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Regulating the degradation rate of silk fibroin films through changing the genipin crosslinking degree

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ABSTRACT

Degradation behavior is a key fundamental topic in the field of silk-based biomaterials. In this study, genipin-crosslinked silk fibroin films with varying crosslinking degrees were generated to investigate the effect of the crosslinking degree on the degradation behavior. Higher crosslinking degrees resulted in increased inter-intramolecular network crosslinking density through covalent bonds, which restricted enzymatic attacks and the release of enzyme-degraded polypeptides from silk fibroin molecular networks, providing greater resistance to enzyme degradation. Furthermore, genipin crosslinking induced the conformational transition from random coil to β-sheet due to the structural rearrangement of the chains to form covalent bonds. High β-sheet content contributes to a more crystalline structure that also enhances resistance to enzyme degradation. Consequently, changing the crosslinking degree had a substantial impact on the degradation rate. The high-crosslinking films (with a crosslinking degree greater than 90%) showed a similar degradability to 75% ethanol-treated films, which were barely degraded by collagenase IA and subcutaneous implantation in SD rats. However, decreasing the crosslinking degree from 90% to 78% significantly increased the degradation ratio from 4 wt% to 18 wt% after 28 days *in vivo* degradation, respectively. In particular, the degradability was strongly correlated to the crosslinking degree in the low-crosslinking (lower than 90%) films. These results reveal that the crosslinking process can efficiently control molecular structures and regulate the crystallization of silk fibroin materials, in turn providing control of the degradation rate.

1. Introduction

The desired scaffold degradation rate depends on the specific tissue engineering application. Ideally, the rate of scaffold degradation should mirror the rate of new tissue regeneration [1]. Silk fibroin (SF) is a promising biomaterial for tissue engineering and regenerative medicine due to its abundance, biocompatibility, mechanical robustness, and tunable degradation properties [2,3]. The degradability of silk biomaterials depends on morphological features, processing modes and β-sheet content [4–10]. The pore structure of silk materials and the molecular weight of regenerated SF affect the degradation rate [4–6]; however, these profiles provide only a narrow range of degradation regulation. A wider range degradation rates and tighter control are desirable for varied tissue regeneration needs; for example, a scaffold for bone repair requires a slow degradation rate, while rapid degradation is required for dermal tissue repair.

Natural silk fibers must be regenerated and processed into different products such as films, nanofibers and porous scaffolds for various biomedical applications. In general, the regenerated SF materials are water-soluble and should be treated to induce water-resistant crystallization and chemical cross-linking. To induce β-sheet crystallization, several methods are used in SF biomaterials, such as organic solvent immersion and water vapor annealing [11–13], and the most common method based on structural change is the induction of methanol or ethanol immersion. The structural transition from random coil to the β-sheet results in aqueous insolubility after treatment; however, a significant increase in β-sheet structure leads to lower degradation rates [7–9]. Furthermore, these SF materials tend to be stiff and brittle in the dry state [14]. To maintain water-insolubility and improve the mechanical properties such as flexibility, chemical crosslinking reagents such as genipin, polyethylene glycol diglycidyl ether (PEG-DE) and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride

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(EDC) are widely used to obtain water-insoluble SF materials for tissue engineering purposes [15–19]. However, none of these studies examine the effect of the crosslinking degree on degradation rates in great detail.

Genipin is an effective natural crosslinking agent that can react spontaneously with amino acids or proteins [20]. It has been reported that genipin-crosslinked biological tissues and biopolymers show good mechanical properties and significantly reduced cytotoxicity compared to synthetic crosslinking agents such as glutaraldehyde and epoxy compounds [15,21]. Genipin has been used to crosslink SF-based biomaterials such as films [21], porous scaffolds [15,16] and nanofibers [22,23]. In this study, SF films with varying crosslinking degrees were prepared by changing the genipin ratio to examine the impact of the crosslinking degree on the degradation rate. These SF films had varied crosslinking densities and β-sheet contents, which are very useful in regulating the degradation rate. This method provides new approaches to modulate the degradation rate of SF materials for various tissue repair needs.

2. Materials and methods

2.1. Preparation of regenerated silk fibroin films

A SF solution was prepared as described previously [4]. Briefly, *Bombyx mori* raw silk fibers (Huzhou, China) were boiled for 30 min in an aqueous solution of 0.05% Na_2CO_3 and then rinsed in distilled water to remove sericin. The extracted fibers were subsequently air dried and dissolved in $CaCl_2$:CH_3CH_2OH:H_2O solution (molar ratio 1:2:8) at 72 °C ± 2 °C for 1 h. This solution was dialyzed against distilled water (MWCO 9–12 kDa) in cellulose tubes for 4 days. The resulting SF solution was stored at 4 °C after filtration. To generate genipin-crosslinked SF films with varying crosslinking degrees, genipin (Sigma–Aldrich) was added to the SF solution at 5%, 10%, 20% and 30% of the SF weight in solution, respectively. Subsequently, the mixture solutions were stirred slowly for 12 h at 37 °C. Next, 40 mL of solutions were cast into polyethylene plates at 60 °C ± 2 °C for 2 h, and then further dried at room temperature for 24 h to obtain genipin-crosslinked films. The pure SF films were prepared at the same conditions and became insoluble after treatment with 75% ethanol for 2 h.

2.2. Determination of crosslinking extent

The crosslinking degree was determined by the ninhydrin assay according to a previously reported method [16]. The films were weighed (0.05 g, $n = 3$ per group) and immersed in 1.5 mL distilled water for 1 h. Subsequently, a 450 μL 0.1% ninhydrin (Sigma–Aldrich) solution was added to each sample and heated at 100 °C for 20 min. The number of free amino groups in the sample was proportional to the absorbance of the solution. The absorbance was recorded at 450 nm using a microplate reader (Bio-Tek Synergy HT, USA). Glycine solutions of various known concentrations were used as standards. The crosslinking degree was expressed as the percentage of reacted free amine number relative to the initial free amine number.

2.3. In vitro enzymatic degradation

The SF films were weighed and incubated at 37 °C in PBS solution (PBS; 0.05 M, pH 7.4) containing 1.0 U/mL Collagenase IA (from *Clostridium histolyticum*, EC 3.4.24.3, Sigma–Aldrich). The samples ($n = 3$ per time point) were incubated in enzyme solution (bath ratio 1:50) for 1, 3, 6, 12, 18 and 30 days under slow shaking and in PBS under otherwise identical conditions as a control. The degradation solution was replaced with a fresh enzyme solution every 3 days. At the designated time points, the degradation products and remains were collected for analysis. The remaining samples were rinsed in deionized water and then dried at 105 °C to constant weight. Quantitative changes were expressed as the percentage of weight retained relative to the initial dry weight.

2.4. The morphological and structural change

The surface morphologies of SF films after degradation for 0, 18 and 30 days were observed by scanning electron microscopy (SEM; S-4800, Hitachi, Japan). For the molecular conformation measurements, Fourier-transform infrared (FTIR) spectroscopy analysis was performed using a Nicolet 5700 spectrometer (Thermo Scientific, USA). The secondary structure content of the SF films was measured as described previously [4,24]. Briefly, Fourier self-deconvolution of the amide I region (1595–1705 cm^{-1}) was performed using Opus 6.5 software (Bruker, Germany), and the Fourier self-deconvolution spectra were curve-fitted to measure the relative areas of the amide I region components. Furthermore, X-ray diffraction (XRD) was performed to investigate the changes in the crystal structure of the samples using an X-ray diffractometer (X'Pert-Pro MPD, PANalytical B.V. Holland) with Cu Kα radiation at 40 kV and 30 mA and a scanning rate of 0.6/min.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The degradation products at 6 days and 30 days were examined using SDS-PAGE. The samples were run on 5% and 8% polyacrylamide gel in running buffer (0.25 M Tris–HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol and 5% 2-mercaptoe-thanol, pH 8.3). The stacking gel contained 5% acrylamide, 0.1% ammonium persulfate and 0.1% SDS in 1.0 M Tris–HCl buffer (pH 6.8), and the separating gel contained 8% or 10% acrylamide, 0.1% ammonium persulfate and 0.1% SDS in 1.5 M Tris–HCl buffer (pH 8.8). Prestained protein served as the MW markers.

2.6. In vivo degradation

To study the relationship between the *in vivo* degradation and the crosslinking degree, the 78%-, 90%- and 94%-degree-crosslinking films were implanted in Sprague–Dawley (SD) rats, and the 75% ethanol-treated films were used as controls. The animal experiments were in accordance with the Management Ordinance of Experimental Animals of China ([2001] No. 545) and were approved by the Jiangsu Province in experimental animal management rules ([2008] No. 26). The animals were divided into four groups of 5. The SF films were cut into 20 × 20 mm pieces and subcutaneously implanted in the back of male SD rats (180–200 g, SPF grade). Pentobarbital sodium (30–60 mg/kg body weight) was administered pre-surgically. After shaving and disinfection, two perpendicular incisions were created on the back of the rats, and then a blunt probe was inserted through the incision to form a subcutaneous implantation space (approximately 20 mm × 20 mm). The samples were implanted into the subcutaneous space. The wounds were closed with 6-0 silk sutures and covered by Vaseline carbasus and dry carbasus. At 28 days, the specimens were harvested. A portion of harvested samples were immediately fixed in 4% formaldehyde in PBS at room temperature and embedded in paraffin to cut tissue sections for hematoxylin and eosin (H & E) staining and optical microscope observation (Olympus BH-2, Japan). The other portion of harvested specimen ($n = 3$ per group) were explanted and freed from the surrounding tissue. The specimen were rinsed in deionized water and then dried at 105 °C to constant weight. The degradation ratio was expressed