Materials Science and Engineering C 51 (2015) 287–293

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Silk fibroin/sodium alginate fibrous hydrogels regulated hydroxyapatite crystal growth

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ARTICLE INFO

Article history:
Received 9 November 2014
Received in revised form 29 January 2015
Accepted 9 March 2015
Available online 11 March 2015

Keywords:
Silk fibroin
Sodium alginate
Hydroxyapatite
Fibrous hydrogel

ABSTRACT

Use of organic templates for controlling the growth of inorganic crystals is one of the research topics in biomimetic field. In particular, oriented growth of hydroxyapatite (HAp) in organic fibrous matrix is provided a new view angle to study biomineralization of bone and its potential biomedical applications. The crystallization of HAp in fibrous hydrogels could mimic such biomineralization. In this paper, we report HAp nanorod crystal synthesized successfully by a biomimetic method using calcium chloride and ammonium dihydrogen phosphate as reagents in the presence of silk fibroin/sodium alginate (SF/SA) fibrous hydrogels. The effects of influence factors such as mineral times, pH, and temperature on controlling HAp nanorod crystals are discussed. The elongated HAp nanorods with rectangular column are grown with the increase of mineral times in biomimetic process. By changing pH, HAp nanorod crystals are obtained at alkaline condition in fibrous hydrogels. Moreover, compared to other temperatures, rod-shaped HAp crystals were formed at 20 °C. The results imply this to be an effective method for preparing HAp crystals with controllable morphology for bone repair application.

1. Introduction

Natural mineral organization such as bone and tooth is a hierarchically structured composite material containing biological macromolecules (protein, polysaccharide, etc.) and inorganic minerals, which has been well studied by the materials engineering community because of its unique structure and mechanical properties [1,2]. Hydroxyapatite (HAp), the mineral constituents of human bones and tooth, has been studied in biomedical applications for many years [3,4]. Many chemical methods have been used for the preparation of HAp crystals with controllable size and morphology, such as solution–precipitation [5], sol–gel synthesis [6], solid-state reaction [7], and hydrothermal method [8]. However, these methods mostly prepare irregular forms of powder [9]. Presently, the preparation of HAp crystals is developed by biomimetic approach, which is a research topic in the field of bone tissue engineering [10]. In biomimetic preparation process, organic templates are used to control the morphology and structure of HAp crystals [11]. Masanori Kikuchi and co-workers reported a bone-like HAp/collagen nanocomposite prepared by self-organization [12]. The results showed that the *c*-axes of blade-shaped HAp nanocrystals 50–100 nm in size were aligned along collagen fibers up to 20 μm in length. At the same time, the mechanical properties of composites had 40 MPa in bending strength and 2.5 GPa in Young's modulus. Wei et al. studied HAp crystals depositing on regenerated silk fibroin (SF) nanofibers by a biomimetic Ca–P method [13]. The results exhibited HAp crystals with 30 nm diameter distributing on the surface of SF nanofibers.

Recently, HAp has been intensely studied with the aim of understanding how crystal polymorph and structural features can be controlled by natural polymers [14]. Some natural polymers containing polysaccharides (alginate, chitosan, and cellulose) and proteins (collagen, gelatin, and silk), are used to control the crystallization of HAp in recent research works [15–17]. However, the mineral growth environment rarely occurred in gel state [9,18]. In our previous study, we reported silk fibroin/sodium alginate (SF/SA) fibrous hydrogels to regulate and control the growth of HAp crystals. This fibrous hydrogels containing SF protein and SA polysaccharides can better mimic the real mineralization system of bone more than a single protein system [9,14,19,20]. At the same time, rectangular column and size-controllable HAp nanorods were controlled in SF/SA fibrous hydrogels at room temperature.

In this study, we aim to analyze the effects of influence factors on controlling HAp nanorod growth in SF/SA fibrous hydrogels. By studying the crystallization process in fibrous hydrogels, the probable mechanism of HAp nanorod crystal growth is analyzed. Through this study, it provides clues to understand HAp biomineralization process as it occurs in bone formation and suggests a pathway for the biomimetic fabrication of biomaterials with controllable morphology and structure.

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http://dx.doi.org/10.1016/j.msec.2015.03.014
0928-4931/

2. Experimental

2.1. Materials

Bombyx mori silk was bought from Zhejiang province, China. Sodium alginate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and used without any further purification. All chemical reagents (lithium bromide, sodium carbonate, calcium chloride, formic acid, ammonium dihydrogen phosphate, ethanol, etc.) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), which were analytical grade, and also used without any further purification.

2.2. Preparation of regenerated SF solution

B. mori silk fibers were boiled in 0.05 wt.% Na_2CO_3 solution for 30 min and then rinsed thoroughly with deionized water to extract the glue-like sericin proteins. Each step was repeated twice. Degummed silk was then dissolved in formic acid/$CaCl_2$ (FA/$CaCl_2$, 5 wt.% concentration) solvent at room temperature. Compared to traditional dissolved method (LiBr aqueous solution and $CaCl_2$/ethanol/water solution), the FA/$CaCl_2$ dissolved silk by breaking hydrogen bonds in the crystalline region while preserving the nanofibril structures [21]. During the dissolution process, the dissolution behavior of silk from fiber to nanofibrils was regulated by $CaCl_2$ concentration. This solution (SF–FA–$CaCl_2$) was used for film formation. The formed films were redissolved in 9.3 M LiBr solution at room temperature for 4 h, yielding a 10% (w/v) solution. This solution was dialyzed against distilled water using Slide-A-Lyzer dialysis cassettes (Sigma, USA, molecular weight cut-off 3500) for 72 h to remove salt ions. The final concentration of aqueous silk solution was ~1.0 wt.%, determined by weighing the remaining solid after drying. This preparation process was two-step dissolution method, and its obtained aqueous solution was stored at 5 °C for use.

2.3. Preparation of SF/SA fibrous hydrogel

SA (2 g) was dissolved in deionized water at room temperature. The solution was mixed under constant stirring in a blender for 1 h, standing 24 h at 5 °C to obtain a uniformly 0.5 wt.% SA solution. And then, SF and SA aqueous solutions with 70/30 ratio were mixed by stirring, and the concentration of the mixture solution was controlled at 1.0 wt.%. The mixed solution was stored overnight at 5 °C to achieve homogeneity and to avoid any premature precipitation of the protein, which occurred at room temperature. Finally, hydrogels were prepared by adding 5 mL of blend solution in 24 well plates (Coring, USA). The solutions were allowed to gel in an incubator at 37 °C for obtaining fibrous hydrogel (Fig. 1a). SF/SA fibrous hydrogels had nanofiber network morphology with β-sheet structure (Fig. 1b, c). According to our published procedures [22], the porosity of fibrous hydrogels was 97.0 ± 1.3%, which its compressive stress was 31.9 ± 3.6 kPa (Fig. 1d). In addition, SA hydrogels were gelled by adding 10 mM Ca^{2+} solution. The morphology of SA hydrogels was interconnected nanofiber networks [9].

2.4. Preparation of HAp crystals in SF/SA fibrous hydrogels

The mineral process was used to grow HAp crystals in SF/SA fibrous hydrogels. Firstly, SF/SA fibrous hydrogels were treated in 75% (v/v) ethanol aqueous solution for 30 min to prepare the water-insoluble hydrogels. The water-insoluble hydrogels were immersed directly in 0.1 M $CaCl_2$ supersaturated solution for 1 h at room temperature and washed twice with deionized water to remove free ionic calcium. Then, the samples were placed in 0.1 M $(NH_4)_2HPO_4$ supersaturated solution. After soaking for different conditions (mineral time, pH, temperature), cycles were repeated every 2 h. The HAp-deposited SF/SA fibrous hydrogels were rinsed with deionized water and freeze-dried.

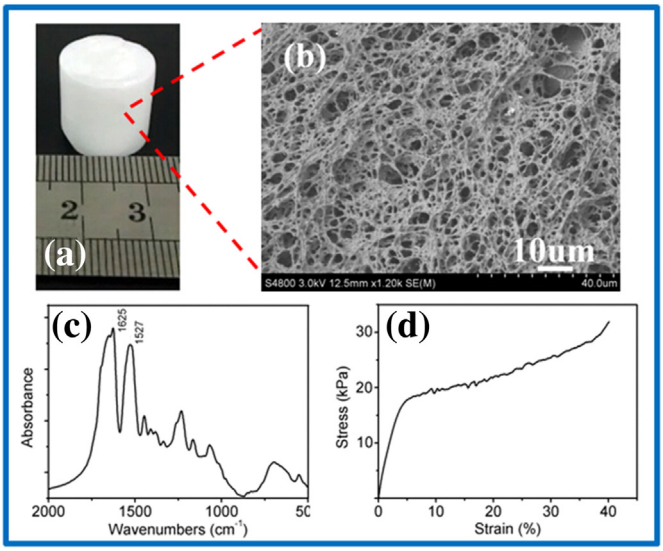

Fig. 1. SEM images (a, b), FTIR (c), and mechanical properties (d) of SF/SA fibrous hydrogels prepared through 70/30 proportions of SF/SA aqueous solution at 37 °C.

As control, HAp crystals were prepared according to our previous procedures [23]. HAp crystals synthesized by solution–precipitation approach exhibited needle-like morphology with 25.7 ± 2.9 nm.

2.5. Characterization

2.5.1. Scanning electron microscopy (SEM)

The morphology of SF/SA fibrous hydrogels and its HAp crystal growth was examined by SEM (S4800, Hitachi, Japan). Samples for SEM experiment were observed with gold coating. At the same time, energy dispersive X-ray spectroscopy (EDX) was employed to determine the elemental composition.

2.5.2. X-ray diffraction (XRD)

The experiment was recorded on X Pert-Pro MPD (PANalytical, Netherlands) with CuKα radiation working at 40 kV and 40 mA in the interval range from 10° to 60° with a scan rate 2° min^{-1}.

2.5.3. Fourier transform infrared (FTIR)

FTIR spectra were obtained using Nicolet 5700 (Thermal Nicolet Company, USA) in absorbance mode at the wave number ranging from 400 to 4000 cm^{-1}.

3. Results and discussion

3.1. HAp crystal growth in fibrous hydrogels

Bone, natural organic–inorganic ceramic composite, consists of collagen fibrils containing embedded, well-aligned nanocrystalline, rod-like HAp crystals [24]. In order to mimic the growth process of HAp crystals in bone formation, SF/SA fibrous hydrogels were used to control HAp crystal growth. Fig. 2 showed the morphology and structure of HAp crystals regulated by different organic templates for a mineral time of 1 h at room temperature. At blank template, needle-like HAp crystals with 25.7 ± 2.9 nm were obtained by solution–precipitation (Fig. 2d) and its crystalline structure was characterized by XRD (Fig. 2e). Compared to JCPDS-ICDD database, the main peaks at (002), (211), (300), (202), (310), (222), (213), and (004) were appeared, attributing to typical HAp characteristic peaks. At the same preparation condition, crystals with flower-shaped morphology were obtained by using SF fibrous hydrogels as template (Fig. 2a). SEM results suggested that hydroxyl, carboxyl, and carbonyl groups in SF molecules had