Silk fibroin modified porous poly(ε-caprolactone) scaffold for human fibroblast culture *in vitro*

GUANG CHEN, PING ZHOU*, NA MEI, XIN CHEN, ZHENGZHONG SHAO
The Key Laboratory of Molecular Engineering of Polymers, Ministry of Education, Macromolecular Science Department, Fudan University, Shanghai 200433, People's Republic of China
E-mail: pingzhou@fudan.edu.cn

LUANFENG PAN*, CHUNGEN WU
Laboratory of Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, People's Republic of China

In order to develop scaffolds with improved biocompatibility for cell culture, hybrid scaffolds were fabricated by modifying poly(ε-caprolactone) (PCL) with silk fibroin (SF) in a porous structure. Scanning electronic microscopy revealed that the morphology of the PCL–SF hybrid scaffold was affected by the concentration of the SF solution. Availability of SF on the surface and the conformational transition induced by methanol treatment were proved by attenuated total reflection Fourier transformed infrared spectroscopy (ATR–FTIR), and wettability of the hybrid scaffold was greatly improved. To evaluate scaffold biocompatibility, human fibroblasts were cultured on the hybrid scaffold with the unmodified PCL scaffold as control. An MTT assay indicated that although fewer cells were initially held on the hybrid scaffold after one day of culture, comparable cell numbers were achieved after four days and significantly more cells proliferated on the hybrid after seven days. The cell morphology also indicated that the PCL–SF hybrid scaffold was favorable for cell culture. This study suggests that surface modification with SF would be an effective way to improve the biocompatibility of PCL, facilitating its application in practical tissue engineering.
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1. Introduction

Enormous efforts have been devoted to construction of the cell culture scaffold in the field of tissue engineering. The scaffold serves as a three-dimensional (3D) template for cell adhesion, proliferation and extracellular matrix (ECM) formation as well as a carrier of growth factors or other biomolecular signals. Besides the obvious requirement of high porosity, the ideal scaffold for practical tissue engineering should have considerable mechanical properties, suitable biodegradability and good biocompatibility. Since the outermost surface of a scaffold is immediately located at the interface with cells or tissue, the surface property will guide cell–material interactions and, in return, affect cell adhesion [1]. Additionally, cell adhesion is an important factor in determining the proliferation rate of cells in culture [2]. Therefore, many methods of surface engineering have been developed to improve material biocompatibility [3–8]. Different biologically active molecules (e.g. RGDs containing peptides) have been chemically or physically immobilized on polymeric matrices [3, 4], and amphiphilic block polymers were employed as surface modification additives [5]. Plasma polymerization has also been used to incorporate functional groups onto the substrates [6]. Among all the methods, modification of synthetic materials with natural products is an easy and effective way to achieve hybrid scaffolds, with the combined advantages of both components. Collagen has been widely used [7, 8], but its potential contamination with the prion protein may restrict its application in the future [9].

Silk fibroin (SF), naturally derived from silkworm *Bombxy mori* silk, is a kind of fibrous protein composed mainly of amino acids such as Gly, Ala and Ser, and it is presently considered as a potential biomaterial for tissue engineering [9–14]. The inflammatory potential of SF observed was limited, compared with poly(styrene) and poly(2-hydroxyethyl methacrylate) [11]. Altman *et al.* [12] reported that a silk–fiber matrix supported the attachment, expansion and differentiation of adult human progenitor bone marrow stromal cells. Additionally, Inouye and his colleagues [9] found that both SF and collagen enhanced equivalently the growth of anchorage-dependent cells. However, the mechanical properties and architecture of SF cannot be easily tailored to meet the requirements of specific applications. The regenerated SF was recently hybridized with those synthetic polymers that possess good processibility and consider-

*Authors to whom all correspondence should be addressed.

able mechanical strength, such as polyurethanes [13] and poly(D-, L-lactic acid) [14], to improve their biocompatibility. Nevertheless, most of the research focused merely on surface modification of two-dimensional (2D) dense films, which evidently cannot meet the requirement of high porosity to be practical scaffolds.

Poly(ε-caprolactone) (PCL) has been regarded as a soft and hard tissue-compatible biodegradable polymer with good mechanical properties [15]. However, due to its intrinsic hydrophobicity and lack of bioactive functional groups, PCL is not very favorable for cell growth [16]. In this study, we attempt to fabricate a hybrid scaffold by modifying PCL with a regenerated SF solution in a porous structure. PCL was used to form a substrate with the desired shape and mechanical strength, whereas SF was expected to enhance its surface hydrophilicity and improve its biocompatibility. The scaffold morphology, wettability and SF conglutination were characterized. The biocompatibility of the hybrid scaffold was also evaluated by culture of human fibroblasts *in vitro*.

2. Materials and methods

2.1. Materials

The PCL pellets (Aldrich) used in this study has a number-average molecular weight of 80 000, with a melting point of 60 °C. Regenerated SF solution was prepared following the method of to Chen *et al*. [17]. Briefly, raw *Bombyx mori* silk was degummed twice with 0.5% (w/w) $NaHCO_3$ solution at 100 °C for 1 h and then washed with water. The degummed silk was dissolved in 9.3 mol/L LiBr solution at room temperature. After dialysis against deionized water for three days to remove LiBr, the solution was filtered to remove impurities and diluted with deionized water. A series of SF solutions was obtained with concentrations of 0.5%, 1.0% and 2.5% g/mL.

2.2. Preparation of scaffolds

2.2.1. Preparation of porous PCL scaffolds

Porous PCL scaffolds were prepared by the particle-leaching technique using sodium chloride (NaCl) particles as the porogen [18]. Briefly, 18 g sieved NaCl particles with diameters ranging from 76 to 100 μm were added into 40 mL 5% g/mL PCL dichloromethane solution. The solution was stirred vigorously to disperse the particles evenly and cast equally into eight circular glass dishes with diameter 6 cm. The solvent was allowed to evaporate for 24 h in a fume hood, yielding PCL/salt composite scaffolds with a thickness of around 0.4 mm. After 10 h vacuum drying to eliminate the residual dichloromethane, the salt in the scaffolds was leached out in deionized water. The porous scaffolds were vacuum-dried and weighed before further use.

2.2.2. Preparation of SF-modified hybrid scaffolds

The freeze-drying technique [7] was used to prepare the PCL–SF hybrid scaffolds. Briefly, the porous PCL scaffolds were dipped in the regenerated SF solutions of different concentrations under a vacuum of 50 KPa at room temperature. The SF-coated scaffolds were frozen in liquid nitrogen for 5 min, followed by lyophilization overnight under a vacuum of 0.1 Torr at − 50 °C. All the freeze-dried samples were immersed in absolute methanol for 1 h to immobilize the SF component, followed by vacuum drying, except for one of the samples modified by 0.5% SF solution, which was kept for analysis by attenuated total reflection Fourier transformed infrared spectroscopy (ATR–FTIR).

2.3. Characterizations of scaffolds

Except for scanning electron microscopy (SEM) analysis, all the characterizations of the PCL–SF hybrid scaffolds were performed on the scaffolds modified by 0.5% SF solution.

2.3.1. ATR–FTIR analysis

ATR–FTIR was performed on a Nexus 470 spectrophotometer (Nicolet) equipped with a multiple reflection horizontal ATR attachment. Each spectrum was recorded at a resolution of 4 cm^{-1} over 128 scans. The hybrid scaffold was analyzed before and after methanol treatment, with the unmodified PCL scaffold as control.

2.3.2. SEM observation

The morphology of the scaffolds was observed with a SEM (Philips XL30) at an accelerating voltage of 15 ~ 20 kV. The cross-sections were obtained by fracturing the scaffolds in liquid nitrogen. Both surfaces and cross-sections were sputter-coated with an ultrathin layer of gold before observation.

2.3.3. SF content and conglutination

The SF content in the PCL–SF hybrid scaffold was calculated using the following expression:

$$SF\% = 100 \times (W_h - W_{PCL})/W_h$$

where W_{PCL} represents the weight of the PCL, whereas W_h represents the weight of the hybrid scaffold after methanol treatment.

The conglutination of SF in the hybrid scaffold was evaluated under physiological-like conditions [13]. Briefly, the PCL–SF hybrid scaffold after methanol treatment was immersed in phosphate buffer saline (PBS, pH = 7.4) and incubated at 37 °C. Sodium azide (0.01 g/100 mL) was added to prevent bacterial colonization. After different durations of incubation, the scaffold was taken out from PBS, washed, vacuum-dried and weighed. The SF maintenance ratio (R_t) was defined as follows to evaluate SF conglutination:

$$R_t\% = 100 \times (W_t - W_{PCL})/(W_0 - W_{PCL})$$

where W_{PCL} represents the weight of PCL and W_0 and W_t represent the weights of the hybrid scaffold before and after incubation in PBS for different durations, respectively. This definition implied that the initial SF maintenance ratio (R_0) before incubation was 100%,

and R_t was measured at different incubation periods up to seven days.

2.3.4. Scaffold wettability
The wettability of the PCL–SF hybrid scaffold was assessed by the water contact angle of a SF-modified PCL film, with a dense PCL film as control. The dense film was prepared by casting 5 ml 5% (g/mL) of PCL dichloromethane solution on a circular glass dish with a diameter of 6 cm, followed by air-drying. One piece of PCL film was eluted in 0.5% SF solution, air-dried and treated with methanol, yielding the SF-modified film. Water contact angles were measured using the sessile drop method with an image analysis system (OCA 15plus, dataphysics) at 25 °C.

2.4. Cell culture
Human fibroblasts (ATCC HFL-1, American Type Culture Collection) were subcultured in a humidified atmosphere with 5% (V/V) CO_2 at 37 °C. The culture medium was Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (V/V) heat-inactivated newborn calf serum (NCS, Gibco), 100 U/mL penicilin and 100 μg/mL streptomycin (Gibco). The cells were digested with 0.25% trypsin (Sigma), and the concentration of cell suspension was 1.0×10^6 cells/mL. Both the PCL–SF hybrid scaffold and control (unmodified PCL scaffold) were cut into small circular samples 15 mm in diameter and sterilized with 70% (V/V) ethanol overnight. The samples were preincubated in the culture medium to remove the remaining ethanol. The sterilized samples were transferred to a new sterile 24-well cell culture plate (Costar), and 100 μL cell suspension was dripped onto each sample. After 1 h incubation in a humidified incubator (5% CO_2, 37 °C), an additional 1 mL culture medium was added for further culture. The medium was replenished every two days.

2.4.1. MTT assay
Changes in the numbers of viable fibroblasts on the scaffolds were quantitatively assessed with 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT; Sigma) at different culture periods up to seven days.

The MTT assay is a rapid colorimetric method to determine viable cell numbers, which is based on mitochondrial conversion of MTT to MTT formazan [19]. Briefly, the cell-containing samples were rinsed with serum-free medium to remove the unattached cells and transferred to a new plate. Then 400 μl serum free medium and 40 μL MTT stock solution (5 mg/mL in RPMI 1640) were added to each sample and incubated for 3 h (37 °C) for MTT formazan formation. Purple formazan on each sample was extracted with 400 μL dimethyl sulfoxide (DMSO), and 200 μL extraction solution was used for optical density (OD) measurement with an automatic microplate reader (ELX 800, Bio-Tek) at a wavelength of 570 nm with DMSO as blank.

2.4.2. Cell morphology
The cell morphology on the hybrid scaffold during the culture time was observed using a SEM. Each sample was rinsed twice with PBS and fixed with 2.5% (V/V) glutaraldehyde for 1 h at 4 °C. The cells on the scaffolds were dehydrated through a series of ethanol with increasing concentration of 30%, 50%, 75% and 99% (V/V) for 5 min each, followed by lyophilization.

2.5. Statistical analysis
Experiments were run at least triple in parallel tests. Data were expressed as means ± standard deviation (SD) for $n \geq 3$. Statistical evaluation was performed by one-way analysis of variance for multiple comparisons using a Bonferroni procedure. The mean values were considered to be significantly different when the probability of difference fell below 5% (i.e. $P < 0.05$).

3. Results and discussions
3.1. Scaffold characterizations
3.1.1. ATR–FTIR analysis
ATR–FTIR analysis was performed to examine the availability and conformational structure of SF on the surface of the PCL–SF hybrid scaffold. The amide I region between 1600 and 1700 cm^{-1} is very useful in the conformational analysis of SF. In general, the amide I mode associating with α-form conformation occurs at 1650–1660 cm^{-1}, whereas the random coil conformation gives bands in the range of 1640–1650 cm^{-1} and the β-form conformation results in bands between 1620 and 1640 cm^{-1} [20].

Fig. 1(A), (B) and (C) represent the ATR–FTIR spectrum of the PCL scaffold and the PCL–SF hybrid scaffold before and after methanol treatment, respectively. Compared with the spectrum of PCL, the peaks at 3270 cm^{-1} (NH stretching), 1622 cm^{-1} (Amide I) and 1526 cm^{-1} (Amide II) on the hybrid scaffolds demonstrate the availability of SF on the surface (Fig. 1(B) and (C)). Notably, the peak at 1653 cm^{-1} is characteristic of the α-form conformation of SF (Fig. 1(B)), which indicates that freeze-drying induces the partial α-form

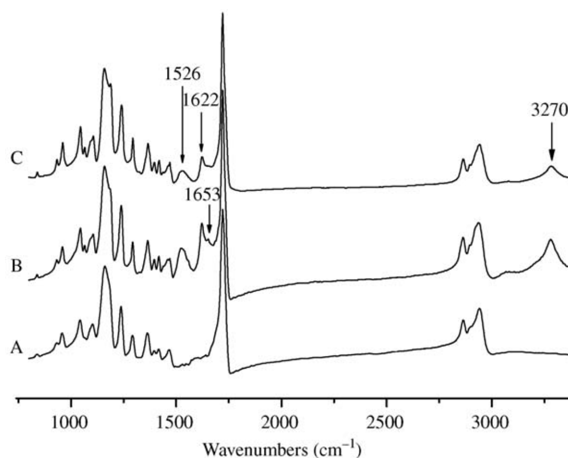

Figure 1 ATR–FTIR spectroscopy of (A) PCL scaffold; (B) the PCL–SF hybrid scaffold modified by 0.5% SF solution before methanol treatment; (C) the same hybrid scaffold after methanol treatment.

crystallization during the scaffold fabrication process. Finally, the complete conformational transition from water-soluble α-form to water-insoluble β-form was confirmed by disappearance of the characteristic peak at 1653 cm $^{-1}$ after methanol treatment (Fig. 1(C)).

3.1.2. Scaffold morphology

The SEM images demonstrate that both the surface (Fig. 2(a)) and cross-section (Fig. 2(c)) of the PCL scaffold before modification had uniformly distributed and interconnected pores and the pore size was almost as same as that of the NaCl particles.

The freeze-drying technique is usually used to generate a sponge structure in the scaffold. But in this study, the hybrid scaffold modified with 0.5% SF solution produced no evident SF sponges, either on the surface or the cross-section (Fig. 2(b) and (d)). This was attributed to the low concentration, at which it was difficult to form sponges with SF itself and, moreover, the numbers of pores and interconnections between pores were somewhat decreased on the surface (Fig. 2(b)). When a SF solution with a higher concentration of 1.0% was used, an SF sponge was observed in PCL pores (Fig. 2(e)). An elongated-pore and layer structure (Fig. 2(f)) was achieved in the hybrid scaffold modified with 2.5% SF solution, where it was difficult to distinguish the PCL and SF components, and the morphology was totally different from that of the control: that is, the morphology of the PCL–SF hybrid scaffold was remarkably affected by the concentration of the SF solution, with the SF with very low concentration (i.e. 0.5%) altering the PCL scaffold morphology least.

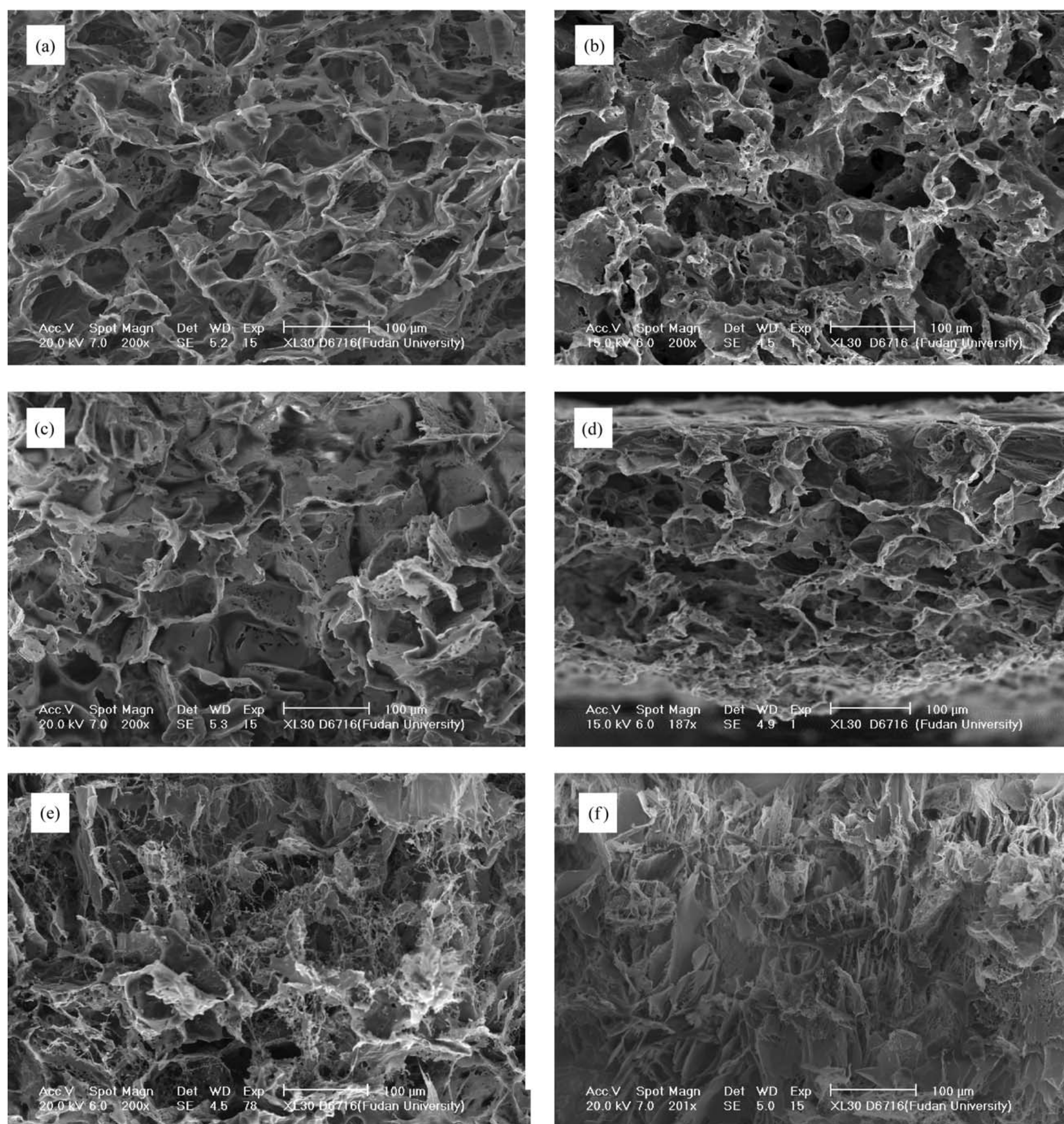

Figure 2 SEM images of different scaffolds. (a) Surface of PCL scaffold; (b) surface of PCL–SF hybrid scaffold modified with 0.5% SF solution; (c) cross-section of PCL scaffold; (d), (e) and (f) were the cross-sections of hybrid scaffolds modified with 0.5%, 1.0% and 2.5% SF solution, respectively.

3.1.3. SF conglutination and scaffold wettability

It was calculated that the SF content was about $5.1 \pm 0.1\%$ (W/W) in the hybrid scaffold modified with 0.5% SF solution. Fig. 3 demonstrates the changes in the SF maintenance ratio (R_t) in the culture medium up to seven days. R_t was $96.6 \pm 1.3\%$ and $92.0 \pm 2.4\%$ after one and four days, respectively ($P < 0.01$). Such slight decreases resulted from the detachment of SF fragments, but no significant decrease was found in the next three days ($P > 0.05$). These results indicated that the conformational transition from the α- to the β-form induced by methanol conglutinated the SF component. This conglutination may be an advantage of SF, compared with the widely used collagen coatings, which could be easily removed from the surface of polymeric materials when exposed to the culture medium [21].

The contact angle of the SF-modified film ($48 \pm 3°$, $n = 6$) was dramatically decreased, compared with that of PCL dense film ($78 \pm 2°$, $n = 6$), indicating that hydrophilicity of the film was greatly enhanced by the SF coating. Therefore it could be reasonably thought that the wettability of the porous SF-modified PCL scaffold was remarkably improved.

3.2. Cell culture on the scaffolds

3.2.1. Cell growth on the scaffolds

Because of the linear correlation between cell numbers and the optical density of MTT formazan [19], the OD_{570} values of MTT formazan on the PCL–SF hybrid scaffold and control shown in Fig. 4 indicate the change in cell numbers during the culture time. After one day of culture, fewer viable cells were held on the hybrid scaffold than on the control ($P < 0.01$). In the next three days, the cell number on the control showed a significant decrease ($P < 0.01$), whereas it remained almost unchanged on the hybrid ($P > 0.05$), resulting in the comparable cell numbers in these two groups ($P < 0.01$). After another three days, the cell numbers were increased ($P < 0.01$), but evidently more cells proliferated on the hybrid scaffold than on the control ($P < 0.01$). Such comparisons demonstrate that the SF-modified scaffold was more favorable for the cell culture. This enhanced growth could be attributed mainly to the improved surface hydrophilicity that was mediated by SF other than the scaffold morphology change, since the 0.5% SF solution used in this study altered the morphology least.

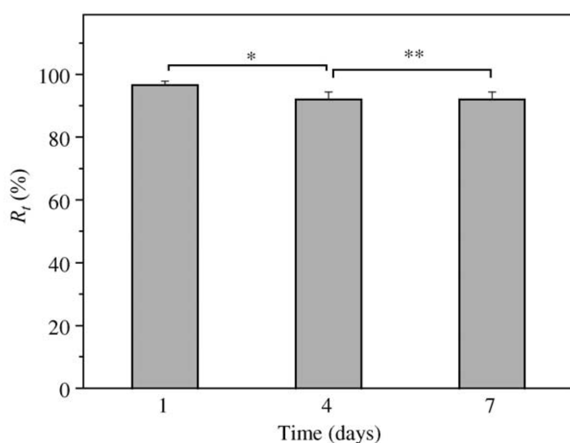

Figure 3 Change of SF maintenance ratio under physiological-like conditions (*$P < 0.01$, **$P > 0.05$, $n = 5$).

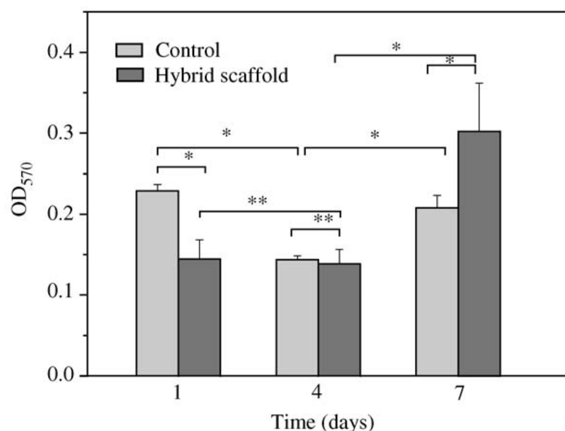

Figure 4 Comparison of optical density of MTT formazan on the PCL–SF hybrid scaffold at different culture periods with the PCL scaffold as control (*$P < 0.01$, **$P > 0.05$, $n = 3$).

3.2.2. Cell morphology

Fig. 5 demonstrates the representative cell morphology on the PCL–SF hybrid scaffold during the culture time. One day after seeding, cells not only adhered to the surface of the scaffold (Fig. 5(a)) but also aggregated in the pores (Fig. 5(b)). Compared with the pore size, the cells were small enough to deposit deep into the scaffold if the pores on the surface were interconnected with the inner ones. The porous scaffold is more efficient for cell seeding than the dense film just because it offers more space to accommodate cells. The slight decrease in the number of open pores on the surface and reduced interconnections between pores (Fig. 2(b) and (d)) hampered cells entering deeper into the hybrid scaffold, whereas it was difficult to wash out the viable cells entrapped in the inner pores even if they did not adhere on the scaffold. Thus, it would well explain why fewer cells were initially held on the PCL–SF hybrid scaffold than on the control. The SEM observation demonstrated that most cells were still spheroidal (Fig. 5(b)), while others showed an ellipsoid-like shape, adhering to the scaffold with many filopodia (Fig. 5(a)) after one day of culture.

Only adhered cells could spread (Fig. 5(c)), proliferate and take the lamellar appearance, partly covering the porous scaffold (Fig. 5(d)), whereas those entrapped without adhesion did not spread at all and did not survive during the culture time. These two reverse processes occurred simultaneously, giving a reasonable explanation for why no cell expansion was found during the first four days. A large area of confluence of tightly packed cells was observed after seven days, totally covering the hybrid scaffold (Fig. 5(e)), which was also in line with the quantitative result from the MTT assay.

4. Conclusions

In this study, novel porous hybrid scaffolds were fabricated by freeze-drying porous PCL scaffolds

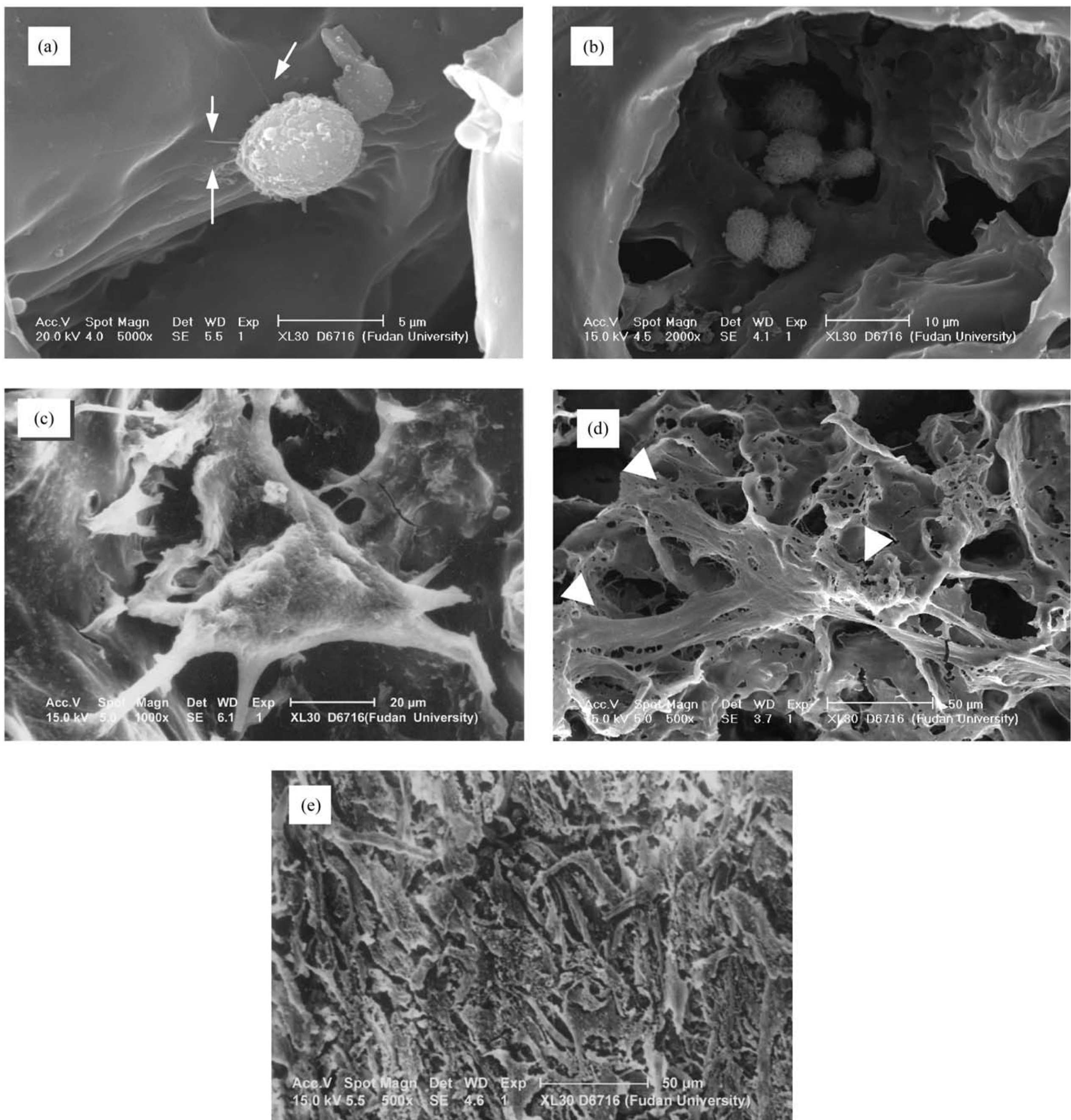

Figure 5 Representative SEM images of fibroblasts cultured on the PCL–SF hybrid scaffold. (a) Cell adhesion on the surface with filopodia (arrow); (b) cell aggregation in the pores; (c) cell spreading between pores; (d) lamellar cell with pseudopodia (arrow head); (e) cell confluence. (a) and (b) for one day, (c) and (d) for four days, and (e) for seven days.

coated with regenerated SF. SEM showed that the morphology of the PCL–SF hybrid scaffold was greatly influenced by the concentration of the SF solution. Methanol treatment could conglutinate the SF component when the hybrid scaffold was exposed to physiological-like conditions. Water contact angle measurement indicated that surface modification of PCL with SF could remarkably enhance PCL wettability. Human fibroblast culture *in vitro* showed a significantly higher cell proliferation on the hybrid scaffold. The morphology change of cells during the culture time also indicated that the hybrid scaffold was favorable for cell adhesion, spreading and proliferation. This study suggests that PCL modified by SF in a porous structure could effectively improve its biocompatibility and facilitate its application in practical tissue engineering.

Acknowledgments

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