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Enhanced cell affinity of the silk fibroin- modified PHBHHx material

Min Sun · Ping Zhou · Luan-Feng Pan · Shui Liu · Hua-Xiao Yang

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Abstract Cell affinity is one of the important issues required for developing tissue engineering materials. Although the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) has been attractive for its controllable mechanical properties recent years, its cell affinity is still necessary to be improved for the requirements. For this purpose, the regenerated silk fibroin (SF) was coated on the PHBHHx films and its porous scaffolds. The mechanical test showed that SF-modified PHBHHx (SF/PHBHHx) film has a maximum tensile strength of 11.5 ± 0.5 MPa and elongation at break of 175 ± 5%. ATR-FTIR spectroscopy demonstrated that SF firmly attached on the scaffold by the hydrogen bonding interaction between SF and PHBHHx even flushed for 21 days in the phosphate-buffer saline (PBS) solution (pH = 7.4). In order to characterize the cell affinity of the SF-modified material, endothelial-like cell line ECV304 cells were seeded on the SF/PHBHHx films and its porous scaffolds. The histochemical analyses of cells stained by the hematoxylin and eosin (HE) as well as cell nuclei stained by the 4′,6-diamindine-2′-phenylindole (DAPI) demonstrated that cell attached and reached nearly 100% confluence on the SF/PHBHHx films when cultured for 4 days, which was much faster than that on the pure PHBHHx film. Moreover, the assay of cell activity by the 3-(4, 5-dimethyl thiazol -2-yl)-2, 5-diphenyl terazolium bromide (MTT) showed quantitatively that the number of cells on the SF/PHBHHx porous scaffolds was significant more than that on the unmodified ones after 4, 8, and 14 days culture, respectively. Scanning electron microscopy (SEM) revealed the similar results. Therefore, the SF-modified PHBHHx material is maybe a potential material applicable in the cardiovascular tissue engineering.

M. Sun · P. Zhou (✉) · H.-X. Yang
The Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Fudan University, Shanghai 200433, People's Republic of China
e-mail: pingzhou@fudan.edu.cn

M. Sun
e-mail: 062044030@fudan.edu.cn

H.-X. Yang
e-mail: 072044017@fudan.edu.cn

L.-F. Pan (✉) · S. Liu
Laboratory of Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, People's Republic of China
e-mail: lfpan@shmu.edu.cn

S. Liu
e-mail: brightls_2000@hotmail.com

1 Introduction

Cell affinity is one of the important issues for developing the tissue engineering materials [1]. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) has been attractive for its controllable mechanical properties recent years [2–4]. As a new member of the polyhydroxyalkanoates (PHAs) family produced by a wide range of bacteria, PHBHHx possesses similar thermoplastic properties to the common polyethylene and polypropylene [5, 6]. The PHAs family (such as PHB, PHBV) is well-known for its no toxicity as well as its therapeutic or nutritional benefits to the cells [2]. In addition, as a degradation product of the PHAs, hydroxyl butyrate (HB) is a normal metabolized component in blood circulation system [7]. It has also been found that HB could promote the L929 cells proliferating well [8, 9]. PHBHHx, a modified generation of PHB, has a low crystallinity, resulting in a higher tensile strength and strain than that of PHB [5, 10]; While

PHB was limited in further application because of its high crystallinity although it was ever used to construct a tri-leaflet heart valve scaffold by Sodian et al. [11]. In addition, Wu et al. transplanted the PHBHHx, PLA, and PHB films into the back of New Zealand white rabbits. They found that PHBHHx showed a very mild tissue response during 6 month test, compared with other two films [12]. Furthermore, because of the adjustable molecular weight and composition ratio, PHBHHx has controllable strength and strain for the requirements of various tissue engineered organs [5], such as tissue engineered heart valve [13], vascular-related tissues [14] and bone [15], etc. P(HB-co-12%HHx) film degraded ~7% higher than PHB, P(HB-co-5%HHx) and P(HB-co-20%HHx) films which degraded <3% after lipase treatment for 7 weeks [16]. Sodian et al. found that it would take 17 weeks to form a new valve tissue when PHB scaffold was implanted in vivo [11]. Therefore, P(HB-co-12%HHx) is a better degradable material proper to the tissue engineered valve or blood vessel which would be eventually replaced by the newly-formed autologous tissue.

Though the PHBHHx is a potential tissue engineering material, its cell affinity is still necessary to be improved for the requirements. Generally, cell affinity undergoes two consecutive processes: attachment and proliferation [1]. The processes are greatly affected by the surface properties of the material, such as electric charge, surface energy, and hydrophilicity [17, 18]. Recently, the regenerated silk fibroin has been used to modify the synthetic polymer materials, including polyurethanes [19] and poly(D-, L-lactic acid) [20, 21], to improve the cell affinity of the materials. Silk fibroin (SF) obtained from the natural silk has a good biocompatibility both in vitro and in vivo [22–26]. Silk fiber has been used as the surgeon suture thread for hundred years [22]. The regenerated silk fibroin could form hydrogels [23], films [24], and scaffolds [25] as well as was used to fabricate the no-woven mats [26]. As a structural protein, silk fibroin is of affinity to various cells, for instance, vascular smooth muscle cells [27], endothelial cells [26], and peripheral nerve cells [28]. Therefore, the silk fibroin has been used as tissue engineered bone [29], cartilage [30], tendon [31], ligament tissue [32], small diameter vessel [33] and peripheral nerve repair [28], etc.

In this work, the regenerated silk fibroin was used to improve the cell affinity of PHBHHx by surface coating. Both SF-modified PHBHHx film and porous scaffold, which were expected to possess a good biocompatibility from the natural protein as well as a good mechanical property from the synthetic polymer, were fabricated. In order to test the cell affinity of the SF-modified material, the endothelial-like cell line ECV304 cells which are often used as a cell model in the medical and tissue engineering fields [34, 35], were seeded on the films and the porous scaffolds of the SF/PHBHHx. The investigation may give ones a helpful guidance to develop a proper material for the usage of tissue engineering.

2 Materials and methods

2.1 Materials and preparations

PHBHHx was kindly donated by Professor Chen G. Q. in Tsinghua University, China. The content of 3-hydroxyhexanoate (HHx) in PHBHHx copolymer is 12 mol%. The molecular weight of PHBHHx is 100,000 in Mw. The PHBHHx compound was purified by following processes: one gram compound was dissolved in 5 ml dichloromethane solution, and then refluxed at 38°C until the compound was completely dissolved. The mixture solution was filtered to remove the unsolvable impurity, and then 30 ml hexane solution was added into the filtered solution, leading to the pure PHBHHx precipitated from the solution, and the PHBHHx solid was dried in fume hood overnight.

The regenerated silk fibroin solution was prepared following the method of Chen et al. [20]. Briefly, the raw *Bombyx mori* silk fiber was degummed twice with 0.5 wt% $NaCO_3$ solutions at 100°C for 1 h and then washed with deionized water. The degummed fiber was dissolved in 9.3 mol/l LiBr solution at room temperature. The silk fibroin solution was dialyzed against the deionized water for three days to remove LiBr, and then filtered to remove the impurities. Thus, the regenerated silk fibroin solution with concentration of ~2 wt% was obtained and then diluted to the concentration of 0.75 wt% for the usage.

2.1.1 PHBHHx and SF/PHBHHx films preparation

A total of 0.35 g purified PHBHHx compound was dissolved in 7 ml dichloromethane. The solution was cast in a glass dish in diameter of 60 mm at room temperature overnight to allow the solvent evaporated. The PHBHHx film in thickness of about 100 μm was thus formed and then dried overnight in vacuum at room temperature.

The PHBHHx films were cut into circular shape in diameter of 10 mm by scalpel. Then they were immersed into a mixture solution of acetone/water (3:1) to be swollen for 10 min for their surface enlarged to absorb the silk fibroin as much as possible, and then rinsed with deionized water three times; afterward the films were immersed into the 0.75 wt% silk fibroin solution overnight to form the SF/PHBHHx films. The SF/PHBHHx films were vacuum dried overnight at room temperature and then immersed into the methanol solution which could change the silk fibroin conformation from soluble random coil to insoluble β-sheet form, making the silk fibroin coating firmly bound

to the surface of films by hydrogen bonding interaction [13, 36]. The SF/PHBHHx films were rinsed three times with distilled water and dried in a fume hood overnight.

2.1.2 PHBHHx and SF/PHBHHx Porous scaffold preparation

A three dimensional (3D) porous PHBHHx scaffold was prepared by the particle-leaching method using sodium chloride (NaCl) particles as porogens with the weight ratio of porogen to polymer of 9:1. In brief, 0.3 g PHBHHx was dissolved in 7 ml dichloromethane solution and then 2.7 g sieved sodium chloride particles in sizes of 50–75 μm were added into the solution. The paste-like particle/polymer mixture was vigorously stirred to allow the particles dispersing evenly, and then the mixture was cast in a glass dish in diameter of 60 mm and dried in a fume hood overnight to ensure the solvent evaporated totally. The formed film was washed by deionized water until the NaCl porogens were leached out completely from the film. The porous scaffold was thus obtained in the thickness of 200–300 μm as well as the porosity of ~85% demonstrated by the water saturation method [13].

The PHBHHx porous scaffold was cut into circular shape in diameter of 10 mm. The 0.75 wt % silk fibroin solution was used to modify the PHBHHx scaffolds, since the low concentration solution could remain the original pore sizes of the PHBHHx scaffold [13]. The scaffolds were immersed into a mixture solution of acetone/water (3:1) to be swelled for 10 min, and then washed with deionized water three times. Afterward the porous scaffolds were immersed into a 0.75 wt % silk fibroin solution overnight under vacuum of <1 mmHg to form a surface modified SF/PHBHHx porous scaffold. The SF/PHBHHx porous scaffolds were frozen in liquid nitrogen for 5 min, followed by lyophilization overnight under vacuum of 0.1 torr at −50°C. The freeze-dried porous SF/PHBHHx scaffolds were immersed into a pure methanol solution for 1 h to fix the SF on the PHBHHx porous scaffold, and then were rinsed three times with deionized water and dried under vacuum.

2.2 Methods of characterizations

2.2.1 Mechanical test of PHBHHx and SF/PHBHHx films

Mechanical properties were determined by an Instron tensile machine (Model 5656, Instron) at room temperature. The constant elongation rate was 5 mm/min for both PHBHHx and SF/PHBHHx films. For comparing with the results of the literature which used the rectangle specimen in the mechanical tests, tests were performed using a method already reported in literature [11]. The films were cut by scalpel into rectangle shape in length × width × thickness of 40 × 5 × 0.1 mm. In addition, because of the inhomogeneous 3D porous scaffold, the tested samples were always ruptured near to the two clamps of the machine when the samples were drawn, leading to the incorrect mechanical results. Therefore, homogeneous 2D films were instead used for the mechanical property tests. The results are still meaningful. Four samples were measured for each film.

2.2.2 ATR-FTIR analysis of SF/PHBHHx porous scaffold

Compared with 2D films, 3D porous scaffolds are used more efficiently in the tissue engineering field since 3D porous scaffold can provide large space to allow the required nutrition transported for the cell growth and proliferation, so study of the 3D porous scaffolds can make sense. The studied 3D porous scaffolds for the ATR-FTIR (attenuated total reflection Fourier transformed infrared) analysis were cut into circular shape in diameter of 10 mm and thickness of ~300 μm. They were put in a mimic flowing blood system in vitro. That means, the scaffolds were flushed by PBS solution (pH = 7.4) in a flowing rate of 55 ml/min driven by a squirm pump (D100A, Shanghai) for 0, 7, 14, and 21 days at 37°C. The chosen rate was same as the blood running rate of an adult under normal physiological condition. The PBS solution was renewed every other day to avoid the fluctuation of pH value. And then, the 3D porous scaffolds were dried and analyzed by an ATR-FTIR spectrometer (Nicolet Nexus 470) with resolution of 4 cm^{-1} and scan number of 128. Four parallel samples were tested at each time point.

2.2.3 Cell culture in vitro

The PHBHHx or SF/PHBHHx films and scaffolds were sterilized by ultraviolet for 30 min and immersed in 75% (v/v) ethanol solution overnight. The sterilized films and porous scaffolds were put in a 24-well culture plate (Coster™) and pre-incubated in PBS solution for 3 h. The PBS solution was changed every 1 h to replace the remained ethanol. Then the films and scaffolds were transferred to another 24-well cell culture plate. The endothelial-like cell line ECV304 cells (ATCC) were cultured in M199 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 100 U streptomycin and 100 μg/ml penicillin in the incubator with the humid air and 5% CO_2 at 37°C. A total of 400 μl cell suspensions with the concentration of 1 × 10^5 cells/ml were added onto the sterilized films or scaffolds in the 24-well cell culture plate. After 3 h incubation, an additional 500 μl culture medium was added for further culture. The culture medium was replaced every 2 days.

2.2.4 Tests of cell attachment and proliferation on the films by histochemical analyses

In the histochemical analyses, the cell morphologies are generally studied by the inverted light microscope to observe the cells stained by hematoxylin and eosin (HE), and the cell nuclei are studied by the fluorescence microscope to observe the cell nuclei stained by 4′, 6-diamindine -2′-phenylindole (DAPI). These methods require the samples thin enough to allow the light penetrating. The studied 2D films were transparent and homogeneous, thus used to do the HE and DAPI analyses for the cell and cell nucleus images, respectively. The cells seeded on the PHBHHx and SF/PHBHHx films for 0, 1, 2, and 3 days were rinsed with PBS, and then fixed by 4% polyformaldehyde buffer solution (pH = 7.4) for 30 min at 4°C. The fixed cells were stained by HE (Sigma), in which the cell nuclei were stained in purple by hematoxylin and the cytoplasma was stained in red by eosin. The HE-stained cell images were taken by the inverted light microscope (XDS-1B, Chongqing) equipped with a camera (D60, Nikon) to show the cell adhesion and proliferation on the films as the culture time increasing. In addition, for the observation of cell nuclei, the cells cultured on PHBHHx and SF/PHBHHx films for 4 days were rinsed with PBS solution, fixed by polyformaldehyde buffer solution (pH = 7.4) for 30 min at 4°C and stained by DAPI (Sigma) for 3 min. The cell nuclei images were taken by the fluorescence microscope (DP70, Olympus) with a CCD camera.

2.2.5 Morphology of 3D porous scaffolds and cells on the scaffolds by SEM observation

Larger space was available on the 3D porous scaffold than that on the 2D film for the cell growth, so the cells cultured on the 3D porous scaffolds for longer time were observed. The cells cultured on the 3D porous scaffold samples for 7 days were rinsed by PBS, and then fixed by 2.5% (v/v) glutaraldehyde for 4 h at 4°C. After dehydrated gradually with the concentration of 60, 80, 90, and 100% ethanol solution, the samples were frozen in liquid nitrogen for 5 min, followed by lyophilization overnight under vacuum of 0.1 torr at −50°C. The scaffolds with and without cells were cut into rectangle shape, sputter-coated with an ultrathin gold layer, and then the morphology of the fabricated PHBHHx and SF/PHBHHx porous scaffolds, as well as that of cells on those scaffolds were observed by scanning electron microscopy (SEM; TS, 5136MM) at an accelerating voltage of 20 kV.

2.2.6 Assay of mitochondrial metabolic activity

The metabolic activity of the cells seeded on the PHBHHx and SF/PHBHHx porous scaffold samples were assessed quantitatively by the 3-(4, 5-dimethyl thiazol -2-yl)-2, 5-diphenyl terazolium bromide (MTT; Merk) assay, which is a very common method to detect specially the live cells. The cells seeded on the porous scaffolds for 1, 4, 8, 14, and 21 days were rinsed three times with PBS solution, and then 300 μl M199 medium and 30 μl 5 mg/ml MTT PBS solution were added into each sample, followed by incubation for 4 h under the humid condition at 37°C. The insoluble purple formazan crystals of live cells and MTT were formed. The crystals were dissolved in the 300 μl dimethylsulfoxide (DMSO; Sigma) solution and then 200 μl of this solution was transformed into a 96-well plate for the optical density (OD) measurement using the spectrophotometer (ELx800, BioTek instrument) at 565 nm. The 200 μl pure DMSO solution was used as control.

2.2.7 Statistical analysis

Data were presented as mean ± SD (standard deviation). Four data were measured for every sample. Statistical comparisons were carried out using ANOVA one-way method. $P < 0.05$ was considered to be a significant difference between two data groups.

3 Results and discussion

3.1 Characterization of the 2D films and 3D porous scaffolds

3.1.1 Mechanical test of PHBHHx and SF/PHBHHx films

The cardiovascular tissue engineering requires the material having high tensile strength and strain to bear the dilatation. Therefore, the proper mechanical properties of the material are very important. Sodian et al. transplanted the PHB scaffold into a lamb for 20 weeks and found that the mechanical properties of the PHB-made new tissue resembled those of the native valve tissue [11]. Here, the results of the tested mechanical properties of SF/PHBHHx and PHBHHx films are shown in the stress–strain graph (Fig. 1). It can be seen from Fig. 1 that SF/PHBHHx (Fig. 1a) and PHBHHx (Fig. 1b) films have the same line shapes which are the typical pattern of the plastic material. SF/PHBHHx films have the maximum tensile strength of 11.5 ± 0.5 MPa and the elongation at break of 175 ± 5%, not significantly lower than those of 11.7 ± 0.5 MPa and 204 ± 5%, respectively, of PHBHHx film. The small decrease in the elongation of SF/PHBHHx films is maybe resulted from the addition of SF in the PHBHHx films which were swelled by the water/acetone solution. It is found that the native cardiovascular tissues have yield strength of about 0.7–8 MPa and a maximum elongation of

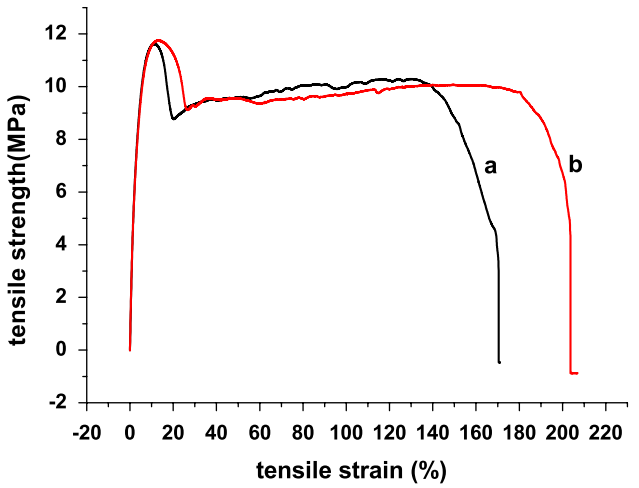

Fig. 1 Stress–strain graph of SF/PHBHHx films (**a**) and PHBHHx films (**b**). The constant elongation rate was 5 mm/min. Four samples in the shape of rectangle in length × width × thickness of 40 × 5 × 0.1 mm were tested for each film

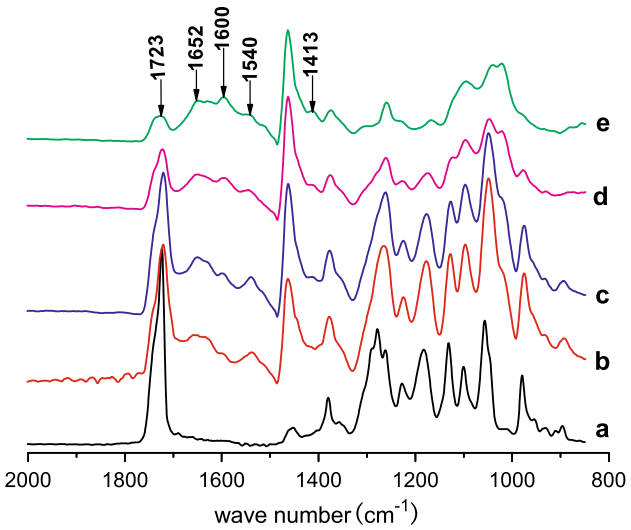

Fig. 2 ATR-FTIR spectra of PHBHHx and SF/PHBHHx porous scaffolds. **a** PHBHHx porous scaffold; **b–e** SF/PHBHHx porous scaffolds flushed with PBS solution (pH = 7.4) at 37°C for 0, 7, 14, and 21 days, respectively. The flushed rate is 55 ml/min which is the blood impulse rate under normal physiological condition

about 150% [11]. Therefore, SF/PHBHHx material is maybe a potential material applicable in the cardiovascular tissue engineering based on its mechanical properties.

3.1.2 ATR-FTIR analysis of the silk fibroin absorption on the PHBHHx porous scaffolds

Here, ATR-FTIR was used to detect the presence of silk fibroin on the surface of SF/PHBHHx porous scaffold before and after flushed with PBS solution for different periods of time. Because the low concentration SF solution could penetrate through the SF/PHBHHx scaffold, we thought that the SF could be absorbed not only on the surface of porous scaffold, but also in the scaffold pores. Figure 2a is the ATR-FTIR spectrum of PHBHHx scaffold and Fig. 2b–e show the spectra of SF-modified PHBHHx scaffolds flushed for 0, 7, 14, and 21 days, respectively. The assignments of FTIR spectrum of PHBHHx and SF/PHBHHx are summarized in Table 1.

Compared with the peaks in the spectrum of PHBHHx scaffold (Fig. 2a), the peaks at 1652 cm^{-1} and 1540 cm^{-1} from SF [13, 36] are found in the spectra of SF/PHBHHx scaffolds (Fig. 2b–e). It demonstrates that SF was successfully absorbed on the porous scaffold, even the scaffolds were flushed by PBS solution for 21 days. We also found that for the PHBHHx scaffold the C=O peak at 1723 cm^{-1} decreased gradually while the new peaks at 1600 cm^{-1} and 1413 cm^{-1} increased after being flushed for 21 days, demonstrating the partial hydrolysis of PHBHHx. Meanwhile, the peak at 1230 cm^{-1} decreased, indicating the helical conformation of PHBHHx changed [37]. The intensity of peak at 1460 cm^{-1} in PHBHHx scaffold slightly increased during flushing process, indicating the hydrolyzed products were still present in the scaffold. The repeated experiments herein found that the peak at 1460 cm^{-1} was much stronger in the flushed SF/PHBHHx scaffold than that in the SF/PHBHHx scaffold, perhaps due to PBS solution changing the polar environment of CH_3 group [37]. It can be speculated that the SF anchored on the scaffold through the hydrogen bonding with PHBHHx [13], even when the PHBHHx was partially hydrolyzed in the mimic flowing blood system.

Table 1 Assignments of FTIR spectra of PHBHHx and SF/PHBHHx

Peaks (cm^{-1})	Assignments	References
1652	Amide I of silk fibroin	[13, 36]
1540	Amide II of silk fibroin	
1723	C=O stretching band in PHBHHx	[37, 38]
1600	Symmetric stretching band of COO^- in PHBHHx	
1460	CH_3 asymmetric bending band in PHBHHx	
1413	Asymmetric stretching band of COO^- in PHBHHx	
1230	C–O–C stretching band in PHBHHx	

3.1.3 SEM observation of the PHBHHx and SF/PHBHHx porous scaffolds

Figure 3 shows the morphologies of 3D porous PHBHHx (Fig. 3a) and SF/PHBHHx scaffolds (Fig. 3b). The pore sizes are about 50–75 μm observed from the SEM images

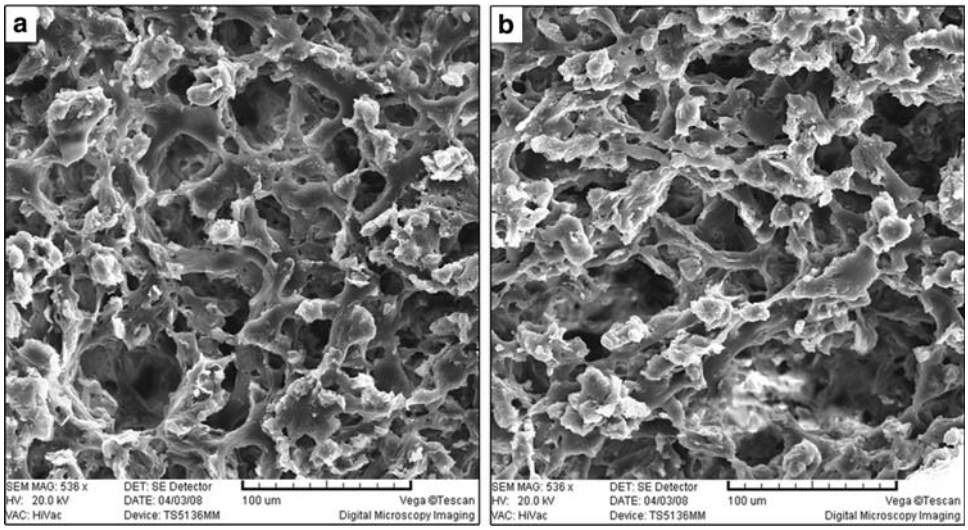

Fig. 3 SEM images of 3D porous PHBHHx (**a**) and SF/PHBHHx (**b**) scaffolds with the pore sizes of 50–75 μm

in Fig. 3, same as the sizes of the used porogens. The morphology of the scaffold had no apparent change after the scaffold was modified by 0.75 wt % SF solution.

3.2 Biocompatibility test

3.2.1 Cells adhesion and proliferation on the PHBHHx and SF/PHBHHx films

Figure 4 shows the process of cells adhesion and proliferation on the PHBHHx and SF/PHBHHx films cultured for different periods of time, studied by the histochemical analysis. From the images of HE-stained cells cultured on PHBHHx (Fig. 4a) and SF/PHBHHx (Fig. 4b) films at beginning, it can be seen that the background of SF/PHBHHx film showed red darker than that of the PHBHHx film, which is due to the silk fibroin stained by the eosin as well. This also demonstrates the existence and homogeneous distribution of the silk fibroin on the PHBHHx surface. The polygon-shaped endothelial cells are observed from HE-stained cells images, where the cell nuclei are in purple and the cytoplasma in red. Apparently, more cells adhered on the SF/PHBHHx film (Fig. 4d, f) than that on the control one (Fig. 4c, e) after seeded for 1 and 2 days, respectively. The cells on the SF/PHBHHx film almost reached confluence (Fig. 4h) in 3 days of culture, while the cells on the control were far away from the confluence (Fig. 4g).

The DAPI-stained cell nucleus images show that the cells on the PHBHHx film (Fig. 5a) are fewer than that on the SF/PHBHHx film in 4 days of culture (Fig. 5b). It demonstrates that the cell proliferation is better on the SF/PHBHHx film than on the control one, indicating that the SF-modified PHBHHx film is more favorable for the cell growth. Additionally, we found that the cells distributed more evenly on the SF/PHBHHx film than on the unmodified PHBHHx film by comparing Fig. 5a with Fig. 5b.

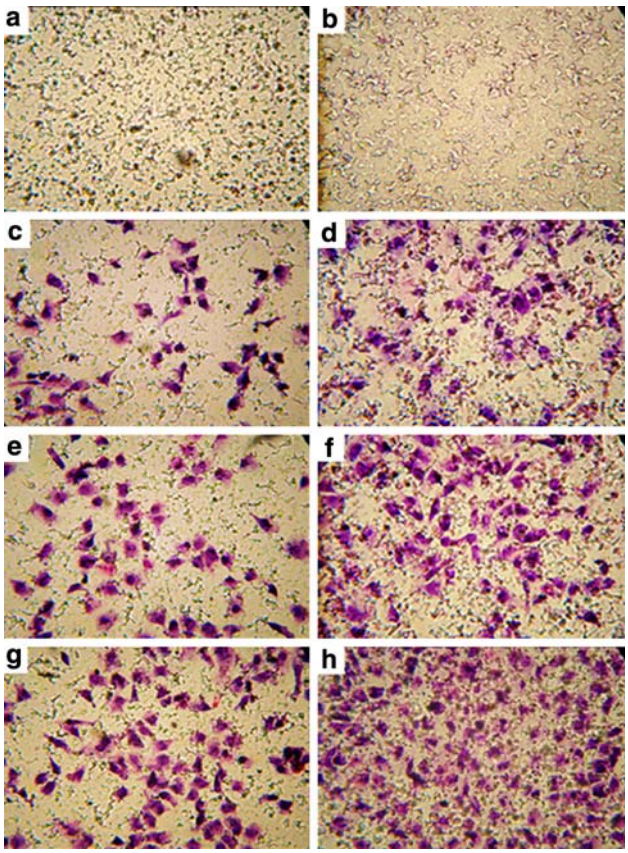

Fig. 4 Images of HE-stained ECV304 cells cultured on the PHBHHx (**a**, **c**, **e**, **g**) and SF/PHBHHx (**b**, **d**, **f**, **h**) films for 0, 1, 2, and 3 days, respectively. The cell nuclei were stained in purple with hematoxylin and the cytoplasma was stained in red with eosin (magnification 200×)

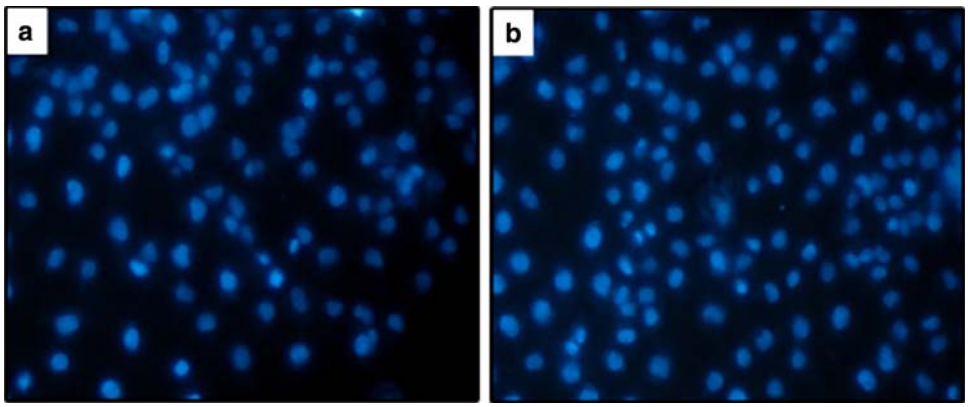

Fig. 5 DAPI-stained nuclei of cells on PHBHHx films (**a**) and SF/PHBHHx (**b**) films for 4 days of culture (magnification 200×)

3.2.2 *Mitochondrial metabolic activity of the cells*

Mitochondrial metabolic activity of cells is usually tested by the MTT essay. The histochemical analyses demonstrated that the cells nearly reached the confluence on the SF/PHBHHx film with the limited space in 4 days of culture, whereas the larger area available to cell growth in the 3D scaffold allowed MTT assay to study the cells cultured for longer time. The optical density (OD) value of MTT formazan solution is proportional to the live cell numbers [20], so we can measure the OD values to evaluate the relative cell amount on the porous scaffold. From Fig. 6, it is found that the OD values show no significant difference between the SF-modified and unmodified scaffolds in 1 day of cell culture; in 8 days, the OD values of cells on the SF/PHBHHx porous scaffolds are significant higher than that on the unmodified ones, which means the cells amount on the SF/PHBHHx porous scaffold is more than that on the PHBHHx scaffold, indicating that the cells on the SF/PHBHHx scaffold proliferated faster; until in 21 days, the OD values of the cells on the SF/PHBHHx porous scaffolds kept increasing while that of the cells on the unmodified scaffold decreased. It demonstrates that the SF/PHBHHx porous scaffold can sustain the cell growth for longer time than the pure PHBHHx scaffold, more suitable for the tissue regeneration.

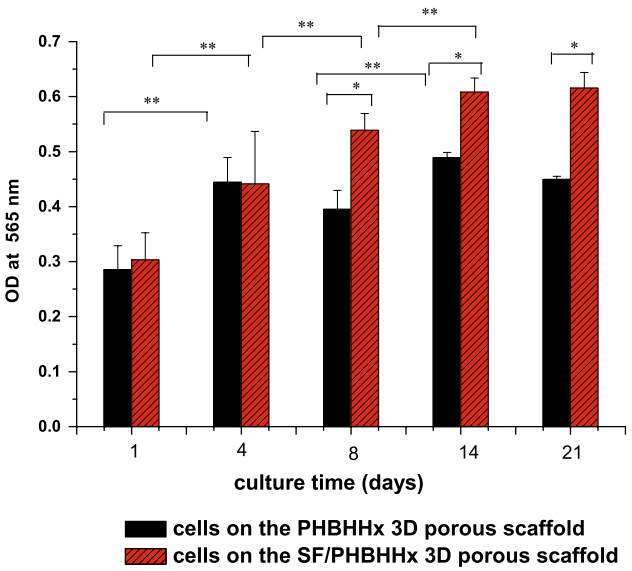

Fig. 6 OD values of cells cultured for different time on the 3D porous PHBHHx and SF/PHBHHx scaffolds (*: comparison between the cells on the different scaffolds for the same culture time; **: comparison between the cells on the same scaffolds for the different culture time, their $P < 0.05$, $n = 4$)

3.2.3 *Cell morphology on the 3D porous scaffold*

The cells on the SF/PHBHHx scaffolds formed a continuous monolayer with closer connection (Fig. 7b) than those cells on the PHBHHx scaffold in 7 days of culture (Fig. 7a). Moreover, the cells grew more evenly on the SF/PHBHHx porous scaffolds and spread with close space between each other. Figure 7c shows an amplified single cell image where the cell grew well and stretched out into the pores of the PHBHHx scaffold in 7 days of culture. The pseudopodium and microvillus were clearly observed in the image. It indicates that there is a proper environment for the cells growth.

4 Conclusions

In this work, the SF-modified PHBHHx material was fabricated by coating SF on the surface of PHBHHx. The mechanical properties of SF/PHBHHx films are comparable with that of the PHBHHx films. ATR-FTIR spectra demonstrated that silk fibroin was firmly anchored on the PHBHHx scaffold even flushed by PBS solution for 21 days at 37°C along with the PHBHHx scaffold was partially hydrolyzed. Histochemical analyses demonstrated that the endothelial-like cells, ECV304 cells, attached and proliferated faster on the SF/PHBHHx films than on the PHBHHx ones. Also, MTT assay and SEM observation

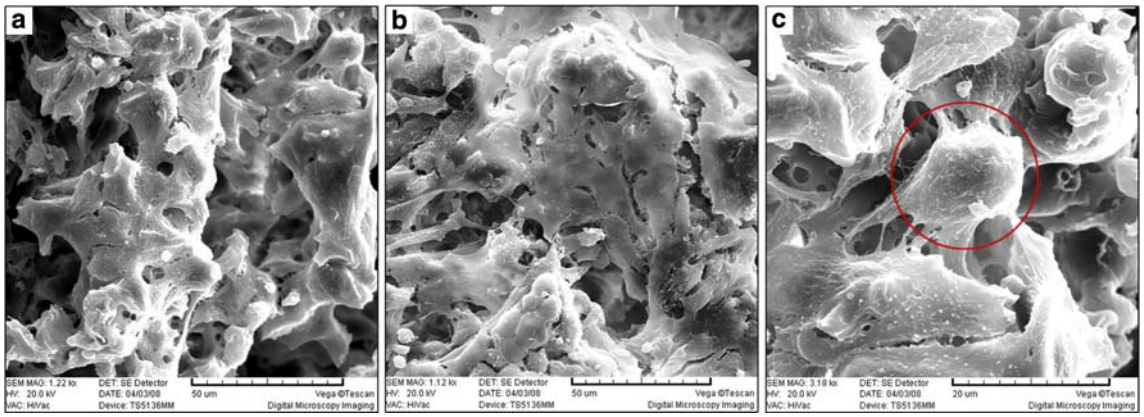

Fig. 7 SEM images of cells on the 3D porous PHBHHx (**a**) and SF/PHBHHx (**b**) scaffolds for 7 days culture as well as an amplified single cell on the 3D porous PHBHHx scaffold for 7 days culture (**c**)

showed that the cell affinity of SF/PHBHHx porous scaffold was significantly better than that of the unmodified ones. With an excellent mechanical properties, the SF-modified PHBHHx material would be potentially used in the cardiovascular tissue engineering.

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