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Silk fibroin composite membranes for application in corneal regeneration

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ABSTRACT: The ordered microstructure of the corneal stroma determines the transparency of the cornea. The difficulty of constructing three-dimensional corneal tissue mainly lies in the reconstruction of the corneal stroma. This article reports propionamide/silk fibroin composite membrane materials for use in corneal regeneration. X-ray diffraction is used to explore the structure of the composite fibroin membrane. Propionamide acts as a crosslinking agent and inhibits the formation of larger crystal grains controlling the crystallization process. Corneal stromal cells are seeded on sterilized composite films. Propionamide/fibroin membranes with different blending proportions exhibit stable transparency and good cell compatibility. The results demonstrate that composite fibroin membranes are suitable potential materials for use in corneal stromal cell proliferation and repair. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42407.

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INTRODUCTION

Corneal blindness is a serious worldwide public health, social, and economic problem. Due to the limited cornea donors, artificial corneas are a reliable and effective alternative for corneal repairs, replacing traditional allergenic human corneal transplantation. At present, the various polymeric materials for preparing the artificial cornea induce strong immune rejection responses. Finding a new biologically engineered tissue material for the development of artificial corneas is the emphasis of this research. Because of the cornea's unique physiological structure and function there is a demand for a new biological material exhibiting important and relevant optical, mechanical and organizationally compatible characteristics as well as other aspects required for its functionality and compatibility.

In corneal transplantation, the corneal epithelium cells are completely replaced by the corneal limbal stem cells of the receptor. The endothelial cells are absorbed and the stromal cells survive for a long time. When the cornea is damaged, the corneal stromal cells convert to fibroblasts to achieve corneal damage repair.[1] The corneal stromal cells play a vital role in the process of repairing cornea. The corneal hierarchical microstructure is principally responsible for the optical transparency and biomechanical properties.[2,3] Necessary characteristics of a corneal stromal cell carrier are biocompatibility and stable transparency. Silk proteins are polymers in structure and functionality.[4] Silk fibroins (SF, purchased from Soho Biotechnology) are obtained from the silkworm cocoon and are widely used as biomaterial matrices for tissue engineering, regenerative medicine, and drug delivery owing to their good biocompatibility,[5] tuneable mechanical properties,[6,7] and low immunogenicity.[8] SF can be engineered to exhibit a water-soluble or water-insoluble structure with the use of a crosslinking agent.[9] SF is used as a cornea repair biomaterial.[10] Human corneal fibroblasts grow on porous membranes and proliferate. It is expected that silk protein can be used to repair damaged human corneas.[11]

The degradation rate of biological material should be consistent with tissue repair rate. Regenerated SF is more susceptible to degradation than the fiber.[12] Degradation rate of SF is related to the secondary structure of the SF.[13] The β-sheet structure has an effect on degradation rate.[14] The SF is difficult to be degraded with the β-sheet structure percentage increase. As a protein, SF is digested easily by enzyme.[15] In general, the degradation process is divided into two steps, first of all, the enzyme was adsorbed on the surface of SF by surface binding region, and second, the hydrolysis of the ester bond. SF degradation products are amino acids that can be absorbed by organism. More importantly, β-sheet structure can be sufficiently degraded and degradation products are nontoxic.[16] In vitro degradation

experiments, different enzyme will produce different degradation behavior for SF. Li *et al.*[15] found the magic proteases can degrade the amorphous region of SF material, and finally get highly crystalline structure. In contrast, other enzymes (especially proteases XIV) are able to degrade crystalline region.[16] In vitro degradation experiments, protease XIV derived from *Streptomyces griseus* is the most widely used degrading enzymes, followed by α-chymotrypsin derived from bovine pancreas.[17–19]

For the successful design of a corneal stromal carrier, the transparency stability, strength, flexibility, and ability to sustain cell adhesion and proliferation must be taken into consideration. A pure SF membrane is water-soluble and brittle, which are two characteristics that limit the applications of pure SF in the field of biological materials.[20,21] Silk/cellulose blend membranes demonstrate increased mechanical strength compared with pure silk membranes,[22] but the stability of the light transmittance is unknown. SF membranes treated with 75% ethanol are brittle and take time to degrade. They are usually unsuitable for practical use and their applications are limited. Hence, improving the transparency properties of SFs and broadening the potential applications of a propionamide/SF (PRO/SF) membrane as a biomedical material through the addition of a crosslinking agent may be effective and promising research avenues.[23,24]

PRO is an amide. Amide can be seen as carboxylic acid derivative formed by carboxylic acid with ammonia or amine compounding. Amide molecules containing a carbonyl group and an amine group are capable of forming hydrogen bonds between their molecules. The 3-mercapto propionamide has been used in antihypersensitive medicine.[25] Amides are the main organic chemistry functional group of protein.[26] It may form critical juncture in natural polymer such as protein and synthetic polymer. Amides carboxylic acid hybrids have an important influence on adjusting order and disorder of proteins molecules.[27] SF, a polyamide with the similar functional group of PRO, is expected to carry out better compatibility with PRO to obtain blend membranes with excellent properties.

In this article, blend films are fabricated by mixing SFs with PRO in different proportions to obtain a fibroin membrane that is water-insoluble and stably transparent. Rabbit corneal stromal cells are used to evaluate the biocompatibility of the PRO/fibroin blend membrane. The results indicate that the blend membrane may potentially be used as a biomaterial for corneal regeneration and repair.

MATERIALS AND METHODS

Preparation of SF Solution

Raw mulberry silk (Soho Biotechnology) obtained from fresh cocoons of *Bombyx mari* was boiled once in a 0.075 wt % sodium carbonate solution at 100°C for 30 min. It was then treated twice with a 0.05 wt % sodium carbonate solution at 100°C for 30 min. Next, the silk material was thoroughly washed with distilled water and dried at 60°C. A silk solution (approximately 4% wt/wt) was obtained by dissolving the silk in a 9.3*M* LiBr solution at 60°C for 1 h. It was then dialyzed in cellulose tubes against deionized water for 3 days until the salt was completely removed. The SF solution was filtered using medical absorbent cotton and centrifuged at 3000 rpm for 10 min. The supernatant was collected at 4°C. The concentration of the final silk solution was determined by gravimetric analysis.

Preparation of PRO/SF Membranes

The concentration of the PRO solution was 10 wt %. PRO/SF solutions with different weight ratios (0 : 10, 1 : 10, 2 : 10, 3 : 10, 4 : 10, 5 : 10, and 6 : 10) were prepared in the same solvent system. They were dried (temperature: 25°C, humidity: 65%) to obtain transparent water-insoluble SF membranes.

PRO/SF Composite Membrane Dissolution Loss Rate

The composite membranes were equilibrated for 24 h at a constant temperature and humidity (temperature: 25°C, humidity: 65%). The membranes (0.1 g) were put into tubes, and distilled water was added at a bath ratio of 1 : 100. The contents were incubated in a water-bathing constant-temperature vibrator at 37°C for 24 h. The absorbance (A) of the supernatant liquid was tested using an ultraviolet spectrophotometer at 278 nm. The weight loss of silk (D) in the blend membrane was determined using Eq. (1):

$$D = (K \times A \times V/m) \times 100\%, \quad (1)$$

where K is the ultraviolet absorption light constant of the SF solution ($K = 1.10$), V is the volume of the solution (mL), and m is the weight of the sample (g).

Structural of the Composite Membranes

An automatic X'PERT PRO MPD-ray diffractometer (XRD) was used to obtain diffraction curves. The diffraction data were recorded for 2θ ranging from 5° to 45°. Other parameters taken into consideration were tube current (35 mA), voltage (40 kV), and scan speed (10°/min).

Light Transmittance Test

PRO/SF (1/10 w/w) composite membranes were placed in the bottom of 24-well plates. A Multiscan spectrophotometer (BIOTEK SYNERGY) was used to measure the absorbance values (A) at 492 nm, 550 nm, and 700 nm. For each mixture ratio, the membrane absorbance was recorded four times to calculate the average. The absorbance of the PRO/SF (1/10 w/w) fibroin membrane immersed in a 0.9% sodium chloride solution for 9 weeks was measured. The transmittance was calculated according to the following equation:

$$T = 1/10^{A} \times 100\%. \quad (2)$$

Mechanical Properties of PRO/SF Membranes

A US Instron3365 testing machine at a tensile speed of 20 mm/min and a clip distance of 28 mm was used to measure the strength and elongation at the break of the composite membranes. The strips were pressed out with a type 3 knife according to the GB/T1040-2006 protocol. The membranes were soaked in physiological saline for 24 h. They were removed and weighed on blotting paper before the breaking strength was measured and the elongation of the wet membrane was performed.

In Vitro Biodegradation of PRO/SF Composite Membranes

Each membrane (100 mg) was placed separately into a tube containing 10 mL of a phosphate buffered saline (PBS) solution with Protease XIV (pH 7.4, 37°C, 2 U/mL). A similar sample

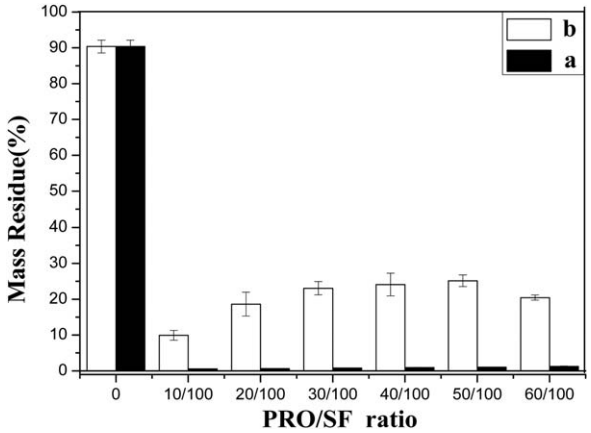

Figure 1. Solubility of the blend films. (a) The rate of protein loss and (b) the rate of mass loss.

was kept in PBS as a control. The protease XIV solution was replaced daily with freshly prepared solution. The membranes were collected at a predetermined time, and the excess solution was removed from the surface using a tissue.

Cell Culture

The PRO/SF blend solution (80 μL/100 μL) was placed in 96-well plates/24-well plates and blown dry. The dry composite membranes (in 96-/24-well plates: diameter: 6/16 mm, thickness: 40/40 μm) were soaked for 3 days with deionized water and sterilized by Co^{60}. The rabbit corneal stromal cells at four to six generations were augmented in a culture medium comprising 90% Dulbecco's modified eagle medium (DMEM)/F12, 10% foetal bovine serum, 100 U/mL penicillin, and 1000 U/mL streptomycin and were seeded on the membranes. The cells were cultivated at 37°C in a carbon dioxide cell incubator with 95% air, 5% CO_2, and 95% humidity. An aliquot of 0.1 mL cells (1×10^5 cells/mL) was seeded onto the surface of the pre-wet membrane in each well. To detect the cell adhesion rate at different times (1 h, 3 h, and 5 h), the cells were seeded on silk membranes that were precast in 24-well plates with 30,000 cells per well in 1.5 mL of complete medium. Alamar blue was added to the silk membranes that were precast in 96-well plates with 4000 cells per well in 0.2 mL of complete medium to measure the fluorescence values at different times (1, 3, 5 and 7 days).

RESULTS AND DISCUSSION

Dissolution Loss Rate of PRO/SF Composite Membrane

The pure SF films were dissolved in water, which limited their application in vivo (Figure 1). The addition of PRO modified the water-soluble SF films to form water-insoluble SF films. The protein loss rate of the PRO/SF films was reduced to 2% or less. When the ratio of PRO/SF was 1/10, the mass loss rate was 9.9%. When the ratios of PRO/SF were 2 : 10 and 3 : 10, the PRO still precipitated out and remained a suitable carrier material. Swelling mirrored the material's water-holding capacity and the interaction of water molecules with the SF surface and the interior region of the films.[28] Crosslinking affected the water absorption properties of the material through a variety of complex and often interrelated processes.[29] The expected swelling characteristic of the material indicated good water holding capacity, which assisted in cell adhesion and nutrients transmission from the surroundings. The range of the degree of swelling of the PRO/SF membranes was between 20% and 30%.

Transparency of SF Composite Membrane

After cornea damaged or missing, a high transparency material can be more conducive to the corneal regeneration and maintenance of normal function.[30,31] A suitable cornea material requires high material strength, biodegradability, biocompatibility, and long-term transparency. To obtain the best visual acuity, cornea must efficiently transmit incident light by maintaining its transparency.[32] The transmittance of the composite membrane in the dry state was approximately 90% [Figure 2(A)]. With an increase in PRO, there was no significant change observed. In wet state, when the ratio of PRO/SF was 5/10, the excessive PRO made SF molecule slight assemble resulting in the size of the crystalline grains increasing. Transmittance of SF membranes decreased slightly. The crystallization peak at 9.1° became a little sharp indicating that there was an increasing size of crystalline particles (Figure 3). When PRO was increased to 60% of SF, the role of PRO between molecules weakened cohesion of PRO with fibroin molecules. So, the transmittance of PRO/SF composite membrane appeared increasing. Overall, the luminousness of the SF composite membrane in the wet state was above 90%. The SF composite films in the wet state exhibited optical stability and remained transparent, which might be attributed to the stability of the interspace structure of the fibroin molecules with the addition of PRO. Although the SF composite films had crystalline domains, the crystal grain size was less than the wavelength of visible light and thus did not cause diffraction or scattering of light. PRO/SF membranes soaked in the saline solution for 9 weeks remained transparent for the duration of soaking [Figure 2(B)]. There was no sign of blanching from the first week to the ninth week indicating that the crystallinity of the composite films did not increase and the film structure did not transform into a new crystalline structure. The blend membranes demonstrated stable transparency up to 9 weeks and after. The luminousness of cornea stromal was $87.1 \pm 2.0\%$.[33] Corneal cells -films implanted after 30 days, the corneal cells completely covered materials and secreted basement collagen.[34] So the material should maintain transparency before it was covered by cells. The stabilized transparency of the composite films provided the preconditions necessary to be considered as a potential corneal cell carrier material.

The Structure of PRO/SF Composite Membranes

In previous studies, two SF conformations have been identified by XRD. The principal diffraction peaks of silk I crystal structure are exhibited at 12.2°, 19.7°, 24.7°, 28.2°, 32.3°, 36.8°, 40.1°.[35] Silk II diffraction peaks can be seen at 9.1°, 18.9°, 20.7°, and 24.3°.[36] The pure SF had random coil (amorphous) structure (Figure 3). When proportions of PRO/SF were 1/10 or 2/10, the silk I crystal structure appeared at 12.2°, 19.6°, and 24.7°. When the ratios of PRO/SF were 3/10 or 4/10, the crystallization peak at 12.2° decreased. PRO may crosslink SF further to transform the crystalline regions into amorphous regions and inhibiting crystallization of the SF. When PRO accounted for 50% or 60%, the silk I crystalline peak of the SF composite

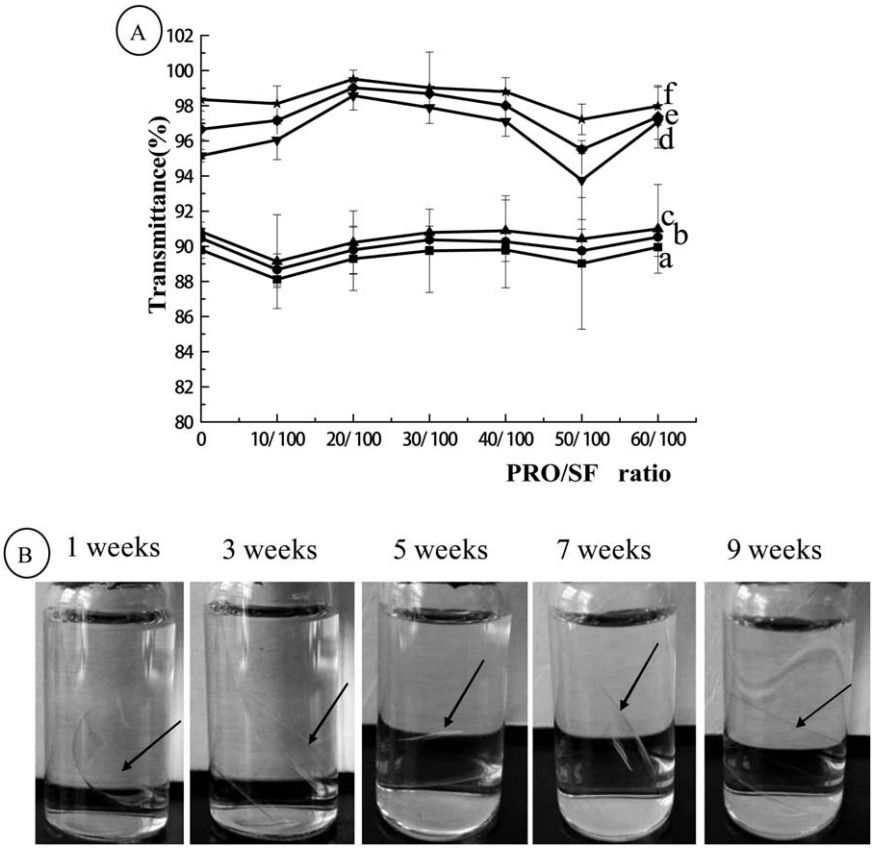

Figure 2. The transparent of SF composite membrane. (A): The transmittance of silk films. a, 492 nm; b, 550 nm; c, 700 nm in dry state; d, 492 nm; e, 550 nm; f, 700 nm in wet state. The pure SF film was treated with 75% ethanol. (B): Transparent of SF composite membrane. from left to right in order: 1 week; 3 weeks; 5 weeks; 7 weeks; 9 weeks. Arrows indicate the films.

membrane decreased and a new silk II crystalline peak appeared at 9.1°. The segmental motion of macromolecules formed a new crystalline region (with the addition of PRO). Therefore, with the content of PRO increasing, the crystalline structure of the composite film changed from silk I to silk I coexist with silk II, silk II crystal structure and more silk II crystal structure. PRO changed the spatial structure of the SF molecules to obtain a composite membrane with partial crystallinity. The SF molecules formed crystalline particles with smaller grain area when PRO was added. Crystalline regions inhibited water molecules from permeating into the material and thereby prevented the binding of fibroin molecules with water molecules resulting in the water-insoluble characteristic of the PRO/SF composite membranes.

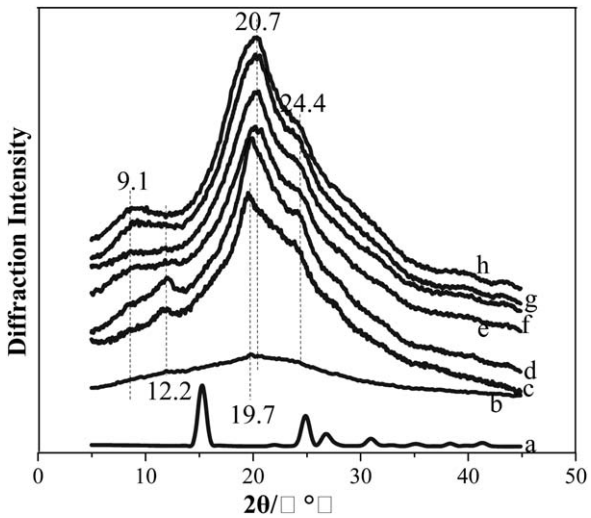

Figure 3. XRD curve of silk membranes, a, PRO; b–h, PRO/fibroin = 0/10–6/10.

Mechanical Properties of the Composite Membrane

The addition of PRO improved the mechanical properties of the pure SF membrane by increasing its flexibility and breaking strength. With the addition of different amounts of PRO, the breaking strength of the PRO/SF composite membrane decreased initially (Figure 4), then increased and decreased again. This was due to the influence of protein-amide-induced molecular motion. When PRO comprised 30% or 40% of the composite film, the breaking strength of the film increased. The above analysis indicated that the PRO with fibroin molecules achieved greater crosslinking, and the SF molecule mobility was reduced. When PRO accounted for 50% or 60%, the SF composite membrane formed a new crystal structure. This was due to an excessive amount of PRO enhancing crosslinking thus

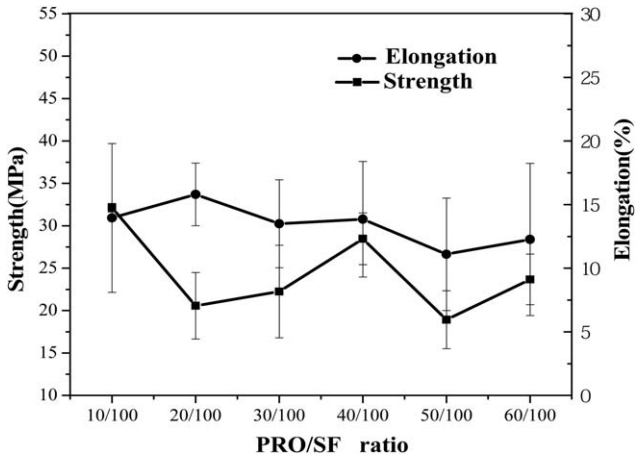

Figure 4. The strength of the silk blend films (wet state).

making the distance between the SF molecules shorter. Additionally, the blend membranes produced no significant change in elongation with the change in PRO amount. Therefore, PRO did not introduce any obvious changes in the flexibility of the SF molecules. The breaking strength of the human cornea is 3.81 ± 0.41 MPa;[24] therefore, PRO fibroin composite membranes are a suitable option as an alternative corneal cell carrier material.

Biodegradability of the Composite Membranes

Biodegradation is a necessary requirement for any biological material used for corneal repair. After 7 days, the PRO/SF composite membrane was subjected to treatment in protease XIV solution. Following 7 days of the protease XIV treatment, the composite membranes exhibited 40% degradation (Figure 5). The remaining quantity of the pure SF film (in protease XIV solution treated with 75% ethyl alcohol) was approximately 65%. The change in the silk film in the PBS solution was negligible in comparison to the membranes in the protease XIV solution. The PRO/SF film demonstrated the ability to biodegrade and exhibited a faster degradation rate than the pure silk film mentioned above.

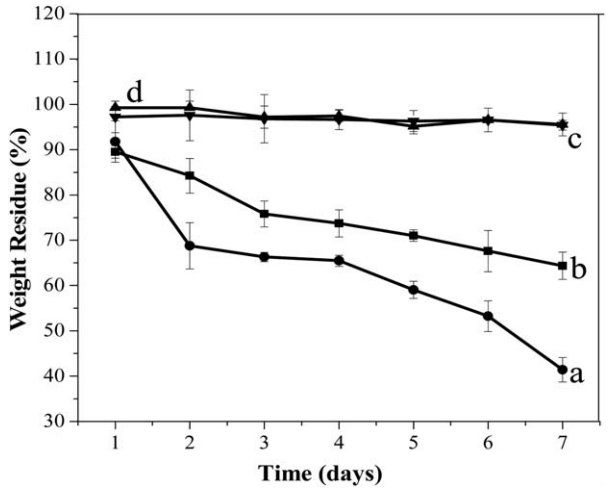

Figure 5. The degradation of the composite membrane. a, PRO/SF (PRO/SF) (Protease XIV); b, SF (Protease XIV); c, PRO/SF (PBS); d, SF (PBS).

Table I. The Adhesion Rate of the Corneal Stromal Cell on Different Materials (%)

Materials	1 h	3 h	5 h
PRO/SF (w/w) = 1/10	85.38 ± 11.09	86.00 ± 3.14	94.45 ± 6.71
Pure SF	89.17 ± 10.89	89.18 ± 7.49	92.46 ± 7.84
96-well plates	90.18 ± 1.85	98.49 ± 1.45	100.00 ± 1.00

Cell Culture and Morphology on the Composite Membranes

The adhesion rate of PRO/SF (w/w = 1/10) membrane exceeded 80% after 1 h and then gradually increased (Table I). The cell adhesion rate on the blend membrane was greater than 90% after 5 h. The corneal stromal cells adhered well to the PRO/SF membrane.

The fluorescence value of the cells seeded on the PRO/SF membrane was approximately 2000 after 1 day (Figure 6). There was no significant difference observed between PRO/SF membranes and pure silk films on the seventh day, and there were no obvious differences when compared with the control group. The PRO/SF membranes supported the growth of corneal stromal cells. The stromal corneal cells planted on the membranes started proliferating on the first day, and, on the third day, the cells seeded on the PRO/SF membranes began to proliferate faster. The long strips of corneal stromal cells seeded on the pure silk films and in the 96-well plates became regular on the fifth day (Figure 7).

So far, there have been many studies on corneal tissue repair. Lawrence *et al.*[37] had grown cornea limbal epithelial cells on SF membrane surface engraved parallel lines and ring. The results showed that epithelial cells had better adhesion, proliferation, and control the alignment of cells. Madden *et al.*[38] studied SF film using vacuum treatment in high humidity environments to improve transparency. Human corneal endothelial cells could proliferate on the water-insoluble SF films. Lai[39] cultured corneal stromal cells on hyaluronic acid-modified gelatin

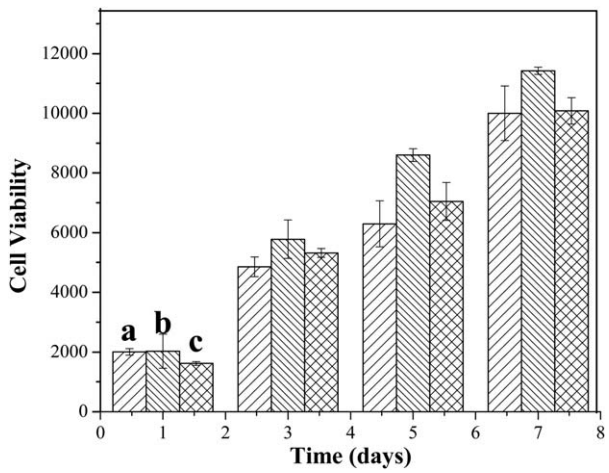

Figure 6. The activity of the corneal stromal cells. (a) Cells on pure silk films; (b) cells in 96-well plates; (c) cells on PRO/SF membrane.

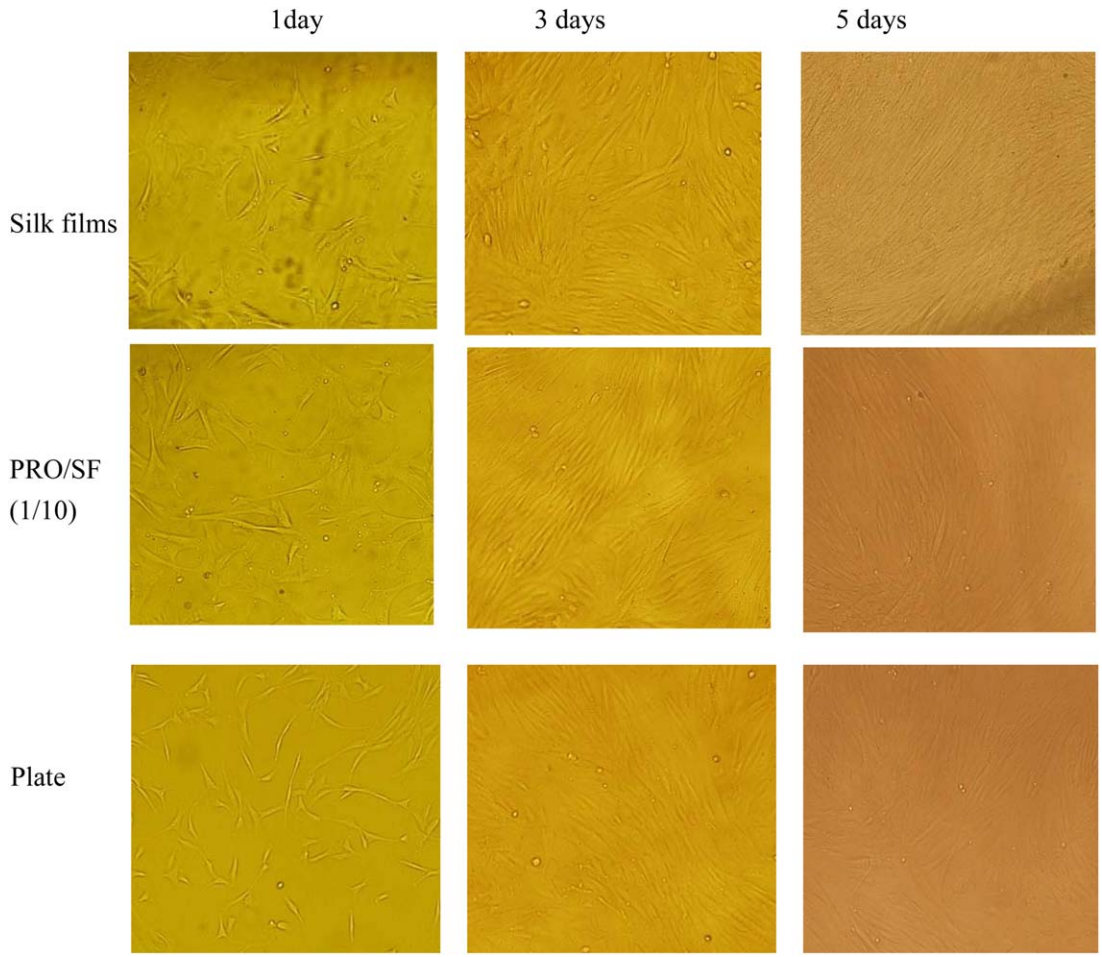

Figure 7. The cell morphology grown on the membranes and in the tissue culture plate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

microcarriers and results showed that hyaluronic acid modified gelatin carrier could effectively support the growth of stromal cells and increase their collagen synthesis. Zhang *et al.*[40] seeded rabbit corneal stromal cells on porcine cornea then transplanted corneal. Renewable corneal nerves were observed, cornea remained clear, and no immune rejection after 8–10 weeks. As for our films, the preparation of PRO/SF films is convenient and simple. Besides, the composite membranes are easy to operate and have a variety of excellent performance especially for the long time transparency.

CONCLUSION

SF composite membranes are fabricated by adding PRO to pure silk films. The blend materials show good cell compatibility, steady optical transparency, appropriate mechanical properties, and good cell growth. The PRO/silk composite membrane characteristics indicate that the blend materials may be suitable potential materials for use in corneal regeneration and repair applications.

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