

BINDING OF THERMO-SENSITIVE AND pH-SENSITIVE BUTYLATED POLY(ALLYLAMINE)S WITH LYSOZYME*

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Abstract Butyl modified poly(allylamine)s with butyl substitution degrees of 15% to 70% were prepared. The polymers show pH sensitive property and lower critical solution temperature (LCST) behavior. The LCST appears at lower temperature, lower pH and lower polymer concentration for the polymer with higher butylated degree. The binding of native lysozyme with the polymers depends on the hydrophobicity of the polymers at the pH range that the protein and the polymer carry the same positive charges. The increase of polymer hydrophobicity can increase the binding with lysozyme, but the self-aggregation of the polymer decreases the binding. The bound lysozyme molecules can recover their native activity completely after the dissociation of the complexes. Compared with native lysozyme, the denatured one which exposes the hydrophobic residues can increase the binding with the polymer and form stable complex nanoparticles.

Keywords: Hydrophobic interaction; LCST; Polyelectrolytes; Protein binding.

INTRODUCTION

The applications of polyelectrolyte/protein complexes are rapidly increasing due to simple and efficient encapsulation of protein^[1], detection of protein^[2], separation of protein^[3], fabrication of fancy materials by layer by layer method^[4] and stabilization of emulsion^[5], *etc.* However, the protein containing polyionic complexes (PIC) in aqueous solutions are not stable, which limits their applications^[6].

One effort to overcome this disadvantage is introducing short-range hydrophobic interaction in the polyionic complexation. The hydrophobically modified polyelectrolytes show more stable binding with the oppositely charged species. A recent research compared poly(γ -glutamic acid) (γ -PGA)/poly(ε -lysine) (ε -PL) PIC micelles with those composed of L-phenylalanine (L-Phe) modified γ -PGA and ε -PL in phosphate buffered saline (PBS)^[7]. The γ -PGA-Phe/ ε -PL nanoparticles showed high stability for a longer storage and over a wider pH range, while γ -PGA/ ε -PL complexes disassembled in PBS. The introduced hydrophobic segments not only improve the stability of the complexation but also increase the binding amount of proteins with polyelectrolytes^[8, 9]. The association between bovine serum albumin (BSA) and a set of alkyl-grafted poly(acrylic acid)s (HMPA) in aqueous solution revealed that the binding amount of the protein per HMPA chain increased with the increase of the alkylate degree when the protein was excess^[9]. Furthermore, the hydrophobic regions of BSA or lysozyme interacted strongly with the alkyl groups of one or more HMPA

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chains. In the later case, the macromolecules were crosslinked, which led to viscosity enhancement and even gelation^[10]. We previously studied the complexation of lysozyme with poly(isobutylene-alt-maleic acid) (PIMA) and poly(1-tetradecene-alt-maleic acid) (PTMA) at pH 7.4^[11]. The electrostatic interaction of PIMA with lysozyme resulted in a loose complex structure in which some of the lysozyme molecules retained a part of activity and native structure. At the same time, the electrostatic and hydrophobic interactions between PTMA and lysozyme produced a more compact complex structure and a complete disappearance of lysozyme tertiary structure and activity. In the release process, the denatured lysozyme molecules were able to avoid hydrophobic aggregation and able to fully regenerate their native structure and activity.

Gao and Dubin have demonstrated that a minimum alkyl chain length of 3–4 methylenes is required for significant hydrophobic interaction between protein and polymer^[12]. In a mixture of protein and polymer, the complexation is a balance result involving polymer-polymer, polymer-protein and protein-protein interactions in solution^[12–16]. The protein-polymer binding usually needs to overcome polymer-polymer interactions in which the molecular weight, concentration and chemical composition of the polymer play important roles. When the concentration of a polymer with certain hydrophobic degree reaches to its critical aggregation concentration, the polymer chains tend to self-aggregation, which is unfavorable for protein binding^[12]. Furthermore, the polyelectrolyte/protein complexes can dissociate when the polyelectrolyte and protein carry the same net charges. These tunable and reversible binding properties are essential for protein delivery systems and protein separation systems. For the applications of hydrophobically modified polyelectrolyte/protein complexes, it is important to investigate conscientiously the influence of the hydrophobicity of polyelectrolyte on the binding with protein. In the present research, butyl-modified poly(allylamine)s with variant substitution degrees were prepared. The polymers with higher substitution degrees exhibit pH sensitive and thermo-sensitive self-aggregation behaviors. The binding of the polymers with native and denatured lysozyme at a temperature range of 4–30°C and pH range of 7–11 was investigated to understand the influence of the polymer hydrophobicity.

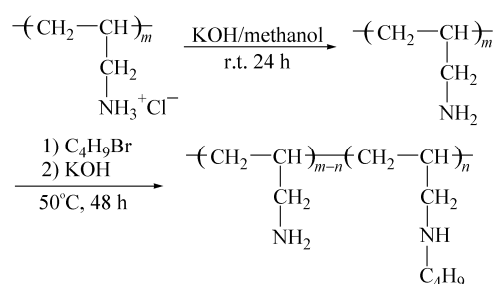
EXPERIMENTAL

Materials

Poly(allylamine) hydrochloride (PAHCl, molecular weight 15000, Sigma), egg white lysozyme (high pure, Sino-American Biotechnology Co.), *Micrococcus lysodeikticus* (Sigma), *n*-butyl bromide (C₄H₉Br, analytical grade, Sinopharm Chemical Reagent Co., Ltd.), guanidine hydrochloride (GdHCl, high pure, Sangon Biotech Co., Ltd.) and dithiothreitol (DTT, 99%, Bio Basic Inc.) were used as received. Bicinchoninic acid (BCA) protein assay kit was from Pierce Chemical Co. All solutions were prepared using deionized water and the pH of the solutions was adjusted with 1 mol·L⁻¹ HCl or 1 mol·L⁻¹ NaOH.

Preparation of Butylated Poly(allylamine)s

Butyl-modified poly(allylamine)s were prepared as reported in literature^[17, 18], and the approach is shown in Scheme 1. For the polymer with 15% butyl substitution degree (C4-15), the preparation process is as follows. PAHCl (1 g) and KOH (0.662 g) were dissolved together in 50 mL of methanol. The mixture was stirred for 24 h and then was concentrated to about 15 mL by rotary evaporation. After adding 25 mL of ethanol, the mixture was evaporated to about 15 mL again. The salt in the mixture was removed by filtration with a polytetrafluoroethylene (PTFE) filter. Butyl bromide (0.118 mL) was added into the filtrate and then the mixture reacted under stirring at 50°C for 48 h. The resultant solution was cooled to room temperature, followed by adding KOH (0.066 g in 10 mL of methanol), stirring for 1 h, adding 10 mL of ethanol and evaporating the mixture to about 15 mL in succession. After removing the salt using a PTFE filter, the polymer was obtained by precipitation in hexane/ethyl ether mixture (1/1, *V/V*) or petroleum ether two times, and then was dried under vacuum at room temperature for 48 h. The butylated poly(allylamine)s were characterized by elemental analysis (elementar VARIO ELIII) and ¹H-NMR (400 MHz, BRUKER AVAVCE DMX500) in D₂O with tetramethylsilane as an internal standard. For the preparation of the other butylated poly(allylamine)s, the feeding amounts of PAHCl, butyl bromide and KOH are shown in Table 1.

**Scheme 1** Reaction scheme of the preparation of butylated poly(allylamine)s**Table 1.** Preparation condition and substitution degree (SD) of butylated poly(allylamine)s

Polymer	W_{PAHCl} (g)	$V_{\text{C}_4\text{H}_9\text{Br}}$ (mL)	$W_{\text{KOH (1st)}}$ (g)	$W_{\text{KOH (2nd)}}$ (g)	Precipitant (V/V)	SD (EA) ^a	SD (NMR) ^b	SD (average) ^c
C4-15 ^d	1	0.118	0.066	0.068	Hexane/ethyl ether (1/1)	0.172	0.136	0.154
C4-30	1	0.236	0.066	0.136	Hexane/ethyl ether (1/1)	0.308	0.293	0.301
C4-44	1	0.353	0.066	0.201	Hexane/ethyl ether (1/3)	0.427	0.442	0.435
C4-53	1	0.470	0.066	0.268	Hexane/ethyl ether (1/3)	0.546	0.504	0.525
C4-58	1	0.588	0.066	0.332	Petroleum ether	0.567	0.596	0.582
C4-70	1	0.705	0.066	0.402	Petroleum ether	0.702	–	0.702

^a Substitution degree obtained by elemental analysis; ^b Substitution degree obtained by ¹H-NMR; ^c Average substitution degree calculated from the data of elemental analysis and ¹H-NMR; ^d C4-15 denotes the butylated poly(allylamine) with butyl substitution degree of 15%.

Preparation of Polymer Solutions and Turbidity Measurements

Butylated poly(allylamine) was dissolved in water to prepare a stock solution with the concentration of 2–5 mg·mL⁻¹. The turbidity of the stock solution in a temperature range from 10°C to 70°C was measured on a spectrophotometer (Lambda 35, Perkin-Elmer) by recording the absorbance at 450 nm. In each temperature, the solution was equilibrated for 10 min before the measurement. For the turbidity measurement at pH 11.0, the polymer solutions with a final concentration of 0.2 mg·mL⁻¹ were adjusted to pH 11.0. After overnight equilibrium at 4°C, the pH was adjusted again. Then, the absorbance of the solutions at 450 nm was measured in a temperature range of 6–55°C.

Steady-state Fluorescence Measurements

Fluorescence emission spectra were measured on a fluorescence spectrophotometer (FLS-920, Edinburgh). The polymer solutions were adjusted to pH 7.0 and pH 11.0, respectively, followed by the addition of pyrene acetone stock solution (2×10^{-5} g·mL⁻¹). The final pyrene and the polymer concentrations were 1×10^{-7} g·mL⁻¹ and 0.2 mg·mL⁻¹, respectively. The resultant mixtures were incubated at 4°C or 30°C for 48 h before measurements. The spectra were recorded at 4°C or 30°C according to the incubation temperature with an excitation wavelength of 330 nm. Three scans were accumulated for each measurement. Both of the excitation and emission slits were 1 nm.

Preparation of Lysozyme Solutions

Lysozyme was dissolved in water, or denaturant solution (pH 9.0, 6 mol·L⁻¹ GdHCl, and 30 mmol·L⁻¹ DTT) to prepare stock solutions. The concentration of lysozyme stock solutions was measured spectrophotometrically at 280 nm using an extinction coefficient of 2.63 mg⁻¹·cm⁻¹ for native lysozyme and 2.37 mg⁻¹·cm⁻¹ for denatured lysozyme^[19].

Preparation of Complex Solutions and Separation of Unbound Lysozyme Molecules

The complex solution of native lysozyme with butylated poly(allylamine) was prepared by dropwise adding lysozyme solution into polymer solution under shaking. The final concentration of lysozyme was $10\ \mu\text{mol}\cdot\text{L}^{-1}$ ($0.143\ \text{mg}\cdot\text{mL}^{-1}$); the final polymer concentration was $0.2\ \text{mg}\cdot\text{mL}^{-1}$. The pH of the solution was adjusted to 11.5 then the solution was equilibrated at 4°C or 30°C overnight. After the pH was adjusted to 11.0 or 7.0, the solution was equilibrated at 4°C or 30°C overnight again. The free lysozyme molecules in the solution were separated from the complexes by ultrafiltration (cutoff molecular weight 100 kDa; MicroconYM-100, Millipore) at 4°C or 30°C . The lysozyme activity and concentration in the ultrafiltrate were analyzed to investigate the binding ability of the polymer with lysozyme at various conditions. The relative activity and relative concentration of the free lysozyme in the complex solution were calculated by comparing the activity and concentration of the lysozyme in the ultrafiltrate with those of $10\ \mu\text{mol}\cdot\text{L}^{-1}$ free lysozyme solution which had the same pH adjustment, equilibration and ultrafiltration process. Our control experiment confirmed that the activity of $10\ \mu\text{mol}\cdot\text{L}^{-1}$ free lysozyme solution does not change significantly in the pH range of 3–11. Higher percent of the relative activity in the ultrafiltrate means less binding of lysozyme with the polymer.

The complex solution of denatured lysozyme and butylated poly(allylamine) was prepared by mixing denatured lysozyme with the polymer at the condition of $300\ \mu\text{mol}\cdot\text{L}^{-1}$ lysozyme, $3\ \text{mg}\cdot\text{mL}^{-1}$ polymer, $6\ \text{mol}\cdot\text{L}^{-1}$ GdHCl, $30\ \text{mmol}\cdot\text{L}^{-1}$ DTT at pH 9 under shaking. After incubation overnight, the mixture was dialyzed (cutoff molecular weight 3 kDa) against water with different pH values to remove the denaturants (GdHCl and DTT) completely, and then the solution was diluted to lysozyme concentration of $200\ \mu\text{mol}\cdot\text{L}^{-1}$ ($2.86\ \text{mg}\cdot\text{mL}^{-1}$) and polymer concentration of $2\ \text{mg}\cdot\text{mL}^{-1}$ with the same pH solution. The solution was further diluted to lysozyme concentration of $10\ \mu\text{mol}\cdot\text{L}^{-1}$ for activity and concentration analyses.

When the complex solution was turbid or the absorbance of the polymer at 280 nm could not be ignored, the lysozyme concentration was determined by BCA assay^[20]. Typically, 0.1 mL of the sample was mixed with 2 mL of BCA solution and the mixture was heated at 60°C for 30 min. The absorbance at 562 nm was recorded after cooling the mixture to room temperature in about 25 min. The concentration of lysozyme was calculated through the working curve measured by the standard protein solutions at the same conditions.

Lysozyme Activity Measurements

Lysozyme catalyzes the hydrolysis and transglycosylation of certain β -linked *N*-acetylated oligosaccharides, including those of *N*-acetylglucosamine in bacteria cell wall. The activity of lysozyme was assayed at 30°C according to the method of turbidity decrease of lysozyme substrate, *Micrococcus lysodeikticus* suspension^[21]. The cells were first suspended in $60\ \text{mmol}\cdot\text{L}^{-1}$ sodium phosphate buffer of pH 6.2. Then, 0.05 mL of sample was mixed with 2.45 mL of the cell suspension. The final concentration of the substrate was $0.24\ \text{mg}\cdot\text{mL}^{-1}$. The mixture has an initial absorbance of 1.2–1.3 at 450 nm. The decrease of the absorbance was recorded on a spectrophotometer (Lambda 35, Perkin-Elmer).

Dynamic Laser Scattering (DLS) Measurements

DLS measurements were carried out on a commercial laser light scattering spectrometer (ALV/CGS-8F, Laservertriebsgesellschaft). The measurements were performed at 25°C and a fixed scattering angle of 90° . Apparent *z*-average hydrodynamic radius (R_h) and polydispersity index (PDI) were obtained by Cumulants analysis.

Estimation of Lysozyme Charges in Different pH Solutions

The charges of lysozyme carried at different pH conditions were estimated using free software ANTHEROT 4.3 (Institute of Biology and Chemistry of Proteins, France). The amino acid sequence of hen egg white lysozyme was obtained from Protein Data Bank (PDB code 2LYZ).

RESULTS AND DISCUSSION

Characterization of Butylated Poly(allylamine)s

Figure 1 shows ^1H -NMR spectra of butylated poly(allylamine)s and corresponding peak assignments. The signal of the protons of $-\text{CH}_2\text{Br}$ group ($\delta \approx 3.3$) was not detected, indicating that the unreacted butyl bromide was completely removed. The signal of methyl protons increases while the signal of methylene protons nearby nitrogen decreases with the increase of butyl bromide in feed. These phenomena can be explained by the increase of the butyl substitution. The butyl substitution degrees of the polymers measured through ^1H -NMR and elemental analysis are shown in Table 1. The NMR spectrum of C4-70 sample is not shown in Fig. 1 because of the low solubility of C4-70 in D_2O and therefore weak NMR signals.

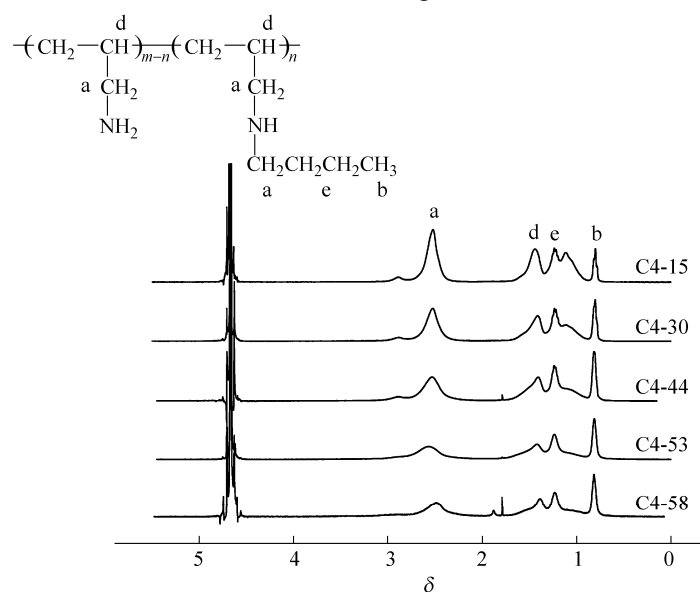


Fig. 1 ^1H -NMR spectra of C4-15, C4-30, C4-44, C4-53 and C4-58 polymers in D_2O

Solution Properties of Butylated Poly(allylamine)s

Poly(allylamine) is a weak polyelectrolyte and its ionization degree is pH dependent. Choi and Rubner reported that poly(allylamine) is about 100% unprotonated at pH 12.0, about 50% protonated at pH 8.8 and about 100% protonated at pH 4.0^[22]. Hydrophobically modified poly(allylamine) derivatives have been reported in literature. Long-alkyl and other hydrophobic pendant group substituted poly(allylamine) derivatives are amphiphilic and form a compact micellar conformation in aqueous solutions. The pendant species, substitution degree and solution pH influence the aggregation ability of the hydrophobic domains^[18, 23–26].

In this study, hydrophobic aggregation of the polymers may happen when the butylated degree increases. At a concentration of $2 \text{ mg}\cdot\text{mL}^{-1}$, the original pH of the butylated poly(allylamine) aqueous solutions is about 9.6; when the substitution degree is 58% and less, the solutions have no significant scattering under a laser irradiation at room temperature, whereas the scattering is significant for C4-70 solution. Figure 2 shows that the absorbance at 450 nm of C4-70 solution with the concentration of $2 \text{ mg}\cdot\text{mL}^{-1}$, which is close to its saturated solubility, increases from 17°C, that is, the polymer solution exhibits a lower critical solution temperature (LCST) behavior. Decreasing C4-70 concentration to $0.5 \text{ mg}\cdot\text{mL}^{-1}$, the absorbance increases at 30°C (data not shown). C4-58 solution with a concentration of $2 \text{ mg}\cdot\text{mL}^{-1}$ does not have LCST in the temperature range studied, nor do C4-44 and C4-53 solutions at the same concentration. However, for C4-58 solution with a concentration of $5 \text{ mg}\cdot\text{mL}^{-1}$ the absorbance at 450 nm increases rapidly from 35°C (Fig. 2).

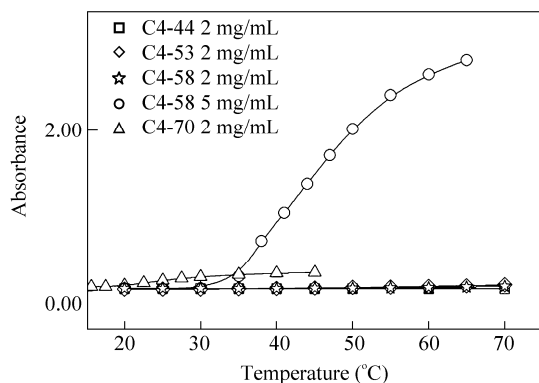


Fig. 2 Temperature dependence of the absorbance at 450 nm of butylated poly(allylamine) aqueous solutions

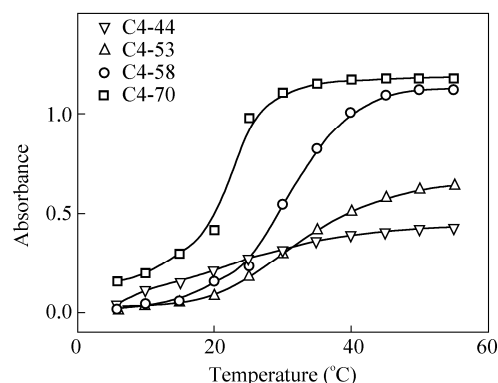


Fig. 3 Temperature dependence of the absorbance at 450 nm of butylated poly(allylamine) aqueous solutions at pH 11.0

The concentration of the polymers was $0.2 \text{ mg} \cdot \text{mL}^{-1}$.

Obviously, the butyl-modified poly(allylamine)s are more hydrophobic and tend to self-aggregation at higher pH. We investigated the turbidity of the polymer solutions at pH 11.0 with a concentration of $0.2 \text{ mg} \cdot \text{mL}^{-1}$. Figure 3 shows that the polymers with the butylated degree of 44% and higher exhibit temperature dependence behavior. The turbidity of the polymer solution increases with the increases of temperature and butyl substitution degree; the polymer with higher butyl substitution degree has a lower LCST at pH 11.0. On the other hand, at pH 7.0, the polymers carry more positive charges. No temperature dependent aggregation was observed; the samples were transparent for all of the polymer solutions with the concentration of $2 \text{ mg} \cdot \text{mL}^{-1}$ in the temperature range of 20–70°C.

As we know, poly(*N*-isopropylacrylamide) (PNIPAM) is a typical thermo-sensitive polymer. The folding or aggregation of polymer chains is controlled by the balance of polymer-solvent hydrogen bonding and inter/intra polymer hydrogen bonding and hydrophobic interaction^[27]. When the temperature is above its LCST, the interactions of polymer units overcome the hydrogen bonding between polymer and water, inducing the release of translational entropy of water molecules^[28]. The critical temperature of PNIPAM can be modulated by the copolymerization with hydrophilic or hydrophobic units^[29, 30]. For butylated poly(allylamine) aqueous solution, polymer-water hydrogen bonding and inter/intra polymer hydrogen bonding and hydrophobic interaction also exist because of the amine groups and butyl groups. Figure 3 shows the turbidity of C4-58 and C4-70 starts to increase remarkably at about 20°C and 10°C, respectively, implying that the polymer chains start to aggregate at these temperatures. The sizes of C4-58 and C4-70 aggregates at 25°C are 150 and 143 nm, respectively, measured by DLS. C4-70 solution has a stronger scattering light intensity than C4-58 solution, indicating more C4-70 chains involved in the aggregates. For C4-44 and C4-53 polymers, the changes of the absorbance are mild on heating, indicating the decrease of the polymer chains in the aggregates due to the lower hydrophobicity. C4-15 and C4-30 polymer solutions are transparent in the temperature range measured, suggesting their hydrophobicity is too low to induce intermolecular aggregation at this concentration. The turbidity changes of butylated poly(allylamine) solutions demonstrate that the polymer with higher butyl substitution degree tends to aggregate at lower pH, lower concentration and lower temperature due to the higher hydrophobicity.

Pyrene was used as a fluorescence probe to further characterize the hydrophobic aggregation of the polymers at $0.2 \text{ mg} \cdot \text{mL}^{-1}$ concentration. Pyrene has a much lower solubility in water (about $10^{-6} \text{ mol} \cdot \text{L}^{-1}$) than in hydrocarbons ($7.5 \times 10^{-2} \text{ mol} \cdot \text{L}^{-1}$). It migrates from water phase into hydrophobic regions once the latter are formed, causing remarkable changes in photophysical character^[31, 32]. Such changes include the increase of the quantum yield and the decrease of the intensity ratio of the first to third band (I_1/I_3) in its emission spectrum. The I_1/I_3 ratio of pyrene in water is about 2.0. Table 2 shows that the I_1/I_3 ratio of pyrene in the polymer solutions decreases with the increase of butyl substitution degree. The ratios of C4-15 and C4-30 polymers are close to the

ratio of pyrene in water, indicating that they do not form hydrophobic aggregates in the conditions studied. The I_1/I_3 ratios measured at 30°C are significantly smaller than those measured at 4°C, further supporting that the hydrophobicity of the polymers increases at higher temperature. The ratios do not increase significantly at pH 7.0, compared with the ratios at pH 11.0 with the same temperature. Considering that at pH 7.0 the polymer solutions are transparent and the polymers carry much more positive charges^[22], the smaller I_1/I_3 ratios can be explained by the fact that single-chain hydrophobic domain structure exists in C4-44, C4-53, C4-58, and C4-70 polymer solutions at pH 7.0 due to the high hydrophobicity and strong electrostatic repulsion of the polymers^[12, 33].

Table 2. I_1/I_3 ratio of pyrene fluorescence in butylated poly(allylamine) aqueous solutions

Polymer	I_1/I_3		
	30°C		4°C
	pH 7.0	pH 11.0	pH 11.0
C4-15	1.98 ± 0.10	1.92 ± 0.01	1.96 ± 0.13
C4-30	1.93 ± 0.22	1.84 ± 0.06	1.96 ± 0.17
C4-44	1.13 ± 0.04	1.13 ± 0.02	1.52 ± 0.13
C4-53	1.06 ± 0.04	1.03 ± 0.03	1.33 ± 0.10
C4-58	1.00 ± 0.03	0.95 ± 0.01	1.25 ± 0.12
C4-70	0.88 ± 0.01	0.86 ± 0.01	1.19 ± 0.16

Complexation of Native Lysozyme with Butylated Poly(allylamine)s

Lysozyme has a molecular weight of 14.3 kDa, an isoelectric point (pI) at pH 11.0, 129 amino acid residues and four disulfide bridges. Lysozyme as a model protein has been widely studied in protein/polyelectrolyte complexation^[34–36]. In order to understand the hydrophobic interaction between lysozyme and the polymer, we investigated the complexation of lysozyme with the polymers at pH 11.0 and 7.0. At pH 11.0 the net charge of lysozyme is about zero, while at pH 7.0 a lysozyme molecule carries about 7 net positive charges estimated by Software Antheprot 4.3. According to the ionization degree analysis of poly(allylamine) reported by Choi and Rubner^[22], butylated poly(allylamine)s we synthesized should carry positive charges when the solution pH is lower than 12.0. The mixture solutions of lysozyme and the polymers were first equilibrated at pH 11.5 to form complexes through hydrophobic interaction and electrostatic attraction, in which lysozyme carries net negative charges. Then, the complex solutions were adjusted to pH 11.0 or 7.0 and equilibrated again.

The data in Table 3 show that the binding of lysozyme with the polymers depends on pH and temperature, as well as butylated degree of the polymers. At pH 11.0 and 4°C, C4-15 and C4-30 polymers with a concentration of 0.2 mg·mL⁻¹ cannot bind lysozyme strongly as the relative activities in the ultrafiltrates are in the range of 82%–99%, *i.e.*, at least 82% of the lysozyme molecules in the mixture solutions are in free state.

Table 3. Relative activity of lysozyme after ultrafiltration separation from the complexes of lysozyme and butylated poly(allylamine) at pH 7.0 and 11.0

Polymer	Polymer concentration (mg·mL ⁻¹)	Relative activity of free lysozyme in ultrafiltrate (%)		
		30°C		4°C
		pH 7.0	pH 11.0	pH 11.0
C4-15	0.1	46 ± 3	42 ± 1	87 ± 1
	0.2	44 ± 1	41 ± 1	89 ± 6
	0.5	56 ± 2	49 ± 1	99 ± 4
C4-30	0.1	43 ± 3	56 ± 4	82 ± 2
	0.2	50 ± 2	47 ± 2	92 ± 1
	0.5	47 ± 1	47 ± 1	90 ± 1
C4-44	0.2	79 ± 10	74 ± 18	66 ± 18
C4-53	0.2	89 ± 1	68 ± 1	49 ± 5
C4-58	0.2	87 ± 1	71 ± 1	57 ± 1
C4-70	0.2	82 ± 3	85 ± 5	70 ± 5

The lysozyme concentration was 10 μmol·L⁻¹ (0.143 mg·mL⁻¹) in feed.

This result implies that the electrostatic attraction and hydrophobic interaction between lysozyme and C4-15 or C4-30 are not significant at pH 11.0 and 4°C. The reasons may be that the ionization degree of the polymers is low at pH 11.0, as well as 15%–30% of the butyl substitutions are not enough to induce significant hydrophobic interaction with lysozyme. Increasing butyl substitution to 53% causes an increase of the binding at 4°C: about half of the lysozyme molecules can bind with C4-53 polymer, indicating an increase of the hydrophobic interaction between the polymer and lysozyme. Further increasing butyl substitution to 70% causes the bound lysozyme molecules to decrease to about 30%. This phenomenon can be explained by the fact that the hydrophobic interaction between polymer and polymer is stronger than the interaction between polymer and lysozyme. The stable hydrophobic aggregates formed by the butyl groups limit the binding of C4-70 with lysozyme.

Changing the binding temperature from 4°C to 30°C, the lysozyme molecules bound with C4-15 and C4-30 increase to about 50%. Both C4-15 and C4-30 do not exhibit LCST in the temperature range studied, the solutions are transparent at 30°C, and their I_1/I_3 ratios of pyrene fluorescence are close to the ratio in water as discussed above. Compared with the binding at 4°C, the increase of the binding at 30°C indicates the increase of the hydrophobicity although no significant aggregates appeared for these two polymers. For the other polymers with higher butyl substitutions, however, the binding ability decreases at 30°C compared with their binding at 4°C. Figure 3 shows that C4-44, C4-53, C4-58 and C4-70 polymer solutions are more turbid, and Table 2 shows that their I_1/I_3 ratios are smaller at 30°C, that is, these polymers form more aggregates and the aggregates are more compact at 30°C compared with these polymers at 4°C. The decreases of the bound lysozyme molecules at 30°C can be attributed to the self-aggregation of the polymers.

Changing the pH of lysozyme and the polymer complex solutions from 11.0 to 7.0 at 30°C, the free lysozyme molecules do not increase much for all of the polymers (Table 3). One reason may be that the electrostatic repulsion between lysozyme and the polymer cannot overcome the hydrophobic interaction completely. Another reason may be that protein can bind with polyelectrolyte on the wrong side of its pI due to the anisotropy of protein charges^[37]. We also investigated the complexation at the polymer concentrations of 0.1 and 0.5 mg·mL⁻¹, separately. The binding does not change significantly when C4-15 and C4-30 concentration increases from 0.1 mg·mL⁻¹ to 0.5 mg·mL⁻¹, while the lysozyme concentration is fixed at 10 μmol·L⁻¹ (0.143 mg·mL⁻¹). The result shown in Table 3 implies that the binding of lysozyme with the polymers is tunable through the hydrophobicity of the polymers, *i.e.*, is tunable through the changes of solution pH, temperature and butylated degree.

The lysozyme activity in the complex solutions without ultrafiltration was also assayed. Our control experiment confirmed that the polymers did not influence the assay. After adding 0.05 mL of complex solution into 2.45 mL of the cell dispersion in 60 mmol·L⁻¹ sodium phosphate buffer of pH 6.2, the relative activity of the lysozyme was close to 100% for all of the complex samples shown in Table 3. As the lysozyme molecules trapped in the complexes cannot react with the substrate, *Micrococcus lysodeikticus* cells^[34–36], whose size is several orders of magnitude larger than the size of lysozyme molecule. The result of about 100% activity indicates that all of the bound lysozyme molecules can be released from the complexes and can recover their native activity when lysozyme concentration decreases to 0.2 μmol·L⁻¹ and the complex solution pH is changed to pH 6.2.

Complexation of Denatured Lysozyme with Butylated Poly(allylamine)s

As a denaturant and a reducing agent, GdHCl and DTT can unfold protein and reduce disulfide bonds, respectively^[38, 39]. Lysozyme was denatured in the solution containing 6 mol·L⁻¹ GdHCl and 30 mmol·L⁻¹ DTT at pH 9. The denatured lysozyme coacervated almost completely after removing GdHCl and DTT by dialysis against water in the pH range of 3–11; there was almost no lysozyme molecules in the supernatant assayed by concentration and activity. The intermolecular hydrophobic interaction and disulfide bonds formed during the dialysis process cause the denatured lysozyme to coacervate.

The interaction between denatured lysozyme and butylated poly(allylamine) was investigated to verify that the unfolded protein can increase the interaction with the polymers. The polymers with butylated degree less than 53% could not prohibit the denatured lysozyme (200 μmol·L⁻¹) from coacervation after removing the

denaturants even when the polymer concentration reached to $5 \text{ mg}\cdot\text{mL}^{-1}$. For C4-53 polymer with the concentration of $2 \text{ mg}\cdot\text{mL}^{-1}$, the coacervation of denatured lysozyme was suppressed completely and stable complex particles with R_h of 159 nm formed after removing the denaturants at pH 3.0 (Table 4). Each lysozyme carries about 17 positive charges at pH 3.0 estimated by Software Antheprot 4.3. The polymers should be 100% protonated at pH 3.0 according to the ionization analysis of poly(allylamine) reported by Choi and Rubner^[22]. Strong electrostatic repulsion exists between C4-53 and lysozyme at pH 3.0. The particle formation indicates that the hydrophobic interaction between C4-53 and denatured lysozyme overcomes the electrostatic repulsion. C4-58 polymer can bind about 50% of the denatured lysozyme forming complex particles with R_h of 152 nm. C4-70 cannot prohibit denatured lysozyme from coacervation at the same condition. At pH 3.0, C4-70 solution is transparent at room temperature, suggesting the polymer is soluble. C4-70 is more hydrophobic than C4-53 and C4-58. It is possible that the compact single-chain hydrophobic aggregates of C4-70 inhibit the binding with the denatured lysozyme. The result in Table 4 exhibits that both polymer and protein play important roles in the hydrophobic interaction between them. Compared with native lysozyme, the denatured one can increase the binding with the polymer after exposing the hydrophobic residues. For the polymers, appropriate hydrophobicity can overcome the electrostatic repulsion, inhibit the coacervation of the denatured protein, and form stable complex particles.

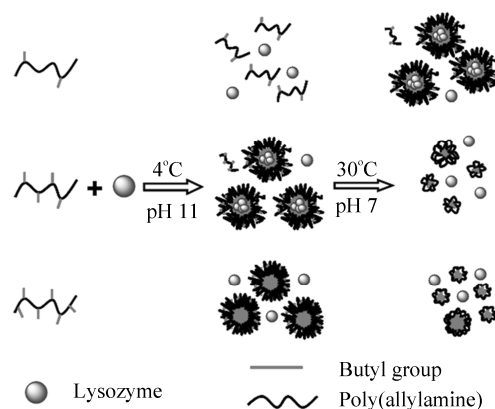
Table 4. Relative concentration and relative activity of lysozyme in the supernatant or nanoparticle dispersion composed of denatured lysozyme and the polymer at pH 3.0

Polymer	Phenomenon	Relative protein concentration (%)	Relative protein activity (%)
C4-44	Coacervation	0.6	7.4
C4-53	Particle with R_h of 159 nm	122	8.7
C4-58	Particle with R_h of 152 nm	49.6	6.7
C4-70	Coacervation	1.8	8.2

The lysozyme concentration was $200 \mu\text{mol}\cdot\text{L}^{-1}$ ($2.86 \text{ mg}\cdot\text{mL}^{-1}$) and the polymer concentration was $2 \text{ mg}\cdot\text{mL}^{-1}$ in feed.

CONCLUSIONS

Butylated poly(allylamine)s were prepared in this report. The butylated degree changes from 15% to 70%. The polymers show pH sensitive property and LCST behavior. The LCST appears at lower temperature, lower pH and lower polymer concentration for the polymer with higher butylated degree. The investigation of the interactions of butylated poly(allylamine)s with lysozyme reveals that the binding of native lysozyme with the polymers is tunable through the hydrophobicity of the polymers, *i.e.*, is tunable through the changes of solution pH, temperature and butylated degree. Scheme 2 illustrates the influence of the polymer hydrophobicity on the binding with lysozyme. The bound lysozyme molecules can recover their native activity completely after the dissociation of the complexes. The results of this report indicate that the dissociation of the hydrophobically modified polyelectrolyte/protein complexes is controllable at wrong side of the pI of the protein.



Scheme 2 Illustration of the interactions between butylated poly(allylamine) and lysozyme at different pH and temperature

Compared with native lysozyme, the denatured one can increase the binding with the polymer after exposing the hydrophobic residues. The polymers with appropriate hydrophobicity can overcome the electrostatic repulsion and can inhibit the coacervation of the denatured lysozyme and form stable polymer/protein complex nanoparticles.

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