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A nanofibrous composite membrane of PLGA–chitosan/PVA prepared by electrospinning

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Abstract

Tissue engineering scaffolds produced by electrospinning feature a structural similarity to the natural extracellular matrix. In this study, poly(lactide-co-glycolide) (PLGA) and chitosan/poly(vinyl alcohol) (PVA) were simultaneously electrospun from two different syringes and mixed on the rotating drum to prepare the nanofibrous composite membrane. The composite membrane was crosslinked by glutaraldehyde vapor to maintain its mechanical properties and fiber morphology in wet stage. Morphology, shrinkage, absorption in phosphate buffered solution (PBS) and mechanical properties of the electrospun membranes were characterized. Fibroblast viability on electrospun membranes was discussed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay and cell morphology after 7 days of culture. Results indicated that the PBS absorption of the composite membranes, no matter crosslinked or not, was higher than the electrospun PLGA membrane due to the introduction of hydrophilic components, chitosan and PVA. After crosslinking, the composite membrane had a little shrinkage after incubating in PBS. The crosslinked composite membrane also showed moderate tensile properties. Cell culture suggested that electrospun PLGA–chitosan/PVA membrane tended to promote fibroblast attachment and proliferation. It was assumed that the nanofibrous composite membrane of electrospun PLGA–chitosan/PVA could be potentially used for skin reconstruction.

Keywords: Electrospinning; Poly(lactide-co-glycolide); Chitosan; Nanofibrous composite membrane; Fibroblast

1. Introduction

The fundamental goal of tissue engineering is to develop biological substitutes that restore, maintain or improve diseased, injured, or congenitally absent tissues or organs [1]. Strategies for the engineered reconstitution of tissues and organs are typically centered three fundamental approaches: cell-based therapy, scaffold-based therapy or bioactive molecule-based therapy [2]. Among the various approaches, biological achievements regarding cell culture using biodegradable materials are more promising techniques.

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Annually, people need skin grafts due to dermal wounds. The skin trauma can be caused by heat, chemicals, electricity, ultraviolet, nuclear energy or diseases and can result in several degrees of skin damage. In the case of wounds that extend entirely through the dermis, such as full-thickness burns or deep ulcers, many skin substitutes such as xenografts, allografts and autografts have been employed for wound healing. However, the disadvantages of these approaches include the limited availability of skin grafts in severely burned patients and the problems of disease transmission and immune response [3,4]. One strategy for dealing with serious skin damage is to develop a tissue-engineered skin equivalent. Therefore, a number of natural and synthetic polymers, including poly(lactide-co-glycolide) (PLGA), collagen and chitosan [5,6], are currently being employed as tissue scaffolds for skin reconstruction. Advanced Tissue Sciences has developed a kind of dermal replacement, named Dermagraft™, which consists of a PLGA scaffold seeded with allogeneic human fibroblasts from neonatal foreskin [3]. Chitosan has been considered to be one of the most promising biopolymers as tissue engineered scaffolds and wound dressing because of its excellent biological properties such as biodegradability, biocompatibility, antibacterial and wound-healing activity [7–9]. An insoluble, flexible hydrogel was prepared by applicating ultraviolet light (UV-) irradiation to a photocrosslinkable chitosan aqueous solution [10]. The chitosan hydrogel could effectively stop bleeding from a cut tail of mice and wound healing experiments using a mouse model have shown that the application of a chitosan hydrogel onto an open wound induces significant wound contraction and accelerates the wound closure and healing. Ueno et al. reported that chitosan in wound healing in dogs stimulated not only the migration of fibroblasts into the wound area and the activity of macrophages, but also the production of type III collagen in addition to the infiltration of inflammatory cells into the wound area [11].

Recently, much work has focused on electrospinning, a novel technique, which can prepare fibers with diameters ranging from 5 nm to 1 μm under a high voltage electrostatic field operated between a metallic capillary of a syringe and a grounded collector [12–14]. With high surface to volume ratio and porosity, non-woven membranes of electrospun nanofibers are thought to mimic natural extracellular matrix (ECM) and thus promote cell adhesion, migration and proliferation [15,16].

In this study, PLGA and chitosan/poly(vinyl alcohol) (PVA) are simultaneously electrospun from different syringes and mixed on the rotating drum to form the PLGA–chitosan/PVA composite nanofibrous membrane, in which PLGA component is expected to provide structural framework and chitosan is useful for its bioactivities. The morphology, mechanical properties, shrinkage and absorption in phosphate buffered solution (PBS) of electrospun membranes are characterized, and the cytocompatibility of fibroblasts are investigated.

2. Materials and methods

2.1. Materials

A sample of crab shell chitosan with 90% of degree of deacetylation was purchased from Yu Huan Ocean Biochemical Co. Ltd., China. The viscosity-average molecular weight (1.65×10^5) of this chitosan was calculated from the intrinsic viscosity $[\eta]$ using Mark–Houwink equation, $[\eta] = K\overline{M}_\eta^\alpha$, where $K = 1.81 \times 10^{-3}$ ml/g, $\alpha = 0.93$ [17]. The intrinsic viscosity was measured in the mixed solvent of 0.1 M acetic acid and 0.2 M sodium chloride at 25 °C. PLGA (LA/GA = 80/20) was kindly donated by Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, China. The molecular weight of PLGA and the polydispersity ($\overline{M}_w = 2.52 \times 10^5, \overline{M}_w/\overline{M}_n = 1.67$) were determined by gel permeation chromatography. PVA with 1750 ± 50 degree of polymerization and 98% of degree of hydrolysis was supplied by Experimental Chemical Plant of Tianjin University (Tianjin, China). PBS was prepared in our laboratory.

2.2. Electrospinning process

PLGA was dissolved at a concentration of 6% (w/v) in an organic solvent mixture composed of tetrahydrofuran (THF) and *N*,*N*-dimethlformamide (DMF) (1:1, v/v). PVA and chitosan were dissolved in 2 wt% aqueous acetic acid respectively and then mixed in the volume ratio of 60:40 to form the chitosan/PVA blend solution. The schematic setup of electrospinning in this study is shown in Fig. 1. For electrospinning of PLGA–chitosan/PVA composite fibers, PLGA and chitosan/PVA solutions were placed in two syringes with metal capillaries respectively, and fed by a double-way syringe pump (WZS-50F, Zhejiang University, China) at a feeding rate of 0.2 ml/h. A grounded rotating drum

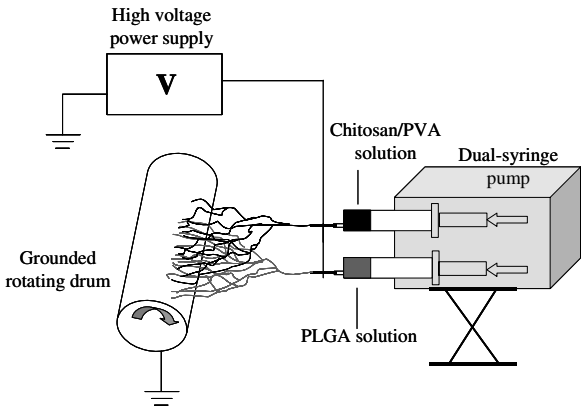

Fig. 1. Schematic setup of electrospinning of PLGA–chitosan/PVA composite membranes.

wrapped with aluminum foil was located at a fixed 10-cm distance away from the capillary tips. Both the capillaries were connected with a high voltage power supply (MGD1-A, Tianjin University, China) applying a high voltage of 15 kV. For electrospinning of PLGA or chitosan/PVA, the same conditions were applied but rather one syringe.

2.3. Characterization of electrospun membranes

2.3.1. Morphology

Electrospun nanofibrous membranes were sputtered with gold, and their morphology was observed under a scanning electron microscopy (SEM, Philips XL-30). The average fiber diameter of the electrospun fibers was measured by Adobe Photoshop 7.0 software from the SEM micrographs in original magnification of $10k\times$.

2.3.2. Shrinkage and PBS absorption

Electrospun membranes of PLGA, PLGA–chitosan/PVA and chitosan/PVA were cut into a square shape with dimensions of 20 mm × 20 mm for shrinkage and PBS absorption. Electrospun PLGA–chitosan/PVA and chitosan/PVA membranes were further crosslinked by glutaraldehyde vapor from a 25% glutaraldehyde aqueous solution at 37 °C for 4 h. After crosslinking, the electrospun membranes were treated with a 0.1 M glycine aqueous solution to block unreacted aldehyde groups [18]. The known weight of specimens were placed in closed bottles containing 20 ml of PBS (pH = 7.40) and incubated *in vitro* at 37.0 ± 0.1 °C for 24 h. The wet weight of the membranes was determined by weighing them immediately in an electronic balance after removing the membranes from PBS and blotting them with filter paper to absorb water on the membrane surface. The water uptake of electrospun membranes in PBS were then calculated from the formula:

$$A\ (\%) = \frac{(W_1 - W_0)}{W_0} \times 100\%$$

where, A is PBS absorption, and W_1 and W_0 are the weights of the membranes before and after immersion in the medium, respectively.

For shrinkage test, three samples in each group were recovered after incubating in PBS and then dried in a vacuum oven for 12 h to remove the water. The sizes of the dried membranes were measured and compared with the initial dimensions. The shrinkage percentage was defined as the ratio of the surface dimensional change of the recovered membranes.

2.3.3. Mechanical properties

The specimens were carefully cut into rectangular strips with dimensions of 10 mm × 60 mm, and tensile properties were characterized by a tensile machine (Testmetric M350-20KN, UK) equipped with a 100-N load-cell. The cross-head speed was 5 mm/min and the gauge length was 40 mm. The reported tensile moduli, tensile strengths, and elongations represented average results of five tests.

2.4. Fibroblast proliferation

2.4.1. Cell culture and seeding

Rabbit dermal fibroblast was isolated from rabbit back skin by sequential dispase and trypsin digestion. The cells were cultured in MEM (Modified Eagle Medium, Gibcol, USA) containing 10% fetal bovine serum, 50 U/ml penicillin and 50 U/ml streptomycin. The medium was replaced every 3 days and cultures were maintained in a tissue culture incubator at 37 °C with 5% CO_2. After reaching about 80% confluence, the cells were detached by 0.05% trypsin/0.05% EDTA (ethylenediaminetetraacetic acid). A density of 3×10^4 cells/cm^2 were seeded onto electrospun membranes, which were sterilized by ^{60}Co γ-irradiation at a dose of 25 kGy, in a 24-well plate for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, and a density of 5×10^4 cells/cm^2 for SEM observation. At 1, 4 and 7 days of culture, three samples were used to assess cell proliferation by MTT assay and SEM observation as follows.

2.4.2. MTT assay

Cell viability was indicated by the reduction of MTT into a formazan dye by living cells. MTT solution (150 μl) at 5 mg/ml in Hank's balanced salt solution was added to each well and incubated for 4 h under the same conditions described. After removal of the medium, the converted dye was dissolved in 250 μl/well DMSO (dimethyl sulfoxide). Solution (200 μl) of each sample was transferred to a 96-well plate. Absorbance of converted dye is measured at a wavelength of 570 nm using an ELISA plate reader.

2.4.3. SEM observation

After 1, 4 and 7 days of culture, cellular constructs were harvested, rinsed twice with PBS to remove non-adherent cells and subsequently fixed with 2.5% glutaraldehyde at 4 °C for 4 h. After that, the samples were dehydrated through a series of graded ethanol solutions and air-dried overnight. Dry cellular constructs were sputtered with gold and observed by SEM.

3. Results and discussion

3.1. Morphology of electrospun fibers

The morphology of electrospun ultrafine fibers is influenced by various parameters such as applied voltage, solution flow rate, distance between capillary and collector, and especially the properties of polymer solutions including concentration, surface tension and the nature of the solvent [19,20]. Fig. 2(a) shows a SEM micrograph of the ultrafine PLGA fibers electrospun from a 6% PLGA solution in a mixture solvent of THF and DMF (1:1, v/v). The average fiber diameter of the ultrafine PLGA fibers was 305 ± 72 nm measured from SEM micrographs.

There were many attempts to prepare chitosan fibers by electrospinning technique. However, electrospun ultrafine fibers could hardly be generated from pure chitosan solutions in aqueous acetic acid, but could be fabricated from blended systems of chitosan with poly(ethylene oxide) [21], silk fibroin

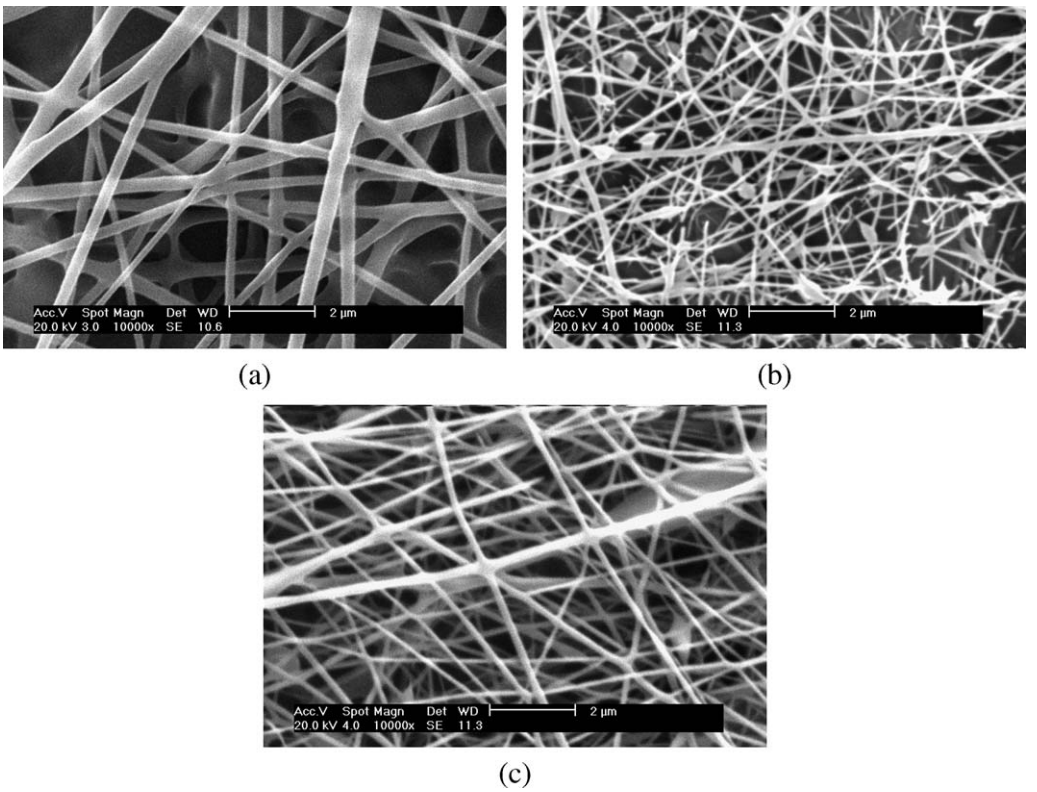

Fig. 2. SEM micrographs of electrospun PLGA (a), chitosan/PVA (b) and PLGA–chitosan/PVA (c) fibers.

[22], PVA [23] or from chitosan solutions in trifluoroacetic acid or 1,1,1,3,3,3-hexafluoro-2-propanol [23,24]. Fig. 2(b) shows a SEM micrograph of the chitosan/PVA blended electrospun fibers. A narrow fiber diameter distribution from 60 nm to 120 nm with an average diameter of 106 ± 27 nm was observed, though with some bead defects. Chitosan is a kind of natural cationic polyelectrolyte. The dilute acetic acid chitosan/PVA solution showed high conductivity and thus possessed high surface charge densities which tended to suppress the varicose instability and enhanced the whipping instability [25,26]. In the high electrical field the chitosan/PVA solution underwent a rapid growth of whipping instability and formed the ultrafine fibers with bead-on-string morphology.

PLGA–chitosan/PVA composite fibers were performed by simultaneously electrospinning of PLGA and chitosan/PVA solutions from two different syringes on the rotating drum. The SEM micrograph of PLGA–chitosan/PVA composite fibers is shown in Fig. 2(c). The composite fibers with average diameter of 275 ± 175 nm contained both smooth fibers with larger diameter and nanofibers with bead defect. The ECM is a component of all mammalian tissues and consists of a network of fibrous proteins such as collagens and elastin, embedded in a viscoelastic gel-like glycosaminoglycans (GAGs). In the electrospun PLGA–chitosan/PVA composite membrane, PLGA and chitosan/PVA nanofibers could serve as substitutes of collagen and GAGs.

PVA is a water-soluble polymer and chitosan possesses hydrophilic hydroxyl and amine groups, therefore the electrospun chitosan/PVA membrane could hardly maintain its morphology after soaking in PBS without crosslinking as shown in Fig. 3(c). On the contrary, the morphology of electrospun PLGA and PLGA–chitosan/PVA composite membranes had a little change as shown in Fig. 3, but the chitosan/PVA component swollen and bonded to a certain extent (Fig. 3(b)). Therefore, the electrospun membranes containing chitosan/PVA were further crosslinked by glutaraldehyde vapor from a 25% glutaraldehyde aqueous solution at 37 °C for 4 h to maintain its morphology and prevent it from dissolution. Fig. 4 presents SEM micrographs showing the morphology of crosslinked electrospun

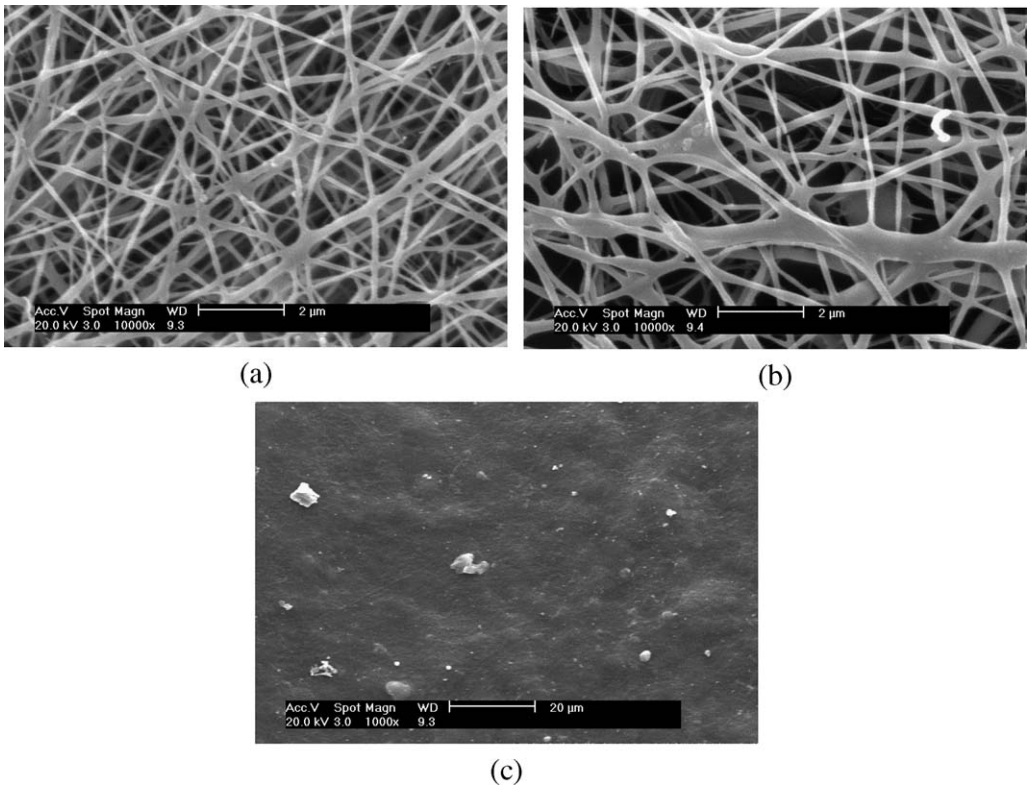

Fig. 3. SEM micrographs of electrospun PLGA (a), PLGA–chitosan/PVA (b) and chitosan/PVA (c) membranes after incubating in PBS solution at 37 °C for 24 h.

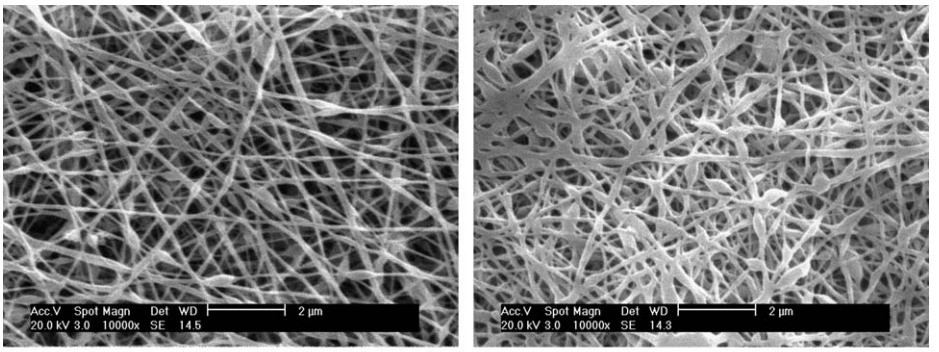

Fig. 4. SEM micrographs of crosslinked electrospun chitosan/PVA membranes before (a) and after (b) incubating in PBS at 37 °C for 24 h.

chitosan/PVA membranes before and after incubating in PBS at 37 °C for 24 h. It can be seen that crosslinked chitosan/PVA ultrafine fibers sustained the fiber structure and nanofibers were fused or bonded at their contact sites.

3.2. Shrinkage and PBS absorption

Optical images of the original and the shrunken membranes after incubating in PBS at 37 °C for 24 h are shown in Fig. 5. Zong et al. reported that the electropun PLGA (LA/GA = 75/25) membrane with a relatively lowered glass transition temperature (T_g), 38–54 °C, shrank dramatically, when the surrounding temperature was higher than T_g [27]. The macromolecular chains can rapidly relax to random coil state that causes a large dimensional change of the PLGA membrane. In this study, the T_g of PLGA (LA/GA = 80/20) is about 65 °C, much higher than the incubation temperature (37 °C). Therefore, the electrospun PLGA membrane exhibited a very small shrinkage (Table 1). Containing hydrophilic groups, chitosan/PVA was expected to swell in water and thus prevent the shrinkage of the electrospun membrane. However, electrospun chitosan/PVA membrane shrunk to 25.0% of its original size after immersing in PBS for 24 h, and 47.4% shrinkage was observed in the PLGA–chitosan/PVA composite membrane. It was probably because chitosan/PVA component was dissolved without crosslinking [28]. The extent of shrinkage in electrospun chitosan/PVA membranes decreased by 45.2% after treatment with glutaraldehyde vapor. Similarly, the electrospun PLGA–chitosan/PVA composite membrane also showed a small shrinkage (3.2%) after crosslinking.

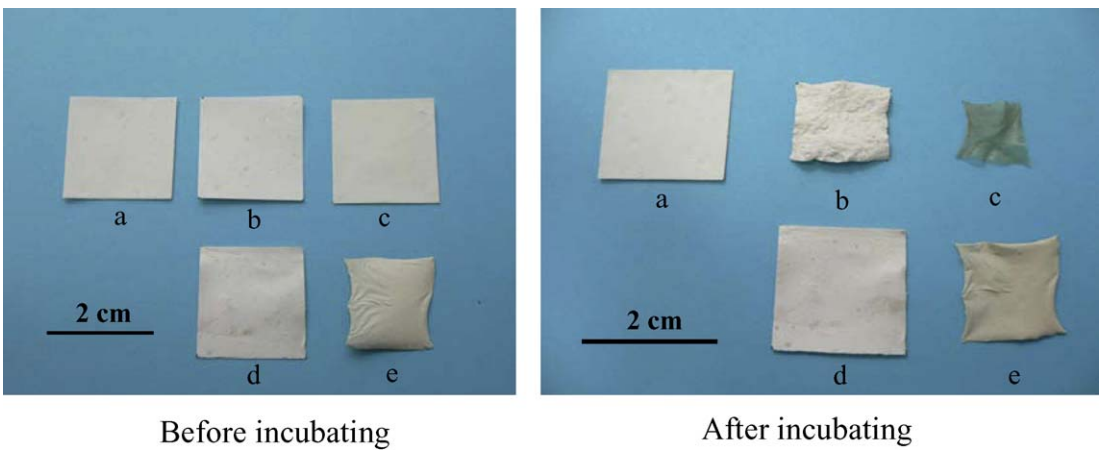

Fig. 5. Optical images of original and shrunken membranes after incubating in PBS at 37 °C for 24 h: (a) PLGA, (b) PLGA–chitosan/PVA, (c) crosslinked PLGA–chitosan/PVA, (d) crosslinked chitosan/PVA.

Table 1
Properties of electrospun membranes

Membrane samples	PBS absorption (%)	Shrinkage (%)	Tensile strength (MPa)	Tensile modulus (MPa)	Elongation (%)
PLGA	41.4 ± 10.9	2.1 ± 1.2	7.3 ± 1.5	419.1 ± 67.4	2.9 ± 0.5
PLGA–chitosan/PVA	218.8 ± 12.9	47.4 ± 1.3	2.6 ± 0.3	88.2 ± 10.6	5.6 ± 0.9
Crosslinked PLGA–chitosan/PVA	109.1 ± 10.9	3.2 ± 0.3	3.8 ± 0.4	106.2 ± 32.9	7.2 ± 1.3
Chitosan/PVA	328.7 ± 49.6	75.0 ± 3.5	4.3 ± 0.4	176.3 ± 26.9	4.3 ± 0.6
Crosslinked chitosan/PVA	146.2 ± 3.9	45.2 ± 6.0	3.1 ± 1.0	195.0 ± 25.7	2.2 ± 0.9

Table 1 also shows the PBS absorption of electrospun membranes. Due to the unique nanofiber morphology with high specific surface area and hydrophilicity, the electrospun chitosan/PVA membrane could absorb about 328.7% PBS after 24 h of incubation. After crosslinking, the PBS absorption of the chitosan/PVA membrane reached to 146.2%. In contrast, the electrospun PLGA membrane exhibited the lowest PBS absorption (41.4%) attributed to its hydrophobic nature. By blending with hydrophilic components, the nanofibrous composite membrane of PLGA–chitosan/PVA clearly increased its PBS uptake to 218.8% without crosslinking and 109.1% after crosslinking, respectively. The crosslinked composite membrane showed moderate PBS absorption and thus could not only absorb wastes of wound but also prevent dehydration.

3.3. Mechanical properties

Fig. 6 presents typical stress–strain curves of electrospun membranes and the detailed data are shown in Table 1. It was observed that electrospun PLGA membrane exhibited higher tensile strength, tensile modulus, and lower elongation than chitosan/PVA membranes. After crosslinking by 25% glutaraldehyde aqueous solution at 37 °C for 4 h, the electrospun chitosan/PVA membrane became brittle, and its ultimate tensile strain decreased from 4.3 ± 0.6 MPa to 2.2 ± 0.9 MPa. The result was similar to the effect of crosslinking on mechanical properties of chitosan/poly(vinyl pyrrolidone) and chitosan/collagen blends [29,30]. With respect to the electrospun PLGA–chitosan/PVA composite membrane, both tensile strength and tensile modulus had dramatically decreased in comparison with PLGA and chitosan/PVA membranes. It was probably because there was no interaction and bonded structure between PLGA and chitosan/PVA fibers where they were simply mixed together. However, the elongation of composite membrane significantly increased compared to the PLGA membrane attributed to the entanglement of PLGA with chitosan/PVA nanofibers. After crosslinking, the tensile strength and tensile modulus of electrospun PLGA–chitosan/PVA composite membrane increased to 3.8 ± 0.4 MPa and 106.2 ± 32.9 MPa, respectively, and the elongation increased correspondently due to the formation of bonded structure between PLGA and chitosan/PVA components. It was suggested that crosslinking could maintain the electrospun composite membrane mechanical properties.

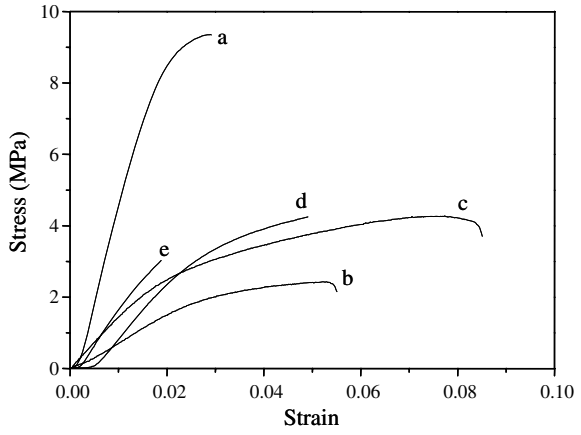

Fig. 6. Typical stress–strain curves of electrospun membranes of PLGA (a), PLGA–chitosan/PVA (b), PLGA–chitosan/PVA after crosslinking (c), chitosan/PVA (d) and chitosan/PVA after crosslinking (e).

3.4. Cell viability

The cell viability measured by MTT assay of fibroblasts cultured on different electrospun membranes within 7 days was shown in Fig. 7. The viability of fibroblasts cultured on each membrane increased on the fourth day compared with the first day, then followed by a decrease on the seventh day. It is possibly because cells may have occupied all available spaces on the electrospun membranes.

The ability of chitosan to enhance cell attachment and proliferation has been contradictory in

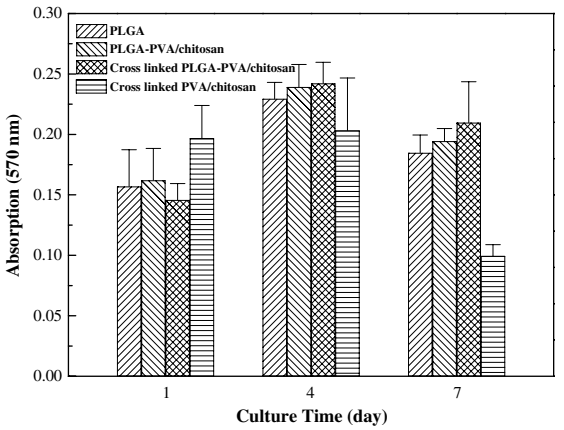

Fig. 7. Formazan absorption (A570 nm) in MTT assay was expressed as a measure of cell viability of fibroblasts seeded on electrospun PLGA, PLGA–chitosan/PVA, crosslinked PLGA–chitosan/PVA and crosslinked chitosan/PVA membranes.

both stimulatory [31] and inhibitory [32,33] effects. Yao [34] also reported that the fibroblast growth rate on the film of chitosan-L-lactic acid graft copolymer was faster than on chitosan. In this study, the cell viability of fibroblasts cultured on crosslinked chitosan/PVA membranes was obviously more than others at first day, but then became significantly less at seventh day. On the contrary, crosslinked PLGA–chitosan/PVA improved cell growth and proliferation after 7-day culture. MTT assay absorption in Fig. 7 suggested that the fibroblast viability on the electrospun PLGA–chitosan/PVA composite membranes, with or without crosslinking, tended to increase but with no significant differences in comparison to the electrospun PLGA and chitosan membranes.

3.5. Cell morphology

Fig. 8 represents the SEM micrographs of fibroblasts cultured for 4 days on different electrospun membranes. It can be seen that fibroblasts attached on all the membranes and changed their original round shape to elongated and spindle-like shape on electrospun PLGA, PLGA–chitosan/PVA and crosslinked PLGA–chitosan/PVA membranes. However, on crosslinked chitosan/PVA membranes,

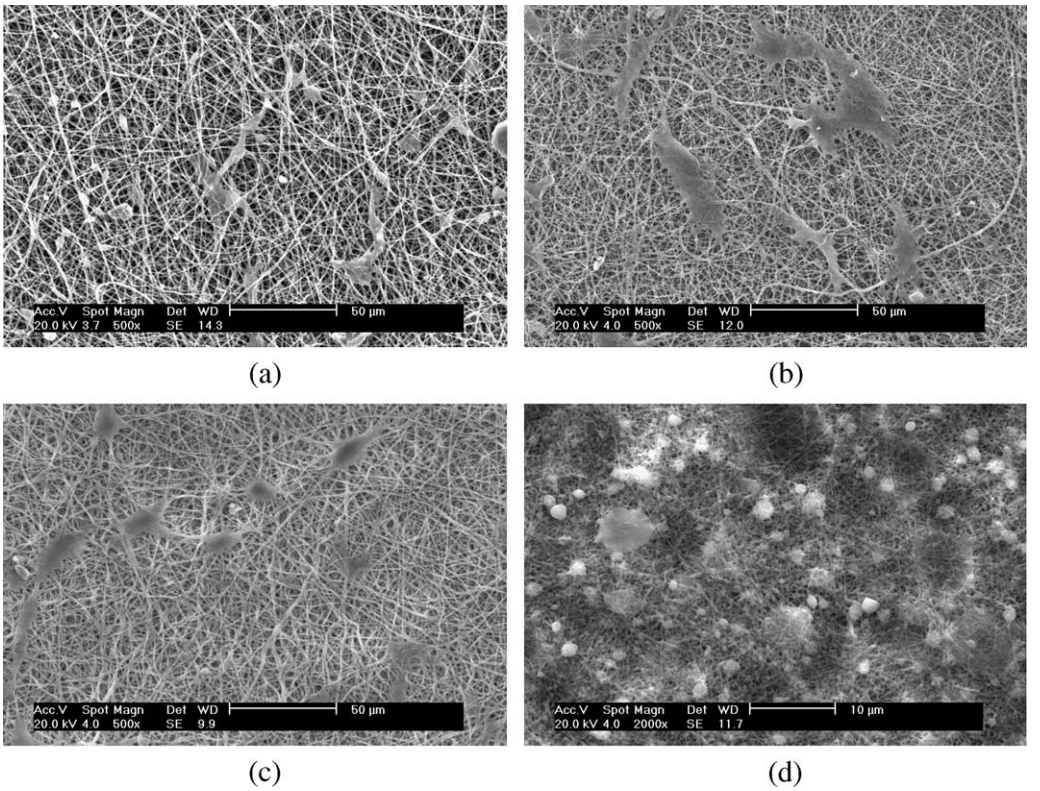

Fig. 8. SEM micrographs of fibroblasts seeded on electrospun PLGA (a), PLGA–chitosan/PVA (b), crosslinked PLGA–chitosan/PVA (c) and crosslinked chitosan/PVA (d) membranes after 4 days of culture.

cells kept in a round shape shown little activity. These results were roughly in accordance with that of cell viability by MTT assay. It has been reported that cells recognize nanometric topologies of fibrous or microporous structure [35]. For electrospun fibers, the decrease in the fiber diameter decreased porosity, but increased fiber density. Kwon et al. [36] studied the cell behavior on electrospun poly(L-lactide-co-ε-caprolactone) (PLCL, 50/50) membranes with different fiber diameter. The results showed that cells were adhered well and proliferated on the small-diameter-fibers with relatively low porosity and high fiber density, whereas reduced cell adhesion and restricted cell spreading were observed in the large-diameter fibers. It was probably because of the large interfiber distance or a very low surface density of fibers, which did not permit cell adhesion across the neighbor fibers. In this study, the composite membranes with average diameter about 275 nm had dense fibric surface and thus enabled the adhesion and proliferation of fibroblasts.

Electrospun nanofibrous membranes provided a high level of specific surface area and pore density for cells to attach and proliferate. SEM micrographs showed that fibroblasts seeded on electrospun PLGA, PLGA–chitosan/PVA and crosslinked PLGA–chitosan/PVA membranes could integrate with the surrounding fibers to form a three-dimensional cellular network. The cells spread along the direction of fibers and filopodium-like extension could be seen on both crosslinked PLGA–chitosan/PVA membranes and those without crosslinking. Therefore, the electrospun composite membranes could mimic the natural ECM and positively promote cell–matrix and cell–cell interactions.

4. Conclusions

PLGA and chitosan/PVA were simultaneously electrospun from different syringes and mixed on the rotating drum to form the PLGA–chitosan/PVA nanofibrous composite membrane which contained both smooth PLGA fibers with larger diameter and chitosan/PVA nanofibers with bead defect. After crosslinking by glutaraldehyde vapor at 37 °C for 4 h, the chitosan/PVA and composite membranes were bonded at their contact sites and thus could maintain mechanical properties and fiber morphology when incubating in PBS for 24 h. PBS absorption of composite membranes, no matter crosslinking or not, was higher than electrospun PLGA membrane due to the introduction of chitosan/PVA components. The crosslinked composite membrane had a little shrinkage after incubation in PBS for 24 h and possessed moderate tensile strength and tensile modulus and larger elongation. It was found that the electrospun PLGA–chitosan/PVA membranes tended to promote the fibroblast attachment and proliferation. Favorable interaction between cell–cell and cell–matrix was demonstrated by cell morphology. It was assumed that the electrospun PLGA–chitosan/PVA composite membranes combined with the advantages of both PLGA and chitosan would have a great potential in the application of skin tissue engineering.

Acknowledgements

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