Interactions of Apo Cytochrome *c* with Alternating Copolymers of Maleic Acid and Alkene

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Apo cytochrome *c* (apo cyt *c*) tends to aggregate at alkali pH. Poly(isobutylene-*alt*-maleic acid) (PIMA) is soluble molecularly, whereas poly(1-tetradecene-*alt*-maleic acid) (PTMA) forms particles that tend to dissociate by increasing pH and decreasing concentration. Dynamic light scattering and surface plasmon resonance are used to investigate the interactions of PIMA and PTMA with apo cyt *c* at different pH values to understand the mechanism of the interactions. When the positive or negative charges are in excess, the copolymer−protein complex particles can be stabilized by the charges on the surface. When the ratio of the positive to negative charges is close to the stoichiometric value, precipitation occurs. At pH 11.8, both PTMA and apo cyt *c* carry negative charges, but the hydrophobic interaction makes them form complexes. A competition exists between the interaction of the copolymer with apo cyt *c* and the self-aggregation of PTMA or apo cyt *c* alone. The interaction of PIMA or PTMA with apo cyt *c* at neutral and alkali pH destroys the aggregation of PTMA or apo cyt *c* and forms new complex particles.

Introduction

The copolymers of maleic acid and alkene in aqueous solution have been investigated widely.[1−3] The conformation of the copolymers is jointly determined by the degree of ionization and hydrophobicity. The former is a function of the media pH, and the latter depends on the length of alkyl side chains. Copolymers with short alkyl side chains at high pH tend to exhibit an extended conformation, while those with long side chains at low pH have a compact conformation and form micelles. Therefore, they offer a good model to investigate the influence of various factors on the interaction of protein with polymer. The interactions of ethylene−maleic acid copolymers and their derivatives with poly-L-lysine and poly-L-ornithine were investigated by studying the conditions of the coprecipitation, which showed that the complex formation was influenced by both the ionization of the carboxyl groups and the hydrophobicity of alkyl side chains of the polyanion.[4] Binding isotherms of β-lactoglobulin or bovine serum albumin with a series of alternating copolymers of maleic acid and alkylvinyl ethers with varying hydrophobicity by capillary electrophoresis were reported.[5] The studies showed that a minimum alkyl chain length of 3−4 methylenes was required for significant hydrophobic interactions between the proteins and copolymers and that a competition existed between intra-polymer micelle formation and protein−polymer hydrophobic interactions.

Apo cytochrome *c* (apo cyt *c*) is the precursor of the mitochondria protein cytochrome *c* (cyt *c*), which is encoded by nuclear DNA and synthesized on free cytoplasmic ribosomes. Apo cyt *c* inserts spontaneously and partially crosses the outer mitochondrial membrane. After or simultaneous with translocation across the outer membrane, apo cyt *c* binds with heme, and then holo cyt *c* folds the polypeptide around the heme into the native structure. In contrast with cyt *c*, which has 39% α-helix content and has a compact well-defined structure in aqueous solution, the heme-free apo cyt *c* has a disordered structure in solution.[6]

The importance of natively disordered proteins in biological systems is only recently being recognized. Proteins that are partially or wholly disordered under physiological conditions can still perform important biological functions, such as molecular recognition, signaling, and regulation.[7,8] The refolding of apo cyt *c* and denatured cyt *c* has been well studied by interaction with various amphiphilic lipids and detergents at certain pH to mimic the interactions of the protein with mitochondrial membrane;[9−14] however, the mechanism is far from being fully understood.[12,15] Rankin et al. reported the folding of apo cyt *c* induced by negatively charged lipid micelles at pH 7.0.[11] Their study found that the extent of secondary structure induced by lipid was dependent on the monomer lipid concentration because the free lipid molecules, which are not involved in the formation of the micelles, can interact with the protein through the ionic headgroups as

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well as through the hydrophobic tail of the lipid. For the lipid concentration above its critical micelle concentration, the helix content remained approximately constant. The recent study of SDS with cyt *c* denaturized by urea at physiological pH also showed that the monomeric surfactant molecules interacted with the protein and this led to the formation of partially folded intermediates of cyt *c*.[12] Moreover, the interactions of apo cyt *c* with lipid micelles found that apo cyt *c* inserted to the lipid micelles after the formation of helical structure on the surface of the micelles,[9−11] and the interaction of protein with lipid disturbed the lipid packing.[16]

The previous work in our lab found that the α-helical structure of apo cyt *c* was induced by highly sulfonated polystyrene nanoparticles at acid pH,[17] and apo cyt *c* might insert into the cores, disrupting the original structure of the particles, but we did not know whether the protein interacted with the free polymer first or interacted with the nanoparticles directly.[18] Our work also found that the alternating copolymers of maleic acid and alkene could interact with apo cyt *c*, inducing an apo cyt *c* structural transformation from a random coil to an α-helix at different pH, even when the copolymer concentration is above its association concentration.[6] The α-helix content of apo cyt *c* induced by the copolymers was dependent on electrostatic interaction, hydrophobic interaction, and hydrogen bonding between the copolymers and protein, which were related to the concentration and hydrophobic chain length of the copolymers, as well as the pH of the solution. As compared to sulfonated polystyrene nanoparticles, alternating copolymers of maleic acid and alkene, alternating polyelectrolytes, and polymer surfactants are more suitable as mimics due to their resemblance to the alternating order of phospholipid biomembranes.[19,20] In this report, the interactions of poly(isobutylene-*alt*-maleic acid) (PIMA) and poly(1-tetradecene-*alt*-maleic acid) (PTMA) with apo cyt *c* are investigated with dynamic light scattering, atomic force microscopy, and surface plasmon resonance to further understand the mechanism of the interactions. The substantial difference in the length of the alkyl chains between the two copolymers makes it possible to estimate the effect of hydrophobic interaction between the copolymer and the protein. The pH values of 2.1, 6.5, and 10.5, with respective ionization degrees of 11%, 53%, and 87% and negative charges of each chain of 8, 41, and 68,[6] are selected for PIMA, while the pH values of 2.1, 6.5, and 11.8, with respective ionization degrees of 8%, 55%, and 100% and negative charges of each chain of 5, 34, and 62,[6] are studied for PTMA. The previous study of the α-helical structure of apo cyt *c* showed that no induction occurred for PIMA, but the induction existed for PTMA at pH 11.8,[6] so we study the case of PTMA at pH 11.8 and PIMA at pH 10.5 here. As compared to Mw 6000 PIMA, PIMA with Mw 60 000 can also induce apo cyt *c* folding, although the helix content is slightly different; therefore, we only study the interaction of apo cyt *c* with low Mw PIMA and PTMA in this paper.

Experimental Section

Preparation of Samples. Poly(isobutylene-*alt*-maleic anhydride) (Mw 6000) with 39 repeat units and poly(1-tetradecene-*alt*-maleic anhydride) (Mw 9000) with 31 repeat units were purchased from Aldrich. Poly(isobutylene-*alt*-maleic acid) (PIMA) and poly(1-tetradecene-*alt*-maleic acid) (PTMA) were obtained by hydrolysis of the corresponding anhydride in the alkali solution. The concentration of PIMA and PTMA stock solutions is 4.5×10^{-4} g/mL with desired pH values, as described previously.[6]

Horse heart cyt *c* (Mw, 12 348; purity, 97%) was from Sigma. Apo cyt *c* was prepared by chemically removing heme group of cyt *c* as described by Fisher et al.[21] The concentration of the apo cyt *c* stock solution is 100 μM, which was measured spectrophotometrically with a molar extinction coefficient of 10 580 M^{-1} cm^{-1} at 277 nm.[22]

Samples for Measurement. The samples were prepared by dropwise adding PIMA or PTMA stock solution into the desired pH aqueous solution, which was then followed by dropping apo cyt *c* stock solution into the copolymers solution under shaking. The samples were allowed to stand at least 12 h before measurement.

Dynamic Light Scattering (DLS) Measurement. DLS was measured using a commercial laser light scattering spectrometer (Malvern Autosizer 4700) equipped with a multi-τ digital time correlator (Malvern PCS7132) and Compass 315M-100 Diode-Pumped Laser (output power ≥ 100 mW, CW at $\lambda_0 = 532$ nm) as light source. All of the DLS measurements were done at 25.0 ± 0.1 °C and at a scattering angle of 90°. The measured time correlation functions were analyzed by the Automatic Program equipped with the correlator, and the *z*-average hydrodynamic diameter $\langle D_h \rangle$ was obtained by a CONTIN mode analysis. The samples for DLS measurement were prepared with the desired pH aqueous solution filtered through a 0.22 μm pore size filter.

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 for at least 12 h.

Surface Plasmon Resonance (SPR) Measurement. SPR was measured using a BIACORE X instrument (BIACORE AB, Uppsala, Sweden). The sensor chip, naked gold slice was cleaned by piranha solution (30% H_2O_2 and 70% H_2SO_4 (v/v)) (*Caution: Piranha solution is extremely corrosive and should be handled appropriately with great care!*) at 90 °C for 1.5 h,[23] and subsequently sonicated in tetrahydrofuran (THF), followed by washing with deionized H_2O and drying under a stream of N_2. After the sensor chip was mounted, apo cyt *c* was immobilized to it through Cys14 and Cys17 residues, the response signal change of about 1100 RU (response unit) was obtained by injection of 100 μL of 10 μM apo cyt *c*, and then the sensor chip was flushed with water of the desired pH. A series of PIMA or PTMA solutions with different concentrations as analyte were passed through the flow cell at a flow rate of 5 μL/min for 20 min. Before each analysis, the sensor chip was equilibrated with water of the desired pH to eliminate the influence of the physical adsorption. All of the SPR measurements were carried out at 25 °C.

Results and Discussion

Apo Cyt *c* Aggregation Studied by AFM and DLS. Generally, protein is a polyampholyte and carries net positive charges below its isoelectric point (pI) and net negative charges above pI. The pI of cyt *c* is 10.6.[24] Apo cyt *c* carries about 24 (19 Lys + 2 Arg + 3 His) and 9 (19 Lys + 2 Arg − 3 Glu − 9 Asp) positive charges at pH 2.1 and 6.5, respectively;[12] the net charge of apo cyt *c* is about zero at pH 10.5, and apo cyt *c* carries negative charges at

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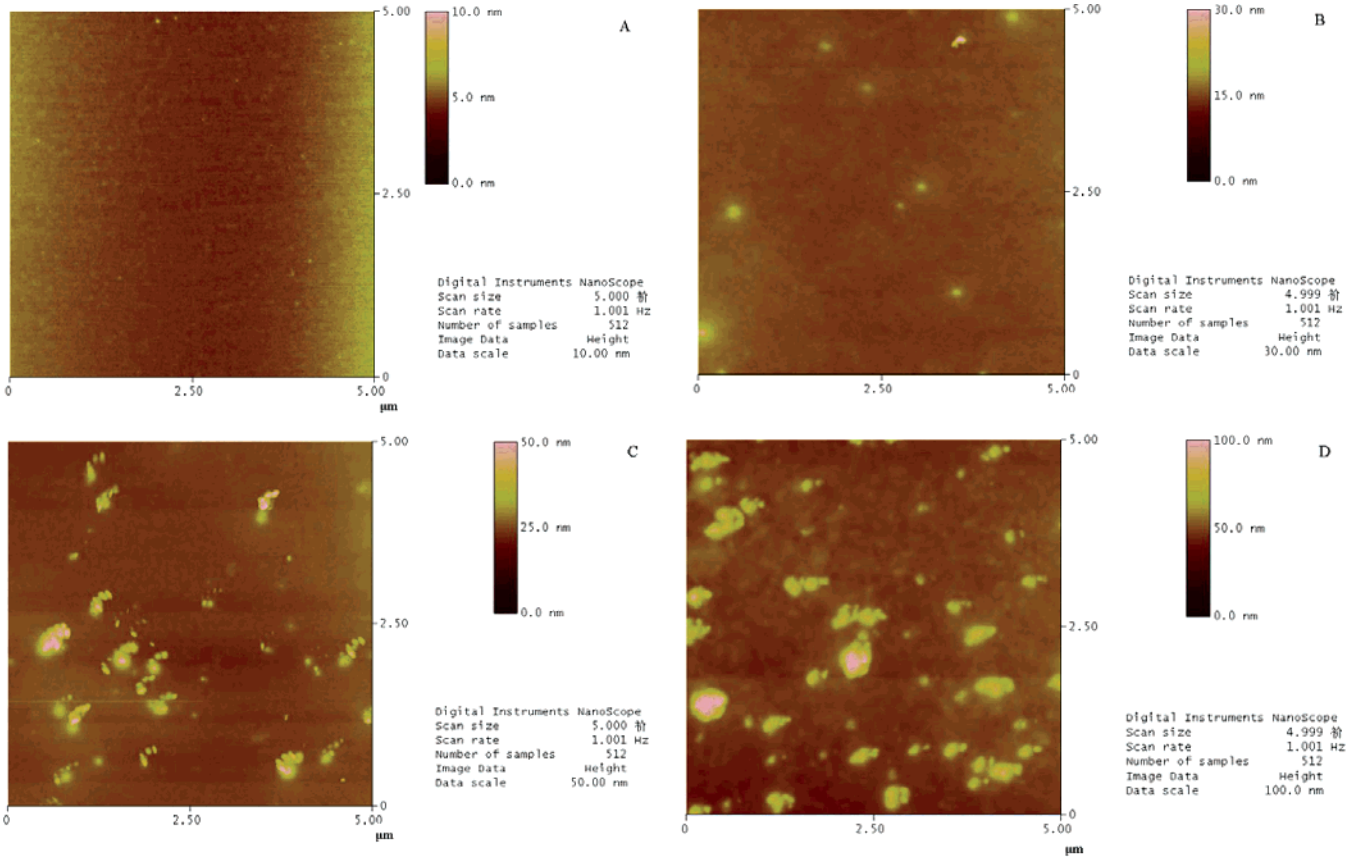

Figure 1. AFM images of apo cyt *c* at pH 2.1 (A), 6.5 (B), 10.5 (C), and 11.8 (D). The concentration of apo cyt *c* is 10 μM.

pH 11.8. The electrostatic attraction between the opposite charges and the hydrophobic interaction make the protein chains collapse, while the electrostatic repulsion of the same charges makes the chains stretch. Figure 1 shows the AFM images of apo cyt *c* at pH 2.1 (A), 6.5 (B), 10.5 (C), and 11.8 (D). At pH 2.1, nothing is seen in the AFM image. Apo cyt *c* carries about 24 positive charges at this pH so that the electrostatic repulsion results in an expanded chain structure. As the pH increases, the net positive charges of the protein decrease and hence the electrostatic repulsion weakens while the electrostatic attraction enhances, and then the protein tends to collapse and aggregate. From the AFM images, the aggregates can be seen at pH 6.5, 10.5, and 11.8. The particles at pH 10.5 and 11.8 are apparently larger than those at pH 6.5. There is a coexistence of the small particles and the larger aggregates, which is apparently the accumulation of small ones at pH 10.5 and 11.8. Generally, the main factor that determines self-association of protein in the vicinity of pI is electrostatic interaction of the charge fluctuations; hydrophobic interaction plays a significant but auxiliary role.[25]

The aggregation of apo cyt *c* in aqueous solution was investigated with DLS. At pH 2.1, the scattering light intensity is too weak to be measured. According to the literature, the radius of gyration of apo cyt *c* is 2.25 nm at pH 3 and salt-free solution.[26] Obviously, apo cyt *c* does not aggregate at pH 2.1; this is also consistent with the result of AFM. Figure 2 shows the apparent diameter (D_a) distributions of apo cyt *c* in aqueous solutions at pH 6.5 (A) and 11.8 (B) at different concentrations, which were obtained by a successive dilution. At pH 6.5, the size distribution shows two peaks: D_a at about 8 nm and 200−300 nm. This result suggests that there is a coexistence of small and large particles, which can also be obtained from the AFM image where the number of aggregates at pH 6.5 is much less as compared to the images of pH 10.5 and 11.8 (Figure 1). After 10 mM NaCl was added, which decreases intermolecule electrostatic repulsion, the size of apo cyt *c* becomes larger at pH 6.5 (data not shown). At pH 11.8, only one peak with a D_a of 200−300 nm occurs in the condition of present or absent 10 mM NaCl.

PTMA Conformation Studied by DLS and AFM. Figure 3 shows the apparent diameter distributions of PTMA in aqueous solutions at pH 2.1 (A), 6.5 (B), and 11.8 (C) at different concentrations, which were obtained by a successive dilution from the stock solution. At pH 2.1, the D_a is basically unchangeable against dilution, suggesting that the hydrophobic aggregates of long tetradecyl side chains are very stable in aqueous solution. The ionization degree of PTMA is 8% at pH 2.1, and the ionized carboxyl groups may locate on the surface of the particles to stabilize them. The influence of the dilution on the D_a distribution at pH 6.5 is evidently different from that at pH 2.1; that is, there are two peaks: one is at about 500 nm and the other reaches about 4 μm when the PTMA concentration is 1.4×10^{-4} g/mL or higher. The larger particles disappear when the concentration decreases to 7.2×10^{-5} g/mL. After the solution was further diluted to 4.5×10^{-6} g/mL, a peak of about 25 nm appears. These results indicate that the larger particles formed at higher concentrations dissociate at lower concentrations. According to the literature, very strong O−H···O$^-$ hydrogen bonding (O···O is 2.2−2.5 Å) usually occurs when the hydrogen bond is stabilized by a negative charge.[27] The ionization degree of PTMA is 55% at pH 6.5; therefore,

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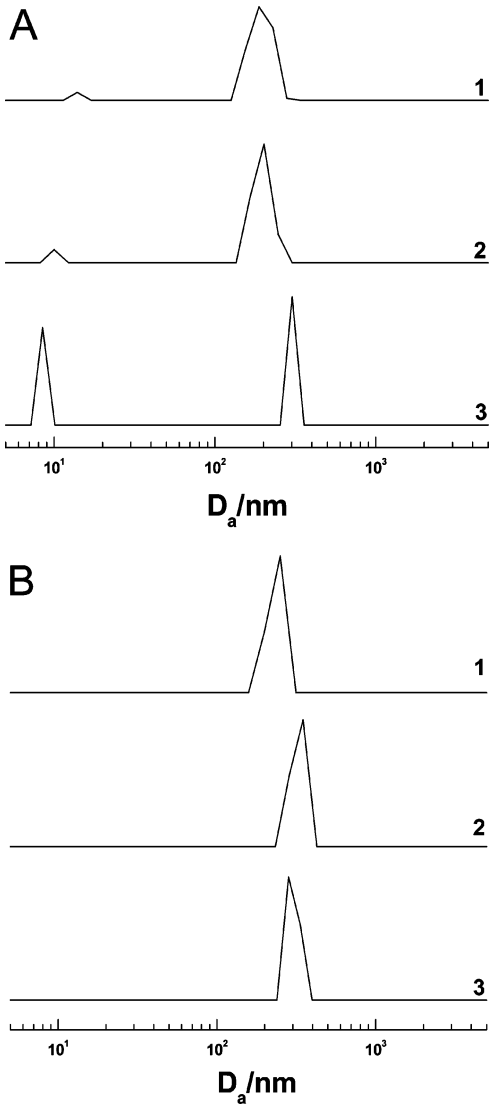

Figure 2. Apparent diameter distributions of apo cyt *c* after a successive dilution at pH 6.5 (A) and 11.8 (B): (1) 0.15, (2) 2.5, (3) 10 μM of apo cyt *c*.

very strong hydrogen bonds may form between ionized and nonionized carboxylic groups and larger aggregates appear at higher concentrations. On the other hand, the electrostatic repulsion within the particles makes the particles dissociate in very dilute solution. At pH 11.8, the D_a is about 230 nm at the concentration of 2.8×10^{-4} g/mL and there are two peaks when the PTMA concentration decreases to 3.6×10^{-5} g/mL. Only one peak at about 10 nm exists at the low concentration, if the calculation is based on the number distribution. These results indicate that the dilution makes PTMA particles dissociate at pH 11.8, too. The ionization degree of PTMA is 100% at pH 11.8, and the electrostatic repulsion is stronger than those at pH 2.1 and 6.5, so the particles begin to dissociate at the concentration of 3.6×10^{-5} g/mL, nearly 1 order of magnitude higher than that at pH 6.5. It is interesting to find that PIMA, which carries much shorter alkyl side chains, shows very different behavior; that is, the scattering light intensities of PIMA are very small at pH 2.1, 6.5, and 10.5 even at a concentration of 1.4×10^{-4} g/mL. This result suggests that PIMA does not

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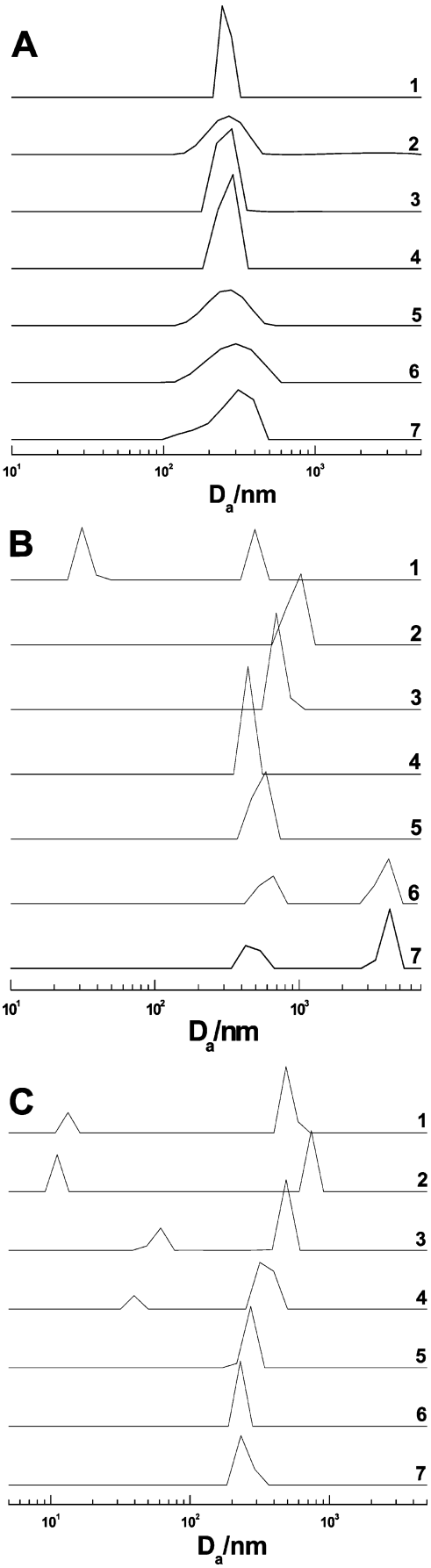

Figure 3. Apparent diameter distributions of PTMA after a successive dilution at pH 2.1 (A), 6.5 (B), and 11.8 (C): (1) 4.5×10^{-6}, (2) 9.0×10^{-6}, (3) 1.8×10^{-5}, (4) 3.6×10^{-5}, (5) 7.2×10^{-5}, (6) 1.4×10^{-4}, (7) 2.8×10^{-4} g/mL of PTMA.

form particles at whole pH, which is consistent with the study of Kitano et al. with the methods of potentiometric titration and the intrinsic viscosity that the apparent hydrophobicity of PIMA was too weak to cause a conformational transition from an extended coil to a compact globule.[28] According to the study of fluorescence probe previously, PIMA is in coiled structure at pH 2.1 and in expanded structure at pH 6.5 and 10.5.[6]

In DLS measurement, the repulsive force among charged particles in very low salt solution may lead to large contributions to the mutual diffusion coefficients, and therefore may cause inaccuracy of size distribution. In our measurement, the total concentrations of COO^- and COOH are 1 mM for PIMA and 2 mM for PTMA, calculated with the maximal concentrations of these two copolymers used. To eliminate the influence of particle charges on the mutual diffusion coefficients, 10 mM NaCl was added to PTMA solutions and then DLS measurement was performed. Previously study has proven that 10 mM NaCl does not influence the structural transition of apo cyt *c* induced by the copolymers.[6] Figure 4 shows the effects of 10 mM NaCl on the solution behavior of PTMA at pH 2.1, 6.5, and 11.8. Like PTMA without NaCl (Figrue 3), the D_a is basically unchangeable against dilution at pH 2.1, and PTMA still dissociates at the lower concentration at pH 6.5 and 11.8. However, there are some differences: the peak of 10 nm apparently decreases for PTMA with 10 mM NaCl at pH 6.5 and 11.8, suggesting that NaCl may restrain the dissociation of PTMA aggregates. Moreover, NaCl may destroy the very strong hydrogen bonds between ionized and nonionized carboxylic groups by screening the charges, and therefore the peak of about 4 μm disappears at PTMA concentrations of 1.4×10^{-4} and 2.8×10^{-4} g/mL at pH 6.5. The size of PTMA particles with NaCl is smaller than that without NaCl at pH 2.1, whereas the size of PTMA particles with NaCl is larger than that without NaCl at pH 6.5 and 11.8 where the particles carry much more charges than that at pH 2.1 and the salt screens the electrostatic repulsion, which leads to larger particle size.

Figure 5 shows the AFM images of PTMA at the concentrations of 4.5×10^{-6} and 7.2×10^{-5} g/mL at pH 11.8. The average size of the particles is about 80 nm at the concentration of 7.2×10^{-5} g/mL, indicating that PTMA aggregates in aqueous solution at this pH. There are two kinds of particles for PTMA at the concentration of 4.5×10^{-6} g/mL at pH 11.8: one is about 80 nm; the other is about 20 nm for the horizontal diameter and about 3 nm for vertical distance, further supporting that PTMA dissociates at the lower concentration at pH 11.8.

The Interaction of PIMA with Apo Cyt *c* Studied by DLS. We now discuss the interaction of apo cyt *c* with PIMA by studying the size distribution of the particles formed in the mixture solutions of apo cyt *c* (10 μM) with different concentrations of PIMA. Figure 6 shows D_a distributions of the mixtures at pH 2.1 (A), 6.5 (B), and 10.5 (C). At pH 2.1, the D_a increases from 110 to 210 nm gradually with PIMA concentration, suggesting that PIMA and apo cyt *c* can interact to form complex particles. PIMA carries about 8 negative charges averagely, and apo cyt *c* carries 24 positive charges at this pH. Therefore, there are multiple intermolecular interactions: the electrostatic attraction between the negative carboxylic groups of PIMA and the positive residues of apo cyt *c*, the hydrophobic interaction between the isobutyl side chains of PIMA and the hydrophobic residues of apo cyt *c*, as well as hydrogen bonds between the ionized carboxyl of PIMA and amide of apo cyt *c*.[6] The ratio of the negative charges of PIMA to the positive charges of apo cyt *c* is 0.4 at a PIMA concentration of 7.2×10^{-5} g/mL. Therefore, the excess positive charges of apo cyt *c* are on the surface of the complexes to stabilize the particles at the concentration

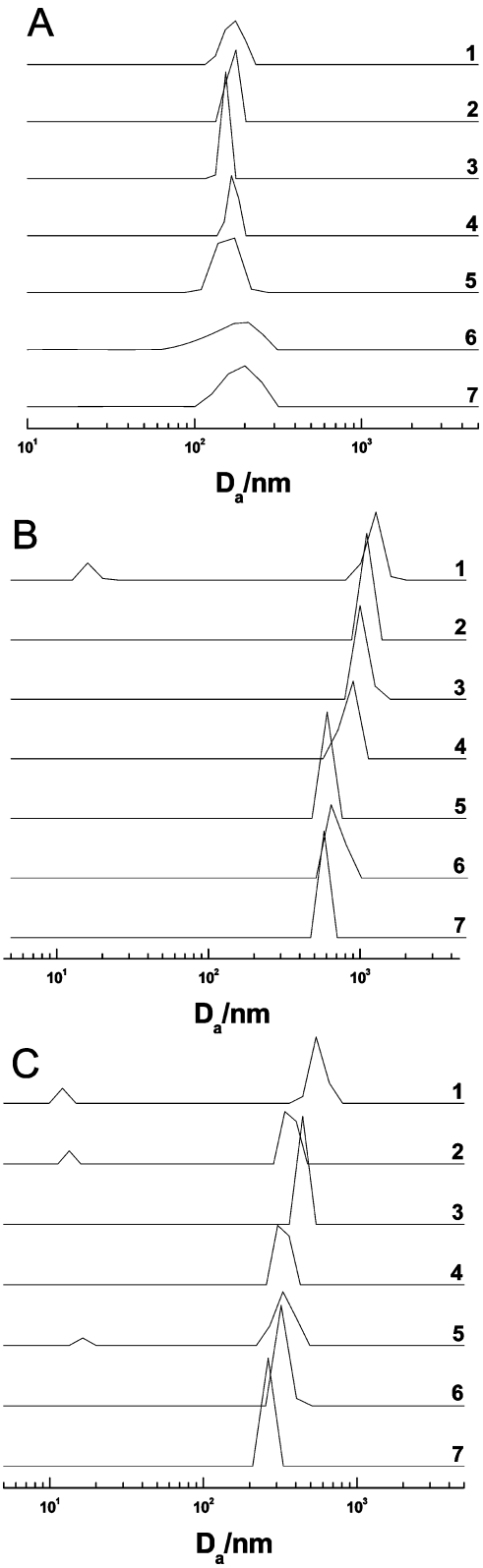

Figure 4. Apparent diameter distributions of PTMA at pH 2.1 (A), 6.5 (B), and 11.8 (C) in the presence of 10 mM NaCl. The concentration of PTMA: (1) 4.5×10^{-6}, (2) 9.0×10^{-6}, (3) 1.8×10^{-5}, (4) 3.6×10^{-5}, (5) 7.2×10^{-5}, (6) 1.4×10^{-4}, (7) 2.8×10^{-4} g/mL.

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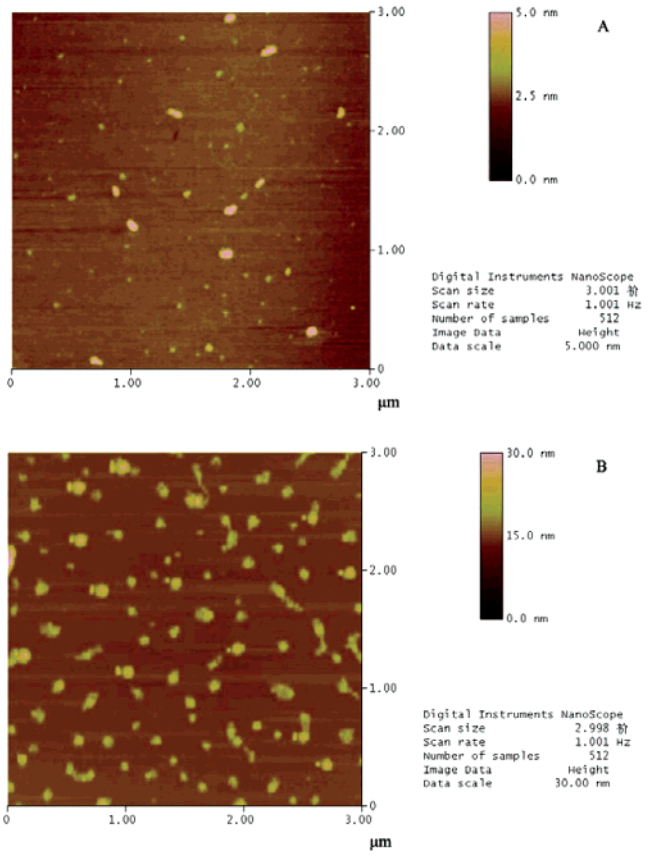

Figure 5. AFM images of PTMA at the concentrations of 4.5 $\times$ 10^{-6} g/mL (A) and 7.2 $\times$ 10^{-5} g/mL (B) at pH 11.8.

range shown in Figure 6A. As precipitation occurs when PIMA concentration reaches 1.4 $\times$ 10^{-4} g/mL, no further studies at higher concentration were performed at pH 2.1.

At pH 6.5, the size of the mixtures shows a complicated change with the PIMA concentration. D_a is about 100 nm when PIMA concentration is 4.5 $\times$ 10^{-6} and 9.0 $\times$ 10^{-6} g/mL. However, much larger peaks and precipitation appear when PIMA concentration increases to 1.8 $\times$ 10^{-5} g/mL. As PIMA concentration increases further to 3.6 $\times$ 10^{-5} g/mL, the D_a distribution returns one peak with the size of about 150 nm. As mentioned above, PIMA carries about 41 negative charges averagely, and apo cyt *c* carries about 9 positive charges at pH 6.5. At low PIMA concentration, excess positive charges of apo cyt *c* are on the surface of the complex particles to prevent further aggregation. When PIMA concentration is 1.8 $\times$ 10^{-5} g/mL, the ratio of the positive charges to the negative charges is about 0.7, and precipitation occurs because there are not enough net charges to stabilize the complex particles. Increasing PIMA concentration further, the excess PIMA negative charges are on the surface of the complexes to prevent the aggregation. The size distribution of apo cyt *c* alone shows two peaks at about 8 and 300 nm at pH 6.5. These results suggest that PIMA can not only interact with apo cyt *c*, but also destroy the aggregation of apo cyt *c* to form the complexes.

At pH 10.5, apo cyt *c* alone forms particles with the D_a of 240 nm. As shown in Figure 6C, D_a for the mixtures increases from 170 to 260 nm with PIMA concentration. It has been pointed out that the charge fluctuations determine an ability of proteins to be bound to polyanions even in the vicinity of pI, resulting in solubilization of protein under such conditions.[25,29] In the mixture solution with PIMA concentration of 4.5 $\times$ 10^{-6} g/mL, the complex

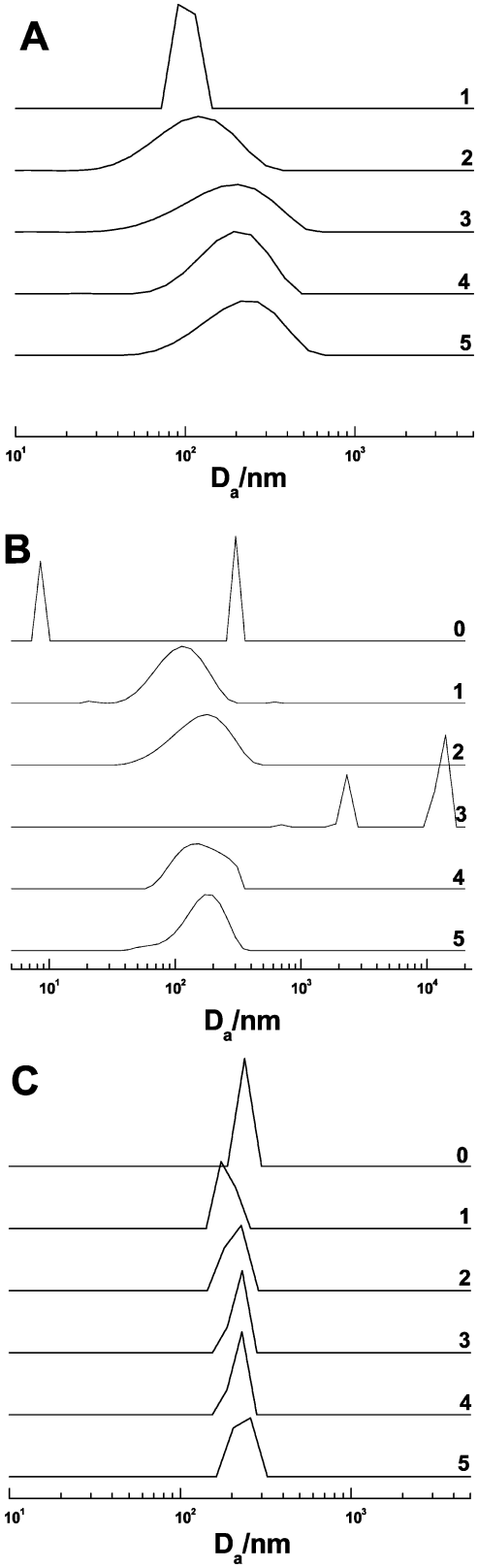

Figure 6. Apparent size distributions of the mixtures of 10 μM apo cyt *c* with different concentrations of PIMA: (1) 4.5 $\times$ 10^{-6}, (2) 9.0 $\times$ 10^{-6}, (3) 1.8 $\times$ 10^{-5}, (4) 3.6 $\times$ 10^{-5}, (5) 7.2 $\times$ 10^{-5} g/mL at pH 2.1 (A), 6.5 (B), and 10.5 (C).

particles become smaller than apo cyt *c*, suggesting that the very low concentration of PIMA may inhibit the aggregation of apo cyt *c*. The net charges of apo cyt *c* are

(29) Gurov, A. N.; Vainerman, E. S.; Tolstoguzov, V. B. *Staerke* **1974**, *26*, 172−176.

about zero and PIMA carries about 68 negative charges averagely, so the negative charges of PIMA are on the surface of the complex particles to stabilize them.

The Interaction of PTMA with Apo Cyt *c* Studied by DLS. Figure 7 shows the size distributions of the mixtures of 10 μM apo cyt *c* with different concentrations of PTMA at pH 2.1 (A), 6.5 (B), and 11.8 (C). At pH 2.1, the size distributions of PTMA with apo cyt *c* are similar to those without apo cyt *c* (Figure 3A). At pH 6.5, the D_a generally increases from 120 to 290 nm with PTMA concentration except for the case of PTMA concentration of 3.6×10^{-5} g/mL. Similar to the case of PIMA with apo cyt *c*, particles with sizes as large as 10^4 nm appear at a PTMA concentration of 3.6×10^{-5} g/mL in which the ratio of the positive to negative charges is 0.7 and the excess charges are not enough to prevent precipitation. In addition, at pH 6.5, the size of the complex particles is smaller than PTMA alone (about 500 nm, Figure 3B), suggesting that the original particle structure of PTMA is possibly destroyed by the addition of apo cyt *c*. At pH 11.8, the D_a of the mixtures of PTMA with apo cyt *c* is in the range of 230−260 nm, smaller than the D_a of 280 nm of apo cyt *c*, implying that the interaction between PTMA and apo cyt *c* inhibits the aggregation of apo cyt *c*. At this high pH, both PTMA and apo cyt *c* carry negative charges, but the hydrophobic interaction between the long alkyl side chains and the hydrophobic residues of apo cyt *c* counteracts the electrostatic repulsion, just as in the report of the interactions of SDS with poly(1-octadecene-co-maleic acid) (POMA) or poly(1-decene-*co*-maleic acid) (PDMA),[30] and the interactions of β-lactoglobulin or bovine serum albumin with a series of alternating copolymers of maleic acid and alkyl-vinyl ethers.[5]

After the interaction of PTMA with apo cyt *c*, 10 mM NaCl was added, which was followed by a few hours of standing and DLS measurements to eliminate the influence of particle charges on the mutual diffusion coefficients as mentioned above. The results are shown in Figure 8. At pH 6.5, the D_a of the mixtures is apparently larger than that without NaCl when the ratio of positive to negative charges is around 1. Increasing the concentration of PTMA to 1.4×10^{-4} g/mL where the negative charge of PTMA is in excess, the D_a of the mixture is about 400 nm, similar to that without NaCl, but smaller than that of PTMA alone with NaCl (about 650 nm, Figure 4B), suggesting that the interaction of PTMA with apo cyt *c* destroys the original particle structure of PTMA. At a PTMA concentration of 2.8×10^{-4} g/mL, there are two peaks: one at about 210 nm, the other at about 600 nm, which possibly corresponds to the particles of PTMA alone. At pH 11.8, the D_a of the mixtures of PTMA with apo cyt *c* is in the range of 210−320 nm, similar to that without NaCl (Figure 7C).

The Interaction of PIMA and PTMA with Apo Cyt *c* Studied by SPR. SPR has been found to be a useful tool for the macromolecular interaction analysis.[31−34] The response signal change is proportional to the change of the refractive index at the chip surface and is generally assumed to be proportional to the mass of substance bound to the chip.[31,32,35] The interactions of apo cyt *c* with PIMA and PTMA are investigated by SPR. The apparent affinity

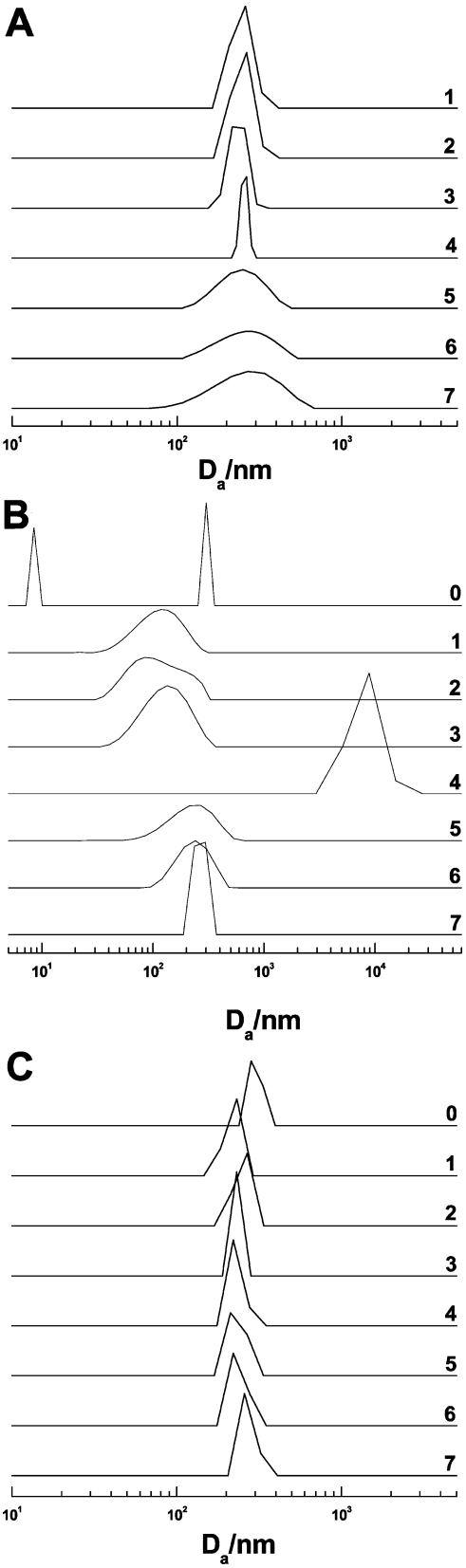

Figure 7. Apparent size distributions of the mixtures of 10 μM apo cyt *c* with different concentrations of PTMA: (1) 4.5×10^{-6}, (2) 9.0×10^{-6}, (3) 1.8×10^{-5}, (4) 3.6×10^{-5}, (5) 7.2×10^{-5}, (6) 1.4×10^{-4}, (7) 2.8×10^{-4} g/mL at pH 2.1 (A), 6.5 (B), and 11.8 (C).

constants (K_a) are calculated as described by Kobayashi et al.,[33,34] from the slopes and intercepts according to the Langmuir equation:

(30) McGlade, M. J.; Randall, F. J.; Tcheurekdjian, N. *Macromolecules* **1987**, *20*, 1782−1786.
(31) Katsamba, P. S.; Park, S.; Laird-Offringa, I. A. *Methods* **2002**, *26*, 95−104.
(32) Nice, E. C.; Catimel, B. *BioEssays* **1999**, *21*, 339−352.
(33) Matsuura, K.; Kitakouji, H.; Sawada, N.; Ishida, H.; Kiso, M.; Kitajima, K.; Kobayashi, K. *J. Am. Chem. Soc.* **2000**, *122*, 7406−7407.
(34) Matsuura, K.; Oda, R.; Kitakouji, H.; Kiso, M.; Kitajima, K.; Kobayashi, K. *Biomacromolecules* **2004**, *5*, 937−941.

$$[C]/\Delta R = [C]/\Delta R_{max} + 1/(\Delta R_{max} \times K_a)$$

where $[C]$ stands for copolymer concentration, ΔR stands for the response signal change after absorption equilibrium for each copolymer concentration, and ΔR_{max} stands for the maximum response signal change. The apparent affinity constants for the interaction of PIMA or PTMA with apo cyt *c* at different pH are listed in Table 1. As we know, molar concentrations of the reagents should be used in the Langmuir equation. In our measurement, the concentrations are in the range of 4.5×10^{-6} to 1.1×10^{-4} g/mL for PIMA, and 4.5×10^{-6} to 2.8×10^{-4} g/mL for PTMA. As discussed above, the structure of PIMA and PTMA is related to the pH of the solution, and PTMA aggregates except at the condition of low concentration and neutral and alkali pH. So, the SPR data shown in Table 1 can only be discussed qualitatively, although very good linear relationships were obtained with Langmuir equation. The K_a values for the interactions of PIMA or PTMA with apo cyt *c* are larger than 10^5 M^{-1}, indicating that both PIMA and PTMA can strongly interact with apo cyt *c* at different pH values, even though the electrostatic interaction, hydrophobic interaction, and hydrogen bonding for these two copolymers are different. K_a for the interaction of PIMA with apo cyt *c* could not be measured by SPR at pH 10.5 where the response signal change was very small, but our previous study found that PIMA could induce apo cyt *c* folding at pH 10.5.[6] The reason may be that the pH of 10.5 is close to the pI of apo cyt *c* and the local positive charges of apo cyt *c* may bind to the surface of sensor chip, naked gold slice, which carries negative charges, and therefore the electrostatic attraction between apo cyt *c* and PIMA is very weak; on the other hand, the hydrophobic interaction between PIMA and apo cyt *c* is not strong because of the short side chains of PIMA.

Gao et al. studied binding isotherms of β-lactoglobulin or bovine serum albumin interacting with a series of alternating copolymers of maleic acid and alkyl-vinyl ethers with varying hydrophobicity at a pH higher than the pI of proteins, showing that the hydrophobic interaction between the protein and polymer could overcome the intra-polymer micellization and the electrostatic repulsion.[5] In our study, the K_a of 1.8×10^6 M^{-1} for PTMA interacting with apo cyt *c* at pH 11.8 indicates that the hydrophobic interaction of PTMA with apo cyt *c* can also overcome the electrostatic repulsion and self-aggregation of PTMA or apo cyt *c* alone. The folding induction of PTMA at this pH also supports this conclusion.[6] Furthermore, DLS and AFM results show that PTMA aggregates dissociate when diluting PTMA solution. The DLS result also shows that the protein−polymer complex particles are smaller than apo cyt *c* aggregates. Therefore, we can obtain a conclusion that the interaction of PTMA with apo cyt *c* destroys the self-aggregation of PTMA or apo cyt *c* at pH 11.8.

At pH 6.5, PTMA particles and apo cyt *c* aggregates dissociate too. DLS shows that the protein−polymer complex particles are much smaller than PTMA particles, as well as smaller than apo cyt *c* aggregates. These results support the conclusion that the interaction of PTMA and apo cyt *c* can destroy the original structure of PTMA particles and apo cyt *c* aggregates. At pH 2.1, PTMA particles do not dissociate at the whole concentration investigated, and we do not find the proof that the interaction of PTMA with apo cyt *c* can destroy the original structure of PTMA.

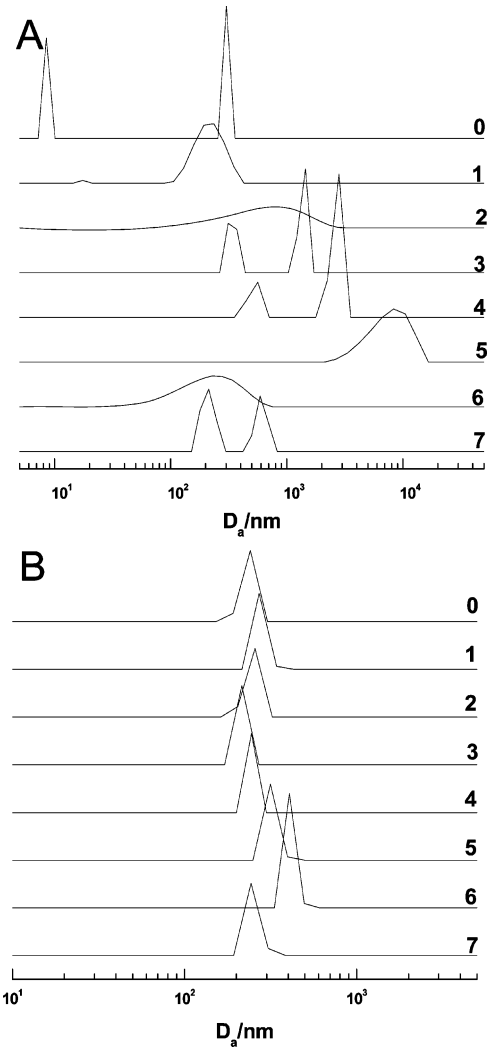

Figure 8. Apparent diameter distributions of the mixtures of PTMA with apo cyt *c* at pH 6.5 (A) and 11.8 (B) in the presence of 10 mM NaCl. The concentration of PTMA: (0) 0, (1) 4.5×10^{-6}, (2) 9.0×10^{-6}, (3) 1.8×10^{-5}, (4) 3.6×10^{-5}, (5) 7.2×10^{-5}, (6) 1.4×10^{-4}, (7) 2.8×10^{-4} g/mL. The concentration of apo cyt *c* is 10 μM.

Table 1. K_a (M^{-1}) for the Interactions of Apo Cyt *c* with PIMA or PTMA

	pH		
	2.1	6.5	11.8
PIMA	4.4×10^5	4.2×10^5	
PTMA	4.3×10^5	2.3×10^5	1.8×10^6

Our previous study found that the I_1/I_3 ratios of pyrene for 7.2×10^{-5} g/mL PTMA solution were 1.23, 1.34, and 1.54 at pH 2.1, 6.5, and 11.8, respectively, and for 10 μM apo cyt *c* solution they were 1.76, 1.46, and 1.46 at pH 6.5, 10.5, and 11.8,[6] which suggests that the PTMA particles and apo cyt *c* aggregates are not very hydrophobic and are somewhat swollen by the water molecules incorporated. Different from PTMA and apo cyt *c* alone, when the PTMA concentration is 7.2×10^{-5} g/mL and apo cyt *c* 10 μM, the I_1/I_3 ratios for PTMA−apo cyt *c* complexes are 1.18, 1.20, and 1.26 at pH 2.1, 6.5, and 11.8, respectively. This result suggests that the interaction of PTMA with apo cyt *c* through the electrostatic and hydrophobic interactions and hydrogen bonding possibly extrudes the water molecules that are originally incorporated in PTMA particles or apo cyt *c* aggregates, forming more hydrophobic structure.

(35) Yamaguchi, H.; Harada, A. *Biomacromolecules* **2002**, *3*, 1163–1169.

Although the copolymers and apo cyt *c* can form complex particles as shown in the studies of DLS and SPR, our previous study found that PIMA and PTMA could not induce apo cyt *c* to form α-helical structure at pH 2.1 when the concentration of PIMA and PTMA was lower than 1.8×10^{-5} and 7.2×10^{-5} g/mL, respectively,[6] where the ratios of the negative charges of the copolymer to the positive charges of apo cyt *c* are 0.1 and 0.2, respectively. It may be explained that the electrostatic repulsion within the apo cyt *c* chain is very strong and the electrostatic attraction between the copolymer and protein is not enough to screen this repulsion; therefore, no α-helical structure is induced when the copolymer concentrations are very low at pH 2.1.[6]

Our previous fluorescence study on apo cyt *c* folding induced by highly sulfonated polystyrene (SPS) nanoparticles found that SPS and apo cyt *c* complex particles at pH 2.0 were more hydrophilic than SPS particles alone, and then we speculated that apo cyt *c* inserted to SPS nanoparticles.[18] Our study on the interaction of apo cyt *c* with PTMA found that PTMA could induce apo cyt *c* to form α-helix.[6] The study in this report exhibits that there is a competition between the interaction of copolymer with apo cyt *c* and self-aggregation of PTMA or apo cyt *c*. There are two possibilities for the interaction of apo cyt *c* with polyelectrolyte particles: one is that apo cyt *c* inserts into the cores of polyelectrolyte particles; the other is that the interchain interaction of apo cyt *c* with the copolymer destroys the primary structure of polyelectrolyte particles and results in an intersecting structure of complex particles. SPS particles are very stable against dilution,[18,36] whereas PTMA particles dissociate at neutral and alkali pH, suggesting that it is easier for apo cyt *c* to destroy PTMA particles than to destroy SPS particles. Therefore, it is possible that apo cyt *c* inserts into SPS particles, whereas apo cyt *c* destroys PTMA particles and forms complex particles at neutral and alkali pH, which is similar to the report of binding study of β-lactoglobulin or bovine serum albumin with a series of alternating copolymers of maleic acid and alkyl-vinyl ethers.[5]

Conclusion

For the interaction of PIMA or PTMA with apo cyt *c*, when the positive or negative charges are in excess, the copolymer−protein complex particles can be stabilized by the charges on the surface. When the ratio of the positive to negative charges is close to the stoichiometric value, precipitation occurs. PTMA and apo cyt *c* carry the negative charges at pH 11.8, but the hydrophobic interaction between the PTMA long alkyl side chains and the hydrophobic residues of apo cyt *c* makes them form complexes. The interaction of the copolymer with apo cyt *c* at neutral and alkali pH destroys the hydrophobic aggregation of PTMA or apo cyt *c* and forms new complex particles. A competition exists between the interaction of the copolymer with apo cyt *c* and the self-aggregation of PTMA or apo cyt *c* alone.

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(36) Li, M.; Jiang, M.; Wu, C. *J. Polym. Sci., Part B: Polym. Phys.* **1997**, *35*, 1593−1599.