Original Article

Fabrication and Evaluation of Porous Keratin/chitosan (KCS) Scaffolds for Effectively Accelerating Wound Healing*

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Abstract

Objective To develop a dressing with desired antibacterial activity, good water maintaining ability and mechanical properties for wound healing and skin regeneration.

Methods The chitosan with different concentrations were added in keratin solution to form porous keratin/chitosan (KCS) scaffolds. The morphological characteristics, chemical composition, wettability, porosity, swelling ratio and degradation of the scaffolds were evaluated. The antibacterial activity was tested by using *S. aureus* and *E. coli* suspension for 2 h. And L929 fibroblast cells culture was used to evaluate the cytotoxicity of the KCS scaffolds.

Results The adding of chitosan could increase the hydrophobicity, decrease porosity, swelling ratio and degradation rate of the KCS porous scaffolds. Mechanical properties of KCS scaffolds could be enhanced and well adjusted by chitosan. KCS scaffolds could obviously decrease bacteria number. The proliferation of fibroblast cells in porous KCS patch increased firstly and then decreased with the increase of chitosan concentration. It was appropriate to add 400 μg/mL chitosan to form porous KCS scaffold for achieving best cell attachment and proliferation compared with other samples.

Conclusion The porous KCS scaffold may be used as implanted scaffold materials for promoting wound healing and skin regeneration.

Key words: Keratin; Chitosan; Porous scaffold; Wound healing; Skin regeneration

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INTRODUCTION

Skin wound caused by accident or burning has seriously affected people's life[1-2]. Moreover, the incidence of skin wound has increased with the development of today's society[3]. The regeneration of skin tissue at the site of injury is still really a challenging task in the field of biomedical science[4]. Generally, the wound repair is a complex process involving a series of overlapping phases, including matrix deposition, inflammation and tissue remodeling. The duration of these individual phases varies depending on the intensity and depth of the wound[5]. Currently, most wound dressing treatments aim to facilitate these stages of wound healing by providing a moist environment, controlling excessive exudate buildup and protecting against infection that would perturb normal

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healing[6]. However, the wound healing effect has not yet met the clinical requirement. Moreover, there may be infections caused by various bacteria[7]. Therefore, it is quite necessary to develop an alternative substitute in order to better repair the skin injury. In recent years, various biomaterials scaffolds have been developed for wound healing and skin regeneration[8-9]. However, the functional recovery is still far from normal skin, and thus much better scaffolds for wound healing and skin regeneration are needed.

In recent years, the porous composite biomaterials, which have many advantages, have been widely applied in the field of tissue engineering and regeneration medicine[10-11]. A porous scaffold consisting of collagen and hydroxyapatite was developed for drug delivery device[12]. The study showed that the endocortical woven and lamellar bone formation could be enhanced with neurotrophic factor loaded by the scaffolds. Thus, the porous scaffolds were believed to be very suitable for filling the irregular defects in cosmetic surgery. In another study, the porous chitosan-siloxane scaffolds for nerve regeneration were fabricated, and the inflammatory response during wound-healing processes was evaluated. It was found that the treatment with porous chitosan-siloxane scaffolds showed low inflammation and better posttraumatic axonal regrowth, indicating a good functional recovery[13]. In addition, both the chitosan-modified poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) porous scaffolds loaded with cord blood (CB)-derived unrestricted somatic stem cells (USSCs) and the collagen/chitosan porous scaffolds crosslinked by glutaraldehyde (GA) were found to have effects to promote skin regeneration[14]. As mentioned above, the porous biomaterials scaffolds could act as protective barrier, absorb excess wound exudate, and maintain a moist environment, which in turn help in pain reduction. It is well known that a moist wound environment could accelerate wound healing[15]. Therefore, the porous biomaterial scaffolds showed a promising future in clinical application for wound healing and skin regeneration.

Keratin could be extracted from silk, feathers, nail, wool, and human hair. It contains cell adhesion sequence, RGD (Arg-Gly-Asp) and LDV (Leu-Asp-Val), which are found in the extracellular matrix (ECM) proteins such as fibronectin[16]. The cellular-binding motifs on keratin could mimic the sites of cellular attachment found in the native ECM. Therefore, keratin could be utilized for the construction of tissue engineering implants. It has been reported that the keratin biomaterials in the form of sponge scaffold have been developed for different biomedical applications such as wound dressings and neural tissue engineering applications[17]. The mouse fibroblast cells were found to proliferate well on the keratin scaffold[18], suggesting the good biocompatibility of keratin. Thus, keratin is expected to be applicable for biomedical use. However, the degradation property and unsatisfied mechanical properties are still the limitation for its sole application as tissue scaffold[19]. Chitosan used in scaffold material with excellent biocompatibility, good mechanical properties, non-toxicity and good antibacterial activity has been intensively studied and widely used in different tissue engineering fields[20]. The collagen-chitosan (CCH) scaffold immobilized with RGD sequences was found to have effect to promote rapid regeneration of injured sciatic nerve in rats[21]. Chitosan combined with organic or inorganic biomaterials could form composite scaffolds, which had been used for bone tissue engineering[22]. The chitosan/collagen scaffold, chitosan/poly(caprolactone) (CS/PCL) nanofibrous scaffold and chitosan/silk fibroin (CS/SF) blend scaffolds were developed by different research groups, and they were found to have effects to effectively promote skin tissue regeneration. In addition, the antibacterial activity of chitosan is also very important for most tissue regeneration. Zhao et al.[23] fabricated chitosan/sericin composite nanofibers by electrospinning, and they found that the composite nanofibers showed good bactericidal activity against both Gram-positive and Gram-negative bacteria, which are promising for wound dressing applications. The chitosan/phosvitin antibacterial scaffolds were fabricated via layer-by-layer deposition by Zhou et al.[24], the result of microbial inhibition assay indicated that the composite nanofibrous mats had excellent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus,* which could be used for antimicrobial packing, tissue engineering, wound dressing, etc. Thus, according to the analysis mentioned above, the combination of keratin and chitosan is anticipated to improve both the mechanical properties and antibacterial activity of the composite scaffolds, which is beneficial for wound healing and skin regeneration. Though the keratin-chitosan composite scaffolds were investigated[25], the effect on wound healing and skin

regeneration has not been reported.

In the present study, a porous composite biomaterial scaffold consisting of keratin and chitosan for wound healing and skin regeneration was fabricated. The physiochemical properties of the composite materials, including morphology, surface chemistry, wettability, porosity, swelling ratio, degradation were evaluated. The antibacterial activity and cytocompatibility were tested by using *S. aureus* and *E. coli* suspension and L929 fibroblast cells culture respectively. Fibroblasts have been widely used in the previous studies of wound healing and skin regeneration[26], especially at the early stage *in vitro*. Thus, in the present study, the KCS scaffolds' cell viability and antibacterial activity were mainly investigated. And the effect of the keratin/chitosan composite materials on wound healing and skin regeneration was discussed.

MATERIALS AND METHODS

Materials and Reagents

Chitosan (average molecular weight 100 kDa, deacetylation degree 90%) were from Sigma-Aldrich, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bacto-trypton, bacto-yeast extract, bacto-agar, 4% formaldehyde and ethanol were from Chengdu Kelong Chemical Company, China. Urea, 2-mercaptoethanol, sodiumdodecyl sulfate (SDS), acetic acid (AA), lysozyme were from Jiancheng Chemical Industries Ltd, China. Dulbecco's minimum essential medium (DMEM), bovine fibrin and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, USA). Phosphate buffered saline (PBS, 0.067 mol/L, pH 7.3), penicillin, and streptomycin were from Hyclone, China. Deionized water (>18.2 MΩ, Millipore Milli-Q system) was used in all the experiments. All the other reagents were of analytical or chromatographic grade and used as received without further purification. Fibroblast (L929, mouse connective tissue) was from Flow Laboratories, USA.

Preparation of Porous KCS Scaffolds

Keratin was prepared by using wool according to the previously reported method by Srinivasan et al.[27]. Firstly, keratins were extracted from wool by incubation with a mixture of SDS, urea, and 2-mercaptoethanol at 60 °C for 12 h. The solution containing wool keratins was thoroughly dialyzed against water to remove urea, and then aqueous keratin solution (100 mg/mL) was stored at -4 °C for further use. Secondly, the lyophilization technique was used to fabricate the porous KCS scaffolds as following. The prepared keratin solution was poured into a beaker, and then chitosan with different weights were dispersed in keratin solution and stirred thoroughly, and thus the keratin/chitosan mixture with different concentrations of chitosan was obtained. In this study, the concentration of chitosan in keratin solution was 50, 100, 200, 400, 600, and 1000 μg/mL, respectively. Finally, the keratin/chitosan mixture was degassed and poured into a 6-well culture plate, frozen at -80 °C and lyophilized for 48 h. Thereafter, the samples were washed repeatedly with PBS and deionized water to remove residual acetic acid excess SDS, mercaptoethanol and urea, and dried naturally for further use. Thus the porous KCS scaffolds were formed and named KCS50, KCS100, KCS200, KCS400, KCS600, and KCS1000. In addition, pure porous keratin scaffold and pure porous chitosan scaffold were also prepared respectively by using the same method and used as controls in the study.

Scanning Electron Microscope (SEM) Observation

The surfaces of the freeze-dried porous KCS scaffolds were observed by using SEM (JSM-T330, JEOL Co., CLRI, Chennai). The KCS porous scaffolds were firstly cut into small pieces (10 mm × 10 mm) from the middle with a razor blade, and then scaffolds were coated with gold under vacuum and observed with SEM at 5 kV of an accelerated voltage.

Contact Angle Measurement

The water contact angles of the prepared samples were evaluated by a water contact angle apparatus (DSA 100, Krüss, GmbH, Germany). Firstly, a droplet of Milli-Q water was added to the samples fixed on a glass slide, then a horizontal microscope was used to record the contact angle of each water drop on the surface of samples. For each droplet the equilibration time was 10 s before the measurement. And at least four individual measurements at different sites on the examined samples were performed for calculating the mean value of the contact angle.

Fourier Transform Infrared Spectrometer (FTIR)

The variation of the chemical composition of the prepared KCS scaffolds was examined using FTIR (Nicolet20 DXB, America) with KBr tablet mode. An

infrared spectrum was recorded from 500 to 4000 cm^{-1} wave numbers with a 4 cm^{-1} resolution. A total of 64 scans were accumulated for each sample.

Porosity of Porous KCS Scaffolds

The porosity of porous KCS scaffolds was detected by ethanol infiltration method. In brief, the known weighed porous KCS scaffolds (W_0) were soaked in ethanol under vacuum condition for at least 10 min to remove the air bubbles. Then the scaffolds were taken out and the surface ethanol was wiped out by using a filter paper. Subsequently, the scaffolds were weighed immediately (W_1). The porosity of the scaffolds was defined as shown in Equation 1:

$$\text{Porosity}=(W_1-W_0)/(\rho Vs) \times 100\% \quad (1)$$

Here, Vs could be calculated from the geometry of the scaffolds according to the height and diameter of the cross-section. And ρ represented the density of ethanol at room temperature (0.789 mg/mL).

Swelling Ratio of Porous KCS Scaffolds

The swelling ratios of the prepared porous KCS scaffolds, pure keratin scaffold, and pure chitosan scaffold were measured respectively as following: The scaffold was firstly cut to a square piece (10 mm × 10 mm) and carefully weighed and placed into a 24-well cell culture plate. Then, 4 mL PBS was added into each well and incubated at 37 °C until the scaffold reached to constant size. Generally, the complete equilibration could be obtained within 2 h. Subsequently, the scaffolds were taken out and carefully weighed after dried with a piece of filter paper. Finally, the equilibrium-swelling ratio was calculated according to the Equation 2:

$$Wsr=W_a/W_d \times 100\% \quad (2)$$

Here, W_a is the weight of scaffold after PBS immersion, and W_d is the weight of dried scaffold.

Degradation of Porous KCS Scaffolds

The degradation of the scaffold was evaluated by the weight loss ratio of the scaffolds as a function of incubation time in PBS and lysozyme at 37 °C. Firstly, the scaffold were cut into pallets (10 mm × 10 mm), weighed (W_b) and placed into a 24-well cell culture plate, and then 2 mL PBS or 2 mg/mL lysozyme (replaced every 2 d) was added into each well and incubated at 37 °C. Then, the samples were taken out at designated times (from 1 d to 30 d), washed repeatedly with deionized water, dried completely and weighed (W_a). The weight loss ratio of the scaffold was calculated according to the Equation 3:

$$(W_b - W_a)/W_b \times 100\% \quad (3)$$

Here, W_b represents the dry weight of the scaffold before PBS immersion, W_a represents the dry weight of the scaffold after PBS immersion.

Mechanical Properties of KCS Porous Scaffolds

The uniaxial tensile measurement was performed to determine the mechanical strength of the prepared porous KCS scaffolds by a digital mechanical tester (Norwood, MA, USA). Specifically, the scaffolds were cut into rectangle pieces with an identical size of 40 mm (length), 40 mm (width) and 2 mm (thickness) and measured at a crosshead speed of 20 mm/min until failure at room temperature. The ultimate elongation, strength and Young's modulus were determined manually by calculating the slope of the linear regression of stress-strain curves from five independent measurements.

Treatment of Porous KCS Scaffolds with Bacteria Suspension

The antibacterial activity of the porous KCS scaffold was evaluated as following: Firstly, LB medium for bacteria culture was prepared by dissolving 10 g bacto-trypton and 5 g bacto-yeast extract and 10 g sodium chloride in 1 L aqueous solution, which was adjusted at pH 7.5 with sodium hydroxide and sterilized by autoclaving. LB plates were prepared by adding 15 g of bacto-agar to 1 L of LB medium, which was dissolved by autoclaving and dispensed to 10 cm plastic petri dishes. Then, the scaffold with a diameter of 10 mm was washed with PBS for 30 min twice, sterilized in 75% ethanol for 30 min and dried in the clean cabinet. After that, fresh overnight culture of Staphylococcus aureus (*S. aureus* and *E. coli*) was diluted to 1000 or 10,000 colony forming units (cfu)/mL. Thereafter, the scaffold was immersed in above prepared *S. aureus* and *E. coli* suspension (100 mL). After 2 h incubation at room temperature, one aliquot of bacteria suspension was spread onto LB plate to determine the bacteria number. The bacteria number was calculated by dividing the bacteria number of the scaffold-treated sample by that of non-treated one. In addition, for negative control, the fibrin scaffold was prepared as follows. Bovine fibrin was dissolved in water solution containing 10 mol/L urea, 0.4 mol/L SDS, and 3 mol/L 2-mercaptoethanol, shaken at 50 °C for 6 h, dialyzed against water for 3 d, and the dialyzate was filtered to remove the precipitates. Thus obtained fibrin solution containing 200 mg of

fibrin was cast onto the polypropylene mold in a manner similar to that mentioned above.

Cytotoxicity Test of Porous KCS Scaffolds

The cell line L929 fibroblast was used to test the cytotoxicity of the prepared porous scaffolds. L929 fibroblast cells were routinely cultured in DMEM supplemented with FBS (10%), penicillin (120 U/mL) and streptomycin (75 mg/mL) in 50 mL-plastic culture flasks and subcultured every five days before use. Before cell seeding, the KCS porous scaffolds were put into a 24-well culture plate, sterilized with 75% ethanol for 30 min and equilibrated with PBS for 20 min. Then, the cells at logarithmic growth phase were treated with 0.2% trypsine-0.02% EDTA, washed with DMEM containing 10% FBS, collected by centrifugation at 1200 rpm for 5 min and re-suspended in DMEM. Then, L929 fibroblasts with concentration of 1×10^5 cells/mL were seeded on the samples and cultivated at 37 °C in a humidified 5% CO_2 incubator for 1 and 3 d, respectively.

For quantitative evaluation of L929 fibroblasts growth on all the samples, MTT assay was performed as following: after culture for 1 and 3 d, respectively, the medium was removed and a new culture medium (500 µL) containing 20 µL MTT solution (5 mg/mL, dissolved in PBS) was added to each well. Then after 3 h of incubation the medium was removed and MTT metabolic product-formazan was dissolved in 200 µL DMSO. Finally, after shaking for 15 min, 180 µL above solution for each sample was added to a 96-well plate. Absorbance at 570 nm was measured by using Epoch microplate reader (BioTek). For further qualitative evaluation of L929 fibroblasts growth on all the samples, the adhesion and proliferation of cells were observed under a phase contrast microscope (Olympus, Japan). Five parallel samples were used for cell evaluation.

Statistical Analysis

The data were expressed in terms of mean±standard deviation (SD) values. The statistical significance of differences was determined by Bonferroni's post hoc comparison tests and One-way ANOVA analysis. A *P*-value of <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

SEM

The morphological characteristics of the prepared porous KCS scaffolds with different chitosan ratios were observed by SEM. It could be seen from Figure 1 that there was obvious morphological difference for all the porous KCS scaffolds with different chitosan ratio. For the pure keratin and chitosan porous scaffolds, a lot of filamentous structure could be observed on the scaffold surface, whereas with the increase of chitosan ratio, the filamentous structure reduced and even disappeared when the largest chitosan (KCS1000) ratio was used. As shown in Figure 1, the surface of keratin and chitosan porous scaffolds were cluttered with plenty of strip- and fiber- like structure, the porous KCS scaffolds with low chitosan ratio did not cause obvious difference of the keratin scaffold surface. Whereas the surfaces of porous KCS scaffolds with higher chitosan ratio showed a skin-like structure with many oval pores on them instead of a fiber-like structure. Moreover, all the KCS scaffolds showed highly porous structure with thin walls and three-dimensional interconnected pores. It has been reported that the pores on scaffolds could supply oxygen and nutrients for the cells, and an average pore diameter from 20 µm to 125 µm was shown optimal morphological active for skin regeneration[28]. As shown in this study, the pore size was from 80 µm to 125 µm, which was consistent well with other studies. The results indicated that the morphology of porous KCS scaffolds could be changed by chitosan addition, but the exact reasons for variation in morphology are still under investigation, which might be ascribed to the re-organization of keratin microstructure during chitosan composition.

FTIR and Water Contact Angle

The chemical structures of pure keratin scaffold, pure chitosan scaffold and porous KCS scaffolds were analyzed by FTIR. As shown in Figure 2A, the spectrum of pure keratin scaffold [Figure 2a (A)] showed characteristic peaks of amide bands, as observed at 1600 and 1575 cm^{-1}, respectively. The peak at 3400 cm^{-1} and 2928 cm^{-1} was approximate to the -OH and -NH groups respectively, and 2818 cm^{-1} corresponding to $-CH_2$ groups. The spectrum of pure chitosan scaffold [Figure 2a(B)] mainly showed characteristic peaks at 3400 cm^{-1}, 2928 cm^{-1}, and 1595 cm^{-1}, corresponding to -OH, -NH and amide bands, respectively. In contrast, the spectrum of porous KCS scaffolds [Figure 2a (C-E)] showed the characteristic peaks of -OH, $-CH_2$ groups and amide I,

II bands, as observed at 3400, 2928, 1595, and 1568 cm^{-1}. Moreover, a new peak at 2363 cm^{-1} was observed. In addition, the strong peak that centered at 675 cm^{-1} could be attributed to the C-S band stretching vibrations. And the band corresponding to carbohydrate moiety was observed at 1175 cm^{-1} as a sharp peak related to C-O and C-O-C groups, which was in agreement well with the previous study[25]. However, after the concentration of chitosan was further increased to 400, 600, and 1000 μg/mL, there was no obvious variation of the peaks except for the tiny enhanced intensity of the four peaks. Thus only the typical FTIR spectra of the porous KCS scaffolds were shown here. All the above absorptions indicated that the addition of chitosan into keratin scaffolds was successful.

The water contact angles of porous KCS scaffolds were measured as a function of different chitosan concentrations. The results are shown in Figure 2b. Compared with pure keratin scaffold (-44°), the water contact angles dramatically increased to -62° after combining with chitosan ($P<0.05$), then further increased to about 100° and kept at a platform when the concentration of chitosan reached 400, 600, and 1000 μg/mL. The pure chitosan scaffold showed a contact angle of about 64°. The contact angles of all the samples increased in the order of single keratin scaffolds < KCS50≈chitosan < KCS100 < KCS200≈KCS (400, 600, 1000). Thus the water contact angle increased with the

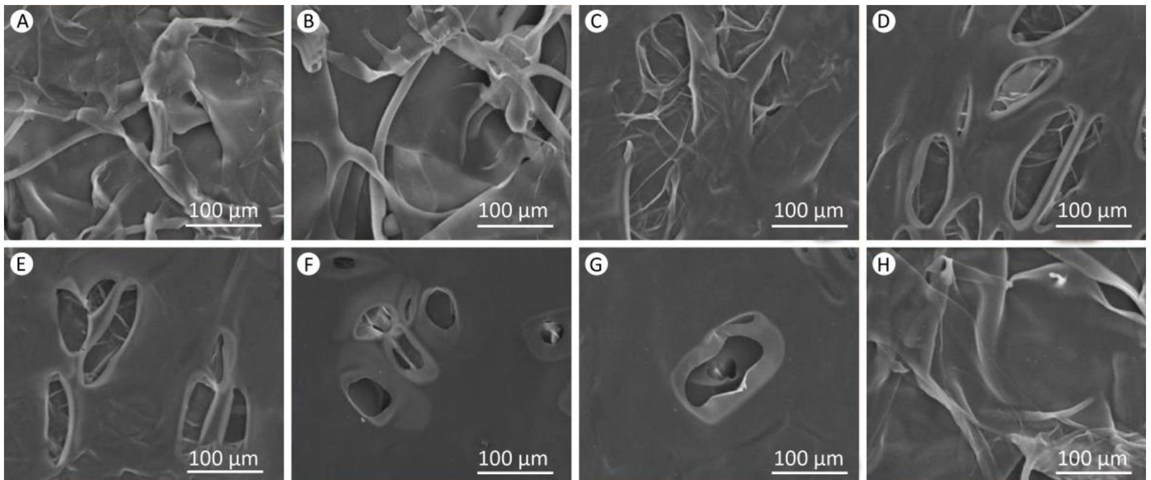

Figure 1. SEM observation of porous KCS scaffolds prepared by freezing for 48 h at −80 °C and subsequent lyophilization. A. keratin, B.KCS50, C. KCS100, D. KCS200, E. KCS400, F. KCS600, G. KCS1000, H.CS.

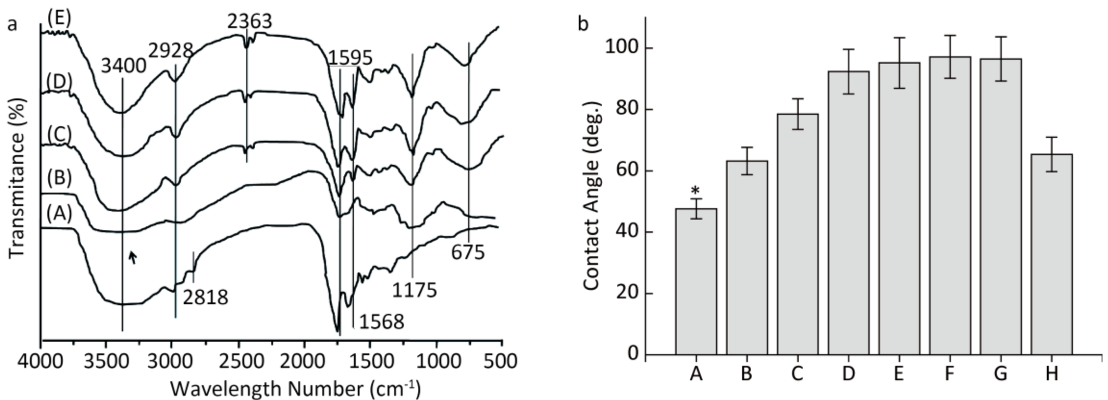

Figure 2. a, FTIR spectra of: (A) pure keratin scaffold, (B) pure chitosan scaffold, (C-E) porous KCS scaffolds, the concentration of chitosan was 50, 100, and 200 μg/mL. b, Water contact angles of the porous KCS scaffolds as a function of chitosan addition. A: pure keratin scaffold, B-G: porous KCS scaffolds, and the concentration of chitosan was 50, 100, 200, 400, 600, and 1000 μg/mL, respectively. H: pure chitosan scaffold. $^*P<0.05$ compared with KCS scaffolds, mean±SD, N=5.

increase of chitosan concentration, indicating that the porous KCS scaffolds were more hydrophobic than pure keratin scaffold and pure chitosan scaffold. The possible reason might be ascribed to the morphological variation and crosslinking of the scaffolds due to the different chitosan ratio as shown in Figure 1.

Porosity and Swelling Ratio

The porosity of porous KCS scaffolds in comparison with pure keratin and chitosan scaffolds was investigated by ethanol immersion method. The results are shown in Figure 3a. The porosity of pure keratin and chitosan scaffolds was both about 92%. The addition of chitosan with concentration of 50 μg/mL to keration solution did not cause large porosity variation. However, after further increasing chitosan concentration from 100 μg/mL to 1000 μg/mL, there was a significant decrease of the porosity from -90% to -72% (P<0.05). And finally, the porosity reached a platform when the chitosan concentration was 600 and 1000 μg/mL, indicating no further porosity change of porous KCS scaffolds occurred when chitosan concentration reached a certain level. The results indicated that the addition of chitosan could cause the porosity change of keratin scaffold, but it was still larger than 70% even with the largest chitosan concentration (1000 μg/mL). It was reported that the porosity could affect the cell behavior[29]. The high porosity of the scaffold was beneficial for cell culture in allowing maximum adhesion and proliferation of cells, leaving space for newly synthesized matrix via a large surface: volume ratio. Thus the high porosity would be helpful for the cells growth on the KCS scaffolds in our study.

The swelling properties of the KCS scaffolds in response to simulated physiological conditions were studied. Figure 3b shows the swelling ratio of the KCS scaffolds in PBS at 37 °C. For pure keratin and chitosan scaffolds, a burst swelling of about 240% and 250% of dry weight was observed, respectively. After chitosan addition to keratin solution, the swelling ratio of porous KCS scaffolds significantly decreased with the increase of chitosan concentration (P<0.05). And the final swelling ratio of the KCS scaffolds with the largest concentration of chitosan was about 125% after 2 h immersion in PBS. The hydration process of the porous KCS scaffolds could be reflected by swelling ratio. Many factors may affect the swelling properties, such as physiochemical properties of biomaterials, swelling medium, surface wettability and different crosslinking method. In the present study, the physiochemical properties of the porous KCS scaffolds and swelling medium were the same without crosslinking treatment, thus the wettability of the scaffolds may mainly contribute to their swelling properties. The pure keratin and chitosan scaffolds showed a higher hydrophilicity than the KCS scaffolds, thus there would be much higher hydration degree, i.e. the higher swelling ratio, in pure keratin and chitosan scaffolds than porous KCS scaffolds. In contrast, the swelling ratio of KCS scaffolds decreased with the increase of hydrophobicity.

Degradation Property

The degradation property of the biomaterial is very important for its normal performance of biological

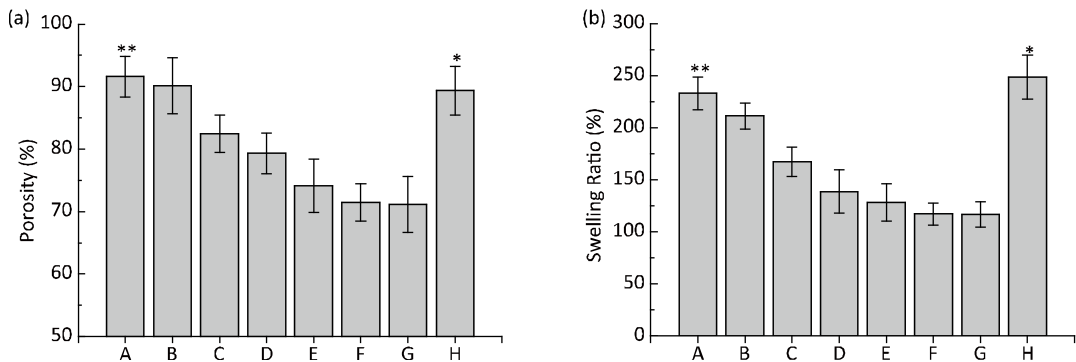

Figure 3. Porosity (a) and swelling ratio (b) of porous KCS scaffolds with different chitosan concentration. A: pure keratin scaffold, B-G: porous KCS scaffolds, and the concentration of chitosan was 50, 100, 200, 400, 600, and 1000 μg/mL, respectively. H: pure chitosan scaffold. $^{*}P$<0.05, $^{**}P$<0.05 compared with KCS porous scaffolds, mean±SD, N=5.

function. A suitable degradation ratio will not only be beneficial for tissue regeneration but also for mechanical support. Too fast degradation may cause worse mechanical property, while too slow degradation may induce potential inflammatory reaction of the host, which may cause implantation failure at last. Thus, it is very important and necessary to study the degradation property of the implanted biomaterial. In the present study, the stability of the KCS scaffolds was evaluated via the in vitro studies of the degradability in PBS solution (pH 7.3) and lysozyme at 37 °C for different periods, respectively. The results are shown in Figure 4, it could be seen that the degradation ratio of pure keratin scaffold increased dramatically to about 6% after immersion in PBS for 1 d (Figure 4a), and then the degradation ratio increased significantly with the prolonged immersion periods. But for the pure chitosan scaffold, there was no obvious variation of degradation ratio during the immersion periods from 1 d to 28 d. Figure 4b shows the degradation properties of KCS scaffolds in lysozyme. It could be seen clearly that the scaffolds degraded quicker than those in PBS due to the enzyme dilution. And the pure chitosan scaffold also showed certain degradation during the incubation time. However, compared with the pure keratin and chitosan scaffolds, all the KCS scaffolds showed a similar trend with degradation property of keratin scaffold. Moreover, it could be seen obviously that the degradation ratios of KCS scaffolds decreased when the concentration of chitosan increased at the same time point. The reason might be ascribed to the addition of chitosan, as it is known that chitosan has possessed poor degradation property[30]. Thus, the addition of chitosan to keratin scaffolds may reduce the degradation ratio. In addition, the change of surface and porosity may also affect the degradation property. The above results demonstrated that the degradation of KCS scaffolds could be controlled by changing the concentration of chitosan.

Mechanical Properties

The mechanical properties of pure keratin, chitosan scaffolds and porous KCS scaffolds are summarized in Table 1. It could be seen that the pure chitosan scaffold showed significantly larger elongation, strength and Young's modulus than pure keratin scaffold. When the concentration of chitosan was 50 μg/mL, the formed porous KCS scaffolds showed similar mechanical properties with the pure keratin scaffold. However, after the further increase of the concentration of chitosan to 100, 200, 400, and 600 μg/mL, all the elongation, strength and Young's modulus of porous KCS scaffolds showed an obvious increase compared with the pure keratin scaffold. Subsequently, the further increase of chitosan concentration to 1000 μg/mL caused little additional variation on ultimate elongation, strength and Young's modulus. The results indicated that the presence of chitosan in keratin scaffolds could effectively enhanced the mechanical properties due to the better mechanical strength of chitosan scaffold than that of pure keratin scaffold, which was consistent well with the reports by Tanabe and Bazargan-Lari et al. that the addition of chitosan could effectively adjust the mechanical properties of keratin scaffold[19]. It is known that there is elasticity and flexibility at the site of wound and skin tissue, which have their own mechanical strength. Each tissue in human body has its own mechanical strength

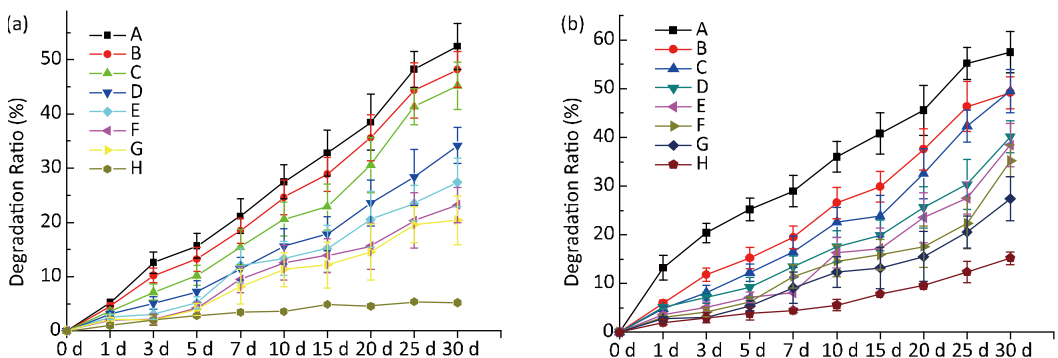

Figure 4. Degradation properties of porous KCS scaffolds with different chitosan concentrations in (a) PBS and (b) lysozyme, respectively. A: pure keratin scaffold, B-G: porous KCS scaffolds, and the concentration of chitosan was 50, 100, 200, 400, 600, and 1000 μg/mL, respectively. H: pure chitosan scaffold. Mean±SD, *N*=5.

to provide a better microenvironment for regeneration and support the cell growth and tissue regeneration. Moreover, when skin is being developed, an enough elongation of the implanted biomaterials will be beneficial for skin regeneration. As wound dressing, an appropriate mechanical property is very important for the physical support of skin tissue regeneration, including the lymphatic system, nerve bundles and extensive vasculatures, etc. Thus, the porous scaffold should have an appropriate mechanical strength after implanted in the wounds in order to support skin regeneration. From our results, the appropriate addition of chitosan was found to has effect to improve the mechanical properties of KCS porous scaffolds, which may have promising future of application in tissue regeneration.

Antibacterial Activity

Chitosan could inhibit the growth of bacteria via binding its positively charged amino groups to negatively charged bacterial cell wall. Thus, in this study, the antibacterial evaluation were conducted to assess whether porous KCS scaffolds have the antibacterial activity of chitosan. The results of the evaluation of antibacterial activities to *S. aureus* and *E. coli* of different samples after immersing into bacteria suspension for 2 h are shown in Table 2, which indicated that the pure chitosan scaffold showed the largest reduction in bacteria number (84% for *S. aureus* and 80% for *E. coli*) compared with all the other samples, but the fibrin scaffold did not show any antibacterial activity (0%). The pure keratin scaffold showed 19% and 21% of reduction in bacteria number, respectively. Then, after further increase of the concentration of chitosan in porous KCS scaffolds, the bacteria number obviously decreased. Notably, the decrease ratio of *S. aureus* and *E. coli* reached more than 70% and 80% respectively when the chitosan concentration was larger than 400 μg/mL. As mentioned above, the antibacterial activity of pure keratin scaffold was limited. Therefore, the antibacterial activity of the porous KCS scaffolds was mainly ascribed to the addition of chitosan[31]. The results indicated that the antibacterial activity of the porous KCS scaffolds could be improved by adding chitosan, which was very important for wound healing and skin regeneration.

Attachment and Proliferation of Fibroblast Cells

Fibroblast cell is an important cell lines during the development of wound healing and skin regeneration[26]. Thus, in this study, the growth of fibroblast cells after culture for 1 and 3 d on different samples were evaluated using MTT assay and optical observation in order to preliminarily evaluate the cytocompatibility of the porous KCS scaffolds. It can be seen from Figure 5a that both the viability and proliferation of fibroblast cells increased firstly and then decreased with the increase of chitosan concentration at 1st and 3rd day of culture. And the cells showed much better viability and proliferation when the concentration of chitosan was 400 μg/mL compared with the other samples at the 3rd day ($P<0.05$), but there was no obvious difference of cell number among different samples at the 1st day ($P>0.05$). Moreover, when the concentration of chitosan was larger than 400 μg/mL,

Table 1. Mechanical Properties of KCS Porous Scaffolds with Different Chitosan Concentrations

Sample ID	Mechanical Properties		
	Elongation (%)	Strength (MPa)	Young's modulus (MPa)
Pure keratin	17±3	46±5	15±5
KCS50	19±2	57±8	21±7
KCS100[a]	31±6	116±16	36±5
KCS200[a]	46±5	138±12	58±8
KCS400[a]	62±6	158±22	86±6
KCS600[a]	78±4	171±19	117±5
KCS1000[a]	81±7	178±14	127±7
Pure chitosan	72±6	136±11	81±4

Note. The data represent mean±SD, [a]significantly different from the scaffold containing no chitosan (i.e. pure keratin) ($P<0.05$).

Table 2. Decrease of *S. aureus* and *E. coli* after Contacting with KCS Porous Scaffolds for 2 h

Scaffolds	Decrease Ratio of *S. aureus* (%)	Decrease Ratio of *E. coli* (%)
Bovine fibrin	0	0
Pure keratin	19±3	21±4
KCS50	24±4	23±2
KCS100[a]	35±3[a]	45±4[b]
KCS200[a]	51±5[a]	63±6[b]
KCS400[a]	71±6[a]	81±4[b]
KCS600[a]	70±3[a]	83±6[b]
KCS1000[a]	73±6[a]	85±3[b]
Pure chitosan	84±5	80±3

Note. The data represent mean±SD, [a,b]significantly different from the scaffold containing no chitosan (i.e. pure keratin) ($P<0.05$).

the cell viability significantly decreased, indicating the bad viability of the cells on these samples. The results here demonstrated that an appropriate chitosan concentration would be beneficial for improving cytocompatibility of porous KCS scaffolds, and chitosan at the concentration of 400 μg/mL showed the lowest cytotoxicity, which is important for wound healing and skin regeneration.

The morphology of L929 fibroblast cells cultured on different samples for 1 and 3 d was further observed by an optical microscopy and the results were shown in Figure 5b. It could be seen clearly that the fibroblast cells were homogeneously distributed on all the samples. At the 1st day of culture, the fibroblast cells mainly showed spindle-like, round and polygon shape, and there was no number and morphological difference on all the samples. However, after cultivation for 3 d, the cells on all samples mainly showed a spindle-shape. And interestingly, fibroblast cells on KCS400 porous scaffold almost formed a confluent layer, which was not observed on other samples. It has been reported that both keratin and chitosan could support the attachment and growth of fibroblast cells[32]. Thus, it is reasonable to believe that the combination of keratin and chitosan could also promote fibroblast

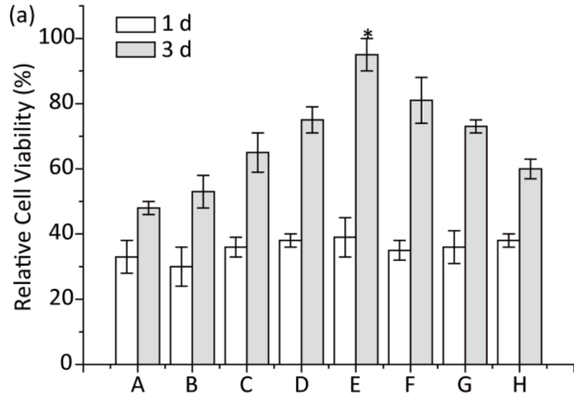

Figure 5. Attachment and proliferation of fibroblast cells on pure keratin, chitosan scaffolds and porous KCS scaffolds with different chitosan concentrations. (a) Viability and proliferation of L929 fibroblast cells after culture for 1 and 3 d evaluated by MTT assay. (b) Morphological observation of fibroblast cells on different samples by optical microscopy. A, A’: pure keratin scaffold; B-G, B’-G’: porous KCS scaffold, and the concentration of chitosan was 50, 100, 200, 400, 600, and 1000 μg/mL, respectively; H, H’: pure chitosan scaffold. $^{*}P$<0.05 compared with other samples, mean±SD, *N*=5.

cells growth. Here, the porous KCS scaffolds were also demonstrated to be a good substrate for fibroblast cells as expected. In our study, the chitosan concentration of 400 μg/mL was found to be appropriate to effectively promote the attachment and growth of fibroblast cells.

CONCLUSION

In conclusion, the porous KCS scaffolds with different chitosan ratios were fabricated by freezing and lyophilizing treatment. The porous KCS scaffolds showed superior characteristics comparing with pure keratin and chitosan scaffolds. The addition of chitosan greatly changed the morphology of the scaffold and decreased the hydrophilicity, porosity, swelling ratio and slowed down the degradation of the KCS scaffolds. However, the mechanical properties of the porous KCS scaffolds could be greatly improved by adding chitosan. Moreover, the porous KCS scaffolds showed enhanced antibacterial activity when the chitosan ratio was increased. Finally, the *in vitro* fibroblast cells culture revealed that the cells could be distributed on KCS scaffolds homogeneously. The proliferation of the cells in the KCS scaffolds increased firstly and then decreased with the increase of chitosan concentration. And the fibroblast cells on the porous KCS scaffolds with 400 μg/mL chitosan showed best attachment and proliferation. Therefore, this study demonstrated that a new wound dressing with better antibacterial activity and cell proliferation property was developed, which might be a promising biocompatible scaffolds for wound healing and skin regeneration.

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