Engineering of Recombinant Spider Silk Proteins Allows Defined Uptake and Release of Substances

ELENA DOBLHOFER,[1] THOMAS SCHEIBEL[1,2,3,4,5]

[1]Thomas Scheibel, Lehrstuhl Biomaterialien, Fakultät für Ingenieurwissenschaften, Universität Bayreuth, Bayreuth 95440, Germany
[2]Bayreuther Zentrum für Kolloide und Grenzflächen (BZKG), Universität Bayreuth, Bayreuth 95440, Germany
[3]Institut für Bio-Makromoleküle (bio-mac), Universität Bayreuth, Bayreuth 95440, Germany
[4]Bayreuther Zentrum für Molekulare Biowissenschaften (BZMB), Universität Bayreuth, Bayreuth 95440, Germany
[5]Bayreuther Materialzentrum (BayMAT), Universität Bayreuth, Bayreuth 95440, Germany

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ABSTRACT: Drug delivery carriers stabilize drugs and control their release, expanding the therapeutic window, and avoiding side effects of otherwise freely diffusing drugs in the human body. Materials used as carrier vehicles have to be biocompatible, biodegradable, nontoxic, and nonimmunogenic. Previously, particles made of the recombinant spider silk protein eADF4(C16) could be effectively loaded with positively and neutrally charged model substances. Here, a new positively charged variant thereof, named eADF4(κ16), has been engineered. Its particle formation is indistinguishable to that of polyanionic eADF4(C16), but in contrast polycationic eADF4(κ16) allows incorporation of negatively charged substances. Both high-molecular-weight substances, such as nucleic acids, and low-molecular-weight substances could be efficiently loaded onto eADF4(κ16) particles, and release of nucleic acids was shown to be well controlled. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:988–994, 2015
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INTRODUCTION

Drug delivery systems can overcome many disadvantages of drugs such as poor solubility, rapid breakdown *in vivo*, unfavorable pharmacokinetics, poor biodispersity, and lack of selectivity,[1] all of which result in lower drug efficacy and unwanted side effects.[2] Colloidal micro- and nanoparticulate carriers have been used extensively as drug reservoirs because they have programmable degradability ranging from a few days to several months.[3] When mobile particulate drug vehicles are decorated with specific recognition motifs on their surface, they can also be used for targeted delivery, for example, to malignant tumor cells.[2]

In addition to being biocompatible, biodegradable, nontoxic, and nonimmunogenic,[3] drug delivery vehicles must fulfill numerous requirements, for example, controlled particle size and mechanical stability.[7] Furthermore, solubility, stability, molecular weight, and charge of the drug substances must be considered for choosing suitable carrier materials.[1] Synthetic polymers are often used for designing of drug carriers, but natural polymers, such as polysaccharides and polypeptides, have also been employed. The advantage of such biopolymers in comparison to synthetic ones is their ability to undergo enzymatic or hydrolytic degradation in natural environments accompanied by release of nonhazardous byproducts that can be biologically eliminated.[5]

Spider silk protein particles have recently been established as drug delivery carriers.[6] As the availability of natural spider silk proteins is limited and farming of spiders is not practical because of their cannibalistic behavior,[7] engineered recombinant spider silk proteins have been developed as a reliable source.[8] It has been shown that engineered spider silk proteins, such as eADF4(C16), can be processed into a variety of assembly morphologies, such as fibrils, hydrogels, capsules, and particles.[9–14] Materials based on spider silk proteins are biocompatible, biodegradable, nontoxic, and do not induce immune reactions,[7,15–19] fulfilling important prerequisites for their use as a drug delivery carrier. Spider silk protein particles have high mechanical stability and can be stored in a dried state because of their fully reversible swelling behavior.[20,21] Furthermore, it has been shown that model drugs, small-molecular-weight molecules as well as proteins, can be loaded onto eADF4(C16) particles in various amounts depending on the physicochemical properties of the loaded substance.[6,22,23] Because of the negative net charge of eADF4(C16) at neutral pH, drugs have to be positively or neutrally charged in order to be taken up.

Currently investigated drug delivery systems that are able to transport negatively charged molecules are cationic liposomes or cationic polymers. Liposomes have the major disadvantage of often inducing rapid immune response and accumulation in the liver without carrying liver-specific target sequences.[24–26] Linear and branched polyethylenimines (PEIs), one of the most extensively studied polymeric drug delivery system, are also commonly used in gene delivery.[27] Although complexes of DNA and PEI are currently the most effective nonviral gene delivery systems, they still exhibit problems, such as toxicity, nonspecificity, and nonbiodegradability. Large amounts of nondegradable PEI remain in free form inside the transfected cells causing cell dysfunction.[28] Recently, silk proteins containing RGD sequences were used to enhance transfection efficiency of PEI/DNA complexes.[29] However, this approach did not

Correspondence to: Thomas Scheibel (Telephone: +49-921-55-7361; Fax: +49-921-55-7346; E-mail: thomas.scheibel@bm.uni-bayreuth.de)

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overcome the stated problems associated with PEI. Therefore, spider silk protein-based hybrids were developed in a separate approach for gene delivery.[12] These hybrid proteins contain 30 lysine residues that are responsible for electrostatic interactions between the hybrid silk proteins and DNA, providing a functional, biodegradable construct. Additionally, again RGD sequences were utilized to enhance transfection efficiency. However, it has been shown that polylysine sequences have an inherent cytotoxicity because of their high-charge density.[30,31]

Here, we engineered a new variant of eADF4(C16), called eADF4(κ16), with a positive net-charge and low-charge density at neutral pH. This approach was accomplished without using additional tags as all charged residues were part of the spider silk core sequence, avoiding side effects as seen in previous attempts. Importantly, most properties of eADF4(C16) were retained in eADF4(κ16), allowing its use as drug carrier material.

MATERIALS AND METHODS

Protein Design and Production

eADF4(C16) containing the sequence T7-(GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGPG)$_{16}$ was produced and purified as described previously.[8] To design eADF4(κ16), glutamic acid residues of eADF4(C16) were replaced by lysine residues within the encoding DNA sequence. A κ2 DNA sequence with *Bam*HI and *Hind*III restriction sites was purchased from GeneArt® Gene Synthesis, and the DNA construct encoding eADF4(κ16) was generated using a previously established cloning strategy.[8] eADF4(κ16) was expressed in *E. coli* BL21 gold (DE3), and the protein was purified using a heat step and ammonium sulfate precipitation as described previously.[8]

Particle Preparation

Lyophilized eADF4(κ16) or eADF4(C16) was dissolved in 6 M guanidinium thiocyanate (GdmSCN) and dialyzed against 25 mM Tris/HCl pH 7.5 (Tris buffer). Samples were dialyzed for 16 h with three buffer changes at 25°C, using a dialysis membrane with a molecular weight cut-off of 6000–8000 Da (Spectrum® Laboratories, Irving, Texas). The resulting spider silk protein solutions were diluted to a concentration of 50 μM using Tris buffer. Aliquots of 200 μL (10 nmol) were mixed at a 1:1 ratio with 2 M potassium phosphate, pH 7.5. The mixtures were incubated for 30 min at 25°C and then centrifuged at 17000*g* for 2 min to obtain a pellet that consisted of the precipitated protein particles.[9,10] The particles were washed three times with MQ-H_2O, and, if needed, stored in MQ-H_2O at 25°C. The eADF4(κ16) particles used in this experiment had diameters of 1533 ± 238, and eADF4(C16) particles diameters of 1286 ± 206 nm.

Particle Size Determination

Particle sizes were determined via dynamic light scattering measurements. These measurements were performed using a ZetaSizer Nano ZS (Malvern Industries Ltd., Malvern, UK). Refractive indices of 1.33 for water and 1.60 for protein were used for computation of particle sizes. Aliquots of freshly prepared and washed particles were resuspended in 2 mL Tris buffer and 500 μL of these suspensions were used to measure particle sizes.

Particle Loading

Protein particles were loaded via diffusion with the model substances carboxy fluorescein (CFl), crystal violet (CV), and acridine orange (AO). CFl, CV, and AO were dissolved in ethanol at a concentration of 25 mM each. Dye solutions were then diluted 500-fold (1 eq), 100-fold (5 eq), 50-fold (10 eq), or 25-fold (20 eq) using Tris buffer. For particle loading, 200 μL of dye solution were added to freshly washed particle pellets and incubated for 30 min at 25°C, followed by centrifugation at 17,000*g* for 2 min. The supernatant was then analyzed using UV/Vis spectroscopy at wavelengths matching the absorbance maxima of the dyes used (CFl: λ_{max} = 492 nm; CV: λ_{max} = 552 nm; AO: λ_{max} = 467 nm) to determine both the loading and the loading efficiency of the particles (Eqs. (1) and (2).

For loading of eADF4(κ16) particles with Rho-ODN [rhodamine-labeled dsDNA: Rho-C6-5′-ggt cac ttc gtg gct aac g-3′ (7387.43 Da)], the labeled DNA was dissolved in MQ-H_2O at a concentration of 125 μM and 200 μL (25 nmol) were added to eADF4(κ16) particles, incubated for 30 min and then centrifuged at 17,000*g* for 2 min at 25°C. The supernatant was analyzed using UV/Vis spectroscopy at the absorption maximum of λ_{max} = 560 nm to determine the loading and the loading efficiency of the protein particles (Eqs. (1) and (2).

$$\text{Loading}\,(\%) = \frac{\text{Amount of substance in particles}}{\text{Amount of silk protein in particles}} \times 100 \quad (1)$$

$$\text{Loading efficiency}\,(\%) = \frac{\text{Amount of substance in particles}}{\text{Amount of dye added}} \times 100 \quad (2)$$

Model Substance Release

To analyze the release kinetics of loaded substances, particles were thoroughly resuspended in release buffer and incubated in a shaker (Thermomixer compact; Eppendorf, Germany) for 30 min at 37°C, followed by centrifugation at 17,000*g* for 2 min at 25°C. Supernatants were carefully removed, and pellets were resuspended in 200 μL of fresh Tris buffer and the corresponding salt concentrations (NaCl or sodium acetate), phosphate buffer or PBS. The removed supernatants were then centrifuged again and analyzed using UV/Vis spectroscopy at the absorption maximum of the loaded substances (see also section *Particle Loading*).

Layer-by-Layer Particle Coating

Freshly washed or freshly loaded eADF4(C16/κ16) particles were resuspended in 200 μL of the corresponding charge counterpart at a protein concentration of 0.5 mg/mL. After 1 h of incubation, particles were centrifuged at 17,000*g* for 2 min and washed once with MQ-H_2O at 25°C.

Microscopy

Scanning electron microscopy (SEM) images were taken using a 1450Es Beam (Zeiss, Oberkochen, Germany) at an accelerating voltage of 3 kV. Particles were pipetted onto Thermanox™ plastic cover slips and washed three times with distilled water. Before imaging, the particles were air dried and sputtered with platinum.

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