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Milled non-mulberry silk fibroin microparticles as biomaterial for biomedical applications

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ABSTRACT

Silk fibroin has been widely employed in various forms as biomaterials for biomedical applications due to its superb biocompatibility and tunable degradation and mechanical properties. Herein, silk fibroin microparticles of non-mulberry silkworm species (*Antheraea assamensis*, *Antheraea mylitta* and *Philosamia ricini*) were fabricated via a top-down approach using a combination of wet-milling and spray drying techniques. Microparticles of mulberry silkworm (*Bombyx mori*) were also utilized for comparative studies. The fabricated microparticles were physico-chemically characterized for size, stability, morphology, chemical composition and thermal properties. The silk fibroin microparticles of all species were porous (~5 μm in size) and showed nearly spherical morphology with rough surface as revealed from dynamic light scattering and microscopic studies. Non-mulberry silk microparticles maintained the typical silk-II structure with β-sheet secondary conformation with higher thermal stability. Additionally, non-mulberry silk fibroin microparticles supported enhanced cell adhesion, spreading and viability of mouse fibroblasts than mulberry silk fibroin microparticles ($p < 0.001$) as evidenced from fluorescence microscopy and cytotoxicity studies. Furthermore, *in vitro* drug release from the microparticles showed a significantly sustained release over 3 weeks. Taken together, this study demonstrates promising attributes of non-mulberry silk fibroin microparticles as a potential drug delivery vehicle/micro carrier for diverse biomedical applications.

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1. Introduction

Materials of natural origin are noteworthy targets for further innovation in biomedical field, which solely rely on the development of different forms of natural polymeric biomaterials [1–3]. The utilization of natural polymeric biomaterials has shown superiority in biomedical applications since they have shown better compatibility with the native extracellular matrix (ECM) [3–5].

Silk fibroin, a fibrous polymer derived from different silkworm species has escalated much interest for biomedical applications [6–9]. Silk fibroin (SF) derived from mulberry silkworm; *Bombyx mori* (*B. mori*) has been widely used as suitable matrices/substrates due to its high oxygen permeability, superior mechanical properties and excellent biocompatibility [10–15]. Additionally, there is a natural abundance of cell binding motifs, arginine-glycine-aspartic acid (RGD) in non-mulberry silk fibroin, which felicitates better cell attachment and proliferation [16–21]. The presence of RGD motifs in non-mulberry silk fibroin based matrices provide a substrate for stem cells differentiation and progenitor cells, which render it as a suitable biomaterial for variety of tissue engineering applications [5,22]. However, non-mulberry silk fibroins are less explored as biomaterials than mulberry silk.

Silk fibroin-based biomaterials in different forms such as films, fibers, nanoparticles, microparticles, hydrogels and scaffolds have been fabricated and utilized for a broad range of biomedical applications [5–7,9,20,22–27]. Among these, micro and nano sized silk particles have received much interest as resorbable vehicles for drugs and growth factors for therapeutics and tissue engineering applications [28–31]. Currently, there is a plethora of different methods for fabrication of silk fibroin microparticles such as emulsion polymerization, phase separation, and solvent evaporation/extraction and others for various biomedical applications [31–37]. These techniques are very effective for the mass production of microparticles, but suffer from some major limitations such as use of high temperature and organic/toxic solvents, which

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ultimately compromise the encapsulation efficiency of certain bioactive materials [38,39]. In order to address the aforementioned drawbacks of conventional techniques, development of mild and cost effective techniques free from the usage of harmful chemicals is utmost required. Therefore, there is significant interest in developing new and improved methods to fabricate biocompatible microparticles for biomedical and drug delivery applications.

In our previous reports, an effective and reproducible method was developed for the preparation of silk fibroin ultrafine particles based on a top-down approach using a combination of wet-milling and spray-drying method [40–43]. This fabrication method is rather simple and reproducible, which allows the production of biocompatible materials with high encapsulation efficiencies [44]. Unlike the bottom-up approach, which requires dissolution of fibers followed by regeneration, this strategy utilizes only mechanical energy for the production of microparticles [41,42]. Compared to the silk fibers, the fabricated ultrafine particles of silk fibroin has demonstrated improved degradation properties, which in turn provide a connection linking modulation of structure with biodegradation [42]. Furthermore, silk fibroin ultrafine particles utilized as filler in silk fibroin scaffolds has also demonstrated enhanced mechanical properties (~40 fold increase) in reinforced scaffolds [45]. However, the physico-chemical characterization, other cellular behavior, cytocompatibility and drug delivery applications of the mulberry and non-mulberry silk fibroin based microparticles are still unexplored to implement these microparticles for specific biomedical applications. Therefore, in order to use these silk fibroin microparticles for specific biomedical applications and/or drug delivery, complete characterization and cellular compatibility evaluation of microparticle are very much required. Herein, we demonstrate the production and characterization of silk fibroin microparticles from different non-mulberry silk (*Antheraea assamensis*, *Antheraea mylitta* and *Philosamia ricini*) to evaluate their suitability for biomedical applications. This study focuses mainly on understanding the effect of milling on silk fibroin microparticles morphology, stability, physico-chemical properties, thermal properties, cell–matrix interactions and drug release behavior.

2. Materials and methods

2.1. Materials

Mulberry and non-mulberry silkworm fresh cocoons were collected from Silk farm, Institute of Advanced Study in Science and Technology, Guwahati, India. Cell culture grade chemicals, including Dulbecco's modified Eagle medium: Ham's F-12 nutrient medium (DMEM/F-12), fetal bovine serum (FBS), Trypsin–EDTA, and penicillin–streptomycin was purchased from Gibco BRL (Rockville, MD, USA). MTT, Rhodamine phalloidin, Hoechst 33342 and alcian blue 8GX were purchased from Sigma. All other chemicals used were of analytical grade.

2.2. Degumming of silk

B. mori, *A. assamensis* and *A. mylitta* silk cocoons were cut to remove pupae whereas *P. ricini* cocoons were open mouthed and were free from pupae. Degumming was done to remove sericin and was performed in a laboratory dyeing machine (Thies, USA) using 2 g/L sodium carbonate and 0.6 g/L sodium dodecyl sulfate (Sigma–Aldrich, Australia) at 100 °C. Degumming was performed for 20 min for *B. mori* and 120 min for *A. assamensis*, *P. ricini* and *A. mylitta* cocoons with a material mass (g) to liquor volume (mL) ratio of 1:25. Further, degummed fibers were thoroughly washed with warm distilled water (dH_2O) followed by cold dH_2O in order to remove all the chemicals completely.

2.3. Preparation of silk fibroin microparticles

Silk microparticles were fabricated using the earlier reported method [40]. Briefly, degummed silk fibers were chopped in a cutter mill (Pulverisette 19 from Fritsch GmbH, Germany) fitted with a 1 mm grid and operating at 2888 RPM followed by wet milling of chopped snippets in a stirred media mill (1S Attritor from Union Process, USA) with a stirring speed of 280 RPM. The milling media comprises of 20 kg yttrium doped zirconium oxide balls of 5 mm diameter and the wet milling time used was 6 h for *A. assamensis*, *P. ricini*, *A. mylitta* and 10 h for *B. mori*. The batch size used was 200 g of snippets and 2 L of deionized (DI) water with cold water circulation (approximately 18 °C) through the vessel jacket was used during milling. Finally, dry powders from the wet milled slurry were recovered using a laboratory spray dryer (B-290, Buchi Labortechnik AG). The conditions used during spray drying includes: inlet temperature, 130 °C; pump setting, 25% (18–20 mL/min); and aspirator setting, 100% (42.5 m^3/h).

2.4. Particle size analysis

The average size of different microparticles was determined by using dynamic light scattering (DLS) (Zetasizer, Nano ZS, 90, Malvern, UK). For the analysis of particle size, the silk fibroin powder was suspended in deionized water (0.1 mg/mL) and vortexed for 15 min in order to obtain the uniformly dispersed microparticles. The dispersity of microparticles was calculated by polydispersity index. The size of the microparticles was measured in a suspension at room temperature, 25 °C ($n = 3$).

2.5. Optical microscopy

Size and morphology of silk fibroin microparticles (0.1 mg/mL) were analyzed by optical microscopy using an inverted microscope (LeicaDMI3000B).

2.6. Scanning electron microscopy (SEM)

Silk fibroin microparticles solution (0.1 mg/mL) was used for SEM analysis. Microparticles solutions were vacuum dried and sputter coated with gold and analyzed with Zeiss Sigma VP FE-SEM. Microparticle size was determined by using Image J software (Wayne Rasband, National Institute of Health, USA). For each type of silk fibroin microparticles a minimum of 50 particles was examined for size determination.

2.7. Transmission electron microscopy (TEM)

The morphology and size of the particles were further characterized by TEM using a JEOL JEM-2100 model transmission electron microscope with an acceleration voltage of 200 KV. The samples were prepared by taking 200 μl of the microparticle dispersion solution (0.1 mg/mL) on carbon coated electron microscopy grids. The grid containing the sample microparticles was air dried in a dust-free environment at room temperature before examination under TEM.

2.8. Structural stability of silk fibroin microparticles

The microparticles solution (0.1 μg/mL) of mulberry and non-mulberry silk fibroin was prepared in PBS (pH-7.4) solution and incubated at 37 °C for 1 and 14 days. After particle incubation for 14 days at room temperature, all samples were assessed by scanning electron microscopy and compared to the day 1 sample for structural/morphological changes. If no difference regarding the

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