Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering

Lie Ma, Changyou Gao,*, Zhengwei Mao, Jie Zhou, Jiacong Shen, Xueqing Hu, Chunmao Han

*Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China
Faculty of Burn, Second Affiliated Hospital of Zhejiang University, Hangzhou 310027, China

Received 1 November 2002; accepted 13 May 2003

Abstract

Porous scaffolds for skin tissue engineering were fabricated by freeze-drying the mixture of collagen and chitosan solutions. Glutaraldehyde (GA) was used to treat the scaffolds to improve their biostability. Confocal laser scanning microscopy observation confirmed the even distribution of these two constituent materials in the scaffold. The GA concentrations have a slight effect on the cross-section morphology and the swelling ratios of the cross-linked scaffolds. The collagenase digestion test proved that the presence of chitosan can obviously improve the biostability of the collagen/chitosan scaffold under the GA treatment, where chitosan might function as a cross-linking bridge. A detail investigation found that a steady increase of the biostability of the collagen/chitosan scaffold was achieved when GA concentration was lower than 0.1%, then was less influenced at a still higher GA concentration up to 0.25%. In vitro culture of human dermal fibroblasts proved that the GA-treated scaffold could retain the original good cytocompatibility of collagen to effectively accelerate cell infiltration and proliferation. In vivo animal tests further revealed that the scaffold could sufficiently support and accelerate the fibroblasts infiltration from the surrounding tissue. Immunohistochemistry analysis of the scaffold embedded for 28 days indicated that the biodegradation of the 0.25% GA-treated scaffold is a long-term process. All these results suggest that collagen/chitosan scaffold cross-linked by GA is a potential candidate for dermal equivalent with enhanced biostability and good biocompatibility.

1. Introduction

The skin loss is one of the oldest and still not totally resolved problems in surgical field. Due to the spontaneous healing of the dermal defects would not occur, the scar formation for the full thickness skin loss would be inevitable unless some skin substitutes are used. In the past decades, many skin substitutes such as xenografts, allografts and autografts have been employed for wound healing. However, because of the antigenicity or the limitation of donor sites, the skin substitutes mentioned above cannot accomplish the purpose of the skin recovery and yet not be used widely [1–5]. Therefore, many studies are turning toward the tissue engineering approach, which utilizes both engineering and life science discipline to promote organ or tissue regeneration and to sustain, recover their functions [6–9]. One crucial factor in skin tissue engineering is the construction of a scaffold. A three-dimensional scaffold provides an extra cellular matrix analog which functions as a necessary template for host infiltration and a physical support to guide the differentiation and proliferation of cells into the targeted functional tissue or organ [10,11]. An ideal scaffold used for skin tissue engineering should possess the characteristics of excellent biocompatibility, suitable microstructure such as 100–200 μm mean pore size and porosity above 90%, controllable biodegradability and suitable mechanical property [12–15]. Collagen is known to be the most promising materials and have been found diverse applications in tissue engineering for their excellent biocompatibility and biodegradability. However, the fast biodegrading rate
and the low mechanical strength of the untreated collagen scaffold are the crucial problems that limit the further use of this material. Cross-linking of the collagen-based scaffolds is an effective method to modify the biodegrading rate and to optimize the mechanical property.

For this reason, the cross-linking treatment to collagen has become one of the most important issues for the collagen-based scaffolds. Currently, there are two different kinds of cross-linking methods employed in improving the properties of the collagen-based scaffolds: chemical methods and physical methods. The latter include the use of photooxidation, dehydrothermal treatments (DHT) and ultraviolet irradiation, which could avoid introducing potential cytotoxic chemical residuals and sustain the excellent biocompatibility of the collagen materials [16]. However, most of the physical treatments cannot yield high enough cross-linking degree to satisfy the demand of skin tissue engineering. Therefore, the treatments by chemical methods are still necessary in almost all cases. The reagents used in the cross-linking treatment recently involve traditional glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), polyglycidyl ether and polyepoxidic resins, etc. [17–21]. GA is a kind of bifunctional cross-linking reagents that can bridge amino groups between two adjacent polypeptide chains and has become the predominant choice in skin tissue engineering because of its water solubility, high cross-linking efficiency and low cost [22].

Chitosan is another biomaterials used in a variety of biomedical fields such as drug delivery carriers, surgical thread, and wound healing materials [23]. Due to its many advantages for wound healing such as hemostasis, accelerating the tissue regeneration and the fibroblast synthesis of collagen, many applications of chitosan in skin tissue engineering have been reported [24–27]. In addition, chitosan can function as a bridge to increase the cross-linking efficiency of GA in the collagen-based scaffolds owing to the large number of amino groups in its molecular chain (Fig. 1). Hence, one can expect that less GA could be used in the presence of chitosan and the potential cytotoxicity of GA might be decreased.

Herein we describe the fabrication of collagen porous scaffold in the presence of 10 wt% chitosan, which functions as a cross-linking bridge in the further treatment of GA cross-linkage. The microstructure, the swelling capacity, as well as the degradability both in vivo and in vitro of the collagen/chitosan scaffold were investigated. In vitro culture of human dermal fibroblasts and in vivo animal tests demonstrated that the scaffolds showed good cytocompatibility and could effectively guide the infiltration and growth of fibroblasts.

2. Materials and methods

2.1. Materials

Chitosan (viscosity average molecular weight \( M_g \): \( 1.0 \times 10^5 – 1.7 \times 10^5 \), 75–85% deacetylation degree), collagenase I (278 U/mg), rhodamine B isothiocyanate, fluorescein isothiocyanate (FITC) and fluorescein diacetate (FDA) were purchased from Sigma. Trypsin (250 U/mg) was a commercial product from Amresco. Glutaraldehyde (GA), 25% water solution, was purchased from Shanghai Pharm. Co. (China). All other reagents and solvents are of analytical grade and used as received.

Collagen type I was isolated from fresh bovine tendon by trypsin digestion and acetic acid dissolution method. Briefly, after removed the fat and muscle impurity substances the bovine tendon was cut into pieces as thin as possible and digested in trypsin solution (0.25%) at 37°C for 24 h. The tendon pieces were then incubated in 0.5M acetic acid (HAc) at 4°C for 48 h. A tissue triturator was employed to agitate the swollen tendon

![Fig. 1. Schematic presentation of collagen cross-linked with glutaraldehyde in the presence of chitosan.](image-url)
pieces violently so that the collagen fibers could be well dispersed. The collagen solution was then centrifuged to get rid of insoluble impurities. The supernatant was precipitated by 5 wt% NaCl solution. The precipitate was then dissolved in 0.5% (w/v) biomacromolecule solutions at 4°C for 48 h to remove insoluble impurities. The supernatant was then centrifuged to remove colloidal impurities. The collagen solution was then centrifuged to remove the precipitated collagen fibers so that the collagen fibers could be well dispersed. The centrifuged suspension was deaerated under vacuum to remove entrapped air-bubbles, the collagen/chitosan blend was injected into a home-made mould (diameter: 16 mm, depth: 2 mm), frozen in 70% ethanol bath at -20°C for 1 h and then lyophilized. The composition and purity of the collagen type I was characterized and confirmed by UV spectroscopy, IR spectroscopy and amino acid analysis.

Rhodamine labeled collagen (Rd-Col) and FITC labeled chitosan (FITC-Chi) were prepared by mixing 0.2 mg/ml rhodamine B isothiocyanate or FITC into 0.5% (w/v) biomacromolecule solutions at 4°C for 48 h, respectively. The free dyes were dialyzed off in 0.05 M acetic acid solution for 4 weeks.

2.2. Preparation of collagen/chitosan scaffold

Collagen or chitosan was dissolved in 0.5 M HAc solution to prepare a 0.5% (w/v) solution, respectively. The chitosan solution was slowly dropped into collagen suspension in the ratio of 9:1 (collagen:chitosan) and homogenized to obtain collagen/chitosan blend. After deaerated under vacuum to remove entrapped air-bubbles, the collagen/chitosan blend was injected into a home-made mould (diameter: 16 mm, depth: 2 mm), frozen in 70% ethanol bath at -20°C for 1 h and then lyophilized for 24 h to obtain a porous collagen/chitosan scaffold.

2.3. Cross-linking treatment

To improve the biostability, the collagen/chitosan scaffolds were treated with GA. All scaffolds were rehydrated in 0.05 M HAc solutions for 15 min firstly, and then were cross-linked in the GA solutions (double-distilled water, pH 5.6) with different concentrations (0.05–0.25%) at 4°C for 4, 16, 24 and 48 h. After washed with double-distilled water (10 min × 5 times), the scaffolds were freeze-dried again to obtain the GA treated collagen/chitosan scaffolds.

2.4. Microstructure observation

The microstructure of the scaffolds was observed under scanning electron microscopy (SEM, Cambridge stereoscan 260) and confocal laser scanning microscopy (CLSM, Biorad 2100). Rd-Col and FITC-Chi were used for CLSM detection with double channels’ mode.

2.5. Swelling test

The collagen/chitosan scaffolds were placed into distilled water at room temperature and the wet weight (w) of the scaffold was determined after incubated for 24 h. The swelling ratio of the scaffolds was defined as the ratio of weight increase (w – w0) to the initial weight (w0). Each value was averaged from three parallel measurements.

2.6. In vitro collagenase degradation

In vitro biodegradation test of the collagen/chitosan scaffolds cross-linked by GA with different concentrations (0–0.25%) was performed by collagenase digestion. Each kind of scaffolds was immersed in phosphate buffered saline (PBS, pH 7.4) containing 100 µg/ml (28 units) collagenase (type I, Sigma) at 37°C for 4, 16, 24 and 48 h. The degradation was discontinued at the desired time interval by incubating the assay mixture in an ice bath immediately. Following centrifugation at 1500 rpm for 10 min, the clear supernatant was hydrolyzed with 6 M HCl at 120°C for 12 h. The content of hydroxyproline released from the scaffold was measured with ultraviolet spectroscopy [29]. The biodegradation degree is defined as the percentage of the released hydroxyproline from the scaffolds at different time to the completely degraded one with same composition and same weight.

2.7. Cell culture

Fibroblasts used in this study were isolated from human dermis by collagenase digestion. Briefly, the epidermis and subcutaneous tissue of human skin were removed by the scalpel. The residual dermis was diced into 0.5–1 mm³ sized tissues, and washed with phosphate buffer saline (PBS, pH 7.4) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) 3 times. Then these dermis pieces were placed in a spinner flask containing 10 ml of 1 mg/ml collagenase (type I, Sigma) in Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) 10% FBS (complete medium). The digesting solution was filtered through a copper mesh (cell strainer, 200 meshes) and then was centrifuged at 1000 rpm for 10 min. The cell suspension at confluence and the 4–8th passage fibroblasts were used for the seeding.

The 0.25% GA treated collagen/chitosan scaffold (both rhodamine-labeled) was immersed in 75% ethanol for 12 h for sterilization, followed with solvent exchange by PBS for 6 times. The scaffold was then placed on a 24-well polystyrene plate and seeded with 200 µl human dermal fibroblast suspension at a density of 5 × 10⁶ cells/ml. After incubation for 4 h, 1 ml complete medium was added.
added and cultured in a 5% CO₂ incubator at 37°C for 3 days. After washed with PBS for 2 times, the fibroblasts were stained with 5 μg/ml FDA solution in the incubator for 15 min. Following with removal of the unreacted FDA with double washing in PBS, 1 ml complete medium was then added. The live fibroblasts can metabolize FDA to form a fluorescence product. Hence, the fibroblasts existed in the scaffolds are distinct from the rhodamine labeled scaffolds (red color) by the generation of green color under CLSM.

2.8. In vivo animal evaluation

Twelve health rabbits weighing about 2 kg were obtained from the animal laboratory and were divided into four groups randomly. The 0.25% GA treated collagen/chitosan (10 wt %) scaffolds were sterilized by immersed into 75% (v/v) ethanol for 30 min and washing with PBS (pH 7.4) (5 times × 5 min). Before implantation, the dorsal surface hairs of the rabbit ears were shaved. Then all rabbits were anesthetized by intravenous administration of 20 mg/kg ketamine-HCl. The ears of rabbits were sterilized with 5% PVP-I, on which subcutaneous pockets were made [30,31]. In every group, four scaffolds (0.5 × 1 cm²) were embedded subcutaneously on the dorsal surface of rabbit ear. Harvests were performed randomly in selected group at 3 days, and 1, 2, 4 weeks after implantation. At harvest, the implantation sites were cut in a full thickness manner (including both sides of the ear skin and cartilage). Paraffin sections were stained with hematoxylin-eosin (HE) reagent for histological observations.

2.9. Immunohistochemistry

Sample of 0.25% GA treated collagen/chitosan scaffold after embedded for 28 days was fabricated into paraffin section. After dewaxed and blocked with 3% (w/v) bovine serum albumin in PBS (pH 7.4) (BSA/PBS) for 20 min at 20°C, sections of the scaffold were incubated for 12 h at 4°C with mouse anti-bovine type I collagen IgG (diluted 1:100) and washed with PBS (pH 7.4) (3 times, each for 5 min). Subsequently, the sections were incubated for 30 min at 37°C with biotinylated goat anti-mouse IgG (diluted 1:300) and washed with PBS (pH 7.4). The slides were then reacted with avidin-conjugated peroxidase (diluted 1:30) at 37°C for 30 min. Finally, the sections were displayed with DAB and embedded by paraffin to yield a positive stain. Sections were observed under light-microscope.

3. Results and discussion

3.1. Distribution of collagen and chitosan

One of the important purposes adding chitosan is providing additional amino groups which function as binding cites to increase the GA cross-linking efficiency. Therefore, the interpenetration of collagen and chitosan in the scaffold is crucial. Exploiting the sequential scanning mode of CLSM, the distribution of FITC-Chi (Fig. 2a) and Rd-Col (Fig. 2b) in their complex scaffold was separately measured at wet state. A merged image is shown in Fig. 2c. The CLSM observations indicate that the scaffold was indeed composed with chitosan and collagen which were evenly dispersed through the scaffold. In acidic solution, both collagen and chitosan are positively charged, either forming a real solution (for chitosan) or suspension (for collagen) [32]. Their mixture in solution is stable and does not precipitate as that for collagen/chondroitin sulfate blend, where chondroitin sulfate is negatively charged [4,7]. Therefore, sufficient mixing of these two hydrophilic biomacromolecules in sub-molecular level can be achieved.

3.2. Morphology

It is known that the microstructure such as pore size and its distribution, porosity as well as pore shape has

![Fig. 2. CLSM images of the distribution of chitosan (a) and collagen (b) in the Rd-Col/FITC-Chi porous scaffold; (c) is the merged image of (a) and (b). × 400.](image-url)
prominent influence on cell intrusion, proliferation and function in tissue engineering. The cross-section morphologies of the collagen/chitosan scaffolds before and after GA treatment are shown in Fig. 3. The interconnected 3D porous structure of the scaffolds was retained after GA treatment; however, some other significant changes occurred with respect to pore size and morphology. The mean pore size increased from $\sim 100 \mu m$, the uncross-linked (Fig. 3a), to $>200 \mu m$, the cross-linked scaffolds (Fig. 3b–e). Accompanying with reduction of the fibers in between pores, more sheet-like structure appeared together with condensed walls. No big difference between the cross-linked scaffolds was observed, except for which treated with highest GA concentration (Fig. 3e), where elongated pores were existed.

The results indicate that the morphology difference is mainly caused by rehydration and relyophilization process in the GA cross-linking treatment. This additional refreeze-drying can induce the collagen fibers to be combined again to form sheets, leading to the fusion of some smaller pores to generate larger ones. It has to be noted that the slight collapse of the scaffold during this process should have an opposite effect to the pore fusion; i.e., reducing the pore size. Hence, one can deduce from the above results that the fusion effect is more prominent than the collapse. As a result, the pores are enlarged. On the other hand, this collapse, if not occurs homogeneously in 3D, will inevitably produce elongated pores as shown in Fig. 3e.

### 3.3. Swelling test

The ability of a scaffold to preserve water is an important aspect to evaluate its property for skin tissue engineering. The swelling ratios of various scaffolds are shown in Fig. 4. The swelling property of the uncross-linked scaffold was doubled than the GA treated scaffolds. However, the cross-linked scaffolds did not show obvious difference regardless of the GA concentration.

The water-binding ability of the collagen/chitosan scaffold could be attributed to both of their hydrophilicity and the maintenance of their three-dimensional structure. In general, the swelling ratio is decreased as the cross-linking degree is increased because of the decrease of the hydrophilic groups [33]. The results in Fig. 4 indicate that the primary factor affected the swelling property is the procedure of the GA treatment other than the GA concentration (hence, the cross-linking degree). As mentioned above, the collapse during the refreeze-drying procedure will cause the reduction of the porosity, hence, the volume for water storage, leading to the decrease of the swelling capacity. However, the absolute value is still over 80 times of its

![Fig. 3. The cross-section SEM images of collagen/chitosan scaffolds treated with different concentration of GA, $\times 100$. (a): control; (b): 0.05% GA; (c): 0.1% GA; (d): 0.2% GA; (e): 0.25% GA.](image-url)
initial weight after GA treatment, which is high enough for skin tissue engineering.

3.4. In vitro biodegradability

The Fig. 5 compares the biodegradation degree of the pure collagen scaffold and the collagen/chitosan scaffold before and after GA treatment. After incubated in collagenase solution for 12 h, the pure collagen scaffold (col) had been thoroughly biodegraded. The addition of chitosan (col/chi) can somewhat increase the biostability, where slight lower biodegradation degree, 92.1%, was found. After cross-linked with 0.25% GA, the biostability of the pure collagen scaffold (col-GA) was greatly enhanced, where only 12.8% was degraded in 12 h. Owing to the expected larger cross-linking degree (Fig. 1), the ability to resist collagenase degradation was further enhanced for the chitosan-combined scaffold. These results reveal that both the addition of chitosan and GA cross-linking are indispensable for improving the scaffold biostability and the presence of chitosan can obviously improve the biostability of the collagen/chitosan scaffold under the GA treatment, where chitosan might function as a cross-linking bridge.

The dynamic degradation of the collagen/chitosan scaffolds cross-linked by different concentrations of GA is illustrated in Fig. 6. The uncross-linked collagen/chitosan scaffold was biodegraded so fast that its biodegradation degree had achieved to 41.5% just treated by the collagenase solution for 2 h. After biodegradation for 16 h, the uncross-linked scaffold had been dissolved in the collagenase solution thoroughly. Fig. 6 shows that the biostability of the GA treated scaffolds were better than the uncross-linked one. For example, even treated with the lowest GA concentration (0.05%), the biodegradation degree of the scaffold was only 6.3% in 4 h. When the GA concentration was up to 0.1%, the biodegradation degree increased very slowly with the degrading. The highest biodegradation degree was just 26.1% after 48 h. Fig. 6 shows also that with the GA concentration increase, the effect of GA concentration on the improvement of the biostability was slowed down.

3.5. Cell culture

Cell infiltration and proliferation are crucial for a scaffold to support and guide tissue regeneration. Fig. 7 represents the CLSM images of the human fibroblasts cultured for 3 days in the collagen/chitosan scaffold treated by 0.25% GA. Exploiting the sequential scanning mode, a great number of fibroblasts (Fig. 7a) were easily distinguished from the scaffold (Fig. 7b). The merged image (Fig. 7c) reveals that the fibroblasts were adhered on the walls of the scaffold tightly with typical
shuttle-like morphology. This result proves that the chitosan-combined and GA-treated scaffold preserves the original good cytocompatibility of collagen. Potential cytotoxicity of GA residue was not evidenced. This ensures the further study of the tissue response to the scaffolds in vivo.

3.6. Histological examination

The histological results of the 0.25% GA-treated scaffold embedded in the rabbit ear for different time are shown in Fig. 8. The pure collagen scaffold was easy to lose its contour structure and biodegraded quickly in 3 days because of its low stability. On the contrary, the structure of the 0.25% GA-treated scaffold was retained entirely and a few of fibroblasts and inflammatory cells could be observed in the scaffold after implanted for 3 days (Fig. 8a). After implanted for 7 days, more fibroblasts were grown into the scaffold and the inflammatory cells were still in existence (Fig. 8b). When the test had processed for 14 days, a large number of fibroblasts were infiltrated into the scaffold. The morphology of the scaffold was similar to the surrounding dermal tissue and its structure could not be obviously observed (Fig. 8c). After 28 days’ implantation, the scaffold had almost disappeared and the blood vessels could be observed (Fig. 8d). These results demonstrate that the collagen/chitosan scaffolds can
effectively sufficiently support and accelerate the fibroblasts infiltration from the surrounding tissue. All the in vitro and in vivo results have shown that the collagen/chitosan scaffold treated with GA has a good biocompatibility.

3.7. Immunohistochemistry

To study the biodegradation behavior of the collagen/chitosan scaffold in vivo, the image of the paraffin section of the 0.25% GA cross-linked scaffold after immunohistochemistry treatment is shown in Fig. 9. The GA cross-linked collagen/chitosan scaffold could not be distinguished from the new-formed collagen fiber under routine paraffin section with light microscope after 28 days’ implantation (Fig. 9a). However, the immunohistochemistric assay shows that the bovine type I collagen had been partially preserved though the scaffold had macroscopically disappeared (Fig. 9b). This result indicates that the biodegrading behavior of the GA-treated collagen/chitosan scaffold is a long-term process. The long-term biodegradation of this kind scaffold in vivo should be studied further.

4. Conclusion

Herein we have described the fabrication of porous collagen/chitosan scaffold in vivo, the image of the paraffin section of the 0.25% GA cross-linked scaffold after immunohistochemistry treatment is shown in Fig. 9. The GA cross-linked collagen/chitosan scaffold could not be distinguished from the new-formed collagen fiber under routine paraffin section with light microscope after 28 days’ implantation (Fig. 9a). However, the immunohistochemistric assay shows that the bovine type I collagen had been partially preserved though the scaffold had macroscopically disappeared (Fig. 9b). This result indicates that the biodegrading behavior of the GA-treated collagen/chitosan scaffold is a long-term process. The long-term biodegradation of this kind scaffold in vivo should be studied further.

Acknowledgements

The authors thank Prof. Yiyong Chen for his valuable discussion. This work was supported by the Natural Science Foundation of China (50173024) and the Major State Basic Research Program of China (G1999054305).

References


