Self-Association of Cholesteryl-Bearing Poly(L-lysine) in Water and Control of Its Secondary Structure by Host−Guest Interaction with Cyclodextrin

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ABSTRACT: A cholesteryl-bearing poly(L-lysine) (CHPLL) was synthesized by the condensation reaction of cholesteryl *N*-(6-isocyanatehexyl) carbamate with poly(L-lysine) (PLL). Sonicated samples of CHPLLs, which were substituted by 0.8, 3.4, and 5.4 cholesteryl groups per 100 lysine units, gave a single peak after sufficient ultrasonication by high-performance liquid column chromatography. The particle sizes (RG) and aggregation numbers (*N*), which were determined by static light scattering, increased with an increase in the DS of cholesterol in CHPLL (R_G = 16−22 nm, N = 1.3−4.2). These CHPLLs form an α-helical structure at a lower pH compared to the parent PLL by circular dichroism spectroscopy. The partial modification of PLL by hydrophobic cholesteryl groups leads to the formation of hydrogel nanoparticles by their self-association, and this induces the α-helical structure of PLL. This α-helicity can be controlled by the degree of substitution of cholesteryl groups. The helical content of CHPLL decreased upon the addition of *β*-cyclodextrins, which were complexed with cholesteryl groups and reached a value similar to that of unmodified PLL. The secondary structure of CHPLL was controlled by host−guest interaction with cyclodextrin.

Introduction

The partial modification of hydrophobes to water-soluble polymers significantly affects their solution properties in water due to association of the hydrophobes.[1,2] This association is influenced by the parent water-soluble polymers, the hydrophobicity of the hydrophobes, and the degree of substitution.[3−7] We previously reported that cholesteryl-bearing nonionic polysaccharides such as cholesteryl-bearing pullulans (CHP), which are partly substituted by hydrophobic cholesterol moieties, form stable and monodispersive nanoparticles (20−30 nm) by intermolecular self-aggregation in dilute aqueous solution.[8−10] In semidilute solution, they form a gel.[11] The size or density of the nanoparticles can be controlled by changing the hydrophobicity or the degree of substitution of the hydrophobic groups. The CHP self-aggregate binds various hydrophobic substances[9] and various soluble proteins.[12,13] The hydrophobic modification of hydrophobic cholesteryl groups plays an important role in the unique properties of CHP in water. This is a new method for preparing monodisperse hydrogel nanoparticles by the self-assembly of amphiphilic polymers. To investigate the scope of this method, we selected a polyelectrolyte, poly(L-lysine) (PLL), as a parent polymer instead of a nonionic polysaccharide. PLL has been widely used not only in basic studies but also in biotechnological and pharmaceutical applications. Complexes of PLL derivatives with DNA have been shown to be useful for delivering DNA into a cell.[14] Cross-linked PLL gel derivatives have been used as a biodegradable matrix.[15] PLL forms unique conformations in solution, such as α-helix and *β*-sheet structures, and it is easy to detect a conformational change in PLL by circular dichroism (cd).[16] In this paper, we focus on the self-association of cholesteryl-bearing poly(L-lysine) (CHPLL) in water and its effect on the secondary structure of CHPLL by the association of hydrophobes. The secondary structures of poly(amino acids) strongly depend on the pH, temperature, solvent, and the substitution of various functional molecules.[17−21] There have been few studies on the conformational change of a polymer chain induced by the association of hydrophobized polymers.

Cyclodextrin (CD) forms an inclusion complex with various small molecules[22] in water and also forms an interesting channel-type complex with water-soluble polymers such as poly(ethylene glycol).[23] Recently, Harada et al. reported the interaction of CD with side-chain guest groups attached to a polymer chain.[24] We also reported that a self-aggregate of cholesteryl-bearing pullulan can dissociate upon the addition of *β*-cyclodextrin (CD) due to capping of the hydrophobic cholesteryl groups by complexation with CD.[25] Reassociation of the cholesteryl groups can be induced by adding 1-adamantanecarboxylic acid (ADC), which is a better substrate for *β*-cyclodextrin. Using this method, we can construct an artificial molecular chaperone system.[26] In this paper, we also describe changes in the secondary structure of CHPLL by host−guest interaction with cyclodextrin (CD).

Experimental Section

Synthesis of cholesteryl-bearing poly(L-lysine) (HCl) (CHPLL) was synthesized by the condensation reaction of cholesteryl *N*-(6-isocyanatohexyl)carbamate (**1**) with PLL. Cholesteryl derivative **1** was synthesized as reported previously.[8] Next, **1** (40 mg, 71.7 nmol) in 1 mL of dry pyridine was added to PLL (HBr) (208 mg, 956 nmol equiv as lysine units) (M_w 49 000, M_w/M_n = 1.22 or M_w 51 000, M_w/M_n = 1.15, Sigma) in 100 mL of dry DMSO containing 1.0 mL of triethylamine at 25 °C for 30 h. The reaction mixture was dialyzed against water, 0.01 N HCl aqueous solution, and finally water using a membrane

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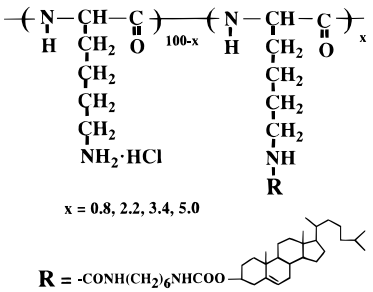

Figure 1. Chemical structure of cholesteryl-bearing poly(L-lysine) (CHPLL).

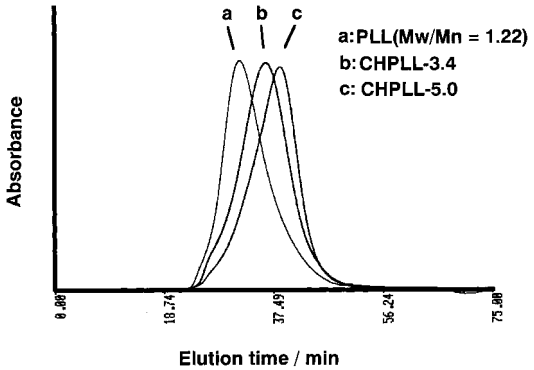

Figure 2. Elution profiles of PLL and CHPLL. High load Q-Sepharose HP column 16/10 (Pharmacia) in 20 mM Tris-HCl buffer pH 8, 30 °C, 250 mL/min detected by UV at 222 nm.

tube (Spectra Pore 6 MWCO2000). The mixture was then lyophilized to give a white powder; yield 168 mg (96%). The product was purified by chromatography (Q Sepharose Fast Flow, Pharmacia Biotech, 25 mm diameter × 50 cm). The degree of substitution by cholesteryl groups was determined by ^{1}H NMR. For example, when poly-L-lysine (M_w 49 000 or 51 000) was substituted by 3.4 cholesterol groups per 100 lysine units, it was coded as CHPLL-3.4 (M_w 49 000). CHPLL-0.8 (M_w 51 000) and CHPLL-5.0 (M_w 49 000) were synthesized by the same methods. All other reagents were commercially available and used without any further treatment. Sample preparation CHPLL(HCl) (1−2 mg/mL) was suspended and swollen in water or buffer solution under stirring for 12 h at 25 °C to give a turbid suspension. The transparent suspension was obtained by further sonication using a probe-type sonifier (TOMY, UR-200P) at 40 W for 10 min and filtration (pore size 0.2 mm). No significant degradation of the polymer was observed during sonication. The reproducibility of the sample preparation was carefully checked by HPLC (High Load 16/10 Q-Sepharose HP column). The HPLC system (Tosoh Ltd., Tokyo) consisted of a CCPD dual pump, a RI-8010 refractive index detector, an UV-8010 UV detector, and a Chromatocorder 12 data-processing system with a GPC extension module.

Static Light Scattering (SLS) Measurements. SLS measurements were performed on a DLS-700 (Otsuka Electronics, Osaka, Japan) equipped with a 5 mW He−Ne laser (vertically polarized, wavelength 633 nm) and a thermoregulated bath (RTE-110, Neslab). The scattering angle was varied from 30° to 130°. The average molecular weight and radius of gyration (R_G) were calculated by the Zimm plot.

Circular Dichroism (cd) Measurement. The cd spectra were obtained using quartz cuvettes (1 mm) on a JASCO J-720 spectrometer equipped with a thermoregulated cell compartment under constant nitrogen flush in a cell with an optical path length of 0.1 cm at 25 °C. The relative α-helix content of PLL or CHPLL was calculated from the ellipticity at 222 nm by using $[\theta_{222}] = -34\,000$ as 100% helix content.

Fluorescence Study. Fluorescence spectra were obtained on a Hitachi F-3010 fluorescence spectrophotometer equipped with a thermoregulated cell compartment. A stock solution of pyrene in ethanol was added to the CHPLL solution. The final concentration of pyrene was 2.5×10^{-6} M. Pyrene was excited at 339 nm. The slit width was set at 5 nm for excitation and 1.5 nm for emission.

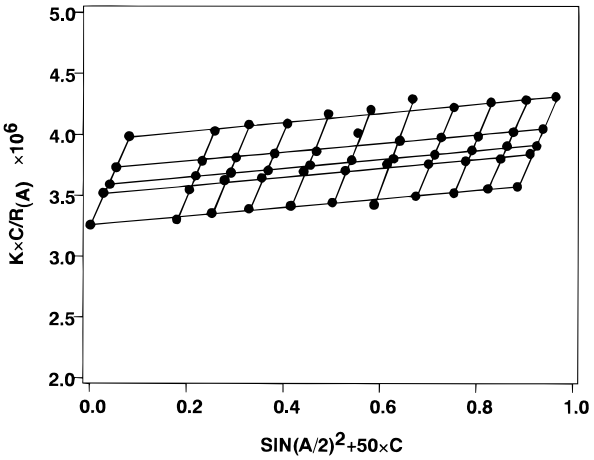

Figure 3. Double-reciprocal Zimm plot for a sample solution of CHPLL-5.0 in water (0.1 M KCl) at pH 7.0 and 25 °C.

Table 1. SLS Measurement of Cholesteryl-Bearing Poly(L-lysine)

polymer	M_w	R_G (nm)	A_2 (mol cm^3 g^{-2})	aggregation no.
CHPLL-51-0.8	0.9×10^5	16	15.1×10^{-4}	1.3
CHPLL-49-3.4	2.2×10^5	22	3.8×10^{-4}	3.1
CHPLL-49-5.0	3.1×10^5	21	2.3×10^{-4}	4.2

Results and Discussion

Self-Aggregation of CHPLL. The solution property of CHPLL in dilute aqueous solution at neutral pH was investigated by high-performance column chromatography (HPLC) and static light scattering (SLS). The unimodality of the associate of CHPLL was investigated by HPLC before the measurement of SLS. The sample of CHPLL, a cationic polyelectrolyte, did not elute well in the usual size exclusion column chromatography (Superose 6 or Superdex 200) in water due to the adsorption of CHPLL. To avoid adsorption, an anion exchange column, Q-Sepharose, which is a cationic gel partly substituted by a trimethylaminomethyl moiety, was used. The Q-Sepharose column provides size exclusion column chromatography at a molecular weight of PLL between 26 000 and 100 000. A unimodal peak was obtained for samples of all of the CHPLLs and the parent PLL after sufficient ultrasonication. Typical examples of the chromatograms are shown in Figure 2. The retention time of the void volume of the column was about 25 min under these conditions. Although we cannot evaluate the exact size of CHPLL by HPLC, the chromatograms show that CHPLL exists as a unimodal distribution in water after sufficient ultrasonication.

SLS was investigated to determine the size and molecular weight of CHPLL. Figure 3 shows a typical example of a Zimm plot of CHPLL-5.0 over the range 0.53−1.58 mg/mL at pH 7.0 in 0.1 M KCl at 25 °C. The results for the cholesteryl derivatives of PLL are summarized in Table 1. The intensity of the light scattering of the parent PLL was too low to obtain significant data. CHPLLs tend to form intermolecular self-aggregates at neutral pH. The particle size and aggregation number increased with an increase in the degree of substitution (DS) of cholesteryl groups of CHPLL. Once the ag-

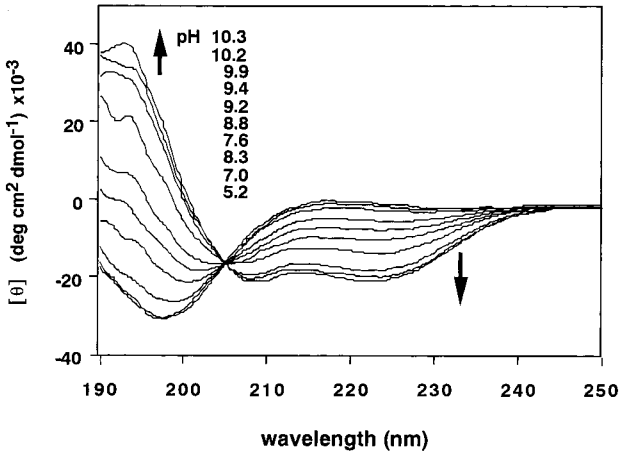

Figure 4. Circular dichroism spectrum of CHPLL-3.4 at various pH in water (0.1 M KCl) at 25 °C. pH was adjusted by 0.1 N KOH and 0.1 N HCl.

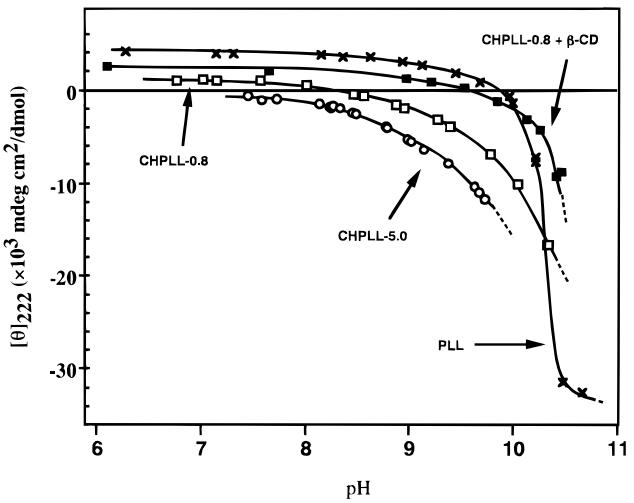

Figure 5. Plots of ellipticity at 222 nm, [θ_{222}], for PLL, CHPLL-0.8, CHPLL-5.0, and CHPLL-0.8 in the presence of β-cyclodextrin (15 mM) as a function of pH in water (0.1 M KCl) at 25 °C. [CHPLL] = 0.4 mg/mL. pH was adjusted by 0.1 N KOH and 0.1 N HCl.

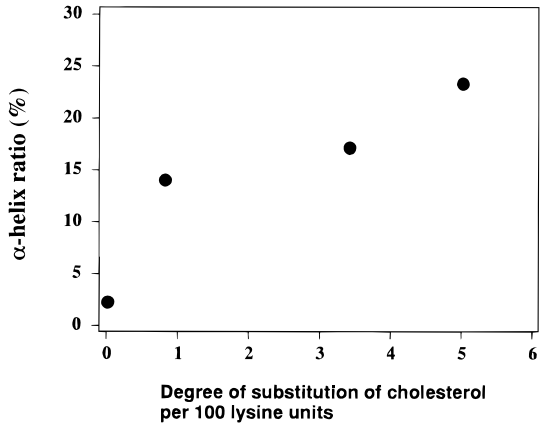

Figure 6. α-Helix content of CHPLLs as a function of the degree of substitution of cholesterol in water (0.1 M KCl) at pH 9.0. [CHPLL] = 0.4 mg/mL.

gregates were prepared at neutral pH, they remained stable at relatively high pH. At higher pH, it is difficult to prepare monodispersive samples for use in the SLS experiment due to their aggregation.

A fluorescence probe experiment using pyrene was carried out to investigate the association of the cholesteryl moiety of CHPLL in water. The ratio of the fluorescence intensity at 384 and 377 nm (I_3/I_1) was used to monitor the microenvironment around the excited pyrene. [27] This value increases in a more apolar microenvironment. The I_3/I_1 value for all of the CHPLLs (0.84) was similar to that for the self-aggregate of cholesteryl-bearing pullulan (0.80) and to those for micellar systems such as Triton X-100 (0.76), cetyltrimethylammonium bromide (CTAB) (0.77), and sodium dodecyl sulfate (SDS) (0.88).[27] Such spectral changes were not observed under controlled conditions with the parent PLL (0.55). These results indicate that the cholesteryl groups of CHPLL associate and form a hydrophobic domain in water. The I_3/I_1 value in CHPLL did not change from pH 7 to pH 10. Pyrene is mainly located in the hydrophobic domain of cholesteryl groups. We previously reported that hydrophobized polysaccharides formed self-assembled hydrogel nanoparticles, in which the associated domains of hydrophobic moieties were cross-linking points.[10] CHPLLs in this study may form a similar nanosize hydrogel.

Secondary Structure of CHPLL. PLL shows a coil-to-α-helix transition depending on pH. [21] CHPLL showed a similar coil-to-α-helix transition with increasing pH (Figure 4). Figure 5 shows plots of ellipticity at 222 nm [θ_{222}] for PLL and various CHPLLs as a function of pH. The plots of CHPLLs were made in the pH range in which sharp isosbestic points were observed (Figure 4), since the ellipticity at higher pH may not be correct due to the light-scattering effect and precipitation of CHPLL, based on its high hydrophobicity. Although we cannot determine the complete helix−coil transition curve for CHPLL for the above reason, the cooperativity in the transition seems to decrease by hydrophobic modification, and the helix begins to form at a lower pH compared with PLL. It is well-known that negative ellipticity at 222 nm corresponds to a helical structure for a PLL. Figure 6 shows the change in the content of the α-helix of CHPLL at pH 9.0 as a function of the DS of cholesteryl groups. CHPLL with a higher DS of cholesteryl groups tended to form an association with a higher α-helicity at lower pH than PLL. The substitution of cholesteryl groups in PLL significantly affects the conformation of PLL below about pH 10.

Control of the Secondary Structure of CHPLL by Cyclodextrin. We reported that a self-aggregate of cholesteryl-bearing pullulan can dissociate upon the addition of β-cyclodextrin (CD) due to capping of the hydrophobic cholesteryl groups by complexation with CD. To obtain information on the importance of the association of cholesteryl groups in the conformational change of CHPLL, the interaction of cyclodextrin with the CHPLL self-aggregate was investigated by cd spectroscopy. Figure 7 shows the cd spectrum of CHPLL-0.8 in the absence and the presence of β-cyclodextrin at pH 9.4. The isosbestic point in the cd spectrum of CHPLL seems to change upon the addition of CD. The helical content decreased with the addition of CD and reached that of unmodified PLL. No spectral change was observed in the case of an unmodified PLL and CD system under similar conditions. Figure 5 shows plots of ellipticity at 222 nm [θ_{222}] for CHPLL-0.8 in the presence of β-cyclodextrin (15 mM) as a function of pH. In the presence of CD, associated cholesteryl group domains are dissociated due to capping of the hydrophobic surface of cholesteryl groups. As a result, the

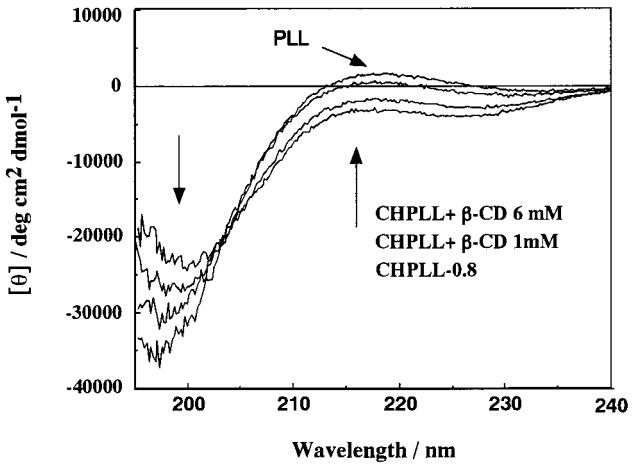

Figure 7. Circular dichroism spectrum of CHPLL-0.8 (0.3 mg/mL) in the presence and absence of β-cyclodextrin at 25 °C and pH 9.4 (50 mM tetraborate buffer solution).

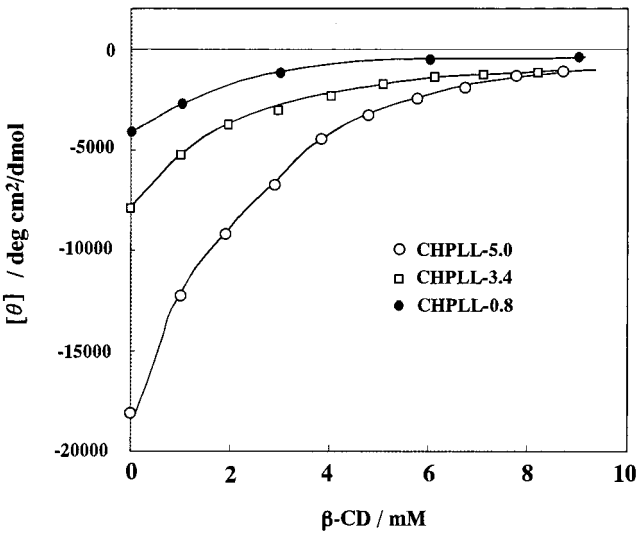

Figure 8. Change in ellipticity at 222 nm for CHPLL-0.8, CHPLL-3.4, and CHPLL-5.0 (0.3 mg/mL) as a function of the concentration of β-cyclodextrin at 25 °C and pH 9.4 (50 mM tetraborate buffer solution).

conformation of the PLL chain of CHPLL seems to return to its original state similar to unmodified PLL. Figure 8 shows the change in ellipticity at 222 nm of various CHPLLs as a function of the concentration of β-cyclodextrin added at pH 9.4. CHPLLs with higher cholesteryl content (CHPLL-3.4 and CHPLL-5.0) show similar behaviors. The helical content of CHPLL-0.8 in the presence of β-cyclodextrin increased upon the addition of 1-adamantanecarboxylic acid (ADC), and finally, the helical content returned to the original value before the addition of β-cyclodextrin (Figure 9). In the case of CHPLLs with higher cholesteryl content, light scattering by the solution increased due to aggregation after the addition of ADC under a similar condition of CHPLL-0.8. The binding constant (3.2×10^4) of ADC with CD is higher than that of cholesterol (1.6×10^4). In the presence of excess ADC, ADC was complexed by β-cyclodextrin instead of the cholesteryl group of CHPLL. The free cholesteryl group of CHPLL then associated again, and the helical content increased. The results indicate that the association of the cholesteryl groups of CHPLL is an important factor in the conformational change of parent PLL.

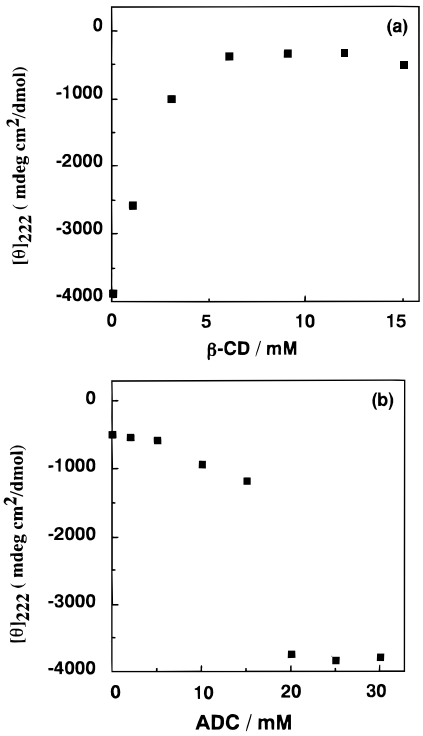

Figure 9. Change in ellipticity at 222 nm for CHPLL-0.8 (0.3 mg/mL) as a function of the concentration of β-cyclodextrin (a) at pH 9.4 (50 mM tetraborate buffer). (b) shows the change in ellipticity at 222 nm for a CHPLL-0.8 (0.3 mg/mL)−CD (15 mM) mixture as a function of 1-adamantanecarboxylic acid (ADC) at pH 9.4 (50 mM tetraborate buffer) and 25 °C.

Conclusion

The partial modification of PLL by strong hydrophobic groups such as cholesterol leads to the formation of hydrogel nanoparticles by self-association. This association can induce an α-helical structure of PLL at lower pH in water. The secondary structure of CHPLL is regulated by host−guest interaction with CD and ADC. Hydrogel nanoparticles of cholesteryl-bearing pullulan have been widely used as a drug-carrier system for anticancer drugs,[28−30] insulin,[31] and vaccine[32] and also as an artificial molecular chaperone.[26] Hydrogel nanoparticles of poly(amino acids) may have similar applications in biotechnology and medicine.

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