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Silk structure and degradation

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ABSTRACT

To investigate the structure of silk and its degradation properties, we have monitored the structure of silk using scanning electron microscopy and frozen sections. Raw silk and degummed raw silk were immersed in four types of degradation solutions for 156 d to observe their degradation properties. The subcutaneous implants in rats were removed after 7, 14, 56, 84, 129, and 145 d for frozen sectioning and subsequent staining with hematoxylin and eosin (H.E.), DAPI, Beta-actin and Collagen I immunofluorescence staining. The *in vitro* weight loss ratio of raw silk and degummed raw silk in water, PBS, DMEM and DMEM containing 10% FBS (F-DMEM) were, respectively, 14%/11%, 12.5%/12.9%, 11.1%/14.3%, 8.8%/11.6%. Silk began to degrade after 7 d subcutaneous implantation and after 145 d non-degraded silk was still observed. These findings suggest the immunogenicity of fibroin and sericin had no essential difference. In the process of *in vitro* degradation of silk, the role of the enzyme is not significant. The *in vivo* degradation of silk is related to phagocytotic activity and fibroblasts may be involved in this process to secrete collagen. This study also shows the developing process of cocoons and raw silk.

1. Introduction

Silk is a protein fiber secreted by the *Bombyx mori* L. juvenile worm, and consists of fibroin [1,2] and sericin [3]. Silk used in biological tissue engineering is mainly regenerated silk fibroin [4–15], or degummed silk [16–19]. Currently, there are only a few reports describing the application of raw silk in tissue engineering. A reason for this paucity is the poor understanding of the structure of silk. This study examines the assembly process from the fibroin to the silkworm cocoon and investigates the structure of silk.

As a natural biological material, the immunogenicity of silk is a topical issue for investigation [4–15,20]. The majority of studies have used regenerated fibroin, in which the main component is silk fibroin. Consequently, the recognized immunogenicity refers to the immunogenicity of silk fibroin. No relevant studies have reported the immunogenicity of silk sericin. Therefore, the present study aims to investigate the immunogenicity of silk fibroin and sericin.

Degradability is an important property of biological materials. The degradation of materials includes both *in vitro* and *in vivo* degradation. *In vitro* degradation experiments are performed in a degradation solution, with PBS (phosphate buffered solution) being the most commonly used [17,21,22]. A PBS solution is characterized by a stable pH value and osmotic pressure, having no oxidative properties [23] and no enzymes present. Thus, the degradation of the materials in PBS relies upon hydrolysis under physiological pH and osmotic pressure [23]. Plasma is a better choice of degradation solution for the evaluation of *in vitro* degradation of materials [22]. The enzyme in tissue solution also play a crucial role in the degradation of the materials in animal tissues [10,24]. Therefore, adding enzymes into the degradation solution will simulate the *in vivo* degradation of materials in tissues [18,21]. In this study, four types of degradation solutions (*i.e.*, deionized water, PBS, DMEM

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and DMEM supplemented with 10% fetal bovine serum (F-DMEM)) were used to investigate the effects on raw and degummed silk degradation.

In the degradation process, cells play a significant role in both the *in vitro* and *in vivo* environments [13,20,25]. At present, only a few studies have focused directly on investigating the degradation mechanism of the materials or the role tissue solution and cells play during the *in vivo* degradation process. In this study, raw silk and degummed silk were subcutaneously implanted into rats in a broader attempt to explore the mechanism of *in vivo* degradation of silk.

Degradation of degummed silk [17,18], degummed silk-prepared surgical sutures [19], or regenerated silk fibroin [1,2,4–20,25,26] is attributed to the degradation of silk fibroin. While the degradation properties of silk fibroin have been the subject of intensive investigation, the degradation properties of raw silk remain unclear. This study aims to elucidate the difference between the degradation mechanisms of raw silk and degummed silk.

2. Materials and methods

2.1. Materials

Silkworm cocoons, raw silk, regenerated silk fibroin and posterior silk gland derived fibroin were provided by Bio-Technology College of Southwest University, China. Sprague-Dawley rats were purchased from the Third Military Medical University of Chinese PLA. Nuclear immunofluorescence kit (DAPI), Beta-actin antibody and Collagen I antibody were produced by Gene Tax Co. Fetal calf serum, PBS and DMEM were produced by Hyclon. Pentobarbital sodium was produced by Merck.

2.2. Analytics

2.2.1. The morphology of natural silk

Silkworm cocoon and raw silk were observed by scanning electron microscopy (SEM) (JSM-6510LV), and samples were sliced into paraffin sections (LEICA RM 2235) or frozen sections (LEICA CM1850) and observed with a biological microscope (Nikon 80i, image-pro plus 5.1).

2.2.2. Degumming of raw silk

Raw silk was prepared as bundles. Bundles were weighed (BL-2204, SHIMAOZU Corporation, Japan) and immersed in a 0.5% (W/V) $NaCO_3$ aqueous solution. The solution was stirred at 100 °C for 1 h using a constant magnetic stirrer (type 85-2, Shanghai Instrument Co., Shanghai, China), then placed in deionized water and stirred at 70 °C for 1 h. The experimental procedures were repeated three times, and the bundles were removed and dried using ventilation equipment (CS101-2A BN, Chongqing Immortalized Experimental Instrument Factory, China) at 40 °C.

2.2.3. In vitro *degradation experiments*

Bundles of raw silk and degummed silk were knotted then trimmed to 10 mm lengths and weighed for further use. Under aseptic conditions, one bundle of raw silk or degummed silk was immersed in a 15 mL ampoule containing 10 mL degradation solution (deionized water, PBS, DMEM or DMEM supplemented with 10% fetal bovine serum (F-DMEM)). The ampoule was placed in biochemical incubator (SHH-2502, Chongqing Immortalized Experimental Instrument Factory), and cultured at 37 °C. An equal amount of degradation solution without degradation materials was taken as the control. At 1, 4, 8, 21, 35, 63, 91, or 156 d the materials were harvested. The ampoule was shaken and then left standing for 30 min. The ampoule was then opened and the bundles were taken out, dried thoroughly using 40 °C ventilation and weighed. The degradation solution was stored at −20 °C. The weight loss rate was calculated according to the following formula:

$$\text{Weight loss}(\%) = [(\text{initial weight} - \text{weight after degradation})/\text{initial weight}] \times 100$$

After ventilation drying the outer surface and cross section of degraded materials were observed by SEM.

2.2.4. In vivo *degradation experiments*

The bundles were immersed in PBS containing antibiotics (250 IU penicillin and 250 IU streptomycin per 1 mL PBS) before subcutaneous implantation. Twenty adult Sprague Dawley rats (half female and half male, weighing 200 ± 10 g) were randomly divided into two groups. Rats were anesthetized with 3% sodium pentobarbital 40–50 mg/kg *via* intramuscular injection. Then materials were subcutaneously implanted, the wound was sutured and the rats were returned to separated cages for feeding. At 7, 14, 56, 84 and 145 d after subcutaneous implantation, the implants were cut into frozen sections and tained with hematoxylin and eosin (H.E.). The regenerated silk fibroin, silk fibroin from posterior silk gland and frozen sections of rat skin were directly observed by laser scanning confocal microscopy (CLSM) (ZEISS LSM200, ZEISS Observer Z1, Lumen Dynamics X-cite 120Q) at 555, 488 and 405 nm to determine their autofluorescence as controls. After 129 d, the implants were cut into frozen sections for DAPI, Beta-actin and Collagen I antibody immunofluorescence staining. The sections were observed under a CLSM.

3. Results

3.1. Morphology of natural silk

Floss consists of two monofilaments with each monofilament composed of silk fibroin and sericin and every two monofilaments are wrapped by sericin to form a floss. In the silk reeling process, each silkworm cocoon provides a floss, and several flosses constitute a raw silk. Since the reeling is carried out in hot water and sericin is water-soluble, several flosses bind together by sericin to form a raw silk.

The area of the monofilament cross sections was found to have large variability, with the width ranging from 12 to 20 μm and an average of 18 μm. The width of each floss ranged between 25 and 45 μm with an average value of 42 μm. The maximum width of each raw silk comprised of 22–27 flosses was 95–120 μm. Raw silk with 24 flosses (48 monofilaments) had a maximum width of 110 μm. In the preparation process of paraffin sections, silkworm cocoons were immersed in 65 °C wax and dewaxed in xylene. Some flosses were separated into the monofilaments while some sericins were isolated from the monofilament surface. In frozen sections the monofilaments maintained the complete structure.

3.2. In vitro *degradation of silk*

The reduced weight of raw silk was regarded as the sericin dissolved in hot water. The results showed that, the amount of sericin removed (W/W) was 16.7–25.0% (n = 12, average value = 18.9%) of the total weight of raw silk.

Incubation of the raw silk for 1 d in the degradation solution showed no significant degradation symptoms on the surface of the silk. After 156 d of degradation (Fig. 1a), the raw silk bundle displayed many monofilaments because of the dissolution of silk sericin. Raw silk degraded in PBS and DMEM were separated