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Synthesis and Conformational Characterization of Oligopeptide–Cyclotriphosphazene Hybrids

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ABSTRACT: Well-ordered and highly-oriented structures were constructed on a cyclotriphosphazene core by the ring-opening polymerization of *N*-carboxyanhydride of γ-methyl and γ-benzyl L-glutamates with hexakis(4-aminophenoxy)cyclotriphosphazene as an initiator. The polymers were found to form α-helix conformation from the positions of amide I and amide II in fourier transform infrared spectroscopy (FT-IR) spectra and negative Cotton effects in circular dichroizm (CD) spectra. Both the γ-methyl and γ-benzyl L-glutamate hybrids displayed conformational stability in their hexafluoro-2-propanol (HFIP) solutions under temperature changes between 5 and 60°C. The oligo(γ-benzyl L-glutamate) with short chain length (average 8 residue per each chain) showed extremely high α-helical content, which was determined to be almost 100%. These results demonstrate that the cyclotriphosphazene core exerts a strong secondary structure stabilizing effect.

KEY WORDS Six α-Helix Bundle Oligopeptide / *N*-Carboxyanhydride / Organic–Inorganic Hybrid / Enhancement of Helicity / Cyclotriphosphazene /

A number of model compounds have been synthesized in an effort to mimic protein tertiary structure. The template-assembled concept is one of the convenient methods that have been used for this aim. Linear or cyclic peptide templates have been used for covalently binding of parallel α-helical peptides.[1–4] Lieberman[5] and others[6] have synthesized a three-α-helix bundle protein using a tris-bipyridine metal complex as a template. Sasaki and Kaiser[7,8] presented porphyrin as a template and established that porphyrin-linked peptide segments induce the amphiphilic α-helical structure and then facilitate a spontaneous formation of the folded tertiary structure. One of the most remarkable characteristics of that synthetic protein is the high α-helical content, while the single peptide alone has a disordered conformation. Akerfeldt *et al.*[9] introduced tetraphenylporphyrin as a more rigid template for a four-helix proton channel building. The nine-residue peptide template has been utilized for chemoselective ligation[10] of unprotected peptide segments resulting in the total chemical synthesis of protein analogs of native topology. An amido amine dendrimer,[11] cyclotriphosphazene[12,13] have been successfully applied for assembling oligopeptide chains by graft polymerization on surface. It was found that the organized oligopeptide onto a dendrimer template results in drastic enhancement in helicity of the peptide segment.

In this study, we focused on the preparation of well-ordered and highly-oriented structures constructed on a cyclotriphosphazene core. Hexakis(4-aminophenoxy)cyclotriphosphazene has been used as initiator for the ring-opening polymerization of *N*-carboxyanhydride of γ-methyl and γ-benzyl L-glutamates. The structure and conformational properties of the resulting oligopeptide chains were investigated by FT-IR, ^{1}H NMR, and CD spectroscopies.

EXPERIMENTAL

Materials

Hexachlorocyclotriphosphazene **1** (Aldrich) was recrystallized twice from *n*-hexane. 4-Nitrophenol, sodium hydride, and PtO_2 (Aldrich), *N*, *N*-dimethylformamide (for peptide synthesis), and aniline (Wako) were used as received. Tetrahydrofuran (THF) was boiled at reflux over benzophenone–sodium, and distilled over lithium aluminum hydride before use. γ-Benzyl L-glutamate (Wako) and γ-methyl L-glutamate (Aldrich) were used without further purification.

Hexakis(4-nitrophenoxy)cyclotriphosphazene **2** was synthesized in according to the method of Allcock *et al.*,[14] mp 263–265 °C. ^{31}P NMR (DMSO-d_6) δ (ppm): 8.32 (s). ^{1}H NMR (DMSO-d_6) δ (ppm): 8.15 and 7.30 (each d_{AB}, each 12 H, aromatic). IR (KBr) (cm^{-1}): 1590 (aromatic), 1520, and 1350 (nitro group); 1210, 1185, and 1160 (cyclotriphosphazene ring).

Hexakis(4-aminophenoxy)cyclotriphosphazene **3**.

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A 500 mL autoclave was charged with hexakis(4-nitrophenoxy)cyclotriphosphazene (5 g, 5.2 mmol), 100 mL aniline and 0.01 g PtO_2. The mixture was barboted with dry nitrogen during 10 min, then vacuumed. Under hydrogen pressure of 2.5 atm and temperature 50 °C mixture was vigorously stirred for 6 h, then temperature was increased to 65 °C and this temperature was maintained for 4 h. After this time the autoclave was heated to 80 °C until no pressure drop was recorded (within 38 h). The catalyst was filtered off and the filtrate was concentrated to 3 mL under reduced pressure. This viscous oil was dissolved in the minimum amount of concentrated hydrochloric acid, decolorizing charcoal was added, and the resulting suspension was filtered. The stirred filtrate was then slowly treated dropwise with 10% aqueous KOH. The solid was collected by suction filtration and washed successively with water, ethanol and hexane. Recrystallization from *o*-dichlorobenzene afforded 2.9 g (72%) of **3** as off-white plates, mp 171–173 °C. ^{31}P NMR (DMSO-d_6) δ (ppm): 11.07 (s). ^{1}H NMR (DMSO-d_6) δ(ppm): 6.44 and 6.54 (each d_{AB}, each 12 H, aromatic) and 4.92 (s, 12 H, amino). IR (KBr) (cm^{-1}): 1625 (amino), 1508 (aromatic), 1195, 1178, and 1165 (cyclotriphosphazene ring).

γ-Benzyl L-glutamate *N*-carboxyanhydride (BLG-NCA) was synthesized by phosgenation of γ-benzyl L-glutamate using triphosgene in anhydrous THF according to the procedure of Dorman,[15] mp 97–98 °C. ^{1}H NMR ($CDCl_3$) δ(ppm): 7.3–7.4 (m, 5 H, aromatic), 6.71 (s, 1 H, NH), 5.14 (s, 2 H, CH_2 benzyl), 4.40 (t, 1 H, CH), 2.61 (m, 2 H, glutamate γ-C), 2.10–2.31 (m, 2 H, glutamate β-C). IR (KBr) (cm^{-1}): 1859 (C=O, anhydride), 1789 (C=O, anhydride), 1730 (C=O, ester).

γ-Methyl L-glutamate *N*-carboxyanhydride (MLG-NCA) was synthesized in the same way as BLG-NCA, mp 99–100 °C. ^{1}H NMR ($CDCl_3$) δ(ppm): 6.48 (s, 1 H, NH), 4.40 (t, 1 H, CH), 3.70 (s, 3 H, methyl), 2.60 (m, 2 H, glutamate γ-C), 2.05–2.28 (m, 2 H, glutamate β-C). IR (KBr) (cm^{-1}): 1855 (C=O, anhydride), 1786 (C=O, anhydride), 1728 (C=O, ester).

Polymerizations of BLG-NCA and MLG-NCA were carried out in a dry THF or DMF solution in a similar manner, and one representative description of the method will be given. A solution of **3** (14 mg, 17.87 μmol) in DMF (3 mL) was added to a stirred solution of MLG-NCA (500 mg, 2.67 mmol) in DMF (5 mL) at room temperature. The mixture was stirred for 24 h, and then was poured into large excess of dry ether. The pure product was obtained by twice reprecipitation from a hexafluoro-2-propanol (HFIP) solution into ether. Yield 317 mg (80%).

Measurement

^{1}H NMR spectra were recorded using a Varian 400 NMR with internal standard-tetramethylsilane. The measurements were carried out with a sample concentration 7–15 mg mL^{-1} in DMSO-d_6 and a mixture of $CDCl_3$/triflouroacetic acid. ^{31}P NMR spectra were recorded on a Varian 400 NMR spectrometer. Chemical shift positions were related to the external standard-0.485 M triphenylphosphate in $CDCl_3$. FT-IR spectra were recorded with the use a Jasco FT/IR–5 M spectrophotometer for KBr disks and films cast from HFIP/$CHCl_3$. Circular dichroism (CD) measurements were carried out with a Jasco J-725 spectropolarimeter. Spectra were taken in a 3 mL quartz cell with a path length of 1 cm, scanning from 300 to 195 nm every 0.5 nm and average 8 scans. Concentration of glutamate unit was 0.1 mM in HFIP. Size exclusion chromatography was performed on a Waters 600 high performance liquid-chromatograph apparatus equipped with TSK gel Super NM-M column, by using DMF eluent and calibrated by polystyrene standards at 30 °C.

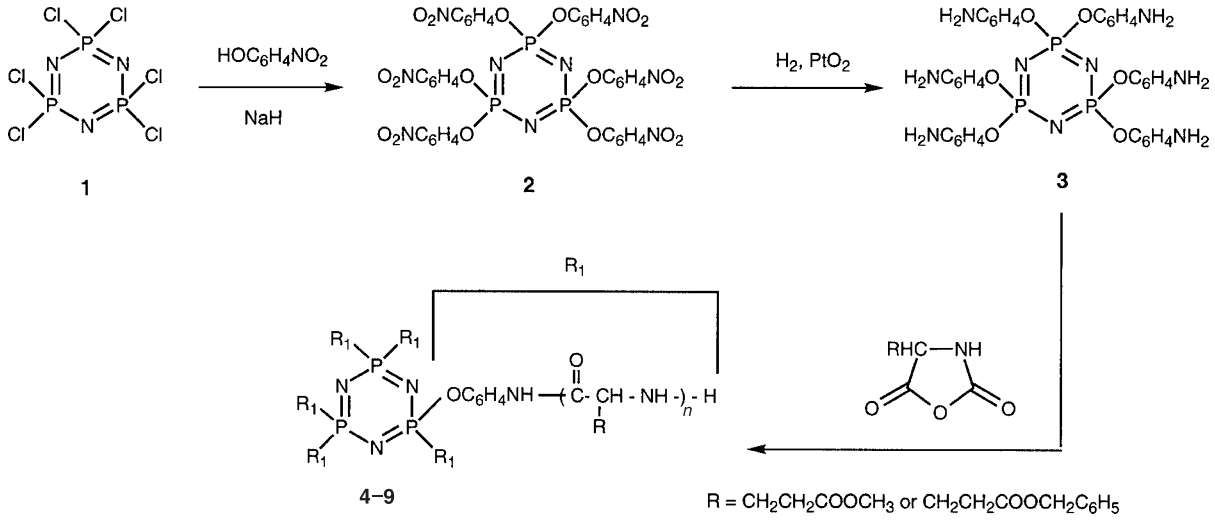
RESULTS AND DISCUSSION

Synthesis and Characterization

The overall reaction sequence for preparation of hexaarmed poly(L-glutamate)s constructed on cyclotriphosphazene core is shown in Scheme 1. The polymerization of *N*-carboxyanhydride of L-glutamates is very delicate because the amine groups are very sensitive to impurities. So, we carefully carried out the synthesize and purification of an initiator, hexakis(4-aminophenoxy)cyclotriphosphazene. Its ^{31}P NMR spectrum showed clear singlet at 11.07 ppm. In the ^{1}H NMR spectrum two doublet peaks for aromatic ring protons appeared at 6.44 and 6.54 ppm and a singlet peak for amine group protons was observed at 4.92 ppm. To prevent undesirable cleavage of anhydride ring, which would lead to oligopeptide non-grafted on cyclotriphosphazene, polymerization was induced at room temperature and in dry solvent.

The monomer to initiator molar ratios had been designed to yield a different length of oligopeptide chains derived from six primary amino groups of hexakis(4-aminophenoxy)cyclotriphosphazene **3**. The results are collected in Table I.

^{1}H NMR spectra of poly(γ-methyl L-glutamate)–cyclotriphosphazene hybrids showed signals at 2.09–2.25 ppm and at 2.60 ppm of β-methylene and γ-methylene protons. The chemical shifts of methoxy protons and α-CH protons appeared at 3.80 ppm and at 4.74 ppm, respectively. The resonance of NH protons resulted in the singlet at 7.95 ppm. The average degree



Scheme 1.

Table I. Ring-opening polymerization of *N*-carboxyanhydride of glutamates initiated by hexakis(4-aminophenoxy)cyclotriphosphazene

Compound	[Monomer] [a] / [Initiator]	Solvent	Yield, %	M_{calcd} ($\times 10^3$) [c]	*DP* [d]
4	720	THF	75 [b]	78.0	90
5	360	THF	67	35.2	40
6	150	THF	78	17.5	20
7	150	DMF	80	17.9	20
8	70	DMF	72	7.9	8
9	70	DMF	70	11.5	8

[a]Monomer is MLG-NCA for **4**–**8**, and BLG-NCA in the case of **9**. [b]Reaction time is 48 h. [c]Molecular weight, calculated as M_{calcd} = 783 + ([Monomer] / [Initiator]) × Yield × $M_{monomer}$ /100. [d]Average degree of polymerization on each ray, calculated as *DP* = ([Monomer] / [Initiator]) × Yield/100/6.

of polymerization (*DP*) was calculated by using intensity ratio of α-CH signal to that of aromatic protons of cyclotriphosphazene moiety. ^{1}H NMR spectra of poly(γ-benzyl L-glutamate)–cyclotriphosphazene hybrids showed signals at 1.97–2.14, 2.49, 4.66, 5.13, 7.27–7.31, and 7.89 ppm, corresponding to β- and γ-methylene, α-CH, benzyl, aromatic, and NH protons, respectively. Aromatic protons of aminophenoxy groups of initiator **3** at 6.44–6.54 ppm disappeared and appeared at 7.20–7.45 in resulting polymer. This indicates that all amino groups of **3** initiated ring-opening polymerization of *N*-carboxyanhydrides without steric hindrance and six oligopeptide chains growing from cyclotriphosphazene center were obtained. Previously, it was reported that in hexakis(4-aminomethylphenoxy)cyclotriphosphazene, all of amino group initiated the polymerization of *N*-carboxyanhydride of γ-benzyl L-glutamate without delay and that the length of polymer chains attached to the phosphazene core are well controlled.[16]

Conformational Characterization

The FT-IR spectra showed that MLG hybrids took β-sheet conformation as well as α-helix conformation: these were assigned from the absorption at 1626 cm^{-1} and 1520 cm^{-1} (amide I and amide II) and at 1652 cm^{-1} and 1544 cm^{-1} (amide I and amide II), respectively. However, in the case of BLG-NCA polymerization only characteristic peaks of α-helical conformation were observed (amide I at 1651 cm^{-1} and amide II at 1545 cm^{-1}). Therefore, it means that the benzyl moiety in the side chain has an important role in stabilizing the α-helix conformation. As shown in Figure 1, the manner of sample preparation also strongly influences the α-helix to β-sheet transition. Absorption bands at 1652 cm^{-1} (amide I) and 1547 cm^{-1} (amide II) of **6** (cast from HFIP) confirm only α-helical conformation (Figure 1a). Though, as it is seen in the spectra of the same compound **6** (KBr disk), considerable amount of the β-sheet form was acquired during the sample preparation (Figures 1b–1c). On the contrary, infrared spectra of **9** show only characteristic bands for the α-helix at 1651 cm^{-1} (amide I) and 1545 cm^{-1} (amide II), independently on manner of the sample preparation (Figure 2). It can be explained by the reason that the α-helix is the most stable conformation for a PBLG chains.[17]

It is not common that the oligopeptides with such short chain length (average degree of polymerization is 8 in the compound **9**) form stable α-helix. The molecular weight of **9** determined by size exclusion chromatography measurement (M_w = 11600 and M_n = 10000), was close to the calculated value of M_{calcd} = 11500. CD analysis performed in an HFIP solution at 20 °C showed strong negative Cotton effects at 218 and 210 nm, which were characteristic for the right-handed α-helix (Figure 3). The helix content calculated from the observed molar ellipticity at 222 nm is determined that as almost 100%.[18] Furthermore, the linear poly(γ-benzyl L-glutamate) **10** having the average degree of polymerization of 8 (initiator of polymerization-propylamine) and the star-shaped poly(γ-methyl L-glutamate) **8** showed the helix content 44 and 67%, respectively. Shoji *et al.*[17] reported that linear poly(γ-benzyl L-glutamate)s with degree of polymerization between 4 and 10 take β-sheet form, and the oligomer with DP = 16 and high polymers adopt the α-helix conformation. Perhaps, in the case of the present star-shaped polymers, additional intramolecular interactions including hydrophobic effect between the helices might be a driving force for the enhancement of the stability of helical structure. Such effect was found by organization of oligopeptide onto a dendrimer template.[11]

The CD spectra of the poly(γ-methyl L-glutamate) hybrids also showed two negative bands at 218 and 210 nm, confirming that the oligopeptide chains took mainly right-handed α-helical conformation. The helix contents for **4**–**7** calculated from the observed molar ellipticity at 222 nm were evaluated to be in the range of 43–78%. Both the γ-methyl and γ-benzyl L-glutamate hybrids displayed stable α-helix conformation in their HFIP solutions. Figure 4 presented the temperature dependence of **7** on CD spectra. The increasing of the temperature induced a negligible conformational transition in the poly(γ-methyl L-glutamate) chains. We prepared a linear poly-MLG **11** by polymerization of MLG-NCA initiated with propylamine (DP calculated by using the intensity ratio of the signal of CH_3 protons

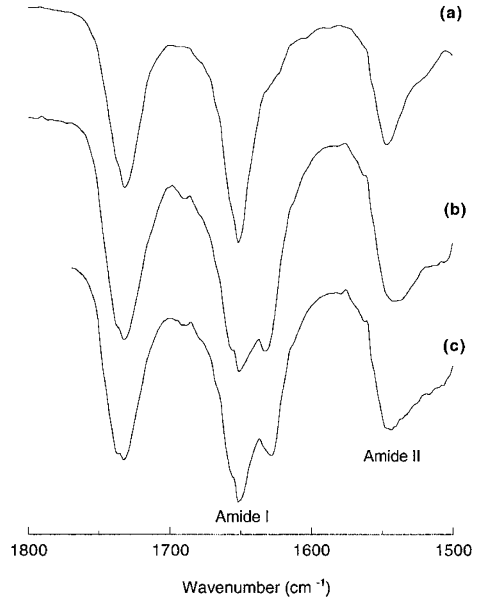


Figure 1. FT-IR spectra of **6**, 1800–1500 cm^{-1} region: (a) casting film, (b) KBr disk using the HFIP cast film, and (c) KBr disk.

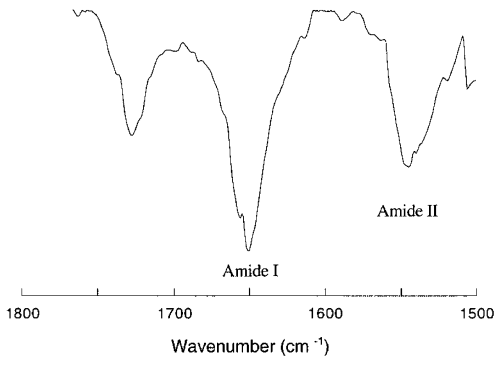


Figure 2. FT-IR spectrum of **9**, KBr disk, 1800–1500 cm^{-1} region.

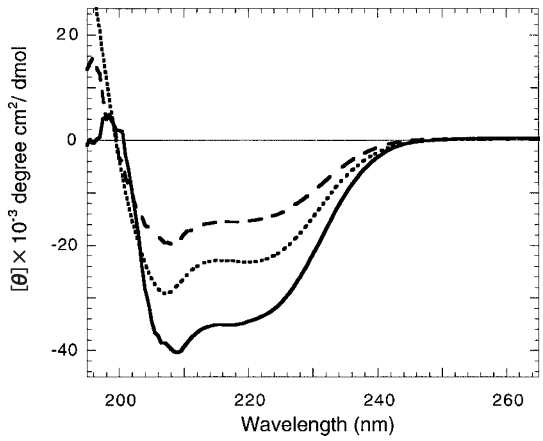


Figure 3. Circular dichroism spectra of **8 (●●●), 9 (—)**, and **10 (- - -)** in HFIP solution at 20 °C.

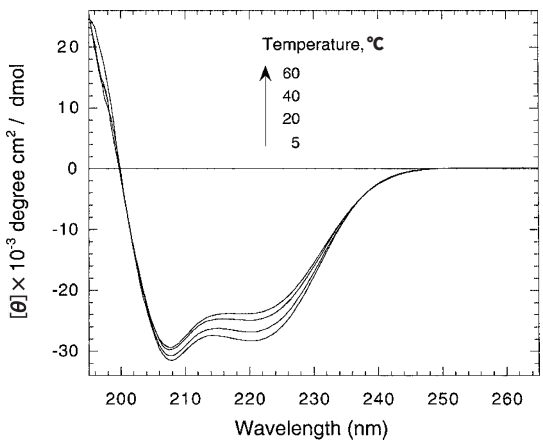


Figure 4. Temperature dependence of **7** on circular dichroism spectra in HFIP solution.

in propylamine to that of O–CH_3 of peptide was found to be 20). The decreasing of helix content of this compound upon increasing the temperature was determined to be almost 22%. However, in the case of compound **7** that was found less than 15%. This is one of the proofs that α-helical conformation is stabilized on the phosphazene core.

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