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Development of silk fibroin modified poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) nanoparticles in supercritical CO_2

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ABSTRACT

Silk fibroin (SF) modified poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) (SF/PLLA–PEG–PLLA) nanoparticles were successfully fabricated in a process of solution-enhanced dispersion by supercritical CO_2 (SEDS). The SF/PLLA–PEG–PLLA nanoparticles exhibited a composite structure with mean particle size of 634 nm and silk fibroin wrapped with PLLA–PEG–PLLA triblock polymer. Fourier transform infrared spectroscopy (FTIR) measurement indicated that minor secondary structural changes of silk fibroin occurred after the SEDS process. X-ray powder diffraction (XRPD) analysis supported the results of FTIR measurement and also revealed that the SEDS process resulted in a notable decrease in crystallinity of the PLLA–PEG–PLLA. *In vitro* cytotoxicity evaluation by MTS assay indicated that SF/PLLA–PEG–PLLA nanoparticles exhibited better biocompatibility than PLLA–PEG–PLLA nanoparticles. Fluorescence microscopy observation and flow cytometric analysis suggested that SF/PLLA–PEG–PLLA nanoparticles could be internalized into fibroblasts in a time-dependent manner and also possessed faster cell adhesion and internalization ability than PLLA–PEG–PLLA nanoparticles. In conclusion, SF/PLLA–PEG–PLLA nanoparticles prepared by the SEDS process could be used as potential biomaterials in the biomedical field, especially nanoparticle drug delivery systems.

1. Introduction

Synthetic biodegradable polymers have been used extensively in biomedical applications as particulate drug delivery systems [1,2]. Among them, poly(L,L-lactide) and its copolymers with polyethylene glycol (PEG) have received considerable attention due to their desirable features such as safety, biocompatibility, flexible structure, controlled particle size, stability, sustained drug release property and avoidance of polymer accumulation *in vivo* after repeated administration [3–5].

The major challenge for these synthetic polymers is undesirable biological responses to cells and/or tissues because of high crystallinity, strong hydrophobicity, and lack of bioactive functions. Particularly, due to the lack of carboxyl or amine groups, synthetic polymers commonly used in tissue engineering are not always suitable for chemical modification [6]. Furthermore, their degradation products are relatively strong acids and cause inflammation [7]. Also, high cost and environmental pollution may result during their synthesis.

An effective strategy to overcome the disadvantages of synthetic polymers and achieve more efficient particle drug delivery systems could be the incorporation of natural polymers with excellent biocompatibility and bioactive group into synthetic copolymers nanoparticles [8,9].

Natural proteins, especially silk fibroin (SF) obtained from *Bombyx mori* is a protein-based biomacromolecule composed of 5507 amino acids [10]. This native biopolymer consists largely of a repeated sequence of six residues (Gly–Ala–Gly–Ala–Gly–Ser)n which form an anti-parallel β-pleated sheet, leading to the stability and mechanical properties of its fibers [11–13]. Silk fibroin exhibits nonthrombogenic, anti-inflammatory, cell-adhesive, cell-responsive and regenerative properties and has been extensively used as a biomaterial in the form of films, three-dimensional scaffolds, hydrogels, electrospun fibers, and spheres. So far, studies on the use of natural silk fibroin to modify synthetic polymers mainly focus on preparing scaffolds and films to improve biocompatibility and cell affinity [14–18]. However, studies on introducing silk fibroin into synthetic polymer particles are rare.

A conventional method of combining natural with synthetic polymers is emulsification because of the limit of the universal solvent. Wang et al. prepared silk fibroin-coated PLGA nanoparticles using layer-by-layer deposition techniques driven by hydrophobic interactions between silk fibroin protein molecules and nanolayer (<100 nm) [19]. However, in such conventional method, coating using layer-by-layer deposition techniques can result in a weak interface between the silk fibroin and inner parts. Also an additional drying step is required to obtain particle powder and the residual organic solvents. Other

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emulsification methods can result in high residual solvent content in the resultant particles and may cause toxic problems.

Supercritical CO_2 ($scCO_2$) has revealed the great potential application in particle formation engineering due to its mild critical conditions (Tc = 31.1 °C, Pc = 7.38 MPa), non-toxicity, non-flammability and lower price [20,21]. The particle formation using $scCO_2$ avoids most of the drawbacks of the conventional methods and presents many advantages for biomedical applications, including small particle size, mild operating conditions, very low/no organic solvent residue, and being environmentally benign [22,23].

A common technique for particle formation using $scCO_2$ is a supercritical antisolvent (SAS) process. In particular, solution-enhanced dispersion by supercritical fluids (SEDS), a modified SAS process, has been widely used to prepare nanoparticles. In this process, a nozzle with two coaxial passages allows the introduction of $scCO_2$ and a solution into the particle formation vessel where $scCO_2$ (anti-solvent) already exists. The $scCO_2$ is used both as an antisolvent because of its chemical properties and as a 'spray enhancer' because of its mechanical effects [24]. When the solution contacts the $scCO_2$, the high velocity of the $scCO_2$ breaks up the solution into very small droplets and enhances mass transfer and mutual diffusion at the interface between $scCO_2$ and the droplets instantaneously, inducing supersaturation of the polymer solution, thus leading to fast nucleation and growth, consequently creating smaller particles [25].

In this study, an amphiphilic block copolymer, poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) (PLLA–PEG–PLLA), was chosen as a synthetic polymer model because it is biodegradable, biocompatible and dissolves easily in a number of organic solvents. The SEDS process was used to prepare silk fibroin modified PLLA–PEG–PLLA (SF/PLLA–PEG–PLLA) composite nanoparticles as a novel biomaterial. The resulting composite nanoparticles were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), a Laser diffraction particle size analyzer, FTIR and XRPD analysis. Besides, a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and flow cytometric analysis were performed to study the biocompatibility of composite particles and their internalization ability in cells.

2. Materials and methods

2.1. Materials

Cocoons of *B. mori* were purchased from the Jiangsu Wujing China Eastern Silk Market Co. Ltd. (China). CO_2 with a purity of 99.9% was supplied by the Hong Kong Specialty Gases Co. Ltd. (Hong Kong). The solvent, DCM, was purchased from the Advanced Technology & Industrial Co. Ltd. (Hong Kong). PLLA–PEG–PLLA triblock polymer (MW 100 kDa, PEG 10%) was purchased from Department of Medical Polymer Shandong Institute (Jinan, China). Human foreskin fibroblasts (HFF-1) were obtained from American Type Culture Collection (ATCC). Cell culture grade chemicals such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin and penicillin, streptomycin antibiotics, were purchased from Invitrogen. All other compounds were of analytical purity.

2.2. Methods

2.2.1. Preparation of pure silk fibroin (SF) raw material

Cocoons of *B. mori* were degummed three times to remove sericin and other impurities enveloping SF, in a water bath at 120 °C for 60 min. Afterwards, the fibroin fibers were washed with deionized water and dried at room temperature. Degummed SF fibers were dissolved in a solution of calcium chloride, water, and ethanol ($CaCl_2$:water:ethanol = 1:8:2, molar ratio) for 6 h at 70 °C to obtain the SF solution. The pure silk fibroin solution was then obtained by dialysis; the solution was dialyzed in distilled water for three days to remove the neutral salts using semi-permeable cellulose tubing (MWCO 12,000–14,000). Subsequently, dry samples of pure silk fibroin were obtained by lyophilizing with a freeze dryer.

2.2.2. Preparation of PLLA–PEG–PLLA and SF/PLLA–PEG–PLLA nanoparticles by the SEDS process

Silk fibroin (SF) nanoparticles can be prepared by the SEDS process given in ref 10. For the preparation of SF/PLLA–PEG–PLLA composite nanoparticles, the SF nanoparticles were firstly immerged into ethanol to induce water insolubility and then the water insoluble SF nanoparticles were dispersed into a 0.5% (w/v) PLLA–PEG–PLLA solution in dichloromethane (DCM) by ultrasonic method to form a suspension for further experiment and the ratio of SF nanoparticles to PLLA–PEG–PLLA was 1:4. Fig. 1 shows a schematic diagram of the SEDS apparatus for preparation of silk fibroin/PLLA–PEG–PLLA composite nanoparticles, which consists of three major components: a CO_2 supply system, a particle suspension delivery system and a high pressure vessel with a volume of 1000 ml. In the particle suspension delivery system an 'injector' was made from a stainless steel cylinder divided into two chambers by a piston with an O-ring seal. Using a SEDS process, when the desired pressure and temperature were stabilized, the silk fibroin nanoparticle suspension in PLLA–PEG–PLLA solution was delivered into the high-pressure vessel through a stainless steel coaxial nozzle (inner diameter (ID) 0.80 mm, and the nozzle of suspension with ID 0.33 mm and length 12.35 mm was used in this study) by using an HPLC pump at a flow rate of 0.5 ml/min. During the process, the pressure, temperature and flow rate of CO_2 were kept at 10 MPa, 35 °C and 25 standard liters per hour (NL/h), respectively. The PLLA–PEG–PLLA nanoparticles without silk fibroin were also prepared in the same process.

2.2.3. Preparation of fluorescent PLLA–PEG–PLLA and fluorescent SF/PLLA–PEG–PLLA nanoparticles by the SEDS process

To evaluate cellular uptake by flow cytometry, fluorescein was incorporated into PLLA–PEG–PLLA and SF/PLLA–PEG–PLLA nanoparticles by the SEDS process. Firstly, 0.5% of fluorescein ($C_{20}H_{12}O_5$, yellowish red) was added to the PLLA–PEG–PLLA organic solution and SF/PLLA–PEG–PLLA suspension. Then the SEDS process was used to produce fluorescent PLLA–PEG–PLLA and fluorescent SF/PLLA–PEG–PLLA nanoparticles. The operating conditions were as described above. The operating conditions were the same as described above.

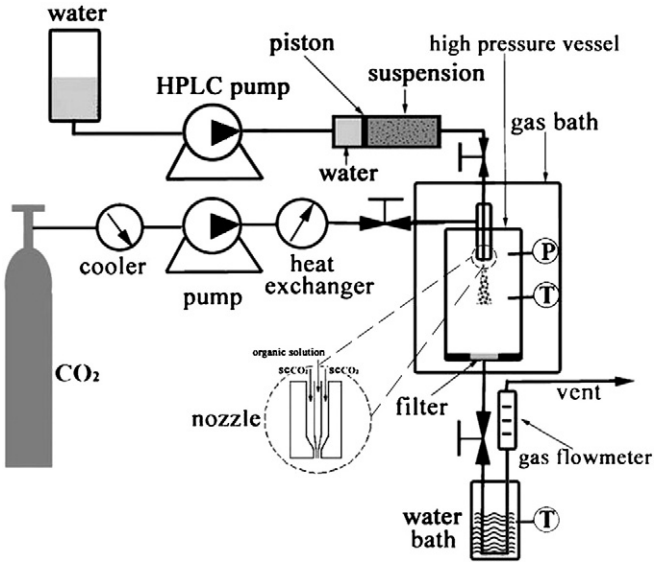

Fig. 1. Schematic diagram of the SEDS apparatus for the preparation of SF/PLLA–PEG–PLLA nanoparticles.