Effect of polyaspartic acid on hydroxyapatite deposition in silk fibroin blend films

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Abstract. Polyaspartic acid/silk fibroin/hydroxyapatite (PASP/SF-HA) composites have been synthesized by biomimetic processing. SF solution was mixed with different contents of PASP to prepare the PASP/SF blend membranes. After ethanol treatment and premineralization process, the blend membranes were immersed into 1.5 simulated body fluid (1.5 SBF) for 24 h to induce apatite deposition at $37\pm0.5^\circ\text{C}$. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) results revealed that a conformation transition of SF occurred after the addition of PASP and ethanol treatment. The FTIR and XRD results also confirmed that the main component of apatite deposition was HA. Scanning electron microscopy (SEM) showed that the content of HA increased with increasing PASP concentration. Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP) results revealed that the Ca/P molar ratio could reach 1.45, which was close to the Ca/P ratio of apatite. It was appropriate to conclude that the increasing content of PASP had a distinct effect on HA deposition in the blend films.

Keywords: biopolymers, biocomposites, hydroxyapatite, silk fibroin, conformation transition

1. Introduction

Hydroxyapatite (HA) with the chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is the major mineral part of natural bones and teeth. HA is the most commonly used apatite-based biomaterial due to its excellent biocompatibility, osteoconductivity and bioactivity [1, 2]. In the past decades, many efforts have been focused on the biomimetic growth of nano-HA through the combination of biodegradable polymers or proteins for bone tissue engineering [3–6]. Among these polymers, collagen is the best known tissue-derived natural polymer and has been used as tissue-culture scaffolds. However, the practical problems with collagen are its cost, and a limited range of physical properties unless extensively crosslinked, which alters biological response [7, 8]. Silk fibroin (SF), used as an appealing alternative polymer, possesses impressive mechanical proper-

ties and appreciable bioactivity that make it attractive for artificial bones [9, 10]. Kong *et al.* [11] employed SF to regulate the mineralization of HA. The effects of SF content on the microstructure and physico-chemical properties of the composite had also been studied [12]. In order to expand its application in biomaterials, the water-insoluble SF films were also prepared by inducing the structure transition from unstable silk I to silk II. The transition was commonly studied through treatment of aqueous ethanol solution [13, 14]. At the same time, biodegradable polymers such as chitosan [15], cellulose [16] and sodium alginate [17] have been widely used in the conformation transition of SF. Recently, Kim *et al.* [18] used aqueous-derived porous SF scaffolds mixing with PASP to deposit apatite, which provided an appropriate osteogenic environment for tissue engineering. However, the

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effect of PASP on the conformation transition of SF has not yet been studied in detail.

In this work, pure SF and various blend ratios of PASP/SF films were prepared. After a premineralization process, the films were immersed into 1.5 SBF for 24 h to synthesize PASP/SF-HA composite. FT-IR, SEM and XRD spectroscopy were used to study the conformation transition of SF and the morphology of the obtained composite films. After the addition of PASP and treatment of ethanol, the silk I conformation had been transformed to silk II, which enhanced the deposition of HA. The results reported here may provide some information about a new strategy for the synthesis of other composite materials.

2. Materials and methods

2.1. Materials

Na₂CO₃, LiBr, NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, HCl, CaCl₂, Na₂SO₄, NaOH, tris(hydroxymethyl) aminomethane (Tris) and ethanol used here were analytical grade and purchased from Huipu Chemical Agents Co. Ltd. (Hangzhou, China). B. mori silkworm cocoons were provided by Anhui Agricultural Research Institute.

2.2. Preparation of SF films and PASP/SF scaffolds

B. mori silkworm cocoons were boiled for 30 min in an aqueous solution of 0.5% (w/v) Na₂CO₃ and then rinsed thoroughly with distilled water to extract the glue-like sericin protein. The treatment was repeated two times to get the pure silk fibroin. The degummed silk fibroin was air-dried at ambient temperature and dissolved in 9.5M LiBr solution for 4 hours at 50°C followed by a dialysis (MWCO 14000) against distilled water for 3 days. The final concentration of aqueous silk fibroin solution was approximately 2.7% (w/v). Then the solutions were stored in a refrigerator at 7–8°C before being used. Then, aqueous SF solution was cast onto polytetrafluoroethylene plates and dried at room temperature for the preparation of SF films. For the preparation of the blend membranes different amounts of PASP solution were added to SF solution under mild stirring for 2 min. The mixing

solutions were poured into the polytetrafluoroethylene plates and air-dried at room temperature. The formed PASP/SF films were then treated with 60% (v/v) ethanol aqueous solution for 20 min.

2.3. Premineralization and mineralization

The alternate soaking process was used to grow apatite on silk fibers. First, SF films and PASP/SF scaffolds were soaked in 50 ml of 0.2M CaCl₂ solution (buffered with 0.05M Tris·HCl, pH 7.4) for 1 min and washed two times with distilled water. The PASP/SF scaffolds were then transferred to 50 ml of 0.12M K₂HPO₄ solution, soaked for 1 min and washed two times with distilled water. The soaking cycles were repeated 3 times. The whole process performed at 37°C.

The premineralized PASP/SF scaffolds were subsequently immersed into 1.5 SBF (Table 1) at 37°C for 24 h, followed by rinsing with deionized water and air-drying at room temperature.

Table 1. Composition of 1.5 SBF (pH 7.4) and order of addition of the reagents to water [19]

Order	Reagent	Weight [g·l ⁻¹]
1	NaCl	12.0540
2	NaHCO ₃	0.5280
3	KCl	0.3375
4	K ₂ HPO ₄ ·3H ₂ O	0.3450
5	MgCl ₂ ·6H ₂ O	0.4665
6	1M HCl	15.00 ml
7	CaCl ₂	0.4440
8	Na ₂ SO ₄	0.1080
9	C ₄ H ₁₁ NO ₃ (Tris)	9.0945
10	1M HCl	50.00 ml

2.4. Characterization

The FT-IR spectra of samples were measured with Fourier transform infrared spectroscopy (FTIR) (Nicolet NEXUS-870, USA), the apatite deposited SF films samples were mixed with KBr in the mass ratio of 1:20–30. Infrared spectra were taken in the range of 4000–400 cm⁻¹ with 4 cm⁻¹ resolution. To investigate the structure and crystallinity, the milled sample powder were analyzed on an X-ray powder diffractometer (XRD) (Philips X'Pert-Pro, Dutch) using a diffractometer equipped for Cu-K_α radiation (λ = 0.15418 nm) in the 2θ range of 10–60° at 40 kV and 30 mA. The morphology of apatite deposited SF films sputtered with gold was

examined using scanning electron microscopy (SEM) (S-4800, Hitachi, Japan) with an accelerating voltage of 5 kV. The content of Ca and P elements in the composites was analyzed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP) (IRIS Intrepid II ICP-OES, Thermo ELECTRON CORPORATION, USA). The milled composites samples were dissolved with HNO_3 and HClO_4 , then they were filled to constant volume, with distilled water to 25 ml.

3. Results and discussion

3.1. FTIR

The conformational transition of SF and the composite films were studied by FT-IR spectroscopy (Figure 1). Figure 1a shows the characteristic peaks of PASP, including the $\text{C}=\text{O}$ peaks at 1733 cm^{-1} and the $\text{C}-\text{N}$ stretching vibrational frequencies at 1398 cm^{-1} , the bands at 3358 and 1586 cm^{-1} corresponding to the stretching vibration and bending vibration of $\text{N}-\text{H}$ bonds, respectively. The bands for pure SF (Figure 1b) at 1658 cm^{-1} (amide I), 1550 cm^{-1} (amide II), 1243 cm^{-1} (amide III) and 670 cm^{-1} (amide V) were attributed to silk I form. After the addition of PASP, the band at 1658 cm^{-1} showed an obvious shift, the 1550 and 1243 cm^{-1} bands shift to lower wave number, the shifts were 7 and 4 cm^{-1} , respectively. Furthermore, the shift

Figure 1. FT-IR spectra of (a) PASP, (b) SF, (c) PASP/SF and (d) PASP/SF treated with 60% (v/v) ethanol aqueous solution. The blend ratio of PASP/SF solution was 10% (w/w). (A) $4000\text{--}500\text{ cm}^{-1}$ range. (B) Detail of the $1800\text{--}600\text{ cm}^{-1}$ region.

of amide peaks was more evident after ethanol treatment. In order to clearly reflect conformation transition of the SF in the blend membranes, the spectral ranges of $1800\text{--}600\text{ cm}^{-1}$ were shown in Figure 1B. The amide I and amide II bands for the PASP/SF composite films were shifted from 1658 to 1662 and 1550 to 1542 cm^{-1} , respectively, and a small sharp peak at 1649 cm^{-1} appeared, indicating that a partial conformation transition from silk I to silk II structure occurred [11]. After treated with ethanol, the amide I bands were shifted to 1634 cm^{-1} with a shoulder at 1694 cm^{-1} , which could be assigned to β -turns [20, 21]. Amide II and amide III bands were shifted to 1526 and 1233 cm^{-1} , respectively. The shift of amide bands demonstrated that intermolecular interactions may be formed between the polymer functional groups, such as hydrogen bonding of carbonyl groups of PASP, hydroxyl groups of ethanol, amide groups of SF, which might be related to the conformational transition of SF. From the above results, it was obvious that the addition of PASP changed the structure of SF, and ethanol treatment can further promote this change.

Figure 2 shows the FT-IR spectra of the mineralized scaffolds prepared from various blend ratios of PASP/SF solutions. It could be seen that the spectra of the mineralized scaffolds displayed the absorption bands at 1040 , 603 and 564 cm^{-1} (Figure 2: a, b, c, d), and the intensity of these peaks gradually enhanced with the increasing content of PASP. Compared with the infrared data of pure HA (Fig-

Figure 2. FT-IR spectra of the mineralized scaffolds prepared from various blend ratios of PASP/SF solutions (a: 0%; b: 5%; c: 10%; d: 15%) and (e) HA

ure 2e), the predominant component of the resultant inorganic phase was HA. The results indicated that HA had been successfully deposited on the surface of PASP/SF blend membranes and the carbonyl groups of PASP in the organic template induced the nucleation of HA [22].

3.2. XRD

Figure 3 displayed XRD patterns of the prepared scaffolds. For the pure SF film (Figure 3a), only a weak and wide halo pattern appeared, this exceedingly discrete powder diagram belonged to silk I [23, 24]. According to the studies on silk fibroin molecular conformation [25], the X-ray diffraction patterns had been determined as follows, 11.5° (2θ) for α -helix structure, and 20.2° (2θ) for β -sheet structure. It could be seen from curve (b) that the composite sample showed two peaks around 11.8° (2θ) and 20.2° (2θ). Its molecular conformation exhibited a coexistence of α -helix and β -sheet structure, indicating that PASP partly induced the conformation of SF from silk I to silk II. The characteristic peaks ($2\theta = 20.2^\circ$) of β -sheet became more intensive with the treatment of 60% (v/v) ethanol aqueous solution (Figure 3b, 3c). It showed that the essential changes of silk fibroin molecular conformation have taken place during the treatment by ethanol.

Figure 3d displays XRD patterns of the PASP/SF-HA composite. As shown in Figure 3d, after soak-

Figure 3. X-ray diffraction of (a) SF, (b) PASP/SF, (c) PASP/SF with the treatment of 60% (v/v) ethanol aqueous solution, and (d) the mineralized scaffolds at 37°C . The blend ratio of PASP/SF solution was 10% (w/w)

ing in 1.5 SBF for 24 h, the clear but weak diffraction peaks were detected at 25.9° (2θ) and 31.9° (2θ) corresponding to the (002) and (211) planes of HA crystal (verified by PDF Card No.09-0432). The result also confirmed that the predominant component of the inorganic phase was HA. The broadened peaks of the (002) and (211) crystal planes showed an imperfect crystallization of HA. This crystallographic structure of HA in the PASP/SF-HA composite was more similar to natural bone mineral. In addition, the organic components in the PASP/SF-HA composite also showed a diffraction peak at 20.2° (2θ) in Figure 3d, which assigned to the β -sheet structure of SF. These results were consistent with the analysis of FTIR.

3.3. SEM

The surface morphology of the prepared materials is shown in Figure 4. After addition of PASP, the surface of SF films became rough (Figure 4A: b, c, d). Through the alternate soaking in CaCl_2 and K_2HPO_4 solutions at 37°C , some inorganic compounds attached to the film surface and much aggregation occurred with increasing content of PASP. When the blend ratio of PASP reached 15%, a smooth porous surface was formed (Figure 4B: d). Figure 4C shows the SEM images of the scaffolds soaked in 1.5 SBF at 37°C for 24 h. The scaffolds formed highly interconnected and porous structure. The general size of the pores got to the maximum with 5% (w/w) blend ratio of PASP and higher content PASP resulted in the formation of needle-like apatite assembled into clusters. These results indicated that the composite films could become more homogeneous along with the increasing content of PASP, which could enhance the deposition of apatite. The ICP results of PASP/SF-HA shown in Table 2 also demonstrated the Ca/P molar ratio in the scaffolds increased gradually along with addition of PASP. When HA deposited on the pure SF films, the Ca/P molar ratio was only 1.38. How-

Table 2. ICP results from the PASP/SF-HA composite

PASP/SF	Element contents in the sample solution*		
	Ca [mg/l]	P [mg/l]	Ca/P
100/0	43.28	24.24	1.38
95/5	53.16	41.20	1.39
90/10	56.74	30.50	1.44
85/15	56.81	30.37	1.45

*All samples are of the same quality

Figure 4. SEM images of the scaffolds (A) prepared from various blend ratios of PASP/SF solutions, (B) after soaking in CaCl_2 and K_2HPO_4 solutions at 37°C , and (C) followed by being soaked in 1.5 SBF at 37°C for 24 h (a: 0%; b: 5%; c: 10%; d: 15%)

ever, it increased to 1.45 when the blend ratio of PASP/SF reached 15% (w/w). As the proportion of PASP in the blend membranes increased, the content of Ca in the composite solution also increased clearly. These results in this study proved that apatite had been deposited on the SF substrates successfully. The carbonyl group of PASP enhanced apatite deposition on membranes [18, 26].

4. Conclusions

The PASP/SF-HA composites were fabricated through a simple pretreatment and biomimetic method. The addition of PASP and treatment of ethanol greatly influenced the molecular conformation of SF. Intermolecular hydrogen bonds between the carbonyl group of PASP and the polarity group of SF played important roles in the formation of the

PASP/SF-HA composite. The present study may further enhance the understanding of biomineralization and promote the development of new biomaterials for bone tissue engineering.

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