Preparation and evaluation of chitosan-silk sericin/hydroxyapatite nanocomposites for bone tissue engineering

Li Chen, Yifei Long, Junli Zhao, Wei Wang, Haiyan Liang, Qiuxin Liu *

Department of Urban Construction, City College, Wuhan University of Science and Technology, Wuhan 430083, China

* Corresponding author

Keywords: chitosan; silk sericin; hydroxyapatite; bone tissue engineering

Abstract: In this study, a simple and effective technique was developed to synthesize chitosan-silk sericin /hydroxyapatite nanocomposites by in situ precipitation. SEM showed that the rod-like hydroxyapatite particles with a diameter of 20-50 nm were distributed homogeneously within the chitosan-silk sericin matrix. The result of XRD indicated that the inorganic phase in the nanocomposite was carbonate-substituted hydroxyapatite with low crystallinity. *In vitro* cytocompatibility of the nanocomposite was evaluated by SEM through MG63 osteoblast cells cultured on the samples, which demonstrated that they are non-toxic and support cell growth. These results suggest that the chitosan-silk sericin/hydroxyapatite nanocomposites are promising biomaterials for bone tissue engineering.

1. Introduction

Bone tissue engineering is a rapidly developing discipline used in order to repair, replace and regenerate injured bone tissue [1]. In bone tissue the extracellular matrix (ECM) consists of an organic phase made of type I and type III collagen and glycosaminoglycans (GAGs) and an inorganic phase made up of hydroxyapatite [2].

Chitosan (CS) is a linear polysaccharide derived by partial N-deacetylationof chitin, which is the primary structural polymer in arthropod exoskeletons, shells of crustaceans, or the cuticles of insects [3]. CS is widely applied in bone tissue engineering because of its special characteristics, such as structural similarity to the various glycosaminoglycans found in the ECM of bone, osteoconductivity to enhance bone formation both *in vitro* and *in vivo*, good biodegradability, and excellent biocompatibility [4,5]. However, its bioactivity isn't good enough for bone tissue engineering and it is frequently combined with biologically active materials like collagen, silk sericin and hydroxyapatite [6].

Hydroxyapatite (HA), which is the mineral constituents of human skeleton, has been currently used in bone tissue engineering due to its excellent bioactivity and biocompatibility [7]. Furthermore, the osteoconduction, non-inflammation and non-toxicity of HA enable osteoblast adhesion, proliferation and differentiation. Nowadays, there are some methods on preparing CS/HA composite materials, including co-precipitation [8], alternate soaking [9] and mechanical mixing [10]. Among these methods, there is a common shortcoming that inorganic particles cannot be distributed homogeneously in the organic matrices at nanolevel, which leads to poor mechanical properties and limits their applications.

Silk sericin (SS) is a protein secreted from the middle silk gland of a mature silkworm larva and acts as the glue for adhesion of fibroin based fibers during cocoon formation [11]. Especially, it has been proved that SS has been shown to enhance functionality in promoting osteoblast adhesion, proliferation, and alkaline phosphatase activity [12]. Hence, SS was introduced into the CS/HA system to enhance the cytocompatibility of CS/HA nanocomposite.

Based on the above knowledge, homogeneous CS-SS/HA nanocomposites were prepared by the in situ precipitation technique, which is totally different from the traditional ones and rarely reported in the synthesis of CS-SS/HA composites. In comparison with other methods, the

  DOI: 10.25236/eeems.2018.058

superiority of in situ precipitation is unique morphology and ultrafine HA particles can be produced, and moreover, distributed homogeneously within the organic template. What's more, this method had another important merit that the products had no other impure inorganic component except HA in composition by comparison with other in situ precipitation methods. In the present study CS-SS hydrogel cross-linked by genipin was constructed. The composition and morphology of as-synthesized nanocomposites were mainly analyzed by X-ray diffraction (XRD) and field emission scanning electron microscopy (SEM). Cytocompatibility of CS-SS/HA nanocomposites was finally evaluated based on MG63 osteoblast cells morphologic changes.

2. Materials and Methods

Chitosan (Mw 1,000,000) was obtained from Golden-Shell Biochemical Co. (Zhejiang, China) with 95% degree of the deacetylation. Bombyx mori silk sericin (Mw 30,000) was purchased from Huzhou Xintiansi Biotechnology Co., Ltd. (China). Genpin was purchased from Chengdu ConBon Bio-tech CO., Ltd. (China). All the reagents used in this work were of analytical grade (AR) and used without any further purification. Deionized ultrapure water was used throughout the experiment.

CS solution was prepared by dissolving 0.16 g CS in 40 ml of acetic acid solution (2 vol.%) with continuously stirring at 45 ºC until it became perfectly transparent. Then 0.16 g SS powder was added into the CS solution and stirred at 45 ºC for 30 min. Afterwards, 1.128 g $Ca(NO_3)_2 \cdot 4H_2O$ and 0.378 g $(NH_4)_2HPO_4$ were together added to the mixed solution under agitation until the salts were entirely dissolved. Subsequently, 0.048 g genpin was added to the mixed solution as a crosslinking agent. The solution was continuously stirred until a blue hydrogel formed. The resulting hydrogel was then stored under ambient conditions for a certain time to reach complete crosslinking. Ammonia solution was then poured on the top of the blue hydrogel at room temperature. Under this alkaline condition, HA precipitated within the hydrogel gradually. The in situ precipitation method can be represented by the following chemical reaction:

$$10Ca^{2+} + 6HPO_4^{2-} + 8OH^- + Ca_{10}(PO_4)_6(OH)_2(\downarrow) + 6H_2O \quad (pH > 10)$$

The nanocomposite was finally washed with disilled water until the pH of eluate was about 7, followed by drying at room temperature to obtain the solid nanocomposite.

CS/HA nanocomposites as control samples were also prepared by in situ precipitation. The procedures are the same as described in Section 2.2.1, but without the addition of SS. An opaque composite of CS/HA was produced.

Morphology of inorganic/organic composite was observed using Environmental Scanning Electron Microscopy (SEM, Quanta200, FEI, Holland and SEM, Sigma, Zeiss, Germany). The crystalline phase and component of obtained products were identified using wide angle X-ray diffraction analysis (XRD, X'pert PRO, Panalytical, Holland).

Samples were made into circular discs suitably sized (diameter 10 mm, height 1 mm). Behaviors of MG63 cells on both CS-SS/HA and CS/HA nanocomposites were studied by SEM. After cultivation for 3 days, composites grown with cells were washed twice with PBS, and cells were fixed with 2.5 wt.% glutaraldehyde under 4 ºC overnight. Fixed samples were dehydrated by ethanol in an increasing concentration gradient, followed by lyophilization. The dried samples were glued onto copper stubs, and sputter coated with gold prior to SEM observation.

3. Results and discussion

Inorganic phase composition of CS/HA (Fig. 1a) and CS-SS/HA (Fig. 1b) nanocomposite were measured by using XRD (Fig. 1). The predominant crystal phase of all samples was HA corresponding to the Powder Diffraction File (PDF Card No. 9-432). The peaks of crystal phases at 25.9°, 32° and 39.7° (2θ) are assignable to (002), (211) and (310) of crystalline HA, respectively. As shown in Fig. 1a,b, two samples revealed broad peaks with poor crystallinity around the characteristic diffraction region near 32° (2θ), which signified that HA had low crystallinity in all

samples. This crystallographic structure of two samples was more similar to natural bone mineral (biological apatite) [13]. The reason for the low crystallinity of precipitated HA in all samples might be the size effect owing to the three-dimensional network microstructure provided by the crosslinked CS-SS hydrogel, where the growth of inorganic crystal was limited. In spite of this, CS-SS/HA nanocomposite possessed higher crystallinity than CS/HA nanocomposite based on (211) peak, indicating the possibility of different preferential orientation growth in the presence of SS.

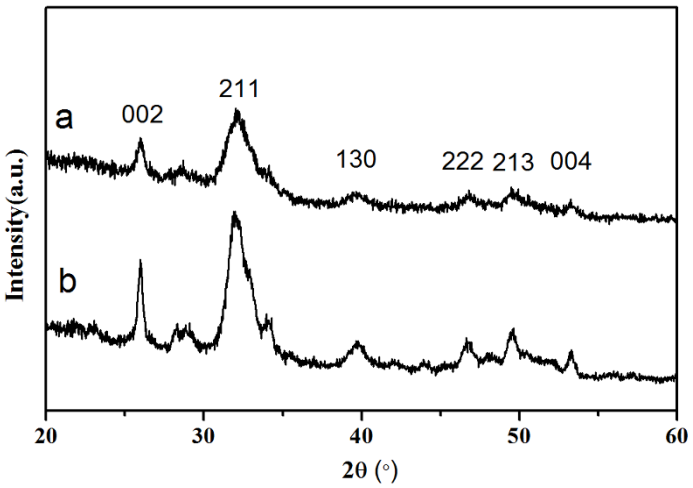

Fig. 1 XRD pattern of (a) the CS/HA nanocomposite; (b) the CS-SS/HA nanocomposite

SEM morphologies of the CS-SS/HA and CS/HA nanocomposites are shown in Fig. 2a-d. From the SEM results of the CS-SS/HA nanocomposite (Fig. 2a,b), it could be observed that inorganic crystals of HA are tightly bonded with the CS-SS matrix, because no interface between the inorganic and organic phases can be distinguished. It is difficult to get this decentralization effect by conventional mechanical mixing or co-precipitation [14]. The inorganic particles exhibited as rod-like crystals whose size was over 200 nm in length and 20–50 nm in diameter. However, the SEM images for the CS/HA nanocomposite where many spherical particles were found are presented in Fig. 2c,d. This signifies that SS was responsible for the formation of the uniform rod-like HA nanoparticles. In our work, CS hydrogel played an important role in the regulation and decentralization of inorganic nanoparticles through its compartment effect. SS was responsible for the formation of the uniform rod-like HA nanoparticles.

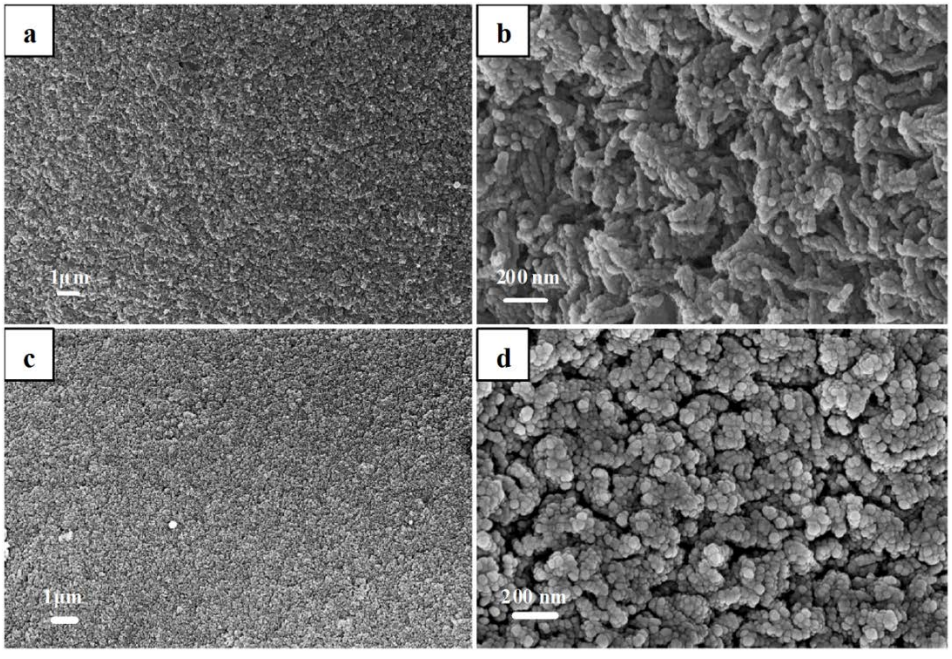

Fig. 2 SEM micrograghs of (a,b) the CS-SS/HA nanocomposite; (c,d) the CS/HA nanocomposite

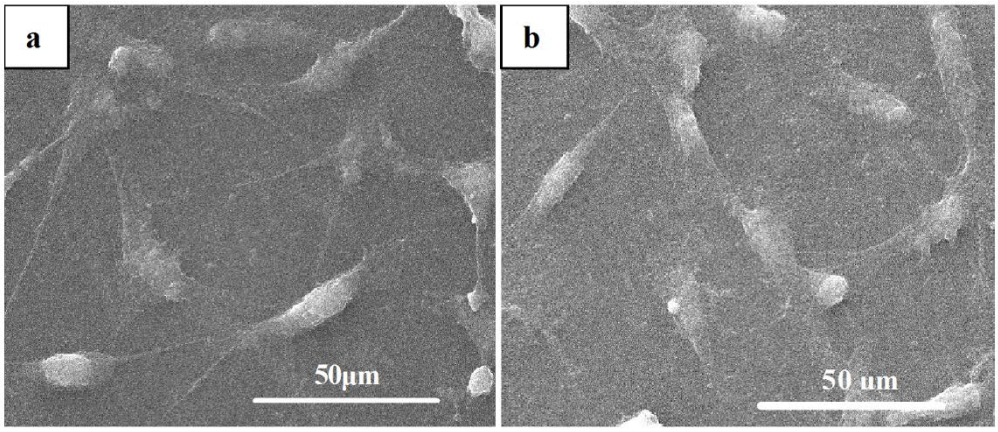

Fig. 3 SEM micrographs of MG63 cell morphology on nanocomposites after incubation for 3 days: (a) CS-SS/HA nanocomposite and (b) CS/HA nanocomposites

In this work, the preliminary biological performance of the CS-SS/HA nanocomposites was evaluated by *in vitro* culturing of MG63 cells. SEM observation of cell cultures to evaluate morphologic changes is most frequently used in cytotoxicity evaluation of biomaterials [15, 16]. Fig. 3a,b reveals that MG63 cells cultured for 3 days adhered on the surface of CS-SS/HA and CS/HA nanocomposites. Clearly, it can be observed that MG63 cells exhibited fusiform or polygonal morphology and distributed well on all the samples. Furthermore, MG63 cells re-established cell-cell contacts and formed aggregates on the CS-SS/HA nanocomposites, which meant that CS-SS/HA nanocomposite was propitious to the attachment and growth of MG63 cells.

4. Conclusions

CS-SS/HA nanocomposites were systhesized by a new in situ precipitation method. The introduction of SS in the CS matrix greatly had a large influence on the nucleation and the growth of HA crystalline. Such a double temple based on the CS hydrogel and the intensive heterogeneous nucleation sites of SS played an important role in the fabrication of the CS-SS/HA nanocomposites. The CS-SS/HA nanocomposite exhibited a homogeneous structure, with special rod-like nanoscale hierarchical features. The osteoblast-like MG63 cells cultured on the CS-SS/HA nanocomposites grew and spread actively. The present study may provide more theory basis for further enhance the understanding of biomineralization and promote the development of new biomaterials for bone tissue engineering.

Acknowledgements

This research was supported by the Science and technology research project of education department of Hubei province (No.B2016440).

References

[1] Li X, Wang L, Fan YB , Feng QL, Cui FZ, Watari F. Nanostructured scaffolds for bone tissue engineering. J Bio med Mater Res A, 2013, 101:2424-2435.

[2] Peter M, Ganesh N, Selvamurugan N, Nair SV, Furuike T, Tamura H, Jayakumar R. Preparation and characterization of chitosan gelatin/nanohydroxyapatite composites scaffolds for tissue engineering applications. Carbohydr Polym, 2010, 80:687-694.

[3] Muzzarelli RAA. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. Carbohydr Polym, 2009, 77:1-9.

[4] Jayakumar R, Prabaharan M, Kumar PTS, Nair SV, Tamura H. Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnol Adv, 2011, 29:322-337.

[5] Yao Q, Nooeaid P, Roether JA, Dong Y, Zhange Q,. Boccaccini AR. Bioglasss-based scaffolds incorporating polycaprolactone and chitosan coatings for controlled vancomycin delivery. Ceram Int, 2013, 39:7517-7522.

[6] Hardy, J.G.; Scheibel, T.R. Composite materials based on silk proteins. Prog. Polym. Sci.2010, 35, 1093–1115,

[7] Hench. L L Bioceramics. J. Am. Ceram. Soc, 1998, 81(7):1705-1728

[8] Li B, Huang LN, Wang XB, Ma JH, Xie F, Xia L. Effect of micropores and citric acid on the bioactivity of phosphorylated chitosan/chitosan/hydroxyapatite composites. Ceram Int, 2013, 39:3423-3427.

[9] Tachaboonyakiat W, Serizawa T, Akashi M. Hydroxyapatite formation on/in biodegradable chitosan hydrogels by an alternate soaking process. Polym J, 2001, 33:177-181.

[10] Zhang XB, Zhu LX, Lv H, Cao YL, Liu Y, Xu Y, Ye WM, Wang J. Repair of rabbit femoral condyle bone defects with injectable nanohydroxyapatite/chitosan composites. J Mater Sci Mater Med, 2012, 23:1941-1949.

[11] Khire T, Kundu J, Kundu SC, Yadavalli VK. The fractal self-assembly of the silk protein sericin. Soft Matter, 2010, 6:2066-2071.

[12] Zhang F, Zhang Z, Zhu X, Kang E, Neoh K. Silk-functionalized titanium surfaces for enhancing osteoblast functions and reducing bacterial adhesion. Biomaterials, 2008, 29:4751-4759.

[13] Elliot JC. Structure and chemistry of the apatites and other calcium orthophosphates. In: Elliot JC, editor. Studies in inorganic chemistry, Amsterdam: Elsevier Science; 1994. pp.111.

[14] Y. R. Cai, D. P. Mei, T. Jiang and J. M. Yao, Mater. Lett., 2010, 64, 2676. Cai YR, Mei DP, Jiang T, Yao JM. Synthesis of oriented hydroxyapatite crystals: Effect of reaction conditions in the presence or absence of silk sericin. Mater Lett, 2010, 64:2676-2678.

[15] Tan HP, Chu CR, Payne KA, Marra KG. Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering. Biomaterials, 2009, 30: 2499-2506.

[16] Martins AM, Santos MI, Azevedo HS, Malafaya PB, Reis RL. Natural origin scaffolds with in situ pore forming capability for bone tissue engineering applications. Acta Biomater, 2008, 4:1637-164