Reversibility of Structural Transition of Cytochrome *c* on Interacting with and Releasing from Alternating Copolymers of Maleic Acid and Alkene

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The interaction of cytochrome *c* (cyt c) with poly(isobutylene-*alt*-maleic acid) (PIMA) and poly(1-tetradecene-*alt*-maleic acid) (PTMA) was studied using circular dichroism, absorption spectroscopy, and atomic force microscopy to investigate the electrostatic and hydrophobic influence of the copolymers on the structure of cyt c. At pH 7.4, the interaction of PIMA with cyt c can only partly disturb the integrity of the heme pocket, while PTMA has very intensive influence on the structure of cyt c. After adding 0.15 M NaCl, PIMA−cyt c complexes dissociate, and the released cyt c recovers its native structure, whereas NaCl has no significant influence on PTMA−cyt c complexes. GuHCl (0.5 M) destroys PTMA−cyt c complexes, forming GuHCl−PTMA precipitates; the cyt c released from the complexes regenerates its native structure. In comparison with electrostatic interaction, hydrophobic interaction leads to more stable polymer−cyt c complexes and more intensive influence on cyt c structure, but cyt c can recover its native state after release.

Introduction

The interactions of globular proteins with oppositely charged polymers can form soluble complexes, coagula, or precipitates,[1−3] depending on the concentrations of protein and polymer, pH,[4,5] and ionic strength of aqueous solution.[6] The interactions of proteins with polymers have been widely studied because of the practical applications of polymers in protein separation and purification, protein immobilization and stabilization,[7−9] protein folding,[4−6,10] and protein encapsulation and release.[11−17] In protein delivery systems, polymers are often used to form polymer−protein microparticles or nanoparticles[11−14] to protect proteins and facilitate proteins crossing critical and specific biological barriers and hitting specific targets.[18]

The native structure is essential for a protein to keep its biological function.[19] The structure of a protein is maintained by electrostatic, hydrophobic, and van der Waals interactions, hydrogen and disulfide bonding.[20] All the interactions, except disulfide bonds, may occur between proteins and polymers. It has been reported that, in cytochrome *c* (cyt c) binding on negatively charged lipid membranes[21−27] or SDS micelles,[28] highly helical structure remained, but tightly packed native tertiary structure disappeared, and Met80 heme iron ligation cleaved. Our previous study found that the interaction between randomly sulfonated polystyrene and cyt c caused an intensive disruption of the native compact structure of cyt c: the cleavage of Fe-Met80 ligand, about 40% loss of the helical structure, and the disruption of the asymmetry environment of Trp59.[6] The denaturation of cyt c is caused by the electrostatic and hydrophobic interactions.[6,28,29] However, up to now, as far as we know, there have not been systematic reports on the structural transition of proteins induced by interacting with polymers and the recovery of the structure of the proteins released from the polymers.

Cyt c plays important roles in the electron-transfer chain and programmed cell death.[30−32] Because of its small size, stability, well-studied structure, as well as the increasing evidence for its physiological roles in both soluble and membrane-bound forms,[33] cyt c has been widely studied in the areas of protein chemistry, protein function, and protein folding.[6,21,34,35] On the other hand, the copolymers of maleic acid and alkene and their derivatives are a series of well-characterized polymers. The structure of these copolymers is jointly determined by the ionization degree of carboxyl and the length of alkyl chain.[36−38] Alternating copolymers of maleic acid and alkene, alternating polyelectrolytes and polymeric surfactants, are suitable biomimetic models due to their resemblance of alternating order of phospholipid biomembranes.[39,40] Our previous study found that, in aqueous solutions, poly(isobutylene-*alt*-maleic acid) (PIMA) was molecularly soluble, whereas poly(1-tetradecene-*alt*-maleic acid) (PTMA) formed nanoparticles which trended to dissociate at low PTMA concentration and at neutral and alkaline pH. PIMA and PTMA could interact with heme-free apo cyt c, the precursor of cyt c, to induce apo cyt c to make a conformational transition from random coil to α-helical structure, and the content of the helical structure depended on the electrostatic and hydrophobic interactions and hydrogen bonding between protein and copolymer. The interactions of PTMA and apo cyt c at neutral and alkaline pH destroyed the aggregates of PTMA and apo cyt c alone, forming apo cyt c−PTMA complex particles.[4,5]

In this report, we study the respective interactions of cyt c with PIMA and PTMA at the physiological pH of 7.4, where electrostatic attraction exists between the positively charged protein and the negatively charged copolymers. The substantial difference in the length of the alkyl chains between PIMA and PTMA makes it possible to estimate the effect of hydrophobic interaction between the copolymer and the protein. NaCl and GuHCl (guanidine hydrochloride) were added to the cyt c−copolymer complex particle dispersion to release cyt c. Circular dichroism, absorption spectroscopy, and atomic force

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microscopy were used to investigate the structural transition of cyt c on interacting with the copolymers and its structure recovery after releasing from the copolymers.

Experimental Section

Materials. Horse heart cyt c (type VI) was purchased from Sigma and used without further purification. Poly(isobutylene-*alt*-maleic anhydride) with M_w 6000 and 39 repeat units and poly(1-tetradecene-*alt*-maleic anhydride) with M_w 9000 and 31 repeat units were purchased from Aldrich. PIMA and PTMA were obtained by hydrolysis of poly(isobutylene-*alt*-maleic anhydride) and poly(1-tetradecene-*alt*-maleic anhydride) at alkaline solution, as reported previously.[4] All samples were prepared using water that was deionized to a resistance of 18 MΩ.

Sample Preparation. Cyt c was dissolved in 10 mM sodium phosphate buffer of pH 7.4. The concentration of cyt c stock solution was 100 μM, which was measured spectrophotometrically using a molar extinction coefficient of 1.06×10^5 M^{-1} cm^{-1} at 410 nm.[41] PIMA and PTMA stock solutions were prepared in 10 mM sodium phosphate buffer of pH 7.4 with the concentration of 4.5×10^{-4} g/mL.

The samples were prepared by titrating PIMA or PTMA stock solution into 10 mM phosphate buffer of pH 7.4, and then titrating cyt c stock solution with shaking. For the samples containing NaCl or GuHCl, NaCl or GuHCl was titrated after the mixture of cyt c and polymer was left at room temperature for 3 h. The final concentrations of NaCl and GuHCl are 0.15 and 0.5 M, respectively. All the samples were left at room temperature overnight before measurement. All the measurements were performed with at least two batches of samples, and the average data were reported.

Circular Dichroism (CD) Measurement. CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Naslab temperature controller. The path lengths were 0.1 cm for far-UV (190−250 nm) and 1 cm for near-UV (250−350 nm) and Soret region (350−500 nm). The ellipticity was recorded at 200 nm/min of speed, 0.2 nm of resolution, 8 of accumulations, and 1.0 nm of bandwidth. At each given temperature, the sample was allowed to equilibrate for 10 min before the spectrum was recorded. The temperature was increased stepwise from 25 to 95 °C. Buffer background was subtracted from the original spectra.

Absorbance Measurement. Absorption spectra were measured at room temperature on a Lambda 35 spectrophotometer (Perkin-Elmer). The path length was 1 cm.

Atomic Force Microscopy (AFM) Measurement. AFM images were obtained using tapping mode on a Nanoscope IV of Digital Instruments equipped with a silicon cantilever with 125 μm and E-type vertical engage piezoelectric scanner. The AFM samples were prepared by dropping the samples on freshly cleaved mica and then drying naturally at room temperature.

Results and Discussion

Horse heart cyt c is a well-characterized small protein. In native cyt c, there are α-helix in its secondary structure, a covalently bound heme group coordinated with two strong-field ligands, His18 and Met80, and single tryptophan (Trp59) whose fluorescence is largely quenched due to resonance energy transfer to the adjacent heme group.[21,42−44] A Soret absorption at 410 nm indicates a strong-field low-spin state of the heme iron, while a Soret absorption at 394 nm and a new band at 620 nm indicate a high-spin state of the heme iron, i.e., the ligands of the heme iron are replaced by two weak-field ligands from a solvent, such as water. The absorption at 695 nm is the feature of Met80−iron ligand.[41,45,46] Two negative peaks at 208 and 222 nm in far-UV CD spectrum show a typical α-helical structure. Two sharp minima at 282 and 288 nm in the near-UV CD spectrum have been assigned to a tight tertiary structural packing in the vicinity of Trp59. The Soret CD spectrum shows a strong negative band at 418 nm and a positive band at 405 nm, which are considered to represent a characteristic fingerprint for the integrity of the heme pocket structure, because they originate from the coupling of the electronic transition dipole moments of the heme and nearby aromatic amino acids. These amino acids (Trp59, Tyr67, Phe82) are located on the Met80 side of the heme so that changes in the Soret CD spectrum should specifically reflect structural perturbations of this part of the heme pocket.[42,47] In the following study, the molar ellipticities of cyt c at 222 (θ_{222}), 288 (θ_{288}), and 405 nm (θ_{405}) were measured to monitor α-helical structure, tight packing in the vicinity of Trp59, and the integrity of the heme pocket of cyt c, respectively.[42,47]

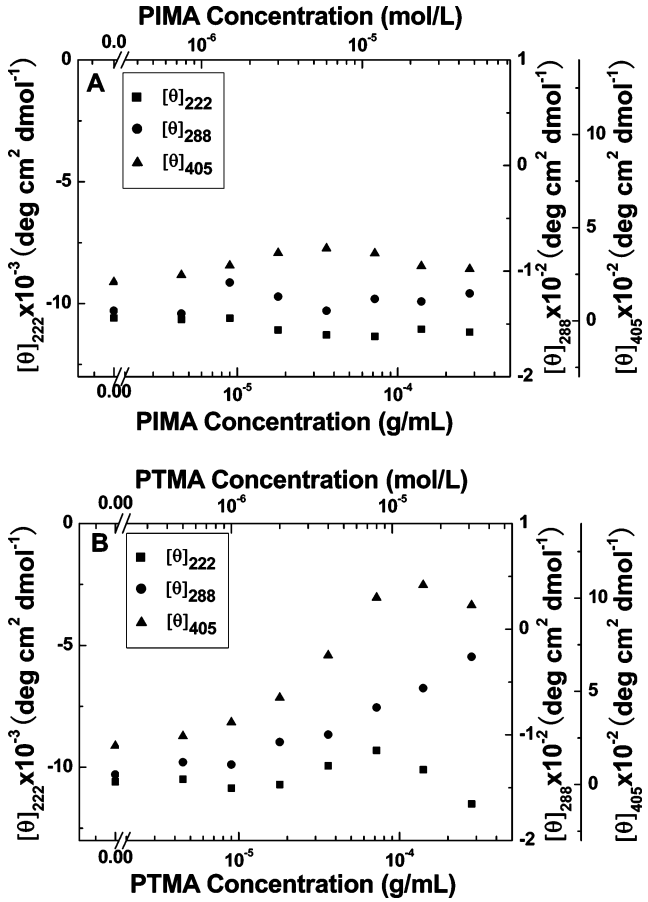

Figure 1. The changes of molar ellipticities of cyt c at 222, 288, and 405 nm with different PIMA (A) and PTMA (B) concentrations at pH 7.4. The concentration of cyt c is 10 μM.

Influence of PIMA or PTMA on the Structure of Cyt c. The isoelectric point of cyt c is 10.6,[48] and cyt c carries positive charges at pH 7.4; the ionization degrees of PIMA and PTMA are about 54% and 65% at pH 7.4, respectively, calculated from the potentiometric titration curves reported previously.[4] Therefore, electrostatic attraction exists between cyt c and the copolymers at physiological pH. There is also hydrophobic interaction between the alkyl chain of PTMA and the hydrophobic residues of cyt c. Figure 1 shows the influence of PIMA and PTMA on the structure of cyt c at pH 7.4. For PIMA, θ_{222} and θ_{288} of cyt c do not change significantly, indicating that the interaction of PIMA with cyt c has little influence on the α-helical structure and tertiary structural packing in the vicinity of Trp59. However, the θ_{405} value increases with the concentration of PIMA and attains its maximum at 3.6×10^{-5} g/mL

PIMA, suggesting that the interaction of PIMA with cyt c affects the heme pocket. The maximal change of θ_{405} of cyt c induced by PIMA is only 22% of that denatured by 4.5 M GuHCl, which was reported in the literature[49] and verified by our experiment, indicating that the interaction of PIMA with cyt c can only partly disturb the integrity of the heme pocket. The θ_{405} value decreases when PIMA concentration is higher than 3.6×10^{-5} g/mL. This phenomenon is similar to the unfolding of cyt c induced by trichloroacetic acid and trifluoroacetic acid,[50] and the reason is not well-known.

The influence of PTMA on the structure of cyt c is apparently different from that of PIMA. The maximal change of θ_{222} at 7.2×10^{-5} g/mL PTMA is 14% of that of cyt c denatured by 4.5 M GuHCl, suggesting that cyt c still maintains a significant amount of α-helical structure. Meanwhile, θ_{288} and θ_{405} increase with PTMA concentration; the maximal changes of θ_{288} and θ_{405} of cyt c are comparable with the changes of cyt c denatured by 4.5 M GuHCl, indicating that the interaction of cyt c with PTMA disrupts the asymmetry environment of Trp59 and the integrity of the heme pocket intensively. All the results above suggest that the copolymers destroy the tertiary structural packing in the vicinity of Trp59 and the structure of the heme pocket more seriously than the secondary structure of cyt c, and PTMA has more intensive influence on the structure of cyt c than PIMA.

The electrostatic and hydrophobic interactions lead to cyt c denaturation.[6,28,29] The electrostatic interaction plays an important role in the interaction between cyt c and its natural redox partners.[31,32] The interaction of cyt c with cyt c peroxidase perturbs about 2% of the optical absorption spectrum in the Soret band, and their association constants are 6.0×10^6 M^{-1} at 0.01 M ionic strength and 2.2×10^3 M^{-1} at 0.20 M ionic strength, and decrease sharply with the increase of ionic strength.[32] Sediak and Antalik reported that a nativelike state of cyt c remained after interacting with poly(vinyl sulfate) through Coulombic interaction, while a profound conformation change occurred when cyt c interacted with poly(4-styrene sulfonate) due to the additional non-Coulombic interaction.[29] PIMA and PTMA have a substantial difference in the length of the alkyl chains. Our previous study found that the hydrophobic interaction of apo cyt c with PTMA was much stronger than that with PIMA.[4] The current study shows that the hydrophobic interaction between PIMA and cyt c is not strong enough to affect the structure of cyt c significantly, while the hydrophobic interaction between PTMA and cyt c has an apparent influence on the tertiary structure around Trp59 and heme of cyt c. Obviously, the hydrophobic interaction between the protein and the copolymer destroys the hydrophobic core of cyt c, resulting in cyt c denaturation.

Influence of Temperature on the Interaction of Cyt c with PIMA and PTMA. As we know, proteins usually unfold at high temperature. The CD results of cyt c without or with the copolymer at a temperature range from 25 to 95 °C are shown in Figure 2. For cyt c alone, θ_{222}, θ_{288}, and θ_{405} vary with the increase of temperature, and the changes of θ_{222} and θ_{405} at 95 °C are 77% and 32% of those of cyt c with 4.5 M GuHCl, respectively. These results suggest that rising temperature can partially destroy the structure of cyt c, which is consistent with the study of Myer.[47] Generally, the influence of temperature on the structure of cyt c in cyt c−PIMA solution is a little stronger than that of cyt c alone, suggesting that the interaction of cyt c with PIMA somewhat promotes cyt c denaturation. On the other hand, from 25 to 95 °C, the maximal change of θ_{222} of cyt c with 2.8×10^{-4} g/mL of PTMA is 33% compared to

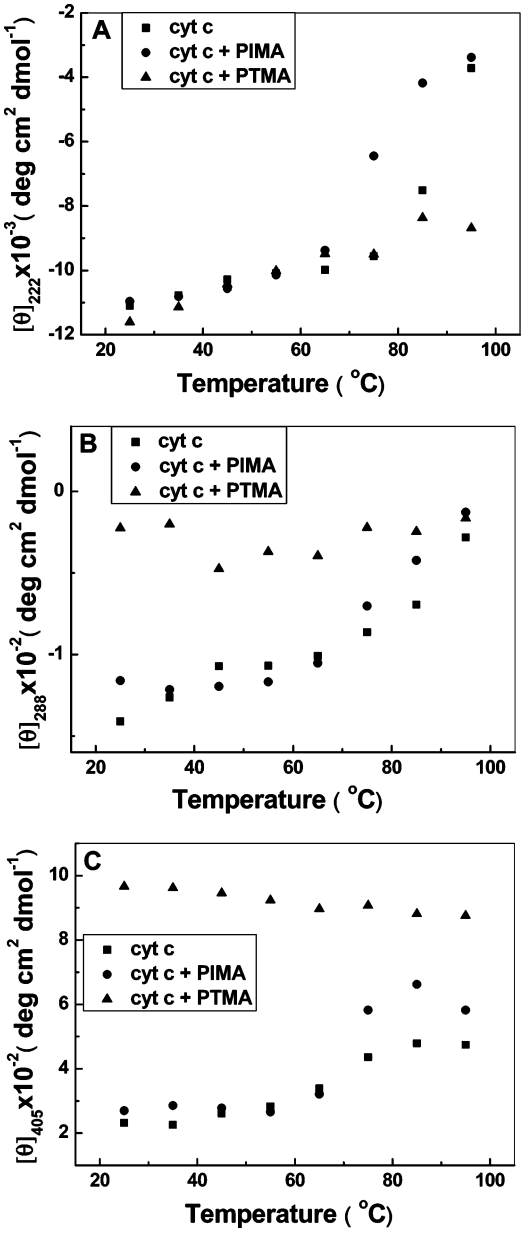

Figure 2. Influence of temperature on θ_{222} (A), θ_{288} (B), and θ_{405} (C) for cyt c alone, mixture of cyt c and PIMA, and mixture of cyt c and PTMA at pH 7.4. The concentration of cyt c is 10 μM; the concentrations of PIMA and PTMA are 2.8×10^{-4} g/mL.

that of cyt c alone, exhibiting that the interaction of cyt c with PTMA protects cyt c secondary structure on heating. The θ_{288} and θ_{405} values of cyt c in cyt c−PTMA solution do not increase with temperature, implying that the interaction of cyt c with PTMA destroys the tertiary structural packing around Trp59 and the heme of cyt c intensively at room temperature.

Our previous study on the structural transformation of apo cyt c induced by PIMA and PTMA found that electrostatic and hydrophobic interactions between the copolymer and apo cyt c excluded water molecules, promoting formation of α-helical structure, whereas the hydrogen bonding between the copolymer and the protein inhibited the formation of the helical structure.[4,5] In comparison with 39% helical structure in native cyt c, at copolymer concentration of 7.2×10^{-5} g/mL, PIMA induces 21% while PTMA induces 43% helical structure of apo cyt c at pH 6.5, and PIMA induces 4% while PTMA induces 26% at pH 10.5 where hydrophobic interaction dominates, exhibiting that the hydrophobic interaction stabilizes the helical structure.[4]

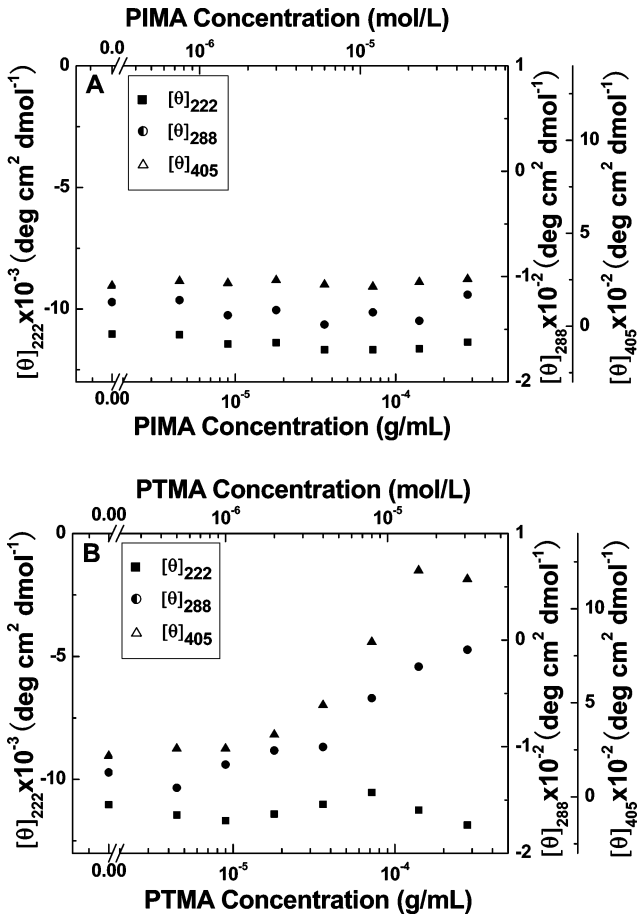

Figure 3. Changes of θ_{222}, θ_{288}, and θ_{405} of cyt c with different PIMA (A) and PTMA (B) concentrations in the presence of 0.15 M NaCl at pH 7.4. The concentration of cyt c is 10 μM.

The current study shows that cyt c maintains a majority of secondary structure at high temperature due to the hydrophobic interaction of PTMA with cyt c, consistent with the previous result.

Influence of the Physiological Ionic Strength on the Interaction of Cyt c with PIMA and PTMA. The complexes formed through electrostatic interaction are not stable in high ionic strength solution because of the electrostatic shielding effects.[51,52] For example, the polyion complex micelles formed by electrostatic interaction between lysozyme, a cationic enzyme, and poly(ethylene glycol)−poly(α,β-aspartic acid) dissociate at 0.15 M NaCl,[14] physiological ionic strength.[53] We studied the influence of 0.15 M NaCl on the respective interaction of cyt c with PIMA and PTMA. NaCl was added into the solutions after the equilibrium of cyt c−copolymer complexes, which was verified by the CD measurements. For cyt c alone, compared with the results in Figure 1, 0.15 M NaCl shows no significant influence on the structure of cyt c (Figure 3). In the presence of NaCl, θ_{222}, θ_{288}, and θ_{405} of cyt c do not vary with PIMA concentration, which are different from the results shown in Figure 1 where θ_{405} exhibits a maximum at 3.6×10^{-5} g/mL of PIMA. In the case of cyt c with PTMA, the changes in θ_{222}, θ_{288}, and θ_{405} of cyt c in the presence of 0.15 M NaCl are similar to the results without NaCl (Figure 1) when PTMA concentration is lower than 7.2×10^{-5} g/mL, while the changes in θ_{288} and θ_{405} are a bit larger than those without NaCl when PTMA concentration is higher.

The total concentration of COO^- and COOH groups is 4 mM at PIMA concentration of 2.8×10^{-4} g/mL, the largest concentration used in this study. Cyt c carries about 8 positive charges at pH 7.4,[28] and the positive charge concentration is about 80 μM in the solution. Therefore, 0.15 M NaCl, which is far more than the total charges of PIMA and cyt c, may restrain the electrostatic interaction between cyt c and PIMA. By analogy, the study indicates that cyt c unfolds in 8 M urea solution and cyt c regenerates its native structure after removing urea by dialysis,[54] PIMA−cyt c complexes possibly dissociate in the solution with 0.15 M NaCl, and the released cyt c regenerates its native structure; therefore, the structure of cyt c does not change with PIMA concentration (Figure 3A).

The study of Kataoka et al. on the stabilization of lysozyme-incorporated polyion complex micelles formed by the ω-end derivatization of poly(ethylene glycol)−poly(α,β-aspartic acid) block copolymers with hydrophobic groups suggested that the stronger hydrophobic interaction might lead to the more stable complexes.[13] In our study, a strong hydrophobic interaction between the hydrophobic residues of cyt c and the long alkyl chains of PTMA exists. Furthermore, the hydrophobic interaction becomes stronger in salt solution.[55] Therefore, PTMA−cyt c complexes may not dissociate in salt solution, and NaCl has no significant influence on PTMA−cyt c complexes; actually, NaCl slightly promotes the denaturation of cyt c in PTMA−cyt c solution.

Influence of GuHCl on the Interaction of Cyt c with PIMA and PTMA. It was reported that GuHCl could weaken the hydrophobic interaction between alkylamides and bovine serum albumin;[56] the electrostatic interaction took place between guanidine cation and negatively charged groups.[57] In this study, 0.5 M GuHCl was added to cyt c−copolymer complexes in 10 mM phosphate buffer, pH 7.4, to investigate its influence on the complexes. GuHCl was added to the solution after the equilibrium of cyt c−copolymer complexes, which was verified by the CD measurements.

Because of the large amount of noise in the range 190−210 nm, only 210−250 nm far-UV CD spectra are shown in Figure 4A for the samples with GuHCl. Figure 4 exhibits that 0.5 M GuHCl has no obvious influence on the structure of cyt c, which is consistent with the result reported by Knapp and Pace.[49] As shown in Figure 1, the CD spectra in Figure 4 display that, in the presence of PIMA, far-UV and near-UV CD spectra of cyt c do not change significantly, but there is a small difference in the Soret region. The far-UV CD spectrum of cyt c−PTMA is very similar to the spectrum of cyt c−lipid.[21] There is no substantial change at 222 nm compared with cyt c alone, suggesting that there is no significant change in α-helix content; the spectral change around the minimum at 208 nm may arise from the changes in other secondary structure elements or may be due to the presence of optically active heme transitions, other than those associated with the amide transitions of the polypeptide chain.[47] The interaction of cyt c with PTMA makes the two negative peaks at 282 and 288 nm in the near-UV region disappear, suggesting that the asymmetry environment of Trp59 is disrupted; the Soret CD spectrum changes to a single positive band with a maximum near 410 nm, indicating a disruption of the integrity of the heme pocket. After adding 0.5 M GuHCl to the mixtures of cyt c−PIMA and cyt c−PTMA, the CD spectra in far-UV, near-UV, and Soret regions return to those of cyt c alone, suggesting that α-helical structure, tight packing in the vicinity of Trp59, and the integrity of the heme pocket of cyt c are recovered.

Figure 5 shows the absorption spectra of cyt c and the mixtures of cyt c with PIMA or PTMA in the absence and presence of 0.5 M GuHCl at pH 7.4. For cyt c alone and cyt c with 0.5 M GuHCl and/or with PIMA, the maximal absorptions

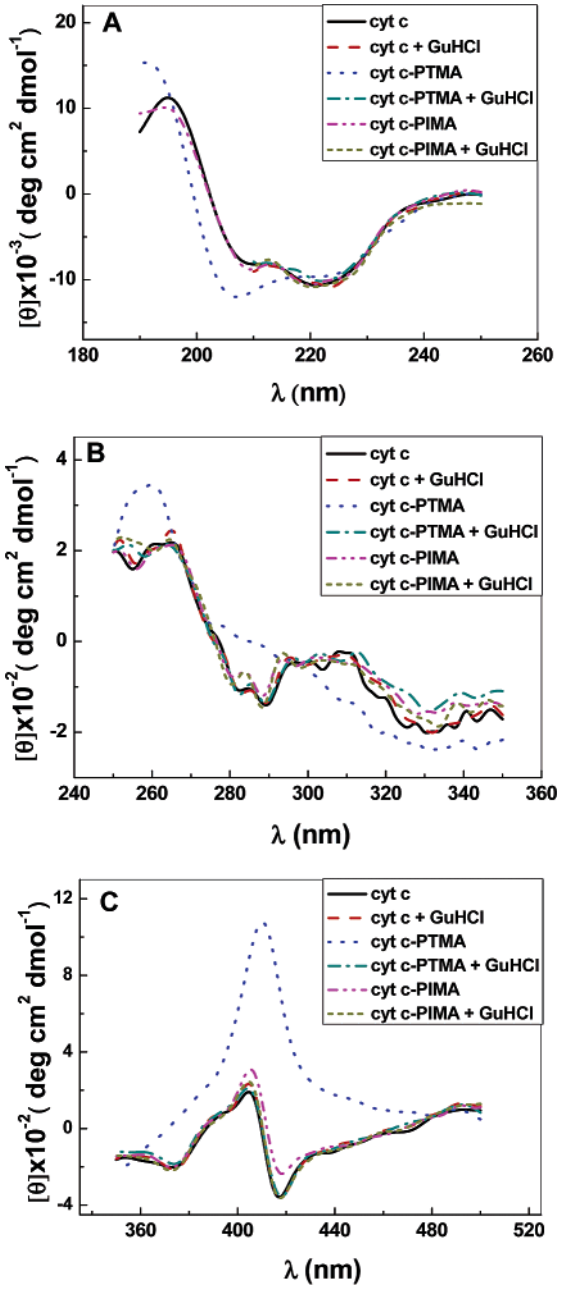

Figure 4. (A) Far-UV, (B) near-UV, and (C) Soret region of CD spectra of cyt c and the mixtures of cyt c and PIMA or PTMA in the absence and presence of 0.5 M GuHCl at pH 7.4 (10 mM phosphate buffer). The concentration of cyt c is 10 μM; the concentrations of PIMA and PTMA are 1.4×10^{-4} g/mL.

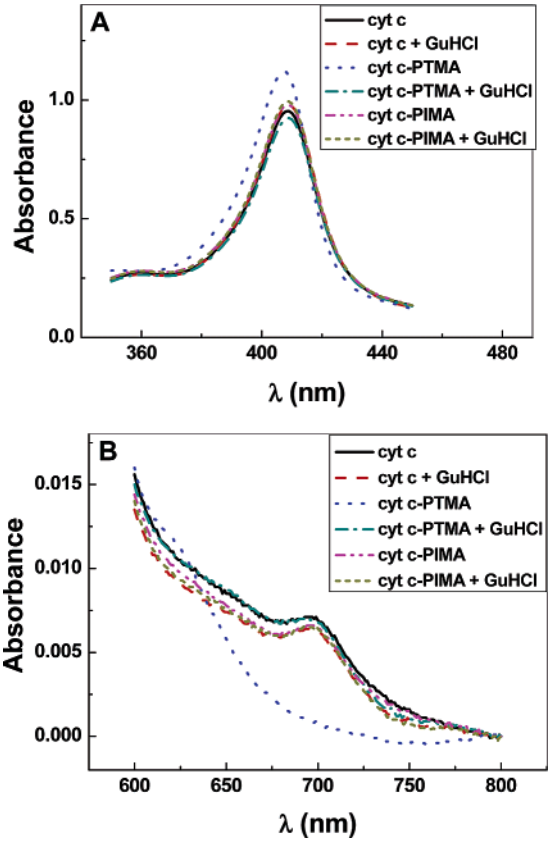

Figure 5. Absorption spectra of cyt c and the mixtures of cyt c with PIMA or PTMA in the absence and presence of 0.5 M GuHCl at pH 7.4 (10 mM phosphate buffer). The concentration of cyt c is 10 μM; the concentrations of PIMA and PTMA are 1.4×10^{-4} g/mL.

at 410 and 695 nm are typical features of the heme iron−Met80 ligand. The interaction of cyt c with PTMA leads to a blue shift from 410 to 407 nm, a new band at 620 nm, and the disappearance of the peak at 695 nm, which are similar to the interaction of cyt c with lipid,[21] indicating the disruption of heme iron−Met80 ligand. After adding 0.5 M GuHCl, the maximal absorption in the Soret region returns to 410 nm, the peak at 620 nm disappears, and the peak at 695 nm reappears, verifying that cyt c regenerates its heme iron−Met80 ligand and the strong-field low-spin state of the heme iron.

The mixtures of cyt c with PIMA or PTMA were transparent. After adding 0.5 M GuHCl, the mixture of PIMA and cyt c remained transparent, whereas precipitates appeared for the mixture of PTMA and cyt c. A control experiment showed that the mixture of PIMA and 0.5 M GuHCl was transparent, but precipitation occurred in the mixture of PTMA and 0.5 M GuHCl. In comparison to the cyt c in 0.5 M GuHCl solution, 94% cyt c was found in the supernatant of cyt c and PTMA in the presence of 0.5 M GuHCl, measured by Soret absorbance.

AFM Study on the Release of Cyt c from the Protein−Copolymer Complexes. AFM was used to study the interactions of cyt c and the copolymers. The gyration radius of cyt c is 1.38 nm at pH 7.0,[58] so nothing can be seen in our AFM image of cyt c. Our previous dynamic light scattering study showed that no particles were detected for PIMA solution,[5] which was verified by AFM image. Figure 6A shows that the mixture of cyt c with PIMA forms aggregates, which is the accumulation of small ones. After adding 0.15 M NaCl into the mixture of cyt c and PIMA, the aggregates in Figure 6A totally disappear, verifying that 0.15 M NaCl makes PIMA−cyt c complex particles dissociate. Therefore, the unchanged structure of cyt c with PIMA concentration in the presence of 0.15 M NaCl (Figure 3A) can be explained in that the released cyt c regenerates its native structure.

For PTMA, the long tetradecyl side chains aggregate in aqueous solution to form the hydrophobic core, and the ionized carboxyl groups prefer to locate on the surface of the particles to stabilize them.[4,5] The AFM image of PTMA is shown in Figure 6B; the particles are spherical, and their diameters are about 40 nm. Figure 6C shows the AFM image of PTMA−cyt c complexes. Different from PTMA alone, there is a coexistence of small particles about 45 nm and some larger particles. As discussed above, PTMA has more intensive influence on the structure of cyt c than PIMA due to stronger hydrophobic interaction. Our previous dynamic light scattering study found that, at neutral pH, the interaction of PTMA with apo cyt c

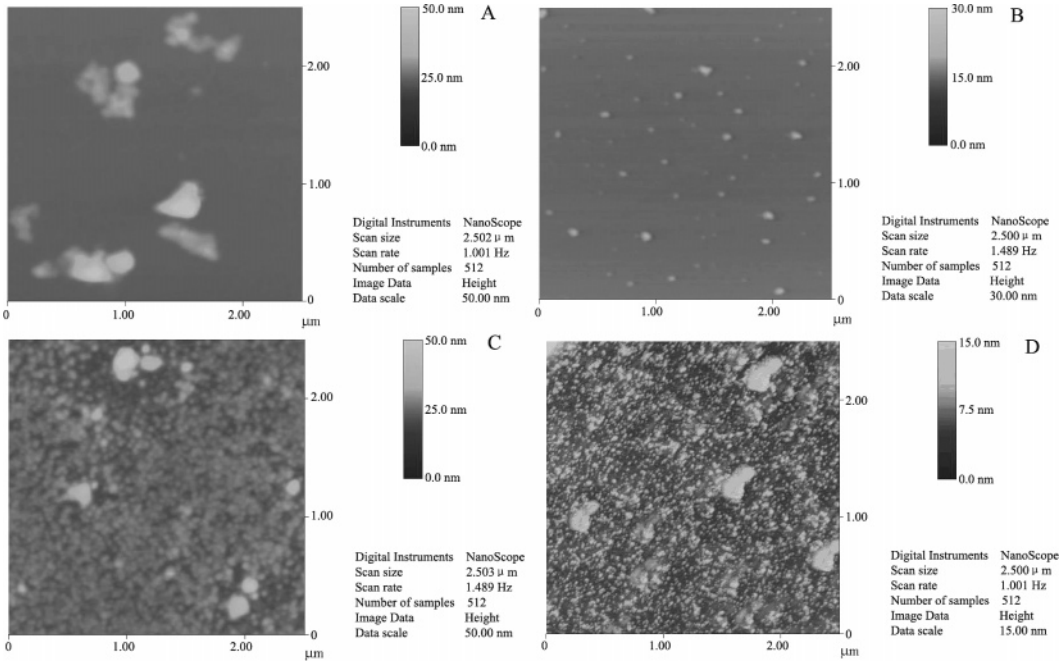

Figure 6. AFM images of (A) mixture of cyt c and PIMA, (B) PTMA, (C) mixture of cyt c and PTMA, (D) mixture of cyt c and PTMA in the presence of 0.15 M NaCl. The concentration of cyt c is 10 μM; the concentrations of PIMA and PTMA are 2.8×10^{-4} and 1.4×10^{-4} g/mL, respectively.

destroyed the aggregation of PTMA or apo cyt c alone, forming new complex particles.[5] By analogy, we speculate that the interaction of PTMA and cyt c destroys the original PTMA particles and the hydrophobic core of cyt c as verified by CD and absorbance measurements, forming new complex particles. The concentration of PTMA in Figure 6C is 1.4×10^{-4} g/mL, which is 1.5 times the cyt c concentration, and the negative charges of PTMA are 7.5 times the positive charges of cyt c. Possibly, PTMA and cyt c form small particles, and the excess of negative charges prevent the particles from further aggregation. After adding 0.15 M NaCl to the mixture of PTMA and cyt c, the AFM image in Figure 6D shows that the particles do not dissociate. This is consistent with the CD study (Figure 3) that NaCl does not influence the interaction of PTMA and cyt c significantly.

With the addition of 0.5 M GuHCl into the mixture of PTMA and cyt c, precipitates occurred, and no particles were detected by AFM in the supernatant. Considering 94% cyt c in the supernatant, we conclude that GuHCl destroys the PTMA−cyt c complex particles and cyt c is released to the solution. As a protein denaturant, GuHCl can destroy the structure of the protein when its concentration is high enough due to the hydrogen bonding, electrostatic, and hydrophobic interactions between the protein and GuHCl.[49,57] Michnik and Sulkowska reported that GuHCl could weaken the hydrophobic interaction between alkylamides and bovine serum albumin.[56] Possibly, GuH^+ can interact with negatively charged PTMA through electrostatic and hydrophobic interactions, forming precipitates and releasing cyt c. This speculation found support in that precipitates occurred when mixing PTMA with GuHCl solutions. On the other hand, no precipitates were detected for the mixture of PIMA and GuHCl, although 0.5 M GuHCl can also make cyt c regenerate its native structure in PIMA solution (Figure 4C) as 0.15 M NaCl does (Figure 3A). These results suggest that electrostatic interaction exists between the copolymer and GuHCl, and hydrophobic interaction is an important factor in PTMA−GuHCl precipitation.

After adding GuHCl to the mixture of cyt c and PTMA, a competition between the interaction of PTMA with cyt c and that with GuHCl is expected. In this study, 0.5 M GuHCl is far more than the concentrations of PTMA and cyt c; therefore, the interaction of PTMA with GuHCl dominates. That 94% cyt c exists and no particles can be found in the supernatant support this speculation. Besides, the results shown in Figures 4 and 5 exhibit that 0.5 M GuHCl cannot denature cyt c, and the study of Dong et al. on lysozyme refolding with immobilized molecular chaperone GroEL column chromatography showed that 1 M GuHCl in the refolding buffer was helpful for the refolding of denatured protein.[59] So, a low concentration of GuHCl may be favorable for the folding of denatured cyt c after it is released from PTMA−protein complexes.

In vivo, efficient folding of many newly synthesized proteins depends on assistance from molecular chaperones through hydrophobic interaction, which also prevents protein misfolding and aggregation in the crowded environment of the cell, and folding in the cytosol is achieved either by controlled chain release or after transfer of newly synthesized proteins to downstream chaperones.[60] The study on the interaction of cyt c with molecular chaperone GroEL found that GroEL could interact with cyt c to form a stable complex, and GroEL-bound cyt c was in a compact, non-native state. After adding adenosine 5′-triphosphate (ATP) and co-chaperone GroES, bound cyt c could be selectively released and then attained a biologically active native state.[61] Akiyoshi et al. found that the nanogels of cholesterol-bearing pullulan and its derivatives could entrap denatured carbonic anhydrase and then could release protein in native state by adding β-cyclodextrin.[62,63] In this study, the cyt c denatured by PTMA can recover its native structure, which may result from the competition between the interaction of PTMA with cyt c and the interaction of PTMA with GuHCl. The controlled release of cyt c from PTMA−cyt c complexes avoids the hydrophobic aggregation of denatured cyt c and assists cyt c to regenerate its native state.

Conclusions

At the physiological pH of 7.4, the interactions of cyt c with alternating copolymers of maleic acid and alkene show that PIMA can only partly disturb the integrity of the heme pocket, whereas PTMA with long alkyl side chains has very intensive influence on the structure of cyt c. After adding 150 mM NaCl, PIMA−cyt c complexes dissociate and released cyt c recovers its native structure, whereas NaCl has no significant influence on PTMA−cyt c complexes. GuHCl (0.5 M) can destroy PTMA−cyt c complexes to form GuHCl−PTMA precipitates, and released cyt c regenerates its native structure. The results obtained in this study may be useful in protein separation and purification, protein immobilization and stabilization, protein folding, and protein encapsulation and release. The increase of hydrophobic interaction leads to more stable polymer−protein complexes which do not dissociate in physiological salt solution. The influence of hydrophobic interaction on the structure of protein is more intensive than that of electrostatic interaction, but protein may recover its native state after release.

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References and Notes

(1) Wen, Y.-p.; Dubin, P. L. *Macromolecules* **1997**, *30*, 7856−7861.
(2) Sato, T.; Mattison, K. W.; Dubin, P. L.; Kamachi, M.; Morishima, Y. *Langmuir* **1998**, *14*, 5430−5437.
(3) Kaibara, K.; Okazaki, T.; Bohidar, H. B.; Dubin, P. L. *Biomacromolecules* **2000**, *1*, 100−107.
(4) Liang, L.; Yao, P.; Jiang, M. *Biomacromolecules* **2005**, *6*, 2748−2755.
(5) Liang, L.; Yao, P.; Jiang, M.; Zhang, G. Z.; Yan, Y. F. *Langmuir* **2005**, *21*, 10662−10670.
(6) Gong, J.; Yao, P.; Duan, H. W.; Jiang, M.; Gu, S. H.; Chunyu, L. J. *Biomacromolecules* **2003**, *4*, 1293−1300.
(7) Fisher, R. R.; Glatz, C. E. *Biotechnol. Bioeng.* **1988**, *32*, 777−785.
(8) Margolin, A. L.; Sherstyuk, S. F.; Izumrudov, V. A.; Zezin, A. B.; Kabanov, V. A. *Eur. J. Biochem.* **1985**, *146*, 625−632.
(9) Wang, Y. F.; Gao, J. Y.; Dubin, P. L. *Biotechnol. Prog.* **1996**, *12*, 356−362.
(10) Liang, L.; Yao, P.; Gong, J.; Jiang, M. *Langmuir* **2004**, *20*, 3333−3338.
(11) Harada, A.; Kataoka, K. *Langmuir* **1999**, *15*, 4208−4212.
(12) Harada, A.; Kataoka, K. *Macromolecules* **1998**, *31*, 288−294.
(13) Yuan, X. F.; Harada, A.; Yamasaki, Y.; Kataoka, K. *Langmuir* **2005**, *21*, 2668−2674.
(14) Harada, A.; Kataoka, K. *J. Am. Chem. Soc.* **1999**, *121*, 9241−9242.
(15) Prokop, A.; Holland, C. A.; Kozlov, E.; Moore, B.; Tanner, R. D. *Biotechnol. Bioeng.* **2001**, *75*, 228−232.
(16) Prokop, A.; Kozlov, E.; Newman, G. W.; Newman, Mark J. *Biotechnol. Bioeng.* **2002**, *78*, 459−466.
(17) Lackey, C. A.; Press, O. W.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2002**, *13*, 996−1001.
(18) Dai, C. Y.; Wang, B. C.; Zhao, H. W. *Colloid Surf., B* **2005**, *41*, 117−120.
(19) Lee, S.; Tsai, F. T. F. *J. Biochem. Mol. Biol.* **2005**, *38*, 259−265.
(20) Dill, K. A. *Biochemistry* **1990**, *29*, 7133−7155.
(21) Sanghera, N.; Pinheiro, T. J. T. *Protein Sci.* **2000**, *9*, 1194−1202.
(22) Pinheiro, T. J. T.; Elove, G. A.; Watts, A.; Roder, H. *Biochemistry* **1997**, *36*, 13122−13132.
(23) Muga, A.; Mantsch, H. H.; Surewicz W. K. *Biochemistry* **1991**, *30*, 7219−7224.
(24) Spooner, P. J. R.; Watts, A. *Biochemistry* **1991**, *30*, 3871−3879.
(25) Spooner, P. J. R.; Watts, A. *Biochemistry* **1991**, *30*, 3880−3885.
(26) de Jongh, H. H. J.; Killian, J. A.; de Kruijff, B. *Biochemistry* **1992**, *31*, 1636−1643.
(27) Pinheiro, T. J. T. *Biochimie* **1994**, *76*, 489−500.
(28) Chattopadhyay, K.; Mazumdar, S. *Biochemistry* **2003**, *42*, 14606−14613.
(29) Sedlak, E.; Antalik, M. *Biopolymers* **1998**, *46*, 145−154.
(30) Liu, X. S.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. D. *Cell* **1996**, *86*, 147−157.
(31) Dopner, S.; Hildebrandt, P.; Rosell, F. I.; Mauk, A. G.; von Walter, M.; Buse, G. *Eur. J. Biochem.* **1999**, *261*, 379−391.
(32) Erman, J. E.; Vitello, L. B. *J. Biol. Chem.* **1980**, *255*, 6224−6227.
(33) Cortese, J. D.; Voglino, A. L.; Hackenbrock, C. R. *Biochemistry* **1998**, *37*, 6402−6409.
(34) Nuevo, M. R.; Chu, H. H.; Vitello, L. B.; Erman, J. E. *J. Am. Chem. Soc.* **1993**, *115*, 5873−5874.
(35) Boussaad, S.; Dziri, L.; Arechabaleta, R.; Tao, N. J.; Leblanc, R. M. *Langmuir* **1998**, *14*, 6215−6219.
(36) Dubin, P. L.; Strauss, U. P. *J. Phys. Chem.* **1970**, *74*, 2842−2847.
(37) Fenyo, J. C.; Delben, F.; Paoletti, S.; Crescenzi, V. *J. Phys. Chem.* **1977**, *81*, 1900−1905.
(38) Chu, D. Y.; Thomas, J. K. *Macromolecules* **1987**, *20*, 2133-2138.
(39) Waugh, D. F.; Wilhelmson, D. F.; Commerford, S. L.; Sackler, M. L. *J. Am. Chem. Soc.* **1953**, *75*, 2592−2600.
(40) Garnier, G.; Duskova-Smrckova, M.; Vyhnalkova, R.; van de Ven, T. G. M.; Revol, J.-F. *Langmuir* **2000**, *16*, 3757−3763.
(41) Babul, J.; Stellwagen, E. *Biochemistry* **1972**, *11*, 1195−1200.
(42) Oellerich, S.; Wackerbarth, H.; Hildebrandt, P. *J. Phys. Chem. B* **2002,** *106*, 6566−6580.
(43) Colon, W.; Wakem, L. P.; Sherman, F.; Roder, H. *Biochemistry* **1997**, *36*, 12535−12541.
(44) Sedlak, E.; Antalik, M. *Biochim. Biophys. Acta* **1999**, *1434*, 347−355.
(45) Tsong, T. Y. *Biochemistry* **1975**, *14*, 1542−1547.
(46) Dyson, H. J.; Beattie, J. K. *J. Biol. Chem.* **1982**, *257*, 2267−2273.
(47) Myer, Y. P. *Biochemistry* **1968**, *7*, 765−776.
(48) Noritomi, H.; Hidaka, Y.; Kato, S.; Nagahama, K. *Biotechnol. Tech.* **1999**, *13*, 181−183.
(49) Knapp, J. A.; Pace, C. N. *Biochemistry* **1974**, *13*, 1289−1294.
(50) Ahmad, A.; Madhusudanan, K. P.; Bhakuni, V. *Biochim. Biophys. Acta* **2000**, *1480*, 201−210.
(51) Wang, C.; Tam, K. C. *Langmuir* **2002**, *18*, 6484−6490.
(52) Lohman, T. M.; DeHaseth, P. L.; Record, M. T., Jr. *Biochemistry* **1980**, *19*, 3522−3530.
(53) Mei, H.; Wang, K.; McKee, S.; Wang, X.; Waldner, J. L.; Pielak, G. J.; Durham, B.; Millett, F. *Biochemistry* **1996**, *35*, 15800−15806.
(54) Stellwagen, E. *Biochemistry* **1968**, *7*, 2893−2898.
(55) Cooper, C. L.; Dubin, P. L.; Kayitmazer, A. B.; Turksen, S. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 52−78.
(56) Michnik, A.; Sulkowska, A. *J. Mol. Struct.* **1995**, *348*, 53−56.
(57) Rashid, F.; Sharma, S.; Bano, B. *Protein J.* **2005**, *24*, 283−292.
(58) Kataoka, M.; Hagihara, Y.; Mihara, K.; Goto, Y. *J. Mol. Biol.* **1993**, *229*, 591−596.
(59) Dong, X. Y.; Yang, H.; Sun, Y. *J. Chromatogr., A* **2000**, *878*, 197−204.
(60) Hartl, F. U.; Hayer-Hartl, M. *Science* **2002**, *295*, 1852−1858.
(61) Smith, C. M.; Kohler, R. J.; Barho, E.; El-Thaher, T. S. H.; Preuss, M.; Miller, A. D. *J. Chem. Soc., Perkin Trans.* **1999**, *2*, 1537−1546.
(62) Nomura, Y.; Ikeda, M.; Yamaguchi, N.; Aoyama, Y.; Akiyoshi, K. *FEBS Lett.* **2003**, *553*, 271−276.
(63) Morimoto, N.; Endo, T.; Ohtomi, M.; Iwasaki, Y.; Akiyoshi, K. *Macromol. Biosci.* **2005**, *5*, 710−716.

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