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Recombinant spider silk particles for controlled delivery of protein drugs

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ABSTRACT

The engineered and recombinant spider silk protein eADF4(C16) has been shown to be a promising biomaterial for the use as drug delivery system. In previous studies, eADF4(C16) particles were loaded with low molecular weight drugs exhibiting a positive net-charge and sufficient hydrophobicity. Here, we demonstrate that also macromolecular drugs like proteins can be loaded on eADF4(C16) particles. Using lysozyme as a model protein, remarkably high loading of up to 30% [w/w] was feasible and high loading efficiencies of almost 100% were obtained. Furthermore, using confocal laser scanning microscopy, it is demonstrated that fluorescently labeled lysozyme is not only adsorbed to the negatively charged particles' surface, but also diffusing into the matrix of eADF4(C16) particles. The release of lysozyme is shown to be dependent on the ionic strength and pH of the release medium. To improve the long-term stability of eADF4(C16) containing dispersions, lyophilization is shown as a suitable tool. Disaccharides (sucrose, trehalose) and mannitol served as stabilizers to prevent aggregation and/or particle degradation during freeze-drying. The slowly biodegradable eADF4(C16) particles are a promising new particulate drug carrier system for the delivery of susceptible drugs like therapeutic proteins.

1. Introduction

Particulate drug delivery systems made of different types of biodegradable polymers have been in the focus of research for decades due to their excellent controllability [1]. Nanoparticles as well as microspheres have been employed for controlled delivery of various therapeutic agents including peptides and proteins. As this class of pharmaceutical compounds requires special formulation due to their susceptibility to proteolysis, chemical modification and denaturation/aggregation during storage [2], the polymer used as drug carrier system has to fulfill a number of requirements: protection of the protein drug from denaturation or degradation, control of drug release, biodegradability, no toxicity, low cost and easy processability [3]. Commonly used polymeric carriers for this purpose are of synthetic origin and polyesters like polylactic-co-glycolic acid (PLGA) are the most extensively studied among them [4,5]. Unfortunately, polyester-based matrices often failed to be suitable drug delivery systems for protein drugs due to the required usage of organic solvents during preparation as well as the acidic microenvironment that is created by polymer degradation [5–8]. Therefore, research on hydrophilic polymers of natural origin, which can be easily processed in an aqueous environment, was accelerated. Herein, especially chitosan was applied as a particulate drug carrier for protein delivery [9–12]. Nevertheless, non-modified chitosan particles very often exhibit a significantly high burst release wherefore cross-linking agents had to be implemented in the preparation process [13]. The same disadvantages exist for gelatin nanoparticles, which are produced by a one or two-step desolvation procedure including acetone as organic solvent and glutaraldehyde as cross-linking agent [14].

To overcome the aforementioned limitations, different types of biomaterials are required. Silk proteins including silk fibroin from *Bombyx mori* and recombinant spider silk proteins are very promising candidates regarding their non-cytotoxic and customizable properties as well as their easy processing into various morphologies like scaffolds, films and spheres [3,15–19]. Using silk fibroin from *Bombyx mori*, microspheres as well as nanoparticles were prepared by different preparation techniques: lipid-templates [20–22], film casting and subsequent sonication [23], vibrational splitting of laminar jet [24] and usage of water-miscible organic solvents [25]. Different kind of therapeutic agents were incorporated and acceptable *in vitro* release profiles were obtained using these systems, but nevertheless the applied preparation techniques were quite sophisticated. Moreover, the used silk fibroin from *B. mori* is a completely natural product and the quality of the protein may vary between species and individuals of the same

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species and can depend on the degumming process. Finally, quality control of such delivery systems will be difficult [3].

Therefore, the engineered and recombinantly produced spider silk protein eADF4(C16), which is adopted from the natural occurring spider silk protein ADF4 from the European garden spider *Araneus diadematus*, represents a more favorable biomaterial [26,27]. Previous studies on the assembly process had shown that smooth protein spheres with high β-sheet content can be formed by a salting-out process with potassium phosphate as lyotropic salt avoiding any organic solvent [28,29]. Lammel *et al.* described that the size of eADF4(C16) particles can be easily controlled by the mixing intensity and the concentrations of potassium phosphate and eADF4(C16) [30]. In order to evaluate these particles as a drug carrier system, small molecular weight drugs were recently loaded on eADF4(C16) particles using a simple incubation step after particle preparation [31]. High loading efficiencies of almost 100% could be achieved with positively charged and sufficiently hydrophobic drug molecules and constant release rates were obtained over a period of two weeks.

The aim of this study was to investigate the applicability of eADF4(C16) particles as drug carrier for high molecular weight drugs like protein pharmaceuticals. First, the colloidal stability of spider silk particles was studied at different pH and ionic strengths as well as during long-term storage. Second, loading with a high molecular weight drug was carried out. Lysozyme was chosen as a model protein to examine the loading behavior and the *in vitro* release behavior in dependence of ionic strength and pH of the release medium. Furthermore, the loading mechanism was investigated using fluorescein isothiocyanate (FITC) labeled macromolecules for loading on eADF4(C16) particles and subsequent analysis of the corresponding particle dispersions by confocal laser scanning microscopy. As liquid particulate formulations often fail during long-term stability studies due to aggregation or drug escape, the applicability of a freeze-drying process for empty and lysozyme-loaded eADF4(C16) particles with different excipients and varying excipient-to-nanoparticle ratios was examined.

2. Materials and methods

2.1. Materials

2.1.1. Production of the recombinant spider silk protein eADF4(C16)

The spider silk protein eADF4(C16) is composed of an amino acid sequence adapted from the natural sequence of ADF4 from *A. diadematus*, comprising 16 repeats of the sequence GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP. eADF4(C16) has a molecular weight of 47.7 kDa and was provided by AMSilk GmbH (Martinsried, Germany). On account of its amino acid sequence, eADF4(C16) has an isoelectric point of 3.48. Hence, the spider silk protein has an overall negative net charge at the physiological pH of 7.4.

2.1.2. Drugs for loading of eADF4(C16) particles

Lysozyme from chicken egg white (protein ≥ 90%, molecular mass: 14.307 kDa), FITC-albumin (from bovine albumin), FITC-dextran (average molecular weight: 21.2 kDa) and FITC (isomer I, ≥ 90%) were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Preparation of eADF4(C16) particles

The dried protein eADF4(C16) was dissolved in 6 M guanidinium thiocyanate and extensively dialyzed at room temperature against 10 mM tris(hydroxymethyl)aminomethane(Tris)/HCl, pH 8, using dialysis membranes with a molecular weight cut-off of 6000–8000 Da (Spectrum Laboratories Inc., Rancho Dominguez, USA). After centrifugation and filtration (0.2 μm cellulose acetate syringe filter), the concentration of the resulting eADF4(C16) solution was determined by UV–Vis spectroscopy using the molar extinction coefficient of eADF4(C16) at 276 nm (ε = 46,400 M^{-1}*cm^{-1}). eADF4(C16) particles were prepared using a phase separation procedure as described previously [28,30].

Particle preparation by using a micromixing system: A syringe pump system consisting of two identical syringe pumps (model 100 DX, Teledyne Isco Inc., USA) and a digital control element (Series D, Teledyne Isco Inc., USA) were implemented for the preparation of particle dispersions with batch sizes of up to a total volume of 200 ml. The syringe pumps were filled with eADF4(C16) solution (c = 1.0 mg/ml) and 2 M potassium phosphate (pH 8), respectively. Micromixing and precipitation was performed by connecting the syringe pumps via a T-shaped mixing element with a circular mixing zone having an inner diameter of 0.50 mm (P-727 PEEK tee, Upchurch Scientific, Oak Harbor, USA). A flow rate of 50 ml/min was used for both syringe pumps.

Particle preparation by dialysis: The eADF4(C16) solution (c = 1.0 mg/ml) was dialyzed against 2 M potassium phosphate (pH 8) in a volumetric ratio of 1:50 at room temperature using the aforementioned dialysis membrane.

Particle dispersions resulting from either the micromixing system or dialysis were centrifuged (7500 g, 10 min and 12,500 g, 15 min) and washed three times with highly purified water. Subsequently, the aqueous particle dispersions were treated with a sonifier (Sonopuls HD 2200, Bandelin electronic, Berlin, Germany) for a maximum of 15 min (cycle 6, amplitude 50%) and filtered through a 1.2 μm or 5 μm syringe filter (Minisart Plus; Sartorius Stedim Biotech S.A., Aubagne, France) to obtain homogenous particle size distributions. Particles obtained by dialysis were also subjected to the ultrasonic treatment but not filtered. The particle concentration in mg/ml was determined gravimetrically.

2.3. Characterization of eADF4(C16) particles

2.3.1. Dynamic light scattering (DLS)

The eADF4(C16) particle size represented by the Z-average value and the width of the particle size distribution expressed by the polydispersity index (PI) were determined by dynamic light scattering measurement using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The samples were diluted directly before the measurement to a concentration of 0.01 mg/ml in the corresponding buffer. The measurements were conducted in triplicate at 25 °C.

2.3.2. Zeta-potential analysis

The zeta-potential of eADF4(C16) particles was equally determined with the Zetasizer Nano ZS at the same particle concentration and temperature as used for particle size measurements.

2.3.3. Laser diffraction spectrometry (SLS)

The size and size distribution of eADF4(C16) particles obtained by dialysis were determined by laser diffraction spectrometry (Horiba Practica LA-950, Horiba Scientific, Japan). Refractive indices of 1.33 for water and 1.60 for eADF4(C16) were used for the calculation of the particle sizes.

2.3.4. Scanning electron microscopy (SEM)

eADF4(C16) particles were immobilized by evaporation at room temperature on Leit-Tabs (Plano GmbH, Wetzlar, Germany), carbon sputtered under vacuum and analyzed by a Joel JSM-6500F field emission scanning electron microscope (Joel Inc., Peabody, USA).

2.4. Colloidal stability of eADF4(C16) particles

The colloidal stability of eADF4(C16) particles was studied at three different pH (3, 5 and 7) at constant ionic strength of 30 mM and at different ionic strength (30 mM, 60 mM, 100 mM and 154 mM) at constant pH of 5 and 7, respectively. All buffers were prepared at concentrations of 10 mM and the ionic strength was adjusted with sodium chloride. The particle concentration was set to 0.25 mg/ml after centrifugation of the stock dispersion and the samples with a total volume of 1.0 ml were allowed to stand at room temperature without agitation. Samples were analyzed directly after redispersion as well as after 1 h and 24 h for particle size, size distribution and zeta-potential using the Zetasizer Nano ZS as described in sections 2.3.1 and 2.3.2. Storage stability of the eADF4(C16) stock dispersion in highly purified water was investigated at 2–8 °C over a period of 6 months.

2.5. Loading of eADF4(C16) particles with lysozyme

Loading with lysozyme was conducted in 10 mM phosphate buffer at pH 7.0 and different total ionic strengths of 30 mM, 60 mM and 100 mM (each buffer was adjusted with sodium chloride). The loading procedure was implemented as follows: A stock dispersion of spider silk particles in highly purified water was centrifuged (15,000 g, 30 min) and redispersed in the desired buffer media before loading. A second stock solution comprising lysozyme was prepared by dissolving lyophilized lysozyme in the identical buffer solution. Appropriate volumes of spider silk particle suspension and lysozyme stock solution were mixed to obtain a final spider silk particle concentration of 0.5 mg/ml and the desired w/w-ratio [%] of lysozyme to spider silk particles. After 30 min of incubation at room temperature under gentle agitation (horizontal shaker with 40 rpm (GFL 3015, Burgwedel, Germany)), 20 μl of the particle suspension were used for dynamic light scattering measurements. Simultaneously, samples were centrifuged (15,000 g, 30 min) and the supernatant was analyzed for residual protein content using the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). A standard calibration curve for lysozyme in the corresponding buffer (0.5–40 μg/ml) and control groups of empty eADF4(C16) particles and lysozyme solutions were used to quantify the residual