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

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ABSTRACT

Drug delivery systems allow tissue / cell specific targeting of drugs in order to reduce total drug amounts administered to an organism and potential side effects upon systemic drug delivery. Most drug delivery systems are polymer-based, but the number of possible materials is limited since many commercially available polymers induce allergic or inflammatory responses or lack either biodegradability or the necessary stability in vivo. Spider silk proteins represent a new class of (bio)polymers that can be used as drug depots or drug delivery systems. The recombinant poly-anionic spider silk protein eADF4(C16), which can be processed into different morphologies such as particles, films, or hydrogels, has been shown to fulfil most criteria necessary for its use as biomaterial. Further, eADF4(C16) particles have been shown to be well-suited as drug carriers for poly-cationic or neutral drugs, but cellular uptake of such particles is low. Novel variants of eADF4(C16) with inversed net charge or incorporated cell penetrating peptides and receptor interacting motifs show an increased cellular uptake. Further, poly-cationic variants allow incorporation of negatively charged drugs including high molecular weight substances, like nucleic acids.

Keywords: spider silk, particles, drug delivery, cellular uptake, biodegradation

1 INTRODUCTION

Conventional drug administration such as intravenous injection or oral administration often requires the application of high drug dosages to attain the biologically effective concentration at the target tissue, since this form of administration lacks selectivity and furthermore suffers from a poor biodistribution of the drug. These disadvantages can be overcome using drug delivery vehicles to which drugs can be bound covalently or non-covalently [1].

The vast majority of drugs have their primary target within cells and tissues [2]. It is thus preferable to deliver drugs into the cytosol especially if significant amounts of the drug are exported out of the cell by ATP-dependent efflux transporters such as the permeability glycoprotein 1 [3]. Drug delivery vehicles which are taken up into cells can act as drug depots and lead to a sustained drug release in order to achieve constant drug levels thereby lowering undesired side effects caused by over- or under-dosage. Moreover, nanoscale drug delivery platforms offer the possibility to protect labile drugs from rapid degradation in the bloodstream and prevent premature renal or hepatic clearance. Thus, the dosage of the drug can be reduced. One major problem during pharmacotherapy is the delivery of a drug to its target site [4]. There, drug delivery vehicles can help to direct the drug to its desired site of action. Cell or tissue specific targeting can be realised if the carrier's surface is modified with biological signalling peptides, antibodies or aptamers to achieve accumulation of the carrier together with its payload in the respective tissues. Thereby, local drug concentrations can be increased several folds within the target tissue and help to reduce the necessary drug concentration. Besides active targeting mechanisms, particles of a size of around 100 nm can be used for the passive targeting of inflamed and cancerous tissues via the EPR (enhanced permeability and retention) effect. Within these tissues, the envelope of the vascular system by endothelial cells is more loose and leaky and therefore supports particle extravasation into the interstitial space, while in healthy tissues particles are retained in the blood stream.

In addition to the tissue specific targeting, drug release from the carrier should be controllable. Many drugs exert their positive effect only within a certain range of concentrations, the so-called therapeutic window. If the drug concentration falls below the lower boundary, no therapeutic effect will be seen while exceeding the upper boundary results in massive toxic side effects. It is therefore desirable to achieve drug delivery systems with defined control properties.

Concerning the application of drug delivery vehicles in humans, the vehicles should fulfil some requirements. For

example, they should be non-allergenic, non-immunogenic, non-toxic as well as biocompatible. Furthermore, control over their structure and morphology is prerequisite [5-7]. A broad range of drug delivery vehicles has been developed including liposomes, micelles, dendrimers, and inorganic or polymeric nanoparticles with a size in the range of a few to a few hundreds of nanometres. In general, polymeric nanoparticles are considered as the preferred form of drug delivery vehicles as they are very stable compared to e.g. liposomes and a variety of different materials can be used [4]. Among natural polymers, polysaccharides such as chitosan or alginate and proteins such as albumin or collagen are widely used [4, 8]. Besides their bio-compatibility and biodegradability, most natural polymers can be processed without any need for harsh processing conditions, like high temperature, high pressure, or toxic (organic) solvents which is furthermore beneficial for their biocompatibility. Silk as a member of natural polymers combines the required features for its use in biomedical applications; it is non-toxic, non-allergenic, biodegradable and exhibits a good biocompatibility. Silks are therefore a suitable material for the use as drug delivery vehicles.

Dragline silk which builds the frame and the radii of an orb web is one of the best studied types of silk but the availability of spider silk is limited as the cannibalistic nature of spiders obstructs farming of spiders in large scale [9]. A recombinantly producible spider silk protein has been established to generate the desired protein in big amounts and constant quality [0]. The engineered spider silk protein eADF4(C16) is derived from ADF4, one of the dragline proteins of the European garden spider *Araneus diadematus.* A consensus sequence (GSSAAAAAAAAS-GPGGYGPENQGPSGPGGYGPGGP), called C module, has been identified and is repeated 16 times in the recombinant spider silk protein eADF4(C16) to design a protein of a molecular mass of 48 kDa (Figure 1) [1]. In every C module, there is one glutamate residue which is negatively charged under physiological conditions. Hence, eADF4(C16) exhibits a negative net charge.
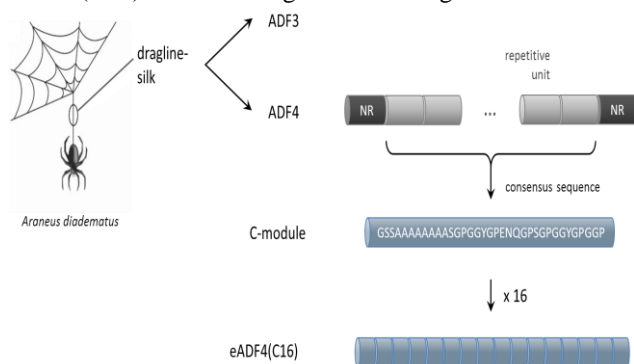
derived from ADF4, and repeated 16 times in the engineered protein eADF4(C16).

In addition to eADF4(C16), a genetically modified variant of eADF4(C16), eADF4(K16) has been engineered. In this protein, the negatively charged amino acid glutamate is exchanged to the positively charged amino acid lysine to yield the so-called K module. This K module is repeated 16 times in the protein eADF4(K16). Under physiological conditions, eADF4(K16) therefore possesses a positive net charge. Furthermore, genetic engineering allows the modification of eADF4(C16) with functional peptides, e.g. cell penetrating peptides which makes theses protein constructs highly interesting for biomedical applications [12].

One feature of recombinant spider silk proteins is their processability. It has been shown, that the recombinant spider silk protein can be processed into different morphologies, for example into fibers, films, hydrogels or particles [13]. Owing to the amino acid composition the recombinant spider silk protein is unstructured when dissolved in aqueous solutions. The addition of potassium phosphate in concentrations higher than 400 mM to spider silk protein solutions leads to a salting out process in which solid particles with no obvious submicrostructure are formed [14]. During the particle formation process, β-sheet rich structures are formed which are responsible for the stability of the particles in aqueous solutions. The size of the produced particles can be controlled by adjusting the protein concentration used during the salting out process and the mixing intensity of the spider silk protein solution during the addition of potassium phosphate (Figure 2). While increasing protein concentration yields bigger particles, the particle diameter decreases with increasing mixing intensity. Using this production method, particles of a size of 250 nm up to 3 µm can be produced [15].
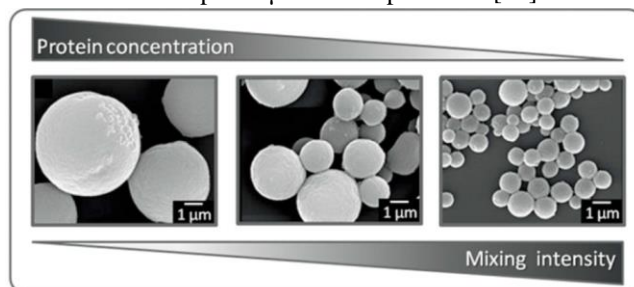
Figure 2: Influence of protein concentration and mixing intensity on the particle size. Decreasing protein concentration and increasing mixing intensity results in a decrease in particle diameter.

Figure 1: The recombinant spider silk protein eADF4(C16).Non-repetitive termini (NR) flank the highly repetitive core domain of ADF4, one of the proteins found in the dragline silk of the European garden spider *Araneus diadematus*. A consensus sequence called C-module is

2 RESULTS

2.1 Cellular uptake of spider silk particles

First, the properties concerning cellular uptake of the recombinant spider silk particles have been assessed. For these experiments, particles of a size from 220 nm to 360 nm have been used. It could be shown that the uptake of particles produced from eADF4(C16) is rather poor which is mainly the result of electrostatic repulsion effects between the negative charge of eADF4(C16) particles and the negatively charged cellular surface. In contrast, the positively charged eADF4(K16) is taken up into cells much more efficiently. Furthermore, hybridizing the recombinant spider silk protein eADF4(C16) with the artificial cell penetrating peptide R_8 (eight arginines) significantly enhanced particle uptake in comparison to eADF4(C16) (Figure 3) [16].
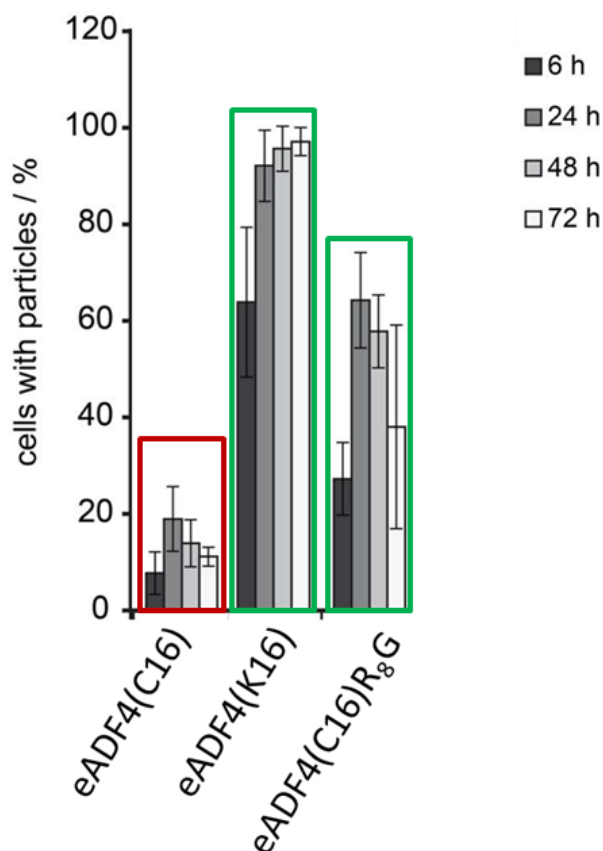

Figure 3: Uptake of recombinant spider silk particles by HeLa cells. Uptake of the respective spider silk particles after the indicated incubation period has been analysed by flow cytometry.

2.2 Drug loading and release of spider silk particles

eADF4(C16) particles are e.g. suitable transport vehicles for low molecular weight molecules and proteins [17, 18]. Uptake and release of water-soluble substances such as rhodamine B serving as a model can be, to some extent, controlled by processing conditions. Co-precipitation of rhodamine B with eADF4(C16) increased the loading efficiency compared to loading of particles by diffusion processes. Crosslinking of eADF4(C16) reduced the rate of rhodamine B release, depending on the production route. The influence of salt on release was little due to the twitterionic behavior of rhodamine B and differed depending on the loading process as well as on crosslinking of eADF4(C16). For non-crosslinked particles no influence of salt on the release of rhodamine B could be detected, while for crosslinked spheres a significant difference in the rhodamine B release was observed in presence of salt. These findings can be explained by changes in protein hydrophobicity due to the formation of di-tyrosines, the osmotic gradients between the particles and the surrounding media, as well as the partially electrostatically shielded rhodamine B molecules.

Particles of the polycationic spider silk protein eADF4(κ16) can be efficiently used to encapsulate negatively charged substances [12]. Nucleic acids are bound to eADF4(κ16) particles by strong electrostatic interactions due to the high charge density of the DNA. Nucleic acids are slowly released in contrast to small molecular weight substances which show a burst release within the first 30 min of incubation. Using Layer-by-Layer-coating of eADF4(κ16) particles with eADF4(C16) to slow down release of small molecular substances was not successful.

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