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Control of silk microsphere formation using polyethylene glycol (PEG)

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ABSTRACT

A one step, rapid method to prepare silk microspheres was developed, with particle size controlled by the addition of polyethylene glycol (PEG). PEG molecular weight (4.0 K–20.0 KDa) and concentration (20–50 wt%), as well as silk concentration (5–20 wt%), were key factors that determined particle sizes varying in a range of 1–100 μm. Addition of methanol to the PEG-silk combinations increased the content of crystalline β-sheet in the silk microspheres. To track the distribution and degradation of silk microspheres *in vivo*, 3-mercaptopropionic acid (MPA)-coated CdTe quantum dots (QDs) were physically entrapped in the silk microspheres. QDs tightly bound to the β-sheet domains of silk via hydrophobic interactions, with over 96% of the loaded QDs remaining in the silk microspheres after exhaustive extraction. The fluorescence of QDs-incorporated silk microspheres less stable in cell culture medium than in phosphate buffer solution (PBS) and water. After subcutaneous injection in mice, microspheres prepared from 20% silk (approx. 30 μm diameter particles) still fluoresced at 24 h, while those prepared from 8% silk (approx. 4 μm diameter particles) and free QDs were not detectable, reflecting the QDs quenching and particle size effect on microsphere clearance *in vivo*. The larger microspheres were more resistant to cell internalization and degradation. Since PEG is an FDA-approved polymer, and silk is FDA approved for some medical devices, the methods developed in the present study will be useful in a variety of biomedical applications where simple, rapid and scalable preparation of silk microspheres is required.

Statement of Significance

The work is of significance to the biomaterial and controlled release society because it provides a new option for fabricating silk microspheres in one simple step of mixing silk and polyethylene glycol (PEG), with the size and properties of microspheres controllable by PEG molecular weight as well as PEG and silk concentrations. Although fabrication of silk microspheres have been reported previously using spray-drying, liposome-templating, polyvinyl alcohol (PVA) emulsification, etc., applications were hindered due to harsh conditions (temperature, solvents, etc.) and complicated procedures used as well as low yield and less controllable particle size (usually <10 μm). Since PEG is an FDA-approved polymer, and silk is FDA approved for some medical devices, the methods developed in the present study will be useful in a variety of biomedical applications where simple, rapid and scalable preparation of silk microspheres is required.

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1. Introduction

Microscale particles with high drug payloads and sustained release features can act as carriers to deliver therapeutic drugs to the body for a variety of disease treatments [1]. Microparticles are normally fabricated into spheres (microspheres), with particle sizes ranging from 1 to 1000 μm depending on the method of preparation. Microspheres encapsulating drugs can be administered through oral or injectable routes, thus less invasive and more practical in the clinic compared to carriers that need surgical implantation, e.g., rods, sheets and sponges. Microspheres should be biodegradable or safe *in vivo*, and degradation or clearance rates should match the release rates of the encapsulated drugs. Degradation products should be non-toxic and should not induce side

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effects in the body after the encapsulated drug is released [2,3]. After injection, microspheres should reside at the injection site in the body as a depot to maximize drug release efficiency, such as under the skin, for extended time frames. Encapsulated drugs are released from the microspheres with rates controllable through passive diffusion and/or microsphere degradation, with subsequent distribution in the circulating blood and body fluids to address the disease sites [4]. Instead of systemic administration, drug-loaded microspheres can also be injected into specific parts of the body to treat local diseases, such as the intraocular injection for age-related macular degeneration (AMD) [5]. Sustained release of drugs from microspheres at therapeutic levels can reduce systemic toxicity of the drug, minimize dosing frequency and improve patient compliance [6]. In addition, drug-releasing microspheres play important roles in screening and diagnosis of cytopathology, gene therapy, immunotherapy and tissue engineering [7].

The size of the microspheres is an important factor that determines degradation and drug release rate. Large microspheres degrade and release drug more slowly than smaller sizes due to the lower surface-to-volume ratio. For instance, poly(D,L-lactide-co-glycolide) (PLG) microspheres with a diameter of 20 μm exhibited faster initial release of rhodamine than the same microspheres at diameters around 65 μm [8]. In addition, small microspheres (<5 μm) can be quickly cleared by macrophages after injection into the body [9]. However, pulmonary inhalation of small microspheres may improve the delivery efficiency of drugs and also avoid being taken up by alveolar microphages [10]. Thus, for different administration routes, microsphere carriers should be designed and fabricated to have sizes and properties to meet specific delivery requirements.

The materials most often used to prepare microspheres are mainly biodegradable polymers from natural sources or chemical syntheses [1]. Natural biomaterials include collagen, chitosan and alginate, among others, and synthetic polymers mainly include polyesters and polyanhydrides [11,12]. Silk fibroin protein is extracted from silkworm cocoons and formatted into fibers, gels, films, nano/microspheres under mild and all-aqueous conditions. These silk-based materials have tunable mechanical properties, biodegradability and excellent biocompatibility [13–17]. Silk nano-/microspheres have been used as carriers for bioactive molecules and cells for tissue engineering, enzyme immobilization and drug delivery [18–22]. The methods most often used to prepare silk microspheres include: (1) organic solvent evaporation/extraction (emulsification), (2) organic solvent displacement, (3) phase separation, (4) organic solvent-assisted self-assembly, (5) rapid expansion using supercritical fluid, (6) microfluidics and (7) spray drying [23–29]. Emulsification methods, e.g., oil-water-oil and silk-polyvinyl alcohol (PVA) blending, are relatively simple and are free of toxic solvents, thus useful to retain the bioactivity of encapsulated drug molecules [30,31]. However, the size and crystallinity of the microspheres are difficult to control by these methods, and oil and PVA have to be removed from the microspheres to minimize *in vivo* responses. In addition, the silk-oil or silk-PVA emulsified solution has to be further treated with methanol or air-dried into films to induce silk β-sheet structure formation to make microspheres water-insoluble. Thus, improved processes to avoid some of the above complications would provide more accessible modes to generate microspheres from silk proteins.

In the present study, silk microspheres were fabricated in a simple, one step process, by mixing polyethylene glycol (PEG) with silk. Particle size and silk crystalline β-sheet structure were tunable via adopting different PEG molecular weights and through the addition of methanol during mixing. Since PEGs have been widely used in the pharmaceutical industry as drug excipients, the microspheres generated possess the advantages of safety, simplicity and scalable processing.

2. Materials and methods

2.1. Materials

Silk fibers that had been processed according to the quality standard of textile industry were purchased from Xiehe Silk Incorporation (Hangzhou, Zhejiang province, China). Lithium bromide (LiBr) was purchased from Aladdin (Shanghai, China). Iodine solution (CAS: 7553-56-2) was purchased from Macklin (Shanghai Macklin Biochemical Co., Ltd). Cd $(ClO_4)_2{\cdot}6H_2O$, Al_2Te_3 powders, and mercaptopropionic acid (MPA) were purchased from Alfa Aesar (MA, USA). Polyethylene glycol (PEG) with different average molecular weights and other analytical grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd, (Shanghai, China). Kunming mice (20 ~ 25 g) were purchased from SLRC laboratory animal Co. Ltd. (Shanghai, China). All the animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Soochow University, China. Ultrapure water from the Milli-Q system (Millipore, Billerica, MA) was used throughout this research.

2.2. Silk solution preparation

The purchased silk fibers were further degummed to remove residual sericin and to extract silk fibroin protein based on our published procedures with some modification [32]. Briefly, 30 g silk fibers were boiled for 30 min in 12 L ultrapure water of 0.02 M sodium carbonate, and then rinsed thoroughly with ultrapure water. After air-drying, the extracted silk fibroin weighing 25 g were soaked in 100 ml of 9.3 M lithium bromide solution, and then placed in an oven at 60 °C for 4 h to completely dissolve the fibers. The protein solution obtained was then transferred into slide-a-lyzer dialysis cassettes (MWCO 3500, Viskase, America) and was dialyzed against 10 L deionized water for three days, with water changed 10 times during the process. The dialyzed silk solution was centrifuged at 9000 rpm for 20 min at 4 °C, (Avanti J-26S XP, Beckman Coulter, America) to remove insoluble aggregates. A small portion of silk fibroin solution was dried and weighed to determine silk concentration. Silk solution (approximately 8 wt%) was diluted with water to prepare low concentration solutions. To make high concentrations, silk solution was freeze-dried using a lyophilizer (CHRIST Alpha 2–4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany), and the lyophilized powder was reconstituted with certain amount of water following the procedure in the literature [33].

2.3. Preparation of silk microspheres

To screen PEGs that induce silk microspheres and hydrogel formation, PEG molecular weights (MW) and concentrations were varied. For PEG MW 0.2–1.0 K, the concentrations used were 40, 50, and 60 wt%; For PEG 1.5–6.0 K, the concentrations were 20, 30, 40, 50, 60 wt%; For PEG 10.0 K, the concentrations were 10, 20, 30, 40, 50 wt%; For PEG 20.0 K, the concentrations were 10, 20, 30, 40 wt%. The PEG solutions prepared were mixed with 5 wt% silk fibroin at a volume ratio 1/1. The mixture was incubated for 30 min at room temperature to observe changes in the solution; if turbid the solution was placed in a tabletop centrifuge (Sorvall Legend Micro21R, Thermo Scientific, Germany) and centrifuged at room temperature for 10 min. The pellet after centrifugation was resuspended in deionized water for microscopic imaging. In some cases the silk/PEG mixture formed gels instead of particles; the tubes were placed upside down and solution flow was observed to determine gelation [34].

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