

Fabrication and Characterization of Chitosan-Silk Fibroin/Hydroxyapatite Composites *via in situ* Precipitation for Bone Tissue Engineering*

Jing-xiao Hu^a, Xuan Cai^a, Shao-bo Mo^a, Li Chen^a, Xin-yu Shen^a and Hua Tong^{a**}
Key Laboratory of Analytical Chemistry for Biology and Medicine, Ministry of Education, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China

Abstract Homogeneous chitosan-silk fibroin/hydroxyapatite (CS-SF/HA) composites were prepared by *in situ* precipitation method driven by a multiple-order template. The morphology of the composites was investigated by scanning electron microscope (SEM) and transmission electron microscope (TEM). The compositional analysis was carried out by X-ray diffraction analysis (XRD) and Fourier transformed infrared spectroscopy (FTIR). The mechanical properties and biocompatibility of the composites were also determined. The results indicated that the inorganic particles of uniform size (50 nm) were well-dispersed among the CS-SF matrices. The compressive modulus of the CS-SF/HA composites was enhanced with the increasing amount of SF. The *in vitro* results suggested that the MC3T3-E1 osteoblast-like cells on CS-SF/HA composite disks displayed strong bonding and spreading, and the cell proliferation cultured on each composite disk increased throughout the culture period for up to 7 days. Especially, the samples with higher content of SF had much better biological properties. The evidences proved that the CS-SF/HA composites possessed excellent biocompatibility. By using the freeze-drying technique, hierarchical porous scaffolds with pores ranging from 50 μm to 200 μm were obtained. This work presented the advantages of *in situ* precipitation method to prepare the organic/inorganic composites, and a multiple-order template was introduced in the system to improve the properties of the composites by combining the merits of each organic template.

Keywords: Composites; *In situ* precipitation; Biocompatibility; Mechanical properties.

INTRODUCTION

For bone tissue engineering, as to prepare load-bearing bone substitutes, the natural bone consisting of inorganic minerals and biomacromolecules, is the best paradigm for biomimetic synthesis. The biomacromolecules in bone are mainly collagen (Col) fibrils, and the inorganic phase is mainly needle hydroxyapatite (HA) crystallizing along the long axis of collagen fibrils^[1]. The nucleation sites on chains of the biomacromolecules can induce the growth of the inorganic crystallites at the nucleation sites, and determine the way of crystallization and the size, orientation and structure of crystallites^[2–4].

As is well known, the natural load-bearing bone is a hierarchically porous composite. The nutrient transport and bone ingrowth can be accomplished through the delicate pore structure. So the three dimensional (3D) bone scaffolding materials with hierarchical pores have been designed to enhance the osteoconductivity and induce the formation of new bone tissue. However, the porous microstructure and interconnected macropores of pure polymer results in poor mechanical properties of the scaffolds. Hydroxyapatite (HA), a typical inorganic component of bone, has been widely used in synthesis of scaffolding materials^[5, 6]. The composites are prepared in a biomimetic way by introducing HA in the system. Due to the presence of HA, the structure and component

* This work was financially supported by the National Natural Science Foundation of China (Nos. 31071265 and 30900297).

** Corresponding author: Hua Tong (童华), E-mail: sem@whu.edu.cn

Received May 6, 2015; Revised May 27, 2015; Accepted May 27, 2015

doi: 10.1007/s10118-015-1710-3

of the composites are almost the same as those of with the natural bone, and the mechanical properties can be enhanced. In such practices, a few methods such as particulate leaching^[5], rapid prototyping^[7], gas foaming^[8], and electrospinning^[9] have been used for 3D porous scaffolds fabrication. But most of these methods were complex, and the reaction was done at high temperatures, or hazardous organic solvents were used; also, the hydroxyapatite particles were not well-dispersed in the matrix and the interaction was weak between the inorganic phase and the organic phase, which resulted in the undesirable mechanical properties of the scaffolds.

Recently, biocompatible polymers, both natural and synthetic, have been chosen to preparing composite scaffolds for bone repair. For bone tissue engineering, natural polymers such as chitosan^[10], collagen^[4, 11], cellulose^[12, 13], gelatin^[9], and silk fibroin^[14, 15] are preferred than synthetic polymers, because the natural polymers have better biodegradability, nontoxicity, biosecurity and biocompatibility.

Silk fibroin (SF) is a natural polymer, with outstanding biocompatible properties and high modulus. In the presence of SF, the osteoconductivity and osteoanagenesis can be improved without causing any inflammation^[16, 17]. Compared with collagen, SF is better in tenacity and also more cost-saving, which can be easily extracted. Moreover, SF belongs to plant based protein, which has better biosecurity than collagen.

Chitosan (CS), a cationic polymer, was extensively investigated and used in a wide range of applications because of its desirable biocompatibility, biodegradability, and nonantigenicity^[18, 19]. Therefore, CS is an ideal template for preparation of biocomposites.

In this work, CS-SF/HA composites with better mechanical properties and desirable biocompatibility have been fabricated successfully. The hierarchical pore structure of the obtained composites potentially provided proper environment for nutrients delivery and cell growth, and the aim of simulating biomineralization was achieved. The little compartments built by dual organic matrices had size effect on the growth of the inorganic crystallites at nanoscale. Meanwhile, the inorganic crystallites were regulated and controlled by the organic matrices with heterogeneous nucleation sites supported by SF molecular chains. The CS-SF/HA composites have potential clinical applications in both traditional bone-defect repairing and bone tissue engineering in future.

MATERIALS AND METHODS

Materials

Chitosan (CS, M_w 100 kDa, DD = 95%) was obtained from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Cocoons were purchased from Jingwei silk Co., Ltd. (Hubei, China) and from which silk fibroin (SF) was extracted. Dialysis membranes were obtained from Shenshi Co., Ltd. (Wuhan, China). Sodium hypochlorite solution, glutaraldehyde, acetic acid, sodium carbonate, lithium bromide (LiBr), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{HPO}_4$, sodium hypochlorite solution and ammonia were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were all of analytical grade. The chemicals were used as received without any further purification. All the aqueous solutions were prepared using ultra-purified water with a resistance of 18.2 M Ω -cm.

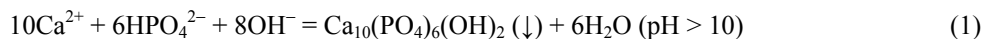
Methods

Fabrication of CS-SF/HA composites

Firstly, the SF was prepared according to the previous literature^[20]. Briefly, cocoons were degummed for 10 min in an aqueous Na_2CO_3 (0.5 W/V%) at 100 °C for three times in order to remove the glue-like sericin protein, then washed with ultra-purified water for several times and dried at room temperature. The degummed SF was dissolved in 9.0 mol/L LiBr solution at 40 °C for 4 h under gentle stirring, and dialyzed against ultra-purified water at room temperature for 3 days. The concentration of regenerated SF solution was 3.5 wt%.

For fabrication of CS-SF/HA composites *via in situ* precipitation, CS powder was dissolved in acetic acid solution (2 vol%) under gentle stirring for 2 h to get a homogeneous solution. After that, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{HPO}_4$ (Ca/P = 1.67) were dissolved in the as-prepared CS solution, and then 0.1 mL glutaraldehyde (25 wt%) was added and crosslinked with gentle stirring at room temperature for 2 min. Finally, SF solution was added drop-wise into the obtained mixture, and ultrasonically treated for 30 min and maintained at room temperature for 24 h to form a homogeneous hydrogel. Afterwards, aqueous ammonia was added onto the

hydrogel for the penetration from top of the gel to the bottom, and the whole penetration process was carried out in a beaker at room temperature for 24 h. HA was precipitated in the network of the hydrogel under the alkaline condition, and the reaction process was represented by the chemical formula:



The ammonia was refreshed every 4 h to maintain the pH value stable. After that, the composite hydrogel was rinsed with ultra-purified water repeatedly until the pH of the eluate was about 7. In the hydrogel, the organic components/HA weight ratio was 40/60, and the weight ratio of the CS/SF in composites was adjusted at 25/15, 30/10 and 35/5.

All the composites were calcinated to remove the organic matrix in muffle at 650 °C for 6 h. The HA precipitates were collected for further characterizations.

Fabrication of hierarchical porous CS-SF/HA composites

The composites were first processed by liquid nitrogen for 10 min, and then kept in refrigerator at −20 °C for 24 h. The hierarchical porous scaffolds were prepared by lyophilizing for 48 h (−50 °C, 3–5 Pa).

Characterization

Fourier transform infrared spectroscopy (FTIR, Nicolet5700, USA) was performed to characterize the samples. Morphological investigations were carried out by using an ESEM (Quanta200, FEI, Holland) and a FE-TEM (2010FEF, JOEL, Japan). The crystal phase was characterized by wide angle X-ray diffraction analysis (XRD, RIGAKU, Japan). The working condition of XRD was $\text{CuK}\alpha$ radiation *via* a rotating anode at 40 kV and 50 mA. The data were collected in step of 0.05 (°)/s and the range of scattering angles (2θ) was from 10° to 90°.

Mechanical Properties of CS-SF/HA Composites

Compressive strength test was performed at room temperature by a universal testing machine (SHIMADZU, AGS-J, Japan) at a crosshead speed of 1 mm/min. Before the test, the wet hydrogels were dried at room temperature with relatively constant humidity. Then the dry samples were planed and rubbed with sandpaper of different surface roughness. The size of the disks was kept with the diameter and height of 5 mm × 5 mm.

In Vitro Tests

Cell culture

The MC3T3-E1 osteoblast-like cells were obtained from the Hospital of Stomatology and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. The medium was refreshed every two days. The cells were treated with trypsinization prior to seeding onto the composites.

Cell adhesion

Cell adhesion is a primary index of biocompatibility. Herein, cell adhesion on the composite disks was observed by SEM. Before cell culture, all dried samples were sterilized by immersing in 75 vol% ethanol aqueous solution for 2 h, followed by ultraviolet radiation for 1 h. After 4 days, disks co-cultured with MC3T3-E1 osteoblast-like cells were washed twice with phosphate-buffered saline (PBS), and the adherent cells were fixed with 2.5% glutaraldehyde at 4 °C overnight. Then the disks were dehydrated by ethanol in an increasing concentration gradient (30 vol%, 50 vol%, 70 vol%, 90 vol%, 95 vol% and 100 vol%), followed by lyophilization. The dried disks were glued onto copper stubs, and sputter coated with gold prior to SEM observation.

Cell proliferation

After air drying for 3 days, the hydrogels were sliced into circular disks (diameter 5 mm, height 2 mm) before tests. MC3T3-E1 osteoblast-like cells (2.0×10^3 cells/well) were seeded on each disk, and the disk co-cultured with cells was placed in each well of the 24-well plates. Then the cell viability was studied using Counting kit-8 assay (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's instructions after culture for 1 day, 4 days, and 7 days. Briefly, 450 μL serum-free culture medium and 50 μL CCK-8 solutions were added to each

sample, after incubation at 37 °C for 3 h, the supernatant was transferred to a 96-well plate, and the optical density (O.D.) was measured at 450 nm using an ELX808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., USA).

Statistical Analysis

All data were reported as means $\pm$ standard deviation. Results were analyzed by using one-way analysis of variance (ANOVA) test (SPSS 19.0 software, USA). Results with p -values of < 0.05 (*) and < 0.01 (**) were considered statistically significant.

RESULTS AND DISCUSSION

Compositional Analysis

As shown in Fig. 1(A), SF has been incorporated into the composite system by comparing the bands at 1642 and 1600 cm^{-1} of pure CS with those of the composites around 1633 and 1541 cm^{-1} . Besides, the inorganic phase displayed quite the same bands with those of the stoichiometric HA around 1095, 1033, 961, 603 and 567 cm^{-1} corresponding to the different vibration modes of phosphate groups of HA, and the bands around 3570 and 633 cm^{-1} were assigned to stretching and bending vibration of hydroxyl group^[21]. Meanwhile, bands around 1450 and 1419 cm^{-1} were assigned to carbonate groups, which was identical with the fact that the HA crystals

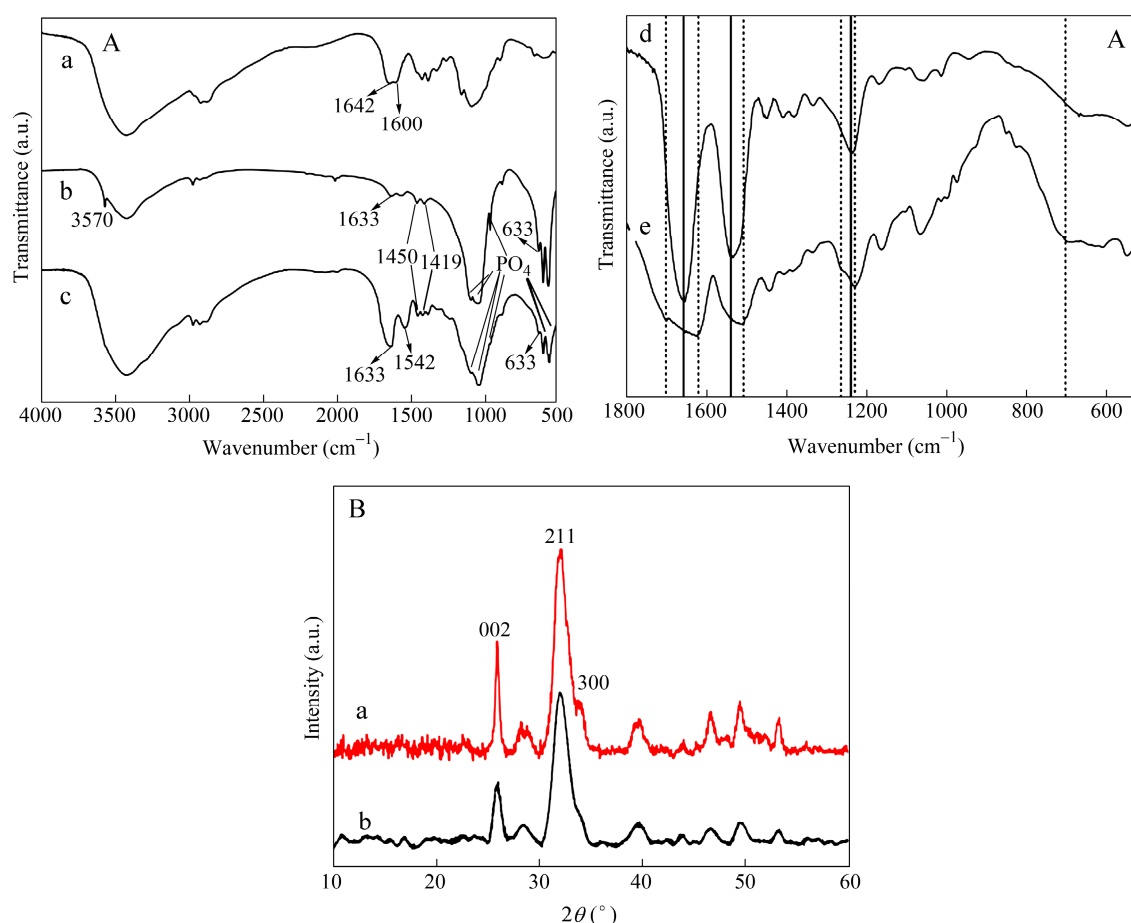


Fig. 1 (A) FTIR spectra of (a) pure CS, (b) the inorganic phase of the CS-SF/HA composite (CS/SF/HA = 30/10/60) after 650 °C, (c) the CS-SF/HA composite (CS/SF/HA = 30/10/60), (d) SF solution, and (e) gelling SF (The solid indicates the random coil or α -helix; the short dash indicates the β -sheet); (B) XRD patterns of (a) the CS-SF/HA composite (CS/SF/HA = 30/10/60) and (b) CS/HA composite (40/60)

prepared *via* the precipitation method contained carbonate ions^[22]. Interestingly, a band at about 1633 cm⁻¹ of inorganic particles after calcination demonstrated that certain amount of residual SF was observed, which verified there was strong bonding between the hydroxyl groups of SF and hydroxyl groups of HA even forming ether bonds.

The conformation of SF at different states was also investigated to find the effect of conformational changes on the properties of CS-SF/HA composites. Herein, the SF solution and gelling SF were studied by FTIR (Fig. 1A, spectra d and spectra e). Apparently, the spectra exhibited significant differences. According to the previous literature about the FTIR analysis of SF^[20], it can be concluded that the conformation of gelling SF was β -sheet, and in solution, the main conformation of SF was random coil or α -helix. Meanwhile, it can be seen that conformations of β -sheet and random coil coexisted in the CS-SF/HA composites, and these conformations were different from that of bulk SF hydrogel. In conclusion, SF presented multi-conformation in the composites due to the intricate interactions including hydrogen bonds and electrostatic effects.

XRD pattern of the CS-SF/HA composite in Fig. 1(B) presented typical apatite crystal structure. This XRD result was verified by the Power Diffraction File PDF (Card No.9-432). The diffraction characteristic peaks around 26° and 32° (2 θ) were corresponded to the peaks of HA. It can be seen that the crystal planes (002, 211, and 300) of CS-SF/HA composite had higher intensity than those of CS/HA composite, especially, the plane (300) was apparently presented in CS-SF/HA composite (Fig. 1B). The results indicated that the HA crystals in CS-SF matrices preferentially tended to grow along the *c*-axis^[4], however the crystals in CS matrix didn't have the tendency. Thus, the SF had effect on regulating and controlling the growth of HA crystals in CS matrix. Meanwhile, the size effect of the 3D CS network microstructure limited the growth of inorganic crystals so that the size of HA was at nanoscale. In addition, the organic components in the CS-SF/HA composites were in amorphous phase and no diffraction peaks could be observed in Fig. 1(B). In conclusion, crystallographic structure of the HA in the CS-SF/HA composites was much alike to that of natural bone mineral (biological apatite)^[22, 23].

Morphology Investigation

As shown in Figs. 2(a) and 2(b), inorganic particles were well-dispersed in the composites, and the average diameter of these particles was about 50 nm, which demonstrated that the inorganic particles were regulated and controlled by the organic matrices. The inorganic crystallites tended to aggregate in the CS/HA composites, however, the particles in CS-SF/HA composites were well-dispersed, which showed that SF played a pivotal role in the crystalline fabrication of the well-dispersed HA nanoparticles even controlling the size of HA. It can be speculated that the intimate combination of inorganic and organic phase at nanoscale could be attributed to the electrostatic interaction and hydrogen bonding effect of the complex gelling system.

Comparing the morphology of inorganic particles in CS-SF/HA composites before/after calcination, we find the residual aggregates still retain the original structure instead of melting into powder after calcination, and the outline of the particles gets more distinct, due to the removal of organic matrix. As shown in Fig. 2(d), the size of the gaps between HA nanoparticles were within several nanometers width. Besides this, the gap between the crystalline region and amorphous region of the composites was less than 0.5 nm (Fig. 2f), which proves again the strong combination between organic and inorganic phases of the CS-SF/HA composites. Therefore, the prominent advantage of *in situ* precipitation method could be further testified.

Furthermore, according to TEM results (Figs. 2e and 2f), the rod-like aggregates were displayed, which was a typical morphology of HA in TEM images. The rod-like crystals proved that the HA crystals grew along the *c*-axis in the presence of SF, which was consistent with the deduction based on the XRD results. Also, the sub-crystallites at nanoscale had no uniform crystallographic orientation in the organic matrices. Meanwhile, the polycrystal diffraction ring and the amorphous spot shown in the inset also accorded with the structure in Fig. 2(f), and the amorphous zones surrounded the lattice stripe regions. The random orientation of the sub-crystallites was presented, which results in the isotropic structure of the composites.

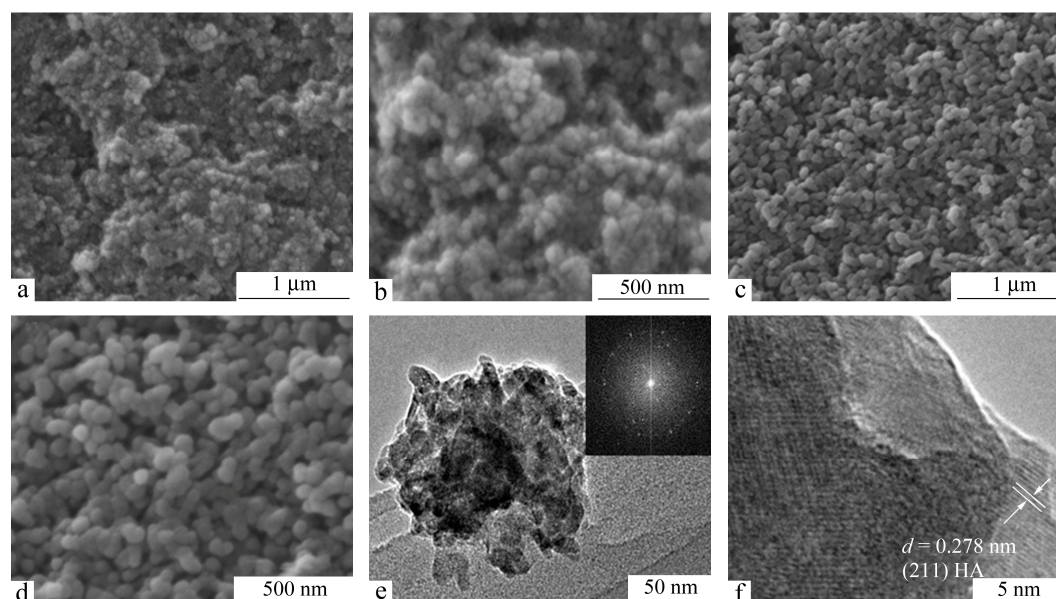


Fig. 2 SEM images of CS-SF/HA composite (CS/SF/HA = 30/10/60) of different magnifications (a) and (b); SEM images of the inorganic phase of the CS-SF/HA composite (CS/SF/HA = 30/10/60) after 650 °C of different magnifications (c) and (d); TEM micrograph of CS-SF/HA composite (CS/SF/HA = 30/10/60) (The inset shows a polycrystal diffraction ring and diffused spots) (e) and highly magnified TEM image of crystal lattice (f)

Synthesis and Formation Mechanisms of CS-SF/HA Composites

From the point of synthesis, it is reported that the metal ions could affect the conformation of SF^[24]. For example, Ca^{2+} can help to maintain the stable structure of SF in solution due to the affinity for Ca^{2+} endowed by carboxyl groups on the side chains of SF. A large number of Ca^{2+} existed in the precursor solution, in order to make *in situ* precipitation method accessible to form stable hydrogels, CS was selected as the main part of organic phase, and SF as the additive.

A formation mechanism of the CS-SF/HA composites was proposed (Fig. 3). The CS template probably supported the whole hydrogel system by forming 3D network, and it had less effect on regulating and controlling the morphology of the inorganic particles than SF. According to this deduction, the inorganic spherical nanoparticles in the composites might be mostly regulated and controlled by SF.

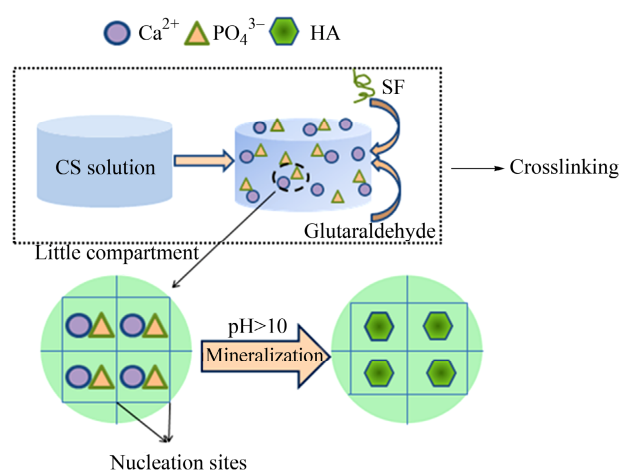


Fig. 3 The schematic of the formation mechanism of homogeneous inorganic/organic composites *via in situ* precipitation

To verify the above hypothesis, the SEM morphology of SF hydrogel was investigated, and the results are shown in Fig. 4. The spherical SF particles with 50 nm average diameter almost occupied the whole gel system (Figs. 4c and 4d). Besides, the intramolecular crosslinking mainly derived from the hydrogen bonds that might cause the fragility and low transparency of SF hydrogel. However, after mixing SF with CS solution, SF possibly participated in the crosslinking behavior of CS with the addition of glutaraldehyde. Tyr, His and Lys residues of SF were crosslinked with CS molecules to form the intermolecular network. To a certain degree, the network limited the crosslinking behavior of SF molecules. According to the FTIR results in Fig. 1, random coil (silk I) conformation still existed in CS-SF/HA composites^[21]. Park *et al.* found a higher content of β -sheet conformation (47%) in pure SF molecules than that (34%) in SF/CS blends (70/30) after methanol treatment^[21]. In our work, the weight content of CS was higher than that of SF (35/5, 30/10, 25/15), so it can be deduced that the content of β -sheet conformation in the CS-SF/HA was lower than that reported by Park *et al.* As a result, when the ratio of β -sheet decreased in CS-SF/HA composites, the ratio of random coil increased, indicating that part of SF molecules could not transform into β -sheet, due to their participation in the crosslinking behavior of CS. In CS-SF/HA composites, parts of SF molecules formed particles distributing uniformly in the composites by crosslinking intramolecularly *via* hydrogen bonding effect, and some other SF molecules participated in the intermolecular crosslinking behavior with CS molecules to form CS-SF hydrogel. The latter sort of SF particles had an effect on heterogeneous nucleation of spherical HA nanoparticles during the entire process of *in situ* precipitation. Since the amount of CS was larger than that of SF in the composites, so the amount of SF particles formed *via* hydrogen bond in the composites should be less than those in pure SF hydrogel, so the entangled SF molecules also became less and the nucleating sites for hydroxyapatite were more uniformly distributed. Besides, electrostatic interaction and strong hydrogen bonding effect might occur between SF and CS, due to the carboxyl groups on the side chains of SF and the hydroxyl groups and amino groups of CS^[14, 21]. This interaction can also affect the crosslinking behavior of the polymers and the formation of HA crystallites.

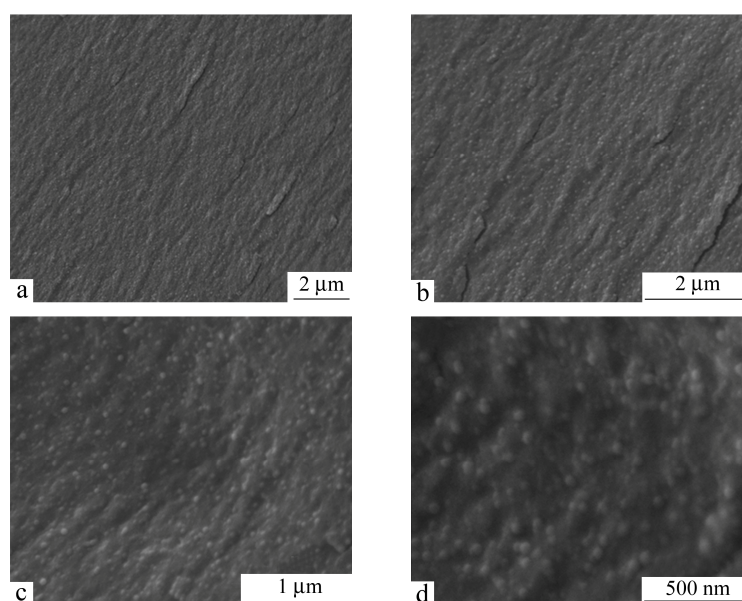


Fig. 4 SEM images of pure SF hydrogel of different magnifications (a), (b), (c) and (d)

Hierarchical Pore Structure

Generally, multilevel lyophilization technique was employed to prepare porous scaffolds. In Fig. 5, CS-SF/HA composites possessed hierarchical pore structure with high porosity. The pores exhibited two kinds of configuration: one is with small pore ($< 5 \mu\text{m}$) in continuous distribution within large area; the other is with large independent pore ($50\text{--}200 \mu\text{m}$) interspersing among the small pores. And it has been reported that macropores

(> 50 μm) can contribute to osteogenesis by facilitating cell and ion transport, while micropores (< 20 μm) can improve bone growth by expanding surface area for protein adsorption^[25]. Through freeze drying method, we found that the CS-SF/HA composites were easy to form more ordered and delicate pores than other composites reported by some researchers^[9, 26]. This characteristic can be attributed to the high strength of SF and the interaction of SF and CS, leading to the satisfying toughness of the CS-SF/HA composites.

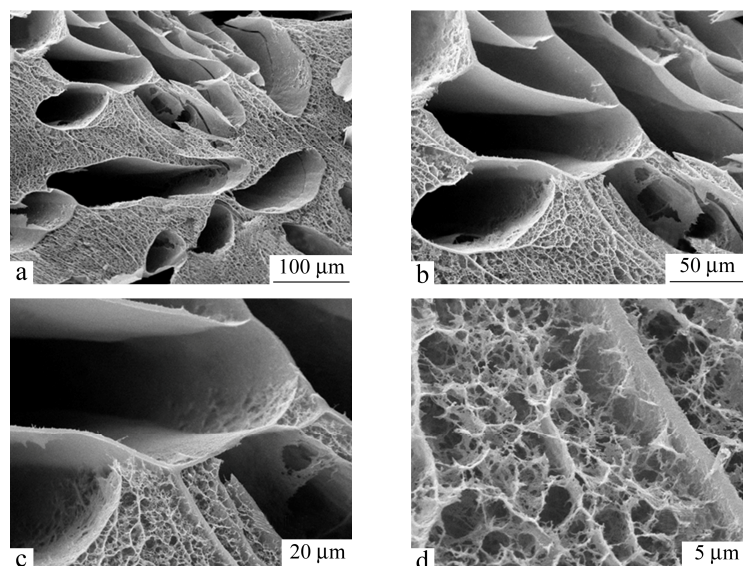


Fig. 5 SEM images of freeze-drying CS-SF/HA composite (CS/SF/HA = 30/10/60) of different magnifications: (a), (b), (c) and (d)

Mechanical Properties

After air-drying, the mechanical properties of the composites were investigated. Figure 6(a) shows the stress-strain curves of the samples. All the samples presented similar stress-strain behavior. The stress increased dramatically during the initial stage and decreased gradually until final fracture. In Figs. 6(b) and 6(c), the CS-SF/HA composites displayed better mechanical properties with the increasing amount of SF. Besides, the elastic modulus of all CS-SF/HA samples was higher than 1000 MPa (1776 MPa when CS/SF ratio was 25/15), indicating that the stiffness was enhanced significantly with the addition of SF, thus the composites would not be liable to deform. Meanwhile, the CS-SF/HA composites exhibited higher compressive strength than that of the CS/HA composite. And the compressive strength was elevated as the SF ratio increased. Obviously, the brittleness also increased after adding SF. The increased brittleness of CS-SF/HA composites should be mainly attributed to the spherical HA nanoparticles which tended to dislocate and shift under compression. Besides, the macromolecular chain of SF had relatively high stiffness. Wang *et al.*^[1] reported that the elastic modulus and ultimate strength of bone were 11.3 ± 1.8 GPa and 194 ± 21 MPa, respectively. In this work, even the lowest elastic modulus of CS-SF/HA composites was more than 10 GPa, and the compressive strength of the CS-SF/HA composites was in the range of 120–143 MPa. In conclusion, the mechanical properties of CS-SF/HA composites can be potentially used for load-bearing bone repair. The homogeneous distribution of HA particles and the β -sheet conformation of SF endowed the CS-SF/HA composites with desirable mechanical properties.

Cell Viability

MC3T3-E1 osteoblast-like cells were seeded on the composite disks. Herein, the CS/HA composite was selected as the control group. Cell adhesion was the first marker for evaluating the biocompatibility of the biomaterials. In Fig. 7, the cells displayed strong bonding and spreading on all the composite disks, and polygonal morphology was presented (Figs. 7a–7d).

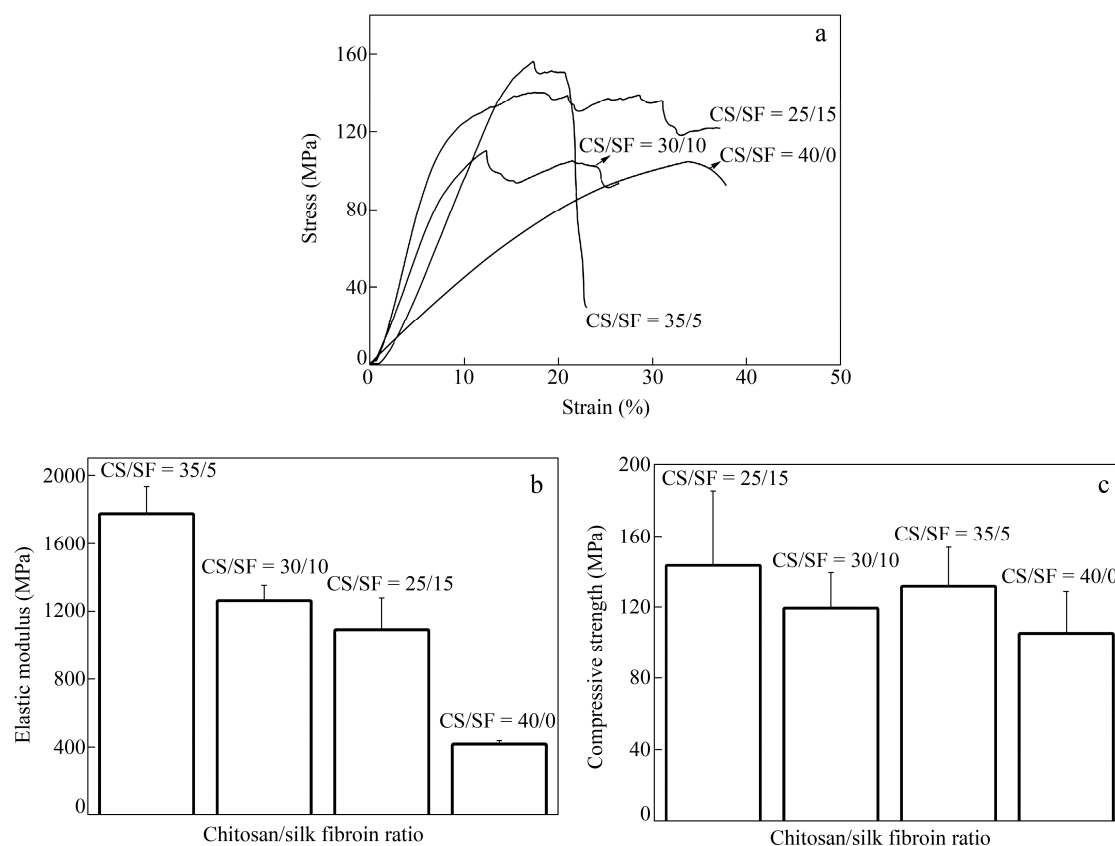


Fig. 6 Mechanical property curves of CS-SF/HA composites (organic/inorganic weight ratio 40/60) with different CS/SF weight ratios: (a) compressive stress-strain curve, (b) elastic modulus-CS/SF weight ratio bar graph and (c) compressive strength-CS/SF weight ratio bar graph

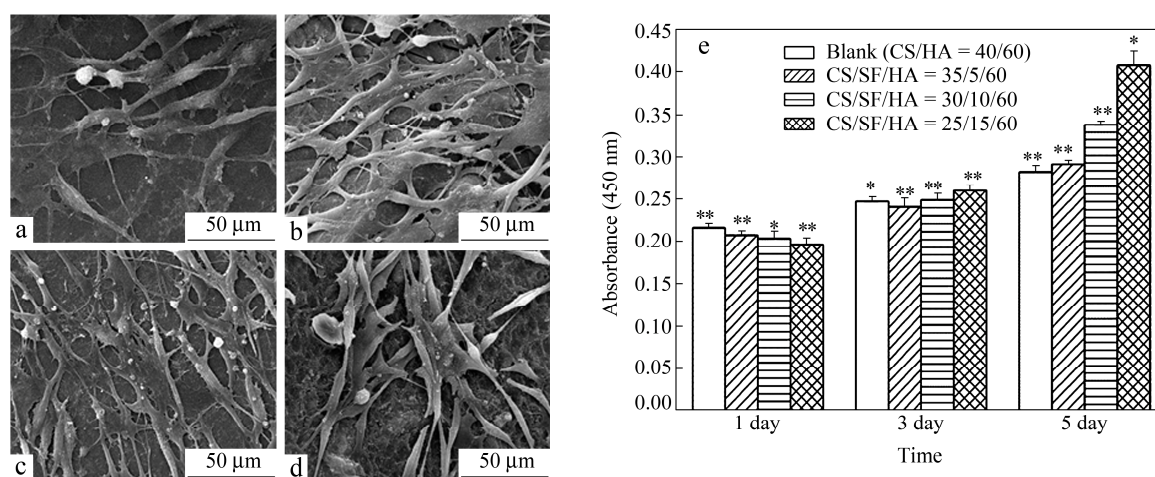


Fig. 7 SEM micrographs of MC3T3-E1 cell morphology on organic/HA composites with organic/inorganic weight ratio of 40/60 after incubation for 4 days: (a) blank (CS/HA = 40/60), (b) CS-SF/HA composite (CS/SF/HA = 35/5/60), (c) CS-SF/HA composite (CS/SF/HA = 30/10/60), (d) CS-SF/HA composite (CS/SF/HA = 25/15/60) and (e) CCK-8 assay of the proliferation of MC3T3-E1 cells cultured on the CS-SF/HA composite disks with different SF weight ratios and blank control ($n = 4$) (p -values of < 0.05 (*) and < 0.01 (**)) were considered statistically significant.)

The CCK-8 assay kit was used to evaluate the cell proliferation (Fig. 7e). After 1 day culture, the cells seeded on the disks with higher CS contents showed relatively higher viability, which proves that CS has desirable biocompatibility indeed. After 3 days post-seeding, the cells seeded on the CS-SF/HA composite disks with the highest SF content presented the highest viability. And the groups displayed the similar behavior after culturing for 5 days. Hence, the cell proliferation was significantly enhanced owing to the satisfying biocompatibility of SF even at a low concentration.

CONCLUSIONS

By simulating the composition of natural bone at the organic/inorganic weight ratio of 40/60, CS-SF/HA composites were prepared *via in situ* precipitation. SF was introduced into CS matrix. As a result, the elastic modulus of CS-SF/HA composites was 2–3 times higher than that of CS/HA. Meanwhile, SF as the heterogeneous nucleation sites regulated and controlled the morphology of HA crystallites. The spherical HA nanoparticles in the CS-SF/HA composites were well-dispersed without aggregation owing to the size effect of CS matrix, and the intimate combination between the inorganic crystallites and organic matrices further proved the advantage of *in situ* precipitation method. Additionally, *in vitro* tests indicated that CS-SF/HA composites had good biocompatibility and cytoactivity. In conclusion, this work will be a meaningful supplement for the researches of bone tissue engineering.

REFERENCES

- 1 Wang, X.D., Bank, R.A., TeKoppele, J.M. and Agrawal, C.M., *J. Orthop. Res.*, 2001, 19(6): 1021
- 2 Wu, S.L., Liu, X.M., Yeung, K.W.K., Liu, C.S. and Yang, X.J., *Mat. Sci. Eng., R: Reports*, 2014, 80: 1
- 3 Gelinsky, M., Welzel, P.B., Simon, P., Bernhardt, A. and Konig, U., *Chem. Eng. J.*, 2008, 137(1): 84
- 4 Zhang, Y. and Lu, J., *Cryst. Growth Des.*, 2008, 8(7): 2101
- 5 Liu, L., Liu, J.Y., Kong, X.D., Cai, Y.R. and Yao, J.M., *Polym. Adv. Technol.*, 2011, 22(6): 909
- 6 Dadsetan, M., Guda, T., Runge, M.B., Mijares, D., LeGeros, R.Z., LeGeros, J.P., Silliman, D.T., Lu, L.C., Wenke, J.C., Baer, P.R.B. and Yaszemski, M.J., *Acta Biomater.*, 2015, 18: 9
- 7 Cirillo, B., Morra, M. and Catapano, G., *Int. J. Artif. Organs*, 2004, 27(1): 60
- 8 Rodrigues, S.C., Salgado, C.L., Sahu, A., Garcia, M.P., Fernandes, M.H. and Monteiro, F.J., *J. Biomed. Mater. Res., Part A.*, 2013, 101(4): 1080
- 9 Gong, H., Agustin, J., Wootton, D. and Zhou, J.G., *J. Mater. Sci. Mater. Med.*, 2014, 25(1): 113
- 10 Swetha, M., Sahithi, K., Moorthi, A., Srinivasan, N., Ramasamy, K. and Selvamurugan, N., *Int. J. Biol. Macromol.*, 2010, 47(1): 1
- 11 Hartwell, R., Leung, V., Chavez-Munoz, C., Nabai, L., Yang, H., Ko, F. and Ghahary, A., *Acta Biomater.*, 2011, 7(8): 3060
- 12 Zadeh, S., Hosainipour, M., Rezaie, H.R., Ghassai, H. and Shokrgozar, M.A., *Mater. Sci. Eng., C: Mater. Biol. Appl.*, 2011, 31(5): 954
- 13 Deng, H.B., Zhou, X., Wang, X.Y., Zhang, C.Y., Ding, B., Zhang, Q.H. and Du, Y.M., *Carbohydr. Polym.*, 2010, 80(2): 474
- 14 He, J., Cheng, Y., Li, P., Zhang, Y., Zhang, H. and Cui, S., *Iran Polym. J.*, 2013, 22(7): 537
- 15 Meinel, L., Fajardo, R., Hofmann, S., Langer, R., Chen, J., Snyder, B., Vunjak-Novakovic, G. and Kaplan, D., *Bone*, 2005, 37(5): 688
- 16 Wei, K., Li, Y., Kim, K.O., Nakagawa, Y., Kim, B.S., Abe, K., Chen, G.Q. and Kim, I.S., *J. Biomed. Mater. Res., Part A*, 2011, 97A(3): 272
- 17 Meinel, L., Hofmann, S., Karageorgiou, V., Kirker-Head, C., McCool, J., Gronowicz, G., Zichner, L., Langer, R., Vunjak-Novakovic, G. and Kaplan, D.L., *Biomaterials*, 2005, 26(2): 147

- 18 Li, W., Li, X.Y., Li, W., Wang, T., Li, X.X., Pan, S.Y. and Deng, H.B., *Eur. Polym. J.*, 2012, 48(11): 1846
- 19 Aguzzi, C., Sandri, G., Bonferoni, C., Cerezo, P., Rossi, S., Ferrari, F., Caramella, C. and Viseras, C., *Colloid Surf., B: Biointerfaces.*, 2014, 113: 152
- 20 Kim, K.H., Jeong, L., Park, H.N., Shin, S.Y., Park, W.H., Lee, S.C., Kim, T.I., Park, Y.J., Seol, Y.J., Lee, Y.M., Ku, Y., Rhyu, I.C., Han, S.B. and Chung, C.P., *J. Biotechnol.*, 2005, 120(3): 327
- 21 Park, W.H., Jeong, L., Yoo, D.I. and Hudson, S., *Polymer*, 2004, 45(21): 7151
- 22 Zhang, Y., Cao, C.B., Lu, Q., Ma, X.L. and Zhu, H.S., *Acta Polymerica Sinica (in Chinese)*, 2004, (4): 556
- 23 Yamaguchi, I., Tokuchi, K., Fukuzaki, H., Koyama, Y., Takakuda, K., Monma, H. and Tanaka, T., *J. Biomed. Mater. Res.*, 2001, 55(1): 20
- 24 Li, G.Y., Zhou, P., Sun, Y.J., Yao, W.H., Mi, Y., Yoa, H.Y., Shao, Z.Z. and Yu, T.Y., *Chem. J. Chinese Univ-Chinese (in Chinese)*, 2001, 22(5): 860
- 25 Mobini, S., Hoyer, B., Solati-Hashjin, M., Lode, A., Nosoudi, N., Samadikuchaksaraei, A. and Gelinsky, M., *J. Biomed. Mater. Res., Part A*, 2013, 101A(8): 2392
- 26 Koshkaki, M., Ghassai, H., Khavandi, A., Seyfoori, A. and Molazemhosseini, A., *Iran Polym. J.*, 2013, 22(9): 653