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Preparation and evaluation of collagen-silk fibroin/hydroxyapatite nanocomposites for bone tissue engineering

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ABSTRACT

A new in situ precipitation technique was developed to synthesize collagen-silk fibroin/hydroxyapatite nanocomposites. The componential properties and morphological of nanocomposites were investigated. It was revealed that the inorganic phase in the nanocomposite was carbonate-substituted hydroxyapatite with low crystallinity. Morphology studies showed that hydroxyapatite particles with size ranging from 30 to 100 nm were distributed uniformly in the polymer matrix. According to the TEM micrographs, inorganic particles were composed of more fine sub-particles whose diameters were between 2 and 5 nm in size without regular crystallographic orientation. The mechanical properties of the composites were evaluated by measuring their elastic modulus. The data indicated that the elastic modulus of nanocomposites was improved by the addition of silk fibroin. Finally, the cell biocompatibility of the composites was tested in vitro, which showed that they have good biocompatibility. These results suggest that the collagen-silk fibroin/hydroxyapatite nanocomposites are promising biomaterials for bone tissue engineering.

1. Introduction

Bone is a dynamic and highly vascularized tissue that is formed from a composite of 70% mineral (mostly nanoscale HA crystals) and 30% organics (including collagen, glycoproteins, proteoglycans, and sialoproteins) by dry weight [1]. Bone repair or regeneration is a key problem in orthopedic surgery. Traditional therapeutic approaches in treating large bone defects include bone grafts [2] and transplants [3] (autologous – from the iliac bone or fibular grafts, allograft – fresh or frozen after cleaning, or xenografts). Autografts have achieved various degrees of success in treating bone defects. However, the autograft is limited by the donor site morbidity, prolonged rehabilitation, increased risk of deep infection and restricted availability. Moreover, allografts might cause potential risks of transmitted diseases such as HIV or contamination [4,5]. Thus, more and more researchers have focused on organic/inorganic artificial biomaterials for treating bone defects in recent years.

Recently, various synthetic polymers, such as poly(glycolic acid) (PGA) [6], poly-L-lactic acid (PLLA) [7] and poly-(lactic-co-glycolic acid) (PLGA) [8], have been thought as potential bone grafts. However, the low strength and inflammatory responses caused by release of degraded acidic products limit their applications in bone repairing. Consequently, some natural polymers, containing polysaccharides (alginate, chitosan and cellulose) and proteins (collagen, gelatin, and silk), have been considered as the most important substrates for bone grafts. Collagen (Col), one of the most important natural polymers and the main organic component of bone tissue and extracellular matrix (ECM), has been widely applied in tissue engineering owing to its excellent bioactivity and degradability. However, the weak mechanical properties and fast degradability of Col may limit its applications. In the most biomaterials applications, the collagen matrix is usually combined with a reinforcing phase such as hydroxyapatite (HA), which should improve both the mechanical properties and the bioactivity of the material [9–11]. More than that, Col/HA composites bear a very close compositional resemblance to natural bone. Hydroxyapatite, the major inorganic mineral of natural bone, is a biologically active calcium phosphate ceramic that is used in surgery to replace and mimic bone.

Currently, there are some techniques concerning preparing Col/HA composite materials, including co-precipitation [12–14], alternate soaking [15,16] and mechanical mixing [17]. 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. Moreover, some researchers attempted to add other components to strengthen mechanical strength of Col/HA composite. Song et al. [18] applied physical crosslinking method to obtain poly(vinyl alcohol)/collagen/hydroxyapatite hydrogel. Poly(vinyl alcohol) (PVA) endowed mechanical strength and safety [19,20]. However, the network of PVA hydrogel prepared through

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physical crosslinking method is not strong enough and easily influenced by external conditions, such as temperature, and so on. Besides, polylactic acid (PLA) [21] was introduced into Col/HA system, which inevitably damaged the biocompatibility of Col/HA composites. Not only to enhance mechanical strength of Col/HA composite, but also to maintain its good biocompatibility, silk fibroin was introduced into the Col/HA system. Nevertheless, there are few reports on the preparation of Col-SF/HA composites. Wang [22] et al. synthesized the Col-SF/HA composite by co-precipitation method. Resulting from mechanical mixing during the pressure casting process through the co-precipitation, inorganic particles couldn't be distributed homogeneously within the organic matrices at nanolevel.

Silk fibroin (SF), a fibrillar protein derived from the silkworm *Bombyx mori*, is composed of 17 amino acids. SF with β-sheet structure has several unique properties such as advantageous processability, long-term biodegradability, superior mechanical strength as well as biocompatibility and good oxygen permeability [23–26]. Thus, it has been widely applied in drug-delivery [27,28], bone tissue engineering [29], wound dressing [30], skin tissue [31,32], and so on. In our previous work [33,34], a novel in situ precipitation method was developed to endow synthesized composites with unique morphology ultrafine nano-HA particles dispersed in organic template homogenously. The aim of this work is to fabricate homogeneous Col-SF/HA composites by the in situ precipitation approach, which is totally different from the traditional ones and rarely reported in the synthesis of Col-SF/HA composites. In this work, the growth of HA in Col-SF hydrogel was compared with that of HA in a single protein (collagen or silk fibroin) hydrogel through in situ precipitation method. The approaches currently used to obtain SF/HA composite materials are based on co-precipitation [35,36], alternate soaking [37,38] and mechanical mixing [39,40]. The mechanical performance and the compositional properties of as-synthesized nanocomposites were investigated. The cell biocompatibility with the composites was also tested in vitro. MG63 osteoblast-like cells were selected to evaluate the biocompatibility of the composites architecture with cells such as cell adhesion, cell spreading, cell proliferation and cell viability. The resulting composite, which combines good biocompatibility with high strength, provides a promising material in bone tissue engineering.

2. Materials and methods

Soluble type-I collagen from porcine dermis was purchased from National Engineering Research Center for Biomaterials of Sichuan university (Sichuan, China). *Bombyx mori* cocoons were obtained from Jingwei silk Co., Ltd. (Hubei, China). Dialysis membranes were obtained from Shenshi Co., Ltd. (Wuhan, China). Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), glutaraldehyde, acetic acid and ammonia were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, 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.

2.1. Preparation of Bombyx mori silk fibroin solution

Silk fibroin solution was prepared according to a previously reported method [41]. *Bombyx mori* cocoons were boiled for 10 min in 0.02 mol/L Na_2CO_3 solution for three times and rinsed thoroughly with distilled water to extract the glue-like sericin protein. The extracted silk was dissolved in 9.3 mol/L LiBr solution at 60 °C. The solution was dialyzed against water with a dialysis membrane (MW = 3500) for 3 days. The final concentration of the silk fibroin in aqueous solution was about 5.0% (w/v), which was determined by weighing the solid after drying. Silk fibroin solution was stored at 4 °C.

2.2. Synthesis of Col-SF/HA nanocomposites by in situ precipitation

Col solution was made by dissolving type-I collagen in 8 ml of acetic acid solution (1.5 vol.%) with continuously stirring at room temperature for 12 h. Then $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ (Ca/P = 1.67) were together added to the Col solution under agitation until the salts were entirely dissolved. SF solution was dropped slowly into the Col solution of Ca^{2+} and PO_4^{3-} with gently stirring at ambient temperature. Subsequently 0.3 ml glutaraldehyde (25 wt.%) was added to the previous mixed solution as a crosslinking agent. The solution was gently stirred until a transparent hydrogel formed. The resulting hydrogel was then stored under ambient conditions for 24 h to reach complete crosslinking. It was then added with ammonia solution for 24 h 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:

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

The nanocomposite was finally washed with distilled water until the pH of eluate was about 7. The starting content of all reagents was scaled according to the final organic/HA weight ratio of 40/60, and the initial amounts of the reagents used in this work are listed in Table 1. The weights of HA, $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ were calculated according to above equation.

2.3. Synthesis of Col/HA nanocomposites by in situ precipitation

The preparing procedures are the same as described in Section 2.2, but without the addition of SF. An opaque composite of Col/HA was produced. The starting content of all reagents was scaled according to the final Col/HA weight ratio of 40/60.

2.4. Synthesis of SF/HA nanocomposites by in situ precipitation

The preparing procedures are the same as described in Section 2.2, but without the addition of Col. An opaque composite of SF/HA was produced. The starting content of all reagents was scaled according to the final SF/HA weight ratio of 40/60.

2.5. Characterization

Morphology of inorganic/organic composite was observed using Environmental Scanning Electron Microscopy (SEM, Quanta200, FEI, Holland) and field emission transmission electron microscope (2010FEF, JOEL, Japan). The crystalline phase and component of obtained products were identified using wide angle X-ray diffraction analysis (XRD, X'pert PRO, Panalytical, Holland) and Fourier Transform Infrared Spectrometer (FT-IR, Nicolet5700, America).

Mechanical properties tests were measured at room temperature by a universal testing machine (SHIMADZU, AGS-J, Japan) at a crosshead speed of 0.5 mm min^{-1}. Elastic modulus was calculated as the slope of the initial linear portion of the stress–strain curve.

Samples of Col-SF/HA nanocomposites were made into circular discs suitably sized (diameter 5 mm, height 2 mm). The MG63 cells (2.0×10^4 cells/well) were seeded on each discs placed in the 96-well plates (Corning Life Sciences). Cells cultivated in the