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Protocols

Multi-scale characterization of thermoresponsive dendritic elastin-like peptides

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ABSTRACT

Elastin like peptides (ELPs)—polypeptides based on the protein elastin—are used widely as thermoresponsive components in biomaterials due to the presence of a sharp soluble-to-insoluble phase change at a characteristic transition temperature (T_t). While linear ELPs have been thoroughly studied, few investigations into branched ELPs have been carried out. Using lysine amino acids as branching and terminal units with 1–3 pentameric repeats between each branch, ELP dendrimers were prepared by solid-phase peptide synthesis with molecular weights as high as 14 kDa. A conformation change from random coil to β-turn upon heating through the T_t, typical of ELPs, was observed by circular dichroism spectroscopy for all peptides. The high molecular weights of these peptides enabled the use of characterization techniques typically reserved for polymers. Variable-temperature small-angle X-ray scattering measurements in dilute solution revealed an increase in size and fractal dimension upon heating, even well below the T_t. These results were corroborated by cryogenic transmission electron microscopy, which confirmed the presence of aggregates below the T_t, and micro differential scanning calorimetry, which showed a broad endothermic peak below the T_t. These results collectively indicate the presence of a pre-coacervation step in the phase transition of ELP dendrimers.

1. Introduction

Branching structures are widely adopted in nature across many length scales. Examples include neural processes, the mammalian vascular system, as well as tree branches, root systems, and leaf veins. Nature-inspired branching systems appear widely in human designs (e.g., art and architecture), analyses (e.g., factor trees, heat maps), and organization (e.g., family trees, company organizational structures). Chemists have also been inspired by branching structures, with highly branched dendritic polymers (dendrimers) being perhaps the most thoroughly studied example [1,2]. Characterized by their globular structure and high surface functionality imparted by exponential branching, dendrimers have attracted much research interest with goals of applying these unique polymers as catalysts [3,4], drug delivery vehicles [5,6], and nano-objects with precisely controlled structures [7–9].

Dendrimers with static branching structures, including Frechét-type dendrimers and polyamidoamine (PAMAM) dendrimers among many others, have been widely investigated [10–12]. In contrast, responsive dendrimers have been less thoroughly studied [13–15]. This is surprising given the large amount of research effort devoted toward responsive polymers, with stimuli including temperature, pH, light, and others [16,17]. Among these, thermoresponsive polymers have gained the most research attention, with potential applications primarily in drug/gene delivery [18–20], tissue engineering [21,22], bioseparation [23–25], and sensors [26–28]. Thus far, most reports on dendrimers with thermoresponsive components have focused on attaching peripheral thermoresponsive moieties to static dendrimer cores [29–33]. Dendrimers with thermoresponsive units built into the entire structure, which are synthetically more challenging, may allow the benefits of branching to be applied broadly in new smart materials. Therefore, it remains interesting and useful to understand how the 3D structures of thermoresponsive dendrimers transform in response to changes in the conformation of branching units throughout.

Elastin is a thermoresponsive extracellular matrix protein that maintains the structure and persistent elasticity of many parts of the human body, including skin, blood vessels, and connective tis-

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sues [34,35]. Elastin like peptides (ELPs) are biomacromolecules derived from the hydrophobic domains of elastin and are widely used as thermoresponsive units [36,37]. With the sequence Xaa-Pro-Gly-Xaa-Gly (XPGXG), ELPs exhibit lower critical solution temperature (LCST) behavior (X represents different amino acids, which can be changed to tune the transition temperature). The LCST behavior of linear and star-shaped ELPs has been thoroughly investigated, including factors such as hydrophobicity balance, salt/peptide concentration, chain length, etc. [38–40].

Evidence suggests that the phase transition in ELPs from soluble peptide to insoluble precipitate proceeds through a two-step process of a secondary structure transition from random coil to type II β-turn followed by coacervation [33,41]. Initial studies from our groups showed that while these two steps also govern the phase transition of branched/dendritic ELPs, the branching structure in the dendrimers slows down the coacervation process, leading to a higher transition temperature (T_t). Furthermore, the change in peptide secondary structure starts well below the T_t, and there are no significant differences in entropy/enthalpy changes for the secondary structure transition upon heating between the dendrimers and their linear counterparts.

Herein we discuss an extension of our previously developed synthetic methods to make ELP dendrimers with molecular weights approaching those of typical polymers, with some exceeding 14,000 Da. This enables the use of characterization techniques suitable for small peptides along with other techniques typically reserved for polymers and nanostructures to evaluate how changes in peptide secondary structure affect the 3-dimensional structure of the ELP dendrimers. Using a combination of turbidity studies, circular dichroism (CD), micro differential scanning calorimetry (micro-DSC), cryogenic transmission electron microscopy (cryo-TEM) and small-angle X-ray scattering (SAXS), we sought to gain insight into the process of coacervation and precipitation in large ELP-based dendrimers.

2. Results and discussion

2.1. Design and synthesis of ELP dendrimers

Scheme 1 shows the synthetic route used to produce the ELP dendrimers. Compared to our previous report [41], peptides with higher branching generation and MW were synthesized to enable characterization using previously inaccessible techniques. Using the divergent synthesis strategy, in which mass and branch number increase exponentially, the synthesis of these peptide dendrimers was less time consuming than their linear counterparts, with satisfactory yield maintained (Fig. S1). The notation "DGx-y" designates a peptide dendrimer of x^{th} generation with y amino acid residues between the branching units. For example, DG3-5 stands for the 3rd generation peptide dendrimer, with the pentameric repeating unit GLPGL between branching Lys resides (Fig. 1). We synthesized the peptides on a microwave-assisted synthesizer, and Fmoc-Lys(Mtt)-OH was used as the branching unit. After deprotection of the Fmoc group, the Mtt protecting group was removed using dilute trifluoroacetic acid (TFA) to reveal two amines. Each generation was grown through the repeated procedure of Fmoc-Lys(Mtt)-OH addition, removal of both protecting groups, and conventional solid-phase peptide synthesis (SPPS) to add $(GLPGL)_n$ repeats (n = 1–3) onto each free amine. We used the sequence GLPGL as the repeating pentamer because ELPs with Xaa = Leu have lower LCSTs compared with traditional Xaa = Val sequences, with good solubility retained [42]. The crude products were purified by preparative HPLC and lyophilized to afford dry powders for storage and analysis. Peptide purity was confirmed by LC–MS (Fig. S2) with purity >90% for all peptides except for DG3-10, which was too large to analyze by this technique. Prior to LC–MS analysis, ELP dendrimers were acetylated by addition of acetic anhydride in order to reduce the complexity of the chromatograms resulting from differing retention of peptides in different ionization states. Analysis of peptide DG3-10 was performed using gel electrophoresis because its molecular weight is close to that of a small protein, over 14 kDa (Fig. S3).

2.2. Characterization of the thermal properties of ELP dendrimers

2.2.1. Secondary structure

ELPs undergo a characteristic conformational transition from random coil to β-turn structure upon heating [39]. To verify this transition in the ELP dendrimers, circular dichroism (CD) spectra of all peptides were obtained at various temperatures. As expected, at temperatures below the LCST all profiles showed minima at 200 nm and maxima near 218 nm (Fig. 2A, Fig. S4) typical of a random-coil conformation [43]. Upon heating, the peak intensity decreased (Fig. 2B), and an isodichroic point was observed at 210 nm, indicating a transition from random-coil to β–turn [44]. For DG2-15 and DG3-10, the signal reached a plateau above 30 °C (Fig. 2B). This was likely not a result of a sudden halt to the conformational change, but rather due to the presence of aggregates, which disrupt the CD signal even at low concentrations [45].

2.2.2. Coacervation

The phase transitions of dendrimers in pure aqueous solutions were examined by turbidity measurements, but none of the solutions became turbid even after heating above 80 °C, indicating the T_t is beyond our detection limit. Kosmotropic salts, which are typically used to salt out proteins from water, lower the T_t of ELPs due to several effects. Kosmotropic anions polarize the water molecules involved in hydrogen bonding to the amide and thereby weaken the hydrogen bonding of the water to the ELP. The cost of hydrating hydrophobic regions of ELPs (i.e., Leu residues) increases with higher salt concentration as well [46,47]. In addition, salt screens the positive charges of the Lys side-chain amines at the periphery of the ELP dendrimers and thus weakens the charge-dipole interaction between ELP dendrimers and water molecules [47]. Because salts such as NaCl lower the T_t of ELPs [48], the turbidity experiments were repeated in phosphate buffered saline (PBS) at pH = 7.4 with various amounts of added NaCl. The phosphate concentration was kept constant (0.01 M) while the NaCl concentration was increased in 0.25 M intervals until turbidity was observed. Fig. 3 shows the dependence of T_t on NaCl concentration for all dendrimers.

In our previous report on branched ELPs, we calculated a T_t^0 value for each peptide, which was obtained by linear extrapolation of the T_t vs. [NaCl] graph to zero salt concentration. Here we observed that the trend deviated from linearity outside of a narrow range for each dendrimer, making T_t^0 calculations unreliable. Instead, we chose to compare the amount of salt required for each peptide dendrimer to have a T_t at 37 °C (Table 1), which we refer to as the critical salt concentration (CSC). In all cases, this was in the linear (or near-linear) range of the plot. Variable-temperature UV–vis spectroscopy measurements of DG2-10, DG2-15 and DG3-10 (Fig. S5) in pH = 7.4 solutions with salt concentration equal to the CSC show an upturn in the O.D at ~37 °C (Table 1), confirming our CSC results. The T_t of ELP dendrimers in salty solution was inversely correlated to the peptide concentration (Fig. S6), which is compatible with the general trend described in literature for linear ELPs [49]. It should also be noted that all turbid solutions became clear after cooling, indicating that the phase transition/coacervation is reversible.

We first aimed to determine whether any correlations existed between CSC and the molecular structure of the ELP dendrimers. Contrary to linear ELPs, in which molecular weight (MW) and pep-