Superoxide Dismutase Binding and Release Behaviors of Dodecylated Poly(allylamine)s: Effects of Self-Aggregation and Organic Solvents

Yunfeng Yan, a Jingjing Liu, a Yubing Xiong, Ye Cheng, Ping Yao*

pH sensitive dodecyl-modified poly(allylamine)s associate with superoxide dismutase (SOD) in aqueous solutions at pH 5.5 and 7.4. The bound SOD can be released completely at pH 10.0 due to the self-aggregation of the polymers. In a mixture of water and organic solvent, the hydrophobic groups of the polymers are exposed. During the process of removing the organic solvent, the polymers increase their hydrophobic interactions with SOD. As a result, the SOD binding of the polymers increases; the release of SOD from the complexes is remarkably extended. The released SOD molecules have full activity. The knowledge gained in this study will be beneficial to the applications of hydrophobically modified polyelectrolytes as delivery carriers for protein/peptide drugs.

1. Introduction

Protein-based drugs are an increasingly attractive component in modern medical industry. It was reported that four of the top fifteen marketable US pharmaceutical products in 2008 were protein drugs.[3] For the pharmaceutical application of protein drugs, a safe and efficient delivery carrier is a prerequisite to improve their stability in serum, transport them into the cytoplasm, and prolong their biological and therapeutic activities.[2–4] During the last decade, various materials have been proposed as delivery carriers for proteins, such as carbon nanotubes, silica nanoparticles, liposomes, and polymeric systems.[5–11]

In 1995, Kataoka and coworkers reported the first core–shell polyeon complex (PIC) micelles by the electrostatic interaction between two hydrophilic diblock copolymers: poly(ethylene glycol)–poly(lysine) and poly(ethylene glycol)–poly(α,β-aspartic acid).[12] Poly(ethylene glycol)-based polyelectrolytes can bind with oppositely charged DNA or RNA to form PIC micelles which can be used as delivery carriers of these biomacromolecules.[13,14] The application of PIC micelles in therapeutic fields is rapidly increasing due to their simple and efficient encapsulation, pH sensitivity, and high biocompatibility.[15] Polyelectrolytes can also form PIC micelles with countercharged proteins. However, due to the low charge density of protein surfaces, the electrostatic attraction between proteins and polyelectrolytes is not strong enough to retain the PIC micelles at physiological salt concentration and in the presence of charged serum proteins in the body which limits the applications of protein-containing PIC micelles.[3,16] To overcome this problem, the strategies used to improve the PIC micelle stability at physiological conditions are chemical cross-linking[17,18] and introduction of hydrophobic segments in the polymer.[19–21]

The introduction of hydrophobic interactions between proteins and polyelectrolytes not only can improve the stability of the complexes at physiological conditions but also can increase the amount of protein binding to the polyelectrolyte.[22,23] Hydrophobic interactions can also induce a sustained and extended release of the protein from the complexes that is favorable for protein drug delivery.[24,25] Furthermore, it was reported that hydrophobically

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modified polyelectrolyte particles can enhance cellular uptake and intracellular trafficking due to the interactions between the particle and cell membrane that increase the bioavailability of the loaded drugs.[26] On the other hand, hydrophobic interactions between protein and polymer usually induce an exposure of the hydrophobic residues originally inside the protein, i.e. cause protein denaturation.[27–30] In our previous studies, the tertiary structure of cytochrome c and lysozyme changed significantly when the proteins were bound to hydrophobically modified polyelectrolytes. However, their structure and activity recovered completely after the proteins were released from the complexes.[27,28,29]

Dubin and coworkers have demonstrated that a minimum alkyl chain length of 3–4 methylenes is required for significant hydrophobic interactions between protein and polymer.[31] Polymers with a low degree of ionization and/or with high hydrophobicity tend to have a compact conformation and form micelles that are usually unfavorable for binding to proteins.[32] In protein/polymer mixtures, complexation is a balance involving polymer–polymer, polymer–protein, and protein–protein interactions in solution. The increased intra-polymer and inter-polymer interactions usually reduce the binding ability of the polymer to the protein.[32,33–35] Compared to native protein, denatured protein with exposed hydrophobic residues has stronger interactions with aggregated polymer, forming stable complex particles after removing the denaturant.[36] On the other hand, the hydrophobic groups in an amphiphilic polymer can also be exposed in organic solvent.[37] Therefore, we speculated that in contrast to in a pure aqueous solution, a hydrophobically modified polyelectrolyte may have a looser structure in a miscible mixture of organic solvent and water, and then the polyelectrolyte can increase the hydrophobic interactions with protein during the process of removing the organic solvent. In this study, we prepared dodecyl-modified poly(allylamine)s with two substitution degrees and investigated their hydrophobic aggregation, protein binding, and release behaviors in aqueous solution and the influence of organic solvents. The results verify our speculation.

2. Experimental Section

2.1. Materials

Poly(allylamine) hydrochloride (PAH, molecular weight 15,000) was from Sigma. Cu,Zn-superoxide dismutase (SOD, specific activity 6000 U mg−1) was from Yili BioChem Company. Bicinchoninic acid (BCA) protein assay kit was from Pierce Chemical Co. Pyrogallol acid, n-dodecyl bromide (C12H25Br), acetic acid, and other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. All solutions were prepared with deionized water except for the organic solvent indicated and the pH was adjusted with HCl or NaOH solution.

2.2. Preparation of Dodecylated Poly(allylamine)

The synthesis procedure of the dodecylated poly(allylamine) is reported in the literature[37,39,40] and is illustrated in Scheme 1. Typically, the polymer with 14% dodecyl substitution degree (C12–14) was prepared as follows. A mixture of PAH (1 g) and KOH (0.662 g) in 50 mL of methanol was stirred at room temperature for 24 h. The mixture was concentrated to 15 mL by rotary evaporation, followed by addition of 25 mL of ethanol, and was concentrated to 15 mL again. After removing the salt in the solution by filtration with a polytetrafluoroethylene (PTFE) filter, 0.257 mL of n-dodecyl bromide 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 (0.068 g in 10 mL methanol), stirring for 1 h, addition of 10 mL of ethanol, and concentrating the mixture to about 15 mL successively. After removing the salt using a PTFE filter again, the mixture was concentrated to 15 mL by rotary evaporation, followed by addition of 25 mL of ethanol, and was concentrated to 15 mL again. After removing the salt in the solution by filtration with a polytetrafluoroethylene (PTFE) filter, 0.257 mL of n-dodecyl bromide 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 (0.068 g in 10 mL methanol), stirring for 1 h, addition of 10 mL of ethanol, and concentrating the mixture to about 15 mL successively. After removing the salt using a PTFE filter again, the polymer was precipitated in a hexane/ethyl ether mixture (3/1, v/v) twice, and then was dried under vacuum at room temperature for 48 h. The yield of C12–14 was about 32%. For the preparation of another dodecylated poly(allylamine) C12–25, the polymer was precipitated in a hexane/ethyl ether mixture (3/1, v/v) twice, and then was dried under vacuum at room temperature for 48 h. The yield of C12–14 was about 32%. For the preparation of another dodecylated poly(allylamine) C12–25, the polymer was precipitated in a hexane/ethyl ether mixture (3/1, v/v) twice, and then was dried under vacuum at room temperature for 48 h. The yield of C12–14 was about 32%.

2.3. Preparation of the Polymer Stock Solutions

Dodecylated poly(allylamine) was dissolved in acetic acid for at least 48 h, and then the solution was dialyzed (molecular weight cut-off 3.5 kDa) against water to remove the acetic acid. PAH was dissolved in water directly. The final concentration of the polymer stock solutions was 2–4 mg mL−1.

2.4. Preparation of SOD Stock Solution

SOD was dissolved in water, 10 mmol L−1 pH 7.4 phosphate buffer, or PBS (10 mmol L−1 pH 7.4 phosphate buffer containing 0.15 mol L−1 NaCl). The concentration of the SOD stock solution was 2–4 mg mL−1.
2.5. Complexation of SOD with the Polymers

The SOD aqueous stock solution was added dropwise into the polymer aqueous stock solution under shaking. The mixture was adjusted to pH 5.5 and equilibrated at room temperature for at least 24 h. The complex solution was adjusted to pH 7.4 or 10.0. The final SOD concentrations were 0.5 or 1 mg mL\(^{-1}\), and the weight ratios of SOD to the polymer were 3:1, 2:1, 1:1, or 1:2 in the various complex solutions.

The preparation of a SOD/polymer complex in a water/organic solvent mixture is as follows. The organic solvent was added into the polymer aqueous stock solution and the mixture was equilibrated at room temperature for at least 24 h. The SOD aqueous stock solution was then added dropwise. The solution was adjusted to pH 5.5 and equilibrated for 48 h. After removing the organic solvent by dialysis (molecular weight cut-off 3.5 kDa) against water, the complex solution was adjusted to pH 5.5, 7.4, or 10.0.

The free SOD in the complex solution was separated from the SOD/polymer complexes by a high-flow ultrafiltration membrane (molecular weight cut-off 100 kDa, MicroconYM-100, Millipore), and was collected in the ultrafiltrate. The SOD activity in the ultrafiltrate was measured by the method of pyrogallol autoxidation. Typically, the relative activity of the SOD in the ultrafiltrate was measured by the method of pyrogallol autoxidation. The control experiment verified that the polymers do not influence the absorbance change of the mixture was measured over 4 min at 25 °C on a spectrophotometer (UV-vis Spectrometer, Lambda 20, Perkin-Elmer). The control experiment verified that the polymers do not react with pyrogallol.

2.6. Release of SOD from the Complexes

A SOD/polymer complex solution of 2 mL with 1 mg mL\(^{-1}\) SOD concentration was dialyzed (molecular weight cut-off 100 kDa) against 20 mL of release buffer (PBS) at 37 °C. Periodically, 2 mL of the release buffer was taken out and the same volume of fresh buffer was added. The SOD concentration and activity in the release buffer were measured.

2.7. Activity Measurement of SOD

The activity measurement of SOD was based on the inhibitory effect of SOD on the reaction rate of pyrogallol autoxidation, which can be monitored spectrophotometrically at 318 nm as described in the literature. Typically, 50 μL of Cu,Zn-SOD solution was added into 2.9 mL of pH 8.2, 50 mmol L\(^{-1}\) Tris-HCl solution containing 1 mmol L\(^{-1}\) EDTA for scavenging any free copper ions. A 50 μL aliquot of a 50 mmol L\(^{-1}\) pyrogallol solution, which was prepared in 10 mmol L\(^{-1}\) HCl just before the measurement, was then added. The absorbance change of the mixture was measured over 4 min at 25 °C on a spectrophotometer (UV-vis Spectrometer, Lambda 20, Perkin-Elmer). The control experiment verified that the polymers do not influence the absorbance in the BCA assay.

2.8. Concentration Measurement of SOD

The concentration of SOD released from the complexes was determined by a BCA assay. Typically, 0.1 mL of the sample was mixed with 2 mL of BCA solution and the mixture was heated at 60 °C for 1 h. The absorbance at 562 nm was recorded after cooling the mixture in a 4 °C water bath for 20 min. The SOD concentration was calculated by the working curve obtained by the standard protein solutions under the same condition. Our control experiment verified that the polymers do not influence the absorbance in the BCA assay.

2.9. Steady-State Fluorescence Measurement

Fluorescence emission spectra were measured on a fluorescence spectrophotometer (RF-920, Edinburgh). The polymer solutions were adjusted to pH 5.5, 7.4, and 8.0, respectively, followed by the addition of pyrene acetone stock solution (1 × 10\(^{-3}\) mg mL\(^{-1}\)). The final pyrene and the polymer concentrations were 1 × 10\(^{-4}\) and 1 mg mL\(^{-1}\), respectively. The resultant mixtures were incubated at 4 °C for 48 h before measurement. The spectra were recorded with an excitation wavelength of 330 nm at 4 °C. Both the excitation and emission slits were 1 nm. Three scans were accumulated for each measurement.

2.10. Dynamic Light Scattering (DLS) Measurement

DLS measurements were carried out at 25 °C on a commercial laser light scattering instrument (Malvern Autosizer 4700, Malvern Instruments) at a 90° scattering angle. The measured time correlation function was analyzed by the automatic program equipped with the correlator. The z-average hydrodynamic diameter (D\(_{z}\)) and polydispersity index (PDI, μ\(_{2}/\Gamma\)) were obtained by cumulant analysis.

2.11. ζ-Potential Measurement

The net surface charges of SOD, C12–14, or their complexes at different pH values were determined using a ZetaSizer Nano ZS90
three times.

Poly(allylamine) is a weak polyelectrolyte and its ionization degree is pH dependent. Choi and Rubner reported that poly(allylamine) is completely unprotonated at pH 3.2. Solution Properties of Dodecylated Poly(allylamine)s

Figure 1 shows the spectra of C12–14 in CD3OD and PAH in D2O. (Malvern Instruments). Electrophoresis mobility (Uζ) was measured and the ζ-potential was calculated by the Dispersion Technology Software provided by Malvern. Each sample was analyzed three times.

3. Results and Discussion

3.1. Characterization of Dodecylated Poly(allylamine)s

Figure 1 shows the spectra of C12–14 in CD3OD and PAH in D2O (note: PAH is not soluble in CD3OD while C12–14 and C12–25 cannot directly dissolve in D2O). There is no methyl peak in the spectrum of PAH. There are three peaks (f, g, h) corresponding to the protons of the methylene groups in the backbone bound to amine, methine, and methylene, respectively. The area ratio of the three peaks is 2:1:2 (f, g, h), which is consistent with the number of protons in the chemical structure. After dodecyl substitution, the peak of methyl protons was observed at δ = 0.9 (b). The substitution degree can be calculated by the area ratio of methyl protons (b) to methylene protons nearby amine (a) and the result was confirmed by the elemental analysis (Table 1).

3.2. Solution Properties of Dodecylated Poly(allylamine)s

Poly(allylamine) is a weak polyelectrolyte and its ionization degree is pH dependent. Choi and Rubner reported that poly(allylamine) is completely unprotonated at pH 12.0, half protonated at pH 8.8, and entirely protonated at pH 4.0.[48] Hydrophobically modified poly(allylamine) derivatives have been reported in the literature. Long-alkyl and other hydrophobic pendant-group-substituted poly(allylamine) derivatives are amphiphilic and have a compact micellar conformation in aqueous solution.[40] It is known that the pendant species, substitution degree, and solution pH can influence the aggregation of the hydrophobic domains.[46–49] In this study, the hydrophobic aggregation of the polymers in aqueous solutions was investigated at pH 5.5, 7.4, 8.0, or 10.0. DLS results (not shown) indicate that the scattering intensities are very weak at pH 5.5 for all three polymers (PAH, C12–14, and C12–25), suggesting that the polymers do not form intermolecular aggregates at pH 5.5. The intensity is also very weak for PAH at pH 7.4, while the intensities of C12–14 and C12–25 increase significantly. At pH 10.0, the intensity increases for PAH, but macroscopic aggregates appeared in the C12–14 and C12–25 solutions.

We used pyrene as a fluorescence probe to characterize the hydrophobic aggregation of the polymers. Pyrene has a much lower solubility in water (about 10−6 mol L−1) than in hydrocarbons (7.5 × 10−2 mol L−1). It migrates from the aqueous phase into hydrophobic regions once the latter are formed, causing the decrease of the intensity ratio of the first to third band (I1/I3) in its emission spectrum.[50,51] Figure 2 shows the I1/I3 ratios of pyrene in the polymer solutions. Between pH 5.5 and 8.0, the I1/I3 ratios in PAH solutions are close to the ratio of 2.06 in water, confirming that there is no aggregation in the PAH solution. For dodecylated poly(allylamine), the I1/I3 ratios are smaller than those in PAH solutions, and the ratio decreases with the increase of dodecyl substitution degree and pH value. The ratios of 1.2–1.4 indicate that the hydrophobic domains exist.[51] C12–25 shows smaller I1/I3 ratios than C12–14, suggesting that C12–25 has a more compact conformation than C12–14 in aqueous solution. This result is consistent with the increase of the hydrophobicity. Both C12–14 and C12–25 present stronger hydrophobic aggregation at higher pH due to the increase of unprotonated amine groups in the polymers.

3.3. Complexation of SOD with the Polymers in Aqueous Solutions

A model protein SOD, a dimer with a molecular weight of 31 kDa and an isoelectric point of pH 5.4,[52] was used in
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This study was carried out to investigate the binding and release behaviors of the dodecylated poly(allylamine)s. It was reported that SOD can protect cells against the harmful effects of oxidative stress, and has clinical applications in the treatment of several diseases, such as rheumatoid arthritis, aging, and cancer. However, its use is limited by the short plasma half-life of only 6 min and poor cellular penetration. It is known that nanoparticles can encapsulate and protect proteins from proteolysis. Furthermore, hydrophobically modified polyelectrolyte particles can enhance the cellular uptake and intracellular trafficking of the loaded protein as mentioned above. In this study, the complexation of SOD with the polymers was carried out at pH 5.5, at which most of the amine groups in the polymers are protonated, and therefore the conformations of C12–14 and C12–25 are less compact; meanwhile, SOD carries negative charges as shown in Table 2 below. After the complexation at pH 5.5, aliquots were taken out and adjusted to pH 7.4 and 10.0. When the complex solution with the desired pH reached equilibrium at room temperature, the free SOD in the solution was separated by ultrafiltration and then assayed to calculate the SOD binding of the polymers.

Figure 3 shows that all three polymers can bind with SOD effectively at pH 5.5; about 75–89% of the SOD was bound to the polymer. At pH 5.5, electrostatic attraction exists between the negatively charged protein and the positively charged polymer. Increasing the hydrophobicity of the polymer does not increase the SOD binding, suggesting that the binding is mainly driven by electrostatic and van der Waals interactions. However, in the presence of 0.15 mol L⁻¹ NaCl, their SOD binding increases to about 90% for all the ratios studied when increasing the NaCl concentration to 0.5 mol L⁻¹ (data not shown), demonstrating that hydrophobic interactions exist between the SOD and C12–14/C12–25. Figure 3 shows that increasing the polymer ratio in the complexes enhances the SOD binding slightly for C12–14 and C12–25, but does not do so for PAH.

The positive charges of the polymers decrease, the negative charges in SOD increase (Table 2), and the hydrophobicity of C12–14 and C12–25 increases (Figure 2) when the complex solution pH was changed from 5.5 to 7.4. Compared with the SOD binding at pH 5.5, the binding of PAH decreases, while the binding of C12–14 and C12–25 does not change significantly at pH 7.4 (Figure 3). When the complex solution pH was further changed to 10.0, the SOD binding of PAH further decreases, but C12–14 and C12–25 cannot bind SOD at all. This result can be explained by the fact that the further decrease of the positive charges and then the self-aggregation of the polymers at pH 10.0 prohibit the C12–14 and C12–25 from binding with SOD.

Table 2. ζ-Potentials of individual SOD, C12–14, and SOD/C12–14 complex solutions with different pH values. SOD concentration was 0.5 mg mL⁻¹ and the weight ratio of SOD to C12–14 was 1:1 in the feed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH 5.5 ± 0.47</th>
<th>pH 7.4 ± 0.8</th>
<th>pH 10.0 ± 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>−9.41 ± 0.47</td>
<td>−20.3 ± 0.8</td>
<td>−32.3 ± 0.2</td>
</tr>
<tr>
<td>C12–14</td>
<td>52.4 ± 0.1</td>
<td>34.3 ± 0.3</td>
<td>macroscopic</td>
</tr>
<tr>
<td>SOD/C12–14</td>
<td>50.4 ± 0.2</td>
<td>19.2 ± 0.2</td>
<td>aggregates</td>
</tr>
<tr>
<td>SOD/C12–14 35% ethanol[4]</td>
<td>45.0 ± 1.4</td>
<td>16.4 ± 0.2</td>
<td>−18.1 ± 0.4</td>
</tr>
</tbody>
</table>

[4] The SOD/C12–14 complex was prepared by mixing SOD and C12–14 in the presence of 35% ethanol, dialysis against water, and pH adjustment in series.
The DLS results of SOD/polymer complex solutions at pH 7.4 and 10.0 are shown in Figure 4. At pH 7.4, SOD/C12–14 and SOD/C12–25 complexes are much smaller than individual C12–14 and C12–25 particles, which are 1441 and 685 nm, respectively. Meanwhile, the scattering intensities increase 3–10 times after binding with SOD. These phenomena may indicate a collapse of the charged segments in the polymer after complexation with oppositely charged SOD, forming more compact aggregates. Another possibility is that the binding of dodecylated poly(allylamine) with SOD can inhibit the self-aggregation of the polymer at pH 7.4, forming smaller complex particles. At pH 10.0, the particles shown in Figure 4 are entirely composed of dodecylated poly(allylamine)s because no SOD was bound to the polymers as demonstrated in Figure 3. Furthermore, these particles tend to form macroscopic aggregates after storage. Therefore, the self-aggregation of dodecylated poly(allylamine)s results in the complete release of the bound SOD after the pH was changed from 5.5 to 10.

The data in Figure 3 and 4 demonstrate that in the complex solution of SOD and dodecylated poly(allylamine), the interaction between SOD and the polymer is dominant at pH 7.4, whereas the hydrophobic self-aggregation of the polymer is dominant at pH 10.0. When the pH changed from 7.4 to 10.0, the deprotonation of amino groups caused a rearrangement of the polymer chains to form self-aggregates. This property may have a potential application in protein purification in which the polymer can be recycled by pH adjustment and centrifugation separation.

The SOD release behavior of the complexes prepared at pH 5.5 was investigated in pH 7.4 PBS. Figure 5 shows that SOD/C12–14 complexes have an extended release behavior compared to individual SOD. Increasing the C12–14 ratio in the complexes can prolong the SOD release. This result indicates that the release rate of the bound protein is tunable by the weight ratio of protein to the polymer in the complexes. For the complexes of SOD/C12–25, a similar SOD release behavior was also observed (data not shown); but the increase of the dodecyl substitution degree does not change the release rate significantly. For the complex of SOD/PAH, at a weight ratio of SOD to polymer of 1:1, the release rate is much faster than that of the SOD/C12–14 complex (Figure 5). This result demonstrates that the hydrophobic interactions between SOD and C12–14 can reduce the burst release of the SOD from the complex at physiological conditions.

Besides the measurement of SOD activity in the release buffer as shown in Figure 5, the SOD concentration in the release buffer was also measured by BCA assay (data not shown). The released SOD percentage obtained from the activity analysis is very close to the percentage obtained from the concentration assay in a whole release process. This result indicates that all the released SOD molecules have their full native activity. Furthermore, the data in Figure 3 also confirm that at pH 10.0, all the SOD molecules can be released from the complexes and the released SOD molecules have full native activity.

Figure 4. Particle size in pH 7.4 and 10.0 SOD/polymer complex aqueous solutions as a function of weight ratio of SOD to polymer. SOD concentration was 0.5 mg mL$^{-1}$ in the feed.

Figure 5. Cumulative release of SOD from the complexes of SOD/C12–14 and SOD/PAH in pH 7.4 PBS. The complexes were prepared at pH 5.5 and the concentration of SOD was 1 mg mL$^{-1}$ in the feed. An individual SOD solution was used as a control.
3.4. Complexation of SOD with the Polymers in the Presence of Organic Solvent

Amphiphilic polymers assemble in a selective solvent. For hydrophobically modified polyelectrolyte, the hydrophobic segments tend to aggregate and form compact domains in aqueous solution, which reduces their hydrophobic interactions with protein. Generally, the solubility of the hydrophobic segments increases when adding a water miscible organic solvent into water. The exposed hydrophobic segments are randomly distributed in C12–25. The data in Table 3 confirm that the ethanol can enrich in the hydrophobic domain of the aggregates of poly(n-butyl acrylate)-block-poly(acryl acid) in a mixture of water and tetrahydrofuran (THF) or ethanol at middle ratios. Possibly, the solvent environment of the hydrophobic segments in C12–14 does not change significantly when increasing the ethanol volume fraction from 35% to 65%. The SOD release from the SOD/C12–14 complex, which was prepared with the SOD to C12–14 weight ratio of 1:1 after removing 65% ethanol, was investigated in PBS at a diluted concentration (SOD concentration 0.02 mg mL⁻¹, data not shown). The result revealed that the SOD binding of the polymers does not change significantly. Galder et al. reported that organic solvent can be enriched in the hydrophobic domain of the aggregates of poly(n-butyl acrylate)-block-poly(acryl acid) in a mixture of water and tetrahydrofuran (THF) or ethanol at middle ratios.

In the presence of 20% (v/v) ethanol, PAH cannot bind with SOD in the pH range of 5.5–10.0. The relative activity of SOD in the ultrafiltrate is 104%, 103%, and 101% at pH 5.5, 7.4, and 10.0. This result also implies that the 20% ethanol does not change the activity of SOD. The dodecylated poly(allylamine)s can bind about 80% of the SOD at pH 5.5 and 7.4, and about half at pH 10.0 in the presence of 20% (v/v) ethanol. When the ethanol was increased to 35% (v/v), and then removed by dialysis against water, and the resultant complex aqueous solutions were adjusted to pH 5.5 and 7.4, all the three polymers show similar SOD binding (Table 3). Importantly, the binding of SOD with the dodecylated poly(allylamine)s increases to more than 70% at pH 10.0, demonstrating an increase of the hydrophobic interactions between the polymer and SOD after removing the ethanol. The binding of SOD with PAH also increases at pH 10.0 after removing the ethanol, although the increase is much smaller than those of C12–14 and C12–25. The data in Table 3 confirm that the ethanol can greatly increase the hydrophobic interactions between SOD and the polymer at pH 10.0.

The surface charges of individual SOD, C12–14, and SOD/C12–14 complexes were characterized by \( \zeta \) potential measurement. The data in Table 2 indicate that when the solution pH was changed from 5.5 to 10.0, SOD carries more negative charges, whereas C12–14 carries less positive charges. For SOD/C12–14 complex solutions, the \( \zeta \) potential values are smaller than the values of C12–14 because of the binding with negatively charged SOD. The complex prepared by containing and then removing 35% ethanol shows slightly smaller \( \zeta \) potentials at pH 5.5 and 7.4 compared with the SOD/C12–14 complex prepared without ethanol. It is worth noting that the charges and hydrophobic segments are randomly distributed in C12–14. Different from the PIC micelles formed by diblock polyelectrolyte and oppositely charged protein, the SOD/C12–14 complex may not have a complete core–shell structure. More SOD molecules may be bound close to the surface of the complex that causes the decreases of \( \zeta \) potentials in the complex solutions prepared by containing and then removing 35% ethanol.

We further increased the ethanol to 65% (v/v), but the SOD binding of the polymers does not change significantly. The SOD/polymer complex solution was prepared by mixing SOD and the polymer in the presence of organic solvent with a desired volume fraction at pH 5.5, followed by dialysis against water, and pH adjustment. It is worth noting that the charges and hydrophobic interactions with protein. Generally, the solubility of the hydrophobic segments increases when adding a water miscible organic solvent into water. The exposed hydrophobic segments are randomly distributed in C12–25. The data in Table 3 confirm that the ethanol can enrich in the hydrophobic domain of the aggregates of poly(n-butyl acrylate)-block-poly(acryl acid) in a mixture of water and tetrahydrofuran (THF) or ethanol at middle ratios. Possibly, the solvent environment of the hydrophobic segments in C12–14 does not change significantly when increasing the ethanol volume fraction from 35% to 65%. The SOD release from the SOD/C12–14 complex, which was prepared with the SOD to C12–14 weight ratio of 1:1 after removing 65% ethanol, was investigated in PBS at a diluted concentration (SOD concentration 0.02 mg mL⁻¹, data not shown). The result revealed that the bound SOD can be released completely and the released SOD has full native activity. This result indicates that the SOD activity was not influenced by the process of adding and removing 65% ethanol, nor was the activity influenced by the process of complexing with and releasing from C12–14.

Table 3. Free SOD in different SOD/polymer complex solutions. The percentage was obtained by ultrafiltration and analysis of SOD activity in the ultrafiltrate. The SOD concentration was 1 mg mL⁻¹ and the weight ratio of SOD to the polymer was 1:1 in the feed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Free SOD [%]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH 5.5</td>
</tr>
<tr>
<td>PAH no organic solvent</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>C12–14</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>C12–25</td>
<td>14 ± 10</td>
</tr>
<tr>
<td>PAH 35% ethanol(3)</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>C12–14</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>C12–25</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>C12–14 65% ethanol(3)</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>65% acetone(3)</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>65% THF(3)</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>65% DMF(3)</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

(3) The SOD/polymer complex solution was prepared by mixing SOD and the polymer in the presence of organic solvent with a designed volume fraction, dialysis against water, and pH adjustment in series.
investigated. The result (Table 3) shows that DMF is more effective in increasing the binding of SOD with C12–14. We performed DLS measurement for SOD/C12–14 complexes after removing the solvent. Table 4 shows that SOD/C12–14 aqueous solutions are almost transparent after removing acetone, ethanol, or DMF; their scattered light is measurable only at maximum incident light intensity. The SOD/C12–14 aqueous solution after removal of THF shows stronger scattering intensity and relatively narrow-dispersed particles with a diameter of 252 nm.

The solubility of polymer in solvent is related to the solubility parameter and the dielectric constant of the polymer and solvent. A higher polarity (high dielectric constant) is favorable for the dissolution of a polar polymer. For a non-polar polymer, a good solvent must have a close solubility parameter to that of the polymer. Table 5 shows the solubility parameters and dielectric constants of the solvents and \( n \)-dodecane. For the polar segments in dodecylated poly(allylamine), DMF is a better solvent due to its higher polarity. For the hydrophobic segments, THF has a closer solubility parameter to \( n \)-dodecane. Considering these two factors, dodecylated poly(allylamine) may adopt looser structures in DMF and THF compared to ethanol and acetone. On the other hand, the polymer may have different conformations in DMF and THF due to the different solubility parameters and the different dielectric constants. The different conformations of C12–14 in these solvents may result in a difference in the structure of the SOD/C12–14 complexes and the difference in SOD binding ability after removing the solvent.

Figure 6 shows the SOD release behavior of SOD/C12–14 complexes in pH 7.4 PBS. The complexes were prepared by containing and then removing 30% (v/v) THF at pH 5.5. The concentration of SOD was 1 mg mL\(^{-1}\) in the feed. An individual SOD solution was used as a control.

### Table 4. DLS result of SOD/C12–14 complexes after removal of 65% (v/v) organic solvent by dialysis against water. The SOD concentration was 1 mg mL\(^{-1}\). The weight ratio of SOD to the polymer was 1:1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Intensity</th>
<th>( D_h ) [nm]</th>
<th>PDI</th>
<th>Slit [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>71.8</td>
<td>283</td>
<td>1.000</td>
<td>300</td>
</tr>
<tr>
<td>Ethanol</td>
<td>21.7</td>
<td>129</td>
<td>0.721</td>
<td>300</td>
</tr>
<tr>
<td>DMF</td>
<td>69.3</td>
<td>371</td>
<td>1.000</td>
<td>300</td>
</tr>
<tr>
<td>THF</td>
<td>78.7</td>
<td>252</td>
<td>0.389</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table 5. Solubility parameter\(^{[62]}\) and dielectric constant\(^{[63]}\) of the solvents and \( n \)-dodecane.

<table>
<thead>
<tr>
<th>Solvent and ( n )-dodecane</th>
<th>Solubility parameter ([\text{MPa}^{0.5}])</th>
<th>Dielectric constant (\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>48</td>
<td>80.1</td>
</tr>
<tr>
<td>DMF</td>
<td>24.7</td>
<td>38.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>26.2</td>
<td>24.5</td>
</tr>
<tr>
<td>Acetone</td>
<td>19.7</td>
<td>20.7</td>
</tr>
<tr>
<td>THF</td>
<td>18.5</td>
<td>7.6</td>
</tr>
<tr>
<td>( n )-Dodecane</td>
<td>16.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

### 4. Conclusion

The hydrophobicity of dodecylated poly(allylamine) C12–14 and C12–25 increases with the dodecyl substitution and solution pH. The polymers associate with SOD effectively in aqueous solution at pH 5.5 and 7.4. The self-aggregation of the polymers at pH 10.0 results in a complete release of the bound SOD and the released SOD molecules have full native activity. The SOD/polymer complexes have an extended release behavior in pH 7.4 PBS and the release rate is tunable by the polymer ratio in the complexes.
the presence of organic solvent, the hydrophobic groups of the polymers are exposed; the hydrophobic interactions between the polymer and SOD increase significantly after removing the organic solvent, which further extends the release of SOD from the complexes. Although the dodecylated poly(allylamine)s are not expected to be used in pharmaceutical and food products, the knowledge gained from this study on the interactions of a protein and a hydrophobically modified polysaccharide can be applied in the fields of protein delivery and protein purification.

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