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Surface Modification of GTA Crosslinked Collagen-based Composite Scaffolds with Low Temperature Plasma Technology

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In this study for preparing the better performance scaffold materials for peripheral nerve repairing, the collagen-based composite scaffolds are crosslinked with glutaraldehyde and their structure and performance are investigated. The results of FTIR indicated that the collagen and chitosan are certainly crosslinked through GTA without any significant change in the chemical property. It was observed under a scanning electron microscope (SEM) that the crosslinked collagen-based composite scaffolds had a porous three-dimensional cross-linked structure. The experiments showed that the biostability of the scaffold is greatly enhanced, but the GTA crosslinking induces the potential cytotoxicity and poor hydrophilic nature. To overcome these disadvantages, the low temperature plasma technology is utilized to modify the surface of the cross-linked collagen-based composite scaffolds in this study. Measurements of water contact angle showed that hydrophilic nature of surface of the scaffolds was improved after low temperature plasma technology modification. The cell proliferation experiments revealed that the modified collagen-based composite scaffolds still kept their bioactivity and benefited the proliferation.

Keywords: collagen-based; scaffolds; surface; modification; plasma

1 Introduction

In clinical peripheral nerve repair, the situation is often encountered when the gap between the nerve stumps is too large to permit repair by direct tensionless suture. Grafting with a segment of autologous nerve is effective in such a situation, but has drawbacks such as donor site morbidity and incomplete recovery of function. Because of this, artificial nerve regeneration conduits have recently been developed to satisfy the need for nerve grafting and to get a better nerve repairing effect. Previous studies have demonstrated that collagen nerve conduits are capable of promoting nerve fiber regeneration and partial functional recovery (1, 2). However, collagen has some disadvantages as nerve conduit materials. They have poor mechanical strengths, fast biodegradation and too much uptake of water, which can result in a slump or deform the nerve conduits during the bridging process. Therefore, these materials were not used for the repair of damaged peripheral nerves. Recently, the triple helix structures of collagen has become one of the most important issues for the collagen scaffolds (3–6). In most cases, the treatments by chemical methods are still necessary. In the chemical method, group glataraldehyde (GTA) is the most convenient and traditional agent used in the treatment of collagen scaffolds and bioprosthetic tissue. It can enhance the biological stability of the collagen scaffolds by bridging amine groups between two adjacent polypeptide chains. This crosslinking can also suppress the immunogenicity of the artificial implant (6). However, accompanying the stability increase of the scaffolds, there are also some problems which arose, such as the potential cytotoxicity and poor hydrophilic nature (7, 8).

In order to overcome these disadvantages, plasma treatment technology was used. Plasma-induced polymerization can easily improve the compatibility of the biomedical tissue materials and the main advantages of this technology are as follows (9, 10): (a) No obvious effect on the substrates as a result of the action depth of only tens of nanometer. (b) Without special demand for the surface shape of the materials. Therefore, the low temperature plasma treatment is a kind of ideal surface-treatment technology for medicine materials.

In this study, the low temperature plasma-induced technology is applied to the cross-linked collagen-based composite scaffolds with GTA and the properties of the scaffold are investigated before and after management of plasma

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treatment, with emphasis on the structure, cell proliferation and hydrophilic nature.

2 Experiment

2.1 Materials

Chitosan (96.7% deacetylated, $\bar{M}_\eta = 1.5 \times 10^5$) were purchased from Shandong Weifang-Kehai, Co. Ltd., China. Collagenase was purchased from Sigma. All other reagents and solvents are of analytical grade and used as received. Collagen was isolated from fresh bovine tendon by pepsin digestion and a citric acid dissolution method. Its purity was confirmed by UV spectroscopy, IR epectroscopy and amino acid analysis. The natural triple helix of collagen was preserved during the extraction process. All of the collagen scaffolds were made of the same batch to avoid influence of the raw materials in this study.

2.2 Preparation and Modification of Collagen-based Scaffolds

1.2% (w/w) collagen solution (1.00 mol/L citric acid solution pH = 2–3) was completely mixed with a 4% chitosan solution (1% of aqueous acetic solution) in certain proportions at 37°C with stirring. The resulting mixture of collagen/chitosan of the quantity ratio 3:1, after it was defoamed in a defoaming centrifuge, was introduced into a mold. The mold was placed in a freeze-drying machine for prefreezing for 4 h at −50°C, and was then kept under vacuum conditions at the same temperature for 24 h. The porous collagen/chitosan composite materials were formed when solid water was vaporized under vacuum. The resulting scaffolds of collagen/chitosan were dipped in a solution of 0.25% of GTA at 25°C for 1 h, and then washed repeatedly with deionized water for 3 h. After that, the scaffolds were dried.

Upon the previous study of selecting plasma treatment parameters (11–13), the treating procedure in this study is as follows:

The plasma treatment of collagen/chitosan composite scaffolds was carried out in a plasma reactor device connected to a capacitance coupling. The samples were treated by glow discharging for 2 min under the power 40 W with the atmosphere environment (20 Pa). In the same vacuum state, the samples was kept in this device for 1 h.

Finally, they were treated with ethylene oxide at 37°C for sterilization. There were three groups of collagen-based composite scaffolds, the first is the uncrosslinked (UCC), the second is crosslinked and untreated with plasma (CC) and the third is GTA crosslinked and treated with plasma (CC/Pla).

2.3 Fourier Transform Infrared Spectroscopy of Collagen-Based Scaffolds

Specimens used for Fourier transform infrared (FTIR) measurements with Nicolet-20sx-B. (film method).

2.4 Scanning Electron Microscopic Analysis of Collagen-Based Scaffolds

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

2.5 Measurements of Water Contact Angle

The three groups of collagen-based composite scaffolds were tested by water contact angle instrument JC2000A to measure contact angle between deionized water and the collagen-based composite scaffolds materials. The contact angles were tested at 30s, 60s and 120s, respectively and at each time seven samples were tested and the results were averaged.

2.6 Cell Poliferation of Collagen-Based Scaffolds

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

2.7 In vitro Collagenase Degradation

The biological stability of the cross-linked collagen-based composite scaffolds was evaluated by an *in vitro* collagenase biodegradation test. Each type of scaffold 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 immediately in an ice bath. Following centrifugation 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 (14). The biodegradation degree is defined as the percentage of the released hydroxyproline from the scaffolds to one the completely degraded with the same composition and weight.

3 Results and Discussion

3.1 Physical and Chemical Properties of GTA Cross-linked Collagen-based Composite Scaffolds

3.1.1 Fourier Transform Infrared (FTIR) Studies

The FTIR spectra obtained from UCC and CC are given in Figure 1. They depict similar characteristic peaks of the parent molecules, with some special observable characteristics.

It is well known that the characteristic absorption bands of pure collagen (15) appear at the frequencies of 3324, 1650, and 1560 cm^{-1}. Generally, amide I bands (1650 cm^{-1})

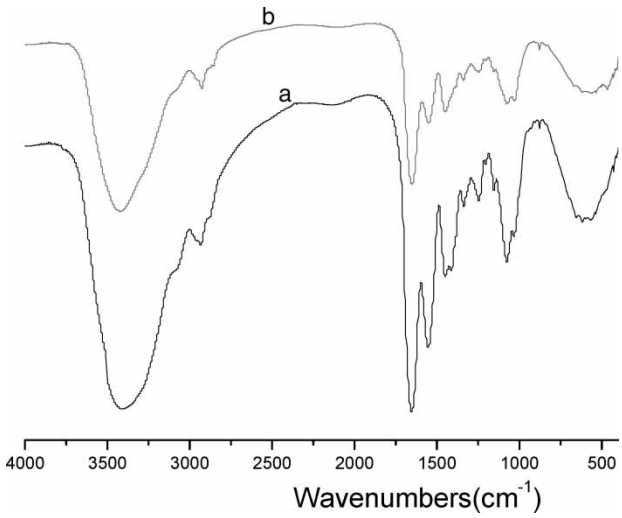

Fig. 1. FTIR spectra of (a) uncrosslinked; (b) GTA cross-linked collagen-based composite materials.

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, appears 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). Also, the absorption bands at 1152 (anti-symmetric stretching of the C-O-C bridge), 1084 and 1040 cm^{-1} (skeletal vibrations involving the C-O stretching) are characteristics of a saccharide structure of chitosan (16).

The FTIR spectra of various scaffolds were divided into various sets and analyzed by comparing their amide II bands.

The spectra of uncrosslinked collagen/chitosan (UCC) (Figure 1a) and GTA cross-linked collagen/chitosan (CC) (Figure 1b) show that the amide II bands (1560 cm^{-1}) and peak at 3439 cm^{-1} corresponding to NH_2 group bands of CC 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 cross-inkages between chitosan and collagen or within collagen molecules formed through GTA).

3.1.2 Scanning Electron Microscopic Analysis

A rough and porous surface of the uncrosslinked collagen-based composite scaffolds was observed under SEM (Fig. 2a). 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 cross-linked collagen-based composite scaffolds (Fig. 2b) was netlike and appeared dense. The average pores size was about a few micrometers and appeared even. The surface of cross-linked collagen-based composite scaffolds was smoother than that of uncrosslinked collagen-based composite scaffolds, and this makes the cells adhere more easily to it. Due to the crosslinking of GTA, the structure of the scaffolds changed from streaks to nets, which led to a great increase in mechanical strength of the conduits. After cross-linking, the pore size of the collagen-based composite scaffolds became smaller and a three-dimensional network structure was formed. This structure can help cells to adhere to the collagen-based composite scaffold and grow on it (17–19).

3.1.3 In vitro Collagenase Degradation

The biodegradation degree of the pure collagen scaffold and collagen-based composite scaffolds, before and after GTA crosslinking, are shown in Figure 3. After being 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, where a slightly lower biodegradation degree, 83.2%, was found. The biostability of GTA cross-linked collagen-based composite scaffolds were greatly enhanced, where only 6.42% was degraded in 24 h. These results reveal that both the addition of chitosan and GTA crosslinking are

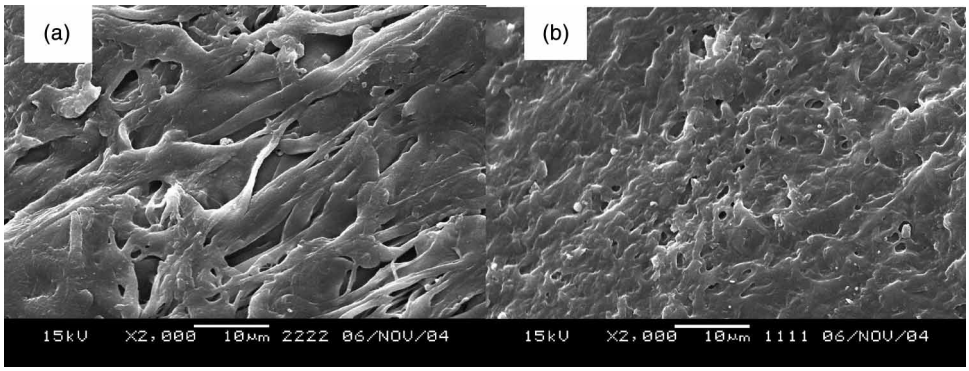

Fig. 2. Scanning electron micrograph of surface scaffolds: (a) uncrosslinked; (b) GTA crosslinked (original magnification × 2000; scale bar: 10 μm).

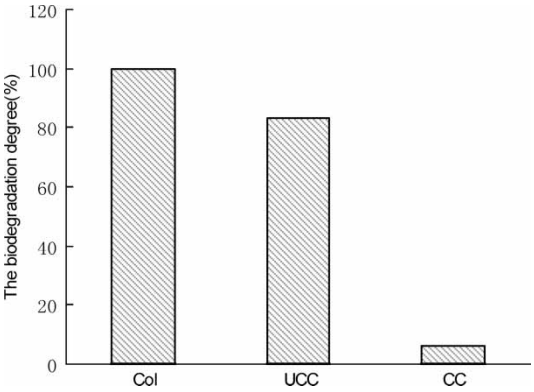

Fig. 3. The biodegradation degree of the pure collagen scaffolds and the collagen-based composite scaffolds. All scaffolds were incubated in 100 μg/ml collagenase for 24 h.

indispensable for improving the biostability of the collagen/chitosan scaffolds.

3.1.4 Hydrophilic Nature of Collagen-based Composite Scaffolds

The hydrophilic nature of materials can be denoted by contact angle. Good hydrophilic nature of materials benefit cell seeding (19). Figure 4 shows that the contact angle between water and GTA cross-linked collagen-based composite scaffold is bigger than the contact angle between water and uncross-linked collagen-based scaffold.

3.1.5 In vitro Cell Culture Studies

The cell proliferation experiments of the scaffold materials were shown in Figure 5. The cell proliferation is evaluated by the absorptancies, which are proportional to the number of cells. We monitored the cell growth on glass plates (control), pure collagen (PCol), UCC and CC by measuring their absorptancies. The results showed that the absorptancies for GTA cross-linked collagen-based composite scaffold materials decreased.

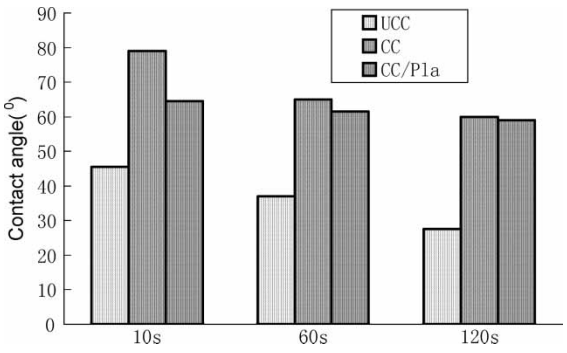

Fig. 4. Effects of the modification on contact angle between water and scaffolds.

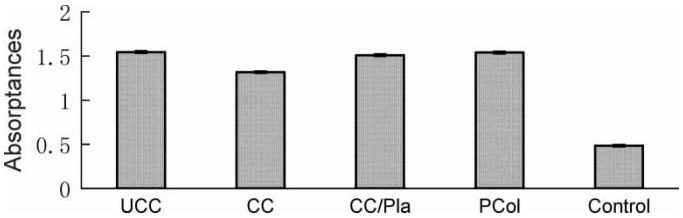

Fig. 5. Direct cytoxicity of dissimilarity materials.

3.2 Surface Modification of CC with Low Temperature Plasma Technology

The experiments presented in section 3.1 showed that the structure of GTA cross-linked collagen-based composite scaffolds is a three-dimensional network structure, which enhances the mechanical stability of the scaffolds, and the biodegradation degree of the scaffolds was greatly increased, demonstrated by the *in vitro* collagenase degradation. But some problems arise, such as the potential cytotoxicity and poor hydrophilic nature. Therefore low temperature plasma treatment is utilized to modify the surface of GTA cross-linked composite collagen-based composite scaffolds.

3.2.1 Hydrophilic Nature of Collagen-based Composite Scaffolds

Figure 4 shows that the contact angle between water and GTA cross-linked collagen-based composite scaffold treated by plasma (CC/Pla) decreased again. It is proven that the hydrophilic nature of materials is improved by plasma treatment.

Contact angle measurements at different times showed that the contact angle between water and the plasma-induced scaffold did not significantly decrease, compared with the other contact angles for UCC and CC. It is indirectly indicated that the plasma-induced technology only modifies the surface of the scaffold.

3.2.2 In vitro Cell Culture Studies

The results in Figure 5 showed that the absorptance for GTA crosslinked and the plasma treated collagen-based composite scaffold (CC/Pla) increased significantly and is close to the absorptance for pure collagen. This absorptance increasing is due to the enhanced hydrophilic nature of the surface of plasma treated materials.

The experiments demonstrated that the plasma treatment of collagen-based composite scaffolds prompts the adhesion and proliferation of retina cells, as well as keep the bioactivities of the native collagen.

Plasma treatment can improve the potential cytotoxicity and poor hydrophilic nature of GTA cross-linked collagen-based scaffolds. The reason for altering the hydrophilic nature of the scaffolds may be that a little -COOH group and -OH group is produced on the surface of the plasma-induced scaffold in rare atmosphere or the surface of the plasma-induced scaffold is corroded. Otherwise, although rinsed many times, remains of aldehyde group inevitably

exist on the GTA cross-linked collagen-based scaffolds and induce the potential cytotoxicity. The reasons for eliminating the potential cytotoxicity by plasma treating are: (a) in the vacuum state, the dissociated GTA continuously volatilizes. (b) GTA, physically aggregated to scaffolds or combined with scaffolds by molecule force, is reacted with the collagen molecule, when glow discharging. (c) The scaffolds are cleaned and sterilized when glow discharging.

4 Conclusions

In this study, plasma treatment technology is utilized to modify the surface of GTA cross-linked collagen-based composite scaffolds for improving the disadvantages, which may brought on by the GTA crosslinking, such as the potential cytotoxicity and poor hydrophilic nature.

Properties of the collagen-based composite scaffolds were analyzed by experiments, before and after the plasma treatment, with emphasis on the structure, cell proliferation, and hydrophilic nature. Measurements of the water contact angle showed that hydrophilic nature of surface of the scaffolds was improved. The cell proliferation experiments revealed that the modified collagen-based composite scaffolds still kept their bioactivity and benefit the proliferation.

The results of the experiments proved that the plasma treatment is an available method to improve the hydrophilic nature of the surface of the scaffolds and subsequently prompt the adhesion and proliferation of cells on the scaffolds.

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