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Silk fibroin layer-by-layer microcapsules for localized gene delivery

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ABSTRACT

Herein, we describe the delivery of plasmid DNA (pDNA) using silk fibroin (SF) layer-by-layer assembled microcapsules. Deposition of fluorescently labeled SF onto polystyrene (PS) template particles resulted in increasing fluorescence intensity and decreasing surface charge in correlation to SF layer number. After removal of the PS core, hollow, monodisperse, and structurally stable SF microcapsules of variable size and shell thickness were obtained. Plasmid DNA encoding for enhanced green fluorescent protein (eGFP) was loaded onto 1 or 4 μm capsules, either by incorporation of pDNA within the innermost layer of the shell or by adsorption to the microcapsules surface, and *in vitro* pDNA release, cytotoxicty and eGFP expression were studied. Sustained pDNA release over 3 days was observed using both loading techniques, being accelerated in the presence of protease. DNA loaded SF microcapsules resulted in efficient cell transfection along with low cytotoxicity after 3 days incubation compared to treatment with pDNA/branched polyethylenimine complexes. Among the tested conditions highest transfection efficiencies were achieved using 1 μm capsules where pDNA was adsorbed to the capsule surface. Our results suggest that SF microcapsules are suitable for the localized delivery of pDNA, combining low cytotoxicity and high transfection efficiency.

1. Introduction

The delivery of nucleic acid aims at introducing exogenous plasmid DNA (pDNA), antisense oligonucleotides, or small interfering RNA (siRNA) into host cells to influence protein expression. Despite the high hopes that have been pinned on nucleic acid delivery to treat a variety of inherited and acquired diseases, safe and efficient delivery still is a major challenge [1,2]. While viral vectors are superior to non-viral, synthetic delivery systems regarding gene transfer efficiency, significant safety concerns limit their usability [3]. Non-viral vectors on the other hand are easy to manufacture and to modify and are associated with fewer safety concerns [4,5]. Non-viral gene delivery systems have undergone significant development over the past decade, resulting in improved transfection efficiency and specificity [6–11] but substantial toxicity, limited physicochemical stability and incomplete protection of encapsulated nucleic acids under physiological conditions still limit their use as viable clinical therapies [12–14].

The majority of non-viral vectors is currently developed for systemic application, requiring complex systems to achieve efficient and specific transfection of target cells. Localized delivery represents an alternative strategy avoiding several major obstacles encountered with systemic delivery, allowing for simpler delivery system design. To date, localized delivery often relies on naked pDNA or simple colloidal preparations, with limited potential for sustained or controlled release [15,16]. However, the application potential for localized gene delivery is immense with regard to tissue engineering as well as local therapy. While scaffold-based delivery of proteins requires the stabilization and controlled release of significant quantities of these fragile molecules [17,18], nucleic acid delivery needs smaller drug quantities and involves molecules being inherently more stable [15]. These considerations and the perceived lack of suitable delivery systems led us to design and characterize a system for the controlled localized delivery of nucleic acids based on silk fibroin (SF).

Silk proteins are a particularly promising biomaterial due to the unique combination of biocompatibility, biodegradability, self-assembly, mechanical stability and control over structure and morphology [19,20]. Silk-based biomaterials have been shown to

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safeguard the activity of sensitive biomolecules in harsh environments, allowing delivering molecules that otherwise quickly lose efficacy [21–23]. While SF as a bulk biomaterial already has interesting properties, the controlled assembly of ultrathin SF films is particularly interesting as it allows further control of the architecture of the drug delivery system and associated properties such as drug release [24,25].

Layer-by-layer (LbL) assembly is a well-established technique for the fabrication of structurally diverse materials, including drug delivery systems, by sequential deposition of complementary species onto a template [26,27]. By using a sacrificial template and subsequent dissolution of the core, hollow microcapsules can be prepared possessing high loading capacity compared to nanoparticles and being able to accommodate a diverse range of molecules [28,29]. Microcapsules further allow incorporation of drugs such as nucleic acids either through preloading, i.e. incorporation of drugs into the capsule shell during shell fabrication or postloading, i.e. ad or absorption of payload after shell assembly [30–32].

The aim of the present study was to develop pDNA loaded SF microcapsules for local application allowing tunable, sustained release of nucleic acids resulting in efficient cell transfection and reduced cytotoxicity. We describe the generation of SF capsules with variable shell consisting of 2–10 layers of SF and establish pre- and post-loading techniques for pDNA incorporation. The effect of loading scheme as well as presence of protease on pDNA release is investigated and the biological response *in vitro* regarding cell viability and transfection efficiency is described.

2. Materials and methods

2.1. Materials and reagents

Bombyx mori (silkworm) cocoon was obtained from Trudel Ltd. (Zurich, Switzerland). Polystyrene (PS) particles of 1 μm (Polybead® Microspheres 1.00 μm, CV 3%, Polysciences Europe GmbH, Eppelheim, Germany) and 4 μm diameter (Te0400, CV 5%, BS-Partikel GmbH, Wiesbaden, Germany) were used as templates, respectively. Cellulose ester dialysis tubing (Spectra/Por®, MWCO 3.5–5 kDa) were purchased from Spectrum Europe B.V. (DG Breda, The Netherlands). Dulbecco's Modified Eagle's Media (DMEM), fetal bovine serum (FBS), antibiotics and Quant-iT™ PicoGreen® dsDNA reagent, used in the pDNA quantification were purchased from Life Technologies GmbH (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4′-6-diamidino-2-phenylindole (DAPI) were obtained from Sigma–Aldrich (Schnelldorf/Taufkirchen, Germany). The plasmid was labeled with the Cy5 fluorescent probe (pDNA-Cy 5) using Label IT® nucleic acid labeling kit from Mirus Bio (Madison, WI). Unless otherwise specified, all other reagents were purchased from Sigma–Aldrich (Taufkirchen, Germany) and were at least of analytical quality.

2.2. Purification and fluorescent labeling of silk fibroin

Cocoons from *B. mori* were boiled twice in an aqueous solution of 0.02 M Na_2CO_3 for 1 h, rinsed several times with ultrapure water (Millipore Milli-Q system) and dissolved in 9.3 M LiBr at 60 °C to generate a 10% (w/v) solution. This solution was dialyzed (MWCO 3.5–5 kDa) against ultrapure water for 3 days by changing water daily to remove the ions and other impurities. The solution was collected, filtered and stored at 4 °C.

Fluorescein isothiocyanate (FITC)-labeled SF was prepared as described before with slight modifications [33]. SF was diluted to 2% (w/v) with ultrapure water and dialyzed against 500 ml of 0.1 M 2-(morpholino) ethanesulfonic acid (MES) buffer, pH 5.6 containing 150 mM NaCl. The buffered SF solution was mixed under stirring with 80 mg of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (2 mM) and 220 mg *N*-hydroxysuccinimide (NHS) (5 mM) and the reaction was continued for 15 min. To quench the reaction, 20 mM α-mercaptoethanol was added. Activated carboxyl groups of SF were modified with 1.5 mg ethylenediamine for 2 h. After dialysis against 500 ml 0.1 M MES buffer, 10 mg of FITC was added to the solution, yielding a molar ratio of about 40:1 between fluorescent probe and SF. The reaction progressed for 2 h under slow stirring at room temperature. Finally, the solution was dialyzed against ultrapure water, yielding a fluorescent SF concentration of approx. 1% (w/v).

2.3. Fabrication of SF LbL microcapsules

According to the literature on SF-based LbL coating processes, the maximum deposition was achieved once the protein concentration reached 1 mg/ml [24,34]. Therefore, all the coating experiments presented herein were performed at an SF concentration of 1 mg/ml. Two hundred μl of a 5% w/v suspension of 1 or 4 μm PS particles was centrifuged at 10,000× *g* or 1500× *g*, respectively, for 2 min and the supernatant was removed and replaced with fresh ultrapure water. The pellet was agitated and subjected to a centrifugation and redispersion wash cycle. Prior to SF deposition, 1 ml of a 0.5 mg/ml aqueous solution of branched poly(ethylene imine) 25 kDa (bPEI25) was added to the particle suspension and incubated for 15 min at room temperature. After being washed three times, the particles were agitated to obtain a homogeneous suspension and incubated with 1 mg/ml SF solution for 15 min at 4 °C. Assembly at low temperature was used to ensure SF protein stability and to decrease protein precipitation. After incubation, the particles were washed as described before. However, 1 μm particles required ultrasonication at 10% amplitude for 10 s to redisperse particles after centrifugation. The SF coated PS particles were immersed in 90% methanol for 15 min to induce silk crystalline β-sheet structure formation. The washed particles were then dried by a flow of nitrogen gas at 20 kPa (Turbovap LV, Zymark, Hopkinton, MA) and subjected to the next coating procedure. When a desired number of layers were deposited, the particles suspensions were washed several times with ultrapure water. To produce hollow microcapsules, PS cores were dissolved by shaking the dispersion for 4 h in THF solution. The dispersion of the microcapsules was then dialyzed against ultrapure water for 2 days.

2.4. Field emission scanning electron microscopy (FESEM)

The morphology of SF microcapsules was characterized by FESEM (JSM-7500F, JEOL, Japan) with an accelerating voltage of 5 kV after coating with gold. For sample preparation, a drop of microcapsules suspension was applied to a mica film and dried under ambient conditions overnight.

2.5. Fourier-transform infrared spectroscopy (FTIR)

The LbL assembly and structure analysis of SF microcapsules in dry state were performed by FTIR spectroscopy over a range of 4000–400 cm^{-1}. FTIR spectra of different samples were obtained by an FTIR-6100 spectrometer system (FTIR-6100, JASCO, Gross-Umstadt, Germany). Background measurements were taken twice with an empty cell and subtracted from sample readings. The samples were analyzed without any further preparation.

2.6. Confocal laser scanning microscopy (CLSM) and fluorescence microscopy

Fluorescence images of SF microcapsules were obtained with a Leica DMI6000 (Wetzlar, Germany) confocal microscope or a Zeiss Axio Observer Z1 (Oberkochen, Germany) fluorescence microscope. Hollow microcapsules for microscopy were prepared as described above using FITC-labeled SF. Samples were prepared by embedding 20 μl of the capsules suspension using standard glass slide and coverslips. Samples were stored for at least 30 min at room temperature prior to imaging to allow sedimentation of capsules.

2.7. Flow cytometry

To follow film buildup on PS particles, FITC-labeled SF was deposited as described before. The fluorescence intensity with different layers was monitored on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using an 488 nm laser line of an argon-ion laser and emission bandpass filter 530/30 nm for detection of FITC fluorescence. Cy5 was excited using a 635 nm diode laser and emission was detected using a bandpass filter between 653 and 667 nm. Approximately 10,000 particles were analyzed in each experiment.

2.8. Zeta-potential measurements

Zeta potentials of unmodified and SF coated 1 and 4 μm PS particles were measured in aqueous solutions using a Delsa Nano HC (Beckman Coulter, Brea, CA). Results were obtained at ambient conditions and by averaging three independent measurements of 6 sub-runs each.

2.9. Loading of pDNA using the pre- and post-loading approach

Two different approaches to loading pDNA onto microcapsules, preloading and postloading, were evaluated [32]. Plasmid DNA was labeled with Cy5 using Mirus Label IT Nucleic Acid Labeling Kit according to the manufacturer's instructions. The Cy5-labeled pDNA was purified through a microspin column to remove any unbound dye. The recovered labeled plasmid was stored protected from light at −20 °C until use.

For the preloading method, 200 μl of 5% (w/v) suspension of bPEI25 modified 1 or 4 μm PS particles in ultrapure water were incubated with 1 ml of 10 μg/ml Cy5-labeled pDNA solution for 20 min. Subsequently, 4 layers of FITC-labeled SF were deposited. Finally, the PS cores were dissolved using THF to produce capsules containing pDNA. For postloading, 4 layers of FITC-labeled SF were deposited on bPEI25 modified PS particles and then incubated with 0.1 mg/ml bPEI25 solution, resulting in a positive surface charge of particles. After core removal microcapsules were incubated with 1 ml of 10 μg/ml Cy5-labeled pDNA for 20 min while shaking at 1200 rpm (Thermomixer comfort, Eppendorf, Wesseling-Berzdorf, Germany) to avoid sedimentation and agglomeration. Subsequently, the capsules were washed