Preparation of Insoluble Fibroin/Collagen Films Without Methanol Treatment and the Increase of Its Flexibility and Cytocompatibility

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ABSTRACT: It is still a critical challenge to increase the flexibility of regenerated fibroin materials in dry and near dry states. In this study, a novel biocompatible and water-stable film composed of fibroin and collagen was successfully prepared from aqueous fibroin solution without methanol treatment. The result of contact angle measurement indicates that hydrophilicity is evidently increased when collagen was added to fibroin film. The elongation at break in wet state is also increased because of the blending of collagen, which implied the improvement of flexibility. More importantly, the blend films containing 20% collagen become flexible when placed at above 65% humidity in atmosphere. It means that the blend films could be fabricated to different conformations easily through adjusting humidity in atmosphere. HepG2 cells were cultured on fibroin and fibroin/collagen films to investigate the cytocompatibility of these films. Scanning electron microscopy and MTT analysis demonstrated that the adding of collagen evidently improved HepG2 proliferation in over 10 days culture. The excellent cytocompatibility, the flexibility in the near dry state as well as the green preparation process of fibroin/collagen blend films make them become the promising biomaterials for different medical applications. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 1577–1584, 2008

Key words: fibroin; collagen; flexibility; biocompatibility

INTRODUCTION

Silk fibroin is the protein that forms the filaments of silkworm and has been recognized as a potential new biomaterial because of its unique mechanical properties as well as its biodegradability and biocompatibility.[1–3] Many researchers have extensively studied the influences of casting temperature,[4,5] drying rate,[6] solvent,[7] and heat treatment[8,9] on the molecular conformation; and the insoluble fibroin materials have been prepared through methanol treatment.[10,11] However, the brittleness of fibroin in dry state is still a thorny problem, which makes it difficult to prepare different fibroin configurations. Many other polymers such as poly(ethylene glycol),[12,13] polyacrylamide,[14] poly(vinyl alcohol),[15,16] cellulose,[17] sodium alginate,[18] and chitosan[19] have been blended with fibroin to increase fibroin mechanical properties in dry state, but it is still a challenge to obtain synchronously excellent biocompatibility and mechanical properties in dry state.

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Collagen is one of the most excellent biomaterials used in tissue engineering, but its fast biodegradation rate and low mechanical strength cannot match the demand of *in vitro* and *in vivo* applications. Considering the hydrophilicity and biocompatibility of collagen, the fibroin/collagen blend materials having both excellent biocompatibility and mechanical properties may be obtained through controlling different preparation conditions such as concentration, pH, temperature, and so on. Cirillo et al.[20] ever prepared fibroin/collagen blend films and investigated adhesion and function of rat liver cells adherent on the blend films; however, the fibroin/collagen films were obtained from acidic conditions, which might decrease the mechanical properties. On the other hand, methanol, a poisonous solvent, is also not suitable to be used in biomaterial preparation process.

In our previous studies, insoluble fibroin films have been directly prepared in aqueous solution without methanol treatment.[21–23] Moreover, many other bioactive molecules such as heparin have been added into fibroin solution, and then fibroin/heparin blend films, having blood compatibility and biocompatibility, were prepared in mild conditions.[24] In this study, we prepared the insoluble fibroin/collagen blend films from near neutral solution without methanol treatment to obtain the flexible films with excellent cytocompatibility in near dry state. By adjusting collagen content, the

excellent cytocompatibility was preserved, and the elasticity of the fibroin/collagen blend films was really improved. *In vitro* HepG2 cell culture was also carried out to assess the cytocompatibility of the blend films.

EXPERIMENTAL

Materials

Bombyx mori silkworm silk was purchased from Yi Xian raw silk factory in China. Bovine collagen type I gel (collagen content 1%) was supplied by Medical and Health Biological Company in Beijing, China. HepG2 was obtained from martial and medical science academy. The Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FCS) were purchased from HyClone Laboratories, Inc., Logan Utah. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), L-glutamine, sodium hydrogen carbonate, penicillin-streptomycin, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO).

Preparation of regenerated fibroin solution

B. mori silk fibroin was prepared just as described in our earlier procedure.[21] Silk was boiled for 1 h in an aqueous solution of 0.5 wt % Na_2CO_3 and then rinsed thoroughly with distilled water to extract the sericin proteins. The degummed silk was dissolved in $CaCl_2$/H_2O/CH_3CH_2OH solution (mole ratio, 1/8/2) at 80°C. Then, the fibroin solution was filtered and dialyzed against distilled water for 3 days to yield fibroin water solution. The final concentration of the aqueous silk fibroin solution was about 4%, which was determined by weighing the remaining solid after drying.

Preparation of blend films of fibroin and collagen

Various silk blends in water were prepared by adding different ratio of collagen gel into fibroin aqueous solutions. Through adjusting the content of fibroin solution and collagen gel, the blending ratios of pure silk fibroin and collagen were 100/0, 90/10, and 80/20, respectively. When heated up to 50–60°C with mildly stirring, the collagen gel dissolved in fibroin solutions. The pH value of the solution was about 6, and then was adjusted to 7 through adding 1 mol/L sodium hydroxide. The aqueous solution of fibroin and collagen was concentrated at 50–60°C with mildly stirring until fibroin concentration was up to 4%. Then, the blends was cast on polystyrene petri dishes and dried at different temperatures for the preparation of insoluble blend films. The drying time of all samples was controlled in about 24 h through adjusting the drying rate.

Characterization

The infrared spectra of silk fibroin and silk fibroin/collagen blend films were measured with an ATR-FTIR (NICOLET 560, American) spectrophotometer. Each spectrum was acquired in transmittance mode by accumulation of 256 scans with a resolution of 4 cm^{-1} and a spectral range of 4000–400 cm^{-1}.

Fibroin/collagen blend films were sputter coated with gold. The morphology of the films was observed with JEOL JSM-6460LV SEM (Japan).

The contact angle, using Millipore purified water droplet, was measured to determine surface hydrophilicity. The water droplet was applied using a syringe and 22-guage needle, and the static contact angle was measured using a JY-82 goniometer (ChengDe, China). With each specimen, the measurement was repeated five times at different sites, and average values were obtained for contact angle.

Mechanical properties of blend films

The tensile strength and elongation at break in wet state was measured by using an Instron 6022 machine after the samples soaked in water for over 24 h and then removed the excess water on the surface. The experimental conditions were as follows: extension rate: 10 mm/min; and dimension of the sample: $4 \times 15 \times 0.15$ mm^3.

Cell culture

HepG2 cells were maintained in DMEM medium and supplemented with 10% fetal bovine serum, 200 m*M* L-glutamine, 2 mg of sodium hydrogen carbonate/mL, and 100 mg penicillin-streptomycin/mL. Then, cells were cultured in 37.5 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. Confluent monolayers were split by treatment with sterile phosphate-buffered saline (PBS) and 0.05% trypsin/EDTA solution, and the culture medium was replaced every 3 days.

Samples of fibroin and fibroin/collagen blend films were cut into circular discs suitably sized (diameter 14 mm, height 3 mm) for 24-well tissue culture plate wells. The circular matrices were sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively three times with sterile PBS. Before cell culturing, scaffolds were prewetted by immersion in DMEM for 12 h in the 37°C incubator.

Cells were trypsinized, counted, and plated at a density of 2×10^5 cells/cm^2 onto the surface of prewetted matrices that were placed in 24-well culture plates precoated with 0.3% poly-HEMA to prevent cell attachment to the tissue culture polystyrene surface. SEM was used to determine cell morphology seeded on fibroin and fibroin/collagen blend films. Following harvest for 3 and 9 days, seeded fibroin and fibroin/collagen blend films were immediately rinsed in 0.2*M* sodium cacodylate buffer, fixed in Karnovsky fixative (2.5% glutaraldehyde in 0.1*M* sodium cacodylate) overnight at 4°C. Fixed samples

TABLE I
Effect of Preparation Conditions on Water-Stability of Blend Films

Samples	Preparation conditions	Water-stability
1	Fibroin concentration 4%, drying temperature 70°C, collagen content 0%	Water stable
2	Fibroin concentration 4%, drying temperature 50°C, collagen content 10%	Water soluble
3	Fibroin concentration 4%, drying temperature 70°C, collagen content 10%	Water stable
4	Fibroin concentration 2%, drying temperature 70°C, collagen content 10%	Water stable
5	Fibroin concentration 4%, drying temperature 50°C, collagen content 20%	Water soluble
6	Fibroin concentration 4%, drying temperature 70°C, collagen content 20%	Water stable
7	Fibroin concentration 2%, drying temperature 70°C, collagen content 20%	Water soluble

were dehydrated through exposure to a gradient of alcohol and allowed to air dry in a fume food. After sputter-coated with gold, samples were examined with a scanning electron microscope (SEM, JSM-35C, JEOL, Japan).

MTT assay

MTT assay is a quantitative colorimetric assay for mammalian cell survival and cell proliferation. It is an indirect method for assessing cell growth and proliferation, since mitochondria oxidize the MTT solution, giving a typical blue-violet end-product. O.D. value of 490 nm can be quantified to cell number.

Briefly, HepG2 cells were cultured inside the samples ($n = 4$) of fibroin and fibroin/collagen blend films for 3, 6, and 10 days, then the culture medium was replaced with serum free culture medium containing thiazolyl blue (MTT) (0.5 mg/mL). Cultured for 4 h, the samples were transferred to 2 mL plastic tubes. Tubes were centrifuged for 5 min at 8000 rpm, and then the supernatant was aspirated. After DMSO was added into each tube, samples were cut into pieces and disintegrated using a Microbeater. Tubes were centri-

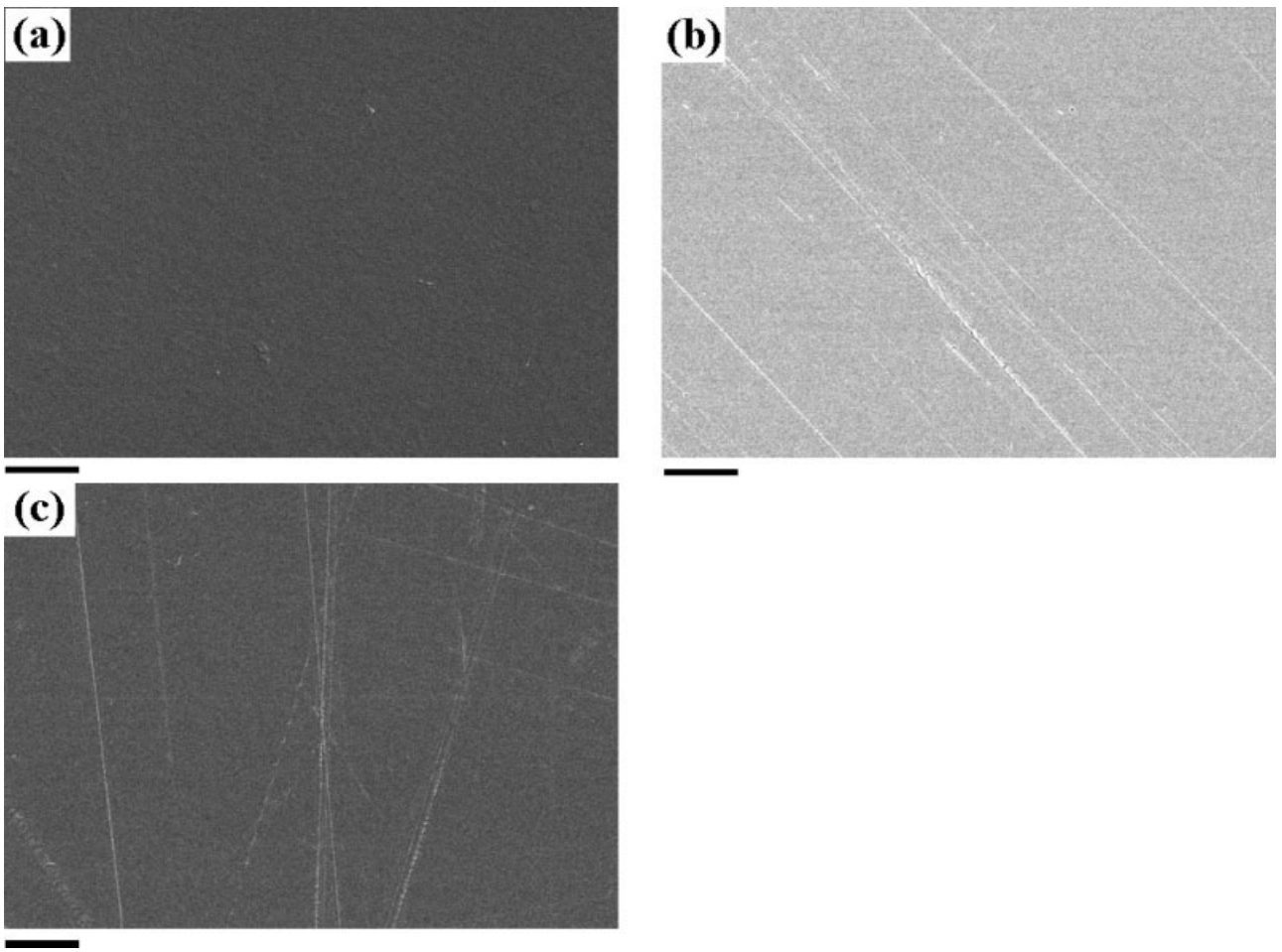

Figure 1 SEM morphologies of fibroin and fibroin/collagen blend films. (a) fibroin film, (b) blend film containing 10% collagen, and (c) blend film containing 20% collagen (scare bar 10 μm).

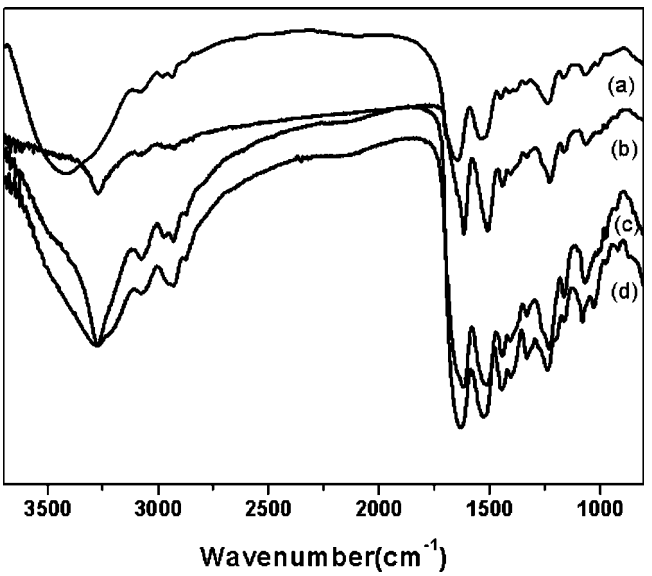

Figure 2 ATR-FTIR spectra of (a) pure fibroin film, (b) blend film containing 10% collagen, (c) blend film containing 20% collagen, and (d) pure collagen film.

fuged at 8000 rpm for 10 min. The solution of each sample was aspirated into a microtiter plate and the absorbance at 490 nm was measured on a SS-3000 Immunoanalyser.

RESULTS AND DISCUSSION

Optimization of insoluble fibroin/collagen films

Several conditions were tested to optimize the insoluble film preparation without methanol treatment. Some of the results are presented in Table I. It can be inferred that fibroin concentration and drying temperature are pivotal for the preparation of insoluble blend films. In our previous researches, we have prepared the insoluble fibroin films without methanol treatment, and then studied the influence of the different conditions such as concentration and drying temperature and the probable mechanism of insolubilization.[21,22] Simply, the increase of drying temperature enhanced the activity of globule-containing micelles that were the pivotal prestate of insoluble crystal β-sheet in the concentrated fibroin solution[25] and made the fibroin transform into a more stable state. In this research, it is found that adding appropriate amount collagen to fibroin solution has no negative effect on the water-stable film formation. Through adjusting the fibroin concentration and drying temperature, the water-stable blend films containing different content of collagen were directly prepared without methanol treatment.

SEM morphology

Figure 1 shows the morphology of fibroin and fibroin/collagen films. It can be seen that the morphology is uniform in the entire film through observing different places in the film except that some fringes appear on the composite film surface resulted from the nicks of the mold since fibroin and collagen were both dissolved in water and then sufficiently blended at molecular level.

ATR-FTIR

The conformational characterization of pure and blend films, as well as the study of specific interactions between fibroin and collagen were carried out by means of infrared spectroscopy. The FTIR spectrum of silk fibroin [Fig. 2(a)] shows strong absorption bands at 1639, 1530, 1260, and 1230 cm^{-1}, which indicates that both β-sheet and random conformation existed in fibroin films.[7,26–28] When fibroin was blended with collagen, the absorptions bands of 1639 and 1530 cm^{-1} shifted to 1615 and 1515 cm^{-1}, respectively, [Fig. 1(b) and 1(c)], which indicates that β-sheet conformation increased in blend films because the bands at 1615 and 1515 cm^{-1} represent β-sheet conformation while that at 1639 and 1530 cm^{-1} represent the α-helix/random coil conformation. On the other hand, many absorption bands which only appear in collagen spectrum [Fig. 1(d)] are also found in the blend films such as the peaks at 1434 and 1329 cm^{-1}.

Water contact angle

To investigate the hydrophilicity of pure and blend films, water contact angle measurement is shown in Table II. The pure fibroin films are relatively hydrophobic. When collagen contents are 10 and 20% in blend films, the contact angle obviously decreases from 90 ± 2° to 44 ± 1°, and 40.5 ± 1°, respectively. It indicates that the introduction of collagen improves the hydrophilicity of fibroin films, which is also conformed by the results of Hepg2 cells adhesion on these films. These results are obviously different when compared with those of other researcher,[20] which indicated that fibroin films was very hydrophilic and the adding of collagen would increase the hydrophobic property of blend films. These differences may be due to the different preparation conditions such as higher fibroin concentration and dry temperature in our studies. The effects of fibroin concentration and drying temperature

TABLE II
Contact Angle of Silk Fibroin and Silk Fibroin/Collagen Films (N = 5, Average ± SD)

Sample	Angle(°)
Silk fibroin	90 ± 2
Collagen 10[a]	44 ± 1
Collagen 20[a]	40.5 ± 1

[a] Collagen weight percent in silk/collagen blends.

TABLE III
Mechanical Properties of Silk Fibroin and Silk Fibroin/Collagen Blend Films

Sample	Tensile modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
Silk fibroina[a]	277.6 ± 15	13.8 ± 0.5	29.0 ± 2
Fibroin containing 10% collagen[a]	125.2 ± 20	8.75 ± 1	52.5 ± 7.2
Fibroin containing 20% collagen[a]	31.2 ± 6	3.39 ± 0.2	56.8 ± 6.5
Fibroin containing 20% collagen[b]	516.7 ± 43	30.0 ± 2.8	10.0 ± 2.1

[a] Wet state.
[b] Dry state in 65% humidity in atmosphere.

on film hydrophilicity have been investigated in detail in the previous studies.[21,24] The increase of fibroin concentration and drying temperature both facilitate the formation of β-sheet crystals, and then make films become water-stable and hydrophobic without methanol treatment. The fibroin/collagen blend films prepared by other groups obtained from much lower fibroin concentration and drying temperature have to be treated with methanol, a poisonous solvent, to make films insoluble in water.[20] So, our method to prepare water-stable fibroin/collagen films fits very well in the striving to green process.

Mechanical properties

Mechanical properties are very important for determining the performance of materials expected to undergo various types of stresses during using in many biomedical applications. Although silk has the excellent mechanical properties, how to make regenerated fibroin flexible in dry state is still the major challenge in the striving to fabricate fibroin biomaterials.

Table III shows the specific mechanical properties of different films. In the previous studies,[12–19] high content of other polymers had to be added in blend films (generally above 50%) to improve the mechanical properties of fibroin, which might sacrifice the biocompatibility of fibroin. More importantly, the above blend films are still too brittle in dry state. In our research, the fibroin/collagen blend films become flexible in near dry state without the losing of biocompatibility. The tensile strength and the elongation at break of pure fibroin film in wet state are 13.8 MPa and 29.0%, respectively. When 10 and 20% collagen was mixed with fibroin film, the elongation increased to 52.5% and 56.8%, while the tensile strength decreased to 8.75 and 3.39 MPa, respectively. The results indicate that the adding of collagen increases the flexibility of blend films. Although the strength and stiffness of the films decrease, the strength can still satisfy the requirement of most biomedical applications, and the increase of flexibility as well as the good strength is more suitable for the biomedical applications. The mechanical properties of different films in dry state are also investigated. Although other films are still brittle in dry state (data not shown), the blend films containing 20% collagen become flexible when placed at above 65% humidity in atmosphere. The tensile strength and elongation at break are 30.0 MPa and 10.0% respectively, at 65% humidity. It is a very interesting progress because we

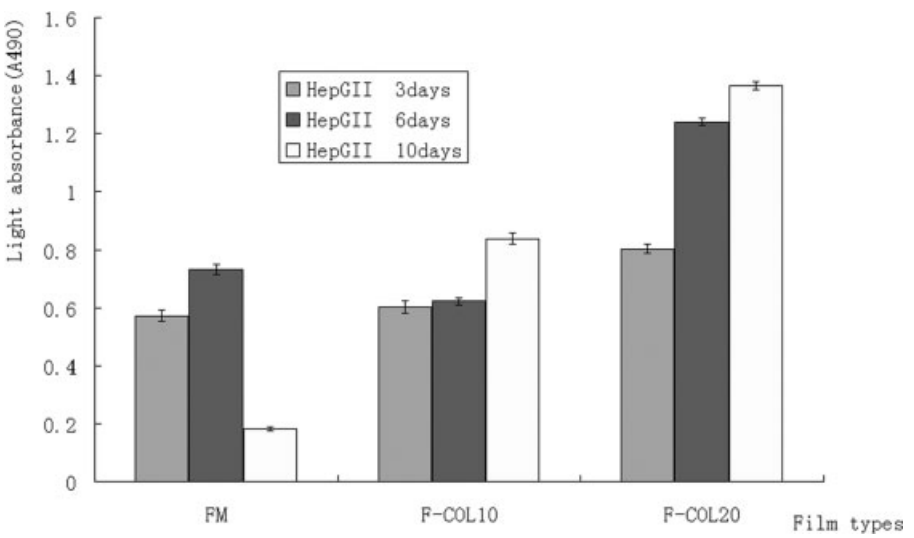

Figure 3 MTT assay after the HepG2 cells cultured on fibroin and blend films for 3, 6, and 10 days (FM: fibroin films; F-COL10: blend films containing 10% collagen; F-COL20: blend films containing 20% collagen).

can easily control the humidity with instruments. As soon as the humidity was above 65%, the fibroin/collagen blend materials would easily form different figures.

Cytocompatibility

HepG2 cells are used to study the preliminary biocompatibility of different films. Since collagen films have to be crosslinked to make them insoluble, many properties such as biocompatibility have changed. So, the fibroin films rather than the collagen films were used as control. The proliferation of HepG2 in fibroin and fibroin/collagen blend films after being cultured for 3, 6, and 10 days, was compared by MTT assay. The data are shown in Figure 3. It is evident that the blend film containing 20% collagen is more suitable for HepG2 proliferation. More interestingly, most cells on fibroin films died after 10 days, which indicates that fibroin is not suitable for HepG2 culture in long term, the blend films containing collagen, especially blend films containing 20% collagen, still exhibit the excellent biocompatibility and the cell number still obviously increases when cultured for 10 days. The results indicate that collagen, having the typical cell binding domains, improves the biocompatibility and proliferation of cells in long term.

SEM was also used to investigate the biocompatibility of different films. Figure 4 reveals that HepG2 cultured for 3 days has occupied most of surface of these three different films. It indicates that all samples are favorable for attachment and proliferation of HepG2. When cultured for 9 days as shown in Figure 5, HepG2 cells on blend films containing 20% collagen continued to proliferate and form multilayer aggregates, which was commonly found on three-dimensional scaffolds rather than films,[29,30] while the cells on fibroin films almost disappeared, leaving a smooth surface. The result is consistent with that of MTT. It confirms further that fibroin/collagen blend films maintain excellent biocompatibility. The excellent biocompatibility in long term, as well as the excellent strength and

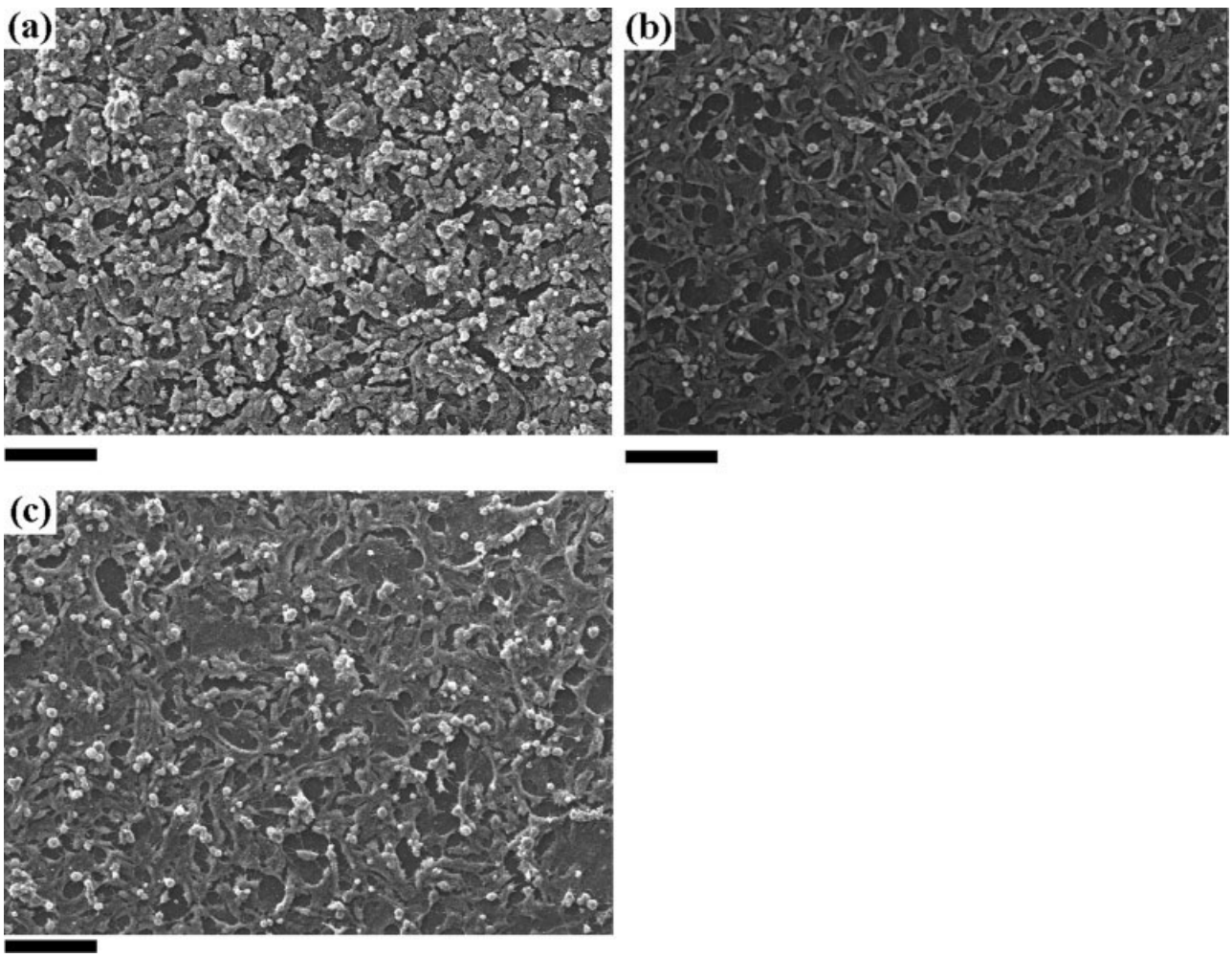

Figure 4 SEM photomicrographs of HepG2 cells cultured on different films at 3 days (a) fibroin film, (b) blend film containing 10% collagen, and (c) blend film containing 20% collagen (scare bar 100 μm).

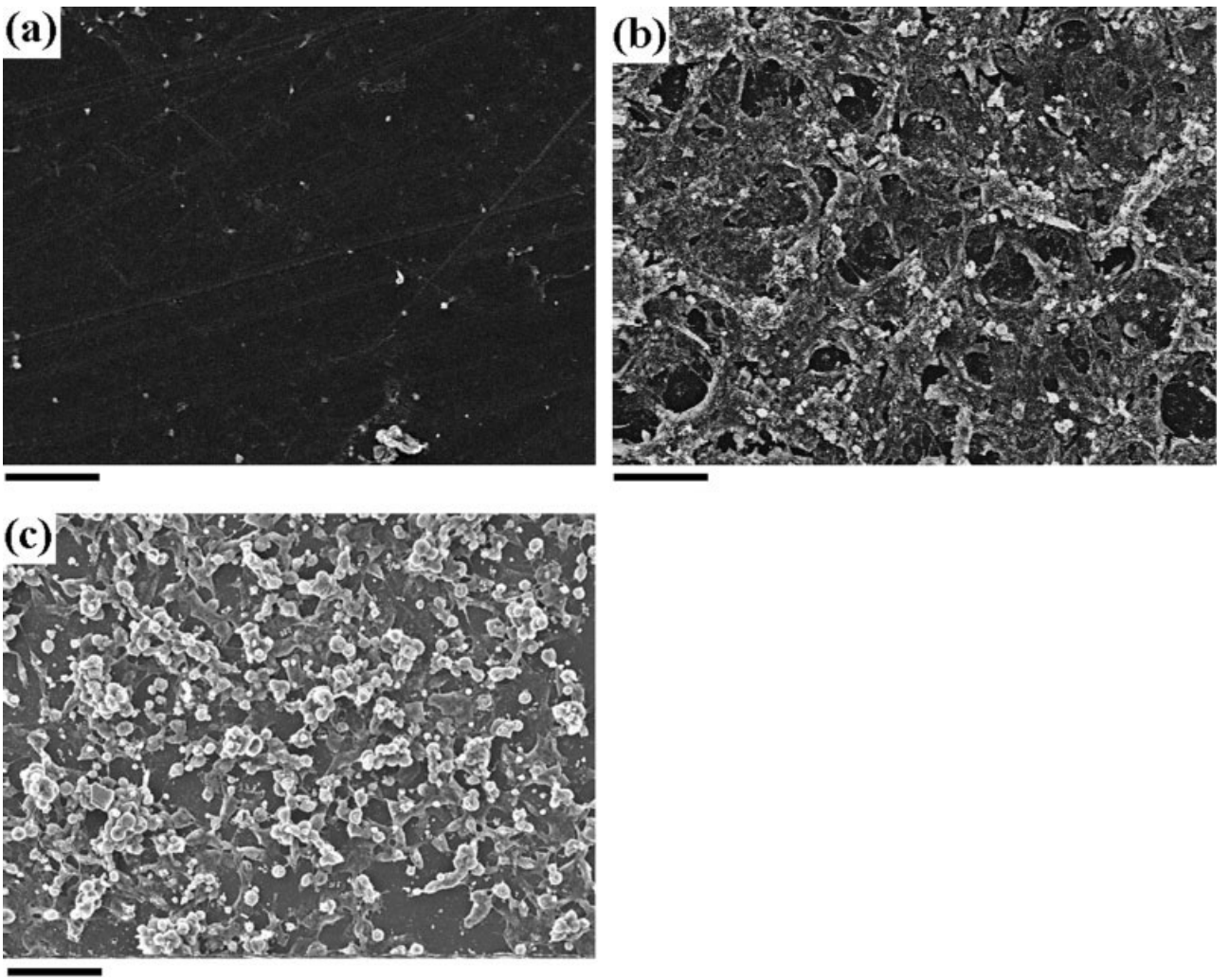

Figure 5 SEM photomicrographs of HepG2 cells cultured on different films at 9 days (a) fibroin film, (b) blend film containing 10% collagen, and (c) blend film containing 20% collagen (scare bar 100 μm).

flexibility of blend films containing 20% collagen in above 65% humidity, imply that this kind of blend films would become an outstanding biomaterial for different biomedical applications.

CONCLUSIONS

Solution blending of two biocompatible polymers was used to construct novel biomaterials with homogeneous microstructures. Silk fibroin and collagen, the two fibrous protein biopolymers, were blended in water and then the insoluble fibroin/collagen blend films were obtained directly from aqueous solution. By blending 20% collagen with fibroin, the blend film was evidently more hydrophilic, furthermore, it became flexible when the humidity was above 65%, which solved the brittle problem of regenerated fibroin materials without the decrease of biocompatibility. All these results support the concept that fibroin/collagen blend material would serve as an excellent candidate for different biomedical applications.

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