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Hydroxyapatite/sericin composite film prepared through mineralization of flexible ethanol-treated sericin film with simulated body fluids

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Abstract

A flexible ethanol-treated sericin film was prepared and used to form a hydroxyapatite/sericin composite film by mineralizing it with simulated body fluids solution. The hydroxyapatite mineral deposited on the surface of the ethanol-treated sericin film was characterized by Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy, Energy Dispersive X-Ray Spectroscopy, and X-ray diffraction analysis. Results indicated that the quantity of deposited minerals increased with increasing mineralization time and showed a three-dimensional structure. This structure was determined to be calcium-deficient carbonate apatite with poor crystallinity and growth in the direction of the *c*-axis similar to natural bone mineral. The mechanical properties of the hydroxyapatite/sericin composite film decreased with increasing mineralization time. XTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tests showed that the hydroxyapatite/sericin composite film promoted cell viability, which is beneficial for its application in bone tissue engineering.

Keywords: A. Films; B. Composites; C. Mechanical properties; D. Apatite

1. Introduction

Hydroxyapatite [$Ca_{10}(P0_4)_6(OH)_2$, HA] is an inorganic salt with excellent biocompatibility, osteoconductivity and bioactivity, and it is widely used in bone tissue engineering such as orthopedics and dental implants [1]. However, pure hydroxyapatite has limited use as a load-bearing implant because of its poor fracture strength and toughness. One prospective method for obtaining load-bearing implants is the combination of HA with an organic substance [2]. Based on the composition of natural bone, many studies on HA/collagen composite materials have been conducted [3–5]. However, the clinical application of collagen-type implants is still limited because of the drawbacks of collagen such as high cost and the use of collagen increases the risk of cross-infections. Further investigations on HA/organic composite materials is necessary for the development of applicable bone implant materials.

Silk sericin is a widely distributed protein secreted by silkworms; it exists on the surface of the silk fiber and can be extracted using hot water without any toxic solvent. Additionally, it has been reported that sericin can efficiently improve cell proliferation and wound healing [6–8]. Therefore, sericin is a promising protein for the development of biomaterials. The large number of acidic amino acids in sericin facilitates hydroxyapatite deposition by combining tightly with calcium icons in simulated body fluids (SBF) [9], which indicates its potential application in the preparation of HA/sericin composite materials. Takeuchi et al. [10] found that the deposition of hydroxyapatite was regulated by the structure of sericin. Hydroxyapatite was successfully deposited on sericin films that had both high molecular weight and high β-sheet structure, whereas no hydroxyapatite formation was observed on sericin films with lower molecular weight or low β-sheet structure [10]. It has also been reported that sericin-type materials showed improved mechanical properties after ethanol treatment by increasing the β-sheet structure [11–13].

In the present study, we prepared a hydroxyapatite/sericin composite film by mineralizing a flexible ethanol-treated sericin film (Et-sericin film) in simulated body fluids (1.5SBF). The

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structure of the deposited apatite on the surface of the Et-sericin film was characterized through Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Energy Dispersive X-Ray Spectroscopy (EDX), and X-ray diffraction analysis. The mechanical properties and the cell viability of the hydroxyapatite/sericin composite film were also evaluated.

2. Materials and methods

2.1. Materials and reagents

Cocoon shells of the *Bombyx mori* silkworm were provided by the Institute of Huzhou Cocoon Testing (PR China). Tris (hydroxymethyl)aminomethane (Tris) was purchased from Sigma (Singapore). MG-63 cells were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Fetal bovine serum, trypsinase, and an XTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit were purchased from Hangzhou Kezhe Biotechnology Co., Ltd. The high-glucose Dulbecco's Modified Eagle's Medium (DMEM) culture containing a double antibody and the phosphate buffered saline (PBS) solution were purchased from Jinuo Biopharma Technology Co., Ltd. Dehydrated alcohol was purchased from Sinopharm Chemical Reagent Co., Ltd. All chemicals were of analytical grade and were used as received without any further purification.

2.2. Preparation of flexible ethanol-treated sericin film

B. mori cocoons were cut into 1 cm^2 pieces and immersed into boiling water (1:30 weight ratio of sericin to water) for about 30 min to extract the sericin solution with the molecular weight mainly higher than 66 kDa [14]. The extracted sericin solution was filtrated with gauze and concentrated to 9 wt% with a rotary evaporator. It was then cast on a polystyrene dish and dried at room temperature to form a sericin film. The obtained sericin film was then soaked in 40% (v/v) ethanol solution for 10 min to form a flexible ethanol-treated sericin film (Et-sericin film) as the template for mineralization. Additionally, sericin film without ethanol treatment was prepared as the control. The thickness of the Et-sericin film was controlled at 93.5 ± 28.7 μm.

2.3. Mineralization of Et-sericin film in simulated body fluid

Simulated body fluid (1.5SBF, pH 7.4) was prepared according to the composition of human blood plasma [15]. The Et-sericin film was immersed in a beaker containing the simulated body fluid and put into a chamber with a rotation speed of 60 rpm for mineralization. The temperature was kept at 37 °C and the SBF solution was refreshed every day. After a certain number of days (1, 3, 5, or 7), the Et-sericin film was taken out and air-dried at ambient temperature after being rinsed with deionized water.

2.4. Scanning electron microscopy (SEM)

The morphology of the film samples was observed using a scanning electron microscope (XL30-ESEM, Philip, The Netherlands) with an accelerating voltage of 10 kV after sputtering with gold.

2.5. Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared analysis (FTIR) spectra were collected with an FTIR instrument (8400S, Shimadzu, Japan) in the range of $2000-400\ cm^{-1}$ with KBr pellets. The resolution was $4\ cm^{-1}$ and the number of scans was 120.

2.6. X-ray powder diffraction (XRD) and energy dispersive X-ray spectroscopy (EDX)

The crystallographic properties of the samples were analyzed using a X-ray powder diffractometer (XRD, X'Pert PRO, PANalytical, The Netherlands) equipped with a Cu Kα radiation source ($\lambda = 1.5418$ Å) in the 2θ range of $20-60°$. The step size was 0.02° and the scan rate was 5.0 °/min. The element composition of the Et-sericin film mineralized for 7 days was analyzed with an energy dispersive X-ray analyzer (EDS) attached to the XRD instrument.

2.7. Tensile test

The film was cut into $40 \times 10\ mm^2$ samples and soaked in deionized water for 5 min before the test. The tensile properties were measured on a mechanical tester (AGS-J, Shimadzu, Japan) equipped with a 50 N load cell at a speed of 5 mm/min. The length between two gauges was controlled at 2 cm. The data reported are the means ± SD of three consecutive examinations.

2.8. Cell viability

The cell viability of the film samples was evaluated with human osteosarcoma MG-63 cells through the XTT method as described below. The Et-sericin film and Et-sericin film mineralized for 7 days were cut into circular pieces with diameters of 6.4 mm and sterilized with 75% ethanol solution. The sterilized film samples were put into 96-well cell culture plates. Next, 100 μL of 5.0×10^3 cells/mL MG-63 cells were seeded and 1 mL high-glucose DMEM culture medium with a double antibody were added in each well. The plates were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 1, 3, and 5 days. When the initial incubation was completed, 50 μL XTT reagent was added into each well and the plates were incubated for another 4 h. Then, the cell viability was detected by measuring the optical density at 450 nm (OD_{450}) with an ELISA instrument. During the experiment, the culture medium was exchanged every 2 days and the wells without sericin film samples were used as the blank controls. The experiment was repeated three times and the results are presented as mean ± standard deviation (SD)

($n=3$). One-way analysis of variance (ANOVA) was conducted with SPSS 16.0 software. The difference of statistical results was considered significant when $p<0.05$ (labeled with one asterisk) and as extremely significant when $p<0.01$ (labeled with a double asterisk). The morphology of cells cultured on substrates was observed by using inverted phase contrast microscope.

3. Results and discussion

3.1. Morphology

Fig. 1 shows the surface and cross-sectional images of the ethanol-treated sericin film (Et-sericin film) before and after mineralization. The surface morphology of the Et-sericin film before mineralization is smooth (Fig. 1(a)). After mineralizing for 1 day, an irregular-shaped substance was observed on the surface of the Et-sericin film (Fig. 1(b, c)). This substance was thought to be due to the deposition of inorganic matter through the combination of ions in the simulated body fluid with the functional groups of the sericin film. With the increase in mineralization time, the quantity of the deposited inorganic substance increased, showing a three-dimensional aggregated structure (Fig. 1(d–i)) which provide the larger surface area and is beneficial for the attachment of cells. When the mineralization time was 7 days, the morphology became globular (Fig.1(h, i)) and the thickness of the deposited inorganic substance reached 6.22 ± 2.13 μm (Fig. 1(j, k)). It was interesting to find that the morphology of the inorganic substance deposited on Et-sericin film after 7 days mineralization was obviously different from those on sericin film without any treatment (Refs. [9,10]). Additionally, its density was extremely higher than those on raw silk fiber (Ref. [9]) and sericin film kept at 4 °C for 2 weeks (Ref. [10]), although they have the similar globular morphology. These results indicated the different molecular arrangement of sericin molecules in raw silk fiber, sericin film kept at 4 °C for 2 weeks, sericin film without any treatment and Et-sericin film.

3.2. Analysis of deposited components

In order to confirm the components of the deposited substances, FTIR analysis was conducted and the results are shown in Fig. 2. The spectra of the Et-sericin film (Fig. 2(a)) and the HA synthesized in our lab (Fig. 2(e)) [16] were used as the controls for the component analysis. In the spectra of the Et-sericin film, two characteristic peaks, one of amide I (C=O stretching vibration) and the other of amide II (N–H bending and C–N stretching vibrations), are observed at 1650 cm^{-1} and 1517 cm^{-1}, respectively (shifted from 1663 cm^{-1} and 1534 cm^{-1} of sericin without ethanol treatment [17], respectively, not shown in figures). The peak shifts of amide I and amide II indicate an increase in

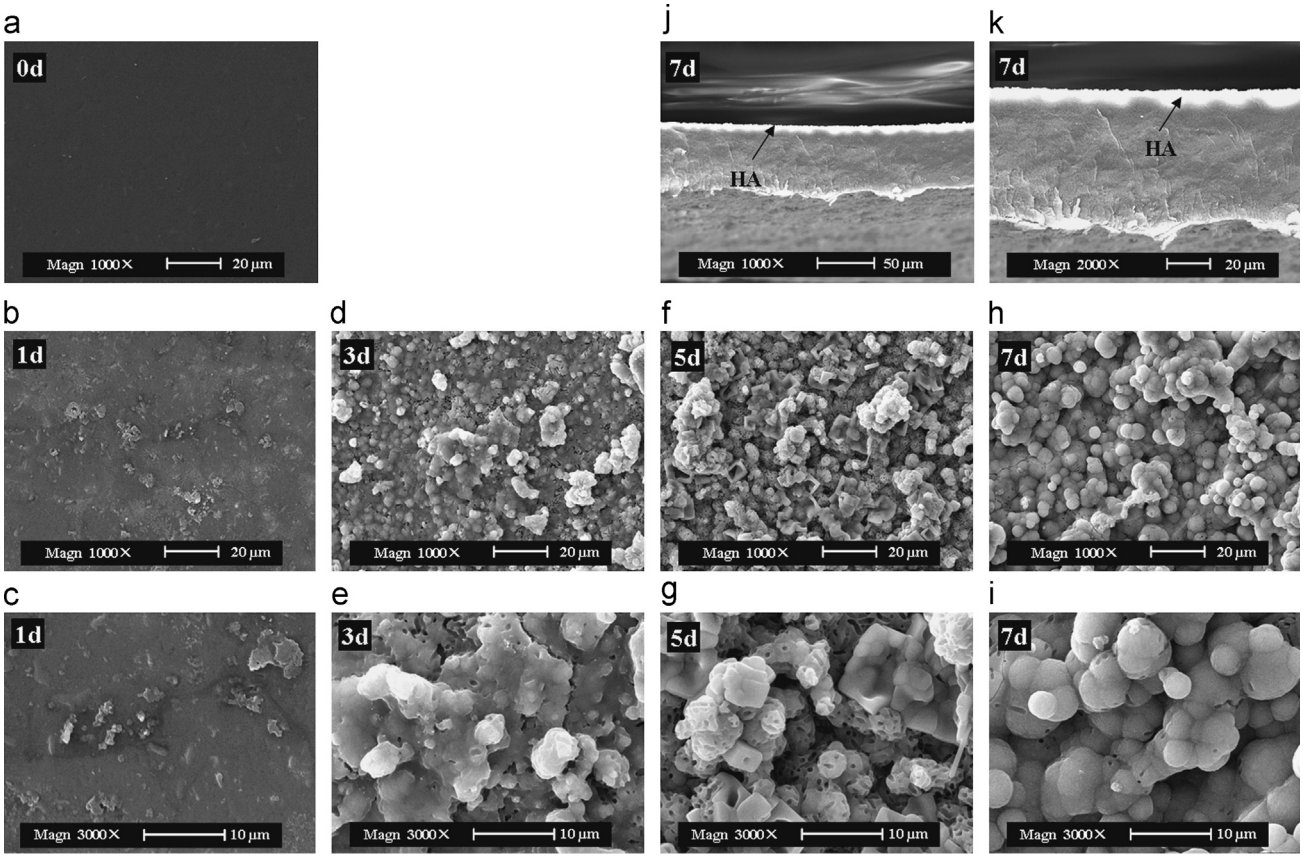


Fig. 1. SEM micrographs of Et-sericin film before and after mineralization. Surface morphology of Et-sericin film (a), Et-sericin film mineralized for 1 day (b, c), Et-sericin film mineralized for 3 days (d, e), Et-sericin film mineralized for 5 days (f, g), Et-sericin film mineralized for 7 days (h, i) at magnifications of 1000 × and 3000 ×, respectively. Cross-sectional morphology of Et-sericin film mineralized for 7 days at magnifications of 1000 × (j) and 2000 × (k).

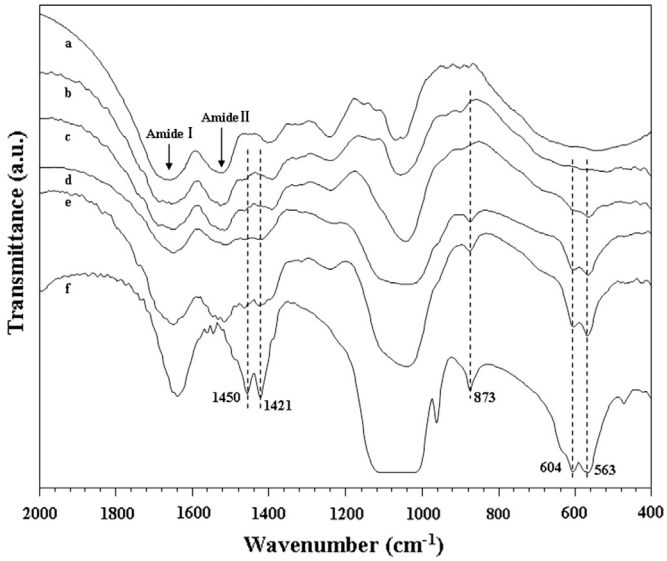


Fig. 2. FTIR spectra of Et-sericin film before and after mineralization and HA. (a) Et-sericin film; (b) Et-sericin film mineralized for 1 day; (c) Et-sericin film mineralized for 3 days; (d) Et-sericin film mineralized for 5 days; (e) Et-sericin film mineralized for 7 days; and (f) HA.

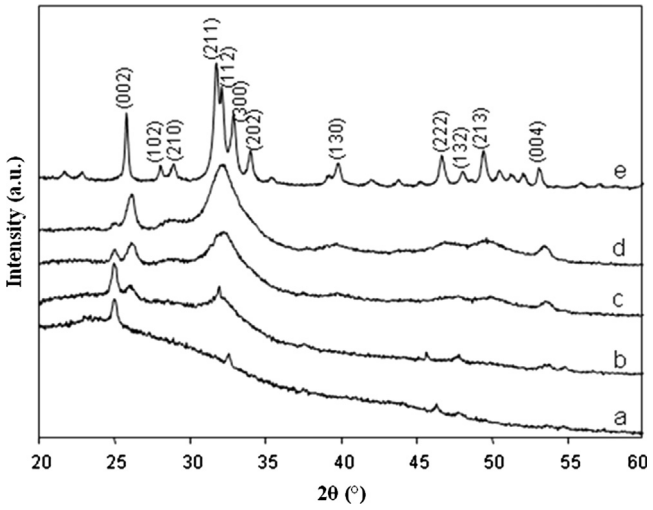


Fig. 3. X-ray diffraction patterns of mineralized Et-sericin film and HA. (a) Et-sericin film mineralized for 1 day; (b) Et-sericin film mineralized for 3 days; (c) Et-sericin film mineralized for 5 days; (d) Et-sericin film mineralized for 7 days; and (e) HA.

the aggregated structure in the sericin film [11,18]. Five characteristic peaks in the HA spectra are observed at 563 cm^{-1}, 603 cm^{-1}, 874 cm^{-1}, 1421 cm^{-1}, and 1450 cm^{-1}. The peaks at 563 cm^{-1} and 603 cm^{-1} are induced by the O–P–O bending vibration from the PO_3^{4-} group [19]. The peaks at 874 cm^{-1}, 1421 cm^{-1}, and 1450 cm^{-1} are due to the C–O stretching vibration of the CO_3^{2-} group, which originated from the dissolved CO_2 during agitation [20]. Therefore, the HA synthesized in our lab was a carbonate apatite similar to natural bone mineral [21].

Peaks of the Et-sericin film and HA were observed in the mineralized Et-sericin films without the appearance of new peaks (Fig. 2(b–e)). These results indicate that the inorganic matter on the surface of the mineralized Et-sericin film is HA containing CO_3^{2-} ions, that is, carbonate apatite. In addition, the characteristic peaks of HA in the mineralized Et-sericin films gradually strengthened with increasing mineralization time, indicating an increase in the number of HA molecules. These results are consistent with the SEM observations.

3.3. XRD analysis

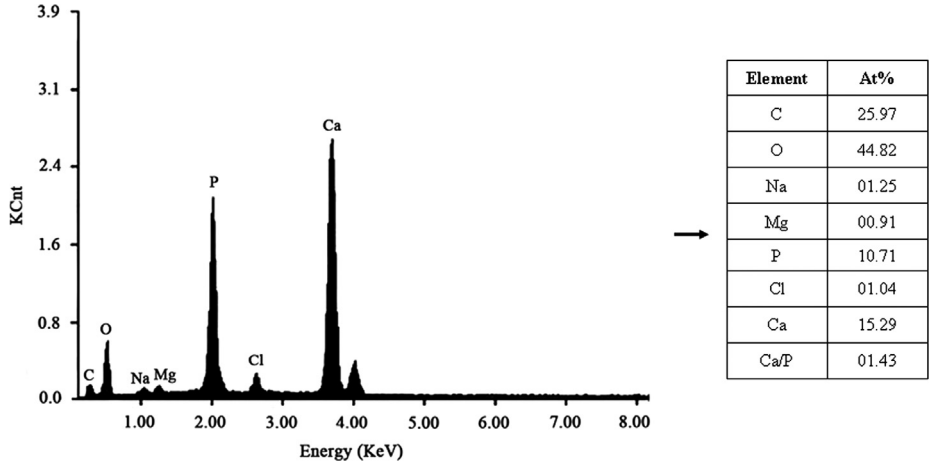
The crystallographic properties of the mineralized Et-sericin films and HA were characterized using X-ray diffraction patterns and the results are shown in Fig. 3. The diffraction peaks of HA were designated according to the International Center for Diffraction Data (ICDD) standard card (Fig. 3(e)). After mineralization, the characteristic peaks of the 002 and 211 planes of the HA crystal gradually appeared and were strengthened in the mineralized Et-sericin film samples (Fig. 3(a–d)). The results indicate that the degree of crystallinity of HA on the Et-sericin film surface gradually increased. The diffraction peaks were broader and overlapped, suggesting that the obtained HA had poor crystallinity similar to that of native bone mineral [22]. Meanwhile, the XRD patterns showed the anisotropic growth of HA crystals along the *c*-axis according to the greater intensity of the 002 peak, which is similar to the growth of apatite minerals in natural bone. By comparing with sericin film without any treatment and sericin film kept at 4 °C for 2 weeks (Ref. [9,10]), it was found that Et-sericin film can induce the formation of bone-like HA more easily according to the characteristic peaks of the 002 and 211 planes of the deposited apatite. This phenomenon can also reveal the difference arrangement of sericin molecules in these materials as shown in SEM results.

3.4. EDX analysis

To further understand the inorganic matter deposited on the surface of the Et-sericin film, energy dispersive X-ray analysis (EDX) was used for the element analysis. The Et-sericin film mineralized for 7 days was mainly composed of C, O, Ca, and P (Fig. 4), which correspond to the elements in sericin and HA. A small number of Na, Mg, and Cl ions from the SBF solution were also deposited on the surface of the Et-sericin film. The Ca/P molar ratio of the deposited mineral was calculated to be 1.43, which is less than that of stoichiometric HA (Ca/P = 1.67). The deposited mineral was presumed to be calcium-deficient hydroxyapatite because of the substitution of Ca^{2+} with other ions such as Na^+ and Mg^{2+} [23,24]. The Ca/P ratio of apatite on Et-sericin film was similar to sericin film kept at 4 °C for 2 weeks (Ref. [10]) while obviously different from sericin film without any treatment (Ref. [9]).

3.5. Mechanical properties

Tensile tests were conducted to clarify the effect of mineralization on the mechanical properties of the Et-sericin film. Table 1 shows the elastic modulus, the tensile strength, and the elongation at break of the Et-sericin film before and after mineralization.



Element	At%
C	25.97
O	44.82
Na	01.25
Mg	00.91
P	10.71
Cl	01.04
Ca	15.29
Ca/P	01.43

Fig. 4. EDX of Et-sericin film mineralized for 7 days.

Table 1
Tensile properties of Et-sericin film before and after mineralization.

Duration of mineralization	Elastic modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
0 day	4.99 ± 2.56	1.58 ± 0.53	112.83 ± 49.02
1 day	3.21 ± 1.17	0.87 ± 0.48	44.14 ± 19.09
3 days	4.02 ± 1.32	0.67 ± 0.19	40.66 ± 16.12
5 days	23.02 ± 10.83	0.39 ± 0.12	3.32 ± 1.25
7 days	57.02 ± 1.04	0.24 ± 0.18	2.01 ± 1.81

The ethanol-treated sericin film showed flexible properties, with an elongation at break of approximately 112%.When the film was mineralized for 1 day and 3 days, the elastic modulus showed little change, whereas the elastic modulus increased significantly after 5 and 7 days. The tensile strength and the elongation at break of the Et-sericin film decreased with increasing mineralization time. The results showed that the rigid and brittle properties of the Et-sericin film increased with mineralization time due to the gradual deposition of inorganic molecules on the surface of the Et-sericin film, as observed with SEM (Fig. 1). Et-sericin film showed the flexible properties while becomes extremely fragile after mineralization, which was presumably due to the decrease in the binding ability among sericin molecules for its combination with HA and the solubility of few sericin molecules during the mineralization period.

3.6. Cell viability

The cell viability of human osteosarcoma MG-63 cells on the Et-sericin film and the Et-sericin film mineralized for 7 days was determined through the XTT method (Figs. 5 and 6). Both the Et-sericin film and the mineralized Et-sericin film showed higher optical density (OD_{450}) and cell density than the blank control (cells grown on the surface of the plate well), indicating their excellent promotion of cell viability. However, the promotion of cell viability of the Et-sericin film mineralized for 7 days was significantly greater than that of the Et-sericin film. With Et-sericin film mineralized for 7 days, cell

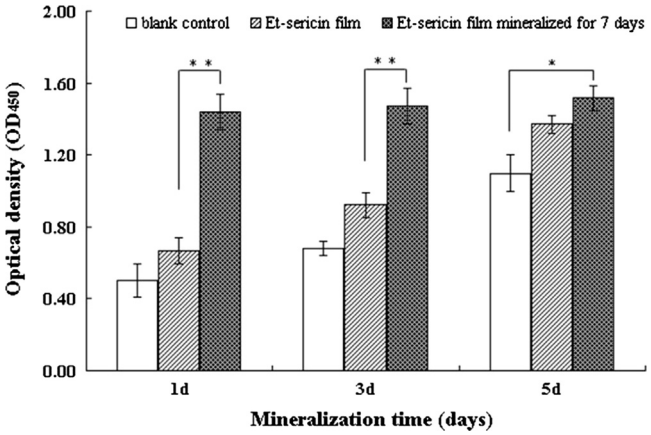


Fig. 5. OD_{450} values of MG-63 cultured on cell plate (blank control), Et-sericin film and Et-sericin film mineralized for 7 days. Asterisks indicate the significant difference between the indicated groups ($*p < 0.05$, $**p < 0.01$).

viability reached a maximum after being cultured for 1 day and little growth was observed after 3 and 5 days possibly owing to the density inhibition (Fig. 6(g–i)); with Et-sericin film, cell viability was significantly lower than that of the Et-sericin film mineralized for 7 days after being cultured for 1 day and 3 days (Figs. 6(d–f), $p < 0.01$) and was proximal to it only after being cultured for 5 days with high cell density (Fig. 6(f)). All these results revealed that the mineralized Et-sericin film showed excellent promotion of cell viability, which could facilitate the development of sericin-type biomaterials.

4. Conclusions

This study has shown that HA can be successively deposited on a flexible ethanol-treated sericin film to form an HA/sericin composite film through SBF mineralization. FTIR, XRD, and EDX results showed that the deposited HA is a calcium-deficient carbonate apatite with poor crystallinity and a *c*-axis direction growth similar to natural bone mineral. The quantity of HA increased with increasing mineralization time, which induced rigid and brittle properties of the flexible ethanol-treated sericin

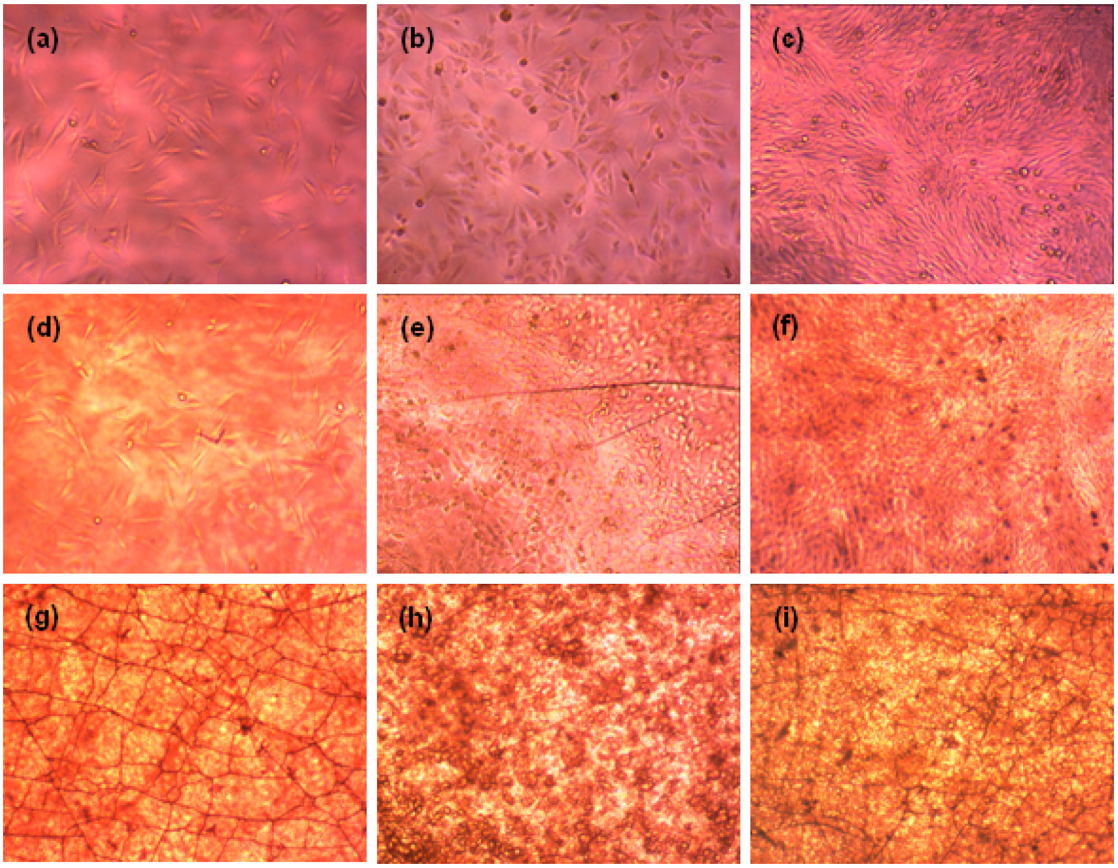


Fig. 6. Morphology of MG-63 cultured on cell plate (blank control) (a–c), Et-sericin film (d–f) and Et-sericin film mineralized for 7 days (g–i), respectively. (a, d and g) represent cells cultured on substrates for 1 day; (b, e and h) represent cells cultured on substrates for 3 days; (c, f and i) represent cells cultured on substrates for 5 days.

film. The HA/sericin composite film showed excellent cell viability resulting from the deposition of HA and its three-dimensional structure.

Acknowledgments

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