Micelles

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Self-Assembly of Thermally Responsive Amphiphilic Diblock Copolypeptides into Spherical Micellar Nanoparticles**

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As structure–property relationships for protein self-assembly have been elucidated, advances in chemistry and structural biology have facilitated the development of biologically inspired polypeptides through chemical and biosynthetic schemes that have afforded novel protein-based films, fibers, micelles, and gels.[1–3] In a number of instances, reversible protein self-assembly has been driven by well-defined conformational changes of peptide units induced in response to an external stimulus.[4,5] Indeed, designed molecular assembly of stimuli-responsive peptides has emerged as a "bottom-up" approach for creating complex, but ordered, hierarchical structures from simple amino acid building blocks.[6] As illustrated by the design of di- and triblock polypeptides, micro- and nanoscale features can be tuned by control of the amino acid sequence, molecular weight, and secondary structure of the peptide.[5,7,8] In particular, amphiphilic block copolypeptides can self-assemble into a variety of diverse structures, including rods, cylinders, spheres, and vesicles.[2,5] Although diblock copolymers consisting of chemically and conformationally distinctive individual polypeptide blocks have been produced by chemical and biosynthetic schemes, to date, relatively few recombinant amphiphilic diblock polypeptides have been synthesized.[2,5b,8] Given the capacity to incorporate targeting ligands, cell membrane fusion sequences, receptor activating peptides, fluorescent or chelating groups, as well as the ability to tailor pharmacokinetics, biodistribution, and peptide stability, significant opportunities exist for micelles or vesicles produced from recombinant protein block copolymers.

Elastin-mimetic polypeptides based on the pentameric repeat sequence (Val-Pro-Gly-Xaa-Gly) undergo thermal and pH-responsive self-assembly in aqueous solution.[8,9] Spontaneous phase separation of the polypeptide coincides with a conformational rearrangement of local secondary structure above a unique transition temperature (T_t) determined by the chemical identity of the fourth amino acid (Xaa) in the pentapeptide repeat. Recent studies have demonstrated the potential of engineered materials derived from elastin in a broad range of biomedical and biotechnological applications and, in particular, drug delivery.[5b,c,7,8]

Characteristically, elastin-mimetic blocks that contain hydrophobic amino acids in the fourth amino acid position, such as tyrosine, display a conformational transition from random coil to repetitive type II β turns at temperatures well below 37 °C, whereas blocks that contain a charged amino acid in this position, such as glutamic acid, persist as a random coil throughout the physiologic temperature range.[9d,10] Thus, we postulated that amphiphilic diblock copolymers bearing glutamic acid and tyrosine residues in N- and C-terminal blocks, respectively, would promote micelle formation by temperature-induced self-assembly with a core–shell structure. Moreover, we speculated that at a sufficiently high density of glutamic acid units, charge repulsion would limit the association of the hydrophilic blocks and minimize micelle aggregation. Micelles stabilized by self-assembly alone are typically unstable in a complex environment containing naturally occurring amphiphiles, such as plasma proteins, glycolipids, and lipopeptides. Therefore, by positioning cysteine residues between blocks, we hypothesized that high-molecular-weight protein aggregation or uncontrolled micelle–micelle association would be avoided by nanoparticle stabilization through disulfide cross-linking. These studies represent the first report of thermally responsive and cross-link stabilized protein micelles produced through the tailored design of recombinant amphiphilic diblock copolymers.

Two amphiphilic diblock polypeptides (ADP1 and ADP2) were synthesized and self-assembled into micellar structures with consecutive cysteine residues incorporated at the core–shell interface (Scheme 1). Expression of the diblock synthetic genes in *E. coli* expression strain, BL21(DE3), afforded recombinant protein polymers in high yield after immobilized-metal-affinity chromatography (IMAC) purification from the cell lysate. Mass spectrometry confirmed a correspondence between the observed and expected masses of the respective diblocks with consistent sequence composition by amino acid analysis. The presence of cysteine residues within the polypeptide chain was characterized by the use of a thiol-reactive fluorescent dye (see the Supporting Information).

Differential scanning calorimetry (DSC) demonstrated an endothermic transition at around 10 °C for both diblock copolymers, which conforms to the established relationship between the position of the transition temperature and the mole fraction of tyrosine in elastin-mimetic protein poly-

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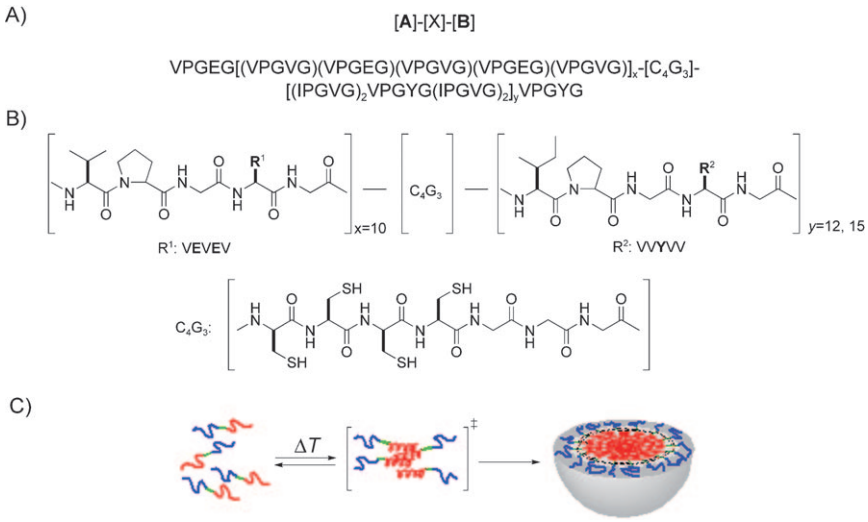

Scheme 1. A,B) Complete amino acid sequence of elastin diblock polypeptides (ADP1 ($x_{10}y_{12}$) and ADP2 ($x_{10}y_{15}$)) and chemical structure. C) Amphiphilic micelle formation of a thermally responsive elastin diblock polypeptide.

mers.[9] The endothermic transition is ascribed solely to the self-assembly of the hydrophobic blocks, which is similar to the lower critical solution temperature (LCST) observed for synthetic polymers such as poly(*N*-isopropylacrylamide),[11] because a model hydrophilic block, [(VPGVG)$_2$VPGEG-(VPGVG)$_2$], did not demonstrate a transition in the examined temperature range. Consistent with these observations, temperature-dependent circular dichroism (CD) spectroscopy revealed positive ellipticity at 210 nm above 10 °C, indicating induction of a type II β-turn conformation in the hydrophobic block. Similarly, negative ellipticity at 197 nm confirmed that the hydrophilic block persists as a random coil above the transition temperature of the hydrophobic block (Figure 1 A).

Temperature-dependent NMR spectroscopy was employed to examine the intensity changes of tyrosine and isoleucine peaks above the transition temperature. These residues were present only within the hydrophobic block sequence and, therefore, could be used as an indicator of its degree of mobility upon thermally responsive micellization above the phase transition temperature. Peaks at $\delta = 6.79$, 7.09, and 0.85 ppm, assigned to tyrosine and isoleucine residues, were observed in the ^{1}H NMR spectrum acquired at 5 °C (Figure 1 B), but decreased at 10 °C and disappeared at 25 °C. Proton peaks from the hydrophilic block were unchanged or decreased slightly over the same temperature range. Dynamic light scattering (DLS) was also used to monitor thermally driven micellization of ADP (Figure S5 in the Supporting Information). At low temperature (5 °C), the intensity of scattered light was very low, suggestive of the presence of only free chains in solution. As the transition temperature was approached, light scattering was suggestive of the formation of hydrated, loose micellar structures. Above 25 °C, well-defined micelles were clearly observed as evidenced by a significant increase in scattered light intensity and a dramatic decrease in the polydispersity index. It is apparent that these amphiphilic diblock polypeptides, present as single polypeptide chains at low temperature, self-assemble into core–shell micelles. The driving force for self-assembly is directly correlated to the conformational change of the polypeptide chain above the transition temperature.

Hydrophobic fluorescent molecules, such as 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) and Nile Red, fluoresce in a hydrophobic environment and have been used to investigate the capacity of amphiphilic block copolymers to encapsulate hydrophobic drugs, as well as the kinetics of drug release.[10b,12–14] Aqueous protein solutions of individual hydrophobic or hydrophilic blocks were examined by fluorescence spectroscopy in the presence of 1,8-ANS. Fluorescence intensity increased only for the hydrophobic block upon heating above its transition temperature (Figure S6 in the

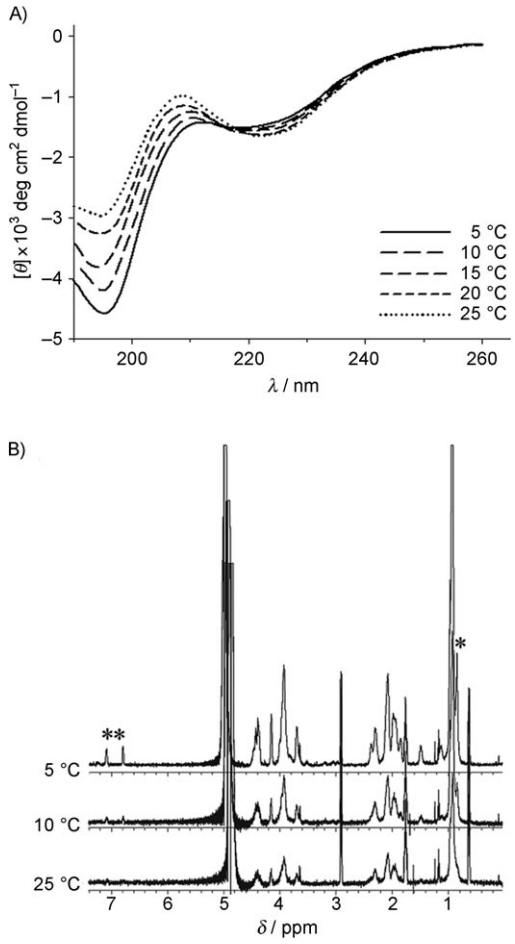

Figure 1. A) Temperature-dependent CD spectra displaying conformational changes for the amphiphilic diblock polypeptide, ADP2. B) Variable-temperature ^{1}H NMR spectra of ADP2 in H_2O/D_2O (70:30) (* = isoleucine methyl peak, ** = tyrosine aromatic ring peaks).

Supporting Information). For diblock polypeptides, the fluorescence intensity increased with the concentration of the protein polymer, which is consistent with the notion that an increased number of micelles in the solution leads to a larger reservoir for the hydrophobic fluorophore (Figure 2).

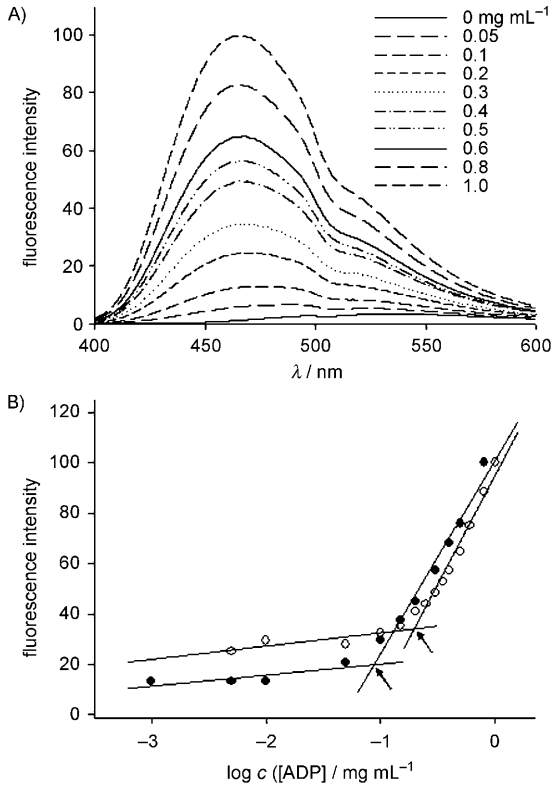

Figure 2. A) Fluorescence spectra of 1,8-ANS (80 μM) with various concentration of ADP2 in phosphate buffer saline at 25 °C. B) Fluorescence intensity of 1,8-ANS as a function of the logarithmic concentration of the diblock ADP1 (○) or ADP2 (●). Arrows indicate the critical micellar concentrations.

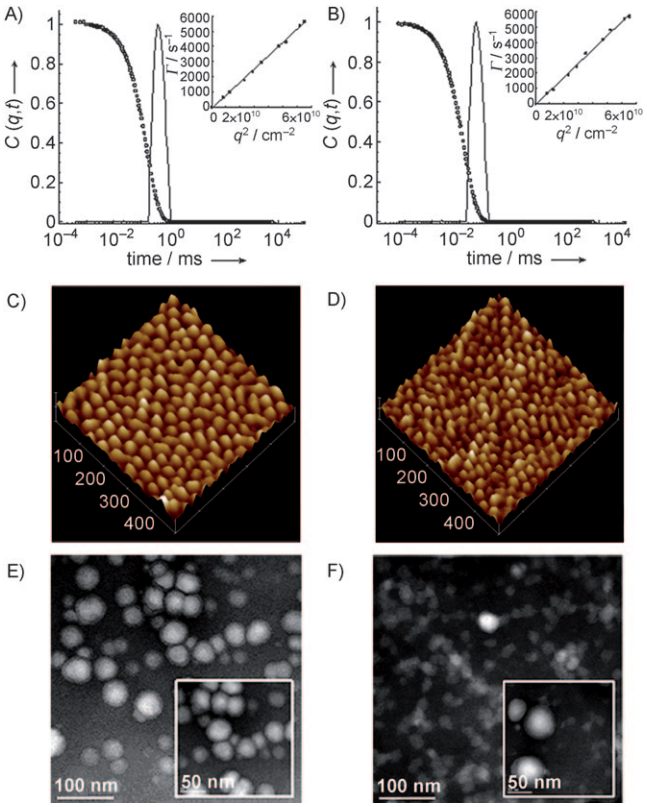

Figure 3. Dynamic light scattering autocorrelation functions of A) ADP1 (2.97 μM) and B) ADP2 (5.27 μM) micelles in water (25 °C) and their time–relaxation distribution at a 90° scattering angle. The inset shows the decay rate Γ dependency to the square scattering vector q^2. AFM images demonstrate a 2D array of micelles with micelle heights and the compact spacing for C) ADP1 and D) ADP2. TEM images of negatively stained micelles in dehydrated state for E) ADP1 and F) ADP2.

Indeed, critical micelle concentrations (CMC) for the ADP1 and ADP2 diblocks were 2.0 and 3.5 μM, respectively, which is in agreement with the formation of stable micellar structures.[15] These values are lower than the CMC values reported for other EMP diblocks.[5c] The incorporation of tyrosine residues into the C-terminal domain affords a more hydrophobic block relative to previously reported elastin-mimetic copolymers. The resulting substantial difference in block polarity may limit aggregation and result in a lower CMC value.

Dynamic light scattering (DLS) analysis demonstrated a single relaxation mode, which is typical of a monomodal size distribution (Figure 3 A,B). The linear variation of Γ versus q^2 passing through the origin is typical of a translational diffusive process of spherical objects. R_H values of 28 and 23 nm were determined with quite low polydispersity ($\sigma = 0.15$ and 0.17, respectively). The morphology of micelles prepared in aqueous solution at 25 °C was further characterized by atomic force microscopy (AFM) and transmission electron microscopy (TEM, Figure 3 C–F). AFM imaging attests to the formation of spherical nanoparticles of low polydispersity with dimensions in close agreement with DLS measurements. TEM imaging confirmed that the diblock polypeptides formed uniform spheres that ranged in diameter from 20 to 40 nm with electron transparent cores and gray-colored shells.

Between pH 3 and 9, only small changes of scattered light intensity were observed, whereas at pH 13 a significant decrease in light intensity was suggestive of micelle disruption. Indeed, at pH 10 or higher, both glutamic acid ($pK_a = 4.2$) and tyrosine ($pK_a = 10.0$) are deprotonated and negatively charged. Without a significant difference in polarity, the transition temperature T_t vanished.

As the hydrophobic blocks self-assemble, cysteine residues in neighboring polypeptide chains have the capacity to form disulfide bonds.[3,16] The proportion of thiol groups participating in disulfide bond formation after micelle formation was estimated using Ellman's reagent with fewer than 10 % of cysteine residues remaining in reduced form.[17] DLS was performed to elucidate whether hydrophobically self-assembled micelles were stabilized by intermolecular disulfide bond formation at low temperature or in the presence of dimethylsulfoxide (DMSO) or bovine serum albumin (BSA) (Table 1, and Figures S7 and S8 in the Supporting Informa-

Table 1: DLS size measurement of micelles under various conditions.

	ADP1		ADP2	
	R_H [nm]	PDI[a]	R_H [nm]	PDI[a]
5 °C	24.9	0.19	20.8	0.06
10 °C	24.2	0.04	25.7	0.02
25 °C	24.0	0.02	24.8	0.02
37 °C	23.8	0.03	24.6	0.01
BSA[b]	25.4	0.01	35.0	0.12
BSA + TCEP[c]	161.0	0.02	86.1	0.01
DMSO[d]	45.3	0.36	50.7	0.25
DMSO + TCEP	–		–	

[a] Polydispersity index; [b] Bovine serum albumin (3 mg mL^{-1}), 37 °C; [c] 1 mM TCEP; [d] 10% DMSO.

tion). Disulfide cross-linked micelles did not display a significant change in size or polydispersity over a temperature range between 5 and 37 °C, but were destabilized in the presence of a reducing agent (tris(2-carboxyethyl)phosphine, TCEP) at 5 °C. Likewise, albumin induced a significant increase in micelle size only after addition of TCEP, which presumably facilitated the intercalation of albumin with these protein particles. Although micelles also increased in size when incubated with 5% DMSO, they could not be detected by DLS analysis under similar conditions, but in the presence of TCEP.

In conclusion, we have demonstrated that recombinant amphiphilic diblock polypeptides based on elastin-mimetic sequences can form thermally responsive micellar nanoparticles in aqueous solutions that exhibit a spherical core–shell structure. Self-assembly is driven by a conformational transition of the hydrophobic block as confirmed by temperature-dependent ^{1}H NMR and CD spectroscopy. Amphiphilic micelles can undergo intermolecular covalent cross-linking through disulfide bond formation at the core–shell interface. Furthermore, hydrophobic fluorescent molecules can be easily encapsulated, demonstrating their potential to serve as a carrier for hydrophobic drugs.[5b,8c,12] Our results demonstrate the generation of a versatile new class of protein-based nanoparticles with significant potential for controlled drug delivery and bioimaging applications. In particular, the presence of interfacial disulfide bonds facilitates the design of micelles that would be stable under complex, physiologic conditions, but otherwise disassemble in response to a thiol-reducing microenvironment.[18]

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