Temperature-Sensitive Elastin-Mimetic Dendrimers: Effect of Peptide Length and Dendrimer Generation to Temperature Sensitivity

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ABSTRACT:

Dendrimers are synthetic macromolecules with unique structure, which are a potential scaffold for peptides. Elastin is one of the main components of extracellular matrix and a temperature-sensitive biomacromolecule. Previously, Val-Pro-Gly-Val-Gly peptides have been conjugated to a dendrimer for designing an elastin-mimetic dendrimer. In this study, various elastin-mimetic dendrimers using different length peptides and different dendrimer generations were synthesized to control the temperature dependency. The elastin-mimetic dendrimers formed β-turn structure by heating, which was similar to the elastin-like peptides. The elastin-mimetic dendrimers exhibited an inverse phase transition, largely depending on the peptide length and slightly depending on the dendrimer generation. The elastin-mimetic dendrimers formed aggregates after the phase transition. The endothermal peak was observed in elastin-mimetic dendrimers with long peptides, but not with short ones. The peptide length and the dendrimer generation are important factors to tune the temperature dependency on the elastin-mimetic dendrimer. © 2013 Wiley Periodicals, Inc. Biopolymers 101: 603–612, 2014.

Keywords: *elastin; dendrimer; temperature-sensitive; peptide; phase transition*

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INTRODUCTION

Elastin is one of the main components of extracellular matrices and has been used as a temperature-sensitive biomaterial. Elastin is composed of repeat sequences such as valine-proline-glycine-valine-glycine (Val-Pro-Gly-Val-Gly, VPGVG).[1–3] This kind of peptide is called an elastin-like peptide and is applied to elastin-mimetic materials. Previous reports showed that elastin-like polypeptides induced the temperature-dependent structure change from random coil into β-turn and/or β-spiral, leading to coacervation because of the hydrophobic interactions.[1–3] Most elastin materials have been extracted from animals or produced by using recombinant protein technology.[2,3] Since pathogens can become contaminated in these preparation methods, chemically synthesized elastin materials are desirable. However, it is very difficult to synthesize

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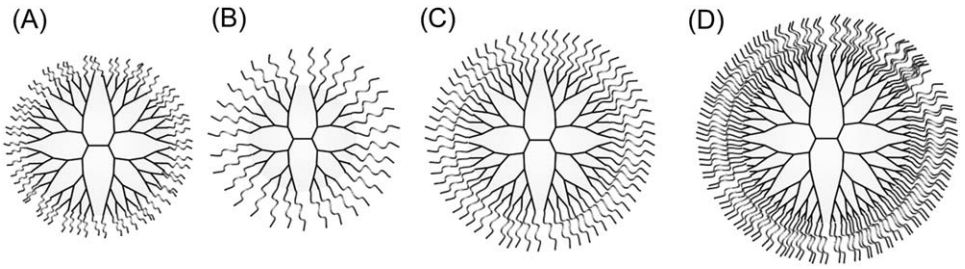

FIGURE 1 Elastin-mimetic dendrimers [ELP1-G4 (A), ELP2-G3 (B), ELP2-G4 (C), and ELP2-G5 (D)].

polypeptides chemically. Short elastin-like peptides, less than four repeats, do not exhibit the temperature-dependent properties.[4] Elastin-mimetic polypeptides are compounds that link elastin-like peptide in tandem, and the parallel-linked elastin-mimetic materials exhibit temperature-dependent properties. Elastin-like peptide-grafted synthetic polymers with one repeat exhibit the temperature-sensitive properties.[5–7] Since synthetic polymers generally have molecular weight distribution, uniform elastin-mimetics are difficult to produce using synthetic linear polymers. Gold nanoparticle was also used as a platform of elastin-like peptides to produce temperature-sensitive nanoparticles.[8] However, it seems difficult to control the binding number of the peptides on the nanoparticle. Consequently, the phase transition temperature may be difficultly controlled in this system.

Dendrimers have highly controllable size, topologies, and surface properties and are quite different from linear polymers.[9–12] Dendrimers can be used as a platform for peptides because they have multiple functional groups at the periphery. Multiple antigen peptides, called antigen peptide-conjugated dendrimers, have been reported.[12–14] Dendrimers conjugating collagen model peptides, such as $(Pro\text{-}Pro\text{-}Gly)_n$ and $(Pro\text{-}Hyp\text{-}Gly)_n$, formed collagen-like triple helical structures and temperature-dependent hydrogels for drug delivery.[15–18] One repeat elastin-like peptide (VPGVG) has been conjugated to the polyamidoamine (PAMAM) dendrimer and exhibited the temperature-dependent properties.[19] Koga et al reported that VPGVG and $(VPGVG)_4$ peptides have been conjugated to the PAMAM dendrimers to produce elastin-mimetics.[20] However, a systematic investigation of this kind of elastin-mimetic dendrimer remains to be examined. In this study, we investigate the effect of peptide lengths and dendrimer generations in the elastin-mimetic dendrimer to the temperature dependency. We synthesized various elastin-mimetic dendrimers with one and two repeats of elastin-like peptide [VPGVG (ELP1) and $(VPGVG)_2$ (ELP2)] and generation 3, 4, and 5 (G3, G4, and G5): ELP1-G4, ELP2-G3, ELP2-G4, and ELP2-G5 (Figure 1). The synthesized compounds were characterized by nuclear magnetic resonance (NMR), circular dichroism (CD) measurements, solution transmittance assay, dynamic light scattering (DLS), transmission electron microscopy (TEM) analysis, and differential scanning calorimetry (DSC) to elucidate the phase transition mechanism of the elastin-mimetic dendrimers and the influence of peptide lengths and dendrimer generations on the temperature dependency.

RESULTS AND DISCUSSION

Synthesis of Elastin-mimetic Dendrimers

We previously prepared ELP1-G4.[19] ELP2-conjugated dendrimers (ELP2-G3, ELP2-G4, and ELP2-G5) were synthesized as shown in Figure 2. The Boc-VPGVG peptide conjugation to the dendrimers (PAMAM G3-G5) and the following de-protection were performed repeatedly. Since amine-terminated elastin-mimetic dendrimers did not exhibit temperature-dependent properties under the physiological condition, the N-termini of the peptide-conjugated dendrimers were acetylated.[19] The synthesized compounds were characterized by ^1H NMR (Supporting Information Figure S1). The spectra of elastin-mimetic dendrimers reveal signals derived from the PAMAM dendrimer (2.5, 2.7, 2.9, and 3.3 ppm), the peptide unit (0.96, 1.9–2.3, and 3.7–4.4 ppm), and

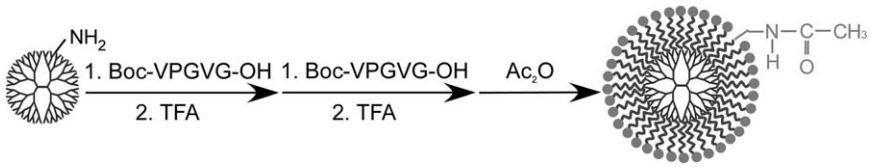

FIGURE 2 Synthetic pathway of elastin-mimetic dendrimers with two repeats of peptide. TFA and Ac_2O are trifluoroacetic acid and acetic anhydride, respectively.

Table I Elastin-mimetic Dendrimers in This Study

Sample	PAMAM Dendrimer (Generation/Terminal Number)	Peptide (Kind/Binding Number[a])	Molecular Weight[b] (Da, ca) (Peptide[c]/Dendrimer)	Transition Temperature[d] (°C)
ELP1-G4	G4/64	Ac-VPGVG/65	43112 (26207/14151)	48±0.9
ELP2-G3	G3/32	Ac-$(VPGVG)_2$/32	34461 (26207/6877)	39±3.7
ELP2-G4	G4/64	Ac-$(VPGVG)_2$/63	69320 (52415/14150)	38±4.8
ELP2-G5	G5/128	Ac-$(VPGVG)_2$/127	139035 (104829/28697)	30±3.3

[a] Estimated from NMR spectra.
[b] Given that target peptides and acetyl groups were fully modified.
[c] Acetyl group is not included.
[d] Average data estimated from temperature-dependent transmittance measurements using three different lots.

the acetyl unit (2.0 ppm). The integral ratio of the peptide signals to the PAMAM dendrimer indicates that virtually all terminal amino groups of dendrimer were modified with the peptide twice. From the integral ratios of the acetyl signal to the dendrimer signals, essentially all terminal groups were acetylated. HPLC analysis of ELP2-modified dendrimers was performed (Supporting Information Figure S2). Broad peaks were observed in chromatograms of ELP2-modified dendrimers, and there were no contaminations of free peptides. Therefore, we synthesized fully ELP-modified dendrimers. The obtained elastin-mimetic dendrimers in this study are listed in Table I.

Secondary Structure of Elastin-mimetic Dendrimers

CD spectrometry is useful for the estimation of protein secondary structure. Elastin-mimetics can form β-turn by heating, which is elucidated when the CD spectrum has a negative cotton effect around 218 nm.[4,19,21–23] The CD spectra of the elastin-mimetic dendrimers were measured, and the ELP peptides were used as a control. Since ELP1-G4 and the one repeat peptide were reported in our previous report, ELP2-conjugated dendrimers (ELP2-G3, ELP2-G4, and ELP2-G5) were analyzed in this study (Figure 3 and Supporting Information Figure S3). The elastin-mimetic dendrimers and the ELP2 exhibited the negative cotton effect that corresponds to β-turn structure (218 nm) and random coil (196 nm), similar to ELP1-G4 and the ELP1.[19] Since conformation of elastin-mimetics is changed from random coil to β-turn structure by heating, the CD spectra were measured at different temperatures from 5°C to 65°C. A strong negative cotton effect at 196 nm was observed at low temperature, corresponding to random coil structure. The negative cotton effect at 218 nm that corresponds to the β-turn structure increased at higher temperature in all elastin-mimetics. Since ELP2-conjugated dendrimers were turbid after heating above 45°C under our conditions, the data from 5°C to 35°C were obtained and compared. The molar ellipticities at 218 nm (β-turn) and 196 nm (random coil) were plotted against temperature (Figures 3C and 3D). The gradual increase of the 218 nm-cotton effect and the gradual decrease of the 196 nm-cotton effect were observed with increasing temperature, indicating that the random coil structure was gradually changed into the β-turn structure by heating. All of the elastin-mimetic dendrimers were similar to the elastin-like peptide, thus the elastin-mimetic dendrimers are an artificial elastin-mimetic material with temperature-dependent conformation change. The negative cotton effect of elastin-mimetic dendrimers at 218 nm was slightly lower than that of the corresponding peptides, indicating that conjugation of the ELPs to the dendrimers slightly interfered with the formation of β-turn structure. However, the dendrimer generation and the peptide length did not affect the negative cotton effect at 218 nm very much. This suggests that the peptide lengths as well as the dendrimer generations did not influence the formation of the β-turn structure. The negative cotton effect of elastin-mimetic materials at 196 nm was affected by peptide lengths, but not by the dendrimer conjugation and the dendrimer generation. It is likely that longer peptides can fix the conformation due to the multiple interactions, which promoted the random coil structure formation.

Phase Transition of Elastin-mimetic Dendrimers

The phase transition of aqueous solutions containing the elastin-mimetic dendrimers (1 mg/mL, pH 7.4, 150 m*M* NaCl) was examined. The solution containing elastin-mimetic dendrimers became turbid by heating, but the corresponding peptides did not. The change in the transmittance of the solution was monitored with increasing temperature. ELP1-G4 was examined in the previous report.[19] ELP2-conjugated dendrimers were analyzed in this study. Figure 4A shows the temperature-dependent transmittance of ELP2-G3, ELP2-G4, and ELP2-G5 in addition to ELP1-G4. The drastic transmittance change at a specific temperature, called a phase transition temperature, was observed in elastin-mimetic dendrimers. The

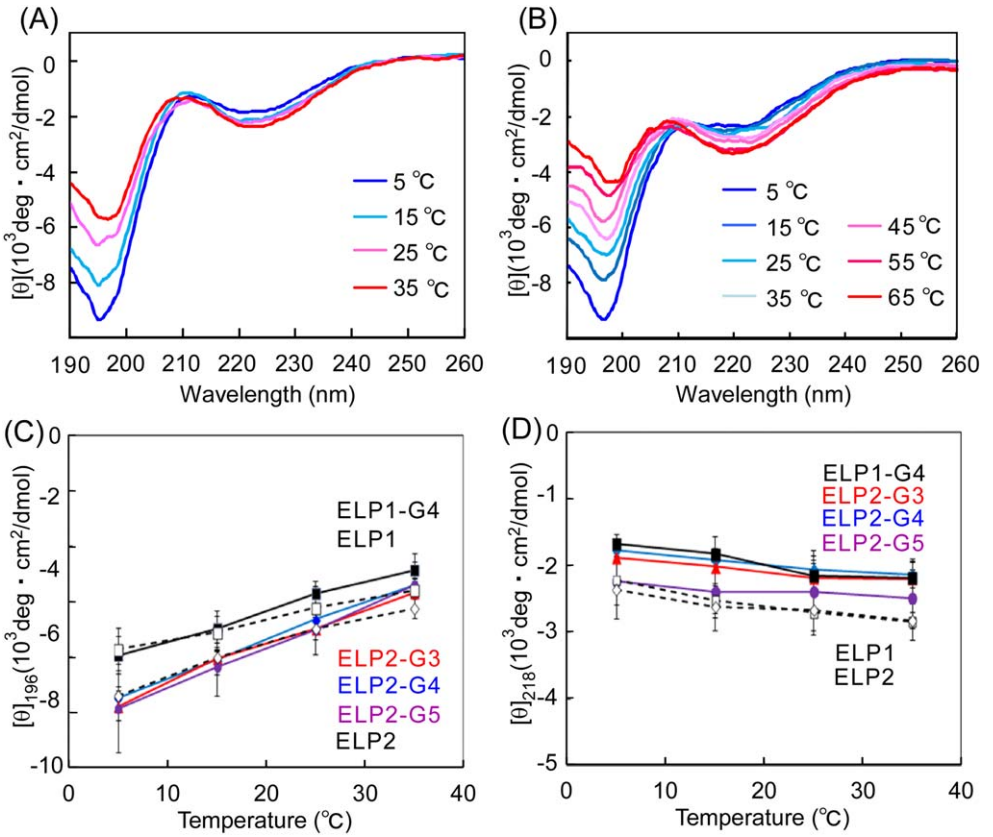

FIGURE 3 Temperature-dependent CD spectra of ELP2-G4 (A) and the ELP2 peptide (B). Correlation of the molar ellipticity that corresponding to random coil (196 nm, C) and β-turn (218 nm, D) to temperature. Data on ELP1-den and Ac-VPGVG-OH were referred from the reference.[19] (C,D) Open squares and diamonds are ELP1 and ELP2 peptides, respectively. Closed squares, closed triangles, closed diamonds, and closed circle are ELP1-G4, ELP2-G3, ELP2-G4, and ELP2-G5, respectively.

phase transition temperature of ELP2-G3, ELP2-G4, and ELP2-G5 were 37°C, 33°C, and 29°C, respectively, which was estimated from Figure 4A. For the experiment, three independent lots of each sample were used. The averaged phase transition temperatures are listed in Table I. Even though the phase transition temperature of ELP1-G4 was 48°C, ELP2-conjugated dendrimers became turbid at less than 40°C. This suggests that longer peptides promoted the phase transition. Compared with the dendrimer generation, the averaged phase transition temperatures of G3 and G4 were similar, but that of G5 was lower than other dendrimers. This suggests that the dendrimer generation partly affected the phase transition behavior. Interestingly, the phase transition temperature of ELP2-G3 was lower than ELP1-G4, even though the weight of peptide moieties of ELP1-G4 and ELP2-G3 were the same (Table I). This suggests that the peptide length in the elastin-mimetic dendrimer dominantly contributed to the decrease of the phase transition temperature. It was reported that elastin-mimetics indicated the salt-dependent thermosensitive property.[4,22,24] We examined the influence of the salt concentration on the thermosensitivity of the elastin-mimetic dendrimers. Figure 4B shows the correlation between salt concentration and the phase transition temperature. The linear relationship was observed in all elastin-mimetic dendrimers, whose slopes were as follows: −19 for ELP1-G4, −17 for ELP2-G3, −16 for ELP2-G4, and −15 for ELP2-G5. The linear decrease of phase transition temperature with increasing salt concentration is consistent with previous observations in other elastin-mimetics.[4,19,22,24] It is thought that salt might suppress the hydration on ELP, which is called the Hofmeister effect.[22,24] The slope of the elastin-mimetic dendrimer was similar to ELP polypeptides and less steep than for short ELP.[4,22,24] These suggest that the elastin-mimetic dendrimer has similar properties to elastin polypeptides but not to ELP. The peptide length and the dendrimer generation did not influence on the slope, indicating that the salt effect happened at a similar level in the elastin-mimetic dendrimers.

We investigated the influence of pH on the thermosensitivity of the elastin-mimetic dendrimer (Figure 4C), since ELP1-G4 largely responded to pH.[19] ELP2-conjugated dendrimers

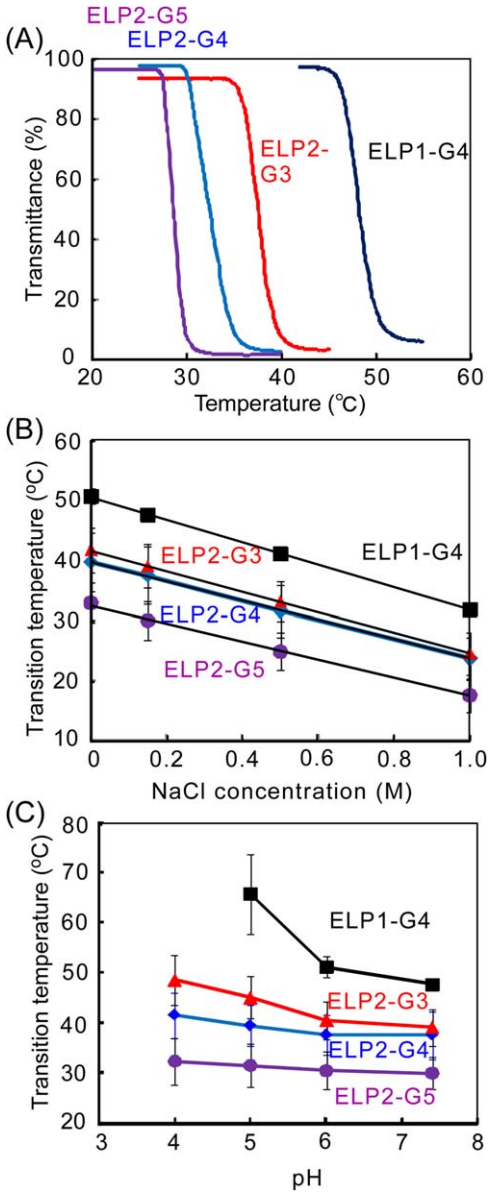

FIGURE 4 Phase transition of elastin-mimetic dendrimers. (A) Temperature-dependent transmittance for solutions of elastin-mimetic dendrimers (ELP1-G4, ELP2-G3, ELP2-G4, and ELP2-G5) in phosphate buffer (pH 7.4 containing NaCl 0.15*M*). (B) Correlation of the phase transition temperature to NaCl concentration (pH 7.4) in elastin-mimetic dendrimers. The results are shown as an average from three different lots of elastin-mimetic dendrimer. (C) Correlation of the phase transition temperature to pH (NaCl 0.15*M*) in elastin-mimetic dendrimers. The results are shown as an average from three different lots of elastin-mimetic dendrimer. The data on ELP1-G4 were referred from our previous report.[19]

were affected by pH. The higher phase transition temperature was observed at low pH. However, the pH sensitivity in ELP2-conjugated dendrimers was lower than the ELP1-conjugated dendrimer, and was attenuated with increasing generation. The pH sensitivity was caused by the protonation of inner tertiary amine in the dendrimer. Since the dendrimer content in the ELP2-conjugated dendrimer became less than in the ELP1-dendrimer, the pH sensitivity may become lower.

Self-assembly of Elastin-mimetic Dendrimer

As described above, the solutions of elastin-mimetic dendrimers became turbid. The size of elastin-mimetic particles at different temperatures was examined by DLS. The intensity-weighted size distribution and the number-weighted one of elastin-mimetic dendrimers (ELP1-G4 and ELP2-G4) at different temperatures are shown in Supporting Information Figures S4–S5. The intensity-weighted data contained multiple peaks at low temperature, indicating that there are several kinds of particles in the solutions. The diameter of the PAMAM dendrimer of G3, G4, and G5 are known to be 3.6 nm, 4.5 nm, and 5.4 nm, respectively.[10,25] Thus, small sized particles were a unimolecular state, and large ones were the assembly. Since the weighted intensity is calculated from the Rayleigh approximation ($I \propto d^6$, where I is the scattering intensity and d is the particle size), larger particles were dominantly observed in the intensity-weighted estimation. The number-weighted size distribution showed a single peak around 6.0 nm and 7.3 nm at low temperature for ELP1-G4 and ELP2-G4, respectively (Supporting Information Figures S4B and S5B). These suggest that small portions of elastin-mimetic dendrimers were assembled at low temperature.

The increase in the size of the elastin-mimetic dendrimers was also examined after heating. Portions of the large particles increased with increasing temperature in all samples. To understand the population change of these two types of particles, average diameters were estimated by cumulant analysis. The averaged diameters of all elastin-mimetic dendrimer were changed by increasing temperature (Figure 5). Interestingly, the averaged diameters of ELP2-conjugated dendrimers drastically increased above 35°C up to 150 nm (ELP2-G3), 680 nm (ELP2-G4), and 1010 nm (ELP2-G5). Since the phase transition temperatures of these dendrimers were 37°C (ELP2-G3), 33°C (ELP2-G4), and 29°C (ELP2-G5), these dendrimers formed the self-assembly around the phase transition temperature. Figure 4 shows that the transmittance in the ELP2-G3 starts decreasing at 35°C, even though the phase transition temperature of ELP2-G3 is 37°C. Thus, the size increase occurred at 35°C. However, the averaged diameter of ELP1-G4 gradually increased with increasing temperature. Even though ELP1-G4 became turbid around 48°C, the large particles were formed at relatively large amount below the phase transition temperature. Thus, the self-assembly of ELP1-G4 gradually occurred and was independent of the phase transition temperature. The averaged diameter of elastin-mimetic dendrimers

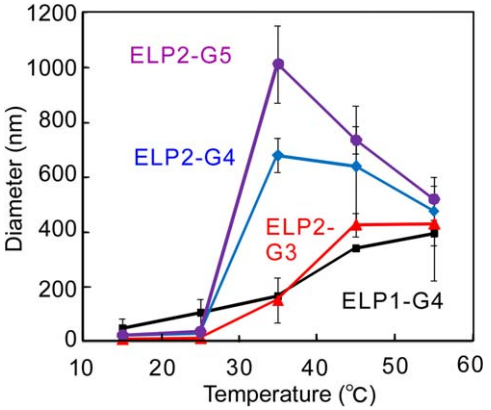

FIGURE 5 Average diameter of elastin-mimetic dendrimers at different temperatures. The same lots shown in Figure 4A were used in this analysis.

after the phase transition increased with increasing the peptide length and the dendrimer generation. This suggests that larger molecules formed larger aggregates after the phase transition. TEM analysis of ELP2-G4 was performed at room temperature

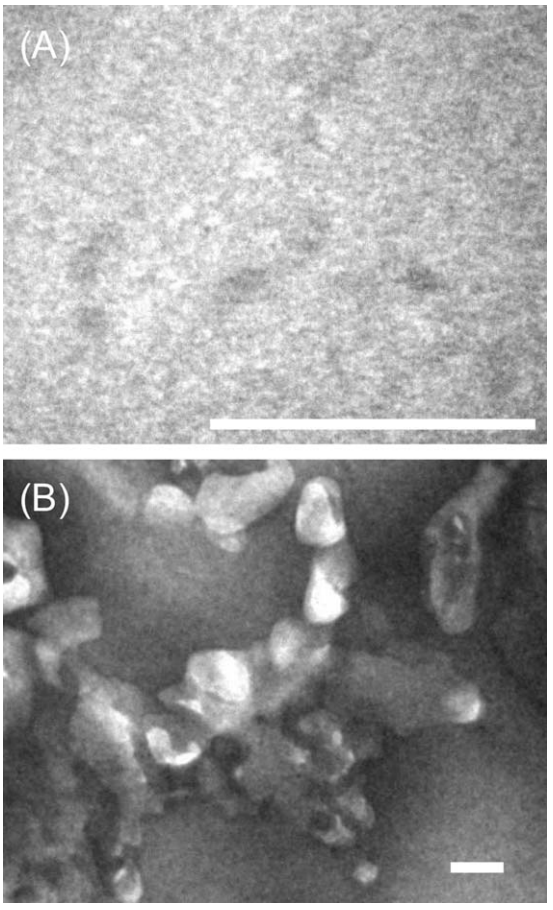

FIGURE 6 TEM images of ELP2-G4 at room temperature (A) and 50°C (B). Bar. 100 μm. The same lots shown in Figure 4A were used in this analysis.

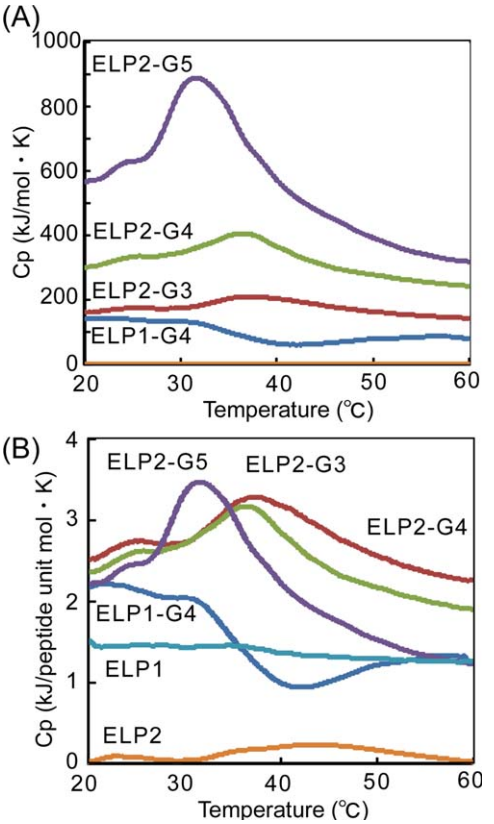

FIGURE 7 DSC analysis of elastin-mimetic dendrimers, which was normalized to dendrimer mole (A) and peptide unit mole (B). The same lots shown in Figure 4A were used in this analysis.

and 50°C (Figure 6). Spherical structures with around 10 nm in diameter were observed at room temperature, but any aggregates were not. On the other hand, large aggregates were observed at 50°C, consistent with our DLS data.

Thermal Process of Elastin-mimetic Dendrimers

Temperature-dependent polymers such as poly(N-isopropylacrylamide) (poly(NIPAM)) exhibited the endothermal peak around the phase transition temperature, at which the dehydration of bound water to the hydrophobic moiety of the polymer occurred.[26] It was reported that elastin-mimetics had an endothermal reaction corresponding to dehydration.[22,27] The thermal behavior of elastin-mimetic dendrimers was examined by DSC and compared with the previous elastin-mimetics. The mole heat capacity (C_p) to dendrimer and ELP unit in the elastin-mimetic dendrimers is shown in Figure 7. ELP2-G3, ELP-G4, and ELP-G5 exhibited endothermal peaks at 38°C, 36°C, and 32°C, respectively, which corresponds to the phase transition temperature. This observation was consistent with the previous elastin-mimetics.[22,27] The data, normalized to dendrimer mole, yield larger endothermal peaks on larger generation dendrimers (Figure 7A). The larger generation

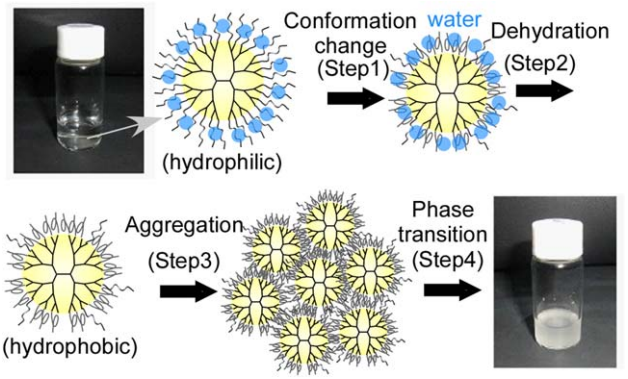

FIGURE 8 Possible mechanism of phase transition in elastin-mimetic dendrimers.

dendrimer contains large amounts of peptide, which is a cause of the large endothermal signal. However, normalized to peptide unit (VPGVG) mole, the endothermal peaks of all ELP2-conjugated dendrimers look similar, suggesting that the peptide induced the dehydration. However, ELP1-G4 did not show any significant peaks under our condition, suggesting that the dehydration did not occur around the phase transition temperature. It was reported that dendrimers conjugating NIPAM units at the termini did not show any significant endothermal signals.[26] It is likely that short peptide units at the surface of the dendrimer may provide only small amounts of bound water.

Current Model of Phase Transition Mechanisms in Elastin-mimetic Dendrimers

The possible mechanism on the temperature dependency of the elastin-mimetic dendrimers is shown in Figure 8. Our CD results indicated that the conformation of the ELP at the dendrimer surface was changed from random coil to β-turn by heating. Since it was reported that the β-turn structure induced the assembly,[2,3,21] the phase transition occurred by increasing temperature. In other words, the conformation change was a cue for the phase transition. Interestingly, the conformation change was gradual by heating, but the turbidity was changed drastically. This suggests that the phase transition occurred when the hydrophobicity of the dendrimer with β-turn structure was beyond the threshold. The size of the elastin-mimetic dendrimers with the long peptides aggregates increased drastically around the phase transition temperature. The phase transition corresponded to the dehydration of the ELP, except in the ELP1-conjugated dendrimer. These results indicate that the dehydrated ELP induced the self-assembly of the elastin-mimetic dendrimers. In summary, conformation of ELP at the termini of elastin-mimetic dendrimers changed from random coil to β-turn by heating (Step 1); the dehydration occurred above the threshold (Step 2), which led to the aggregation (Step 3); and consequently, the turbidity rapidly increased around the phase transition temperature (Step 4).

The effects of peptide length and dendrimer generation to the phase transition behavior are discussed. First, ELP1-G4 and ELP2-G4 are compared to understand the effects of peptide length. The step 1 conformation change was not significantly affected by the peptide length, but the following steps were largely affected. Our DSC experiments detected endothermal signals in ELP2-G4, but not in ELP1-G4, which suggests that the drastic dehydration occurred in ELP2-G4, but not in ELP1-G4 (step 2). This result may be because the amount of hydrated water is small in ELP1-G4 owing to the limited length of the ELP. Accordingly, the average diameter of the ELP1-G4 gradually increased with increasing temperature. Our DLS data suggest that ELP1-G4 assembled each other even at low temperature and the equilibrium was changed from unimolecular form to assembled one by heating (Step 3). When the assembled form reached the threshold, the turbidity changed drastically. The phase transition temperature significantly decreased with elongated peptide length (Step 4). The balance of the hydrophobicity is a key factor of the temperature-dependent polymers.[11,26] It was reported that molecular weight of elastin-mimetics affected the phase transition behaviors.[2,3] Thus, the decreased phase transition temperature came from the increased hydrophobic ratio and the increased molecular weight. The pH sensitivity was also different: ELP1-G4 was more sensitive to pH than ELP2-G4. The pH dependency came from the protonation of the inner dendrimer. The dendrimer moiety in ELP1-G4 is larger than that in ELP2-G4. Since the peptide components in the material increased by the longer peptide, ELP2-G4 did not affect the protonation in the dendrimer very much.

ELP2-G3, ELP2-G4, and ELP2-G5 are compared to understand the effects of dendrimer generation. The Step 1 conformation change was not affected by the dendrimer generation very much. The Step 2 dehydration was essentially unchanged, normalized to the peptide unit mole. These results indicate that the conformation change and dehydration were contributed to the ELP unit. The Step 3 self-assembly was affected by the dendrimer generation. The temperature to exhibit the drastic change in diameter decreased with increasing the dendrimer generation, which corresponds to the phase transition temperature. The diameter after the phase transition became larger in the elastin-mimetic dendrimer with larger dendrimer generation. Even though the hydrophobicity balance is unchanged in the different generation dendrimers, the molecular weights are increased. The large molecular weight induced the larger aggregates. The phase transition temperature decreased with enlarging the dendrimer generation (Step 4). Thus, the decreased

phase transition temperature came from the increased molecular weight. It was reported that the occupied surface areas per peptide at the dendrimer periphery (G3, G4, and G5) can be calculated as 1.3 nm^2, 1.0 nm^2 and 0.8 nm^2, respectively, given that the dendrimers are spherical.[28] Thus, the terminal crowding effect may occur at the dendrimer periphery to induce the cooperative behavior of the ELPs. However, the difference of the phase transition temperature is limited, and thus, the phase transition temperature may largely depend on the hydrophobicity balance.

MATERIALS AND METHODS

Materials

Amino-terminated ethylenediamine core PAMAM dendrimers of G3, G4, and G5 were purchased from Sigma-Aldrich (St. Louis, MO). *o*-(7-Benzotriazol-1-yl)–1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *o*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and Ac-VPGVG were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Triethylamine (TEA), trifluoroacetic acid (TFA), acetic anhydride, and celite (545RVS) were obtained from Nacalai Tesque (Kyoto, Japan).

Synthesis of Elastin-mimetic Dendrimers

Boc-VPGVG and ELP-G4 were produced according to our previous report.[19] Other elastin-mimetic dendrimers (ELP2-G3, ELP2-G4, and ELP2-G5) were synthesized as shown in Figure 2. Three lots of each elastin-mimetic dendrimer were synthesized. The typical synthetic methods are described below.

Synthesis of Boc-VPGVG-conjugated Dendrimers

Boc-VPGVG conjugation to dendrimer was performed according to our previous report.[19] Boc-VPGVG-conjugated G3 and G5 dendrimers were synthesized as follows. PAMAM dendrimer of G3 (2.4 μmol), Boc-VPGVG (0.098 mmol), and HOAt (0.061 mmol) were dissolved in dimethylsulfoxide (DMSO)/N,N-dimethylformamide (DMF)/chloroform at the ratio of 3/3/2 (344 μL). HATU (0.12 mmol) was then added under nitrogen. TEA (0.53 mmol) was added, followed by stirring at room temperature for 3 days. After the addition of deionized water to the mixture, it was purified using a Sephadex LH-20 column with methanol as the eluent. The final product was obtained by freeze-drying. Yield 81%. The same procedure was used for the PAMAM dendrimer of G5 (0.56 μmol), Boc-VPGVG (0.11 mmol), HOAt (0.068 mmol), HATU (0.13 mmol), and TEA (0.53 mmol) in DMSO/DMF/chloroform mixture (344 μL). Yield 63%.

Boc Removal From Boc-VPGVG-conjugated Dendrimers

Boc removal was performed according to our previous report, and VPGVG-conjugated G4 dendrimer was reported.[19] VPGVG-conjugated G3 and G5 dendrimers were synthesized as follows. Boc-VPGVG-dendrimers (G3 1.9 μmol; G5 0.35 μmol) were dissolved in TFA (3 mL) and incubated at 4°C for 2 h. The reaction mixture was evaporated after the addition of water, which was repeated four times. Yield ≈ 100%.

Synthesis of Boc-$(VPGVG)_2$-conjugated Dendrimers

Boc-VPGVG conjugation to VPGVG-dendrimer was performed according to the same procedure described in *Synthesis of Boc-VPGVG-conjugated dendrimers* except the quantities of the materials and the reaction times. For Boc-$(VPGVG)_2$-G3, VPGVG-G3 (2.0 μmol), Boc-VPGVG (0.078 mmol), HOAt (0.047 mmol), HATU (0.095 mmol), and TEA (0.38 mmol) in the DMSO/DMF/chloroform mixture (344 μL) were reacted for 5 days. Yield 77%. For Boc-$(VPGVG)_2$-G4, VPGVG-G4 (0.75 μmol), Boc-VPGVG (0.065 mmol), HOAt (0.047 mmol), HATU (0.094 mmol), and TEA (0.40 mmol) in the DMSO/DMF/chloroform mixture (344 μL) were reacted for 5 days. Yield 73%. For Boc-$(VPGVG)_2$-G5, VPGVG-G5 (0.34 μmol), Boc-VPGVG (0.067 mmol), HOAt (0.079 mmol), HATU (0.079 mmol), and TEA (0.32 mmol) in the DMSO/DMF/chloroform mixture (344 μL) were reacted for 6 days. Yield 97%.

Acetylation of Elastin-mimetic Dendrimers

Boc-$(VPGVG)_2$-dendrimers (G3 1.5 μmol; G4 0.51 μmol; G5 0.31 μmol) were dissolved in TFA (3 mL) and incubated at 4°C for 2 h. The reaction mixture was evaporated after the addition of water, which was repeated four times. Freeze-dried $(VPGVG)_2$-dendrimers were dissolved in acetic anhydride (5 mL) and stirred at 40°C for 2 h. The reaction mixture was evaporated. The mixture was dialyzed (pore: MW1000) in aqueous solution (pH 8) for a day. The final product was obtained by freeze-drying. Yield 74% for ELP2-G3, 92% for ELP2-G4, and 91% for ELP2-G5. ^{1}H NMR (400 MHz, D_2O): δ 0.96 (*m*, H_γ for Val), 1.92–2.31 (*m*, H_β, and H_γ for Pro and H_β for Val), 2.03 (*s*, Ac), 2.45 (br, —$NCH_2CH_2CONHCH_2CH_2$ for dendrimer), 2.69 (br, —$NCH_2CH_2CONHCH_2CH_2N$— for dendrimer), 2.87 (br, —$NCH_2CH_2CONHCH_2CH_2$ for dendrimer), 3.32 (br, —$NCH_2CH_2CONHCH_2CH_2$ and —$NCH_2CH_2CONHCH_2CH_2$ NHCO-peptide for dendrimer), 3.71–4.44 (*m*, H_δ for Pro, H_α for Val, H_α for Gly and H_α for Pro).

Synthesis of ELP2 (Ac-$(VPGVG)_2$)

Boc-VPGVG-OBzl was synthesized according to procedures in our previous report.[19] Boc-VPGVG-OBzl (1.65 mmol) was dissolved and incubated in TFA (3 mL) for 3 h at 4°C. The reaction mixture was evaporated. After the addition of water, the subsequent evaporation was performed, which was repeated four times. Yield (VPGVG-OBzl) ≈ 100%. ^{1}H NMR (400 MHz, D_2O): δ 0.88–1.12 (*m*, H_γ for Val), 1.97-2.34 (*m*, H_β, and H_γ for Pro and H_β for Val), 3.66- 4.19 (*m*, H_δ for Pro, H_α for Val and H_α for Gly), 4.51 (*m*, H_α for Pro), 5.22 (*s*, Bzl), 7.44 (*s*, phenyl). Boc-VPGVG (0.29 mmol) and VPGVG-OBzl (0.28 mmol) were dissolved in distilled acetonitrile (10 mL). HBTU (0.284 mmol) was added to the mixture and stirred for 4 days. After the addition of deionized water, the mixture was purified using a Sephadex LH-20 column with methanol as the eluent. The final product was obtained by freeze-drying. Yield 55%. ^{1}H NMR (400 MHz, DMSO): δ 0.86 (*m*, H_γ for Val), 1.36 (*s*, Boc), 1.83-2.08 (*m*, H_β, and H_γ for Pro and H_β for Val), 3.57-4.17 (*m*, H_δ for Pro, H_α for Val and H_α for Gly), 4.30 (*m*, H_α for Pro), 5.11 (*s*, Bzl), 7.34(*s*, phenyl).

Boc-$(VPGVG)_2$-OBzl (0.16 mmol) was dissolved in ethanol (5 mL). Pd-C (0.060 mmol) was added to the mixture and reduced with H_2 gas for 1 day. The mixture was filtered with celite (545RVS) and evaporated. The final product was obtained after vacuum drying. Yield 98%. ^{1}H NMR (400 MHz, DMSO): δ 0.86 (*m*, H_γ for Val), 1.37

(*s*, Boc), 1.82–2.08 (*m*, $H_{\beta,}$ and H_{γ} for Pro and H_{β} for Val), 3.57–4.16 (*m*, H_{δ} for Pro, H_{α} for Val and H_{α} for Gly), 4.30 (*m*, H_{α} for Pro).

Boc-$(VPGVG)_2$-OH (0.032 mmol) was dissolved and incubated in TFA (3 mL) for 2 h at 4°C. The reaction mixture was evaporated. After the addition of water, the subsequent evaporation was performed, which was repeated four times. The product was dissolved in 2 mL of deionized water to adjust to pH 9. NaOH (4*N*, 0.28 mL) solution and acetic anhydride (0.14 mL) were added in a drop-wise manner and stirred for 12 h at 4°C. The solution pH was adjusted to 7 and the mixture was purified using a Sephadex LH-20 column with methanol as the eluent. Yield 62%. ^{1}H NMR (400 MHz, D_2O): δ 0.92–1.19 (*m*, H_{γ} for Val), 1.98–2.34 (*m*, $H_{\beta,}$ and H_{γ} for Pro and H_{β} for Val), 2.04 (*s*, Ac), 3.72–4.23 (*m*, H_{δ} for Pro, H_{α} for Val and H_{α} for Gly), 4.41 (*m*, H_{α} for Pro). HPLC: Rt 16.6 min, purity 94%.

CHARACTERIZATION

The obtained products were characterized by ^{1}H-NMR (JEOL, 400 MHz).

The HPLC system was equipped with a Cosmosil 5C18-MS-II column (Nacalai Tesque) and a UV detector (220 nm; UV-2075Plus, Jasco, Tokyo, Japan). Samples (5 μL) were injected with an autosampler (AS-2057Plus, Jasco) and eluted with methanol/20 m*M* phosphate buffer = 10/90 at 1.0 mL min^{-1}. Acetonitrile was increased to 60% over 30 min.

DLS analysis was performed, as follows. Solutions for elastin-mimetic dendrimers (1 mg/mL, 10 m*M* phosphate buffer (pH 7.4) containing 0.15*M* NaCl) were prepared and filtered (0.45 μm), followed by DLS analysis using ELSZ-DN2 (Otsuka Electronics, Osaka, Japan). The temperature was adjusted to 15°C, 25°C, 35°C, 45°C, and 55°C. After 15 min incubation at each temperature, the data were measured three times.

The same solutions were used for the DSC. DSC measurements over the range 20–60°C were carried out with a Nano-DSC 2 (Calorimetry Science Corporation (CSC), Salt lake City, UT). The heating rate was 1°C/min.

TEM analysis was performed, as follows. The solution for ELP2-G4 (1 mg/mL, 10 m*M* phosphate buffer (pH 7.4) containing 0.15*M* NaCl) was prepared and incubated at 4°C and 50°C. The sample solutions were dropped on carbon-coated copper grids at room temperature and 50°C, negatively staining with phosphotungstic acid TEM images were obtained using JEM-2000FMX (JEOL, Tokyo, Japan).

CD MEASUREMENTS

CD spectra were measured by using a J-820 spectropolarimeter (Jacso) from 5°C to 65°C. Before the measurements, the sample solutions [0.05 mg/mL, 10 m*M* phosphate buffer (pH = 7.4)] were incubated at each temperature for 10 min. The CD spectra were obtained using a 0.1 cm path length cell, by signal integrating 10 scans from 190 to 260 nm at a scan speed of 50 nm/min. Data were processed by the simple moving average method. The molar ellipticity at 196 nm and 218 nm was estimated from the spectra, and the averaged data of three different lots of dendrimer were reported.

MEASUREMENT OF PHASE TRANSITION

Various solutions for elastin-mimetic dendrimers [1 mg/mL, 10 m*M* buffer (pH 4, 5, 6, and 7.4) containing 0–1*M* NaCl] were prepared by using 100 m*M* phosphate buffer (pH 7.4) or citrate buffer (pH 4, 5, and 6), 4*M* NaCl solution, and 10 mg/mL dendrimer solution. The turbidity was measured at 500 nm using a Jasco Model V-630 spectrophotometer equipped with a Peltier-type thermostatic cell holder coupled with an ETC-717 controller. The heating rate of the sample cell was maintained at 1.0°C min^{-1}. The phase transition temperature was taken as temperature at a transmittance of 50%. The averaged phase transition temperature of three different lots of dendrimer was reported.

CONCLUSION

We synthesized and characterized the fully ELPs-conjugated dendrimers with different peptide lengths and dendrimer generations. The conformation of elastin-mimetic dendrimers changed from random coil into β-turn by heating, essentially independent of the dendrimer structure. Elastin-mimetic dendrimers underwent a temperature-dependent phase transition, which was affected by salt concentration and pH. The phase transition temperature was largely influenced by the peptide length and slightly influenced by the dendrimer generation. The diameter of the elastin-mimetic dendrimer enlarged around the phase transition temperature, at which the dehydration occurred in long peptide-conjugated dendrimers. The enlarged diameter and the endothermal peak normalized to dendrimer mole became greater with increasing peptide length and dendrimer generation. Thus, the peptide length and dendrimer generation are important factors to tune the temperature dependency on the elastin-mimetic dendrimers. Since the ELP2-conjugated dendrimers exhibited the phase transition around the body temperature, they are potent temperature-dependent biomaterials.[11,19]

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