Thermoswitchable Nanoparticles Based on Elastin-like Polypeptides

Bettina Kracke,† James T. Cole,‡ Christoph J. O. Kaiser,§ Björn Hellenkamp,‖ Stefanie Krysiak,† Ali Ghoorchian,‡⊥ Gary B. Braun,#, Nolan B. Holland,‡ and Thorsten Hugel*‖

†Central Institute for Medical Engineering (IMETUM), TU München, Boltzmannstraße 11, 85748 Garching, Germany
‡Chemical and Biomedical Engineering Department, Cleveland State University, 2121 Euclid Avenue, Cleveland, Ohio 44115, United States
§Chemistry Department, TU München, Lichtenbergstraße 4, 85748 Garching, Germany
∥Institute of Physical Chemistry, University of Freiburg, Albertstraße 23a, 79104 Freiburg, Germany
⊥NSF Research Triangle Materials Research Science and Engineering Center, Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, United States
#Cancer Research Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 N. Torrey Pines Rd., La Jolla, California 92037, United States

ABSTRACT: The design of biocompatible particles with defined size on the nanometer scale has proven to be a challenging task in current biomedical research. Here we present an approach toward temperature-responsive nanoparticles by covalently cross-linking micelles based on trimeric constructs of elastin-like polypeptides. These trimers can be triggered to assemble into micelles by heating the solution above a specific transition temperature (Tt) which was shown in previous studies. Here we show that the disassembly of the micelles below the Tt can be prevented by the incorporation of covalent cross-links in the core of the micelles. This facilitates a temperature-triggered swelling and collapsing by around 35% in diameter, as determined by dynamic light scattering. Size distribution was confirmed by fluorescence correlation spectroscopy, atomic force microscopy, and transmission electron microscopy. We show switchable nanoparticles with reversible volume changes in the temperature region between 30 and 40 °C, making these particles promising candidates for switchable drug delivery carriers.

INTRODUCTION

The design of stable nanoparticles featuring stimuli-responsive-ness has gained increased interest in biomedical research during the past decades. Multiple approaches have been carried out toward the development of particles sensitive to environmental changes including pH, ionic strength, CO2, or redox state,1–6 to name a few examples. An outline about the progress in responsive carrier design can be found in the review by Mura et al.7 Particularly interesting biomaterials for the design of stimuli-responsive nanoparticles are elastin-like polypeptides (ELPs) because of their tunable temperature-responsiveness8–10 and their broad range of potential clinical applications, e.g., as drug delivery systems.11 Former studies on ELP-based constructs included polydisperse microaggregates which were stable due to their hysteresis behavior12 or microparticles that had been unspecifically cross-linked with glutaraldehyde.13,14 These systems gave first indication of the high potential of ELP-based carrier systems. Here we show monodisperse particles on the nanoscale, which were specifically cross-linked and show a well-defined temperature response.

ELPs are genetically engineered proteins based on the principal sequence motif from elastin which is a protein in the extracellular matrix that facilitates the shape recovery of many tissues in the body after stretching and contracting.15–17 The sequence consists of polypentapeptide repeats which can be written in a generalized form as (GaGβP)n with α and β as substitutable amino acid positions.10 Genetic engineering of ELPs is well-established so the protein sequence and its properties can easily be modified.18 A particular characteristic of ELPs is their lower critical solution temperature (LCST) behavior. It is marked by the formation of a separated protein-rich phase above a distinct transition temperature Tt which is known as the phase transition.10

A proposed model for the ELP phase transition describes the association and structure formation as two strongly interactive processes that are highly concentration dependent.19 In order

Received: May 2, 2015
Revised: July 31, 2015
Published: August 13, 2015

DOI 10.1021/acs.macromol.5b00932
Macromolecules 2015, 48, 5868−5877
to promote the self-assembly process by mediating local proximity of the chains, ELP sequences have been fused to a trimer forming globular domain called the foldon.20 The foldon domain is derived from the C-terminal domain of the bacteriophage T4 fibrin protein, and in its folded state it has a net negative charge.21 As shown by Ghoorchian et al.,20 an ELP sequence genetically fused to a foldon domain will spontaneously assemble into a trimeric structure. These trimers form micelles when heated above a specific $T_c$. The micelle formation is based on the hydrophobic collapse aggregation of ELP chains above $T_c$ and is facilitated by the subdivision of the construct into a long ELP sequence acting as hydrophobic chain tail and the charged foldon domain as hydrophilic headgroup. The micelles had sizes around 25 nm in diameter which can be tuned by adjusting salt and cosurfactant concentrations. However, as the process of this micelle formation is reversible, these particles are only stable at elevated temperature. Below the $T_c$ they rapidly dissociate into the individual trimers.20

This work represents a new system of responsive nanoparticles which can be reversibly switched in size by a well-defined temperature stimulus in the clinically relevant temperature range between 30 and 40 °C. The system presented here differs in its temperature-responsiveness from other approaches. It is not based on a thermally driven accumulation of ELP like in other studies22−25 but constitutes an assembled particle which is stabilized by covalent links and shows a switching in size upon the phase transition. It constitutes a stable nanocarrier system which might be loaded with small molecule drugs, thereby increasing their concentration. A cross-linked drug-loaded particle could release the drug agents upon its changes in volume, induced by a specific and well-defined change in temperature stimulus.

A protocol for chemoselective cross-linking of ELP fusion protein micelles was developed to obtain stable protein-based nanoparticles. For this, a well-known maleimide chemistry was utilized which is a popular method for cysteine modification.27−29 Bismaleimide poly(ethylene glycol) (PEG) linkers were used as cross-linkers. These linkers are known for their cross-link chemoselectivity and their biocompatibility.29−31 Hereby the maleimide groups were reacted with the cysteines located in the particle interior, enabling a volumetric transition of cross-linked particles. Upon changes in temperature, the particles swell and collapse around 35% in diameter, which is attributed to the reported change in solubility of the ELP chains.20 The change in volume was confirmed by dynamic light scattering (DLS), and sizes at room temperature were confirmed with fluorescence correlation spectroscopy (FCS) measurements. Particle morphology was visualized via transmission electron microscopy and atomic force microscopy images and first loading with FITC-dextran was demonstrated.

**Experimental Details**

**Gene Design.** The gene encoding the ELP-foldon was produced as previously reported20 using a modification of the recursive directional ligation methods described by Meyer et al.18 The results yielded the amino acid sequence $\text{MGH(GVGVG)}_{10}(\text{GGCGV)(GVGVG)})_{12}\text{GVGYPEAPRDQAYVRKDEGWLLSTFL-PGP}$. This sequence allowed for restriction enzyme digestion for further modification with the NdeI, PmlI, and BglII enzymes (New England Biolabs). Briefly, a construct composed of $(\text{GVGVG})(\text{GVGVG})(\text{GVGVG})_{12}$-foldon was double digested with NdeI and PmlI while a construct of $(\text{GVGVG})_{10}$ was double digested with NdeI and BglII. Each of these constructs was run on agarose gel and purified using a gel extraction kit (Qiagen). The two pieces were then ligated together using a quick ligation kit (New England Biolabs) and transformed into a BL21* (DE3) strain of E. coli.

The final construct in the plasmid was verified by DNA sequencing (Cleveland Clinic Genomics Core), and the translated sequence featured 327 amino acids in length and a calculated molecular weight of 27 811 g/mol for a monomer. The molar extinction coefficient $\varepsilon$ was defined according to the method of Gill and von Hippel39 as $14 865 \text{ M}^{-1} \text{ cm}^{-1}$ and was used to determine concentration.

**Protein Expression and Purification.** Bacteria of the E. coli strain BL21 (DE3)-T1 (Sigma) were transformed with the plasmid described above. A starter culture of these was made by the addition of a frozen stock (250 mL) to LB0 (150 mL) containing 75 $\mu$L/g/mL ampicillin (LB$_{amp}$). After overnight incubation at 37 °C and 120 rpm, the culture was transferred to LB$_{amp}$ in a 2 L Erlenmeyer flask and incubated at 37 °C, 120 rpm. At an OD of 0.8 the expression was induced by the addition of IPTG (0.1 mM). After 5 h incubation the cells were harvested by centrifugation (3000g, 30 min). The pellet was stored overnight at −20 °C and resuspended in PBS (15 mL) when thawed. The cells were lysed by sonication for 6 min with a Branson Sonifier 250 (Heimheim Ultraschall- and Labotechnik) with a duty cycle 30% and output control 6. Purification was carried out by the inverse transition cycling (ITC),40,41 starting with centrifugation at 4 °C for 20 min at 14000g. The supernatant, containing the ELP, was recuperated for a second centrifugation step at 40 °C for 20 min at 14000g. After this the supernatant was discarded while the pellet featured the coacervated protein. It was resuspended in PBS. The procedure was repeated another two times, followed by a resuspension of the pellet in phosphate buffer (10 mM, pH 8.0−8.4) and filtering via 0.2 $\mu$m filters with membranes of regenerated cellulose (Corning). Concentration determination was carried out by UV absorption at 280 nm with a spectrophotometer ND-1000 (peqlab Biotechnologie GmbH). The expression yield was around 20 mg/L. The purity of the sample was confirmed by SDS-PAGE with a 4−20% Mini-PROTEAN TGXTM. For this the samples were diluted 1:1 in Laemmli sample buffer (Bio-Rad) with 250 mM DTT and 7.1% (w/v) SDS final concentrations. The SDS in the sample buffer caused denaturation of the trimerization domain and the consecutive disassembly of the ELP chains while reduction of the disulfide bonds was mediated by the addition of DTT. The samples were heated to 90 °C for 5 min prior to loading on the gel. As marker Kaledescope Precision Plus (BioRad) was loaded on the gel.

**Dynamic Light Scattering.** Particle size measurements were carried out with a Zetasizer nano series instrument (Malvern Instruments, United Kingdom) with a 4 mW He Ne gas laser at 632.8 nm and a 175° scattering angle (backscatter detection). The temperature was adjusted with a Peltier temperature control. Each data set was adjusted with a Peltier temperature control. Each data set represented a measurement containing a sequence of 10 runs 30 s each. The correlation function was analyzed by the Protein Analysis algorithm provided by the Malvern software. Sizes with standard deviations are derived from the PSD by intensity. The software version 7.03 provided by Malvern was used for data analysis.

For proof of reversible micelle formation, the protein stock solution of $(\text{GVGVG})_{10}(\text{GGCGV)(GVGVG)})_{12}$-foldon was diluted in double distilled water to 20 $\mu$L protein concentration in 0.4 mM phosphate buffer. The pH was adjusted to 10.2−10.3 by the addition of 0.1 M NaOH.

The concentration-dependent $T_c$ was defined with DLS measurements with heating steps of 0.2 °C. Mean sizes of trimers and assembled nanoparticles were derived from the PSD by intensity. The software version 7.03 provided by Malvern was used for data analysis.
The cross-linker was dissolved in MES (10 mM, pH 7.5) and diluted 1:10 in double distilled water. After equilibration of the cross-linker solution above the specific $T_c$, the cross-linker (100 μL) was added to the protein solution (200 μL), resulting in a 1:2 (v/v) ratio of cross-linker to protein. The cross-link reaction was run in 0.3 mM phosphate/0.3 mM MES with a protein concentration of 13 μM. Upon the addition of the cross-linker solution, the thiol-reactive maleimide reacts with the cysteines, forming stable thioether linkages in the inside of the particle. The cross-linking reaction was carried out overnight above $T_c$. The temperature was kept constant above $T_c$ during the cross-linking. After cross-link the solution was dialyzed against phosphate buffer (10 mM, pH 8.2) in Slide-A-Lyzer MINI dialysis devices (Thermo Scientific) with a 20 kDa cutoff membrane. The dialysis was carried out overnight for around 15–20 h above $T_c$ in a shaker at 70 rpm. Cross-linked nanoparticles were kept at room temperature or above $T_c$.

**Statistical Analysis.** Statistical analysis was carried out using the R Studio software. Analysis was based on DLS-derived mean particle sizes featuring a Gaussian distribution of the particle population. In cases where samples showed minor aggregation which was not visible in the number-weighted distribution, it was attributed negligibly and the main population of the sample was used for statistical analysis.

Requirements for Anova analysis, including Levene test for homogeneity of variances and Shapiro-Wilk test for normality, were tested prior to analysis. Applicability of Tukey test was indicated by an Anova p-value <0.05. Results of Tukey test are indicated in box plot with corresponding levels of significance (0−0.001; ***/0.001−0.01; ***/0.01−0.05; *).

**Fluorescence Labeling and Fluorescence Correlation Spectroscopy.** Fluorescence correlation spectroscopy (FCS) measurements were carried out to support data derived from DLS measurements. A solution of cross-linked nanoparticles was dialyzed against phosphate buffer (10 mM, pH 8.2) and spiked with Attoplex-NH3 (in DMDSO) in a volume ratio 10:1. The nanoparticle solution had a final dye concentration of 12 μM. The solution was incubated on a shaker for 40 min at room temperature, followed by incubation above $T_c$ for 15 min. Free dye was removed via dialysis against phosphate buffer (10 mM, pH 8.2) with Slide-A-Lyzer MINI dialysis devices (Thermo Scientific) with a 20 kDa cutoff membrane. Dialysis was carried out for 4 days above $T_c$ while the buffer solution was exchanged several times.

FCS measurements were carried out with a home-built confocal microscope which resembled other published setups.2–4 Attoplex-NH3-labeled nanoparticles were dialyzed 1:100 in phosphate buffer (10 mM, pH 8.2). The solution was incubated at room temperature for 2 days to remove possible aggregates by sedimentation. Before measurement, the sample was equilibrated at room temperature for approximately 10 min. The nanoparticles were excited with a pulsed 640 nm laser, and time traces of photons were recorded with a single photon avalanche photodiode (PDM-50 μm, PicoQuant). A commercial data acquisition system was used (HydraHarps 400, PicoQuant) which features a picosecond time resolution. The diffusion time $τ_c$ can be derived from a fit on the correlation function by using eq. 1.

$$G(r) = (1 − T + T e^{−r/τ_c}) \left(1 + \frac{1 + r}{τ_c} \right)^{−1/2} \left(1 + r/τ_c \right)^{−1/2}$$

The first part of the equation which describes the triple time is only used for fit optimization and is not part of the analysis. The geometrical parameter $κ$ is held constant for each fit, and the relative diffusion time $τ_c$ through the confocal volume can be extracted from the fit parameters. The hydrodynamic radius is directly proportional to the experimental defined diffusion time according to Stokes’ law. For size definition, the viscosity with 0.8948 cP at 25 °C derived from the dispersant (10 mM phosphate buffer) defined in the Malvern Software was used.

**Imaging by Atomic Force Microscopy.** The nanoparticle solution was imaged 3 days after cross-link in fluid at room temperature below $T_c$. For this, the sample was applied to a freshly cleaved surface of highly oriented pyrolytic graphite (HOPG) with a mosaic spread of 0.4°−0.7° and a thickness dispersion of ±0.2 mm (NT-MDT Service and Logistics Ltd., Limerick/Ireland). The sample was adsorbed to the surface below $T_c$ in a fluid cell which was then filled up with double distilled water (2 mL). Imaging was carried out via tapping mode with a MLCT cantilever (Bruker SPM Probes, Camarillo, CA).

Particle analysis was carried out using the Igor Pro Software, Version 6.1.2.1, from WaveMetrics (Oregon 9, USA). All particles with spherical shape in the $x$−$y$ plane were analyzed, and a procedure was established to deduce the corresponding diameter of the nanoparticles in solution. Each particle was analyzed via its section line by which the particle maximum height $h$ was defined. The particle diameter was derived from the full width at half-maximum (fwhm) of a Gaussian fit on the particle section line.

**Imaging by Transmission Electron Microscopy.** Solutions with cross-linked nanoparticles were negatively stained on previously glow-discharged carbon-coated grids. Negative staining was carried out at room temperature as follows: a solution of cross-linked nanoparticles (5 μL) with around 0.4 mg/mL protein concentration was applied to the carbon layer and was incubated for 2 min. Excess solution was blotted off with filter paper (Whatman grade 1), and the sample was quickly transferred to a drop of ammonium molybdate stain solution (5 μL, 2% w/v, pH 7.0). After 1.5 min incubation, excess stain solution was again blotted off and grids were allowed to air-dry. Micrographs were recorded with a JEOL JEM 100CX electron microscope operated at 100 kV at a nominal magnification of 20000x and a slight negative defocus on Kodak SO-163 electron image films. The images were digitized using a FlexTight Precision II array scanner (Hasselblad) at a calibrated pixel size of 7.9 Å at the sample level. A median filter with a 2 pixel radius was applied. Particle size analysis was performed with ImageJ (Version 1.44e) by manually approximating a circle of which the diameter was determined.

**Loading of Cross-Linked Nanoparticles.** For loading analysis, particles were labeled with DLS94 and loaded with neutral fluorescein isothiocyanate (FITC)-dextrans with 4 kDa molecular weight (Sigma-Aldrich). Trimmers were labeled by addition of DLS94 to the protein solution (73 μM), leading to an end concentration of 18 μM dye. The sample was incubated rotating at room temperature for 2 h. The sample was then dialyzed for 6 h against 1.5 L of phosphate buffer (10 mM, pH 8.2) with Pur-A-Lyzer Maxi dialysis devices (MWCO 12 KDa, Sigma Life Sciences, including one buffer exchange). DLS94-labeled trimmers were added to unlabeled protein in a 1:7 (v/v) ratio. The solution was then spiked with FITC-dextrans (2 mM in 10 mM phosphate buffer) and NaCl (1 M), leading to a final concentration of 125 μM FITC-dextrans and 1.5 M phosphate buffer after cross-linking. The final NaCl concentration after cross-link was 32 or 16 mM, respectively. Salt was added to increase particle size, thus facilitating analysis via confocal microscopy (Leica Type TCS SPS II, Leica Microsystems CMS GmbH). Laser wavelengths for dye excitation were 488 and 633 nm, and emission was detected at 500–550 and 653–695 nm, respectively.

**AFM Force Spectroscopy.** The measurements were performed with a MFP-3D SA (Asylum Research, Santa Barbara, CA) in a temperature-controlled fluid cell at 25 and 55 °C. ELPs were covalently coupled to the AFM tip of the cantilever (MLCT, Bruker SPM Probes, Camarillo, CA), and the glass surface was functionalized with maleimide groups (for details see section S2). Extension–retraction cycles were measured with a constant pulling velocity of 0.5 μm/s and a dwell time on the substrate of 1 s. The dwell time facilitated bond formation between the maleimide on the surface with the cysteine groups of the ELP-functionalized tip. During the retraction worm-like chain (WLC) stretching of PEG chains and rupture were observed in the force–distance curves. For the measurement of hysteresis a smaller force distance was chosen. As soon as an ELP stretching between tip and surface occurred, the starting point of the force distance was stepwise moved away from the surface. Several extension relaxation cycles were recorded until a rupture occurred.
RESULTS

Protein Expression, Purification, and Characterization. The ELP-trimer (GVGVP)$_{36}$(GCGVP)(GVGVP)$_{21}$-foldon construct was successfully expressed, and its purity was confirmed after the protein purification using inverse transition cycling (ITC) technique, by SDS-PAGE gel electrophoresis, showing the monomer band consistent with the calculated molecular weight of 27 811.7 g/mol (Figure S1). To test the temperature-responsive of individual trimers, AFM force curves were measured. To confirm that indeed trimers were stretched, the curves were fitted with the worm-like-chain model and the persistence length was compared to the monomer persistence length. The trimers were stretched and relaxed below and above $T_c$. The absence of a hysteresis above $T_c$ indicates that the trimers, as individual subunits, did not have a distinct folding transition (Figure S2).

Reversible Micelle Formation. Micelle formation and its reversibility was confirmed for the ELP-trimer (GVGVP)$_{36}$(GCGVP)(GVGVP)$_{21}$-foldon in analogy to previous reports of (GVGVP)$_{40}$-foldon. The micelles were shown to form in phosphate buffer (0.4 mM) at pH 10.2 above their $T_c$. This was observed by DLS measurements where particle sizes were derived from the intensity weighted distributions below and above $T_c$ (Figure 1).

![Figure 1. Reversible micelle formation of the elastin-like polypeptide (GVGVP)$_{36}$(GCGVP)(GVGVP)$_{21}$-foldon at 20 μM in 0.4 mM phosphate buffer, pH 10.2. The sizes are derived from the intensity-weighted distribution of DLS measurements. Diameters were 14 ± 2 nm below the transition temperature before heating (red), 29 ± 6 nm above the transition temperature (green), and 16 ± 2 nm below the transition temperature after heating (blue).](image)

Individual trimers with an apparent diameter of 14 ± 2 nm were detected below $T_c$. Above their $T_c$ they assembled into micellar nanoparticles of 29 ± 6 nm in diameter with a polydispersity index (PI) of around 0.1. This illustrates the homogeneity of the micelle solution, as a value of less than 0.1 is considered a monodisperse sample. The observed micelle diameter of 29 ± 6 nm is slightly larger than the reported size of protein micelles of (GVGVP)$_{40}$-foldon which is expected because of the longer ELP chains used in the current study, with additional 18 pentapeptides.

The analysis of micelle formation was carried out with three different protein concentrations. For this, sizes of the main population (with trimer diameters around 15 nm and nanoparticle diameters below 50 nm) were tracked over a temperature range of 27–45 °C in temperature steps of 0.2 °C. The assembly into particles is shown in Figure 2 with 20 μM protein solution in Figure 2a while concentrations of 60 and 4 μM are illustrated in Figure 2b.

It can be seen that $T_c$ for the assembly into particles is concentration-dependent and that it can fluctuate up to around 2 °C for lower concentrations. This could be caused by slight variations in protein concentration which seem to have a larger effect on $T_c$ in the lower concentration range. The mean $T_c$ for 4, 20, and 60 μM are 38.5, 36.6, and 32.5 °C, respectively.

Cross-Linking and Particle Size Measurement. A cross-linking protocol was used to create nanoparticles stable both below and above their $T_c$. The cysteine residues used as cross-linking points were located in the ELP sequence, more precisely in the C-terminal third of the ELP sequence which is followed by the foldon headgroup. This location of the cross-link point should enable swelling of the cross-linked micelles below their $T_c$. The cysteines were successfully covalently linked via bismaleimide PEG linkers.

Figure 3 shows the sizes of cross-linked nanoparticles featuring temperature-responsiveness. A PI of 0.03–0.04 underlines the high homogeneity of the nanoparticle solution. A temperature increase above the $T_c$ triggers a change in diameter from 13° ± 27 to 83 ± 10 nm. The degree of swelling and collapsing was consistent where the diameter decrease was typically around 35%. The difference in volume relative to the volume at low temperature is around 75%.

Particle Stability and Statistical Analysis of Particle Sizes. Particles were analyzed in terms of their stability and significant differences in particle size. Figure 4a shows a boxplot derived from four independent experiments including 14 dialyzed samples in total, where one sample was a pool of 2–3 cross-links.

The mean particle diameters in this data set are 66 ± 8 nm before heating, 46 ± 9 nm above $T_m$ and 64 ± 6 nm after heating. A corresponding box plot showing samples directly after cross-link in reaction buffer can be found in section S3. Figure 4b shows exemplarily three cross-linked samples in phosphate buffer (10 mM, pH 8.2) where the size change as a result of the phase transition was monitored via DLS. Sizes were measured after storage above $T_c$. The heating is assumed to facilitate a restructuring of the nanoparticles, resulting in a decrease in particle size and an increased homogeneity of the solution.

Typically, dialyzed samples feature a broader size distribution directly after buffer exchange. The storage over several weeks can also be accompanied by the coalescence of individual particles which can be detected by an increase in PI, the apparent increase in particle size or the formation of a second larger size population. Samples can occasionally feature a second larger size population after several weeks of storage. Centrifugation can remove occasional larger particles, and sample dilution and incubation above $T_c$ can redissolve aggregated particles and increase solution homogeneity. Possible sources for particle aggregation are discussed later.

Fluorescence Correlation Spectroscopy. Cross-linked nanoparticles were labeled with Atto647N-NHS. The labeling was performed at pH 8.2 for optimal reaction conditions for the N-terminus which has a reported $pK_a$ value of around 7.6–8.3. The N-termini of the ELP trimers were located in the interior of the particles while amine groups from lysines are located in the headgroup. The measurement shown here was of 10 min length and aggregates were cut out, resulting in 30–60 s long intervals. One correlation function of a sample 10 days after cross-link is presented in Figure 5. In this case the derived diffusion coefficient below $T_c$ was 7.7 μm$^2$/s.

Six diffusion coefficients were determined at room temperature to calculate the hydrodynamic radii using the Stokes–Einstein equation $R_H = k_B T/6πηD$, where $k_B$ is the Boltzmann
constant, $T$ the temperature, $\eta$ the viscosity, and $D$ the diffusion coefficient. The value for the viscosity of 10 mM phosphate buffer was the same as used by the DLS software, with 0.8948 cP at 25 °C. The average mean diameter was $60 \pm 13$ nm below $T_t$. 

Figure 2. Definition of concentration-dependent transition temperature $T_t$ for (GVGVP)$_{36}$(GCGVP)(GVGVP)$_{21}$-foldon (pH 10−10.2) with (a) 20 μM protein concentration and (b) 4 and 60 μM protein concentration. Sizes were derived by intensity-weighted DLS measurements, and data were fitted with a sigmoidal fit and definition of $T_t$ at the turning point.

Figure 3. Cross-linked nanoparticles based on ELP fusion proteins showing temperature-responsiveness in temperature-cycling measurement. The diameters are derived from intensity-weighted distributions of dynamic light scattering measurements on the first day after cross-link before dialysis. Sizes were $137 \pm 27$ nm below the transition temperature ($T_t$) before heating (red), $83 \pm 10$ nm above $T_t$ (green), and $133 \pm 25$ nm below $T_t$ after heating (blue). The heating cycle verifies the absence of hysteresis behavior.

Figure 4. (a) Box plot of particle diameters derived from mean diameters of DLS intensity-weighted measurements in a heating cycle. Samples were in phosphate buffer (10 mM, pH 8.2) and had been stored above $T_t$ before measurement. Mean values and standard deviation are $66 \pm 8$ nm before heating, $46 \pm 9$ nm above $T_t$, and $64 \pm 6$ nm after heating. (b) Three exemplary particle sizes with standard deviations derived from intensity-weighted DLS measurements.

Figure 5. Correlation functions obtained by fluorescence correlation spectroscopy measurements of Atto647N-labeled nanoparticles below $T_t$. The resulting diffusion coefficient is $7.7 \mu$m$^2$/s.

buffer was the same as used by the DLS software, with 0.8948 cP at 25 °C. The average mean diameter was $60 \pm 13$ nm below $T_t$. 

Figure 2. Definition of concentration-dependent transition temperature $T_t$ for (GVGVP)$_{36}$(GCGVP)(GVGVP)$_{21}$-foldon (pH 10−10.2) with (a) 20 μM protein concentration and (b) 4 and 60 μM protein concentration. Sizes were derived by intensity-weighted DLS measurements, and data were fitted with a sigmoidal fit and definition of $T_t$ at the turning point.
Imaging by AFM and TEM. The cross-linked particles were imaged by atomic force microscopy in fluid tapping mode on a surface of highly oriented pyrolytic graphite (HOPG) in double distilled water. Figure 6 displays a sample imaged 3 days after cross-linking. Since the sample had not yet been dialyzed, residual trimers are also observed.

Figure 6b shows the size distribution after cross-link before dialysis. The graph displays the presence of two populations: one representing remaining trimers with 40 ± 6 nm and nanoparticles with 85 ± 10 nm in diameter. Deconvolution with a tip radius of 10 nm resulted in diameters of 20 ± 6 nm for the trimers and 65 ± 10 nm for the nanoparticles. Altogether, there are 911 particles for population 1 (attributed to trimers) and 27 particles for population 2 (attributed to cross-linked nanoparticles). When considering a packing factor of 41 trimers per particle, derived from geometrical arguments (section S5), these trimers are equivalent to 22 disassembled nanoparticles. This represents an apparent cross-link efficiency of 55% in this experimental set.

Negative stains of cross-linked nanoparticles for TEM imaging were prepared 2 days after cross-linking. The images obtained (Figure 7a,b, complete images in Figure S6) are comparable to the AFM images, showing both small and large particles.

Size distributions of the undialyzed sample show two populations: one with a diameter of 13 ± 3 nm attributed to the trimers and the other with a diameter of 69 ± 11 nm attributed to the nanoparticles. The ratio of trimers to nanoparticles in the sample is 813:173, which corresponds to an apparent cross-link efficiency of 90%. The particle size distribution of the dialyzed sample (Figure 5d) was subdivided into two populations at a threshold of 50 nm, resulting in 53 trimers with 29 ± 4 nm diameter and 85 cross-linked nanoparticles with 72 ± 13 nm.

Loading of Cross-Linked Nanoparticles. Cross-linking of DLS94-labeled ELP-nanoparticles was carried out with FITC-dextran in solution to produce dextran-loaded nanoparticles. The sample was dialyzed against phosphate buffer (10 mM, pH 8.2) to remove free leftovers of FITC-dextran. Colocalization of FITC-dextran in labeled nanoparticles was analyzed by confocal microscopy. Figure 8 illustrates that loading was successfully carried out for samples with two different final NaCl concentrations (32 and 16 mM, respectively).

**DISCUSSION**

The goal of this work is to develop responsive nanoparticles for targeted drug delivery. Toward this end, this work demonstrates the formation of polypeptide-based nanoparticles that exhibit a dramatic change in volume with a modest change in temperature in the physiologic range. This was accomplished by cross-linking a temperature-responsive polypeptide micelle. It was first demonstrated that the polypeptide forms reversible micelles and after cross-linking retains its temperature-responsive behavior.

Cross-Linking and Particle Size Measurement. We show here a temperature-triggered volume change of cross-linked nanoparticles based on ELP-foldon fusion proteins. While un-cross-linked micelles disassemble below a specific $T_c$, these cross-linked nanoparticles are stable and show an increase in diameter upon cooling. The swelling of the particles below $T_c$ is attributed to hydration of the ELP chains, while the collapse of the nanoparticle above its $T_c$ is induced by dehydration driven by hydrophobic chain associations of the ELP.

The samples of one exemplarily cross-linked set are consistent in size which is illustrated by a low standard deviation for the diameter of three samples on the first day after cross-link with 133 ± 4 and 82 ± 1 nm below and above $T_c$, respectively. The high degree of sample homogeneity is underlined by a PI below 0.1 on the first day after cross-linking. It is observed that particles appear to be larger directly after cross-linking and diminish in size upon storage above $T_c$. It is assumed that this increase in particle size could be caused by the reintegration of PEG molecules with an approximate contour length of 11.5 nm in helical conformation, assuming a mean PEG monomer length of 2.8 Å. 

Significant differences in sizes below and above $T_c$ were shown with the Anova analysis followed by the posthoc Tukey test, with the levels of significant differences indicated in the box plot (Figure 4a). Representative nanoparticle solutions after dialysis are illustrated in Figure 4b where the first sample shown features a diameter of 36 ± 7 nm above $T_c$ and 55 ± 16 nm below $T_c$ after heating. Most notably, the size determined by DLS for the representative sample 1 in phosphate buffer (10 mM, pH 8.2) in Figure 4b at room temperature (56 ± 17 nm) is in excellent agreement with the sizes obtained by FCS measurements (60 ± 13 nm). Samples that were dialyzed against phosphate buffer (10 mM) and stored above $T_c$ for 2 weeks before measurement feature very defined sizes even 9 weeks after cross-linking, which is shown in section S4. The increase in sample homogeneity and particle restructuring upon storage above $T_c$ is especially noteworthy. The difference in absolute particle size between samples after dialysis and samples in reaction buffer (section S4) could be explained by the remaining PEG molecules in the reaction buffer, leading to larger apparent particle sizes. This could be caused by a change in ionic strength.
in solution viscosity caused by potential leftovers of PEG reactants and trimers, which were not included in the dispersant specifications in DLS measurements.

It should be noted that sizes derived by DLS measurements can fluctuate between experimental sets, but the degree of swelling still remains comparable throughout the individual

---

**Figure 7.** Analysis of cross-linked nanoparticles by transmission electron microscopy (TEM). (a, b) TEM images at 20K magnification showing negatively stained nanoparticles (a) before dialysis and (b) after dialysis. (c, d) Corresponding particle size distributions with mean diameter and standard deviation resulting from a Gauss fit to the distribution. (c) Particle distributions before dialysis with mean particle diameters of 13 ± 3 nm for trimers (population 1) and 69 ± 11 nm for nanoparticles (population 2), derived from 986 particles. (d) Particle distributions after dialysis with mean particle diameters of 29 ± 4 nm for trimers (population 1) and 72 ± 13 nm for nanoparticles (population 2), derived from 138 particles.

**Figure 8.** Colocalization experiment of FITC-dextran-loaded particles. The samples had been spiked with NaCl before cross-link to increase particle sizes, where final salt concentration was (a–c) 32 mM NaCl and (d–f) 16 mM NaCl. The scale bar for all images is 5 μm.
measurements. Hence, DLS measurements serve as evidence for particle volume transitions. The reaction solution contains specific buffering salts which can affect the micelle sizes which are salt- and pH-dependent.\textsuperscript{35} The hydrophobic interaction between the side chains may also be altered and disrupted by electric charges.\textsuperscript{36} A minor fraction of cross-linkers might also react with the lysines in the foldon headgroup as pH conditions have to be kept sufficiently high to guarantee micelle formation above $T_c$.

**Stability.** The formation of sporadic large particles after long-time storage can be observed by a wider size distribution which is accompanied by an increase of PI in DLS measurements. The diameter derived by DLS is an intensity-weighted size. The change in buffer conditions via sample dialysis and long-time storage could be causing the formation of occasional larger particles which can mask smaller populations in the measurement. Concomitantly, the average size of smaller particles can be biased to larger sizes in the presence of a few larger particles.

The process of clustering upon long-time storage could be explained either by a tendency of the particles to adhere to each other via their headgroups or via hydrophobic attraction of the tails. The first theory is based on the assumption that particles with an unstructured interior below the $T_c$ tend to lose their uniformity. The slow transformation to nonspherical particles would promote salt-mediated particle coalescence facilitated by larger surface areas of individual particles. The second theory focuses on the N-terminal ELP chains which are in a random coil formation below their $T_c$ that might disentangle and diffuse out of the nanoparticles. If one accounts for the high water content in the micelles, the attractive forces of ELP sequences in a swollen micelle would have to be sufficiently low to enable this process. Hydrophobic sequences protruding out of the micelle interior would then have the tendency to adhere to each other, supported by the larger surface area of nonspherical particles. Keeping the particles above $T_c$ appears to have a positive effect on sample homogeneity. The heating is assumed to cause a restructuring of the particles to a lower energy and more stable conformation with a smaller particle size.

**Imaging Cross-Linked Nanoparticles by AFM and TEM.** Cross-linked nanoparticles were visualized by two different imaging methods. AFM tapping mode in fluid depicted particles with $85 \pm 10$ nm in diameter besides the remaining trimers which are $40 \pm 6$ nm in diameter. Obviously these sizes are larger than expected which is a result of the convolution of the tip radius with the imaged spheres. To obtain a more realistic value, the sizes can be subtracted with twice the tip radius which was estimated to a value of $10$ nm (as the nominal tip radius given by the manufacturer with $20$ nm seemed to be overestimating the radius of the used tip). Concomitantly, the nanoparticle diameters of about $65$ nm determined by AFM images might also be underestimated to some extent as they were deduced from the fwhm of a Gaussian fit. It has to be understood that the derived value represents an approximation to the real particle diameter.

Particle sizes determined by TEM images were $69 \pm 11$ nm in diameter and thus comparable to sizes defined by AFM measurements. The same is true for sample homogeneity. The sporadic clustering seen on TEM images is most likely caused by the ammonium molybdate in the negative staining procedure as the particles are very sensitive to salt conditions. The dialyzed sample features particles of small size with diameters of $29 \pm 4$ nm. They most likely represent associations of remaining trimers in the sample whose diffusion through the dialysis membrane is hindered. The population of small size represents a minor fraction in the dialyzed sample. Because of its rare occurrence, it is only randomly observed in DLS measurement where it is then observable in the number-weighted size distribution. Naturally, when analyzing size data deduced from TEM images, one has to consider the influence of the negative staining procedure on final size where changes in solution conditions of the cross-linked particles may favor fusion via their headgroups. Additionally, flattening of the particles due to the adsorption and staining process and a loss of water upon the exposure of the sample to high-vacuum conditions has to be considered.\textsuperscript{37} Presumably the effect of water loss is less pronounced in the presence of PEG molecules as the particles appear more spherical and consistent before dialysis. This observation supports the assumption that micelles are loaded with the excess remaining PEG molecules, hinting at its potential as drug carrier. Interestingly, former studies on elastin already pointed at the sensitivity of elastin samples to water loss upon the drying process. Hence, in an attempt to reduce drying effects, PEG molecules were added to fill the water spaces in the elastin network.\textsuperscript{38}

Naturally, as AFM and TEM have different preceding sample preparations, the derived particle size will differ to some extent. Therefore, the cutoff in particle size distributions is different for both methods. It was chosen at values where a clear distinction between the populations was observed.

The divergence in the apparent cross-link efficiency derived from AFM and TEM images can also be explained by the differences in sample preparation. Imaging in fluid by AFM brings about a decreased concentration of micelles which might have a smaller tendency to adsorb to the hydrophobic surface as they have a hydrophilic particle shell. It has further been observed that particle densities on HOPG can fluctuate, and the particles appear to have variable tendencies to adsorb on HOPG and glow-discharged carbon layer. Nanoparticles and trimers differ largely in size and thus require a different stain layer thickness in TEM negative stain preparation. Small particles might also be present in other layers or hidden by other particles, impeding a definitive quantitative analysis.

### CONCLUSION

We present here a homogeneous solution of biopolymer-based, water-soluble, thermally switchable stable nanoparticles with well-defined size. The swelling and collapsing of the particles was analyzed by DLS measurements and supported by FCS measurements. Their size and shape were confirmed by AFM measurements in fluid and TEM negative stain imaging. Being based on elastin polypeptide sequences found in nature, these particles are likely to feature high biocompatibility and their subunits can be easily expressed and purified. These properties together with their reversible size transition when heated above their specific $T_c$ makes these particles extremely promising candidates as drug carriers. As the nanoparticles are cross-linked via incorporated PEG-linkers, this gives first indications of their permeability for loading molecules. The potential of loading these nanoparticles was specifically demonstrated with FITC-dextrans, and release studies will be the focus of future experiments.
REFERENCES