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Regenerated egg white/silk fibroin composite films for biomedical applications

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ABSTRACT

Protein-based composites have always been desirable biomaterials as they can be fabricated into a wide range of biomaterials with tunable properties, including modulation of mechanical properties and control of cell responses. Both egg white protein (EW) and silk fibroin (SF) are biocompatible, biodegradable, non-toxic and naturally abundant biopolymers. In order to obtain biocompatible composite films with tunable performance, EW and SF were blended at various ratios. Raising the SF ratio in the composite films significantly increased breaking strength, but impaired flexibility. Conversely, increasing the EW ratio remarkably enhanced elasticity of the composite films. Furthermore, the biological assays based on endothelial cells showed that the incorporation of EW promoted cell viability. These make them potential materials with controllable mechanical property and enhanced bioactivity, providing useful options for the fabrication of tissue engineering scaffolds.

1. Introduction

Protein-based materials hold great promise in biomaterial field because of their intrinsic and unique properties, including nontoxicity, biocompatibility and biodegradability [1]. Considering their unique properties, many natural proteins, including elastin, collagen, silk, keratin and soy protein, have been considered as the desirable matrix for developing innovative materials, including films, porous sponges, fibers, particles and gels, in particular for biomedical applications such as drug delivery systems, biosensors and scaffolds for tissue regeneration [1–7].

Among many different natural polymers available for scaffold preparation, those proteins with high bioactivity and availability, easy handling and low production costs should be preferred. Egg white (EW), a kind of ubiquitous nutritious food in our daily life, is traditionally used in food, and cosmetics industries as a food matrix [8], packing materials [9], emulsifier and thickener [10]. It is a complex system mainly constituted by a globular protein solution, in which ovalbumin (47 kDa), ovotransferrin (77 kDa), ovomucoid (28 kDa) and lysozyme (14 kDa) represents about 54%, 12–13%, 11% and 3.4–3.5% of its protein content, respectively [11]. Ovotransferrin and lysozyme both showed antimicrobial activities. Spectacularly, it was interestingly found that ovalbumin or ovomucoid can stimulate the proliferation of myoblasts and growth of myotubes [12]. With bioactive properties in wound healing, antibacterial [13], anti-inflammatory, and cell growth stimulatory properties [14], it was suggested that EW-based matrixes can be explored for utility in biomaterial applications. However, the use of EA in biomedical applications remains limited. Previous studies have reported EW-based biomaterials were prepared by combining it with synthetic polymers, such as polyvinyl alcohol, cellulose acetate, starch, cellulose and poly(ethylene oxide) (PEO) etc. [15–18]. Recently, Martin et al. demonstrated that EW-based macroporous sponges showed negligible immune reaction and efficient cell and tissue ingrowth after subcutaneous implantation in mice [19]. Despite the progress, more work is needed to improve their performance and stability, etc., of the regenerated EW materials. For instance, the globular molecular structure of EW causes low tensile strength, leading to the limitation of regenerated EW materials.

Silk fibroin (SF) is a typical fibrous protein produced by a variety of insects including silkworm. Because of large-scale cultivation of silkworms for the textile industry, there are abundant and reasonable cost sources for this natural polymer. SF has been explored for numerous biomedical applications involving drug delivery, tissue engineering, or implantable devices, because of their biocompatibility, degradability, mechanical robustness, and versatile processing options from either aqueous solution or an organic solvent [20–22]. Although regenerated SF has been shown to be a biocompatible material, a challenge remains to improve the performance SF materials, such as flexibility and cell activity, to promise its applications within a broad area.

Blending is an effective approach to generate protein-based biomaterials with a more complete set of specific properties [23]. By optimizing molecular interactions between proteins, composite with the

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tunability of mechanical properties, degradability, biocompatibility, and functionalization can be fabricated [24]. Obviously, the molecular structures of EW (globular protein) and SF (fibrous protein) are totally different although they are both proteins. Therefore, the mechanical characteristics and biological properties can be modified by blending EW and SF molecules, which is desirable for biomedical applications. Until now, only silk fibroin-albumin blend nanoparticles, carriers for drug delivery, have been reported [25]. In this study, we first fabricate a composite film via blending EW and SF at various ratios. We then investigated the mechanical properties of composite films and the in vitro cell-material interactions of this new biomaterial by using human umbilical vein endothelial cells.

2. Materials and methods

2.1. Materials

Egg white (EW) was removed from the shell and separated from the egg yolk, which was then freeze dried to obtain EW powder according to reported procedures [26]. The regenerated SF solution was obtained as previously described [27]. Briefly, *Bombyx mori* raw silk fibers (Huzhou, China) were boiled three times to remove sericin in 0.05 wt% Na_2CO_3 for 30 min. The dried fibroin extract was dissolved in a solution of $CaCl_2$/EtOH/H_2O (mole ratio, 1:2:8) at 72 ± 2 °C for 1 h, and then the mixed solution was dialyzed (MWCO 9–12 kDa) against deionized water for 3 days to remove salts. The purified SF solution was frozen at −40 °C for 6 h, followed by lyophilization for 48 h to obtain SF sponge solids.

2.2. Preparation of EW/SF composite films

The concentrations of SF and EW were both fixed at 6 wt% by dissolving them in formic acid (98.0 wt%, Aladdin) respectively. Then EW/SF films were obtained by casting mixed solution onto polyester plates and dried at the room condition with the loading content of EW at different ratio, 0 wt%, 25 wt%, 50 wt%, 75 wt% and 100 wt% based on total weight, referred as SF, 25EW/75SF, 50EW/50SF, 75EW/25SF and EW, respectively. The hybrid EW/SF composites were immersed in 75% ethanol for 1 h to enhance the crystallization.

2.3. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of samples were recorded on the Nicolet 560 FTIR spectrometer using Attenuated Total Reflection method. Each spectrum was obtained with 32 scans in the frequency range of 4000–600 cm^{-1} with 2 cm^{-1} resolution.

2.4. Morphological observation

The surface and cross section of films were examined using a scanning electron microscope (SEM; Hitachi S-4800, Japan). For the observation of cross section, the samples were immersed in the liquid nitrogen to obtain freeze-fractured surfaces. All samples were fixed on top of a conductive tape mounted on a sample stub and coated with a thin gold layer before SEM imaging.

2.5. Mechanical properties of wet EW/SF films

Before measurement, all the samples were immersed in deionized water for 24 h to reach the equilibrium of water uptake. The tensile strength, Young's Modulus and Elongation at break of pure and hydride films were measured using an Instron 5567 Universal Testing machine with a 10 N sensor. Gauge length was 50 mm, and the test was done at 10 mm/min cross-head speed. The thickness average values of films with 1 cm width were measured with a micrometer at least five times. Young's Modulus was automatically calculated by Instron software. Five species were performed for each sample.

2.6. X-ray diffractograms (XRD)

The crystallinity of the various blends was determined by using an X-ray diffractometer (X'Pert-Pro MPD, PANalytical B.V. Holland) with CuKα radiation at 40 kV and 30 mA. Scan was performed in the 2θ range of 5–45° and scanning rate of 0.6 min^{-1}.

2.7. Culture of human umbilical vein endothelial cells (HUVECs)

HUVECs (ATCC, USA) were cultured in Dulbecco's modified Eagle medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin-penicillin (Gibco). Cells were cultured in a humidified incubator at 37 °C and 5% CO_2, and culture medium was replaced every 3 days. The films were punched into 15-mm-diameter samples and then placed into 24-well plates. The samples were sterilized by 75% ethanol for 30 min, and then rinsed three times with sterilized phosphate buffered saline solution (PBS; 0.1 M). After reaching 80% confluence, cells were detached and seeded onto the films at a density of 1×10^5 per well. After cell seeding, cell viability and cytocompatibility assessment were evaluated by CCK-8 assay [27].

At the designated time points, the cell-seeded films were washed three times with PBS and fixed in 4% paraformaldehyde in PBS for 30 min, then rinsed three times with PBS. The cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then blocked with 2% BSA in PBS for 30 min. The F-actin were stained with FITC-phalloidin (5 μg/mL, Sigma-Aldrich) for 2 h at room temperature. After thoroughly rinsing with PBS, the cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/mL, Sigma-Aldrich) for 10 min, rinsed three times with PBS for 10 min each. The cell morphology was observed by confocal laser scanning microscopy (CLSM; IX81/FV1000, Olympus, Japan).

2.8. Statistical analysis

Statistical comparisons were performed using SPSS version 16.0 software (SPSS Inc., Chicago, Illinois). The deductive statistics (*t*-test, ANONA) were conducted and the data were expressed as mean ± standard deviation, and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Structural characterization of EW/SF films

The FTIR spectrum has been widely adopted for characterizing the secondary structures of protein. Here, the typical absorptions of amide I, amide II and amide III structures in pure SF, EW protein and their hybrid films were analyzed by FTIR, as shown in Fig. 1. Commonly, Amide I bonds (—C═O stretching) ranging from 1600 to 1640 cm^{-1} were considered to be characteristic of β-sheet structure [28]. Obviously, the main structure of Amide I in pure EW film which was casted from formic acid is β-sheet. Likewise, the peak at 1618 cm^{-1} in the spectrum of pure SF film also confirmed the β-sheet structures. The similar results have also been found in the keratin, which was due to the regeneration of β-sheet crystallization at slow evaporation of formic acid under room temperature [29]. As the increasing loading of EW, the absorption band of Amide I did not show any significant differences. As for amide II absorptions (—NH bending), the peaks were located at 1513 and 1520 cm^{-1} in pure SF and EA, respectively, indicating that SF and EA films crystallized predominantly in the form of the β-sheet secondary structures by casting from formic acid. A blue shift was observed with the incorporation of EW, suggesting the interactions occurred between EW and SF. After 75% ethanol treatment, the amide II peak absorption