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Effect of Main Chain Conformation to Thermosensitivity in Elastin-Like Peptide-Grafted Polylysine

Chie Kojima[1]*, Ugir Hossain Sk[2$], Daichi Fukushima[1], Kotaro Irie[2], Naotoshi Akazawa[3], Masafumi Umeda[3], Takuro Niidome[3]

[1]Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan.

[2]Nanoscience and Nanotechnology Research Center, Research Organization for the 21st Century, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan.

[3]Department of Applied Chemistry and Biochemistry, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto 860-8555, Japan.

*To whom correspondence should be addressed. Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan

Tel: +81 72 254 8190; Fax: +81 72 254 8190; E-mail: kojima@chem.osakafu-u.ac.jp

[$]Present address: Natural Products Chemistry and Process Development Division, Institute of Himalayan Bioresource Technology, Palampur, H.P. 176 061, India

ABSTRACT

Stimuli-responsive polypeptides can be used in a wide variety of biomedical applications because of the biocompatibility. Elastin is a thermosensitive protein which contains unique repeat sequence such as VPGVG. Although short elastin-like peptides (ELPs) do not exhibit the temperature-dependent phase transition, grafting of ELP to a polymer scaffold provided the temperature-dependent properties. In this study, ELPs were conjugated to linear poly-L-lysine (PLL) and polylysine dendrimer (PLD) for the preparation of synthetic elastin-mimetic polypeptides. Polyallylamine and polyamidoamine dendrimers were also used as scaffolds of elastin-mimetic polymers and compared their thermosensitivity. ELP-grafted PLL exhibited a lower phase transition temperature than ELP-grafted PLD, even though the molecular weight of ELP-grafted PLL was smaller. The conformations of the elastin-mimetic polymers changed from random coil to β-turn structures when they were heated. However, ELP-grafted PLL formed an α-helix when it was heated, and this effect was dependent on the pH. These results suggest that conformation changes of the main chains on the polypeptide contributed to their phase transition temperatures.

Keywords: peptide; stimuli-sensitive polymer; thermosensitive; elastin; dendrimer

Introduction

Stimuli-responsive polymers have attracted considerable attentions from researchers working in a variety of different fields because of their broad range of potential applications. These polymers can respond to various stimuli, including temperature, pH, light and presence of specific ions.[1-5] Thermosensitive polymers have been used in a wide range of biomedical applications such as biosensors, bioseparation processes, drug delivery systems, gene delivery systems and tissue engineering platforms because variations in the temperature can be easily controlled by using a local heating system such as clinically approved hyperthermia.[1-8] Poly(N-isopropylacrylamide) (PNIPAM) is a representative temperature-sensitive polymer. This polymer exhibits a phase transition temperature of around 32 °C, at which point its solubility decreases significantly.[5-8] Polypeptides are more useful than synthetic polymers in biomedical applications because they are biodegradable and biocompatible. Short peptides can be precisely synthesized by solid phase peptide synthesis. Polypeptides have been synthesized by ring opening polymerization of amino acid N-carboxyanhydrides (NCA), although control of the molecular weight and the sequence is difficult. Many kinds of stimuli-responsive synthetic polypeptides have been studied by incorporating the stimuli-responsive moieties into polypeptides.[9-11]

Elastin is a temperature-sensitive protein and one of the major components of the extracellular matrix (ECM). Elastin is composed of several repeat sequences of amino acids, such as valine-proline-glycine-valine-glycine (VPGVG). Peptides containing the sequences are known as elastin-like peptides (ELPs) and have been used to produce elastin-mimetic materials.[12-15] Most elastin-mimetic materials have been prepared by using recombinant protein technology.[12-15] There are some reports on chemically synthesized elastin-mimetic materials such as ELP-conjugated polymers and ELP-modified nanoparticles, which exhibited the temperature-dependent phase transition properties.[16-23] However, these elastin-

mimetic polymers contain unnatural moieties or phages, which probably lose the biocompatibility.

In this study, we selected polylysine as a scaffold for the preparation of elastin-mimetic materials. Polylysine is one of well-studied synthetic polypeptides for drug delivery and gene delivery.[9,10] The amino groups in the lysine side chains can be used for the conjugation of ELPs. Given that lysine can be used as a building block for the construction of dendrimers,[24] it is possible that both dendritic and linear polylysine compounds could be used as backbones for the preparation of elastin-mimetic polypeptides. With this in mind, we synthesized ELP-grafted linear poly-L-lysine (PLL) and polylysine dendrimer (PLD), and compared the thermosensitivity properties of these two materials. Polyallylamine (PAA) and polyamidoamine (PAMAM) dendrimers were used as controls (Figure 1). The synthesized compounds were characterized by proton nuclear magnetic resonance (^{1}H NMR), circular dichroism (CD) and temperature-dependent transmittance measurements. Uncommon thermosensitive properties of linear polylysine (PLL)-based elastin mimetic polymer were observed.

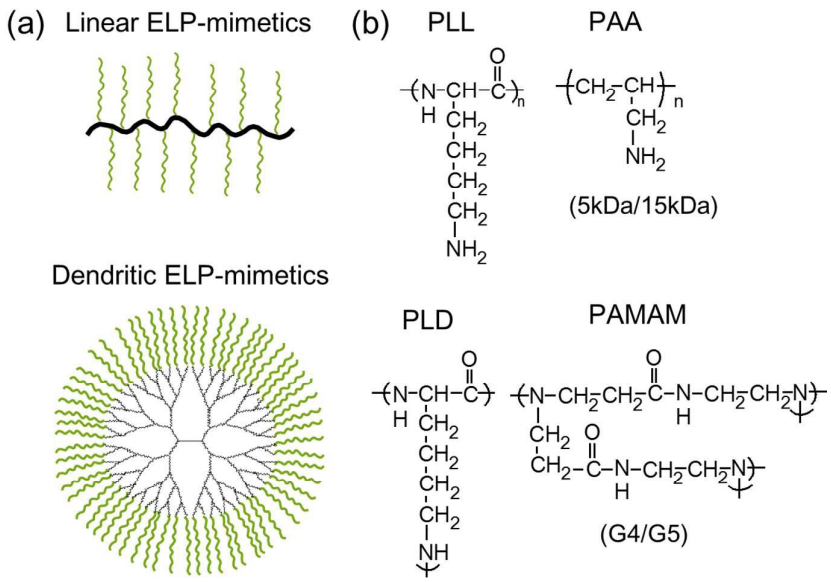

Figure 1. (a) Structures of the ELP-grafted linear and dendritic polymers. (b) Chemical structures of PLL, PLD, PAA and PAMAM dendrimer.

Experimental

Materials

PAAs hydrochloride salt with 5 kDa and 15 kDa were kindly provided from Nittobo (Tokyo, Japan), and PLL with degree of polymerization of 50 was purchased from Alamanda Polymers (Huntsville, AL). o-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) were obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). DMT-MM (4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride) was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). Boc-VPGVG-OH, Boc-protected polylysine dendrimer (PLD), ELP-grafted PAMAM dendrimers were prepared according to our previous papers.[21,22,24]

Synthesis of ELP-grafted polymers

ELP-grafted polylysine dendrimer (ELP-PLD)

The first step in this process was the Boc deprotection of the polylysine dendrimer (PLD). Twenty-six milligrams (0.9 μmol) of the Boc-protected PLD of G6 was treated with 10 ml of trifluoroacetic acid (TFA) at 4 °C, and the resulting mixture was agitated for 2 h. The reaction mixture was then evaporated to dryness to give a residue, which was co-evaporated from water four times. The desired amino-terminated PLD was obtained following the lyophilization of the resulting residue. The amino-terminated PLD (28 mg, 0.9 μmol), Boc-VPGVG-OH (93 mg, 0.18 mmol) and HOAt (15 mg, 0.11 mmol) were dissolved in a 3:3:2 (v/v/v) mixture of DMSO, DMF and $CHCl_3$ (493 μL), and the resulting mixture was treated with HATU (79 mg, 0.21 mmol) and trimethylamine (TEA) (121 μL, 0.0867 mmol) before being stirred under an atmosphere of nitrogen for 3 days. The mixture was then quenched by the addition of 1.5 mL of water, and the crude product was purified using a Sephadex LH-20 column with methanol as the eluent. The purified material was lyophilized to give Boc-ELP-PLD in its pure form. The deprotection of the Boc group was performed by using TFA to obtain ELP-PLD, as described above. Yield 47 mg (62%).

The acetylation was optionally performed using acetic anhydride. Thirty-three milligrams (0.4 μmol) of ELP-PLD was dispersed in 5 ml of acetic anhydride, and the resulting mixture was stirred at 40 °C for 2 h. The reaction mixture was then cooled to ambient temperature and evaporated to dryness to give a residue, which was basified to pH 8.5 by using an aqueous NaOH solution. The mixture was then purified by dialysis (COMW 1 kDa) to obtain Ac-ELP-PLD. Yield 28 mg (98%).

ELP-grafted poly-L-lysine (ELP-PLL)

Boc-VPGVG-OH (176 mg, 0.33 mmol) and DMT-MM (185 mg, 0.668 mmol) were dissolved in acetonitrile (5 mL), and the resulting solution was stirred for 1 h. A solution of

PLL (50 mg, 6 μmol) and TEA (200 μL, 1.4 mmol) in water (1.0 mL) was then added, and the resulting mixture was stirred for 5 days under an atmosphere of nitrogen. The crude product was purified by dialysis (COMW 2 kDa) in DMSO and water before being lyophilized to obtain Boc-ELP-PLL. The subsequent deprotection of the Boc groups was performed according to the method described above to give the amino-terminal ELP-PLL. Yield 150 mg (93%). The amino-terminal ELP-PLL was then acetylated optionally according to the procedure described above to obtain Ac-ELP-PLL. Yield 100 mg (62%).

ELP-grafted polyallylamines (ELP-PAAs)

Boc-VPGVG-OH (289 mg, 0.55 mmol) and DMT-MM (304 mg, 1.1 mmol) were dissolved in acetonitrile (5 mL) and the resulting solution was stirred for 1 h. 1 mL of aqueous solution containing PAA of 5 kDa (50 mg, 0.01 mmol) and TEA (200μL, 1.4 mmol) was then added, and the reaction mixture was stirred for 5 days under an atmosphere of nitrogen. The reaction mixture was purified by dialysis (COMW 1 kDa) before being lyophilized. The subsequent deprotection of the Boc groups was performed according to the method described above. Yield 320 mg (~100%). The amino-terminal ELP-PLL was then acetylated optionally according to the procedure described above to obtain Ac-ELP-PAA-5k. The ultrafiltration was performed with the mixed solvent of MeOH/water at the equal volume for the purification. Yield 160 mg (53%). The same method was performed by using PAA of 15 kDa to prepare ELP-PAA-15k and Ac-ELP-PAA-15k.

Characterization

The synthesized polymers were characterized by ^{1}H-NMR (JEOL, 400 MHz). The High performance liquid chromatography (HPLC) system was equipped with a Cosmosil 5C18-MS-II column (Nacalai Tesque Inc., Kyoto, Japan) and a UV detector (220 nm; UV-

2075Plus, Jasco Inc., Tokyo, Japan). Samples (5 μl) were injected with an autosampler (AS-2057Plus, Jasco Inc., Tokyo, Japan) and eluted with methanol/2% phosphoric acid = 10/90 at 1.0 ml·min^{-1}. Methanol was increased to 70% over 30 min. Log P values were estimated by Crippen's fragmentation using ChemBioDraw Ultra 13.0.[25]

CD Measurement

CD spectra were measured by using a J-820 spectropolarimeter (JASCO, Japan) from 5 ℃ to 65 ℃. Before the measurements, the sample solutions (0.05 mg/ml, distilled water and 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.

Measurement of phase transition

Various solutions for elastin-mimetic polymers (1 mg/ml, 10 mM carbonate and phosphate buffer (pH 10 and 7.4) containing 0-2 M NaCl were prepared. 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 ℃ min^{-1}. The turbidity of the CD sample solutions of Ac-ELP-PLD and Ac-ELP-PLL (0.05 mg/ml) was measured by the same procedure.

Results and Discussion

Synthesis of ELP-grafted polymers

The synthetic routes of the ELP-grafted polymers are shown in the Figure 2. Boc-protected VPGVG was conjugated to the amino groups of PLD and PLL using a suitable condensation agent (e.g., HATU or DMT-MM), followed by the removal of the Boc groups using TFA. Optionally, the resulting amino groups were subsequently acetylated with acetic anhydride (Ac_2O). The ELP-PLD compounds were characterized by ^{1}H NMR (Figure 3). The ^{1}H NMR data revealed that the methylene protons adjacent to the primary amine (2.8 ppm) of the starting materials had shifted downfield to 3.0 ppm, which indicated that the primary amino group had been converted to an amide bond. Furthermore, the absence of a signal around 2.8 ppm suggested that all of the amino groups had reacted with Boc-VPGVG. The reactivity of the peptide conjugation was estimated from the integral ratios between Hγ of valine in ELP and the PLL. The ^{1}H NMR spectra of the material following the sequential Boc deprotection and acetylation steps confirmed the disappearance of the signals belonging to the Boc group (1.3 ppm) and appearance of a signal corresponding to an acetyl (Ac) group (~2.0 ppm). The reactivity of the acetylation was estimated from the integral ratio of Ac group. Similar NMR spectra were observed for the ELP-grafted PLL compounds (Figure S1). We previously reported the synthesis of the ELP-grafted PAMAM dendrimers of G4 and G5. ELP-grafted PAA polymers with different molecular weights were synthesized and characterized by ^{1}H NMR (Figure S2). The NMR spectra did not show the main chain signals of PAA (1-2 ppm), because the polymer main chain was not solvated.[26] Thus, the bound number of ELP could not be determined from the NMR data. However, the shift of a signal from 2.8 ppm to 3.0 ppm suggested that the primary amino groups had converted into the amide groups, suggesting that essentially all amino groups were reacted with the ELP. A list of the elastin-mimetic compounds prepared in the current study is shown in Table 1.

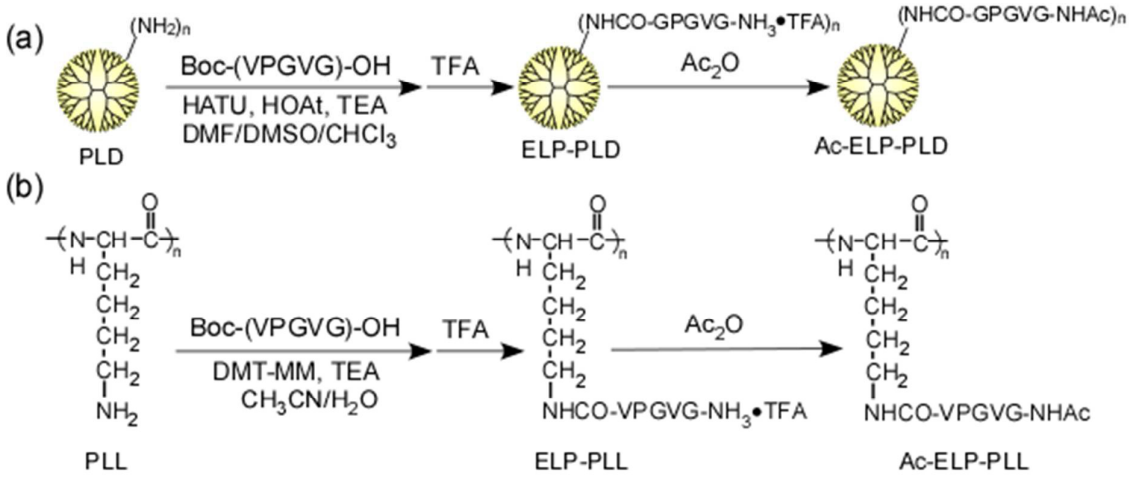

Figure 2. Synthesis of ELP-grafted polymers using PLD (a) and PLL (b).

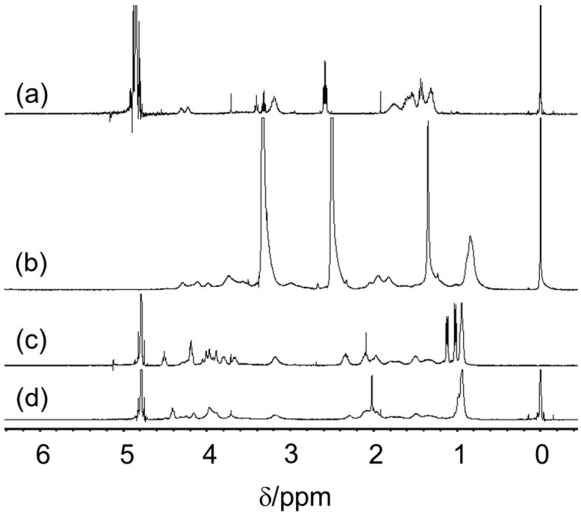

Figure 3. ^{1}H NMR spectra of (A) PLD, (B) Boc-ELP-PLD, (C) ELP-PLD and (D) Ac-ELP-PLD. These spectra were obtained in D_2O, expect for the spectrum of Boc-ELP-PLD which was obtained in DMSO-d6.

Table 1. Summary of elastin-mimetic polymers synthesized in this study

Compound	#amino group	Reactivity (%)		Molecular weight (kDa)	Phase transition temperature in pH 7.4/ pH 10*
		Peptide conjugation	Acetylation		
Ac-ELP-PLL	50	96	82	29	10°C / 80°C
Ac-ELP-PLD	128	100	102	76	34°C / >90°C
Ac-ELP-PAA-5k	53	ND	80	30	14°C / 75°C
Ac-ELP-PAA-15k	160	ND	NA	90	NA / 65°C
Ac-ELP-PAMAM-G4	64	99	100	43	48°C / NA
Ac-ELP-PAMAM-G5	128	101	101	69	45°C / NA

*The non-acetylated polymers were measured at pH 10. NA: not applicable. ND: not determined.

Phase transition temperature of ELP-grafted polymers

The elastin-grafted polymers prepared in the current study showed phase transition properties, which were analyzed using solution turbidity measurements. Figure 4 shows the changes in the transmittance of the synthesized elastin-mimetic polymers (acetylated and non-acetylated polymers) as they were heated. The phase transition temperature was taken as the temperature at which the solution gave a transmittance value of 50%, and the results are listed in Table 1. The phase transition temperatures of the acetylated elastin-mimetic materials were measured at physiological pH (i.e., pH = 7.4). The phase transition temperature of Ac-ELP-PLD was lower than that of Ac-ELP-PAMAM. The phase transition temperatures of the linear PLL- and PAA-based elastin-mimetic polymers were much lower than those of the dendritic PLD- and PAMAM-based polymers, and found to be less than room temperature. Thus, the phase transition temperatures of the amino-terminal elastin-mimetic polymers were also examined at pH 10. ELP-PLL and ELP-PAA (5 and 15 kDa) showed phase transition temperatures of 80°C, 75 °C and 65 °C, respectively, while ELP-PLD did not exhibit the phase transition behavior under our condition. ELP-PLL showed a

higher phase transition temperature than ELP-PAA. And, the phase transition temperature of ELP-PLL was higher than that of Ac-ELP-PLL. It is well known that the phase transition of a polymer can be influenced by the balance between its hydrophobicity and hydrophilicity. Hydrophobic thermosensitive polymers have been reported to exhibit lower phase transition temperatures than hydrophilic thermosensitive polymers of similar molecular weight.[15,27] Octanol-water partition coefficients, that is log P values, are widely used to determine the molecular hydrophobicity.[25] Log P values of the polymer unit and acetylated and nonacetylated ELP were estimated by using ChemBioDraw software, and listed in Table 2. The log P values of PAMAM, PLD and PAA increased, suggesting that the hydrophobicity increased. The phase transition temperature of ELP-grafted PAMAM, PLD and PAA decreased in this order, which was correlated to the hydrophobicity. Because the log P values of acetylated and nonacetylated ELP were similar, these peptides were analyzed by reversed phase liquid chromatography (Figure S3). In this analysis, the retention time is affected by the hydrophobicity: Hydrophobic compounds are eluted later. The retention time of ELP and Ac-ELP were 10 min and 17 min, suggesting that Ac-ELP were more hydrophobic than ELP. Thus, the acetylation of the polymers led to an increase in their hydrophobicity, which resulted in a decrease in their phase transition temperatures. Although the log P values of linear and branched polylysine were the same, the phase transition temperatures of Ac-ELP/ELP-conjugated PLD and PLL were much different. Thus, this phenomenon could not be explained by the hydrophobicity. The molecular weight of a polymer can also have a significant effect on its phase transition temperature.[27] In general, a large molecular weight leads to a low phase transition temperature. This phenomenon was observed for the PAMAM dendrimer and PAA series of polymers. Interestingly, the phase transition temperature of Ac-ELP-PLL was lower than that of Ac-ELP-PLD, even though the molecular weight of Ac-ELP-PLL was smaller than that of Ac-ELP-PLD. Thus, this phenomenon could not be

explained by the molecular weight. Previously, Ghoorchian et al compared the thermosensitivity of linear elastin-like polypeptides and three-armed ones. Branched ELPs exhibited lower phase transition temperature than linear ones,[28] which is inconsistent with our results. These results therefore suggest that linear polylysine-based elastin mimetic polymers show uncommon thermosensitive properties.

The influence of the salt concentration on the phase transition temperatures of these elastin-mimetic polymers was also examined (Figure 5). Irrespective of their polymer backbone and their polymer molecular weight, all of the elastin-mimetic polymers prepared in the current study showed a linear relationship between their phase transition temperature and the salt concentration. These results were consistent with those reported for several other elastin-mimetic polymers, which suggested that ELP worked well in these elastin-mimetic materials.[21,22,29,30]

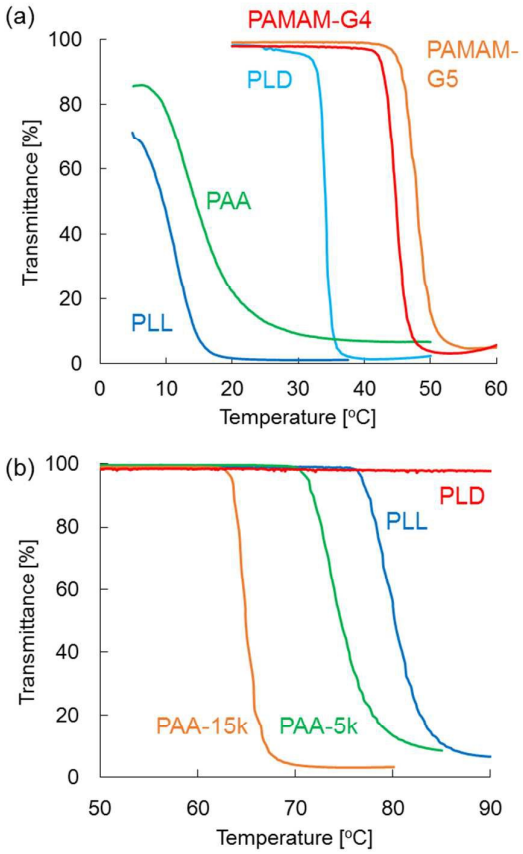

Figure 4. Phase transition temperatures of the elastin-mimetic polymers. (A) Temperature-dependent transmittance for solutions of the acetylated elastin-mimetic polymers at pH 7.4. (B) Temperature-dependent transmittance for solutions of the amino-terminal elastin-mimetic polymers at pH 10.

Table 2. Comparison in Log P values of moieties of ELP-mimetic polymers in this study

Moieties	Log P*[1]
PAMAM unit*[2]	-1.47
Polylysine unit*[2]	-0.26
PAA unit*[2]	0.99
Ac-ELP	-1.75
NH_2-ELP	-1.53
Linear trilysine-$CONH_2$	-3.64
Branched trilysine-$CONH_2$	-3.64

*1 SD = 0.47

*2 The unit structure is shown in Figure 1(b).

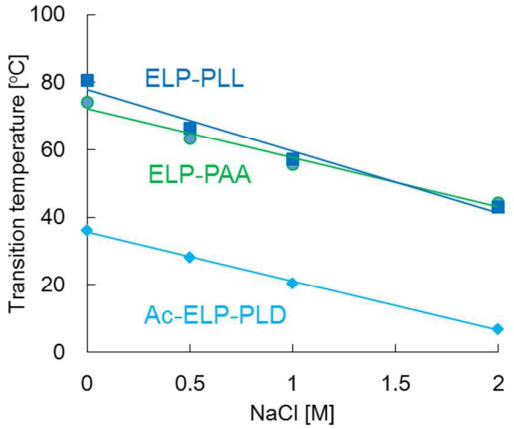

Figure 5. Phase transition temperatures of the elastin-mimetic polymers plotted against NaCl concentration. The amino- and acetyl-terminal polymers were examined at pH 10 and 7.4, respectively.

Higher order structure of ELP-grafted polymers

CD is a spectroscopic method that can be used to determine the secondary structure of proteins. It has been reported that the secondary structures of elastin-mimetic polymers change from random coils to type II β-turn structures when they were heated, with the corresponding CD patterns giving negative Cotton effects of around 197 and 225 nm, respectively.[31] ELP-grafted PAMAM dendrimers have been previously shown to undergo similar conformational changes to ELP.[21,22] Figures 6 (a-d) and S4 show the CD spectra of the acetyl- and amino-terminal elastin-mimetic polymers in water at different temperatures (5–65 °C). All of the elastin-mimetic polymers prepared in the current study except for Ac-ELP-PLL showed similar profiles with a decrease in their negative Cotton effect around 197 nm and an increase in their negative Cotton effect around 225 nm, when they were heated. This result suggested that the conformation of the ELP of the polymers was changing from a random coil to a β-turn structure when it was heated. In contrast, the CD spectra of Ac-ELP-PLL showed different CD patterns. The CD spectra contained negative Cotton effects around

208 and 222 nm and a positive Cotton effect around 193 nm, which corresponded to an α-helix structure. Yan et al. reported that conjugation of dendritic oligoethylene glycol (OEG) to PLL induced a change in its conformation to give an α-helix structure, with OEG behaving as a thermosensitive moiety.[32] Our results are therefore consistent with the previous study. Figure 6 (a) and (b) indicated that the CD spectra of Ac-ELP-PLD and Ac-ELP-PLL did not intersect the isodichroic point above 45 ^{o}C and 25 ^{o}C, respectively. The transmittance of Ac-ELP-PLD and Ac-ELP-PLL solutions started to decrease around 44 ^{o}C and 20 ^{o}C (Figure S5). Thus, these suggested that the solution turbidity above the cloud points affected the CD measurement.

It has been reported that native PLL formed a random coil structure under acidic conditions and an α-helix structure under basic conditions, and that these changes in the conformation could be attributed to changes in the protonation state of the amine groups on the side chains of the lysine residues.[33] The CD spectra of ELP-PLL and ELP-PLD were also measured in phosphate buffer (pH 7.4), because the pH of aqueous solutions of amino-terminal elastin-mimetic polymers was around 5 due to the trifluoroacetic acid salt. The CD spectra of ELP-PLL significantly changed as the pH was changed from acidic to neutral, but that of ELP-PLD did not. The CD spectrum corresponding to the α-helix structure was observed in ELP-PLL at higher temperature. The CD patterns corresponding to the α-helix and β-turn structures increased in ELP-PLL when it was heated at pH 7.4, although the α-helix structures were not observed in water (~pH 5). This result therefore suggests that the PLL in the main chain was forming an α-helix structure at pH 7.4, while the ELP in the side chain was also forming a β-turn structure. It has been reported that the α-helical structures of native PLL and OEG-conjugated PLL decreased when they were heated.[32,34] ELP-PLL showed the opposite behavior to these polymers. It is possible that the observed changes in

the conformation of ELP led to an increase in the hydrophobicity of the side chain and the formation of the α-helix structure. Given that α-helices are more hydrophobic than random coil structures, the formation of an α-helix in a polymer should lead to an increase in its hydrophobicity. Furthermore, the ELPs conjugated to the side chain were aligned with the lateral surface of the α-helix, which could lead to clustering effects. It is therefore possible that these effects could have resulted in the low phase transition temperature of the ELP-grafted PLL. It is well known that the phase transition of a given polymer can therefore be controlled by varying its chemical composition and molecular weight characteristics.[27,35,36] Changes in the pH and salt concentration can also have a significant effect of the thermosensitivity of polymers.[35-37] The encapsulated compounds had a significant impact on the thermosensitivity of the dendrimers.[38] In this study, we have demonstrated that the conformation of the main polymer chain in thermosensitive polymers is another important factor capable of controlling their phase transition temperature.

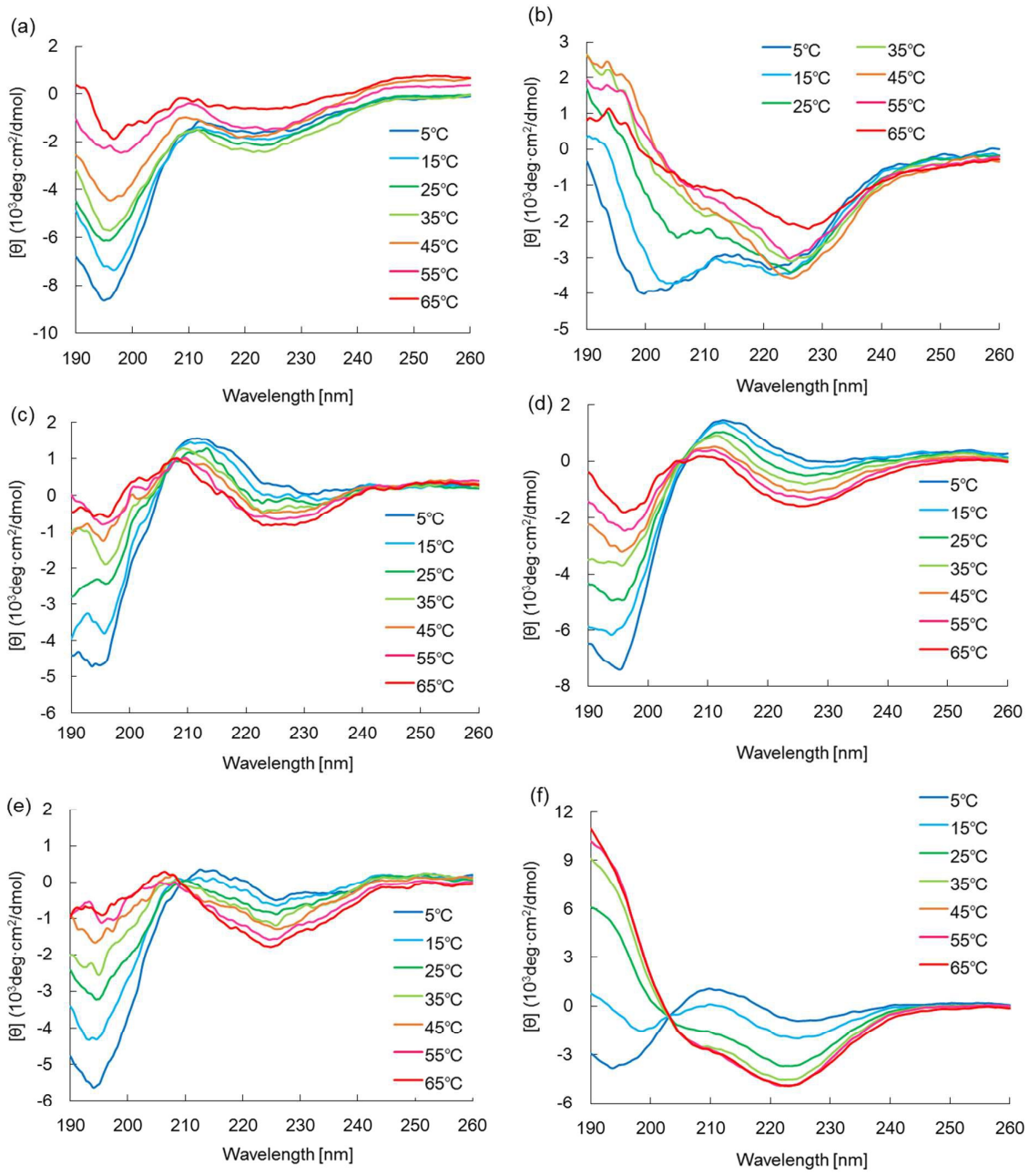

Figure 6. CD spectra of Ac-ELP-PLD (a), Ac-ELP-PLL (b), ELP-PLD (c and e) and ELP-PLL (d and f) in water (a–d) and phosphate buffer (pH 7.4, e and f). The solutions of Ac-ELP-PLD and Ac-ELP-PLL appeared to be turbid at temperatures above 55 and 25 °C, respectively.

Conclusions

We have synthesized linear and dendritic elastin-mimetic polylysines as a thermosensitive material. The linear polylysine in the backbone structure formed an α-helix to induce its phase transition. Polypeptide-based temperature-sensitive polymers could potentially be used to design increasingly sophisticated intelligent polymer materials. Furthermore, ELP-grafted PLD exhibited its phase transition temperature around body temperature, which could be useful for biomedical applications.

NMR spectra of the ELP-grafted PLL and PAA compounds, HPLC chromatograms of Ac-ELP and ELP, CD spectra of the ELP-grafted PAA compounds and the solution turbidity of Ac-ELP-grafted polymers were shown in the Supporting Information.

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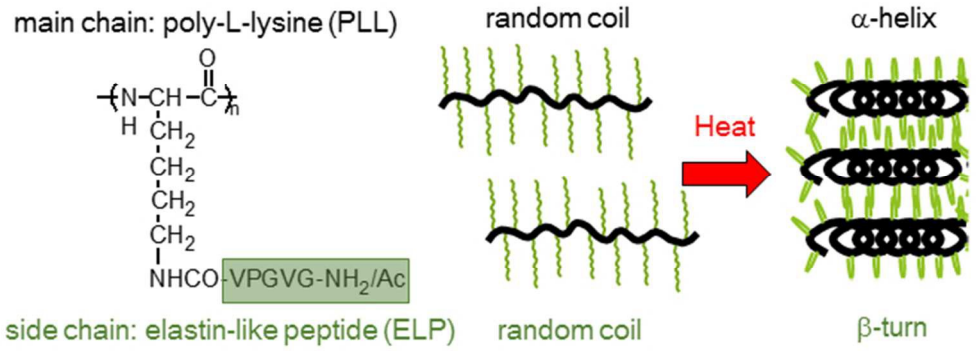

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