Enantioselective Binding and Stable Encapsulation of α-Amino Acids in a Helical Poly(L-glutamic acid)-Shelled Dendrimer in Aqueous Solutions

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A novel water-soluble peptide-shelled dendrimer containing a poly(L-glutamic acid) segment grafted on the third-generation poly(amido amine) dendrimer was successfully synthesized, and its secondary structural properties and interaction with α-amino acids (Trp, Phe, and Tyr) were revealed by spectroscopic measurements. In the lower pH region, this peptide – dendrimer adopted an α-helix conformation with almost 100% helicity resulting from the three-dimensional aggregation of the segment. Interactions with α-amino acids proceeded with positive cooperativity on the basis of a Hill plot, and as a result, D isomers preferentially bound to the α-helical segments relative to L isomers. The bound α-amino acids were not released into the water phase but were transferred into the inner core of the dendrimer where they remained stable, even when a conformational change of the helix segment was caused by pH variation.

KEYWORDS:

amino acids · enantioselectivity · encapsulation · dendrimers · poly(L-glutamic acid)

Introduction

We report here the first synthesis of a water-soluble poly(L-glutamic acid)-shelled dendrimer as a model protein, along with its conformational properties and the specific binding of α-amino acids.

Much attention has been focused on the molecular design and synthesis of model proteins to clarify interactions involved in protein folding[1, 2] and to develop protein-based materials.[3, 4] Protein tertiary structures can be viewed as assemblies of secondary structural elements(for example, α helices, β strands, and reverse turns). This model has been the basis for the design of artificial proteins. A recent approach to protein design is to use a rigid template molecule.[5, 6] A number of artificial proteins have been prepared in aqueous solutions by attaching peptide blocks to templates that direct the component helices into a protein-like packing arrangement.[7–10] We have devised a strategy in which purely synthetic polypeptides are aligned on two-dimensional media[11] such as water and Au surfaces. Poly(L-glutamic acid) (PLGA) has been chosen as a structural element because of its ease of synthesis and well-defined conformational characteristics in water. The two-dimensionally organized PLGA assemblies were expected to offer a field on which guest molecules could be specifically captured. In fact, it has been reported that surface monolayers carrying oligopeptide moieties as polar head-groups can bind guest peptides specifically.[12] In addition, enantiomeric permeations of α-amino acids were accomplished through thick polymer films based on an α-helical PLGA with the oligo(oxyethylene) side chain[13] and a bundle structure of helical PLGA templated with phosphazene.[14] In these papers, the authors emphasized the importance of the assembled structure of PLGA helices to the enantioselectivity. We have discovered that the monolayer of an amphiphilic PLGA can capture α-amino acids enantioselectively, while the laterally attenuated monolayer with a PLGA segment free amphiphile leads to less selectivity.[15] More recently, we proposed an assembly of poly(γ-benzyl-L-glutamate) on a three-dimensional dendrimer template as a new approach to model protein structure.[16] Several groups have also described the synthesis of peptide – dendrimers.[17–20] These studies are important, especially in view of artificial protein technology.

Dendrimers are hyperbranched macromolecules with a very high concentration of surface functional groups.[21] A variety of dendrimers have been developed by introducing functionalities into these terminal groups. For instance, dendrimers terminated with an amino acid,[22] a sugar,[23] and a perfluoroalkyl[24] or alkyl[25] chain show encapsulation functions towards guest molecules. In view of their biological and pharmaceutical applications, dendrimers are highly effective agents for the delivery of genetic materials into a variety of cell lines.[26] Another structural feature of dendrimers is the high degree of control over molecular weight and shape. The diameters of the spherical

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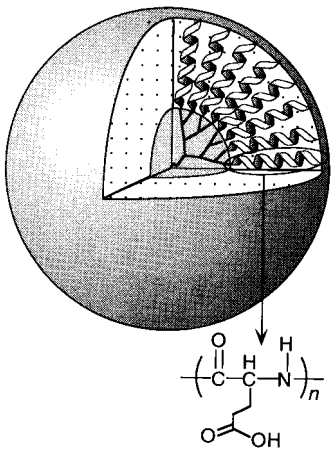
dendrimers range from 3 – 10 nm.[21] Taking account of these features in shape, the polypeptide-attached dendrimer would become a more relevant candidate for model proteins. As a protein model in aqueous media, amphiphilic polypeptides that can spontaneously form assemblies in water, such as micelles and vesicles, have been widely used.[27, 28] Unfortunately, the stability of such self-assemblies may be inferior to that of covalently assembled dendritic molecules.

The aim of the present study is, therefore, to prepare a water-soluble polypeptide-based dendrimer that can adopt an α-helical conformation without losing its applicability as a specific encapsulation system for biomolecules such as α-amino acids. For this purpose, a novel peptide-shelled dendrimer (**G3-PLGA**) has been prepared, which contains the PLGA segment ($n = 34$; Figure 1) as the receptor moiety for α-amino acids grafted onto the periphery of the third generation poly(amido amine) dendrimer (**G3-NH$_2$**). The segment length (n) must be long enough to take the α-helical conformation. The conformational properties of the peptide-shelled dendrimer were examined at different pH values by using circular dichroism (CD) spectroscopy. The dendrimer was found to capture α-amino acids enantioselectively and to incorporate them stably into the dendrimer core.



G3-PLGA (n = 34)

Figure 1. *Spherical model of poly(L-glutamic acid)-shelled dendrimer* ***G3-PLGA*** *(n = 34).*

Results and Discussion

Secondary structure and molecular size of G3-PLGA in water

The secondary structure of **G3-PLGA** in aqueous solution was first examined by CD spectroscopy. Figure 2 A shows CD spectra measured at various pH values. At pH 3.9, the spectrum gives a typical pattern of right-handed helical polypeptides with two negative peaks at 208 and 222 nm. By elevating the pH value to 8.9, the spectrum changes to that of random coil structure with a broad positive peak at 217 nm. These spectral changes with pH value are found to proceed through an isodichroic point at around 204 nm. Figure 2 B displays the pH-dependence of the

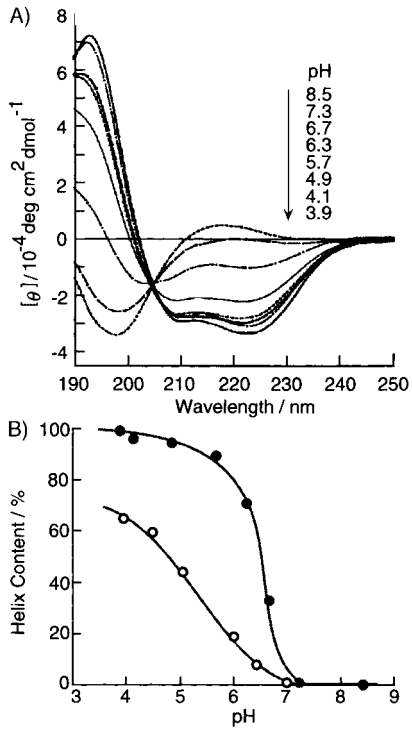


Figure 2. *A) CD spectra of* ***G3-PLGA*** *in water at various pH values. B) The pH dependence of the helix content for* ***G3-PLGA*** *(●) and* ***Pr-PLGA*** *(○). The unit concentration of L-glutamic acid is constant at* $[Glu_{unit}] = 5 \times 10^{-5}$ M.

helix content that was evaluated on the basis of the molar ellipticity per α-amino acid residue at 222 nm.[29] In the lower pH region (< 5), **G3-PLGA** adopts an α-helical conformation with almost 100 % helicity. The helix content decreases steeply in the relatively narrow pH range of 6.5 – 7.0 as a result of the conformational transition of PLGA segments from helix to coil. In this figure, the data for linear **Pr-PLGA**, which was prepared by propylamine-initiated polymerization of γ-benzyl-L-glutamate-*N*-carboxy-anhydride (BLG-NCA) and subsequent debenzylation, is included for comparison. The helix content/pH value curve for **Pr-PLGA** is apparently shifted to the lower pH side, compared with that for **G3-PLGA**, and the conformational transition of **Pr-PLGA** takes place in the broader pH range of 4.5 – 6.5. In addition, it should be noted that the helix content of **G3-PLGA** is much higher than that of **Pr-PLGA** at pH 4.0. These results strongly suggest that the apparent pK_a value of the COOH group of the PLGA segment increased due to the segment interaction induced by attaching the segment onto the dendrimer surface; as a result, the helical conformation of **G3-PLGA** would be more stabilized than that of **Pr-PLGA**, which might not interact over each other. Furthermore, such an aggregation of PLGA segments would significantly contribute to the enhancement in helicity.

The variation in molecular size of **G3-PLGA** with pH value was subsequently examined by a dynamic light scattering (DLS) technique. Figure 3 shows the pH dependence of the **G3-PLGA** particle size (diameter). The observed particle size in the lower pH region (< 6), in which **G3-PLGA** adopts an α-helical conformation, is about 13 nm. This value is in agreement with the theoretical value of 13.2 nm that was calculated by using the

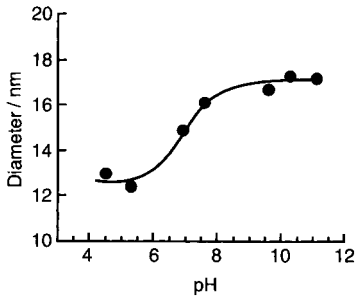


*Figure 3. pH Dependence of the diameter of **G3-PLGA** measured in water at 20 °C by means of a dynamic light scattering.*

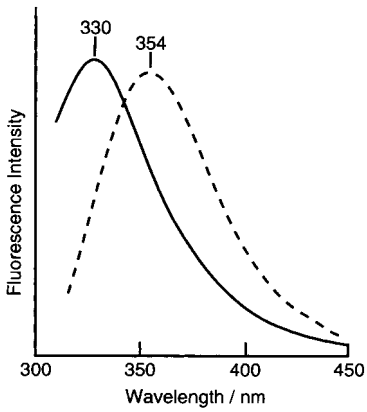


*Figure 4. Fluorescence spectra of D-Trp, excited at 270 nm, in water at pH 4.0 (- - - -) and in **G3-PLGA** aqueous solution at pH 4.0 (——).*

diameter of the **G3-NH$_2$** core (3.0 nm) estimated by DLS and the segment length of PLGA that was evaluated by assuming a complete α-helix conformation; the helix length with $n = 34$ can be computed by using the occupied length (0.15 nm) of one α-amino acid residue along the helix axis. With increasing pH value, the particle size increased sigmoidally and reached a value of about 17 nm. The steep increase observed at around pH 7 must be assigned to the conformational transition of the PLGA segment, since such a pH region is in fair agreement with that observed in the pH dependence of the CD spectra. Therefore, the larger particle size at higher pH values after transition would be due to the extended random coil conformation of the PLGA segment.

Enantioselective binding of α-amino acids to G3-PLGA

The helical **G3-PLGA** was subjected to binding experiments of α-amino acids of tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), all of which have an aromatic ring in the side chain. The interactions were examined by mixing **G3-PLGA** (4.6×10^{-2} μM) with D- or L-amino acids. The amino acid concentration was up to 2 mM on the basis of an equilibrium-binding experiment described later. The pH value was adjusted to 4.0 during the experiment because at this pH value the PLGA segment would adopt an α-helix conformation, and at pH 8.0, where the PLGA segment took anionic random coil structure, no binding of α-amino acids to the **G3-PLGA** had been observed. After an appropriate incubation, the solution was dialyzed to remove unbound α-amino acids prior to spectroscopic measurements. Figure 4 displays a typical fluorescence spectrum of the **G3-PLGA** solution, mixed with D-Trp, in the dialysis tube. The emission of Trp is clearly observed at 330 nm. When the same dialysis experiment was carried out in the absence of **G3-PLGA**, no emission of Trp was observed in the spectrum. Furthermore, the observed emission maximum of 330 nm markedly shifted to a shorter wavelength (354 nm) than that for Trp in bulk water. It has been known for the emission maximum (λ_{em}) of Trp to be considerably affected by its microenvironment. Maste et al.[30] have proposed a classification for the microenvironment of Trp in proteins: 1) Trp residues are placed at a hydrophobic site in the protein ($\lambda_{em} = 330$ nm), 2) Trp residues are placed at a site in which they partly contact water ($\lambda_{em} = 340$ nm), and 3) Trp residues are placed at a site in which they are completely exposed to water ($\lambda_{em} = 354$ nm). We can therefore conclude that the **G3-PLGA**-bound Trp would be present in a hydrophobic environment.

The value of fluorescence depolarization (*P*), which is closely related to the surrounding microviscosity of the fluorescent molecule, for the bound D-Trp (0.19) was found to be much larger than that of D-Trp in bulk water (0.02), which indicates tight binding of D-Trp to **G3-PLGA**. The **G3-PLGA**-bound L-Trp gave a similar *P* value of 0.17. The amounts of **G3-PLGA**-bound Trp were then evaluated by using the absorbance difference of Trp at λ_{max} in UV spectra before and after binding of Trp; these were 4.2 mM for D-Trp and 2.1 mM for L-Trp and can be converted into the number of bound Trp molecules, 91 and 46 per **G3-PLGA** molecule, respectively. This means that D-Trp binds to **G3-PLGA** by a factor of 2 times more preferentially than L-Trp. When the same experiment was performed with **G3-NH$_2$**, no binding of Trp was observed. Thus, Trp binds to the PLGA segments at the dendrimer surface.

The selectivity of D and L isomers is estimated as a ratio (*R*) of the amount of bound D to bound L isomer and is listed in Table 1, together with the data for Phe and Tyr. For each α-amino acid employed, **G3-PLGA** is found to capture D isomers preferentially, although the value of *R* varies depending on the kind of α-amino acid. This effect on the *R* value is supposed to be due not only to steric factors but also to differences in hydrophobicity of the α-amino acid side chains. In fact, our previous work,[31] in which a

*Table 1. Enantioselective interaction between **G3-PLGA** and α-amino acids.*

α-Amino acids	Enantiomer	[Bound α-amino acids] [μM]	R[a]	Hydropathy[b] [kJ mol^{-1}]
Phe	D	9.5	3.0	2.5
	L	3.2		
Trp	D	4.2	2.0	1.5
	L	2.1		
Tyr	D	8.3	1.5	0.08
	L	5.7		

[a] *R* is the ratio of the amount of **G3-PLGA**-bound D isomer and that of L isomer. [**G3-PLGA**] = 4.6×10^{-2} μM. [b] Free energy changes for the transfer of amino acid side chains from a hydrophobic to a hydrophilic phase.

two-dimensional assembly of α helices at the air–water interface (instead of **G3-PLGA**) was used to examine interaction with α-amino acids, emphasized the importance of a hydrophobic atmosphere in enhancing the enantioselectivity. Table 1 includes the value of hydrophathy that indicates a hydrophobicity scale corresponding to the free energy for the transfer of the side chain from an apolar to a polar environment.[32] A larger value of hydrophathy implies that the side-chain group is in a more hydrophobic atmosphere. The observed *R* value is in the same order as that of hydrophathy, which strongly suggests that hydrophobic interaction plays an important role in bringing about such selectivity.

Positive co-operativity in Trp binding to G3-PLGA

The equilibrium ligand (D-Trp) binding experiments have been carried out at constant **G3-PLGA** concentration and increasing ligand concentrations. Figure 5 A shows the titration of a constant concentration of **G3-PLGA** (4.6×10^{-2} μM) with increasing Trp concentrations. The titration curves show saturation behavior at a D-Trp concentration above 2 mM, which indicates that the **G3-PLGA** dendrimer provides a specific binding site. The result was analyzed by using the Hill plot,[33] according to Equation (1) where *N* is the maximum number of D-Trp residues that can bind to **G3-PLGA** (the number of binding sites per **G3-PLGA** molecule), υ is the number of bound D-Trp residues per **G3-PLGA** molecule, [L] is the free ligand (D-Trp) concentration, *K*′ is the apparent affinity constant, and *c* is the Hill coefficient as the index of cooperativity.

$$\log[\upsilon/(N-\upsilon)] = c \times \log[L] + \log K' \qquad (1)$$

Figure 5 B shows the Hill plot. The slope of this plot (*c*) is 1.50, a figure that suggests that D-Trp binds to **G3-PLGA** with considerable positive cooperativity. Such a cooperative binding is probably due to the characteristic shape of **G3-PLGA**, in which the helical rods are radially aligned on the three-dimensional core surface.

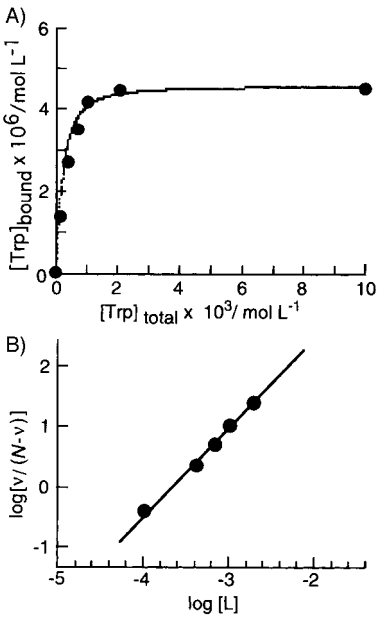


Figure 5. *A) Binding curve of D-Trp to* ***G3-PLGA*** *([****G3-PLGA****] = 4.6×10^{-2} μM) at pH 4.0. B) A Hill plot as described in Equation (1) in the text.*

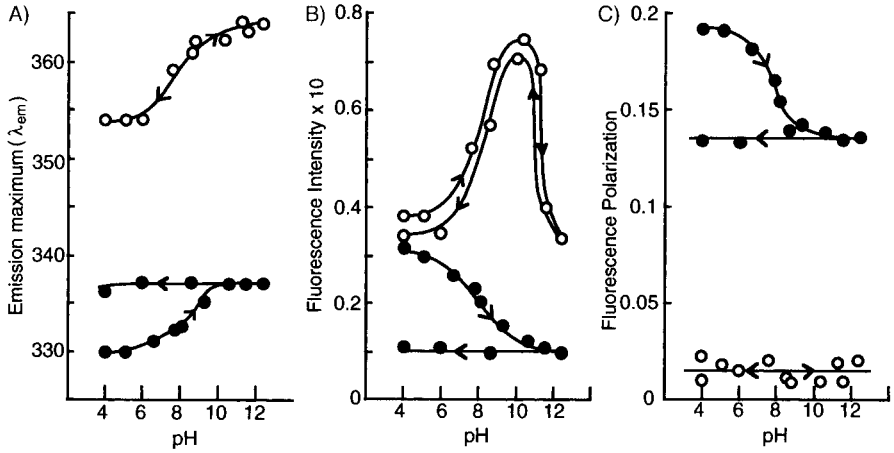


Figure 6. *A) pH Dependence of emission maximum, B) fluorescence intensity, and C) fluorescence depolarization of Trp in* ***G3-PLGA*** *aqueous solution (●) and in pure water (○).*

Conformational effect of PLGA segments on dendrimer-bound Trp

In view of the stable encapsulation of α-amino acids into **G3-PLGA**, it is important to elucidate the effect of conformational change of PLGA segments that was caused by varying the surrounding pH value as described above. Figure 6 displays the pH dependences of fluorescence properties (such as emission maximum (λ_{em}), fluorescence intensity, and fluorescence depolarization (*P*)) of the aqueous solution containing **G3-PLGA**-bound D-Trp. The fluorescence data for free D-Trp residues are also included for comparison. The free D-Trp has strong pH dependence in λ_{em} and fluorescence intensity, but no pH dependence in the *P* value; with increasing pH values, λ_{em} exhibits a marked red-shift and fluorescence intensity gives a maximum at around pH 10. This spectral behavior can be assigned to the microenvironmental change of Trp, which corresponds to the differences in its ionized state and quantum yield.[34] When the pH value was lowered, these values returned reversibly to their original ones. On the other hand, the pH dependences of fluorescence spectra for **G3-PLGA**-bound D-Trp are quite different from those for free D-Trp. Focusing on λ_{em} and *P* values, they exhibit a drastic red-shift and a steep decrease, respectively, with elevating pH values, in particular at pH 6–9 where the PLGA segment of **G3-PLGA** has a conformational transition from α helix to ionized coil. These variations are found to be irreversible, that is, the values at higher pH values were maintained and did not return to their original ones even when

the pH value was lowered to 4.0. It is known that λ_{em} and P value indicate microenvironment and microviscosity of D-Trp, respectively. Thus, the result suggests that the increase in pH value makes the surrounding environment of bound D-Trp more hydrophilic and more fluid (easier in molecular motion). In the higher pH region, above pH 7, the side-chain COOH groups of **G3-PLGA** are deprotonated and behave as anions, and D-Trp also becomes anionic since its isoelectric point (pI) is 5.8. It is, therefore, presumed that in such a pH region the anionic D-Trp will be released from the ionized **G3-PLGA** into the bulk phase owing to electrostatic repulsion. However, the observed spectroscopic data for λ_{em} and P value (λ_{em} = 337 nm, P = 0.13 at pH 12) in aqueous **G3-PLGA** were considerably different from those (λ_{em} = 364 nm, P = 0.03 at pH 12) in the bulk. The fluorescence intensity in aqueous **G3-PLGA** also exhibited a reverse trend to that in the bulk with elevating pH value. These results strongly suggest that D-Trp did not move into the bulk water phase, but preferentially moved into the inner core of the dendrimer.

To prove such a transfer of D-Trp within **G3-PLGA**, the fluorescence properties of D-Trp (5.0 μM) were examined in the presence of PLGA segment free **G3-NH$_2$** at pH 10. Figure 7 displays the **G3-NH$_2$** concentration dependence of λ_{em}, fluorescence intensity and P value. At pH 10, **G3-NH$_2$** would be completely free because the pK_a values for the tertiary amine at the core and the primary amine at the surface are 3.9 and 6.9, respectively.[21a] With increasing **G3-NH$_2$** concentration, a blue-shift of λ_{em} and an enhancement in P value are observed, which implies that the microenvironment of D-Trp becomes more hydrophobic and viscous than that in bulk due to encapsulation in the inner part of **G3-NH$_2$** dendrimer. The observed decrease in fluorescence intensity must be due to concentration effect and/or quenching by interaction with the tertiary amine groups of the dendrimer core.[35] Interestingly, the values of both fluorescence intensity and P are very close to those for **G3-PLGA**-bound D-Trp in the higher pH region (> 10). These spectral features again support the transfer of bound D-Trp into the inner core, induced with the conformational change of the PLGA segment, and they allow us to propose a transfer mechanism as follows (Figure 8). In the lower pH region (< 5), the Trp and PLGA segment interact with each other without any electrostatic factor since their pI and pK_a values are 5.8 and approximately 7, respectively, as described above. Increasing the pH value up to 7 causes both the Trp and PLGA segment to become anionic. It can be expected that the core side of the PLGA segment is more hydrophobic than the water-facing side because of the characteristic molecular shape of **G3-PLGA**, in which PLGA segments are radially aligned on the core (**G3-NH$_2$**) surface. Therefore, the ionization of the PLGA segment should commence on its water-facing side. The anionic layer thus formed (Figure 8C) would protect the anionic Trp from release into the water phase and would at last drive Trp into the core side. Even when the conformation of the PLGA segment (random coil) was again converted into an α helix by lowering the pH value, Trp remained at the core position.

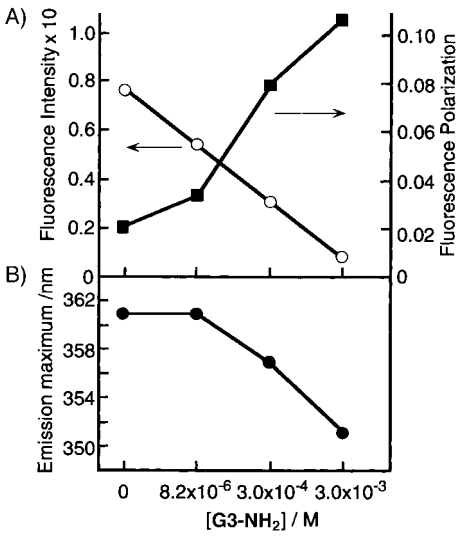


Figure 7. *A) Fluorescence intensity and fluorescence depolarization and B) emission maximum of D-Trp in* ***G3-NH$_2$*** *aqueous solutions at various concentrations.*

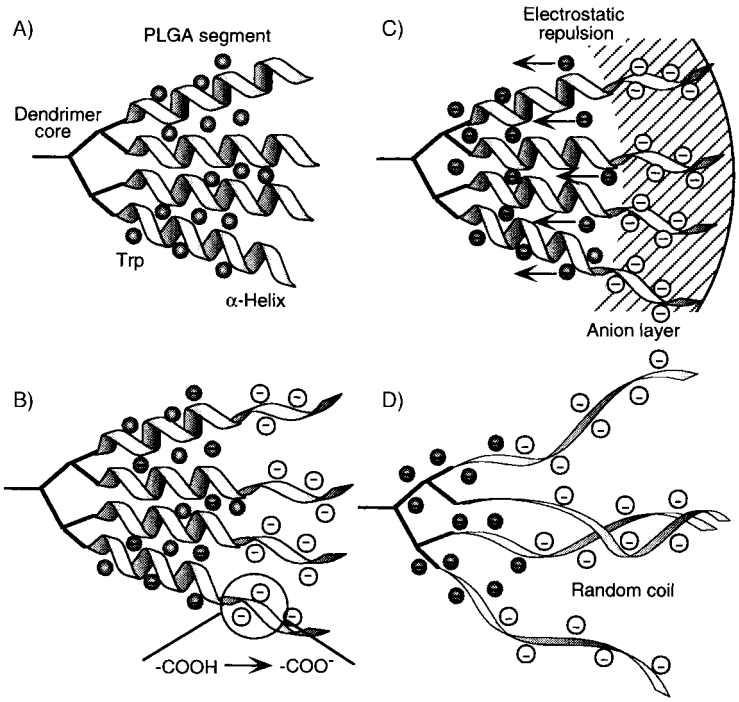


Figure 8. *A possible mechanism for the shift in position of Trp within* ***G3-PLGA****, caused by the conformational change of PLGA segments.*

Conclusion

The present study has described the preparation of a water-soluble helical peptide shelled dendrimer (**G3-PLGA**) and its enantioselective binding and stable encapsulation of α-amino acids. In particular: 1) **G3-PLGA** adopts an α-helix conformation with over 95% helicity in acidic solutions, and at higher pH values has a drastic conformational change to an ionized coil

structure accompanied by an expansion in the molecular size, 2) the equilibrium ligand (α-amino acid) binding experiments indicate that **G3-PLGA** possesses a specific binding site and its process shows positive cooperativity, 3) **G3-PLGA** has the ability to encapsulate D-α-amino acids preferentially and this enantioselectivity strongly depends on their hydrophobicity, 4) **G3-PLGA**-bound amino acids are not released into the outer water phase but are transferred into the dendrimer core by the conformational change of the PLGA segments to an ionized coil structure at increasing pH values.

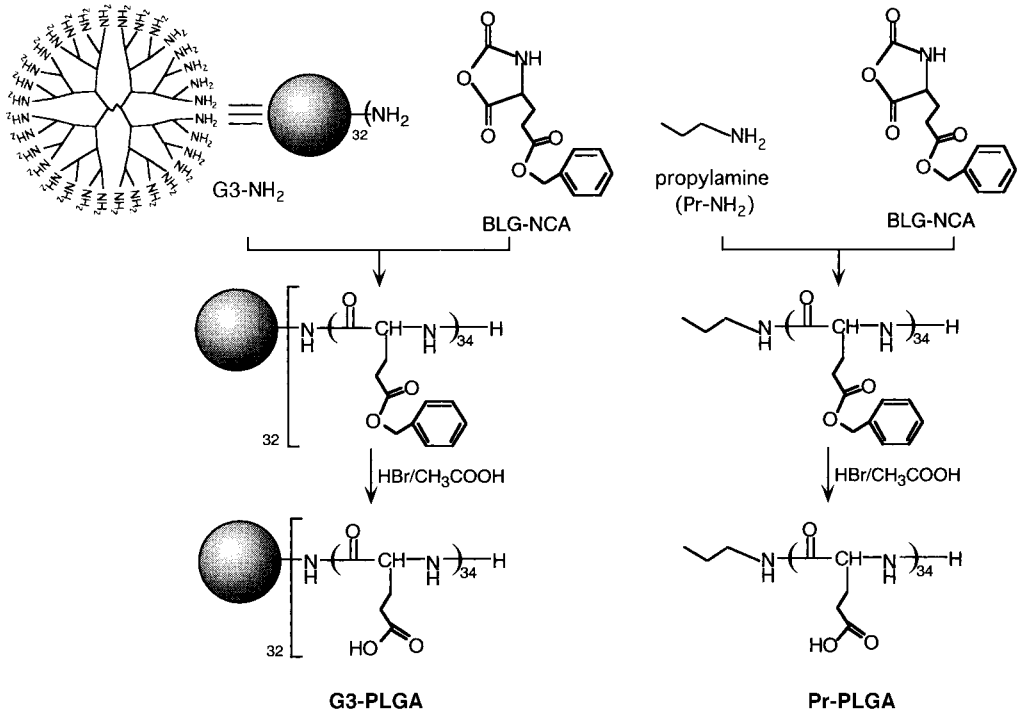
We believe that these findings are of importance not only for a model of higher-order protein structure, but also for a new approach to encapsulation systems of biomolecules based on the characteristic molecular shape of such a peptide-shelled dendrimer.

Experimental Section

Synthesis of poly(L-glutamic acid)-shelled dendrimer: The poly(L-glutamic acid)-shelled dendrimer (**G3-PLGA**) (Figure 1) was prepared according to Scheme 1. Graft polymerization of BLG-NCA was carried out from the primary amino groups of the third-generation poly(amido amine) dendrimer (**G3-NH$_2$**) surface. In the ^{13}C NMR spectra of the polymerized products, no peaks corresponding to the α and β carbons of unreacted terminal amino groups of **G3-NH$_2$** were observed, while those of **G3-NH$_2$** appeared at $\delta = 41$ and 42 ppm, respectively. This means that graft polymerization proceeded from all of the terminal amino groups located at the **G3-NH$_2$** surface. In addition, the degree of polymerization of the polypeptide segment (n) was controlled by adjusting the feed-ratio of monomer (BLG-NCA) to initiator (terminal primary amino groups). As a result, the poly(γ-benzyl-L-glutamate)-modified dendrimer (G3-PBLG, $n = 34$; polydispersity: $M_w/M_n = 1.02$, which was evaluated by means of size exclusion chromatography and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy) was successfully obtained. A detailed description of the synthesis of G3-PBLG has appeared elsewhere.[16] G3-PBLG (1.0 g) thus obtained was dissolved in 5% HBr in acetic acid (50 mL), and stirred for 4 h at 45 °C. The reaction mixture was then poured into a large amount of diethyl ether, and the precipitate was washed with diethyl ether repeatedly and dried. To remove the benzyl groups completely, the same reaction procedure was repeated twice. The structure of **G3-PLGA** was confirmed by means of ^{1}H NMR spectroscopy on the basis of the disappearance of the proton signals of the benzyl groups at $\delta = 5.05$ and 7.25 ppm.

Dynamic light scattering (DLS) measurement: Samples were filtered through Millex-VV filters (with pore sizes of 100 nm, Millipore Ltd.) to remove dust particles in solutions prior to the DLS measurements. DLS measurements were performed in water at 20 °C on a DLS 7000 spectrometer (Otsuka Electric Ltd., Japan) equipped with a He–Ne laser (632.8 nm). The pH value of the sample solution was adjusted with 1 M aqueous HCl or 1 M aqueous NaOH. Scattered light from the sample was analyzed at an angle of 90° from the incident light. The nonnegatively constrained least-squares (NNLS) method was used for correlation analysis to obtain diameter probability distribution functions. The correlation analysis affords directly the diffusion coefficients, from which diameters were calculated by using the Stokes–Einstein relationship.

Circular dichroism (CD) and fluorescence spectroscopy measurements: CD spectra of **G3-PLGA** in water were recorded on a J-720 spectropolarimeter (JASCO Ltd., Japan) under a nitrogen atmosphere ([Glu unit] $= 5.0 \times 10^{-5}$ M). The pH value of sample solutions was adjusted with 0.01 M aqueous HCl or 0.01 M aqueous NaOH. Experiments were performed in a quartz cell with 10 mm path



Scheme 1. *Synthesis of the dendrimer* ***G3-PLGA*** *and the corresponding linear peptide* ***Pr-PLGA****. The PLGA segment length (*n*) of* ***G3-PLGA*** *and* ***Pr-PLGA*** *are controlled to be 34.*

length at room temperature. Fluorescence spectra were measured on an FP-770 fluorescence spectrophotometer (JASCO Ltd., Japan) equipped with polarizers, at an excitation wavelength of 270 nm. Experiments were performed in a quartz cell with 10 mm path length at room temperature. The fluorescence depolarization value (P) was calculated from Equation (2) where I_{vv} and I_{vh} are the fluorescence intensities for the vertical and horizontal components when excited with vertically polarized light and G is a factor for the instrumental correction (that is, $G = I_{vh}/I_{hh}$).

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh}) \quad (2)$$

Binding experiment of α-amino acids: The D- or L-tryptophan (Trp), phenylalanine (Phe), and Tyrosine (Tyr) were purchased from Wako Pure Chemical Industries Ltd. and used without further purification. The binding experiment of α-amino acids was carried out as follows. The D- or L-amino acids (Trp, Phe, Tyr) (2 mM) were mixed with **G3-PLGA** ([**G3-PLGA**] $= 4.6 \times 10^{-2}$ μM) in water (pH 4.0) for 5 h at 25 °C. After incubation, the solution (5 mL) was dialyzed with water (500 mL, pH 4.0, 2 h × 5) with a porous membrane tube (Viskase Co., cellulose, 24 Å, molecular weight cut-off (MWCO) = 12000). In control experiments with **G3-NH$_2$** instead of **G3-PLGA**, a cellulose tube with MWCO = 3500 was used for dialysis. The interaction between α-amino acids and **G3-PLGA** was evaluated by means of UV (Shimazu UV-2100) and fluorescence spectroscopy. The amount of **G3-PLGA**-bound α-amino acid was estimated from an absorbance difference at λ_{max} of each α-amino acid (Trp: $\lambda_{max} = 219$ nm, $\varepsilon = 33994\ M^{-1}cm^{-1}$; Phe: $\lambda_{max} = 206$ nm, $\varepsilon = 8343\ M^{-1}cm^{-1}$; Tyr: $\lambda_{max} = 223$ nm, $\varepsilon = 8456\ M^{-1}cm^{-1}$) in UV spectrum before and after binding of α-amino acid.

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