrolysis ability of this composite membrane was improved.

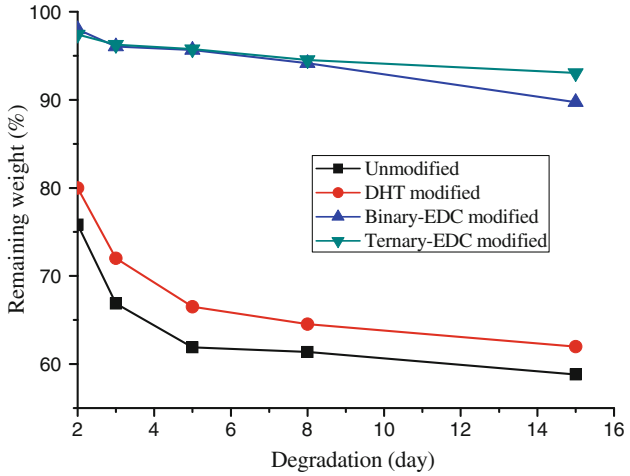

Fig. 6 Degradation of Col-based membrane in lysozyme solution

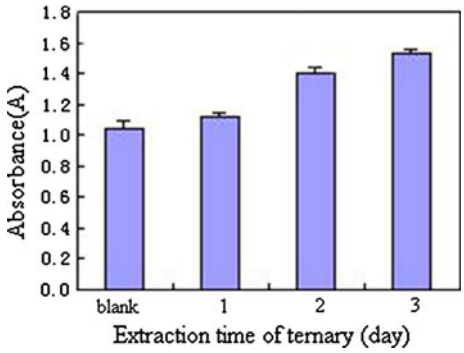

Fig. 7 MTT results of fibroblast culture in extracted solution of Col–Chi–CS membrane

Cytotoxic Test

The cytotoxic test was carried out by culturing fibroblast cells in the fluid extracted from Col–Chi–CS membrane, which contained sustained-release bFGF microspheres, for 1, 2, and 3 days, and a control group; the experimental and control cells were then tested by MTT. More cells were reproduced in the three experimental groups than in the control group (Fig. 7). The cell amount increased with the duration of the extraction period. The extracted fluid was noncytotoxic and promoted cell proliferation. Figure 8 illustrates control and experimental fibroblast cells cultivated for 3 days; cells in the experimental groups grew faster than controls and covered almost the whole culture plate. The reason cell proliferation was promoted may be related to the sustained-release bFGF microspheres. With longer extraction periods, the bFGF amount was enhanced and the cell proliferation rate increased.

Cell Culture

SEM results are shown in Fig. 9 for the self-made bFGF-controlled microsphere-containing Col–Chi–CS membrane cultured for 3 days. The bFGF-controlled microspheres, which were of uniform size, were evenly distributed on the membrane. The three-dimensional structure of membrane was preserved even after soaking for 3 days, which permitted cell proliferation. Figure 10 shows the adhesion growth of the fibroblasts on the membrane, which indicated that the membrane worked well for cell adhesion and growth.

Wound Healing Test

As a holder for cells, membrane based on Col can induce cell proliferation, differentiation, and migration. This membrane is an effective wound healing material that can absorb tissue exudate and adhere to the wound to maintain certain humidity and to avoid mechanical injury and resulting bacterial infection. A novel dressing was created by combining bFGF-controlled microspheres with Col membrane and applied to a rabbit model to observe the wound healing process.

Figure 11 shows the wound reconstruction process. Figure 12 shows the wound covered by composite

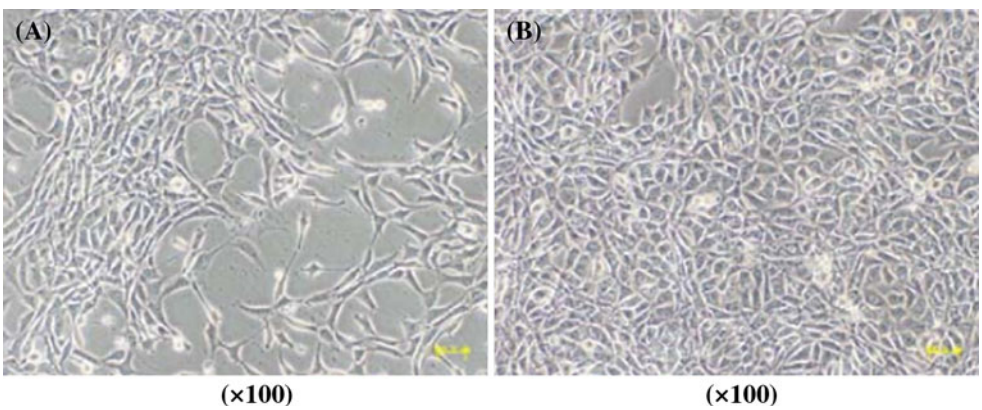

Fig. 8 Fibroblast. **a** Culture medium. **b** Extracted solution of Col–Chi–CS membrane

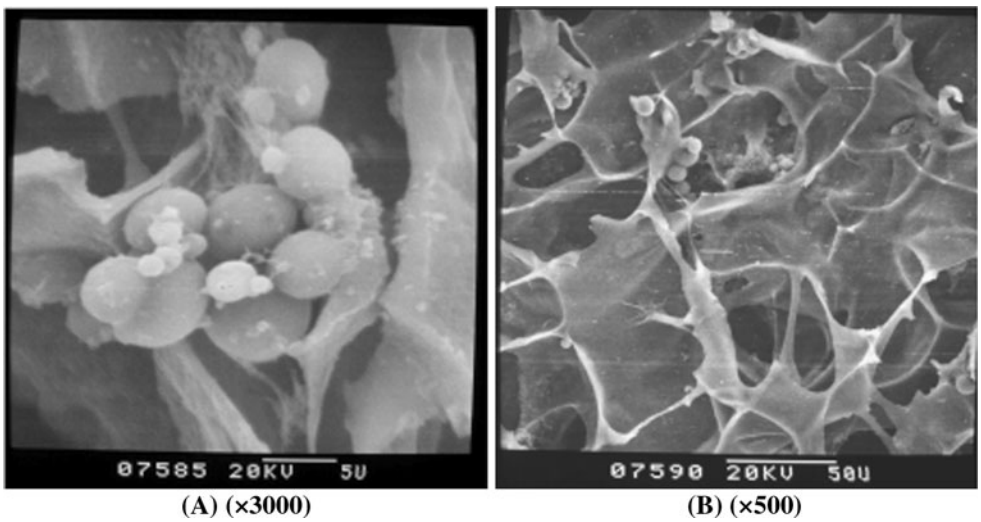

Fig. 9 SEM of Col–Chi–CS membrane impregnated microsphere after 3 days in culture medium

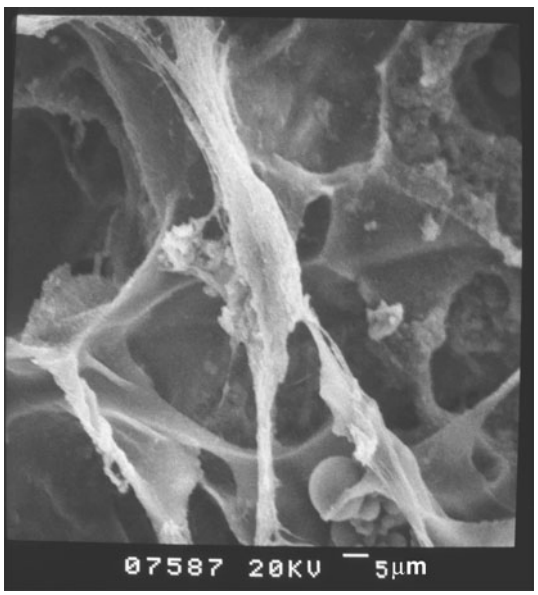

Fig. 10 SEM of fibroblast culture on the Col-based membrane (original magnification, ×3,000)

membrane and an oil swab. After being covered by the composite membrane, tissue exudate and blood were absorbed, and the membrane smoothly adhered to the wound, creating a good microenvironment for the wound healing process. Three days after the wounds were inflicted, the experimental group wound was dry. The wound adhered closely to the membrane, with no adhesion to the paraffin gauze dressings and with no erythema; in addition, no allergy occurred. In the wounds in the control group, on the other hand, tissue exudate and adhesion to the paraffin gauze dressings were observed. Figure 13 illustrates the wound healing of a rabbit 3 weeks after surgery.

Wound healing status and healing are listed in Table 3. There were six cases of bleeding (30 %) in the control group, whereas in the experimental group, there was only one (5 %). There was one case of wound infection in the control group but none in the experimental group. It took 20.76 days of the control group for wound healed but only 18.00 days compared to experimental one. The wounds in the experimental group clearly healed better and faster, and for less cost.

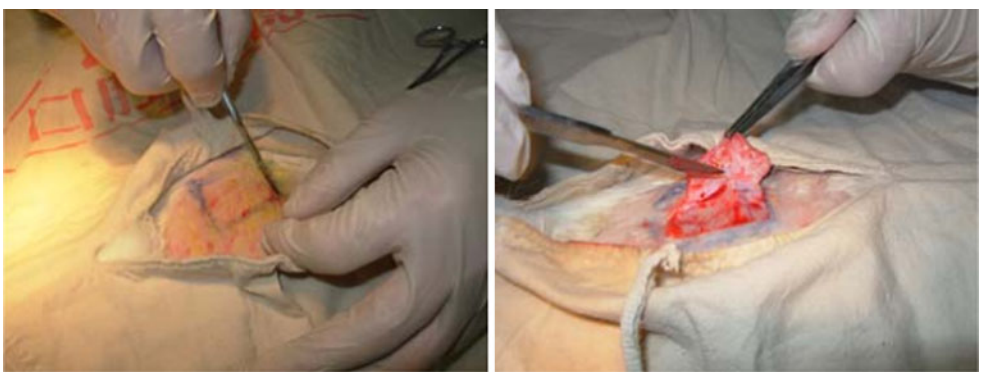

Fig. 11 Establishment of whole-layer skin wound

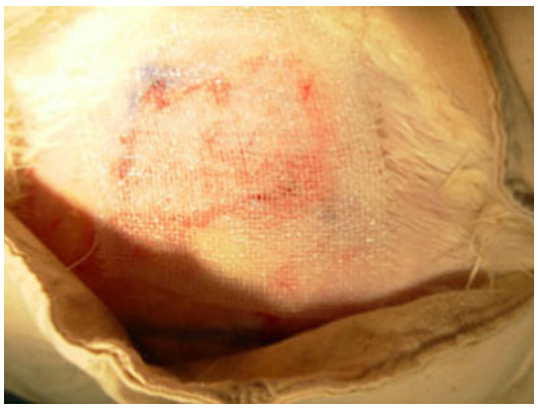

Fig. 12 Wound after covering with repair membrane

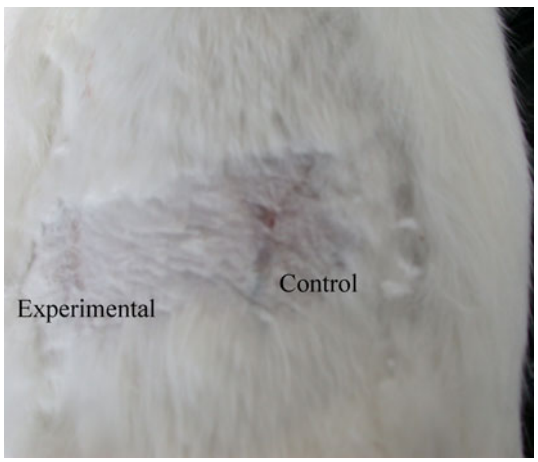

Fig. 13 Wound healing after 3 weeks. The experimental wound is on the *left side* of the rabbit back, and the control wound is on the *right side*. Compared to control wound, which was not fully healed and had a small scab, the experimental wound was entirely healed; regenerated tissue knitted with the surrounding tissue, leaving no scab

Table 3 Observations of wound repairing in rabbits

Wound	Experimental ($n = 10$)	Control ($n = 10$)
Wound bleeding	1	6
Wound with gauze adhesion	0	13
Wound infection	0	1
Average healing time (days)	18.00 ± 1.93	20.73 ± 1.95

The membrane based on Col that we prepared was bonded with histocompatible and biodegradable CS and Chi. Along with cross-linking modified by EDC, the stability and biocompatibility of this composite membrane were improved. We found the novel healing material to be bacteriostatic, hemostatic, and analgesic, and it accelerated the tissue's wound healing abilities.

In this study of Col membrane combined with sustained-release bFGF microspheres, we found that the composite membrane can be used to cover the wound, thus creating a scaffold to assist the migration and proliferation of epidermal cells and granulation tissue. In addition, the wound healing period was markedly shortened, and we found excellent sustained-release function of bFGF microspheres. The primary benefits of this novel composite membrane include protecting the wound, decreasing the amount of exudates, and permitting water-spreading properties. The animal defect model experiment indicated that the membrane based on Col combined with sustained-release bFGF microspheres performs well in terms of wound healing, skin regeneration, hemostasis, antiadhesion, and distinctive knitting abilities.

Conclusions

(1) XPS observation revealed that after EDC modification, CS was firmly bonded in the Col–Chi membrane. Col–Chi–CS membrane of a different polarity was collected.
(2) The modification index of DHT-EDC-modified Col–Chi–CS membrane was higher than that of the Col–Chi membrane, and the tensile strength and degradation of the modified Col–Chi–CS membrane were distinctly higher. The EDC modification technology and the introduction of CS improved the antienzymolysis ability of the composite membrane.
(3) Fibroblast culture experiments indicated the membrane was nontoxic and good for cell proliferation and adhesion.
(4) Wound healing tests suggest the feasibility of promoting the wound healing process and tissue regeneration, as well as promoting hemostasis and preventing postoperative adhesion abilities.

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