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Synthesis and characterization of spider silk calcite composite

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Abstract

Spider silk poses excellent mechanical properties, tenacity and elasticity and it has been used as a template for calcite mineralization to improve load bearing strength of osteoconductive calcite. The samples were obtained by mimicking biomineralization for five days in order to follow formation and growth of calcite on the surface of spider silk. Crystal phase was detected by XRD and FTIR spectroscopy. Microstructure, crystal size and its morphology were studied by means of FESEM. After two days of processing, pure calcite phase was obtained, and a size of the formed crystals increased with prolongation of biomineralization.

Keywords: *spider silk, calcite, composites, biomineralization, crystallization*

I. Introduction

Biomineralization is a process which results in formation of biominerals. Biominerals are composed from organic and inorganic component [1], and are usually superior compared to minerals that does not contain organic component. It is the organic component that influences the mechanical properties of minerals, e.g. nacre and bone are more than 1000 times stronger than pure mineral constituents [2]. By mimicking biomineralization, it is possible to obtain materials with extraordinary properties for different application. *In vitro* biomineralization can be achieved by using biomolecules as a template. There is a growing interest in applying biominerals for tissue engineering, especially for replacement of osseous tissue [3–5]. The application of pure minerals as a replacement in bone tissue repairing failed due to low strength and toughness of brittle mineral phase. On the other side, polymers as a combination of organic and inorganic component would be ideal substitution due to its adjustable load bearing properties derived from both components.

Spider silk (SS) is well known due to its excellent mechanical properties, its tenacity and elasticity [6] and it has already been used as a template for biomineral formation in various applications [7–9]. In addition, spider silk is biocompatible material. Studies conducted on SS showed that it does not cause strong inflammatory response, and that it support proliferation of different cell types. Degradation behaviour of SS can be moderated, depending on the purpose of the material and it can vary from few days to few months [10–12]. These properties make SS an interesting material for application in regenerative medicine.

Calcium carbonate biominerals belong to the most abundant minerals formed in biological systems, in particular in hard tissue of invertebrates [13]. It crystallizes in three forms: calcite, aragonite and rarely vaterite. Among them, calcite is the most thermodynamically stable polymorph of calcium carbonate ($CaCO_3$). It has been widely used in industry of paper, paint, textiles, detergents, adhesives, plastics, cosmetics, food, antacid tablets, as ingredient of cement [14,15]. Calcite is biodegradable material with good osteoconductivity that bonds tightly to bone without a surface apatite layer [16].

Mehta and Hede [9] synthesized spider silk calcite composite by drop-coating spider silk with $CaCl_2$ and obtained spider silk coated with calcite. We believed that it was possible to obtain regular rhombic crystals of calcite on the spider silk, so our work was focused on calcite biomineralization directly from $CaCl_2$ solu-

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tion. Unlike the previous experiments, biomineralization was followed during five days, so the moment of obtaining pure calcite phase without presence of other phases could be detected.

In this paper, composite of spider silk and calcite has been synthesized by mimicking biomineralization process. We believe that this material can show potential *in vitro* and *in vivo* for supporting of bone tissue growth.

II. Experimental

Spider silk (SS) was harvested from the spider *Pholcus phalangoides* which was kept in clean, dust free environment. The spiders were fed with houseflies once a week. We used dragline silk from major ampullate gland that spiders use as a lifeline and building material.

Modified method developed by Xu *et al.* [16] was used for obtaining spider silk/calcite composite. The starting compounds for obtaining calcite were anhydrous calcium chloride ($CaCl_2$) and ammonium carbonate ($(NH_4)_2CO_3$). All used reagents were of analytical grade. Two Petri dishes were put in the desiccator. In one Petri dish, we put solid ammonium carbonate and in the other 0.01 M solution of calcium chloride in which we immersed spider silk (pH of the solution was 6.1). Calcium ions (Ca^{2+}) from the solution bind to carboxylate anions (COO^-) that belongs to amino acids of spider silk proteins. Desiccator was placed in the oven at 60 °C which is the temperature of decomposition of ammonium carbonate to carbon dioxide and ammoniac. Carbon dioxide has dissolved in solution and dissociated to following carbonate species: carbonic acid, HCO_3^- and CO_3^{2-}. Liberated CO_3^{2-} anion formed calcite with calcium ions bonded to carboxylate anions (COO^-). We repeated experiments for five days in order to follow formation of calcite minerals with time.

The samples were characterized by means of X-ray diffraction analysis (XRD), Fourier transform infrared spectroscopy (FTIR) and Field emission scanning electron microscopy (FESEM).

The phase composition of samples was examined by X-ray diffraction (Raguku Ultima IV, Japan). The X-ray beam was nickel-filtered CuKα_1 radiation (λ = 0.1540 nm, operating at 40 kV and 40 mA). XRD data were collected from 20° to 45° (2θ) at a scanning rate of 5°/min. Phase analysis was done by using the PDXL2 software (version 2.0.3.0, 2011, Rigaku Corporation, Tokyo, Japan), with reference to the patterns of the International Centre for Diffraction Data database (ICDD), version 2012.

Infrared spectrum of the sample was recorded at ambient conditions between 1300–650 cm^{-1} (mid-IR region) with a Nicolet IS 50 FT-IR Spectrometer by using the ATR sampling technique.

Prior to the FESEM analysis, samples were coated with Au-Pd alloy using a spatter coater. The morphology of the spider silk calcite composite was studied by field emission scanning electron microscopy (FESEM) TESCAN Mira3 XMU at 20 kV.

III. Results and discussion

In experimental conditions, at temperature of 60 °C, ammonium carbonate has decomposed and liberated gas CO_2. The compound CO_2 was dissolved in water and became source of CO_3^{2-} ions in $CaCl_2$ solution. It is considered that in $CaCl_2$ solution, carboxyl groups of spider silk interact with Ca^{2+} ions, forming the ion complexes, which could further interact with CO_3^{2-} ions due to supersaturation effects [9]. This sites present nucleation centres, forming critical size nuclei for the nucleation, growth and orientation of calcite crystals [17].

Figure 1 shows XRD patterns of different SS/calcite composites after biomineralization for 1, 2, 3, 4 and 5 days. According to XRD, after first day of biomineralization, the most prominent phase is ammonium chloride (NH_4Cl). A low intensity peak at 29.3° is noticed. However, after soaking of SS for 2 days, clear diffraction characteristic peak at 29.3° was detected corre-

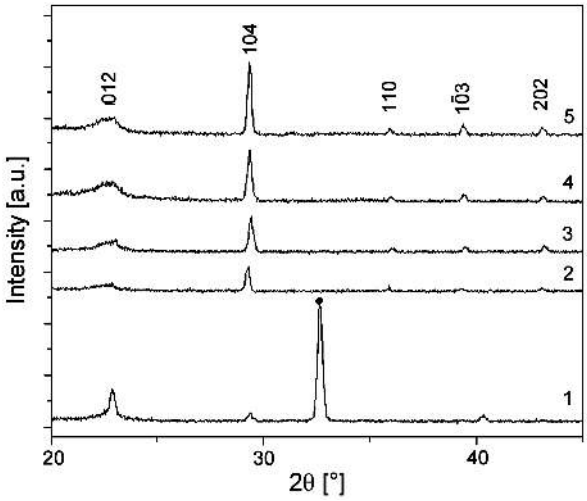

Figure 1. Phase evolution of calcite on the spider silk depending on different numbers of immersion cycles (days = 1-5), • - NH_4Cl

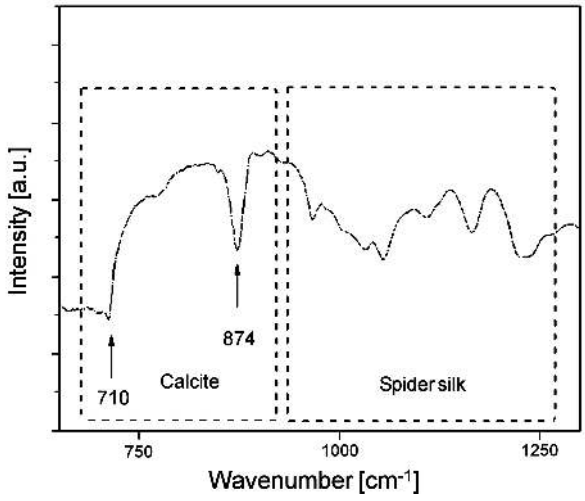

Figure 2. FTIR spectra of biomineralization of calcite on spider silk after fifth day

sponding to (104) plane of calcite crystal, and smaller peaks corresponding to calcite Miller indices (012), (110), (10$\bar{3}$) and (202), respectively [18]. After two days, no other inorganic phases were detected except calcite, so it was concluded that after two days pure calcite was obtained on the surface of spider silk. The intensity of peaks increased with the soaking times.

Since the more intensive XRD reflection is achieved after 5 days of treatment, FTIR analysis is done after the fifth day of biomineralization. Figure 2 shows FTIR spectra between 650 and 1300 cm^{-1}. In that range, calcium carbonate signals can be clearly distinguished from the excitation of organic components. The calcite had the characteristic adsorption bands at 710 and 874 cm^{-1}. Near 710 cm^{-1} the antisymmetrical bending mode E_u occurs in the *ab*-plane of the carbonate group [19,20]. The IR results were in good agreement with XRD, which confirmed the nucleation of calcite on the surface of the spider silk. The excitation mode at 874 cm^{-1} with standard IR spectra of calcium carbonate suggests that structure resembles calcite. The remaining adsorption bands are ascribed to spider silk [21].

The samples were also examined by FESEM and used for comparison of the silks obtained after first, second, third, fourth and fifth day. Figure 3 clearly showed the crystallization of calcite on spider silk substrate. Thus, SEM images of the silk surfaces obtained after first day of biomineralisation (Fig. 3a) depicts small rhombic crystals with size of 1–3 μm on the SS surface. According to Fig. 3a the growth of calcite after the first day was obvious, although XRD pattern indicated that the main phase at that stage was ammonium chloride and only hints of calcite were present. This is probably due to the fact that signals of calcite were low. The micrographs of the samples obtained after the second, third, fourth and fifth day, (Figs. 3b,c,d,e) are in coexistence with XRD patterns. The biomineralization process promoted the formation of rhombic-shaped calcite crystals. There was no significant difference between the SS/calcite composites prepared after immersion within 3, 4 and 5 days except the number and the size of crystals increased with time of biomineralization. The average size of crystals in the SS/calcite composites obtained after 3, 4 and 5 days was 6 μm, 9 μm

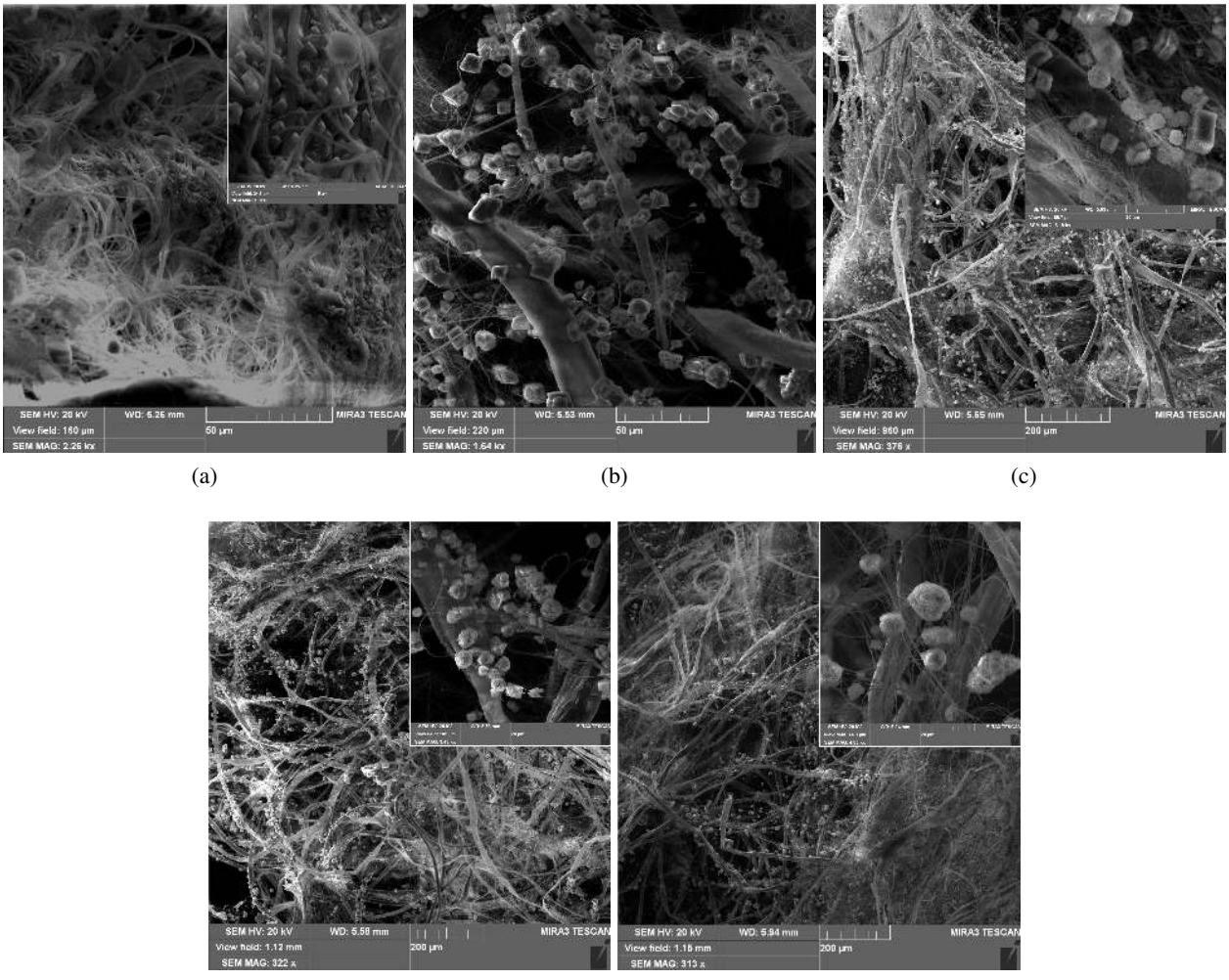

Figure 3. Scanning electron micrographs of spider silk calcite composite after different immersion time: a) first day, b) second day, c) third day, d) fourth day and e) fifth day

and 11 μm, respectively. Regular form of calcite rhombic crystals with perfect cleavage were homogeneously dispersed trough 3D spider mesh.

IV. Conclusions

Composite of calcite and spider silk was obtained by simple method using biomineralization approach. The process was observed during five days and it was found that two days of treatment are sufficient to get the pure calcite phase on the surface of spider silk. Desirable mechanical properties of spider silk complement inelastic calcite and this combination is promising in designing of bone grafts for osseous tissue replacement material.

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