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Structural requirements essential for elastin coacervation: favorable spatial arrangements of valine ridges on the three-dimensional structure of elastin-derived polypeptide (VPGVG)*n*

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The elastin precursor tropoelastin possesses a number of polymeric peptides with repeating 3–9 mer sequences. One of these is the pentapeptide Val-Pro-Gly-Val-Gly (VPGVG) present in almost all animal species, and its polymer (VPGVG)*n* coacervates just as does tropoelastin. In the present study, in order to explore the structural requirements essential for coacervation, (VPGVG)*n* and its shortened repeat analogs (VPGV)*n*, (VPG)*n*, and (PGVG)*n* were synthesized and their structural properties were investigated. In our turbidity measurements, (VPGVG)*n* demonstrated complete reversible coacervation in agreement with previous findings. The Gly^5-deleted polymer (VPGV)*n* also achieved self-association, though the onset of self-association occurred at a lower temperature. However, the dissociation of (VPGV)*n* upon temperature lowering was found to occur in a three-step process; the Val$_i$^{4}-Val$_{i+1}$^{1} structure arising in the VPGV polypeptide appeared to perturb the dissociation. No self-association was observed for (VPG)*n* or (PGVG)*n* repeats. Spectroscopic measurements by CD, FT-IR, and ^{1}H-NMR showed that the (VPGV)*n* and (VPG)*n* both assumed ordered structures similar to that of (VPGVG)*n*. These results demonstrated that VPGVG is a structural element essential to achieving the β-spiral structure required for self-association followed by coacervation, probably due to the ideal spatial arrangement of the hydrophobic Val residues. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: coacervation; elastin; repeat structure; self-association; spiral structure

Introduction

Elastin is a major component of elastic tissues such as arterial walls, lungs, and skin. Tropoelastin, a precursor protein of elastin [1,2], assembles in a regular manner and is then cross-linked by lysyl oxidase to form mature insoluble elastin *in vivo*. The self-association of the tropoelastin molecule, which is called coacervation, is the most important step in the process of elastin biosynthesis. Tropoelastin forms a homogenous solution at temperatures below 25 °C, but upon increasing the temperature to body temperature (37 °C) or above, the solution becomes turbid with coacervating droplets. Eventually, settling occurs to form a dense viscoelastic coacervate layer. This feature is considered to be related to the elastomeric function of elastin [3].

The amino acid sequence of tropoelastins varies among species [4–8]. The typical structural feature of tropoelastin is the mutual repeat of cross-linking regions and hydrophobic regions with β-turns. The hydrophobic regions contain a distinct repetition of peptide sequences. For instance, tripeptide Val-Pro-Gly and heptapeptide Gly-Gly-Leu-Xxx-Pro-Gly-Val, where Xxx is Ala or Val, are characteristic of chick tropoelastin. The tetrapeptide Val-Pro-Gly-Xxx (Xxx=Ala or Gly), the hexapeptide Val-Gly-Val-Ala-Pro-Gly and the nonapeptide Ala-Gly-Val-Pro-Gly-Xxx-Gly-Val-Gly (Xxx=Leu or Phe) are observed exclusively in bovine, porcine, and human tropoelastins.

A common repeating sequence is the pentapeptide Val-Pro-Gly-Val-Gly (VPGVG). This amino acid sequence is conserved in

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many animal species such as bovine, chick, porcine, and human, and is considered to play a chief role in the elasticity of elastin. The polypeptide (VPGVG)*n* has been reported to elicit a coacervation property related to elasticity [9,10]. Urry *et al*. [11–13] carried out conformational analyses of (VPGVG)*n* by CD and NMR techniques, and showed that (VPGVG)*n* has a type II Pro^2-Gly^3 β-turn, resulting in an ordered-state conformation called a β-spiral. However, the structural requirements for formation of such a β-spiral structure have never been reported, and thus the structural elements intrinsic for self-association remain unknown. In order to evaluate the contribution of the pentapeptide sequence to the property of coacervation, a series of polymerized peptides (VPGVG)*n*, (VPGV)*n*, (VPG)*n*, and (PGVG)*n* were synthesized in the present study.

We had specific reasons for choosing these peptides. The truncated polytetrapeptide (VPGV)*n* was used to explore the role of the Gly^5 in the separation of the two Val residues, while the properties of polytripeptide (VPG)*n* had the potential to reveal why the VPGVG unit contains two residues of Val and Gly, in the one case interrupted by Pro. Further, we hypothesized that the polytetrapeptide (PGVG)*n* would reveal the importance of the Val^1-Pro^2 interaction and the type II Pro^2-Gly^3 β-turn in the (VPGVG)*n* repeat. By CD, FT-IR, and ^{1}H-NMR methods, structural analyses of all four peptides were performed together with a coacervation assay.

It is difficult to examine by using spectroscopic analysis the conformation of elastin-derived peptides in the coacervated state because their self-assembly property can make the solution turbid under this state. Therefore, different solution systems were used to overcome the issue of turbidity in this spectroscopic study. A report on the study of elastin-derived polypeptide conformation showed the similarity of CD spectrum of polypentapeptide in TFE with that of the coacervated polypentapeptide [9]. In addition, it is well known that TFE stabilizes structure of peptides into an ordered state in the CD measurements. Therefore, TFE was selected and used as a solvent for CD and FT-IR measurements in this study, like the other researchers did in their studies [14–16]. For NMR measurements, we used DMSO as a solvent since DMSO has been widely used for structural analysis of many peptides including elastin-derived polypeptides [13,17], in addition to the ability of DMSO to prevent the turbidity problem in the NMR study.

We here describe that the parent sequence VPGVG is essential for both the construction of a three-dimensional structure and the reversible self-association or coacervation.

Materials and Methods

Peptide Synthesis

All elastin-derived polypeptides were synthesized by the solution-phase method using the Boc strategy. The condensation methods used were the mixed-anhydride method [18] and the EDC/HOBt method [19,20]. Deprotection of the Boc group was carried out in 4 M HCl/dioxane at 0 °C.

The polypentapeptide (VPGVG)*n* was synthesized according to the polymerization technique reported previously [14,21]. Briefly, the monomer unit H-VPGVG-*p*-nitrophenyl ester (-ONp) was first prepared by coupling between a peptide-free acid and *p*-nitrophenol. The resulting H-VPGVG-ONp was polymerized in concentrated DMSO solution for 2 weeks at 25 °C. The polymerized peptide (VPGVG)*n* was collected by dialysis using a 3500 molecular-weight cut-off membrane. The synthetic scheme of the novel polytetrapeptide (VPGV)*n* is shown in Figure 1.

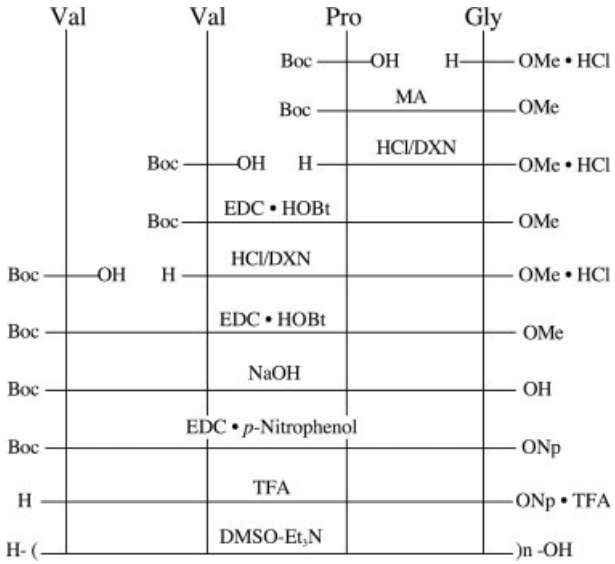

Figure 1. Synthetic scheme of polytetrapeptide (VPGV)*n*, an elastin-derived polymerized analog. To avoid racemization, the monomer VVPG was polymerized; thus, the exact structural denotation should be V-(VPGV)*n*-1-VPG.

The monomer unit of (VPGV)*n* was prepared as VVPG, with Gly located at the *C*-terminal position to avoid racemization in the polymerization reaction. Therefore, the exact structure of polymerized tetrapeptide is denoted by V-(VPGV)*n-1*-VPG. The purity of each monomer unit peptide, H-VPGVG-ONp, H-VVPG-ONp, H-VPG-ONp, and H-PGVG-ONp, was verified by HPLC, and the molecular weights of these peptides were confirmed by MALDI-TOF-MS (Voyager-DE, Applied Biosystems, Foster City, USA). Molecular weights of polymerized peptides were estimated by SDS-PAGE.

The monomers of VPGVG, VPGV, VPG, and PGVG used for ^{1}H-NMR analysis were synthesized by the solid-phase method using the Fmoc strategy. For the coupling reaction, 0.45 M HBTU-HOBt in DMF was used. Fmoc-amino acids, Fmoc-amino acid alko resins, and other reagents for peptide synthesis were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima, Japan). All other chemicals were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Each coupling reaction was monitored by the Kaiser test [22]. After completion of the peptide chain elongation, a final cleavage was carried out by 95% TFA. The purity was confirmed as described above. $(VPGVG)_2$ was also prepared by solid phase synthesis as described above.

Coacervation Measurement

The coacervation property was analyzed by measuring the turbidity of the aqueous solution of each polypeptide. The temperature-dependent turbidity was monitored and traced at 400 nm on a JASCO Ubest-50 spectrophotometer (JASCO Co., Tokyo, Japan). The concentration of the polypeptide solutions was adjusted to 0.40 mg ml^{-1}, and the measurements for turbidity were carried out in function of increasing temperature (from 5 to 60 °C at 0.5 °C min^{-1}) and then of decreasing temperature (from 60 to 5 °C at the same rate). These measurements were repeated through several (at least two) temperature change cycles.

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CD Measurements

CD measurements were carried out in a 0.1 mm cuvette using a JASCO J-725 spectropolarimeter (JASCO). Polypeptides were dissolved in TFE (1.2 mM; estimated by using the molecular weight of each peptide monomer unit) and measured at 25 °C. The spectrum of the neat solvent was subtracted from the spectrum of each sample. Measurements were carried out in the 190–280 nm wavelength range.

FT-IR Measurements

FT-IR spectra of polypeptides in TFE (2.0 mg ml^{-1}) were measured in 0.2 mm cuvettes on a JASCO FT/IR-615 spectrometer (JASCO). Measurements were performed at 25 °C in the range of 400–4000 cm^{-1} with 4 cm^{-1} resolution. Data from the amide I region (1600–1800 cm^{-1}) arising from the absorption caused by C═O stretch vibrations were collected, and the secondary structures were determined by deconvolution (resolution enhancement) and the curve-fitting method [23–26]. In particular, the amide I region was analyzed into individual structural components with reference to the wavenumbers reported [27–29].

^{1}H-NMR Measurements

^{1}H-NMR spectra of peptides were recorded on either a Bruker AM-400 spectrometer (400 MHz) or a JEOL JNM-A500 (500 MHz) at 298 K. Chemical shifts were determined using TMS as an internal standard. All peptides were dissolved in DMSO-d_6 (5 mg ml^{-1}), and the signals were assigned by 2D phase-sensitive double quantum-filtered COSY (DQF-COSY).

Results

Polymer Preparation and Their Average Polymerization Degree

Elastin is distributed in elastic tissues to impart elasticity to the tissues, and the pentapeptide VPGVG takes a major role in these elastic functions. The repetition of (VPGVG)*n* is approximately four to ten times, varying according to species [4–7]. The molecular weights of monomeric peptides used in the polymerization reaction were confirmed by MALDI-TOF-MS as follows; H-VPGVG-ONp: 549.20 (549.57 calcd), H-VVPG-ONp: 491.62 (492.52 calc), H-VPG-ONp: 393.10 (393.39 calcd), and H-PGVG-ONp: 449.20 (450.44 calcd). The molecular weight of synthetic (VPGVG)*n* was measured by SDS-PAGE, and its average molecular weight was estimated to be approximately 60,000. Given this value, the degree of polymerization was calculated to be about 150, much larger than that in native tropoelastin. The polymerization degrees of other polymers, (VPGV)*n*, (VPG)*n*, and (PGVG)*n*, were estimated by the same method and were approximately 170, 120, and 190, respectively.

Coacervation Property of (VPGVG)*n* and Its Analogs

Figure 2 shows the temperature-dependent coacervation profiles of the elastin-derived polypentapeptide and its analogs. It is evident that the (VPGVG)*n* repeat possesses a distinct coacervation property (Figure 2A). It exhibited completely reversible coacervation as reported previously [21]. When the temperature was raised continuously up to 60 °C, starting from 5 °C, no turbidity was observed until 35 °C. Immediately after reaching a temperature around 37 °C, the solution became suddenly turbid, and the turbidity reached a maximal level by 60 °C. This turbidization simply indicates the onset of coacervation in that temperature range.

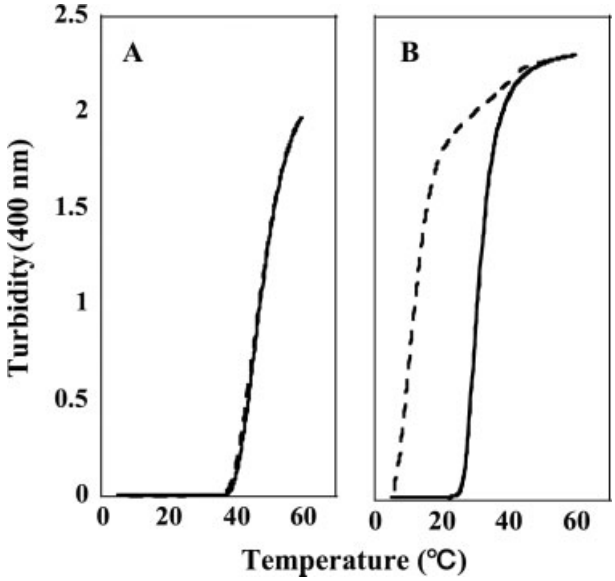

Figure 2. Coacervation profiles of elastin-derived polypeptides (VPGVG)*n* (A) and (VPGV)*n* (B) in the measurement of turbidity at 400 nm. The concentration of each polypeptide was 0.40 mg ml^{-1}. Measurements were carried out by increasing (solid line) and decreasing (dotted line) the temperature between 5 and 60 °C at a rate of 0.5 °C min^{-1}.

The turbidity curve rises with a sharp slope as shown in Figure 2A. When the solution was allowed to cool down, the turbidity curve traced exactly the same path. Thus, the curves of the elevation and decline overlapped completely (Figure 2A). This measurement was repeated several times, and the curves always overlapped.

On the other hand, such an overlap was not observed in the case of the polytetrapeptide (VPGV)*n* repeat. As shown in Figure 2B, the increase in turbidity occurred at 25–50 °C, i.e. at a much lower temperature than that of (VPGVG)*n*. This is a very prominent and definite difference; the temperature difference for the start of the ascent was approximately 10 °C lower than that of (VPGVG)*n* (Figure 2A and B). Another important difference was the steeper slope (*ca* 1.7-fold) of the ascent by (VPGV)*n*. When the slopes of the elevations were calculated, that of (VPGV)*n* was 0.24/ °C, while that of (VPGVG)*n* was 0.14/ °C. Since the molecular size of these polypeptides is almost same, these results clearly indicate that the formation of a highly ordered structure of (VPGV)*n* proceeds much more readily than that of (VPGVG)*n*.

This process of formation of the highly ordered structure can be correlated to the recorded course of decline shown in Figure 2B. The dissociation process consists of at least three distinct steps over a rather broad temperature range (60–5 °C). The first step (60–42 °C) was very slow (0.0003/ °C), but relatively short. The second step (42–20 °C) was moderately slow, at the rate of 0.02/ °C. By contrast, the third step (20–5 °C) was very fast and considerably steep (0.18/ °C). It should be noted that this last steep of dissociation is just like the decline observed for (VPGVG)*n*. These two descent curves appear very similar, based on the similar slopes.

In contrast, no coacervation was observed for (VPG)*n* or (PGVG)*n* (data not shown). Absolutely no turbidity was observed when the temperature was raised to 60 °C. These results suggest that both the Val^1 and Val^4-Gly^5 residues are essential for coacervation. Also,

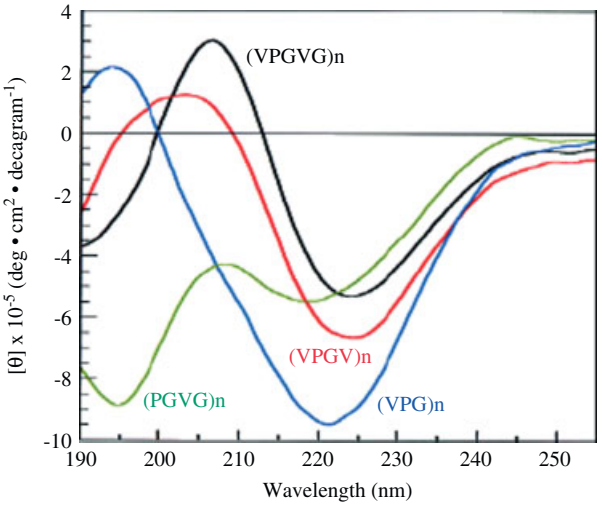

Figure 3. CD spectra of elastin-derived polypeptides (VPGVG)*n* (black), (VPGV)*n* (red), (VPG)*n* (blue), and (PGVG)*n* (green). All polypeptides were dissolved in TFE at a concentration of 1.2 mM (estimated by the molecular weight of each unit), and measurements were carried out at 25 °C.

the Gly^5 residue appears to be important for the reversibility or equilibrium in coacervation.

CD Measurements to Prove the Presence of an Ordered Structure with β-Turn

All the CD spectra were measured at 25 °C, and thus under the solution condition with no coacervation or aggregation. It was previously reported that (VPGVG)*n* exhibits a β-spiral structure, in which Pro^2-Gly^3 forms a type II β-turn at the corner of the spiral [30]. Indeed, in the present study, the spectrum of (VPGVG)*n* exhibited a type II β-turn structure with a distinct negative peak at 224 nm together with a positive peak around 208 nm (Figure 3), just as reported by Urry *et al*. [11]. This profile is certainly a characteristic of the β-spiral structure adopted by elastic polymerized peptides.

The CD spectrum of (VPGV)*n* was found to be very similar to that of (VPGVG)*n*, although a slight blue-shift (*ca* 5 nm) was observed for the positive peak. The total profile indicated the presence of a type II β-turn-like structure as shown by (VPGVG)*n*. Since the positive Cotton effect with a peak at 203 nm was judged to be broad compared to that of (VPGVG)*n*, it is possible that the spiral structure with a β-turn is slightly modified in the polytetrapeptide.

When the spectrum of (VPG)*n* was compared with those of (VPGVG)*n* and (VPGV)*n*, it was clear that its CD profile has a larger negative band at 221 nm, with a maximum blue-shifted approximately by 3 nm compared to the negative maxima of the other two spectra (Figure 3). Since there is a positive band at 195 nm (*ca* 13 nm blue-shifted from that of (VPGVG)*n*), the spectrum shows the predominant presence of a β-structure, probably with consecutive type II β-turns.

On the other hand, the spectrum of (PGVG)*n* was found to be distinctly different from the spectra of any of the other polymeric peptides measured in this study. The curve suggests the presence of an unordered structure characterized by a strong negative band below 200 nm. All the results from CD spectra suggest that the two polypeptides (VPGV)*n* and (VPG)*n* form ordered structures as (VPGVG)*n* does, whereas (PGVG)*n* does not form such an ordered structure.

Table 1. Characteristic frequencies in amide I region of curve-fitted FT-IR spectra of polypeptides

Polypeptide	Band frequency (cm^{-1})	Relative intensity (%)	Assignment
(VPGVG)*n*	1615	20	β-Aggregation
	1633	28	β-Sheet structure
	1656	28	Random coil
	1680	24	β-Turn structure
(VPGV)*n*	1615	14	β-Aggregation
	1633	32	β-Sheet structure
	1656	26	Random coil
	1680	27	β-Turn structure
(VPG)*n*	1615	16	β-Aggregation
	1633	34	β-Sheet structure
	1656	27	Random coil
	1680	23	β-Turn structure
(PGVG)*n*	1605	32	Unknown
	1641	32	Random coil, β-turn and β-sheet
	1672	36	β-Turn structure

Confirmation of the Presence of Carbonyl Group in the β-Spiral Structure by FT-IR

Figure 4 shows the FT-IR spectra of elastin-derived polymeric peptides in the range of 1500–1800 cm^{-1}. In this region, the FT-IR spectra show the presence of absorption bands brought about by a series of amide I carbonyl groups (C═O). Polypeptides (VPGVG)*n*, (VPGV)*n*, and (VPG)*n* exhibited similar spectra, and each curve was decomposed into four components at 1615, 1633, 1656, and 1680 cm^{-1} in the same pattern (Figure 4A–C and Table 1).

The band of 1615 cm^{-1} is considered to indicate the existence of the β-aggregation [28,29]. The absorption band at 1633 cm^{-1} is attributed to the amide C═O groups involved in the hydrogen bonding of the β-sheet structure [27], and intense absorptions were observed for all of the VPG-containing polymeric peptides. The band at 1656 cm^{-1} was attributed to the amide C═O groups of the random coil structure [28,29]. This band was observed in all the repeats, namely, (VPGVG)*n*, (VPGV)*n*, and (VPG)*n*, and thus these polypeptides must contain such non-hydrogen bonding C═O groups in an ordered structure, that is, in a β-spiral structure. In fact, this band is considered to be attributed to Pro^2-C═O and Gly^3-C═O. The band that is attributed to the amide C═O groups in a β-turn emerged at 1680 cm^{-1} [27]. Since the absorption band was elicited by the hydrogen bond that stabilizes the β-turn structure, it was supposed that (VPGVG)*n*, (VPGV)*n*, and (VPG)*n* share a β-turn, where Val$_i$^{1}-C═O is very likely a hydrogen acceptor in the hydrogen bond with Val$_i$^{4}-NH or Val$_{i+1}$^{4}-NH. Judging from the amounts of each decomposed component by deconvolution and the curve-fitting method (Table 1), it was suggested that these three polypeptides contain a similar ordered structure as indicated in the CD measurement. In contrast, the (PGVG)*n* exhibited a different spectrum from those of the other three polypeptides (Figure 4D), and it decomposed into three different absorption bands at around 1605 (unknown), 1641 (random coil, β-turn, and β-sheet), and 1672 cm^{-1} (β-turn).

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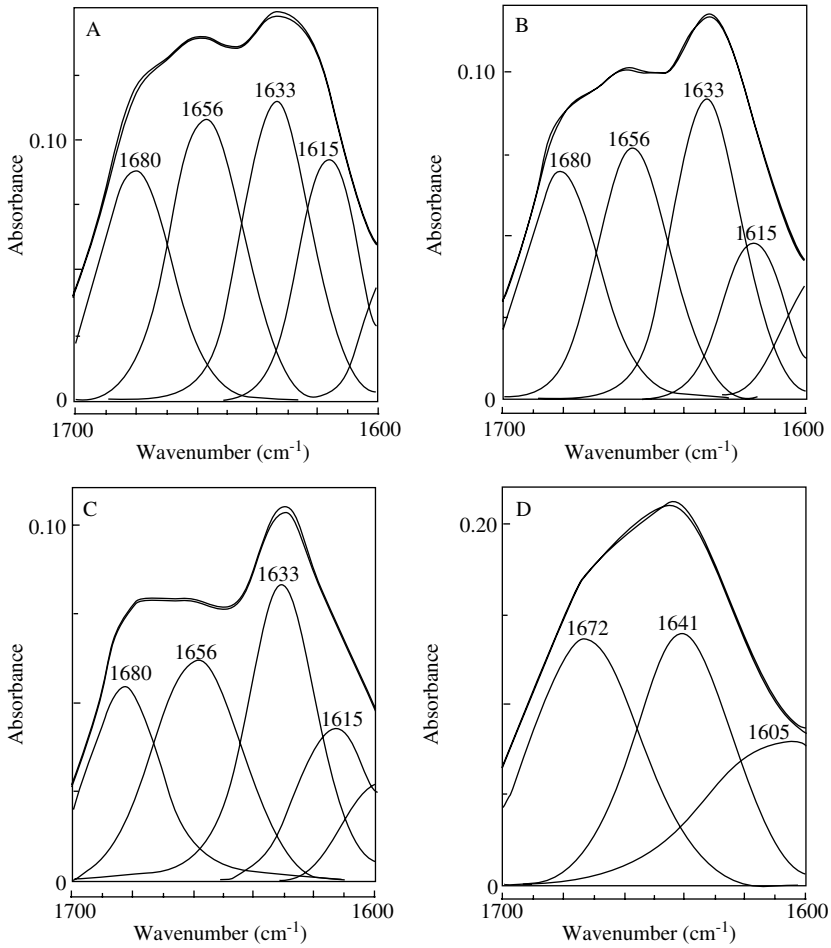

Figure 4. FT-IR spectra of elastin-derived polypeptides (VPGVG)*n* (A), (VPGV)*n* (B), (VPG)*n* (C), and (PGVG)*n* (D). All polypeptides were dissolved in TFE (2.0 mg ml^{-1}), and measurements were carried out at 25 °C in the 400–4000 cm^{-1} wavenumber range.

^{1}H-NMR Analysis of Val-γ-Methyl Groups in an Ordered Structure

In the present study, we measured the NMR spectra of mono-, di-, and polymeric derivatives of the pentapeptide sequence of VPGVG. As shown in Figure 5, for example, very finely resolved resonance signals were observed for all of these derivatives. It was very surprising to attain well-resolved spectra for the polymeric (VPGVG)*n* repeat. The methyl protons of Val^1 and Val^4 were clearly separated and finely resolved (Figure 5). Also, the amide N–H protons of Val^1, Gly^3, Val^4, and Gly^5 were distinctly separated at the baseline and assigned to their respective signals (data not shown). These results definitely indicate that (VPGVG)*n* assumes an extremely highly ordered structure. Such a clear separation of the methyl and amide protons was also identified in the spectra of (VPGV)*n* and (VPG)*n*.

In the (VPGVG)*n* repeat, in which the monomeric peptide VPGVG is repeated over and over again, two different kinds of Val residue emerge: i.e. Val^1 in the G^{5}V^{1}P^2 sequence and Val^4 in the G^{3}V^{4}G^5. The exception is only the *N*-terminal Val residue, which is truncated directly without the preceding Gly^5 residue. Given that these Val^1 and Val^4 residues are present in a strictly ordered structure, they should be grouped distinctively with different structural circumstances. This was indeed demonstrated in the ^{1}H-NMR spectroscopy as shown in Figures 5 and 6. Val is characterized by its side chain isopropyl group, and it can be observed by the NMR-proton signals of methyl groups attached to the β-carbon. The NMR signals of these methyl (CH_3) groups were recorded at the range of 0.7–1.1 ppm. It was immediately apparent that the signals of Val^1 (doublet–doublet; dd) and Val^4 (dd) were completely separate: i.e. 0.84 ppm for the CH_3 of Val^4 in G^{3}V^{4}G^5 and 0.98 ppm for the CH_3 of Val^1 in the *N*-terminal V^{1}P^2 (Figure 5, monomers).

Since the *N*-terminal Val^1 is an exception in the polymeric (VPGVG)*n* repeat, we then measured the dimeric (VPGVG)$_2$ (=$\underline{\text{V}}^1$PG$\underline{\text{V}}^4$G$\underline{\underline{\text{V}}}^{1'}PG\underline{\text{V}}^{4'}$G) to investigate the chemical shift of Val^1 in the GVP sequence. As shown in Figure 6, Val^1 in G$\underline{\underline{\text{V}}}^{1'}$P was easily distinguished. The signal (dd) of this methyl group emerged at the intermediate resonance site of 0.88 ppm. Thus, as seen clearly in the spectrum of the (VPGVG)*n* polymer (Figures 5 and 6), there are two major Val-CH_3 signals: namely, the doublet–doublet at 0.84 ppm (for Val^4-CH_3) and the doublet–doublet at 0.88 ppm (for Val^1-CH_3), although the doublets completely overlap at 0.85 ppm. This is certainly a demonstration that all the Val^1 residues are in the same structural circumstances as the Val^4 residues. Thus, (VPGVG)*n* must have an extremely ordered structure, namely the β-spiral structure.

In the case of the tetrapeptide VPGV and its polymer, the signals of Val^1 (dd) and Val^4 (dd) were found to be much more clearly separated (Figure 5): i.e. the CH_3 of Val^4 in G$^3\underline{\text{V}}^4$V^1 at 0.78 ppm and the CH_3 of Val^1 in V$^4\underline{\text{V}}^1$P^2 at 0.88 ppm. There is no overlapping

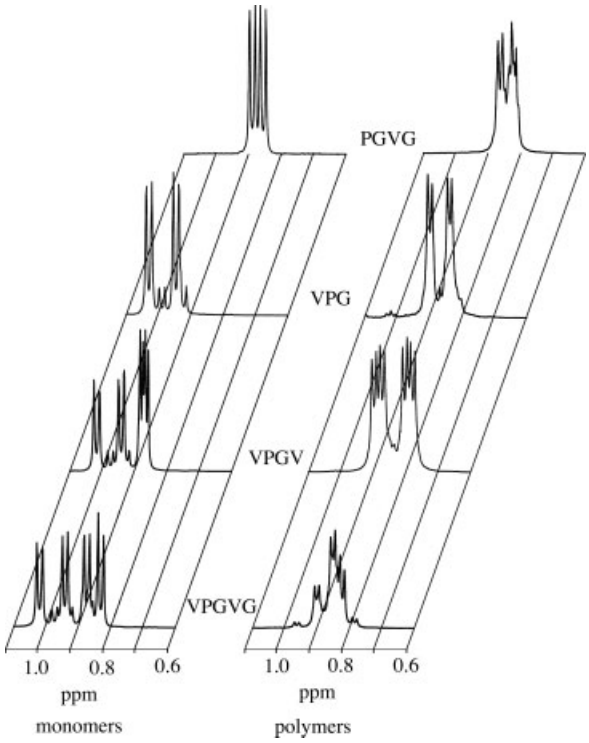

Figure 5. ^{1}H-NMR spectra of Val-γCH$_3$ proton signals of the monomer and polymer of VPGVG together with a series of their shortened derivatives, VPGV, VPG, and PGVG. All peptides were dissolved in DMSO-d_6 (5 mg ml^{-1}).

of signals, and the signals of the doublet–doublet are distinct, indicating that both Val residues are placed in an extremely ordered and restricted conformation.

VPG and its polymer (VPG)*n* exhibited very simple proton signals of Val (dd) at 0.98 and 0.87 ppm, respectively (Figure 5). Since this tripeptide repeat possesses only a single type of Val in the $G^3V^1P^2$ sequence, it is quite reasonable to assume that (VPG)*n* also adopts a highly ordered uniform structure. In contrast, the single-Val-containing (PGVG)*n* repeat exhibited a rather complicated signal pattern as shown in Figure 5. Its monomer showed a very fine *dd* signal at 0.87 ppm. In spite of the presence of a single class of Val in the GVG sequence, the NMR profile suggests that (PGVG)*n* possesses at least two different orientations of Val residues, and thus this repeat adopts a conformation completely different from those of the other repeats using in this study.

The number of repeats in the polymer (VPGVG)*n* sample was estimated to be approximately 150 as mentioned above. Those of the (VPGV)*n* and (VPG)*n* samples were estimated as 170 and 120, respectively. In spite of the extremely high degree of polymerization, their ^{1}H-NMR spectra were finely resolved. These results clearly indicate that the polymers (VPGVG)*n*, (VPGV)*n*, and (VPG)*n* are probably in a uniformly restricted conformation, that is, a β-spiral structure, a β-spiral-like structure, and an ordered structure possibly a β-structure, respectively.

Discussion

The pentapeptide repeat $(V^1P^2G^3V^4G^5)n$ exhibits a coacervation property characterized by reversible association/dissociation. Clearly, the self-association is initiated by not only the intramolecular Val^1-Pro^2 side chain association but also by the intermolecular

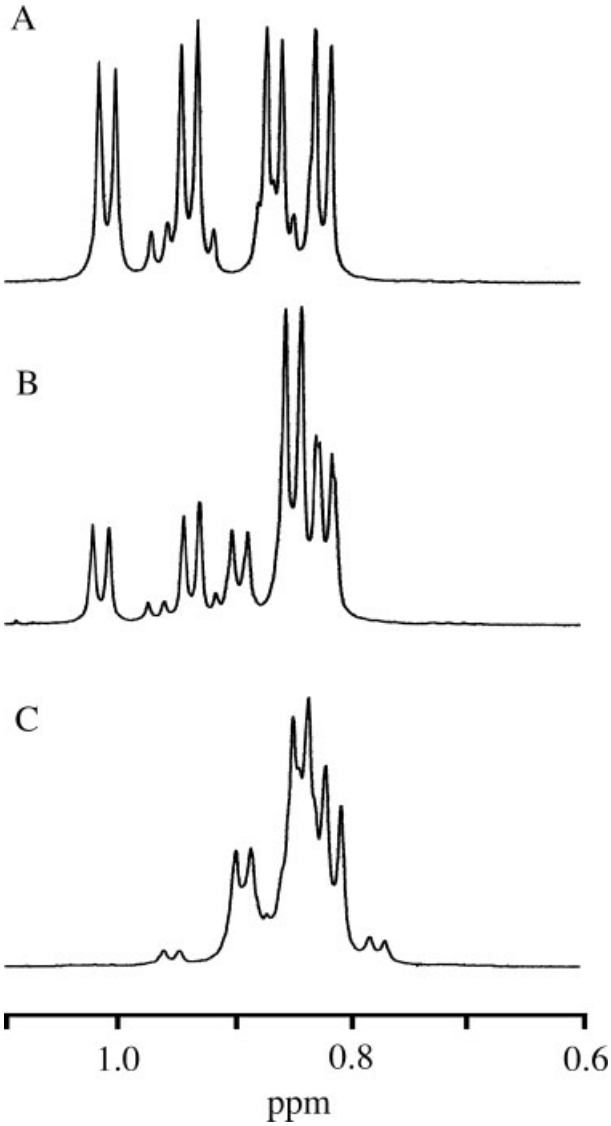

Figure 6. ^{1}H-NMR spectra of Val-γCH$_3$ proton signals of pentapeptide repeats VPGVG (A), (VPGVG)$_2$ (B), and (VPGVG)*n* (C). All peptides were dissolved in DMSO-d_6 (5 mg ml^{-1}).

hydrophobic interactions between Val residues as well. The chief structural characteristic of the pentapeptide repeat $(V^1P^2G^3V^4G^5)n$ is the two line-ups of hydrophobic Val residues. Contact of the Val^4-Val^4 side chain between intermolecular polypeptide is important for proper intermolecular hydrophobic association in $(V^1P^2G^3V^4G^5)n$ [30]. It was reported that there are two basic elements for the β-spiral structure of $(V^1P^2G^3V^4G^5)n$ [31–33]. One is the presence of a type II Pro^2-Gly^3 β-turn as the dominant secondary structure [34,35], and the other is the utilization and optimization of intramolecular hydrophobic contacts resulting in a loose helical structure. It was also described that β-turns are suspended by the Val^4-Gly^5-Val^1 peptide segment with large-amplitude, low-frequency rocking motions of the $(V^1P^2G^3V^4G^5)n$ molecule itself. These characteristics were confirmed in the present study by a series of spectroscopic measurements, i.e. CD, FT-IR and ^{1}H-NMR. The type II Pro^2-Gly^3 β-turn is stabilized by the hydrogen bond between Val^1-CO and Val^4-NH (Figure 7A), and also by the side chain–side chain interaction between Val^1-γCH$_3$ and

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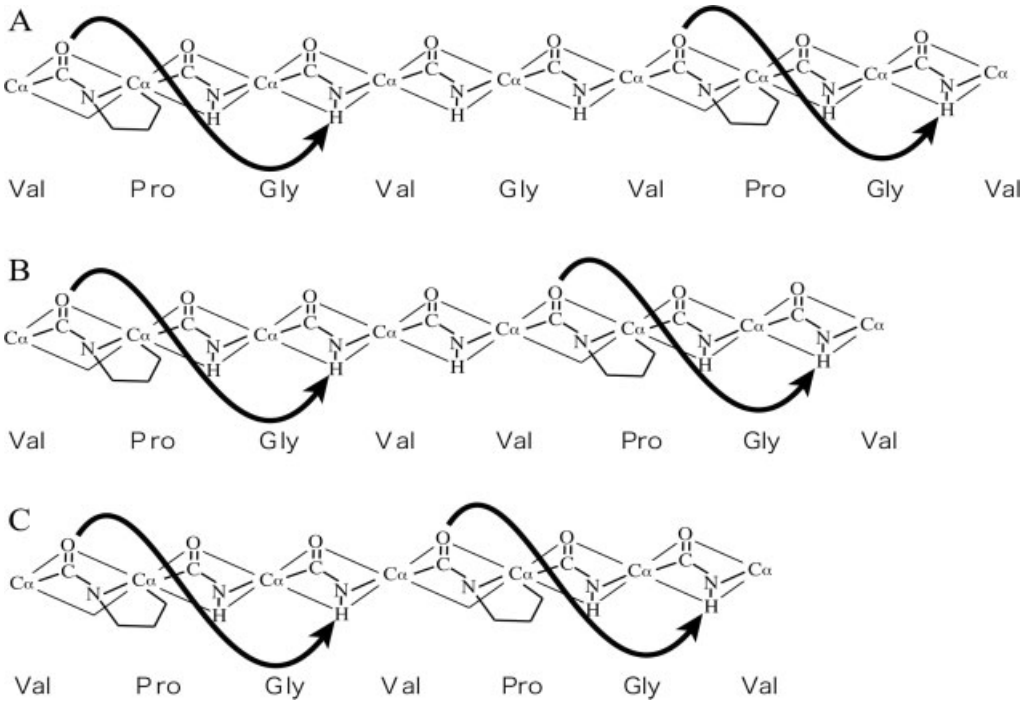

Figure 7. Schematic diagram of the backbone structure of polymeric peptides (VPGVG)*n* (A), (VPGV)*n* (B), and (VPG)*n* (C). Wavy arrows depict the hydrogen bonding between the peptide bonds: i.e. A and B, between the amide-carbonyl group of Val^1 and the amide-imino group of Val^4; and C, between the amide-carbonyl group of Val^1 and the amide-imino group of the next Val^1. Between the two arrows, there are seven linking atoms in (VPGVG)*n* (A), four in (VPGV)*n* (B), and one in (VPG)*n* (C).

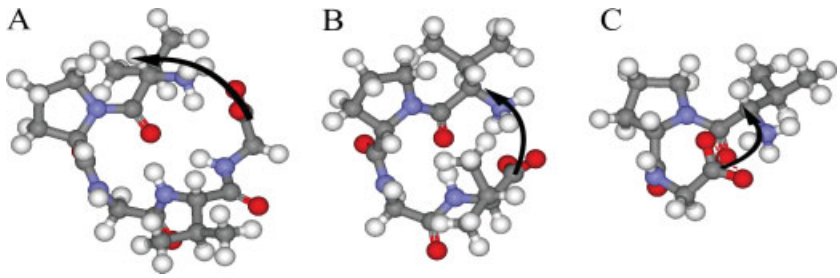

Figure 8. Illustration of a type II β-turn structure putative for polymeric peptides (VPGVG)*n* (A), (VPGV)*n* (B), and (VPG)*n* (C). The first peptide unit structures are depicted, and the curving arrows show the direction of the spiral structure.

Pro^2-βCH_2 as shown in Figure 8A. The hydrogen bond between Val^1-CO and Val^4-NH brings about a ring structure with ten atoms, and the resulting β-turn is repeated over and over by inserting the Val^4-Gly^5-Val^1 peptide segment (Figure 7A). The β-turn hydrogen bonds are separated by seven atoms in the Val^4-Gly^5-Val^1 suspension as shown in Figure 7A. Collectively, the β-spiral structure of $(V^1P^2G^3V^4G^5)n$ is very similar to 3_{10} helix structure.

In this study, the tetrapeptide repeat $(V^1P^2G^3V^4)n$ was found to adopt a β-spiral-like structure. This polymer possesses a type II Pro^2-Gly^3 β-turn stabilized by a hydrogen bond between Val^1-CO and Val^4-NH. The β-turn with the hydrogen bond also makes a ten-atom ring, which is separated from the next ring by only four atoms, namely, $C\alpha^4$-C(=O)-N(-H)-$C\alpha^1$, in the Val^4-Val^1 residues (Figures 7B and 8B). The shortening of the suspension is attributed to the deletion of Gly^5, and this deletion causes the spiral structure to tighten. Further, the deletion of Gly^5 causes the Val residues to be adjacent to each other, resulting in the formation of a very rigid spiral structure. Since the ^{1}H-NMR measurement indicated that all of the Val^1 residues are in the same circumstance, these residues would be placed on the same ridge. Meanwhile, all the Val^4 residues are located on a ridge just adjacent to it; hence, the consecutive Val_i^4-$Val_{i+1}{}^1$ residues create a large ridge consisting of the four methyl groups.

For the Val_i^4-$Val_{i+1}{}^1$ widened row, we denote the line-up with four γCH_3 groups as the *face* and the line-up with one or two γCH_3 groups as the *edge*. In this scheme, the *edge*-to-*edge* intermolecular interaction would require the least energy and be the weakest among the possible interactions of the tetrapeptide repeat $(V^1P^2G^3V^4)n$. On the other hand, the *face*-to-*face* interaction would require the most energy and would also be the strongest. The *edge*-to-*face* interaction would be the intermediate of these interactions. Thus, in the interactions between the tetrapeptide repeat $(V^1P^2G^3V^4)n$ molecules, three different mechanisms, namely *edge*-to-*edge*, *edge*-to-*face*, and *face*-to-*face* interactions, occur in this order with increasing temperature. The first mechanism would be much faster and larger than the latter two mechanisms. It is likely that these mechanisms take place at the same time for the self-association of the Gly^5-deleted polymer $(V^1P^2G^3V^4)n$.

Indeed, this appeared to happen in our turbidity measurement experiments shown in Figure 2B. The tetrapeptide repeat achieved a self-association at a much lower temperature range compared with that by the pentapeptide repeat $(V^1P^2G^3V^4G^5)n$ (Figure 2).

After achievement of self-association, dissociation should occur with the decrease of temperature. It is reasonable to assume that the dissociation would occur in the reverse energy order of the mechanisms of the three self-association interactions; i.e. the order *edge*-to-*edge, edge*-to-*face,* and *face*-to-*face* is reversed. It should be noted that three-step dissociation was in fact observed in the lower temperature range (Figure 2B). The dissociation of the *face*-to-*face* interaction occurred first, and it was the slowest and the smallest in terms of numbers of the interactions among the three mechanisms. Collectively, the consecutive Val_i^4-Val_{i+1}^1 structure arising in the $(V^1P^2G^3V^4)n$ repeat appeared to perturb the dissociation by the additional two mechanisms, the *edge*-to-*face* and *edge*-to-*edge*.

The tripeptide repeat $(V^1P^2G^3)n$ also assumes an ordered structure with a type II Pro^2-Gly^3 β-turn stabilized by a hydrogen bond between Val^1-CO and the NH of the next residue (denoted as $Val^{1'}$ hereafter) $Val^{1'}$-NH (Figure 7C). The ten-atom β-turn ring structures are separated by only a single atom, namely, $Val^{1'}$-Cα, in the Val^1-$Val^{1'}$ residues as shown in Figure 8C. The deletion of Val^4-Gly^5 makes the spiral structure of $(V^1P^2G^3)n$ much tighter than that of $(V^1P^2G^3V^4)n$ (Figure 8). CD spectra of $(V^1P^2G^3)n$ reveal a type II β-bend structure under concentrated conditions even in water at room temperature as reported before [36], suggesting that $(V^1P^2G^3)n$ tends to have ordered rigid structure. Because of the deletion of Val^4-Gly^5, there are successive, uninterrupted β-turns, and this puts the Val residues on a single ridge only. Although each molecule is able to produce something like a fiber by continuous associations, it is impossible to make an assembly owing to the presence of only a single line-up of Val residues. This is compatible with the result of the turbidity experiment. Since the tripeptide repeat $(V^1P^2G^3)n$ is a component of chick tropoelastin as mentioned before, this tripeptide repeat might have a different role in the elastomeric function of elastin.

In the case of the tetrapeptide repeat $(P^2G^3V^4G^5)n$, we did not find any evidence that this polymer adopts an ordered structure. This polypeptide can be regarded as a mutant of for $(V^1P^2G^3V^4)n$, with G^5 in $(P^2G^3V^4G^5)n$ replacing V^1 in $(V^1P^2G^3V^4)n$ when considering the continuous sequence of polymer. Interestingly, this substitution caused a big difference in coacervation property of each polypeptide. This result clearly indicated that this big difference in coacervation property is caused by losing the sequence, VPGV, which is necessary for the coarcervation property. As mentioned above, the presence of $V^1P^2G^3$ is important to stabilize the type II Pro^2-Gly^3 β-turn structure by the hydrogen bond together with the side chain–side chain interaction between Val^1 and Pro^2. Instead, the $(P^2G^3V^4G^5)n$ repeat possesses $G^5P^2G^3$, which lacks the side chain–side chain interaction of Pro^2 because Gly^5 has no side chain. A NOE study has demonstrated the importance of Val^1 and Pro^2 by an increase in the intramolecular hydrophobic association of the γCH_3 of Val and the δCH_2 of Pro at increasing temperatures [37].

In the turbidity measurement experiment, a comprehensible molecular mechanism has emerged to explain the coacervation property characterized by completely reversible association and dissociation of polymeric $(VPGVG)n$. The mechanism can be described as follows. For completely reversible association and dissociation, polymeric peptide repeats should contain a β-turn stabilized by a hydrogen bond between the amide-carbonyl group (C═O) of Val^1 and the amide-imine group (N-H) of Xaa^4 in VPGX; then, the polymeric peptide repeat builds a β-spiral structure. The β-spiral structure creates a line-up of Val^1 residues in addition to a line-up of Val^4 residues to guarantee the intermolecular interaction followed by self-association. This additional line-up of the Val^1 residues is not directly adjacent to the line-up of the Val^4 residues; the two line-ups of Val^4 and Val^1 residues are separated from each other by one or two amino acids.

All of these conditions are realized by the wild-type pentapeptide $(VPGVG)n$ repeat, but not by its shortened analogs $(VPGV)n$, $(VPG)n$, and $(PGVG)n$. All the results in this study demonstrated that VPGVG is a structural element essential to achieving the β-spiral structure that is superlative for self-association followed by coacervation, due to the ideal spatial arrangement of hydrophobic Val residues. In conclusion, only the wild-type $(VPGVG)n$ repeat can play an intrinsic essential role in the elastomeric function of elastin.

There are some reports [29,38] contradicting Urry's studies that $(VPGVG)n$ has β-spiral structure. Although it was suggested that $(VPGVG)n$ has β-turn structure, the clear mechanism of coacervation of elastin-derived peptide was not shown definitely in their reports. Furthermore, it is difficult to explain elastin's completely reversible coacervation property by considering only water molecule adaptation and the β-strand structure of polypeptides. As shown in Urry's recent report [39,40], more detailed structural analysis is needed for determination of the structural change of elastin-derived peptides affected by temperature. A slight modification of his model as presented in this study may explain the precise dynamic mechanism of coacervation.

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