Water-Driven Thermoresponsive Peptohelical Cushion

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ABSTRACT: Oligopeptides were conjugated to the thermoresponsive polymer poly(*N*-vinylbutyramide) (PNVIBA) by the graft polymerization of the corresponding *N*-carboxyamino acid anhydrides from the amino side groups of P(NVIBA-*co*-vinylamine)s. The P{NVIBA-*graft*-L-aspartic acid (LAA)} with random-coiled graft chains showed a quick coil−globule transition accompanied by an endotherm due to chain dehydration across its low critical solution temperature (LCST) upon heating. In contrast, the P{NVIBA-*graft*-L-glutamic acid (LGA)}s with α-helical graft chains showed a slow transition accompanied by not only an endotherm but also an exotherm. A circular dichroism (CD) study indicated that the oligoLGA grafts changed their conformation accompanying the coil−globule transition of the PNVIBA backbone but recovered the α-helix at just above the exothermic temperature. ^{1}H nuclear magnetic resonance (^{1}H NMR) indicated that the proton signals from the PNVIBA backbones and oligoLGA grafts were clearly detected just above the LCST, but only the PNVIBA protons reduced their intensity above the exotherm temperature. Thus, the present peptide grafts with a α-helical conformation interfered with the thermoresponsive shrinking of the PNVIBA backbone by acting like spring cushions.

Introduction

The conformational control of biopolymers is very important for investigating the biological roles of their secondary structures. In particular, when molecules change their structure to resist to the motion or structural transformation of other molecules, these molecules act as so-called "molecular cushion" and have attracted researchers' attention. DNA,[1] RNA,[2] and proteins[3] sometimes play a significant role as a molecular spring in biological systems, whereas in synthetic systems, small molecules such as helicene,[4] vitamin A,[5] and porphyrin derivatives[6] have also functioned as molecular springs. Some of them[3] functioned as the molecular cushions. As far as we know, a synthetic polypeptide cushion working in water has not been previously reported, although a heptapeptide spring driven by changing the composition of an organic solvent mixture has been reported.[7] The polypeptide conformation was controlled by changes in the pH,[8−10] ionic environment,[11,12] temperature,[13,14] and so on, inducing an α-helix → coil transition and an α-helix → *β*-sheet transition. In addition, specific poly- and oligopeptides showed helix−helix transitions including a screw-sense inversion[15,16] and a helix-pitch change,[7] for example, upon a change in environmental polarity and hydrophilicity. These conformation changes of the polypeptides may be related to their actions as molecular cushions.

Thermoresponsive polymers have been studied as one of the most simple and relevant protein models because they show a coil−globule transition which is one of the most drastic conformational changes accompanying dehydration, just like protein shrinking during denaturation.[17] However, the coil−globule transition was much quicker than general protein denaturation via a molten globule transient state. This may be due to the different primary structure of the thermoresponsive polymers from the proteins. The conjugation of the peptide chain with the thermoresponsive polymer may give a more relevant model than the conventional ones. Here, we prepared peptide−thermoresponsive polymer conjugates to develop the protein denaturation model, with the peptide spring triggered by the hydrophilicity change during the thermoresponsive coil−globule transition in water. Synthetic poly(amino acid)s have been prepared by a ring-opening polymerization of the amino acid *N*-carboxyanhydride (NCA) initiating from the amino groups. If the poly(amino acid) chain was grafted onto a thermoresponsive polymer backbone, then the conformational information of the backbone structure may be transmitted to the graft chains. For example, Maeda et al. reported that the helical chirality of poly(L-glutamic acid) chains grafted onto the polyacetylene derivative backbones could be induced to helically wind the achiral backbones.[18] However, the combination of a thermoresponsive backbone and poly(amino acid) grafts has never been made, presumably because the most widely studied thermoresponsive polymers were polyacrylate derivatives, including poly(*N*-isopropylacrylamide) (PNIPAM),[19] which is difficult to derive amino groups from as the NCA initiator because of the poor copolymerizability of acrylate monomers with allylamine or vinylamine (VAm). On the other hand, we have prepared another thermoresponsive polymer poly(*N*-vinylisobutyramide) (PNVIBA)[20] which showed a sharp coil−globule transition at ambient temperature and easily allowed the incorporation of amino groups. In this article, we synthesized graft copolymers with PNVIBA backbones and oligo{L-aspartic acid (LAA)} or oligo{L-glutamic acid (LGA)} graft chains and investigated their structural transformation behavior. We found that the coil−globule transition of the backbones effectively affected the poly(amino acid) secondary structure, and conversely only the α-helical grafts interfered with the backbone shrinkage.

Experimental Section

Materials. Poly(vinylamine) (PVAm; M_w = 60 000) was kindly gifted by Dia-Nitrix Co. Ltd. and used after purified by neutralization and 1 week dialysis in pure water for removal of the impurities. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC; Wako Pure Chemical Industries, Ltd.) used as a condensation

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reagent, triethylamine (Tokyo Kasei Kogyo Co., Ltd.) used as a pH controller, isobutyric acid (Wako Pure Chemical Industries, Ltd.) used for PVAm modification, and triphosgene (Tokyo Kasei Kogyo Co., Ltd.) used for *N*-carboxyamino acid anhydride syntheses were used as received. β-Benzyl-L-aspartic acid (BLA; Wako Pure Chemical Industries, Ltd.) and γ-benzyl-L-glutamic acid (BLG; Wako Pure Chemical Industries, Ltd.) used as monomers for graft polymerization were used as received. *N*,*N*′-Dimethylformamide (DMF), tetrahydrofuran (THF), ethyl acetate, and hexane used as solvents were used after purification by distillation over drying reagents such as calcium hydride and sodium metal. Methanol used as a solvent was used as received. PNVIBA ($M_w = 15\,000$) prepared by the previously reported procedure[20] was used as a control sample.

Synthesis: P(NVIBA-*co*-VAm). P(NVIBA-*co*-VAm) was synthesized by a modification of PVAm by isobutyric acid in the presence of EDC by the procedure reported previously.[20] The composition of the NVIBA units to the total repeating units was 95 mol % as confirmed by proton nuclear magnetic resonance (^{1}H NMR) in D_2O by the reported method (yield: 60 wt %). The structures were confirmed by infrared (IR) spectroscopy. IR (ATR): 1623 (amide I) and 1548 cm^{-1} (amide II). ^{1}H NMR (400 MHz, D_2O): $\delta = 1.1$ ppm (CH_3 in NVIBA side chain), $\delta = 1.4-1.8$ ppm (CH_2 in main chains), $\delta = 2.3-2.6$ ppm (CH in NVIBA main chain), $\delta = 2.8-2.9$ ppm (CH in VAm main chain), $\delta = 3.7-4.0$ ppm (CH in NVIBA side chain).

***N*-Carboxyaminoacid Anhydrides.** *N*-Carboxyamino acid anhydrides (NCA; 45 mmol) of BLA and BLG were synthesized by a reaction with triphosgene (15 mmol) in THF (400 mL) for 4 h at 45 °C and recrystallized in a mixed solvent of hexane/ethyl acetate repeatedly using widely reported methods (yields: 72 and 76 wt %, respectively). Their structures were confirmed by IR and ^{1}H NMR spectroscopy. The IR spectra of the NCAs were almost the same as each other. IR (ATR): $\nu = 3250$ (−NH), 1836, 1789 (C═O of acid anhydride), 1724 cm^{-1} (C═O of ester). ^{1}H NMR of NCA of BLA (400 MHz, $CDCl_3$): $\delta = 2.87$, 3.10 ppm (m, 2H; β-CH_2), $\delta = 4.58$ ppm (t, 1H; α-CH), $\delta = 5.15$ ppm (s, 2H; CH_2-ϕ), $\delta = 6.19$ ppm (s, 1H; NH), $\delta = 7.36$ ppm (m, 5H; aromatic). ^{1}H NMR of NCA of BLG (400 MHz, $CDCl_3$): $\delta = 2.13$, 2.31 ppm (m, 2H; β-CH_2), $\delta = 2.61$ (t, 2H; γ-CH_2), $\delta = 4.37$ ppm (t, 1H; α-CH), $\delta = 5.15$ ppm (s, 2H; CH_2-ϕ), $\delta = 6.30$ ppm (s, 1H; NH), $\delta = 7.36$ ppm (m, 5H; aromatic).

PNVIBA Grafted by Poly(amino acid) Benzyl Esters. The P(NVIBA-*g*-BLG) copolymer was synthesized by the following procedure. The ring-opening polymerization of NCA for BLG (2.74 mmol) was performed without any catalyst in a DMF/THF mixture (35.9 mL, 3/7 v/v) for 168 h at room temperature under a nitrogen atmosphere, initiating from an amine group of P(NVIBA-*co*-VAm) (amine group: 0.137 mmol). The polymerization proceeded homogeneously, and the reaction solution was dialyzed in a large amount of THF for 2 days while replacing the solvent repeatedly and then in water for 7 days, followed by freeze-drying to yield the final product (yield: 64 wt %). The P(NVIBA-*g*-BLA) copolymer was synthesized by an analogous procedure (yield: 65 wt %). Their structures were confirmed by IR and ^{1}H NMR spectroscopy. The IR spectra of the NCAs were almost the same as each other. IR (ATR): $\nu = 3280$ (−NH), 1732 (C═O of ester), 1656 (amide I), 1520 cm^{-1} (amide II). ^{1}H NMR of P(NVIBA-*g*-BLA) (400 MHz, DMSO-d_6): $\delta = 1.1$ ppm (CH_3 in NVIBA side chain), $\delta = 1.4-1.8$ ppm (CH_2 in main chains), $\delta = 2.3-2.6$ ppm (CH in NVIBA main chain), $\delta = 2.9-3.3$ ppm (β-CH_2), $\delta = 3.7-4.0$ ppm (CH in NVIBA side chain), $\delta = 4.6$ ppm (α-CH), $\delta = 5.2$ ppm (CH_2-ϕ), $\delta = 7.0-7.4$ ppm (NH of peptides), $\delta = 7.5$ ppm (phenyl). $\delta = 8.0-8.5$ ppm (NH of NVIBA). ^{1}H NMR of P(NVIBA-*g*-BLG) (400 MHz, DMSO-d_6): $\delta = 1.1$ ppm (CH_3 in NVIBA side chain), $\delta = 1.4-1.8$ ppm (CH_2 in main chains), $\delta = 2.0-2.2$ ppm (β-CH_2), $\delta = 2.3-2.6$ ppm (CH in NVIBA main chain and γ-CH_2), $\delta = 3.7-4.0$ ppm (CH in NVIBA side chain), $\delta = 4.3-4.5$ ppm (α−CH), $\delta = 5.2$ ppm (CH_2-ϕ), $\delta = 7.0-7.4$ ppm (NH of peptides), $\delta = 7.5$ ppm (phenyl), $\delta = 8.0-8.5$ ppm (NH of NVIBA).

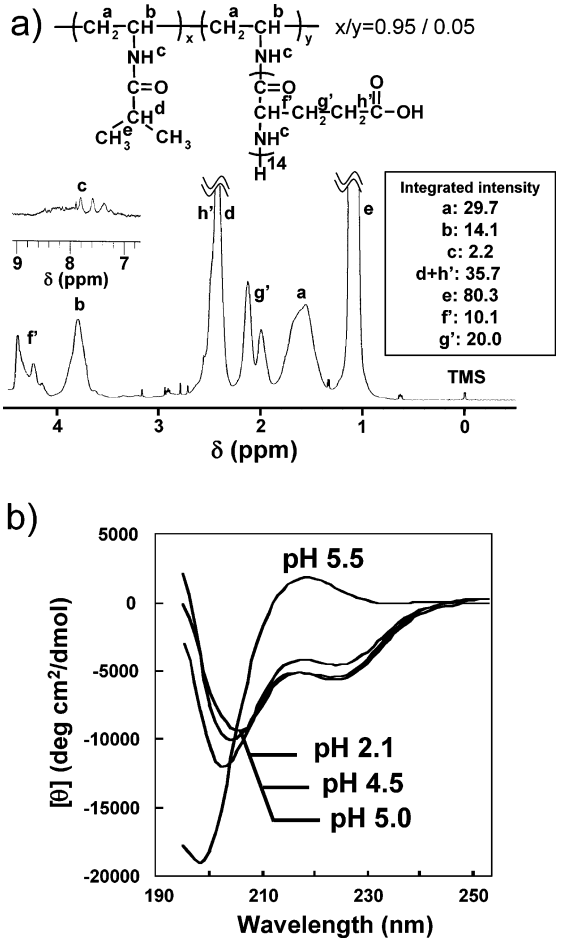
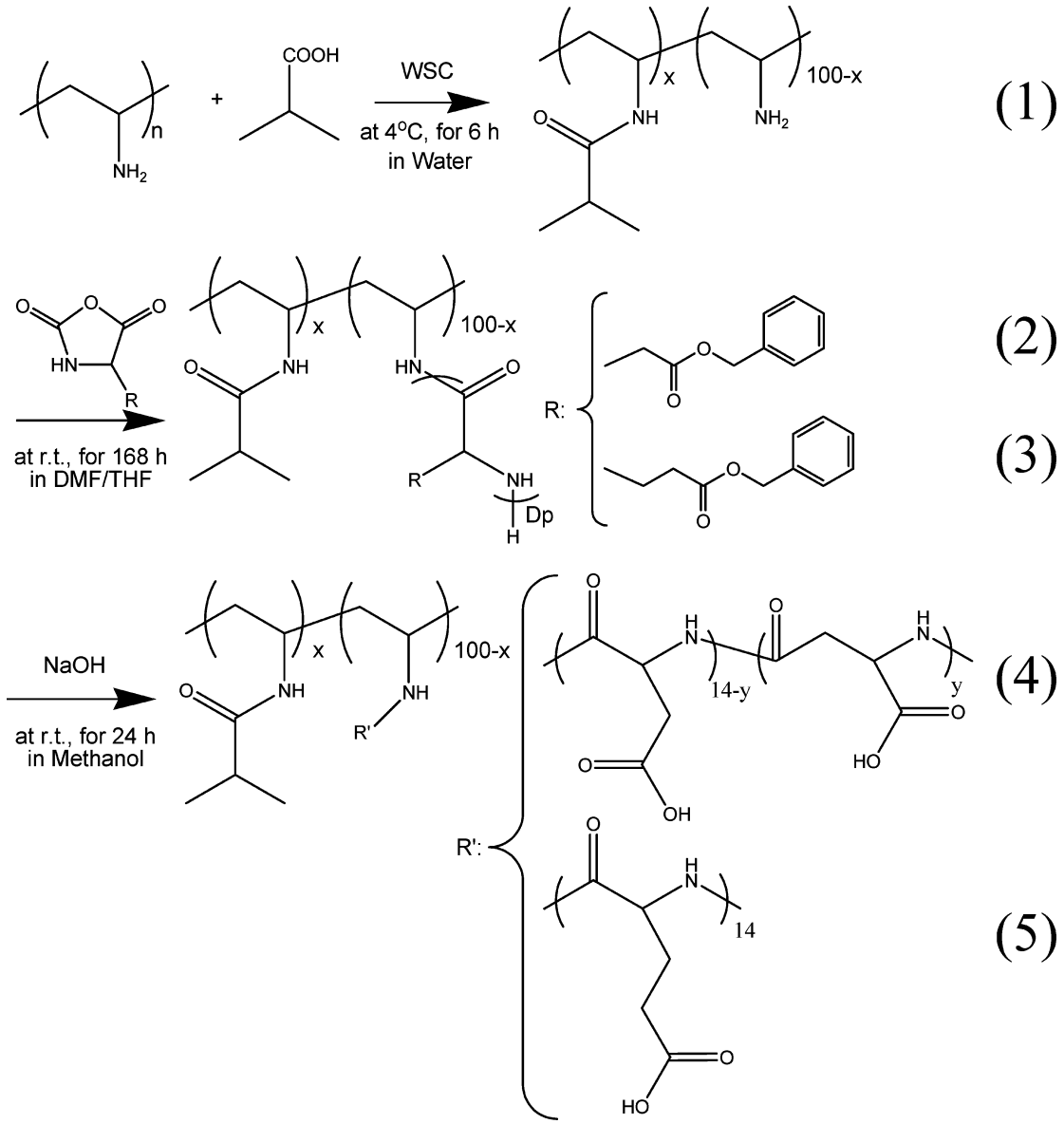


Figure 1. (a) Molecular structure of P(NVIBA-*g*-LGA) and the corresponding ^{1}H NMR spectrum. (b) CD spectra of the P(NVIBA-*g*-LGA) solution measured at pH = 2.1 and pH = 5.5.

P(NVIBA-*g*-amino acid)s. The P{NVIBA-*g*-L-glutamic acid (LGA)} copolymer was synthesized by the following procedure. Deblocking of the benzyl group from BLG was performed by an alkaline hydrolysis in a methanol solution of P(NVIBA-*g*-BLA) (0.1 M, 31.2 mL) using NaOH (1 N, 3.26 mL) at room temperature for 24 h. The reaction solution was then dialyzed in water for 5 h while replacing the solvent repeatedly to remove the low-molecular-weight impurity such as benzyl alcohol. The freeze-drying yields a powder of P(NVIBA-*g*-LGA) (yield: 91 wt %). Since the ^{1}H NMR signal of the phenyl protons at $\delta = 7.3$ ppm and the integrated intensity ratio of isopropyl CH_3 proton peaks (6H × 0.95) to main-chain CH proton ones (1H) was estimated as 5.4 (Figure 1a), we can confirm that the deprotection was completed while little hydrolysis of the NVIBA units occurred. The assignment of P(NVIBA-*g*-LGA) protons was confirmed by another NMR technique such as ^{1}H−^{1}H COSY and HOHAHA. ^{13}C NMR and ^{1}H−^{13}C HMQC also supported the successful synthesis of P(NVIBA-*g*-LGA). The NMR spectra are shown in the Supporting Information. The P(NVIBA-*g*-LAA) was synthesized by an analogous procedure (yield: 90 wt %). Their structures were confirmed by IR and ^{1}H NMR spectroscopy. The IR spectra of the NCAs were almost the same as each other. IR (ATR): 1718 (C═O of carboxylic acid), 1628 (amide I), 1554 cm^{-1} (amide II). ^{1}H NMR of P(NVIBA-*g*-LAA) (400 MHz, D_2O): $\delta = 1.1$ ppm (CH_3 in NVIBA side chain), $\delta = 1.4-1.8$ ppm (CH_2 in main chains), $\delta = 2.3-2.6$ ppm (CH in NVIBA main chain), $\delta = 2.5-3.0$ ppm (d, β-CH_2), $\delta = 3.5-4.0$ ppm (CH in NVIBA side chain and α-CH), $\delta = 4.4-4.6$ ppm (α-CH). ^{1}H NMR of P(NVIBA-*g*-LGA) (400 MHz, D_2O, Figure 1a): $\delta = 1.1$ ppm (CH_3 in NVIBA side chain), $\delta = 1.4-1.8$ ppm (CH_2 in main chains), $\delta = 2.0-2.2$ ppm (β-CH_2), $\delta = 2.3-2.6$ ppm (CH in NVIBA main chain and γ-CH_2),

Scheme 1. Synthetic Roots of P(NVIBA-*g*-amino Acid)s by the Ring-Opening Polymerization of Amino Acid NCAs Initiating from the Amino Side Group of P(NVIBA-*g*-VAm)



δ = 3.7−4.0 ppm (CH in NVIBA side chain), δ = 4.3−4.4 ppm (α-CH).

Measurements. Fourier transformation infrared (FT-IR) spectra of the particle surface were recorded by the attenuated total reflection (ATR) method on a Perkin-Elmer Spectrum One FT-IR spectrometer after 64 scans (4 cm^{-1} resolution) over the range from 4600 to 600 cm^{-1}.

^{1}H NMR spectra of the graft copolymers were measured for the structural determination with a NMR spectrometer (Varian Unity INOVA600 spectrometer) at 600 MHz. The temperature dependence of ^{1}H NMR signals of the graft copolymers in D_2O was measured with a NMR spectrometer (JEOL FX 400) at 400 MHz. ^{1}H NMR chemical shifts in parts per million (ppm) were recorded downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference.

The clouding behavior of the polymer aqueous solution was investigated by ultraviolet−visible spectroscopy (JASCO model V-550 spectrophotometer). The polymer solution was poured into a 1 mm quartz sample cell set in a Peltier-type thermally controlled cell holder. The rate of temperature change was 1 °C min^{-1}. The temperature dependence of the light transmittance at a wavelength of 500 nm was monitored, and the low critical solution temperature (LCST) was determined as the temperature at 50% of the curve bottom.

Differential scanning calorimetry (DSC) studies were performed using a Seiko Instrument Inc. DSC-210. All experiments were performed in a nitrogen gas atmosphere over a temperature range from 20 to 95 °C at a heating rate of 2 °C min^{-1}. The hydrogel specimens were hermetically sealed with water in an aluminum pan.

CD spectra were recorded by a JASCO spectropolarimeter J-725 under a nitrogen atmosphere after the solution was settled for at least 10 min at each temperature. The polymer solution was poured into a 10 mm quartz sample cell set in a Peltier-type thermally controlled cell holder.

Results and Discussion

Syntheses of Graft Copolymers. The P(NVIBA-*g*-amino acid)s were prepared by three-step procedures as follows (Scheme 1). First, PVAm (M_w: 6.0×10^4) was modified by isobutyric acid in the presence of EDC to yield P(NVIBA-*g*-VAm) (**1**) with a VAm composition of 5 mol %, which was determined by a ^{1}H NMR study focusing the methylene protons in the main chains using the previously reported method.[20] Copolymer **1** was water-soluble and showed a pH-dependent coil−globule transition at a lower critical solution temperature (LCST); i.e., it showed a clouding behavior at around 46 °C

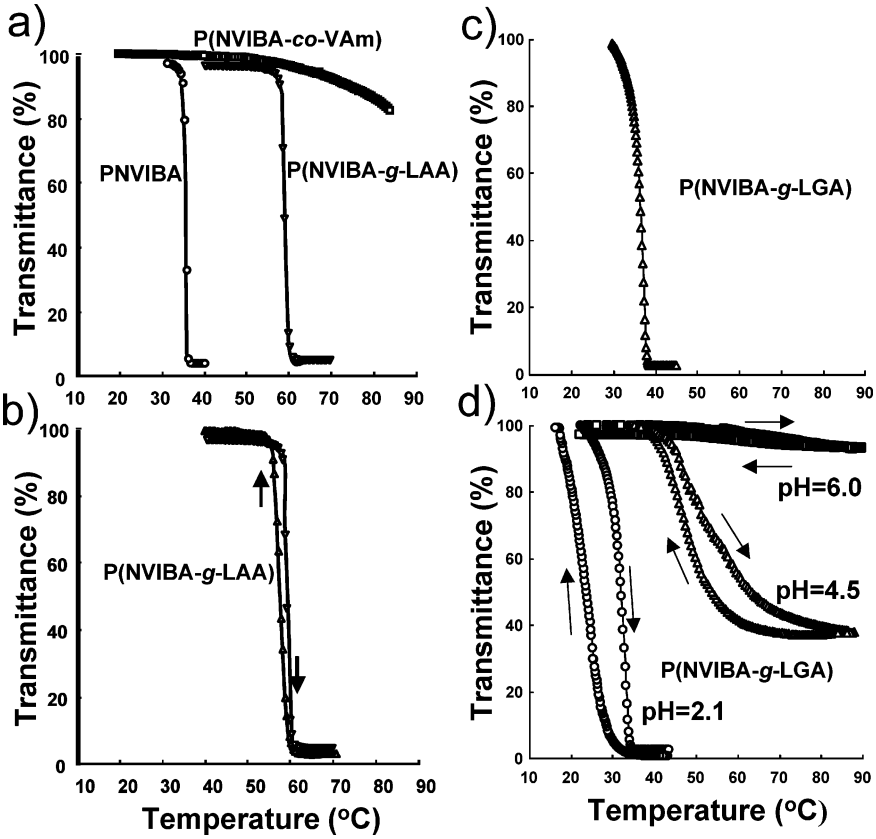


Figure 2. Temperature dependence of light transmittance of PNVIBA and its copolymer solutions. (a) PNVIBA, P(NVIBA-*g*-LAA), and P(NVIBA-*g*-VAm) solution upon heating at pH = 2.1. (b) P(NVIBA-*g*-LAA) solution upon heating and cooling at pH = 2.1. (c) P(NVIBA-*g*-LGA) solutions upon heating at pH = 2.1. (d) P(NVIBA-*g*-LGA) solutions upon heating and cooling at pH = 2.1, 4.5, and 6.0.

upon heating at pH = 12 (pK_a of VAm: 10.0)[20] but showed no thermoresponse at pH = 2.1. This result indicates that the coil−globule transition of PNVIBA was very sensitive to the condition of the side groups attached to the backbone. Second, the polymerization of NCAs for benzyl L-aspartate (BLA) and benzyl L-glutamate (BLG) was initiated from the amino side group of copolymer **1**. The ^{1}H NMR spectra showed that the signals for the VAm methylene protons disappeared completely, suggesting that all of the amino groups played a role as an initiator for NCA polymerization. From the integrated intensity ratio of amino acid side-chain proton peaks to the main-chain proton ones in ^{1}H NMR spectra, the averaged polymerization degree for either poly(amino acid) graft chain was estimated as 14, which was slightly lower than the in-feed ratio of NCA to VAm units (20 mol/mol). Since the VAm composition was only 5 mol %, corresponding to the presence of amino groups at every 20 units, we can hypothesize that the poly(amino acid) graft chains were prepared under negligibly low steric-hindrance. The graft copolymers **2** and **3** were insoluble in water but soluble in methanol. The methanol solution of **2** and **3** showed a CD peak with a negative cotton effect at a wavelength (λ) of 222 nm despite too high noise around λ = 208 nm, suggesting the formation of an α-helical structure for the graft chains of PBLA and PBLG. Third, the benzyl groups of **2** and **3** were deprotected by alkaline hydrolysis using NaOH in methanol. ^{1}H NMR studies demonstrated that the deprotection of the benzyl group was selective, without any damage to the amides of either the NVIBA units or the graft backbones to produce copolymers **4** and **5**. Both P(NVIBA-*g*-amino acid)s **4** and **5** were soluble in water. According to the literature, the deprotection of poly(β-benzyl L-aspartate) using NaOH induced an aspartate isomerization, giving rise to a complex structure with α- and β-peptide bonds to yield the poly(α-/β-LAA)[21] with a random coil conformation. The P(NVIBA-*g*-LAA) prepared here also adopted the random coil conformation, as confirmed by a CD study (data not shown). On the other hand, P(NVIBA-*g*-LGA) was synthesized without such an isomerization and showed right-handed α-helix CD patterns with two minima negative Cotton effects at 222 nm (n → π*) and 208 nm (π → π*) at pH = 2.1 (Figure 1). The ellipticity ratio of $\theta_{222}/\theta_{208}$ was about 0.8, which agreed well with the reported value in the α-helix of the oligoLGA chain with 14.5 repeating units.[22] Although the PLGA grafts adopted the α-helix conformation (clear two minima) over the pH range of 2.1−5.0, the CD spectrum of the copolymer at pH = 5.5 showed the typical Cotton effects of a random coil conformation (Figure 1). Since the π → π* transition peak became shaper at the higher pH, the random-coil content increased by increasing pH. These results indicate that the fixation of the PLGA chain end at the PNVIBA backbone did not interfere with adopting the α-helical conformation or with the helix−coil transformation. Similar transformation phenomena for oligoLGA grafts have also been observed in the poly(phenylacetylene) backbone system.[18] Using these two samples, the mutual effects of the polypeptide conformation and the coil−globule transition of the PNVIBA backbone were investigated.

Thermoresponse of P(NVIBA-*g*-amino acid)s. The temperature dependence of the light (λ = 620 nm) transmittance for a P(NVIBA-*g*-LAA) solution of pH = 2.1 with a high concentration (concentration of NVIBA units: 0.02 M) was measured. The light transmittance showed a steep declination at around a LCST of 59 °C, which was higher than that of PNVIBA (38 °C) (Figure 2a), where the LCST is defined as the temperature at the half point of the minimum transmittance. The incorporation of the hydrophilic graft chain was effective in increasing the LCST as reported in other systems.[19,20] The difference in the LCST between P(NVIBA-*g*-LAA) and PN-

VIBA was 23 °C. On successive cooling, a clear solution of P(NVIBA-*g*-LAA) was recovered at around 58 °C, as shown in Figure 2b. The thermoresponse was very quick upon either heating or cooling, and the LCST was independent of the thermal history. The DSC thermogram of P(NVIBA-*g*-LAA) showed a small but distinct endotherm at 58 °C, indicating that the clouding accompanying the dehydration of the polymeric chain to show the coil−globule transition. The CD spectra showed the Cotton effects of a random-coil state regardless of the temperature, indicating that the PLAA graft chains did not adopt an α-helix, even under the hydrophobic milieu created by the globule backbones. As a consequence, the random coil graft chains barely affected the coil−globule transition, except for increasing the LCST.

Since the P(NVIBA-*g*-LGA) solution also showed clouding behavior upon heating, the temperature dependence of the light transmittance was recorded (Figure 2c). The curve of the change in light transmittance also showed a sudden drop at around 35 °C, but the declination was not so steep. The LCST of P(NVIBA-*g*-LGA) was just 3 °C lower than that of PNVIBA, but 24 °C lower than that of P(NVIBA-*g*-LAA). This result suggests that the PLGA graft chains have hydrophilicity similar to the PNVIBA backbone but lower hydrophilicity than the PLAA grafts, presumably due to the one methylene longer side chain of LGA residues than LAA. In contrast to P(NVIBA-*g*-LAA) and PNVIBA,[23] the LCST of P(NVIBA-*g*-LGA) upon cooling was 10 °C lower than that upon heating (Figure 2d). This large hysteresis may imply a difference in copolymer hydrophilicity between the heating and cooling processes. At pH = 4.5 where the charging of the PLGA grafts should be enhanced, they still adopted the α-helical conformation, and the LCST upon heating increased to 55 °C. Furthermore, the light transmittance was constant at 40% even above the LCST, presumably due to the increased degree of charging, while the thermal hysteresis could still be observed. At pH = 6.0 where the PLGA grafts adopted the random coil conformation due to the repulsion between the charged glutamate side chains, the drop in light transmittance no longer occurred. Since a pH = 2.1 induced the most distinct coil−globule transition under the α-helical conformation of the graft chains, the structural study was made under these conditions.

Thermodynamic Study of P(NVIBA-*g*-LGA). To investigate the reason for the gentle declination curve of the light transmittance around the LCST, we investigated the chain dehydration behavior by DSC (Figure 3a). The aqueous solution of PNVIBA showed an endotherm accompanying the coil−globule transition due to the dehydration of the polymer chains.[20] On the other hand, it was quite unique that the DSC thermogram of the P(NVIBA-*g*-LGA) solution showed not only an endotherm at the onset temperature of 35 °C comparable to the LCST but also a broad exotherm in the temperature range of 40−50 °C. These thermodynamic results imply that a transition of chain rehydration or structural organization may occur just after the coil−globule transition.

The temperature dependence of the polymer structure in a D_2O/DCl solution ([DCl]: 10^{-2} M) was studied by ^{1}H NMR (Figure 3b). The ^{1}H NMR spectra show every C−H proton signals in P(NVIBA-*g*-LGA) over the range of chemical shifts δ = 0.0−4.5 ppm as marked by **a**, **b**, **d**, **e**, **f′**, **g′**, and **h′** where the prime was attached to denote the graft chain protons. The PLGA graft protons at δ = 2.0 and 2.2 ppm were marked as **g′** and kept their intensity constant regardless of the temperature, although the intensity of **f′** at δ = 4.4 ppm was not well recognized because of the overlapping water signal at 40, 50, and 60 °C. On the other hand, CH_2 and CH main-chain protons marked as **a** and **b** around δ = 1.6 and 3.8 ppm, respectively, and around δ = 2.5 and 1.1 ppm marked as **e** (side chain CH) reduced their signal intensity upon increasing temperature to 50 °C and finally disappeared at 60 °C. In the **d** + **h′** signal, the peak reduced their intensity upon increasing temperature, but a small peak was still remained at 60 °C, suggesting that only the main-chain **d** protons may disappear. Since the reduction of the ^{1}H NMR signal means a decrease in molecular mobility,[23] the NVIBA units and main chains reduced their mobility in the globule state at 50 and 60 °C, while the graft chains kept their molecular mobility constant regardless of the backbone state. Here it should be noted that the NMR signals of the main-chain protons kept their intensity strong at 40 °C and did not disappear completely at 50 °C, even above the LCST. This result suggests that the backbone dehydration confirmed by the endotherm in the DSC thermogram might not reduce the molecular mobility over the temperature range of the broad exotherm, presumably due to a force resistant to the main-chain globule. Since the P(NVIBA-*g*-LAA) with random coil grafts did not show such a strange phenomenon, the PGLA grafts adopting the helical conformation may have been resistant, possibly due to the grafts surrounded by the shrinking backbones. If this assumption is true, then the environment of the PGLA grafts may change due to the hydrophobic and nearly globule main chains predictably to induce a conformational change.

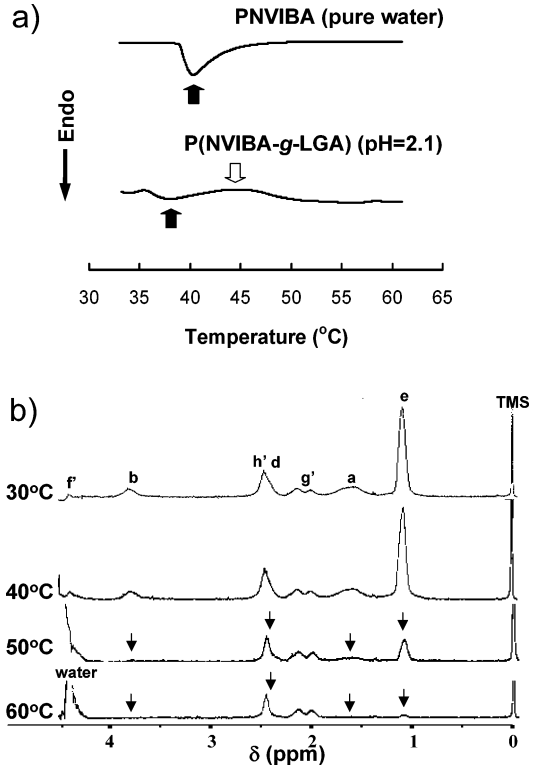


Figure 3. (a) DSC thermograms of PNVIBA in pure water and the P(NVIBA-*g*-LGA) solution at pH = 2.1. The black arrows show the endothermic peaks, whereas the white arrow shows the exothermic peak. (b) ^{1}H NMR spectra of the P(NVIBA-*g*-LGA) solution at various temperatures. The arrows refer to those peaks which reduced their intensity with increasing temperature.

Secondary Structures of P(NVIBA-*g*-LGA). The P(NVIBA-*g*-LGA) retained high transparency regardless of the temperature in the dilute solution at a concentration of 0.01 wt %. We then recorded the temperature dependence of the Cotton effects from the CD spectra of the P(NVIBA-*g*-LGA) solution at pH = 2.1. Even above the LCST, linear dichroism (LD) or

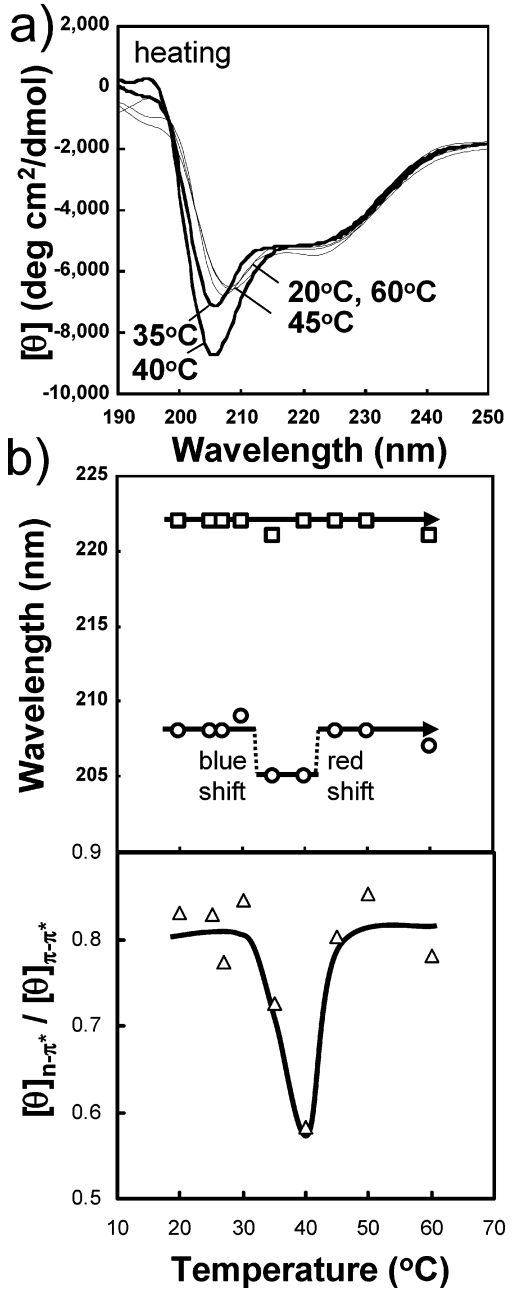


Figure 4. (a) CD spectra of the P(NVIBA-*g*-LGA) solution at pH = 2.1 measured at various temperatures upon heating. (b) Temperature dependence of the wavelengths showing ellipticity minimums and the molecular ellipticity ratios for the $\pi-\pi^*$ and n$-\pi^*$ transitions.

obstructive noise was not detected in the wavelength range over 195 nm, and repeated measurements ($n = 3$) reproducibly showed the same CD spectra. PNIVIBA, of course, showed no Cotton effects regardless of the temperature (data not shown). Figure 4a shows representative CD spectra from a P(NVIBA-*g*-LGA) solution of pH = 2.1 measured at various temperatures. Similar to the PNIVIBA, P(NVIBA-*g*-LGA) showed negative Cotton effects at wavelength minimums of 222 (n $\rightarrow \pi^*$ transition) and 208 nm ($\pi \rightarrow \pi^*$ transition); the $[\theta]_{222}/[\theta]_{208}$ ratio was ~0.8, which is typical for the right-handed α-helical form.[22] We showed the temperature dependence of the wavelengths for the n $\rightarrow \pi^*$ transition and the $\pi \rightarrow \pi^*$ transition and their ellipticity ratios. At 30 °C and lower, similar Cotton effects of the α-helix were detected. On the other hand, the $\pi \rightarrow \pi^*$ transition became more intense and showed a modest blue shift to 205 nm, and the ellipticity ratio of the $[\theta]_{222}/[\theta]_{205}$ was reduced to 0.58 (Figure 4b). From this blue shift and the ellipticity, we can hypothesize two possible conformational changes of the oligoLGA grafts: one is a deformation to a random coil structure, and the other is a transformation to a right-handed 3_{10}-helix structure which is tighter and more elongated than an α-helix and has a different hydrogen-bonding pattern ($i \leftarrow i + 3$ for 3_{10}-helix, $i \leftarrow i + 4$ for α-helix). Besides, it is noteworthy that the minimum of n $\rightarrow \pi^*$ transition peak became a shoulder in the CD spectra recorded at 35 and 40 °C, although the copolymer in the intermediate state showed the clear n $\rightarrow \pi^*$ transition minimum under the condition of 20 °C and pH = 2.1 (Figure 1). According to the literature, on the pH-induced helix−coil transformation of oligoLGAs,[22] the transient state from the α-helix to a random coil showed a blue shift of the $\pi \rightarrow \pi^*$ transition peak similar to our results, but both peaks of the $\pi \rightarrow \pi^*$ and n $\rightarrow \pi^*$ transitions became weak because of a decrease in the helix content. Since the intensity of the peaks at 205 and 222 nm in the present P(NVIBA-*g*-LGA) became stronger and constant, respectively, we can deduce that the possibility of a transformation to a random coil conformation is very low. On the other hand, Formaggio et al. reported that an oligopeptide adopting the 3_{10}-helix conformation in water showed a blue shift of the $\pi \rightarrow \pi^*$ transition peak around 205 nm,[24] although other 3_{10}-helical peptides in organic solvents such as trifluoroacetic acid and hexafluoro-2-propanol showed $\pi \rightarrow \pi^*$ transition peaks at around 208 nm.[7] The present P(NVIBA-*g*-LGA) showed chirospectroscopic patterns in water similar to the above-mentioned 3_{10}-helical peptides but showed a $[\theta]_{222}/[\theta]_{205}$ ratio of 0.58, which is a bit higher than the values in the literature (0.15−0.55),[24] presumably indicating the coadoption of the 3_{10}-helix/random conformation. The adoption of 3_{10}-helix may be supported by the shoulder morphology (not minimum) of the n $\rightarrow \pi^*$ transition peak.[7] Although we attempted to estimate the 3_{10}-helix composition according to the literature,[7,22,24] the blue shift of the $\pi \rightarrow \pi^*$ transition peak and the short length of the graft chain made it difficult to obtain valid values of the molar ellipticity for each helix.[25] Several studies have reported the conditions under which polypeptides preferably adopt a 3_{10}-helix conformation: (1) short length,[7,26] (2) high concentration,[27] and (3) low polarity solvent.[7,28] Since the short chain of the oligoLGA surrounded by shrinking backbones upon dehydration fulfills these three conditions, the adoption of the 3_{10}-helix and/or random-coil conformation seems to be a reasonable phenomenon. The P(NVIBA-*g*-LGA) recovered the CD spectrum of an α-helix over 45 °C. As a consequence, one can hypothesize from Figure 4a that the transition of the α-helix → other conformation (3_{10}-helix or random coil) → α-helix occurred upon heating in water (pH = 2.1).

We additionally studied the chirospectroscopic change of P(NVIBA-*g*-LGA) solution in the cooling process (Figure 5). While the $\pi \rightarrow \pi^*$ transition peak located at a wavelength of 208 nm at 60 °C, the peak went back to 205 nm at 40 °C, indicating the recovery to the intermediate state. The successive heating induced the red shift to 208 nm again, indicating that the oligoLGA transformation occurring over 40 °C is reversible. On the other hand, the $\pi \rightarrow \pi^*$ transition peak kept the wavelength around 205 nm even at 20 °C, and besides the sample settled at 5 °C for 24 h still showed the peak at 205 nm. The result indicates that the oligoLGA conformation did not return to the α-helix. The difference in transformation behavior between heating and cooling process may induce the hysteresis of transmittance change shown in Figure 2d.

Molecular Cushion Effects of Peptide Grafts. We schematically illustrated the thermodynamic structural transformation

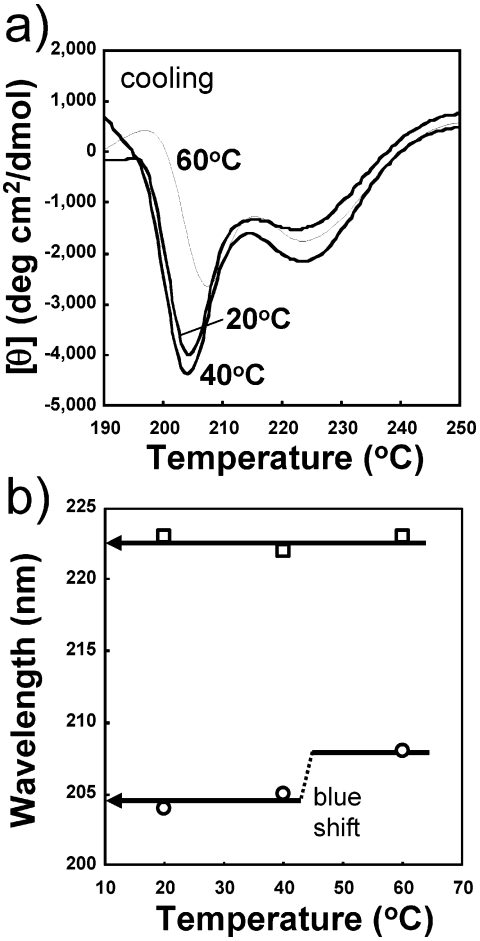


Figure 5. (a) CD spectra of the P(NVIBA-*g*-LGA) solution at pH = 2.1 measured at various temperatures upon cooling. (b) Temperature dependence of the wavelengths showing ellipticity minima.

behavior of a P(NVIBA-*g*-LGA) solution at pH = 2.1 in Figure 6. Below the LCST, P(NVIBA-*g*-LGA) adopted the random coil conformation for the PNVIBA backbone and an α-helix conformation for the oligoLGA grafts as shown in the top left of Figure 6. When the temperature increased slightly higher than the LCST, the P(NVIBA-*g*-LGA) solution showed a light transmittance decline less steep than the PNVIBA and P(NVIBA-*g*-LLA) solutions which had random-coil grafts. The DSC showed an endotherm which was much smaller than that of PNVIBA. Over this temperature range, the backbone was dehydrated and started to shrink, but the thermoresponse was slow, incomplete, and under an intermediate state similar to the molten globule state in protein folding, presumably due to the steric hindrance of the rigid-rod α-helical grafts of the oligoLGA acting like a spring cushion. Inside the shrinking backbone, the oligoLGA grafts were concentrated under the hydrophobic environment, which induced the helix transformation. In this state, the $[\theta]_{222}/[\theta]_{205}$ ratio did not increase despite the graft chain concentration, suggesting that the graft chains were easily jammed and were not regularly associated. The CD study suggests that the oligoLGA may adopt a 3_{10}-helix or random-coil conformation (Figure 6, middle). When the temperature increased slightly higher, the DSC thermogram showed an exotherm, meaning a hydration. The elevated temperature may enhance the molecular motion of the oligoLGA and the hydrophobic effects of PNVIBA backbone, which could induce the segregation shown at the bottom of Figure 6, the hydrophobic core of PNVIBA and the hydrophilic grafts of oligoLGA. At this stage, the oligoLGA grafts imbibe water sufficiently to recover the

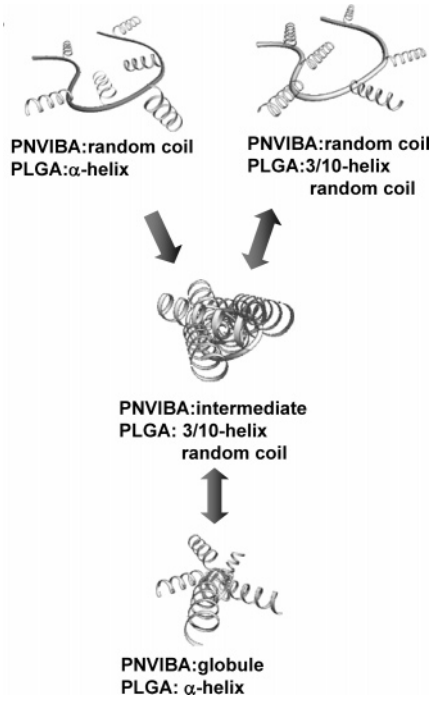


Figure 6. Schematic illustration of the change in the P(NVIBA-*g*-LGA) conformation upon heating at pH = 2.1.

α-helical conformation, thus removing the spring effects. We can hypothesize that the broad exotherm may be due to the hydration of the oligoLGA grafts and the segregation of the backbone and grafts. In the cooling process from 60 to 40 °C, the conformation of oligoLGA grafts changed back from α-helix of to 3_{10}-helical or random coil, indicating the oligoLGA transformation occurring over 40 °C was reversible. However, the 3_{10}-helical or random-coiled structure was maintained at 5 °C even if the main chain adopted the coiled conformation as shown in the top right of Figure 6. The intermediate state may be stabilized by PNVIBA main chains, presumably because the PNVIBA chains had a similar hydrophilicity with the oligoLGA chains as mentioned above (in the explanation of Figure 2c). However, the keep of the copolymer at 4 °C for 1 month recovered the α-helix conformation. Just after cooling, successive second heating of the P(NVIBA-*g*-LGA) solution in the intermediate state showed the thermoresponse, but the clouding temperature was largely increased to around 65 °C, indicating the oligoLGA conformation strongly affects LCST.

For comparison, we additionally synthesized the P(NVIBA-*g*-LGA) having a shorter graft chains (8 LGA units) and studied the conformation and cushion effects. The CD spectrum of P(NVIBA-*g*-LGA) having a shorter graft chains showed the negative Cotton effects (minimum: 216 nm) typical of *β*-strand structure, and the DSC thermogram showed a sharp endotherm but no exotherm (CD and DSC: Supporting Information), denying the cushion effect. Then the cushion effect in the peptides grafted to the thermoresponsive backbones may be specific to the α-helical conformation.

Conclusion

P(NVIBA-*g*-amino acid) copolymers were successfully synthesized by the graft polymerization of the corresponding NCA initiating from the amino side groups of P(NVIBA-*co*-vinylamine)s. Although oligoLLA grafts with 14 repeating units in the P(NVIBA-*g*-LAA) adopted a random-coil conformation, oligoLGA grafts with 14 repeating units in the P(NVIBA-*g*-LGA) adopted an α-helical conformation. P(NVIBA-*g*-LAA)

showed an ordinal coil−globule transition accompanied by a sharp endotherm in its DSC due to chain dehydration across the LCST upon heating, whereas the P(NVIBA-*g*-LGA) with α-helical graft chains showed a slow transition like protein denaturation, accompanied by not only a broad endotherm but also a broad exotherm. Circular dichroism (CD) studies suggested that the oligoLGA grafts transformed from an α-helix to a more highly pitched 3_{10}-helix or random coil. Proton nuclear magnetic resonance (^{1}H NMR) indicated that the proton peaks of the PNVIBA backbones and oligoLGA grafts were clearly detected just above the LCST, but the PNVIBA protons reduced their intensity while the PLGA protons kept their intensity above the exotherm temperature. Then the oligoPGA grafts adopting the α-helical structure may be surrounded by dehydrated and shrinking backbones and can act as a helical cushion resistant to the coil−globule transition of the PNVIBA backbone. At more elevated temperatures, P(NVIBA-*g*-LGA) recovered the α-helix conformation, presumably due to the segregation between the backbones, and the exotherm may be due to graft hydration and segregation. Although the oligoLGA conformation change occurring in the higher temperature range was reversible, 3_{10}-helical or random-coiled conformation was kept even at 5 °C, and the further keeping at 4 °C recovered the α-helix conformation. The present oligoLGA grafts of P(NVIBA-*g*-LGA) are the first synthetic peptide cushions reported to be working in water and may be considered as a good protein denaturation model having one of the most familiar amino acid sequences. For example, we can imagine that the slow denaturation of most proteins via a molten globule state may be associated with the presence of an α-helical spring cushion. In addition, the combination of thermoresponsive backbones with helical biopolymer grafts may lead to the development of a good bioactuating system.

Supporting Information Available: ^{1}H−^{1}H COSY, HOHAHA, ^{13}C NMR, and ^{1}H−^{13}C HMQC spectra of P(NVIBA-*g*-LGA); detailed analysis of CD peak fitting and IR peak shift; (related with ref 25); and CD spectrum and DSC thermogram of P(NVIBA-*g*-LGA) with 8 LGA units which adopted the *β*-strand. This material is available free of charge via the Internet at http://pubs.acs.org.

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(25) Using the peak analyzing application (GRAMS/AI ver.7, Thermo Galactic), the peak fitting of the negative CD peaks recorded at 35 and 40 °C over a wavelength range shorter than 195−260 nm gave three main peaks at 202, 207, and 222 nm (and a negligibly small peak at around 245 nm) with a good fitting correlation of $R^2 = 0.999$ (the fit peaks are shown in the Supporting Information). If we ignore the contribution of random conformation for Cotton effects and focused on the helical conformation, the following calculation can be made. If we can assume that the $[\theta]_{222}/[\theta]_{207}$ value is 0.8 for an α-helix (from ref 27), whereas the $[\theta]_{222}/[\theta]_{202}$ ratio is 0.3 for a 3_{10}-helix (from ref 28); the real value of $[\theta]_{202,\pi-\pi^*}$ is 4240 deg cm dmol^{-1} at 35 °C and 5470 deg cm dmol^{-1} at 40 °C. Then the $[\theta]_{222}$ values from the 3_{10}-helix can be calculated as 1270 deg cm dmol^{-1} at 35 °C and 1640 deg cm dmol^{-1} at 40 °C. In addition, the real values of $[\theta]_{207,n-\pi^*}$ can be calculated as 4050 deg cm dmol^{-1} at 35 °C and 4190 deg cm dmol^{-1} at 40 °C, and the $[\theta]_{222}$ values from the α-helix can be calculated as 3240 deg cm dmol^{-1} at 35 °C and 3350 deg cm dmol^{-1} at 40 °C. The sums of the $[\theta]_{222}$ values for the 3_{10}-helix and α-helix were calculated as 4510 deg cm dmol^{-1} at 35 °C and 4990 deg cm dmol^{-1} at 40 °C, which are in good agreement with the recorded $[\theta]_{222}$ values for the 3_{10}-helix (4533 deg cm dmol^{-1}) and α-helix (4495 deg cm dmol^{-1}). This agreement supports the assumption of the $[\theta]_{222}/[\theta]_{207}$ value. However, we failed to find the molar ellipticity of $[\theta]_{202}$ and $[\theta]_{222}$ for the short oligoLGA chain from the literature, and the helicity for both conformations is under evaluation. Furthermore, we analyzed the hydrogen bonding of the amide groups by their IR/ATR spectra (Supporting Information). However, the differentiation of the LGA grafts from the NVIBA units was very difficult, and the hydrogen-bonding behavior is under evaluation.

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