Binding of Lysozyme to Unimolecular Micelles Formed from Hydrophobically-Modified Polyelectrolytes

Takeshi Sato, Mikiharu Kamachi, Masanobu Mizusaki, Kenjiro Yoda, and Yotaro Morishima*

Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

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ABSTRACT: The binding of lysozyme, a basic protein with the isoelectric point (pI) of approximately 11.5, to unimolecular micelles (unimer micelles) formed from pyrene (Py)-labeled copolymers of sodium 2-(acrylamido)-2-methylpropanesulfonate and *N*-cyclododecylmethacrylamide was investigated by fluorescence spectroscopy, nonradiative energy transfer (NRET), quasielastic light scattering (QELS), and turbidimetry in 0.25 M NaCl aqueous solutions. The unimer micelle is a negatively charged particle with an apparent hydrodynamic diameter of approximately 11 nm, whose hydrophobic surface is partly exposed to the aqueous phase. When lysozyme was bound to the unimer micelles, Py fluorescence was observed upon excitation of tryptophan (Trp) residues at 290 nm due to NRET from singlet-excited Trp to Py labels, thus allowing us to monitor the lysozyme binding by NRET. Lysozyme was found to bind to unimer micelles even at pH 12.5 (>pI), although its net charge was negative. It is known that lysozyme forms an aggregate at this high pH. Immediately after lysozyme was mixed with unimer micelles in a 0.25 M NaCl aqueous solution, the aggregate of lysozyme binds to the unimer micelle mainly due to hydrophobic interaction. The bound lysozyme aggregate dissociates into lysozyme monomers (or dimers) on the surface of the unimer micelle over a period of approximately 12 h, leading to a complex with an apparent hydrodynamic diameter of approximately 20 nm. Effects of pH and ionic strength on the lysozyme binding indicated that even at a pH > pI electrostatic interaction was in effect conjointly with hydrophobic interaction for the binding of lysozyme to unimer micelles. Upon a decrease in the pH to approximately 8.5, the complexes form aggregates with sizes more than 100 times larger than the discrete complex of lysozyme and unimer micelles, leading to bulk-phase separation. This phase separation at pH ≤ 8.5 is due to charge neutralization between the positive charge of bound lysozyme and the negative charge of the unimer micelle.

Introduction

Early studies of protein−polyelectrolyte complexes (PPCs) were reported by Morawetz et al.[1,2] in the 1950s. They investigated the precipitation of liver catalase by interactions with some synthetic polyelectrolytes. Subsequently, the interactions of various proteins with various natural and synthetic polyelectrolytes have been extensively investigated by a number of groups.[3−14]

Protein−polyelectrolyte interactions, which are controlled mainly by electrostatic forces, may yield soluble complexes,[3] complex coacervates,[4−6] and amorphous precipitates.[7−9] These phenomena may be of use for protein separation,[10] immobilization or stabilization of enzymes,[11,12] and the modification of protein−substrate affinity.[13] Moreover, physicochemical studies of protein−polyelectrolyte interactions may provide some insights into interactions between proteins and nucleic acids in the transcription process.[14]

Kabanov et al.[11,12] studied the complexation of bovine serum albumin (BSA) with quaternized poly(4-vinylpyridine)s, and they proposed a model in which the polycation winds around an asymmetrical, approximately cylindrical stack of BSA molecules. A number of free polyion loops maintain a hydrophilic zone along the surface of the stack, promoting the solubility of the complexes. Kokufuta et al.[9] reported that potassium poly(vinyl alcohol) sulfate and various globular proteins form stoichiometric complexes in salt-free aqueous solutions. Subsequently, they proposed a PPC formation mechanism that neutral intrapolymer complexes, consisting of a polyelectrolyte and a number of bound proteins, associate to yield neutral aggregates of a uniform size. Dubin et al.[15−17] investigated interactions between poly(diallyldimethylammonium chloride) and globular proteins, such as ribonuclease, BSA, and lysozyme. Of particular interest is the preferential binding of the lower isoelectric point proteins to the polycation, which leads to selectivity in the coacervation step. Dubin et al.[15−17] focused on the electrostatic interaction between proteins and polyelectrolytes and found that the complex formation resembled a phase-transition phenomenon. They proposed an empirical linear relationship between $\xi\sigma_C$ and $\mu^{1/2}$, where ξ is the polymer linear charge density, σ_C is the protein surface charge density under conditions where complexation commences, and μ is the solution ionic strength. Recently, we reported interactions between hen egg white lysozyme and pyrene (Py)-labeled copolymers of 2-(acrylamido)-2-methylpropanesulfonate (AMPS) and acrylamide (AAm) by quasielastic light scattering (QELS) and nonradiative energy transfer (NRET) from singlet-excited tryptophan (Trp) residues in lysozyme to Py labels.[18]

Studies of protein−polyelectrolyte interactions reported so far have focused on electrostatic interactions. However, in view of the fact that electrostatic and hydrophobic cooperative interactions play an important role in many biological phenomena, hydrophobic effects on PPC formation should be an important problem to be investigated. Although Kabanov et al.[19] first pointed out the contribution of hydrogen bonding and hydrophobic interactions to PPC formation between am-

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phiphilic polyelectrolytes and BSA, no systematic studies of hydrophobic contribution to protein−polyelectrolyte interactions have so far been reported. The lack of systematic studies in this area motivated us to investigate interactions between hydrophobically modified polyelectrolytes and lysozyme.

Random copolymers of AMPS and hydrophobic methacrylamides *N*-substituted with bulky hydrophobes, such as dodecyl, cyclododecyl, and adamantyl groups, form unimolecular micelles (unimer micelles) in aqueous solution independent of the polymer concentration.[20−24] Such unimer micelles can be viewed as a negatively charged compact particle whose hydrophobic surface is partly exposed to the aqueous phase. We chose the random copolymers of AMPS and *N*-cyclododecylmethacrylamide (CDMAm) for a study of interactions with lysozyme. This copolymer forms a compact unimer micelle (e.g., the copolymer of 50 mol % CDMAm with a weight-average molecular weight of 5.1×10^5 forms a unimer micelle with an apparent hydrodynamic radius of 5.5 nm and a mean square radius of gyration of 5 nm).[23] In this paper, we report on the interaction of lysozyme with unimer micelles formed from Py-labeled AMPS−CDMAm copolymers studied by fluorescence, NRET, and scattering (turbidimetry and QELS) techniques.

Experimental Section

Materials. *N*-Cyclododecylmethacrylamide (CDMAm) was prepared as reported previously.[20] 2-(Acrylamido)-2-methylpropanesulfonic acid (AMPS) was purchased from Wako Pure Chemical Co. and was recrystallized from methanol. 2,2′-Azobis(isobutylonitrile) (AIBN), purchased from Nacalai Tesque Inc., was recrystallized from methanol prior to use. Hen egg white lysozyme [EC3.2.1.17] was purchased from SIGMA Chemical Co. and used as received. Hexaoxyethylene mono-*n*-dodecyl ether ($C_{12}E_6$) was purchased from Nikko Chemicals Co. and used as received.

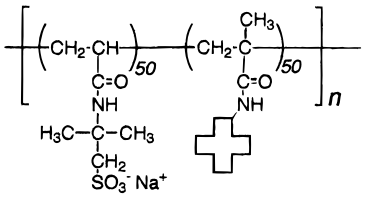
Polymerization. A random copolymer of AMPS with 50 mol % CDMAm, poly(A/CD) (see Chart 1), was prepared by free-radical polymerization initiated with AIBN in *N*,*N*-dimethylformamide (DMF). A DMF solution of the two monomers along with 0.5 mol % AIBN (on the basis of the total monomers) was degassed by five freeze−pump−thaw cycles. Polymerization was carried out at 60 °C for 10 h. The polymer was precipitated with excess ether and purified by reprecipitation from methanol into excess ether three times. The polymer was then air-dried and dissolved in water. After neutralization with aqueous NaOH, the solution was dialyzed against pure water for 3 days. The polymer was recovered by lyophilization. The weight-average molecular weight (M_w) was determined by static light scattering to be approximately 5×10^5.

Fluorescence-labeled copolymers were prepared and purified as reported previously.[21,23]

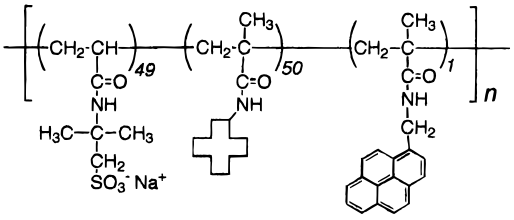
Sample Preparation. Sample solutions were prepared by mixing polymer and lysozyme solutions whose pH and ionic strength (μ) were adjusted to 11.5 and 0.25, respectively. Both the solutions were filtered with an ADVANTECH 0.1-μm syringe filter prior to mixing. The polymer concentration in the resulting solution was constant at 0.05 g/L. To increase the number of net positive charges on the protein surface, the pH was decreased by adding a 0.5 M HCl solution, dispensed from a Gilmont microburet. All fluorescence, NRET, QELS, and turbidity measurements were carried out 24 h after mixing of the lysozyme and polymer solutions (except for time-dependent measurements).

Sample solutions at a pH > 12 were prepared by adding a 0.5 M NaOH aqueous solution, taking into account the contribution of NaOH to the ionic strength.

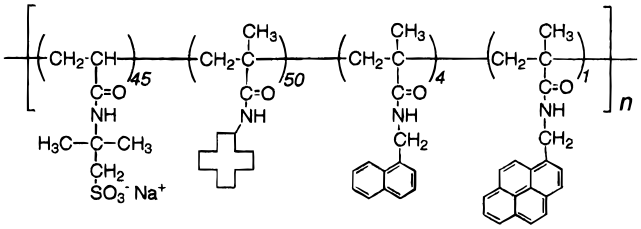
Chart 1. Polymers Employed in This Study



poly(A/CD)



poly(A/CD/Py*1*)



poly(A/CD/Np*4*/Py*1*)

Nonradiative Energy Transfer (NRET). Fluorescence spectra were obtained with a Hitachi F-4500 spectrophotometer. The excitation wavelength was chosen to be 290 nm to excite selectively tryptophan (Trp) residues in lysozyme. The fluorescence intensity of pyrene labels was monitored at 376 nm. A 0.2-mm cell, with the incident light angled at 45°, was used to minimize inner filter effects.[25] The magnitude of NRET was expressed by the relative fluorescence intensity, I/I_0, where I is the fluorescence intensity at a given condition in the presence of lysozyme and I_0 is the intensity at pH 11.5 in the absence of lysozyme.

Turbidimetry. Turbidity measurements were conducted with the same samples for the fluorescence measurements with a Shimadzu UV-2500PC spectrophotometer using a 1-cm path length cell at 420 nm. All turbidity values are reported in the form 100 − % transmittance (100 − %T).

Quasielastic Light Scattering (QELS). QELS measurements were carried out at 25 °C and at a scattering angle of 90° using an Otsuka Photal DLS-7000 light-scattering system equipped with a 75-mW Ar^+ laser (NEC) operating at 488 nm in a vacuum. The autocorrelation decay data were obtained and fitted with a multiexponential decay function using an ALV-5000 wide-band, multi-τ, digital autocorrelator. For samples with the unimodal distribution of relaxation times, apparent diameters were obtained with the first cummulant method.

Results and Discussion

Time Dependence of Complex Formation. Copolymers of AMPS and CDMAm, with or without fluorescence labels, form unimer micelles in aqueous solution.[23] Figure 1 shows fluorescence spectra for solutions of 0.05 g/L of poly(A/CD/Py*1*) (see Chart 1) in 0.25 M NaCl in the absence and presence of 0.05 g/L of lysozyme at pH 11.5. All the sample solutions were excited at 290 nm. In the absence of lysozyme, poly(A/CD/Py*1*) shows weak fluorescence because excitation of Py labels at this wavelength cannot be completely

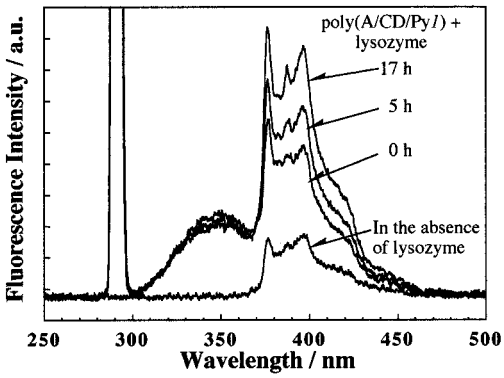


Figure 1. Fluorescence spectra for a mixture of 0.05 g/L of lysozyme and 0.05 g/L of poly(A/CD/Py*1*) in 0.25 M NaCl at pH 11.5 recorded immediately, 5 h, and 17 h after mixing lysozyme and the polymer solutions. A fluorescence spectrum for a solution of 0.05 g/L of poly(A/CD/Py*1*) in 0.25 M NaCl at pH 11.5 is presented for comparison. All the sample solutions were excited at 290 nm.

avoided. In the presence of lysozyme, however, the Py fluorescence is significantly enhanced because of NRET from excited Trp to Py labels. The spectra for the polymer−lysozyme mixture were recorded at varying time intervals after mixing the stock solutions of lysozyme and the polymer. The intensity of Py fluorescence gradually increased with time over a period of 17 h, indicating that the magnitude of NRET increases with time.

In our earlier study,[18] we calculated the Förster radius (R_0)[26,27] for NRET from Trp to Py labels to be approximately 2 nm at pH 11.5 based on the spectral overlap between the lysozyme fluorescence and Py absorption spectra. Since the unimer micelle formed from poly(A/CD/Py*1*) has an apparent hydrodynamic radius of 5.5 nm,[23] which is greater than R_0, some Py labels buried in the unimer micelle may not be able to participate in the NRET event.

These considerations suggest a possibility that the time dependence of NRET shown in Figure 1 is due to a gradual unfold of unimer micelles upon binding with lysozyme. If unimer micelles are unfolded, the number of Py labels that can participate in the NRET event would increase, which would lead to an increase in the magnitude of NRET. In fact, it was reported that such unimer micelles were unfolded in the presence of didodecyldimethylammonium bromide, a double-chain cationic surfactant, in aqueous solution.[22] In unimer micelles formed by poly(A/CD/Np*4*/Py*1*) (see Chart 1), a polymer labeled doubly with naphthalene (Np) and Py, efficient NRET occurs from Np to Py and the polymer exhibits intensive Py fluorescence upon excitation of Np labels at 290 nm (Figure 2). Upon addition of hexaoxyethylene mono-*n*-dodecyl ether ($C_{12}E_6$), a nonionic surfactant, the intensity of Py fluorescence significantly decreases while the intensity of Np fluorescence increases. This is a clear indication of the unfolding of the unimer micelle caused by the addition of $C_{12}E_6$. On the other hand, in the presence of lysozyme at pH 11.5, the fluorescence spectrum remains practically intact except for a slight increase in the Py fluorescence intensity presumably due to NRET from Trp to Py labels. From these results, we conclude that unimer micelles are not unfolded by lysozyme binding and that the micellar structure remains virtually the same before and after the lysozyme binding.

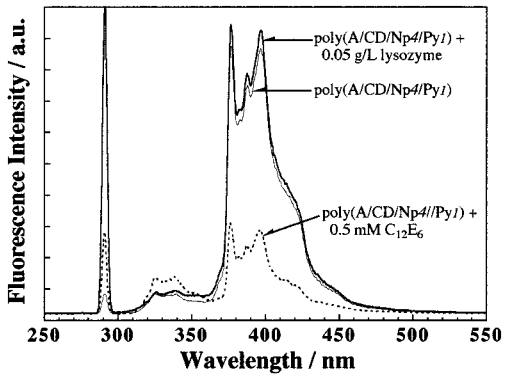


Figure 2. Fluorescence spectra for poly(A/CD/Np*4*/Py*1*) in 0.25 M NaCl at pH 11.5 in the absence and presence of lysozyme or $C_{12}E_6$. The spectra were measured 24 h after mixing with excitation at 290 nm.

It is known that lysozyme, a basic protein with the isoelectric point (pI) of approximately 11.5, exists in a dimer form below pH 10 and in an aggregate form above pH 10.[28,29] In our earlier work,[18] we observed the binding of lysozyme aggregates to a Py-labeled homopolymer of AMPS (polyAMPS) at pH 12.3, but no NRET was recognized until the pH was reduced to approximately 10.5, and thus, we concluded that the bound lysozyme aggregates dissociated near pH 10.5, allowing NRET to occur. In contrast to the unimer micelle of poly(A/CD/Py*1*) in the present study, significant NRET occurs at a pH of 11.5, although the NRET is time-dependent (Figure 1).

To examine whether lysozyme aggregates dissociate when bound to the unimer micelle at pH 11.5, we followed changes in the magnitude of NRET and in the emission maximum (λ_{max}) of Trp fluorescence as a function of time (Figure 3). In these experiments, we employed nonlabeled unimer micelles formed from poly(A/CD) (see Chart 1) for simplicity in the fluorescence spectra of lysozyme bound to the unimer micelle. Figure 3 shows plots of I/I_0 and λ_{max} as a function of time at pHs 11.5 and 8.5. Here, I is the Py fluorescence intensity at 376 nm at a given pH and I_0 is the Py fluorescence intensity in the absence of lysozyme at pH 11.5. The values of I can be determined by subtraction of the contribution from Trp fluorescence at 376 nm.[18] Since the fluorescence intensity of Py labels was confirmed to be independent of pH in the whole pH range examined (pH 4−13), the value of I/I_0 represents the magnitude of NRET. As can be seen in Figure 3a, I/I_0 at pH 11.5 increases with time over a period of approximately 12 h, reaching a constant value of $I/I_0 \approx 3.7$. We previously reported that the dissociation of the lysozyme aggregate could be monitored by λ_{max}.[18] As can be seen from Figure 3a, the increase in I/I_0 is accompanied by a blue shift of λ_{max} in Trp fluorescence (i.e., λ_{max} decreases over a period of approximately 12 h), reaching a constant value of $\lambda_{max} \approx 340$ nm. This implies that lysozyme aggregates dissociate on the unimer micelle over this period of time. In contrast, NRET and λ_{max} are independent of time at pH 8.5 at which lysozyme aggregates dissociate[28,29] (Figure 3b). Therefore, we conclude that the time dependencies of I/I_0 and λ_{max} observed at pH 11.5 arise from a gradual dissociation of lysozyme aggregates on the unimer micelle. Namely, lysozyme binds to unimer micelles in its aggregate form first, and then the aggregate slowly dissociates into dimers or monomers on the surface of unimer micelles in a period of approximately12 h. At

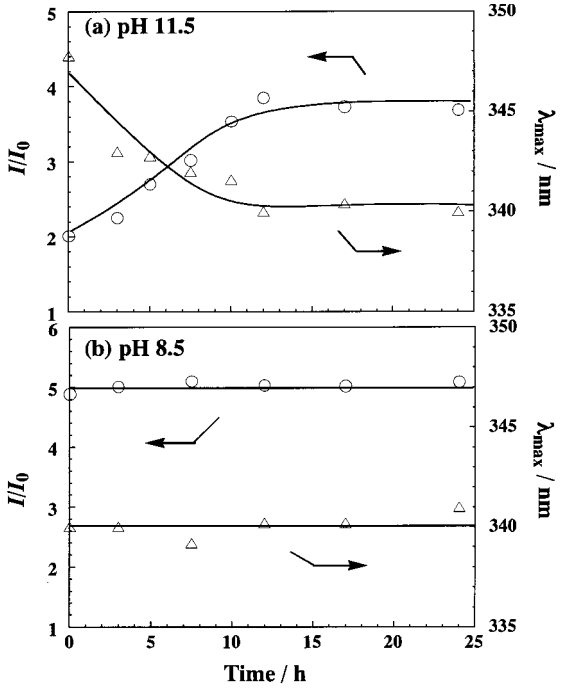


Figure 3. Time dependencies of I/I_0 (○) for a 0.25 M NaCl solution of lysozyme−poly(A/CD/Py*1*) and of λ_{max} of Trp fluorescence (△) for lysozyme−poly(A/CD) at pH 11.5 (a) and 8.5 (b). The concentrations of lysozyme and the polymers are 0.05 g/L, respectively. All the measurements were conducted 24 h after mixing with excitation at 290 nm.

present, we do not know whether lysozyme molecules bound to unimer micelles are dimeric or monomeric because a difference in λ_{max} for the lysozyme monomer and dimer is very subtle.

Figure 4a shows a histogram of size distribution for free poly(A/CD/Py*1*) at pH 11.5. The average diameter for this polymer is approximately 10 nm. Figure 4b shows a size distribution for a lysozyme−poly(A/CD/Py*1*) mixture at pH 11.5 measured immediately after mixing. This mixture shows an apparently unimodal distribution with a peak at approximately 20 nm. This indicates that lysozyme binds to unimer micelles in its aggregate form immediately after mixing, given that lysozyme aggregates show a size distribution with a peak at approximately 10 nm at pH 11.5.[18] Figure 4c shows a size distribution for the same mixture observed 24 h after mixing. The distribution of the size becomes significantly narrower, and particles with diameters below 10 nm are not observed anymore, although the average diameter remains essentially the same as that immediately after mixing. Taken together with the results of NRET, this suggests that lysozyme aggregates initially bound to the unimer micelle dissociate on the unimer micelle within 24 h. A conceptual model for the lysozyme binding to unimer micelles discussed above is illustrated in Scheme 1.

Effect of Lysozyme/Polymer Ratio. To clarify whether the binding of lysozyme to unimer micelles is a stoichiometric phenomenon, the magnitude of NRET (i.e., I/I_0) and the apparent diameter for the complex of lysozyme and unimer micelles were measured at pH 11.5 at varying ratios of the weight concentration of lysozyme to that of the polymer (r = [lysozyme]/[polymer]). All the measurements were performed after equilibration for 24 h. Figure 5 shows plots of I/I_0 and the apparent diameter for the lysozyme−poly(A/CD/Py*1*) system as a function of r. The magnitude of NRET

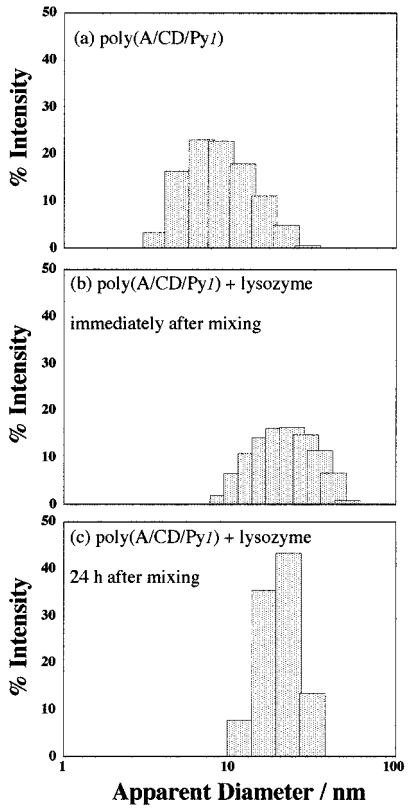
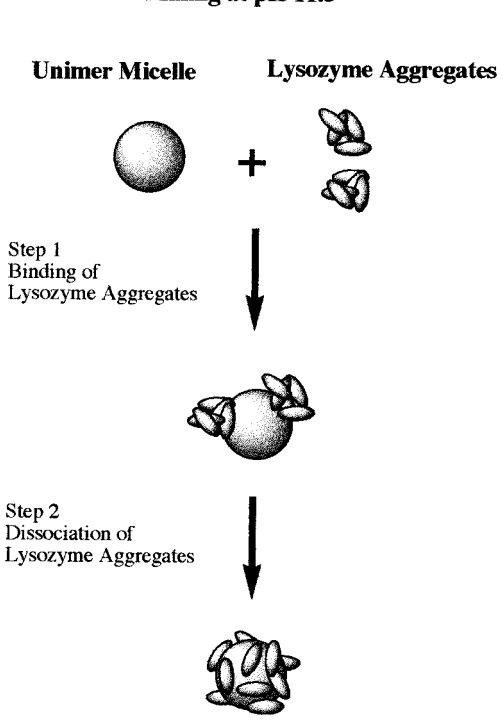


Figure 4. Distributions of apparent diameters for a solution of 0.05 g/L of poly(A/CD/Py*1*) in 0.25 M NaCl at pH 11.5 in the absence (a) and presence of 0.05 g/L of lysozyme immediately after mixing (b) and 24 h after mixing (c).

Scheme 1. Conceptual Illustration of the Binding of Lysozyme on Unimer Micelles



appears to change with r parallel to the apparent diameter (i.e., both I/I_0 and the apparent diameter increase with increasing r up to $r \approx 1$ and then tend to saturate). The I/I_0 ratio increases linearly with r in the regime $r < 1$, suggesting a stoichiometric binding of

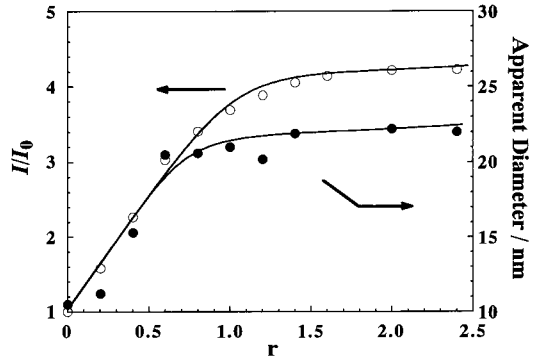


Figure 5. I/I_0 (○) and apparent diameter (●) for poly(A/CD/Py*1*) (0.05 g/L) plotted against r (=[lysozyme]/[polymer]) in 0.25 M NaCl at pH 11.5 after equilibration for 24 h.

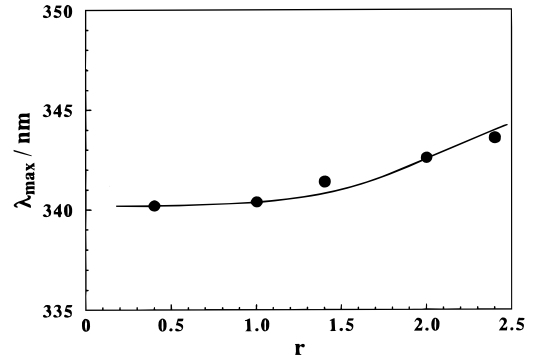


Figure 6. λ_{max} for Trp fluorescence against r (=[lysozyme]/[polymer]) in 0.25 M NaCl solutions of lysozyme−poly(A/CD) at pH 11.5 after equilibration for 24 h.

lysozyme to unimer micelles. The saturation of I/I_0 at $r > 1$ suggests that binding sites for lysozyme on a unimer micelle are saturated with bound lysozyme. Given the diameters for the poly(A/CD/Py*1*) unimer micelle and a lysozyme molecule are approximately 11 and approximately 5 nm, respectively, the saturated apparent diameter at $r > 1$ (i.e., approximately 22 nm) appears to be close to the diameter of a unimer micelle surrounded by monomeric lysozyme molecules.

Figure 6 shows λ_{max} for Trp fluorescence in the lysozyme−poly(A/CD) system plotted as a function of r at pH 11.5. At $r \leq 1$, λ_{max} is almost constant at approximately 340 nm, indicating that lysozyme exists in a dissociated form.[18] At $r > 1$, on the other hand, λ_{max} increases with increasing r. Since lysozyme aggregates show a λ_{max} at 347 nm,[18] the red shift observed at $r > 1$ may be due to excess lysozyme in an aggregate form in the bulk phase.

From these considerations, we conclude that the magnitude of NRET reflects the number of lysozyme molecules bound to the unimer micelle and that in the complex at $r \geq 1$ the unimer micelle is covered with lysozyme molecules.

Effect of pH on the Lysozyme Binding. Figure 7 shows the dependencies of I/I_0 and turbidity (reported as 100 − % transmittance) on pH for the lysozyme−poly(A/CD/Py*1*) system after equilibration for 24 h in 0.25 M NaCl. Significant NRET was observed at pHs > pI (=11.5) for lysozyme, at which lysozyme is negatively charged. This implies that the binding of lysozyme to the unimer micelle is primarily induced by hydrophobic interaction in this high pH regime. Upon decreasing pH from approximately 13.5, I/I_0 shows a sharp increase in a narrow pH region from 13 to 12.5,

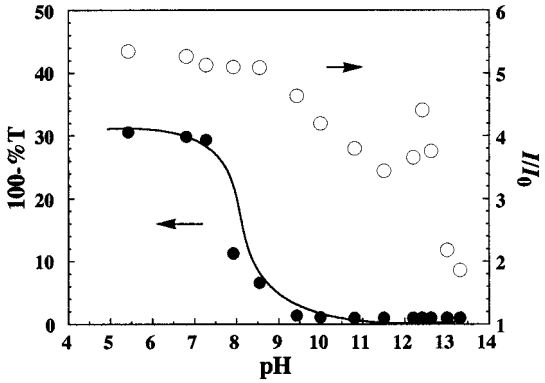


Figure 7. I/I_0 (○) and turbidity (●) as a function of pH for a lysozyme−poly(A/CD/Py*1*) in 0.25 M NaCl after equilibration for 24 h.

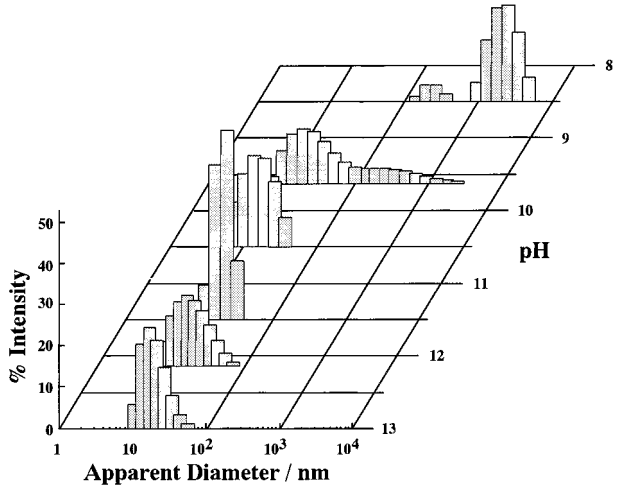


Figure 8. Distribution of apparent diameters for a lysozyme−poly(A/CD/Py*1*) mixture in 0.25 M NaCl at varying pHs after equilibration for 24 h.

although the plots show considerable scatter. At present, why NRET commences abruptly at this high pH is an open question. However, a plausible explanation may be that since this pH is near pK_a (=12.5) for arginyl residues,[30] positively charged arginyl residues may find their way to the negative charge on the unimer micelle and thus electrostatic interaction may interplay with hydrophobic interaction in the lysozyme binding event. In fact, Dubin et al.[16] reported that lysozyme interacted with polyAMPS in 0.10 M NaCl, even though the net charge of lysozyme is negative at a pH > pI. As can be seen in Figure 7, I/I_0 increases gradually in a pH region from 12 to 8.5 upon a further decrease in pH. This is presumably because the number of bound lysozyme molecules increases with increasing positive charges on lysozyme. At a pH of approximately 8.5, where I/I_0 shows a tendency for saturation, turbidity commences to increase. This suggests that charge neutralization in the lysozyme−poly(A/CD/Py*1*) complex occurs near this pH, leading to the aggregation of the complexes.

Figure 8 exhibits histograms showing size distributions for a lysozyme−poly(A/CD/Py*1*) mixture at varying pHs after equilibration for 24 h. At pH ≥ 10.5, the size distribution is unimodal with a peak at approximately 20 nm. It is likely that, at pH ≥ 10.5, the complexes are negatively charged because the positive charge on lysozyme is not enough to neutralize all the negative charge on the unimer micelle in the complex. Thus, each complex can exist as an independent particle with

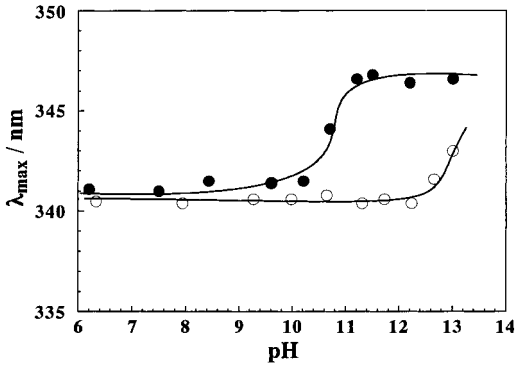


Figure 9. λ_{max} for Trp fluorescence for lysozyme solutions in 0.25 M NaCl in the absence (●) and presence (○) of poly(A/CD) as a function of pH after equilibration for 24 h.

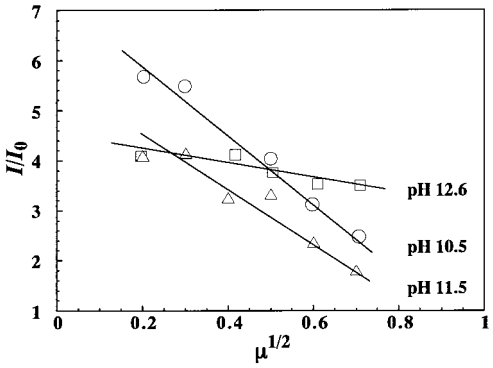


Figure 10. I/I_0 values for mixtures of lysozyme (0.05 g/L) and poly(A/CD/Py*1*) (0.05 g/L) as a function of the square root of the ionic strength ($\mu^{1/2}$) at pH 10.5, 11.5, and 12.6. The I/I_0 values were measured after equilibration for 24 h.

an average diameter of approximately 20 nm. However, at pH < 9.5, particles with much larger sizes (10−100 times larger than that of the independent complex particle) are formed, indicative of the aggregation of the complexes, arising from the charge neutralization. At pH 8.5, there is no discrete complex particle and all the particles are aggregated.

Since the dissociation of lysozyme aggregates can be monitored by λ_{max} for Trp fluorescence,[18] we compared the dependence of λ_{max} on pH in the absence and presence of unimer micelles formed from poly(A/CD) (Figure 9). In the absence of the unimer micelle, λ_{max} is approximately 347 nm at pH ≥ 11.5, indicative of the lysozyme aggregate. Upon decreasing pH, λ_{max} decreases to approximately 341 nm in a pH region of 10.5−11.5, indicating the dissociation of the lysozyme aggregates. In a pH region of 6−10, λ_{max} is virtually constant at approximately 341 nm, reflecting lysozyme in dissociated forms.[28,29] In the presence of the unimer micelle, by contrast, λ_{max} is approximately 343 nm at pH 13, which is a shorter wavelength than that in the absence of the unimer micelle. This implies that lysozyme aggregates bound to the unimer micelle are partially dissociated at pH 13. Upon decreasing pH, λ_{max} immediately decreases to approximately 341 nm and stays with this wavelength at pH ≤ 12.5.

From these results we can conclude that lysozyme molecules are bound to the unimer micelle in an aggregate form at pH 13, and then the aggregate is partially dissociated at this pH. At pH ≤ 12.5, however, the bound lysozyme aggregates completely dissociate, thus leading to a sharp increase in the magnitude of NRET (i.e., I/I_0) near a pH of 12.5 (Figure 7).

Driving Forces for the Binding. To clarify driving forces for the lysozyme binding to the unimer micelle, the effect of the ionic strength on the binding was examined at varying pHs. Since the hydrophobic surface of the unimer micelle is partly exposed to the aqueous phase,[20−24] it is reasonable to consider that hydrophobic interaction is a driving force for the lysozyme binding especially at high pHs. If electrostatic interaction is in effect conjointly with hydrophobic interaction, the binding of lysozyme will be disfavored at higher ionic strengths due to an electrostatic shielding effect. Figure 10 shows plots of I/I_0 against the square root of the ionic strength ($\mu^{1/2}$) (i.e., the Debye's screening parameter (κ)) after equilibration for 24 h. All the plots show linear lines with negative slopes, indicative of the contribution of electrostatic attractive interactions. It is important to note that the slopes are negative even at pHs ≥ pI (=11.5), although the slope decreases with an increase in pH. At pH 11.5, lysozyme has positively charged arginyl residues, although its net charge is zero.[30] Therefore, lysozyme can electrostatically interact with a negative charge on the unimer micelle even at this pH.[16,18] The slightly negative slope at pH 12.6 suggests that there is a slight contribution of electrostatic interaction even at this high pH, although the dominant driving force for the binding at this pH is hydrophobic interaction. Since the p*K*a for arginyl residues[30] is 12.5, nearly half of arginyl residues are positively charged at pH 12.5, thus leading to a weak electrostatic interaction of lysozyme with a negative charge on the unimer micelle conjointly with hydrophobic interaction.

Conclusions

The binding of lysozyme to unimer micelles formed from Py-labeled copolymers of AMPS and CDMAm was investigated by fluorometry, NRET, QELS, and turbidimetry in 0.25 M NaCl. Lysozyme binds to unimer micelles even at pHs above its pI (=11.5) in its aggregate form. The bound lysozyme aggregate dissociates into lysozyme dimers (or monomers) on the surface of unimer micelles over a period of approximately 12 h. Effects of pH and the ionic strength on the lysozyme binding indicate that, even at pHs > pI where the net charge of lysozyme is negative, electrostatic interaction is in effect conjointly with hydrophobic interaction for the binding. The contribution of the electrostatic effect increases with decreasing pH. At pH < 8.5 the complexes form aggregates due to charge neutralization, leading to bulk-phase separation.

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