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# The effect of steam sterilization on recombinant spider silk particles

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ABSTRACT

In this work, the recombinant spider silk protein eADF4(C16) was used to fabricate particles in the submicron range using a micromixing method. Furthermore, particles in the micrometer range were produced using an ultrasonic atomizer system. Both particle species were manufactured by an all-aqueous process. The submicroparticles were 332 nm in average diameter, whereas 6.70 μm was the median size of the microparticles. Both particle groups showed a spherical shape and exhibited high β-sheet content in secondary structure. Submicro- and microparticles were subsequently steam sterilized and investigated with respect to particle size, secondary structure and thermal stability. Sterilization temperature and time were increased to assess the thermal stability of eADF4(C16) particles. Actually, particles remained stable and their properties did not change even after autoclaving at 134 °C. Both, the untreated and the autoclaved submicroparticles showed no overt cytotoxicity on human dermal fibroblasts after incubation for 72 h. The eADF4(C16) particles were already loaded with proteins and small molecules in previous studies. With that, we can provide a highly promising parenteral drug delivery system based on a defined polypeptide carrier, manufactured with an all-aqueous process and being fully sterilizable.



## 1. Introduction

Biocompatible and biodegradable polymers have gained increasing interest for drug delivery applications in recent years (Luckachan and Pillai, 2011). Such biomaterials should have appropriate properties for use in medicine and surgery such as adequate initial strength and controlled degradation rate in addition to the possibility to be processed into different morphologies (Luckachan and Pillai, 2011). According to the requirements of the pharmacopeias, sterility is an indispensable prerequisite for parenterally administered compounds (Council of Europe, 2011). The European Agency for the Evaluation of Medicinal Products and the Evaluation of Medicines for Human Use provides a decision tree for the selection of an optimal sterilization method (EMEA, 2000). In case of aqueous based formulations, sterilization by moist heat at 121 °C for 15 min is the method of choice. Commonly used biodegradable polymers like poly(lactic-*co*-glycolic) acid (PLGA) are often not able to withstand these harsh environments. Steam sterilization of PLGA composites leads to a degradation and hydrolysis of the polymer (Anderson and Shive, 1997; Athanasiou et al., 1996). In addition, PLGA degradation leads to an acidic microenvironment inside the composite and as a consequence an elevated degradation of the loaded protein (Estey et al., 2006). Other biodegradable materials have already been tested for their ability to be autoclaved. For instance, gelatin beads showed degradation after autoclave treatment (Wissemann and Jacobson, 1985), and heating collagen above the shrinkage temperature changed the protein chemistry and physical properties of the material (Bonar and Glimcher, 1970; Wiegand et al., 2009). Ahmed et al. (2013) studied the effect of steam sterilization on polyurethane materials. While the non-degradable polymer withstood the autoclave treatment, the biodegradable poly(caprolactone-urea) urethane polymer degraded during steam sterilization, resulting in the loss of structural integrity. Therefore, aseptic processing was assumed to be the only method to fabricate biopolymers until other sterilization techniques were evaluated for sensitive biomaterials in the past (Wiegand et al., 2009; Yaman, 2001). Physical sterilization methods like gamma irradiation or chemical methods like gas sterilization with ethylene oxide have been tested. Nonetheless, even these techniques are not applicable to many biopolymers. For instance, a general problem of chemical sterilization with ethylene oxide is the risk of toxic residues in the final product (Wiegand et al., 2009). Ahmed et al. (2013) showed that polyurethane

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nanocomposite biomaterials sterilized with gamma irradiation had cytotoxic effects in cell culture. In addition, changes in the physical properties of tested PLGA microparticles were observed (Friess and Schlapp, 2006). On that account, other biopolymers, which can easily be sterilized by autoclave treatment, needed to be considered. Chitosan nanoparticles have been successfully steam sterilized without an effect on particle size or morphology (Qi et al., 2004). As chitosan can be processed in an all-aqueous environment, it is often used as drug carrier for sensitive drugs like proteins (Xu and Du, 2003). One disadvantage is that crosslinking agents are often necessary to control the release kinetics of chitosan based systems (Berger et al., 2004).

Silk proteins represent a promising alternative to the aforementioned biopolymers. It is possible to transform the silk protein into different morphologies such as films, hydrogels, scaffolds, micro- and nanoparticles (Spiess et al., 2010; Wang et al., 2008). Moreover, Hedhammar et al. (2010) have already shown that spider silk fibers can be steam sterilized. They proved that a simple steam sterilization process did not affect the fibers morphology and had no influence on the secondary structure of the protein.

n our study, we used the engineered spider silk protein eADF4(C16). This protein is a recombinant part of the natural spider silk protein ADF4 from the European garden spider *Araneus diadematus*. Lammel et al. (2011) already proved the ability of eADF4(C16) particles to be used as a drug delivery system, which can be easily fabricated in the submicron scale by an all-aqueous micromixing process (Hofer et al., 2012). We fabricated eADF4(C16) submicroparticles using this micromixing system and introduce the ultrasonic atomizer system as an additional method for the preparation of eADF4(C16) microparticles. Furthermore, we systematically investigated the effects of different steam sterilization conditions on both eADF4(C16) micro- and submicroparticles. Starting with the standard autoclave conditions of 121 °C for 15 min (EMEA, 2000), we subsequently increased the thermal load of eADF4(C16) micro- and submicroparticles. We focused on characterization of particle size, secondary structure and thermal stability related to the selected steam sterilization conditions. Cytotoxic effects of steam sterilized eADF4(C16) submicroparticles were evaluated and compared to unsterilized particles.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Recombinantly produced spider silk protein eADF4(C16)

The spray dried eADF4(C16) protein was provided by AMSilk GmbH (Martinsried, Germany). This spider silk protein is a recombinant part of the natural amino acid sequence of ADF4 from *A. diadematus*. A molecular mass of 47.7 kDa is resulting by 16 repeats of the sequence GSSAAAAAAAASGPGGYG-PENQGPSGPGGYGPGGP. Due to this amino acid sequence, eADF4(C16) has a theoretical isoelectric point of 3.48 and a net negative charge at a physiological pH of 7.4.

#### 2.1.2. Chemicals

Trizma base (Tris(hydroxymethyl)aminomethane, primary standard and buffer grade, ≥99.9%) and Dulbecco's Modified Eagles Medium were purchased from Sigma Aldrich (St. Louis, USA). Triton X-100 (analytical grade), di-potassium hydrogen phosphate (EMPROVE bio, European Pharmacopoeia (Ph. Eur.), British Pharmacopoeia (BP)) and potassium dihydrogen phosphate (EMPROVE bio, Ph. Eur., BP, United States National Formulary (NF)) were obtained from Merck KGaA (Darmstadt, Germany). Guanidine thiocyanate (molecular biology grade) was purchased from AppliChem (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was provided from Calbiochem (Darmstadt, Germany). Fetal bovine serum (FKS), L-glutamine, trypsin/EDTA solution (0.05%/0.02% (w/v)) and phosphate buffered saline (PBS) were purchased from Biochrom (Berlin, Germany). Penicillin/streptomycin solution (100×) was obtained from PAA Laboratories (Pasching, Austria).

### 2.2. Particle preparation

The eADF4(C16) protein solution for particle preparation was prepared as described earlier (Hofer et al., 2012). Briefly, eADF4(C16) protein powder was dissolved in a 6 M guanidine thiocyanate solution and subsequently dialyzed against a 10 mM Tris(hydroxymethyl)aminomethane(Tris)/HCl solution at 2–8 °C. A dialysis membrane with a molecular weight cut-off of 8000 Da (Spectrum Laboratories, Rancho Dominguez, USA) was used. After dialysis, the solution was centrifuged and filtered through a 0.2 μm PES filter (VWR International, Radnor, USA). The concentration of eADF4(C16) protein in solution was determined by an Agilent 8453 UV–vis spectrophotometer (Agilent, Waldbronn, Germany) using a molar extinction coefficient of eADF4(C16) at 276 nm ($\varepsilon$ = 46,400 $M^{-1}\,cm^{-1}$). This solution was further adjusted to the desired concentrations for particle preparation with a filtered 10 mM Tris solution.

#### 2.2.1. Particle preparation using a micromixing system

Processing of the spider silk solution into submicroparticle dispersions was carried out by micromixing using a high pressure syringe pump system as described earlier (Hofer et al., 2012). Briefly, two cylinders of the syringe pump system (Model 100 DX and Series D pump controller, Teledyne Isco, Lincoln, USA) were filled with pre-tempered eADF4(C16) solution ($c$ = 1.0 mg/ml) or pre-tempered 2 M potassium phosphate solution (pH 8) at 60 °C. The pumps were connected via a T-shape mixing element (inner diameter 0.5 mm, P-727 PEEK tee, Upchurch Scientific, Oak Harbor, USA) into which the solutions were pumped at a flow rate of 50 ml/min.

#### 2.2.2. Particle preparation using an ultrasonic nozzle

An ultrasonic atomizer system was used for the preparation of microparticles. The ultrasonic nozzle (Sono-Tek 120-00456, Milton, USA) was powered by a broadband ultrasonic generator (Sono-tek, 06-05108) to generate small atomized droplets. A 2 M potassium phosphate solution was fed into the ultrasonic nozzle using a peristaltic pump (Ismatec ISM932, Glattbrugg, Switzerland) at a constant flow rate of 3.0 ml/min. The potassium phosphate solution was atomized at 0.9 W into a stirred eADF4(C16) protein solution ($c$ = 10.0 mg/ml) reservoir ($V$ = 3.0 ml). To ensure a constant flow of the potassium phosphate solution into the nozzle, tubes were completely filled with the potassium phosphate solution prior to the atomization of the potassium phosphate solution. After one minute, the peristaltic pump was stopped and the resulting particle suspension was stirred for an additional minute.

Particle suspensions from both preparation methods were subsequently centrifuged at 14000 rpm (SIGMA 4K15, Sigma Laborzentrifugen, Osterode am Harz, Germany) and washed with highly purified water (HPW) three times. A two minute ultrasonication (Sonopuls HD 3200, Bandelin electronic, Berlin, Germany) step completed the particle preparation procedure. Particles prepared by micromixing were subsequently filtered through a 1.2 μm filter (Acrodisc 32 mm syringe filter, Pall Life Sciences, Ann Arbor, USA) to remove any residual agglomerates. No crosslinking step was applied. The final particle concentration in mg/ml was determined gravimetrically after drying the particles under vacuum (13 mbar) overnight.