ORIGINAL ARTICLE

# Silk fibroin/collagen and silk fibroin/chitosan blended three-dimensional scaffolds for tissue engineering

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**Abstract**

*Introduction* In this study, the silk fibroin blended constructs were produced, scaffold performances of different kinds of scaffold were analyzed, and the better type for tissue engineering was optimized.

*Methods* The silk fibroin/collagen (SF/C) and silk fibroin/chitosan (SF/CS) were made using a freeze-drying technique, porosity, water absorption expansion rate, mechanical properties and pore size of different scaffold was detected. Bone marrow mesenchymal stem cells (BMSCs) of 4-week-old male Wistar rats were separated by density gradient centrifugation, third generation BMSCs were seeded onto scaffolds, cultured 14 days, proliferation and metabolize of cells were detected in different time using the thiazolyl blue tetrazolium bromide (MTT) assay method, and cell morphology and distribution were observed by histological analysis and scanning electron microscopy (SEM).

*Results* Porosity, water absorption expansion rate and Young's modulus of SF/C were significantly higher than SF/CS ($p < 0.05$); pore size of SF/C and SF/CS was $103 \pm 12$ and $76 \pm 11\ \mu m$ and had no significant differences between two types ($p > 0.05$); MTT results showed that the metabolism of cells in the SF/C was better than SF/CS; after cultivation for 14 days, in the inner zone of scaffolds, cells staining were little or absent from SF/CS, lots of cells staining were existing in SF/C; pore size was consistent, holes communicated with each other better, stem cells grew well inside the scaffolds, extended fully and secreted much extracellular matrix under SEM in SF/C scaffold; internal structure of SF/CS was disorder, holes size were not consistent, and did not communicated with each other and cells were partly dead.

*Conclusion* Compared with SF/CS, SF/C scaffold showed better porosity, water absorption expansion rate, elasticity modulus and pore size, cells grow well inside the scaffolds, and was more suitable for tissue engineering.

**Keywords** Silk fibroin · Collagen · Chitosan · Blended scaffold · Bone marrow mesenchymal stem cells

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## Introduction

In clinical, because of trauma, injury, age-related degeneration and other reasons, lots of patients are suffering from bone, cartilage or other tissue defect; moreover, current treatments cannot restore native tissue and the new graft have bad biomechanical properties and other disadvantages, so the clinical need has motivated tissue engineering [1, 2]. In this technique, seeds were seeded onto three-dimensional scaffolds before implanting into body.

To achieve the goal, ideal scaffold should coincide with requirements. Ideal scaffold can guide and promote forming new tissue in tissue engineering. Because of mechanical properties, biocompatibility, nontoxic degradation and suitable mechanical properties, collagen, silk fibroin, etc.,

have been most extensively explored. These biomaterials can promote almost all kinds of cells proliferation, including chondrocytes, osteoblasts and mesenchymal stem cells [3, 4].

Collagen is a natural component of bone and cartilage tissue and rich in the human body. As scaffold, collagen can adhere, support and protect cells, also it is biodegradable biomaterials with low antigenicity, but the disadvantage was that the collagen scaffold degradation speed rate is fast, and mechanical strength is weak. Silk fibroin has been used as biomaterials for many centuries and can be prepared into various forms of material, widely used in tissue engineering scaffolds and had been widely concerned [5, 6]. Due to a crystalline sheets, silk fibers have good mechanical properties, additionally, good biocompatibility and biodegradability is its advantages, but degradation speed rate is slow. Chitosan has been extensively used in tissue engineering. It is good biocompatible and biodegradable and promote cell adhesion and migration [7–10], but lacks sufficient strength to be used and this was intractable to solve this problem using singly unless blended these three materials. In previous study [11, 12], the mechanical properties, thicker pore wall and rougher surface of silk fibroin blending with collagen and chitosan could be improved. In addition to superior physical properties, collagen and chitosan contains RGD sequences that could promote cell attachment and proliferation [13, 14]. But the ability of silk fibroin/collagen (SF/C) and silk fibroin/chitosan (SF/CS) has not been compared.

This study focuses on the advantage of blending these three kinds of scaffold materials, fuse silk fibroin, collagen and chitosan, optimized the better type for chondrocyte tissue engineering. Therefore, in this study, BMSCs were seeded on the three types of constructs. Then, physical characteristics, cell adhesion, cell proliferation and production of the scaffolds were compared; moreover, this study can provide direction of further tissue engineering scaffolds.

## Materials and methods

### Materials

Natural silk worm cocoons, collagen, CS was purchased from the Sigma Chemical Company (Sigma USA); DMEM/F12 medium (Hyclonecompany), fetal bovine serum (FBS Sigma), trypsin (Sigma), lymphocytes separation Medium (Amersham pharmacia biotech), thiazolyl blue tetrazolium bromide (Sigma), toluidine blue (Sigma), Scanning electron microscopy (S3000N Hitachi company), $CO_2$ incubator (78646-2826 Forma Scientific company USA) clean work table (SW-CJ-1f, purification equipment

company Su Zou China), Inverted microscope and camera system (IX70 Olympus company Japan) Ordinary centrifuge machine (LD522A medical centrifuge factory Beijing China) and all other substances were obtained from Sigma.

### Preparation of composite scaffolds

Silk fibroin solution: The methods were different from previous study [11]. Briefly, silkworm silk was boiled in 60 °C, 0.5 % $Na_2CO_3$ solution for 30 min (to remove sericin), repeated three times, then kept at 60 °C to dry, purified fibers was dissolved in $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$ (Mole ratio 1:2:8) at 60 °C about 2 h and was centrifuged 10 min at 8,000 rpm/min to obtain up clear liquid, dialyzed for 72 h, concentrated 7 h in 40 % PEG and got suitable concentration.

SF/C blend and SF/CS blends were prepared using a freeze-drying technique. Scaffolds were added a gradation of ethanol 24 h, then in 0.5 % NaOH solution 15 min, lyophilized, Co60 sterilized and kept at −20 °C.

### Physical characterization

The porosity of the scaffolds was determined using the principle of liquid displacement method [16]. The scaffolds were immersed in distilled water and were calculated from wet ($M1$) and dry weight ($M0$), according to the following equation: $(M0 − M1)/M1 \times 100$ and got the water uptake ability property [11]; inner structure and pore size were detected by randomly measuring at least 30 pores using SEM image analysis program; mechanical properties of the silk/collagen scaffolds were measured using a universal testing machine Instron 5865 equipped with 0.1 N load at 0.5 mm/min in environmental conditions, and compressed 10 %, dynamic displacement was 5 %, amplitude at frequencies of $f = 0.5$ Hz, recycled three times, calculated Young's modulus and drew stress–stain curve diagram. After 14 days of culture, thickness and Young's modulus of constructs were tested again for biomechanical properties compared with nonseeded cells.

### BMSCs culture in scaffolds

BMSCs of 4-week-old male Wistar rats were separated by density gradient centrifugation [6] and maintained in a humidified incubator (5 % $CO_2$, 95 % air) at 37 °C, and third passage cells were used for the further experiments. The third passage (P3) BMSCs were seeded on the three scaffolds. To be brief, scaffolds were cut to 1 mm thickness and 10 mm diameter then were placed in a 24-well plate with 1 ml culture medium. Then 24-well plate was put in the CO2 incubator overnight and discarded culture medium seeded onto scaffold with 100 µl BMSC

suspension containing $2 \times 10^7$ cells. Then 1 ml fresh medium (DMEM/F12, FBS) was added into each well after 4 h, cultured 14 days, media changed (5 ml) every other day.

## Cell viability

The thiazolyl blue tetrazolium bromide (MTT) assay was used to assess cell viability, and its principle was based on metabolic activity of alive cells [16]. The time intervals were 1, 3, 5, 7, 9, 11 and 13 days. At the determined times, removed and transferred the samples to new 24-well plates and per well added 2 ml solution of 2 mg/ml thiazole blue (MTT), and cultured for a further 4 h. The medium was discarded and very well added 2 ml dimethyl sulfoxide (DMSO), dissolved fully and become purple crystals, then 150 µl of each sample was transferred to a 96-well plate and was measured at 520 nm by spectrophotometry.

## Histological study

After 14 days in culture, scaffolds were washed with 0.01 M PBS (pH 7.4) and fixed in 10 % neutral buffered formalin, then embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin–eosin (H&E) and observed cell growth and distribution under light microscope.

## SEM examination

After 14 days in culture, the scaffolds with attached cells were washed with 0.01 M PBS (pH 7.4), fixed with 4 % glutaraldehyde solution and then fixed with 1 % osmic acid in 4 °C for 4 h, and were dehydrated in graded ethanol solutions and air-dried. At last, the scaffolds were coated with aluminum stubs and gold–palladium and were observed under a scanning electron microscope.

## Statistical analysis

Statistical analyses of the data were carried out using SPSS 17.0, all data were reported as mean ± standard deviation (SD), and dates were considered statistically significant when probability values ($p$) was less than 0.05.

## Results

### Physical properties of scaffolds

SEM micrographs of the two kinds of scaffolds illustrated a porous structure, Fig. 1, and SF/C scaffolds with highly interconnecting pores showed a thicker pore wall than SF/CS; Porosity, water uptake ability, Young's modulus and
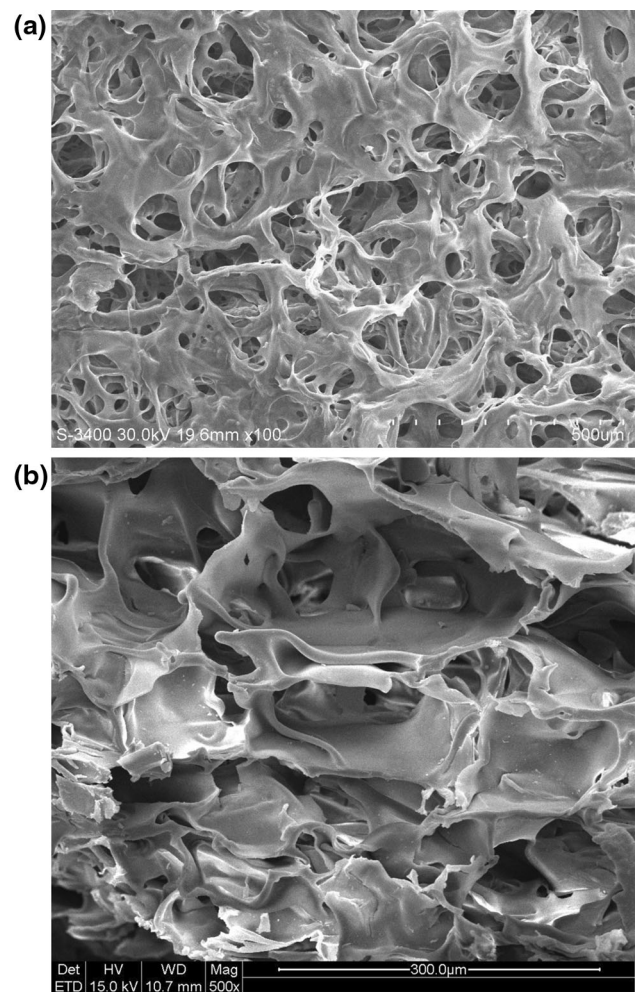


**Fig. 1 a** SEM micrographs of prepared scaffolds after freeze-drying SF/C. **b** SEM micrographs of prepared scaffolds after freeze-drying SF/CS

mean pore size of two kinds of scaffolds are presented in Table 1. The porosity, water absorption rate and Young's modulus of SF/C were significant higher than those of SF/CS ($p < 0.05$), and mean pore size had no significant difference ($p > 0.05$). So it suggested that SF/C scaffolds showed a higher comprehensive property than SF/CS and was suitable for cells cultivation [11]; stress–stain curve diagram suggested the two kinds of scaffolds showed viscoelastic behavior, Fig. 2.

### Biomechanical properties

After 14 days of culture, the thickness and Young's modulus of the scaffolds increased ($p < 0.05$). At the end the culture period, the construct thickness of SF/C and SF/CS was, respectively, 2.5 and 1.6 mm, respectively, corresponding to increases of 250, 160 %, and the construct Young's modulus of SF/C and SF/CS was, respectively, 103.6 and 37.4 kPa, corresponding to increases of 107 and 61 %.

**Table 1** Comparison of mean porosity, pore size, water uptake ability, Young's modulus and mean pore size property of two kinds of scaffolds

| Types of the scaffold | Porosity ± SD (%) | Water absorption expansion ± SD (%) | Young's modulus (KPa) ± SD | Mean pore size (μm) ± SD |
|---|---|---|---|---|
| SF/C | 94.6 ± 1.1 | 1,523.7 ± 186.6 | 49.7 ± 5.0 | 103 ± 12 |
| SF/CS | 81.4 ± 1.3 | 1,071.3 ± 152.3 | 23.1 ± 2.5 | 76 ± 11 |
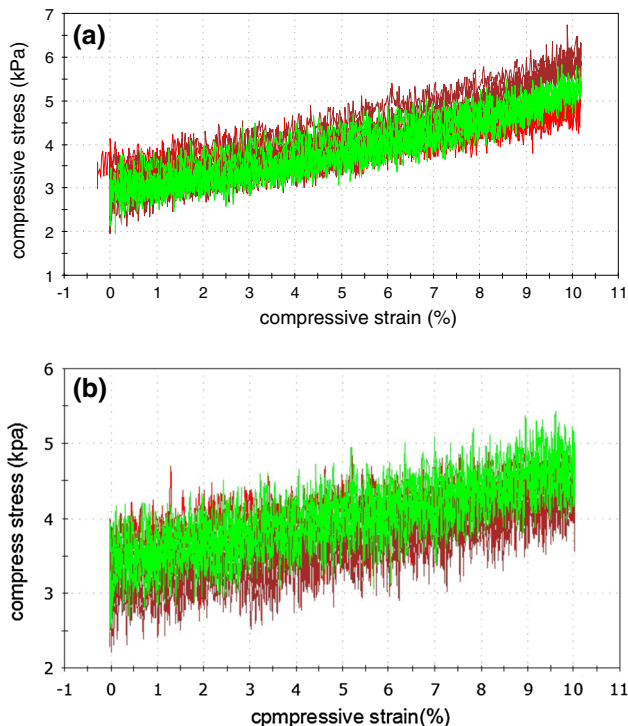


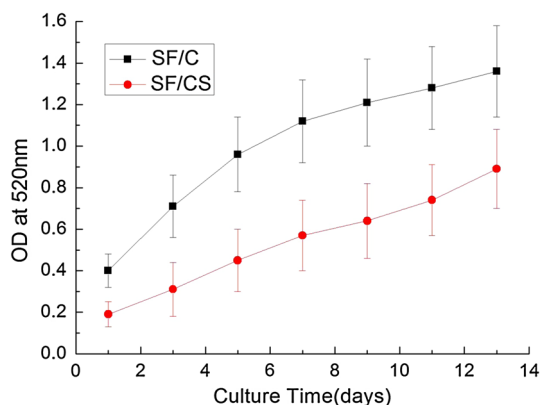**Fig. 2 a** Stress–stain curve diagram SF/C. **b** Stress–stain curve diagram SF/CS



**Fig. 3** MTT result after BMSCs in scaffolds for 1, 3, 5, 7, 9, 11 and 13 days. Significant differences between two groups, at $p < 0.05$

Cell viability and proliferation

Cell viability and proliferation was widely assayed by the MTT, which was a quantitative colorimetric assay. The purple crystals can be formed by metabolically active cells and was detected by spectrophotometry at 520 nm. So, growth and proliferation of active cells can be tested indirectly using this method. And time intervals were 1, 3, 5, 7, 9, 11 and 13 days as is shown in Fig. 3, the OD values of SF/C were significantly higher than SF/CS, at $p < 0.05$, indicating that cells were proliferating fast during the culture period. And obviously, the OD value of SF/C was increasing after 5-day cultivation and gradually increased until 14-day culture period. Also, it suggested only in SF/C scaffold can cells grew and proliferated better.

Cell morphology and distribution in scaffolds

The cell distribution and growth in two scaffolds were assessed using hematoxylin–eosin staining on the surface and in the inner zone, cell morphology and distribution was different after cultivation for 14 days and is shown in Figs. 4 and 5. Outer surface of three scaffolds were full of cells, and more than the inner zone; however, the number of cells in the SF/C that moved into the inner zone were higher than SF/CS and was, respectively, $4 \times 10^2/HP$ and $10^2/HP$ ($p < 0.05$), and this phenomenon was consistent with SEM micrographs. As shown in Fig. 6, on the inside scaffold, it showed lots of cells. From SEM micrographs, two scaffolds were different from the raw native scaffolds, Fig. 1, for the SF/CS, large surface voids still existed from the SEM micrographs but inside SF/C scaffold was covered with cell attachment.

## Discussion

In tissue engineering, a proper physical property scaffolds is crucial and should meet certain criteria, such as its structure should be suitable for cell growth and attachment, water uptake ability was benefit to promote nutrient transport. Additionally, pore size and porosity were optimal to prevent cell loss from the scaffolds [15, 16]. Cells are exposed to a variety of forces such as compression and shear forces and so on in vivo [16]. So to serve and meet this function, we examined the biomechanical properties of blended scaffolds by dynamic compression in this study.

In previous study, silk fibroin, collagen and chitosan scaffolds have been used separately for tissue engineering. Collagen is a biodegradable protein and a most component of cartilage tissue. Silk fibers have good biocompatibility
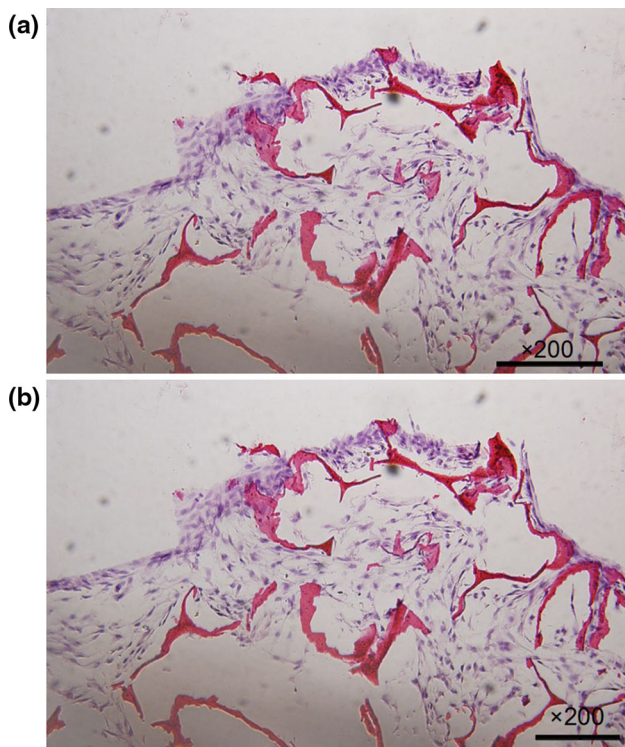
Fig. 4 **a** Photomicrographs of BMSCs growth on the surface area of the SF/C scaffold. The cells were stained with hematoxylin and eosin. Original magnification ×200, *scale bar* 50 μm. **b** Photomicrographs of BMSCs growth on the surface area of the SF/CS scaffold. The cells were stained with hematoxylin and eosin. Original magnification ×200, *scale bar* 50 μm



Fig. 5 **a** Photomicrographs of BMSCs growth on the inner zone of the SF/C scaffold. The cells were stained with hematoxylin and eosin. Original magnification ×200, *scale bar* 50 μm. **b** Photomicrographs of BMSCs growth on the inner zone of the SF/CS scaffold. The cells were stained with hematoxylin and eosin. Original magnification ×200, *scale bar* 50 μm

and biodegradability with containing RGD sequence, which is a cell-recognition signal that promotes cell adhesion and proliferation [17]. CS is good biocompatible and biodegradable and promote cell adhesion and migration [17–20]. In the present study, we evaluated the SF/C and SF/CS blended scaffold by BMSCs cultured in the three-dimensional porous scaffolds. The results showed SF/C is suitable in terms of porosity, pore size, cell viability and mechanic properties. Also, this study demonstrate BMSCs can maintain proliferate and functional mechanical after 2 weeks of culture.

Many methods can fabricate porous biodegradable structures, phase separation, carbon dioxide expansion, solvent casting/particulate leaching and emulsion freeze drying included. In this study, the freeze-drying was used to make blended constructs and was a relatively simple method with the advantage of no organic solvent remained. The present study had shown that BMSCs attached and proliferated inside scaffolds after 2 weeks.

The deposition of matrix within scaffolds was related to viscoelastic properties, and they are important biomechanical function of articular cartilage in vivo [20, 21]. So the dynamic compressive was used to test these constructs.
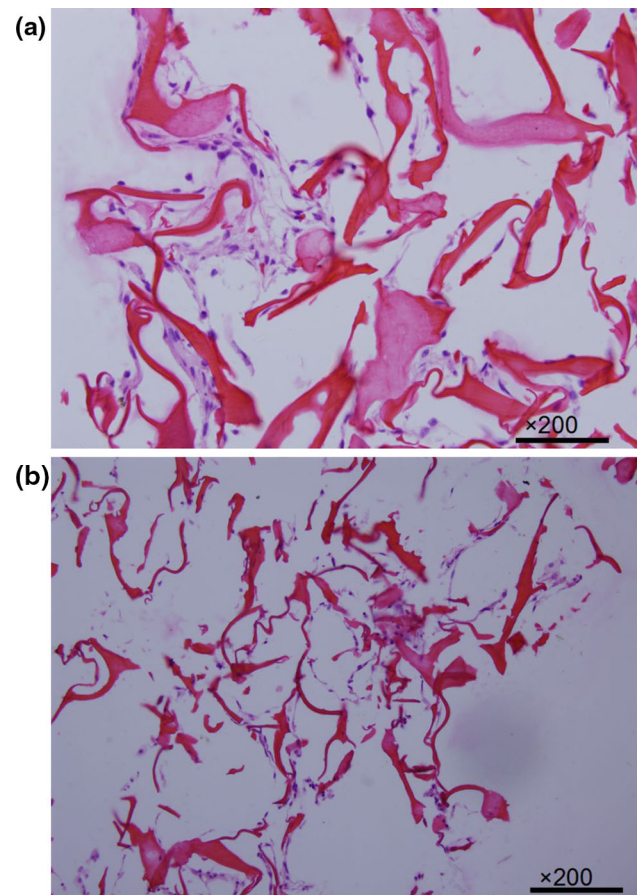
It showed that compressive modulus of scaffolds with cell seeding increased and suggested that deposition of matrix inside the scaffold were functional. Furthermore, compressive properties of constructs are different, scaffolds with higher concentrations of cells develop better mechanical properties. SF/C has highest compressive modules, suggesting more BMSCs attached inside the scaffolds.

MTT results showed that the metabolism of cells in the SF/C was better than SF/CS. When cultured 1 day, the number of live cells in SF/C scaffold was more than that in SF/CS scaffold, and it suggested that better water uptake ability was favorable for cell adhesion; cultured 3, 7, 9, 11 and 13 days, live cells proliferated actively in both scaffolds, but the number of live cells in SF/C was significantly higher than that in SF/CS, which was mainly due to the comprehensive properties—bigger porosity, better water uptake ability and suitable pore size of SF/C.
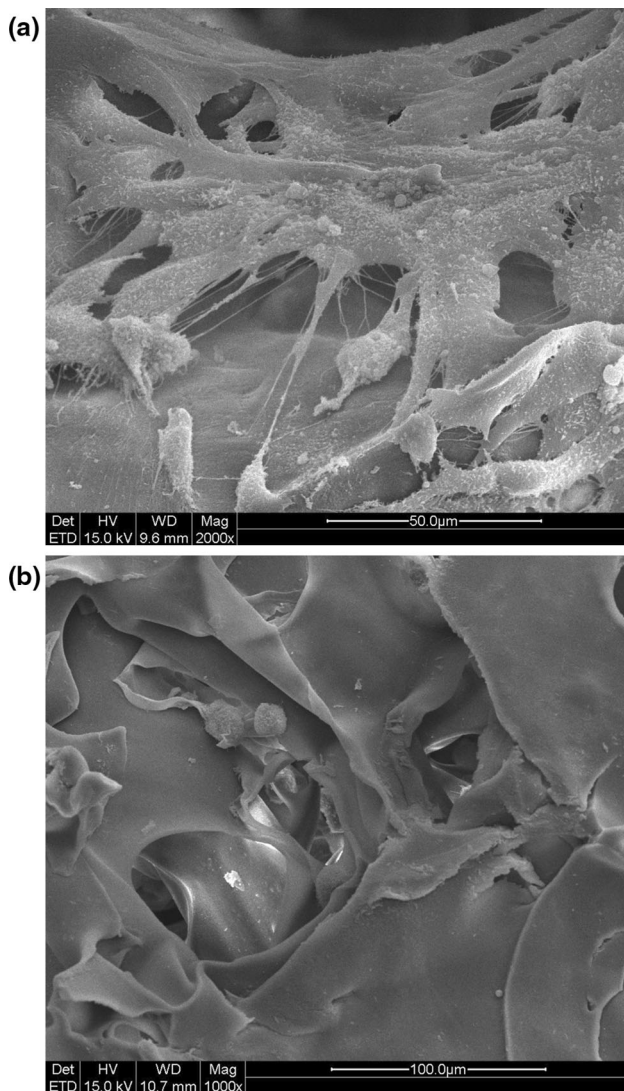
Fig. 6 **a** SEM micrograph of BMSCs growth in the inner area of the SF/C. **b** SEM micrograph of BMSCs growth on the inner area of the SF/CS scaffolds

The HE staining results confirmed the superiority of SF/C construct over SF/CS. BMSCs cultured in SF/C scaffolds were effectively moved into the inner zone. In contrast, cehydrophobicity could limit cell–cell interaction and migration [17].

SEM results showed two kinds of scaffolds with interconnecting pores. Possibility due to different pore size, SF/C scaffold showed the highest level of cells after 2 weeks culture. Pore size and morphology were different can be explained by crystallization of ice [22–24]. Water content of silk fibroin and blending constructs solution depended the pore size. The effect of pore size can attach cells and facilitated the cells proliferating [25] and suggest that porous structures and pore size can be controlled by varying the blending.

## Conclusions

A variety of biomaterial scaffolds have been used in tissue engineering, such as silk, collagen, chitosan and blend scaffolds, but few studies about comparing blend scaffolds functions of SF/C, SF/CS. This study used freeze-drying method to make scaffolds and according to physical properties and cell proliferation to choose optimized better type for tissue engineering. In conclusion, silk fibroin/collagen show the best characteristics and can be served as a new biomaterial. Also cell attachment, proliferation can be guided by RGD signal of silk. Therefore, silk fibroin/collagen scaffolds are suitable for tissue engineering. BMSCs can be induced cartilage osteoblast and other cells, so the scaffold in cartilage, bone tissue engineering and animals study stills remains to be further investigated.

**Conflict of interest** None.

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