Potentiometric Studies on the Interaction Between Superoxide Dismutase and Hyaluronic Acid

Yaofeng Fan,[1,2] Yinong Wang,[1] Yunge Fan,[1] Jianbiao Ma[1,3]

[1]*Key Laboratory of Functional Polymer Materials (Ministry of Education), Institute of Polymer Chemistry, Nankai University, Tianjin 300071, China*
[2]*Patent Examination Cooperation Center, State Intellectual Property Office, Beijing 100190, China*
[3]*School of Chemistry and Chemical Engineering, Tianjin University of Technology, Tianjin 300191, China*

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ABSTRACT: The formation process of soluble complexes and insoluble aggregates between superoxide dismutase (SOD) and hyaluronic acid (HA) was studied using quasielastic light scattering and turbidimetric titration. The electrostatic binding between them was investigated in detail through potentiometric titration and turbidimetric titration carried out from high to low pH. Turbidimetric titration was used to determine the specific pH values at which soluble complex formation was initiated (pH_c) and phase separation occurred (pH_ϕ). An increase of the ionic strength causes a decrease of pH_c and pH_ϕ. With the increase of HA concentrations, pH_ϕ increases but pH_c does not vary. The formed "salt bridges" between $-NH_3^+$ (SOD) and $-COO^-$ (HA) result in the formation of stable SOD-HA complexes and even aggregates. The necessary condition of electrostatic binding was also given for protein-acidic polyelectrolyte systems. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 2583–2589, 2009

Key words: interaction; superoxide dismutase; hyaluronic acid; complex; aggregate

INTRODUCTION

Interactions between globular proteins and polyelectrolytes have attracted much attention from researchers and technologists in recent years. Some research was conducted to investigate biological phenomena, such as nonspecific long-range effects between proteins and DNA.[1] Another research was done to reach industrial applications, including protein purification,[2–4] enzyme immobilization,[5] modulation of a protein affinity for substrates,[6] immunosensing,[7–9] and bioactive sensors.[10]

Many techniques were applied to study the global structure of protein-polyelectrolyte complexes (PPCs), such as sedimentation,[11,12] turbidimetry,[13–16] static light scattering,[17–19] quasielastic light scattering (QELS),[20,21] and electrophoretic light scattering.[22] The experimental results revealed that "primary" soluble complexes were formed before insoluble aggregates. Recently, the frontal analysis continuous capillary electrophoresis was developed to further study the average number of proteins in every PPC and the cooperativity of the binding event.[23–27] Fluorometry was carried out to observe nonradiative energy transfer during the binding process.[28] Isothermal titration calorimetry was performed to measure some important thermodynamic parameters, such as a binding constant, stoichiometry, enthalpy, and entropy.[29] Microcalorimetry experiments performed on the protein-polyelectrolyte systems, such as hyaluronic acid (HA)/poly(L-lysine) and bovine serum albumin/poly(allylamine hydrochloride), showed that counter ions were released in the complexation process, and the binding between polyanions and polycations was mainly entropically driven.[30,31] Generally, information provided by most of the above methods is not about the local (several angstroms) interactions of PPCs, but about their global (10–1000 nm) properties. However, it is important to find out the local interactions since polyelectrolytes are able to bind to proteins with the same charge sign.[32] The potentiometric method was performed to investigate the protein-synthetic strong polyelectrolyte system, and "primary" soluble complexes were observed to form before insoluble aggregates in the course of titration.[33] However, no report was about the protein-natural weak polyelectrolyte system. Influence of pH on polymer charges can be neglected for the former, but the factor should be taken into account for the latter. Thus, the latter is a more complicated (but typical) example.

Differing from protein–DNA interactions, protein-glycosaminoglycan (GAG) ones are relatively nonspecific and low affinity, and the binding behavior is dominated by long-range electrostatic forces.[34–36] HA is the sole GAGs with a single structure, and

Correspondence to: J. Ma (jbma@nankai.edu.cn).

thus it is chosen as a natural polyelectrolyte bound to protein. HA can bind to some proteins including aggrecan, versican, neurocan, link protein, CD44, and RHAMM *in vivo*.[37–42] Long-range electrostatic interactions are likely involved in the protein-HA binding because the hydration and stiffness of HA essentially preclude the intimacy of approach of highly selective ligand binding.[43,44] Therefore, the protein-HA system may be a paradigm for nonspecific complexation between proteins and biological polyelectrolytes.[45]

Superoxide dismutases (SODs) are metalloenzymes that protect cells from oxygen toxicity by catalyzing the dismutation of superoxide anion radicals ($O_2^{\bullet -}$) into molecular oxygen and hydrogen peroxide.[46] However, SODs have a short plasma half-life (for example, $t_{1/2} < 6$) and a poor cellular penetration, which limit their therapeutic potentials.[47,48] Thus, it is necessary to study their controlled release. Here, bovine erythrocytic Cu, Zn-SOD is chosen as a model protein. SOD is a globular protein with two subunits and its isoelectric point (pI) is about 4.95.[49] A combination of SOD and HA is expected to have a good therapeutic effect on some diseases such as rheumatoid arthritis.

EXPERIMENTAL

Materials

SOD (potency > 20,000 IU/mg, $\overline{M}_w$ 32 kD) was the product of Tianjin Life Science Application Institute. HA ($\overline{M}_w$ 1100 kD) with the content of glucuronic acid of more than 42% was a gift of Tianjin Kangting Biological Co., Ltd. (Tianjin, China). The other reagents were of analytic purity and used without further purification. All solutions were prepared with CO_2-free twice-distilled deionized water, and they were purged with a N_2 flow before measurements.

Quasielastic light scattering

SOD (or HA) was dissolved in 0.05*M* NaCl to prepare 0.5 mg/mL SOD, 0.05 mg/mL HA, and 0.5 mg/mL SOD containing 0.05 mg/mL HA. Their initial pH values were 6.2, 5.7, and 6.1, respectively. These solutions were adjusted to a desired pH by a bit of 0.1*M* HCl or 0.1*M* NaOH. Then, they were filtered through a cellulose film with a pore size of 0.45 μm. The resultant solutions were determined by a BI-9000AT photon correlation system (Brookhaven) equipped with an Innova304-4War + laser and a BI-2000SM photometer. The experiment was operated at a wavelength of 532 nm at 25°C. The scattering light intensity was detected through an avalanche photodiode detector at 90° scattering angle. The correlation functions were analyzed by cumulant analysis to obtain the mean apparent translational diffusion coefficient (D_T). The hydrodynamic radius (R_h) of particles was calculated from the Stokes-Einstein equation

$$R_h = k_b T / 6\pi\eta D_T$$

where k_b is Boltzmann's constant, T is the temperature (K), and η is the solvent viscosity. The size distributions were obtained by the CONTIN method.

Turbidimetric and potentiometric titration

SOD and HA solutions were prepared in salt solutions. A total of 0.5 mg/mL SOD solution was obtained by a rapid dissolution of the protein. A total of 0.5 mg/mL HA solution was heated at 40°C and then ultrasonicated for 1 min to ensure complete solubilization. HA was dissolved in 0.5 mg/mL SOD solution to obtain SOD-HA mixtures containing 0.05–0.5 mg/mL HA. The resultant solutions were regulated to pH 12.05 ± 0.01 and then titrated with 0.1*M* HCl at 23 ± 1°C. The titration was performed with the gentle magnetic stirring. Turbidimetric titration was carried out using a UV-762 ultraviolet/visible light spectrophotometer (Shanghai Precision & Scientific Instrumental) at 420 nm. Transmittance (T) of twice-distilled deionized water was set to 100%. Probe drift was recorded to calibrate T, and turbidity was reported as 100% T. Potentiometric titration was done with a PHB-10 pH meter (Shanghai Kangyi Instrumental) equipped with a combination electrode. Buffer solutions with pH 4.0 or 7.0 were used to calibrate the pH meter before the titration. Meter drift was less than 0.02 throughout the titration. pH values were recorded when the meter response was lower than 0.01 in every minute. The time interval between measurements was about 2 min for optically clear solutions, but approximately 5 min for turbid samples.

RESULTS AND DISCUSSION

Quasielastic light scattering

Hydrodynamic diameters of free SOD, free HA, and SOD-HA mixture were determined by QELS in 0.05*M* NaCl at pH 6.5. The diameter is approximately 5.7 nm and 33 nm, respectively for free SOD and HA, but increases to about 55 nm for a SOD-HA mixture. It indicates that soluble SOD-HA complexes are formed since SOD-HA solutions in 0.05*M* NaCl are optically clear at pH 6.5. The "primary" soluble complex consists of a number of proteins bound to a single polymer chain according to the previous report.[28] With the decrease of pH from 6.5 to 3.7, some SOD dimmers with a particle size of

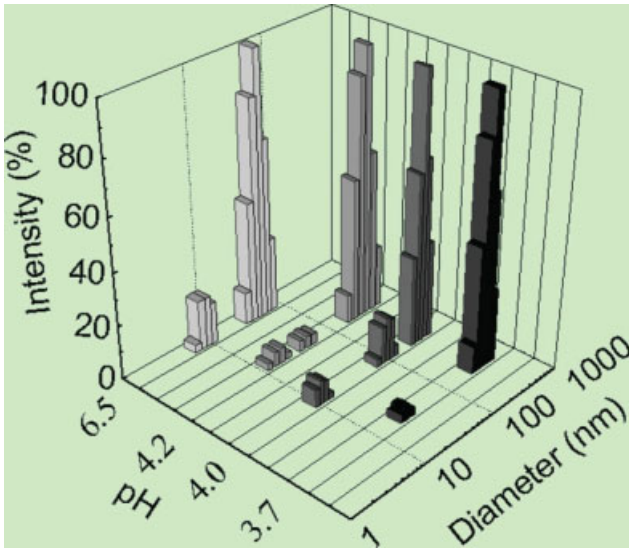

Figure 1 Distribution of apparent diameters for a SOD-HA mixture in 0.05*M* NaCl at various pH values. SOD: 0.5 mg/mL; HA: 0.05 mg/mL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

20–30 nm occur in the free SOD solution, but the size of HA scarcely varies in the free HA solution. Therefore, a larger fluctuation of particle sizes directly indicates a variance of particle constituents for SOD-HA mixtures.

The histograms of SOD-HA mixtures with different pH were given in Figure 1. The mixed liquor mainly has SOD-HA soluble complexes with an apparent diameter of 30–80 nm at pH 6.5. As decreasing pH up to 3.7, these soluble complexes are associated with each other to form "primary" aggregates with about 100 nm, and even higher-order aggregates with around 300 nm. However, some SOD-HA soluble complexes still survive at pH 3.7. Primary and higher-order aggregates are made up of many proteins bound to some polymer chains.[28]

Turbidimetric titration

To distinguish the critical pH of complexation (pH_c) and phase separation (pH_ϕ), a suitable NaCl concentration (i.e., 0.05*M* NaCl) is chosen according the previous report.[33] Domains of noninteraction (the region I at $pH > pH_c$), soluble complexes (the region II between pH_c and pH_ϕ) and aggregates (the region III at $pH < pH_\phi$) for SOD-HA mixtures were shown in Figure 2. SOD-HA aggregates redissolve completely at $pH < 2$, and thus the pH is more than 2 for the domain of aggregates. Both pH_c and pH_ϕ are reduced as increasing NaCl concentrations up to 0.3*M* because the screening effect of salt ions is more pronounced at higher ionic strengths in the range of the experiment.

The HA solution and the SOD one are clear by naked eye at pH = 2–12. Although turbidity hardly varies as a function of pH for the HA solution, it alters slightly at pH 5.0–2.0 for the SOD solution due to formation and separation of SOD homo-complexes, as shown in Figure 3. It accords with the results obtained by QELS. Three SOD-HA mixtures are clear by naked eye at pH 7.0–5.0, but their turbidities commence to increase slowly near pH 7.0 under spectrophotometer. It is attributed to the soluble complex formation according to QELS. Hence, HA concentrations do not affect pH_c (TABLE I), which accords with the previous report.[13] The SOD-HA binding occurs initially at pH = 6.9 ± 0.1 according to Figure 3.

The results obtained by QELS and turbidimetric titration have shown that the SOD-HA binding is mainly electrostatically driven. Interestingly, both SOD and HA bear negative charges at pH_c (6.9 ± 0.1). The phenomenon that protein-polyelectrolyte binding arises "on the wrong side" of the protein pI was found in the other protein-polyelectrolyte systems.[32,33] Actually, charge heterogeneity on protein surfaces may cause the presence of local positive charges (i.e., patch charges) although the integrated protein bears net negative charges. In the SOD molecule, a histidine that is not a metal ligand is positively charged even at high pH and its protonated imidazole has an acid ionization constant (pKa) of more than 9.[50–52] Those "patch charges" may exist around this histidine. They will bind to the $-COO^-$ (HA), leading to the SOD-HA complex formation.

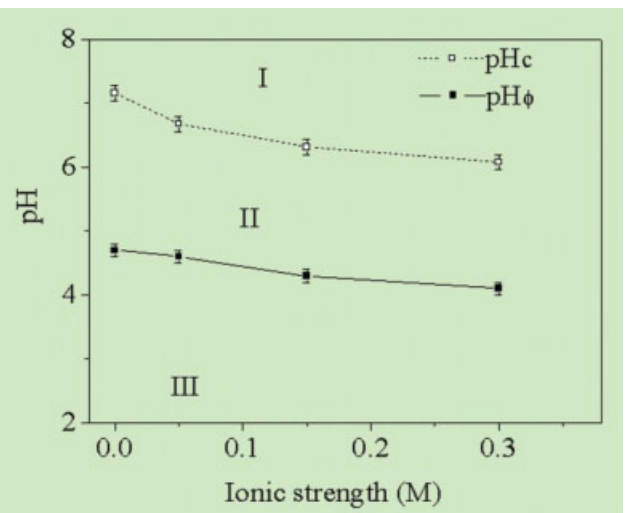

Figure 2 Ionic strength dependence of pH_c and pH_ϕ for the mixtures of SOD and HA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

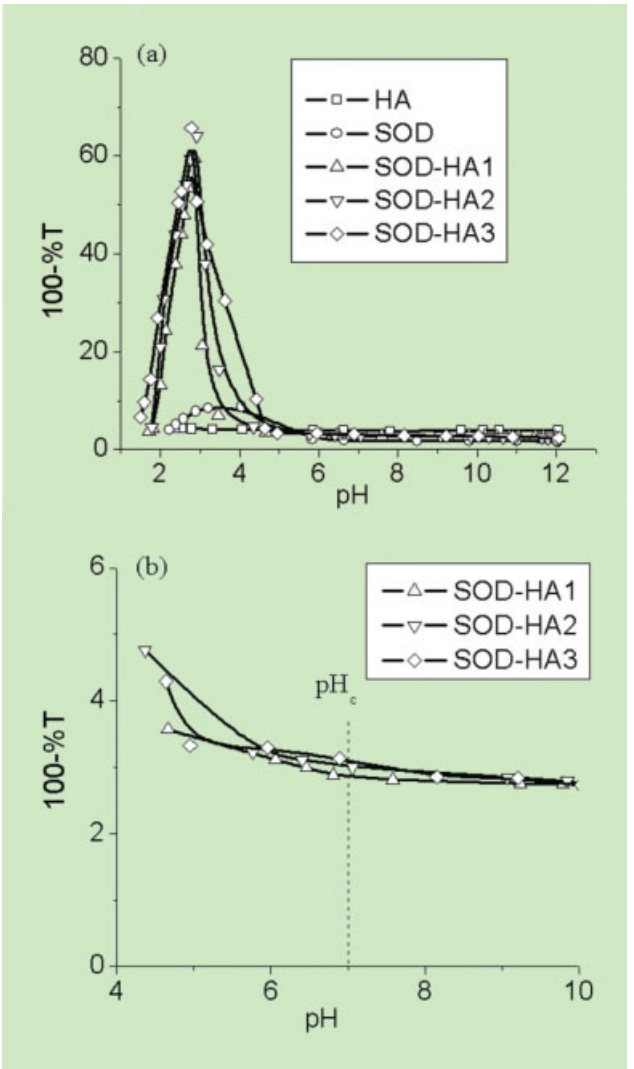

Figure 3 (a) Turbidimetric titration data for 0.5 mg/mL HA, 0.5 mg/mL SOD, and 0.5 mg/mL SOD containing 0.05 mg/mL HA (SOD-HA1), 0.25 mg/mL HA (SOD-HA2), and 0.5 mg/mL HA (SOD-HA3) in 0.05*M* NaCl. (b) Is an enlarged figure. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Therefore, the SOD-HA binding results from charge heterogeneity on protein surfaces.

During the turbidimetric titration, the sediment is not produced in the bottom of a turbidity cell. Turbidity begins to increase significantly at pH $\approx$ 4.6 for SOD-HA3. A remarkable increase of turbidity means phase separation of the mixture. The turbidity reached the maximum at pH $\approx$ 2.9 followed by a rapid decrease, characteristic of redissolution of SOD-HA aggregates. Here, pH_d (about 2.9) is defined as the onset of aggregate redissolution. In the pH range of 4.6–2.9, SOD and HA are oppositely charged since the pI of SOD is approximately 4.95 and the pKa of HA is about 2.9.[49,53] Hence, formation of SOD-HA aggregates may be mostly attributed to electrostatic attractions.

At the fixed SOD concentration and ionic strength, pH_c is almost unchanged but pH_ϕ lowers from 4.6 $\pm$ 0.1 to 3.5 $\pm$ 0.1 with decreasing HA concentrations, as shown in TABLE I. At pH $<$ pH_d, the aggregates redissolve due to protonation of many —COO^- (HA). It is irrespective to HA concentrations.

TABLE I
pH_c and pH_ϕ of 0.5 mg/mL SOD Containing HA with Different Concentrations in 0.05*M* NaCl.

HA concentrations (mg/mL)	pH_c	pH_ϕ
0.5	6.9 $\pm$ 0.1	4.6 $\pm$ 0.1
0.25	6.9 $\pm$ 0.1	4.2 $\pm$ 0.1
0.05	6.9 $\pm$ 0.1	3.5 $\pm$ 0.1

Potentiometric titration

Because electrostatic interactions are driving forces for the formation of SOD-HA soluble complexes and insoluble aggregates, potentiometric titration is helpful to understand this binding process. Figure 4 showed an influence of HA concentrations on the titration curve of 0.5 mg/mL SOD in 0.05*M* NaCl. The effect of polyanion is reported as the pH difference ($\Delta pH_2'$) between the SOD-HA mixture and the corresponding free SOD solution at a given volume of HCl. At pH $>$ 7.0, $\Delta pH_2'$ increases with HA concentrations up to 0.5 mg/mL. Influence of HA concentrations on $\Delta pH_2'$ is weakened at pH $<$ 7.0. $\Delta pH_2'$ scarcely varies with HA concentrations after pH $<$ 6.0. It should be pointed out that pH titration curves display no change at pH_ϕ. It suggests that proteins

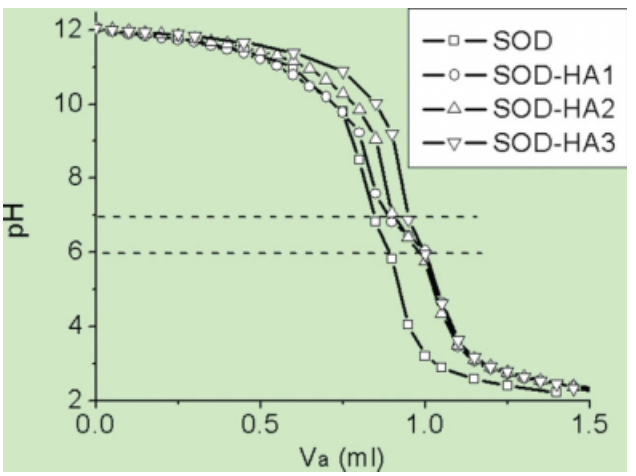

Figure 4 pH titration curves of 0.5 mg/mL SOD in 0.05*M* NaCl, with or without HA at various concentrations C_p. C_p = 0 (SOD), 0.05 (SOD-HA1), 0.25 (SOD-HA2), and 0.5 mg/mL (SOD-HA3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

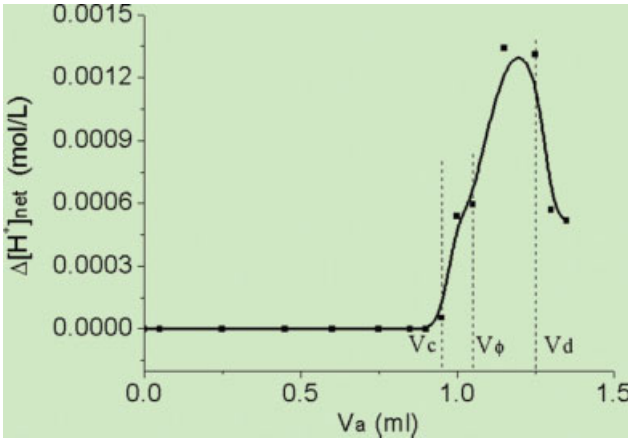

Figure 5 Relationship between of $\Delta[H^+]_{net}$ and the volume of 0.1*M* HCl. The net increment of H^+ of SOD-HA solution in regard to free SOD and free HA solutions $\Delta[H^+]_{net} = [H^+]_{SOD\text{-}HA}\text{-}[H^+]_{SOD}\text{-}[H^+]_{HA}+[H^+]_{Control}$. The solutions used are respectively a blank (control), 0.5 mg/mL HA (HA), 0.5 mg/mL SOD (SOD), and 0.5 mg/mL SOD containing 0.5 mg/mL HA (SOD-HA) in 0.05*M* NaCl. V_c, V_f, and V_d are defined in the previous context. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

within the precipitation phase are fully titratable and precipitation has no effect on an equilibrium electrode response.[33]

Potentiometric experiments have shown that HA enhances the H^+ binding capacity of $-NH_2$ in SOD, i.e., reduces the base ionization constant (pK_b). It is because the configuration of bound polymer may be spontaneously adjusted to maximize the proximity of its anionic repeating units and the $-NH_3^+$ on protein surfaces. Furthermore, those $bond;NH_3^+$ will be stabilized when they are bound to $-COO^-$ (HA) with "salt bridges." SOD seems to enhance the H^+ binding capacity of HA, i.e., reduce pK_b (HA). However, H^+ is firstly bound to $-NH_2$ (SOD) since its pK_b is much lower than that of $-COO^-$ (HA). Therefore, a decrease of pK_b (HA) is just a pseudo image. SOD, which is bound to H^+, can attract $-COO^-$ (HA) by electrostatic interactions in the SOD-HA mixture.

Figure 5 presented a variance of net increment of H^+ ($\Delta[H^+]_{net}$) with the adding amount of HCl solutions. To be convenient for discussion, V_c, V_ϕ, V_d are defined as the adding amount of HCl solutions at pH_c, pH_ϕ, and pH_d, respectively. $\Delta[H^+]_{net}$ directly indicates if electrostatic interactions exist between SOD and HA. It is calculated according to the following equation:

$$\begin{aligned}\Delta[H^+]_{net} &= \{-\Delta[H^+]_{SOD} - \Delta[H^+]_{HA}\} \\ &\quad - \{-\Delta[H^+]_{SOD\text{-}HA}\} = \{[H^+]_{SOD\text{-}HA} - [H^+]_{Control}\} \\ &\quad - \{[H^+]_{SOD} - [H^+]_{Control}\} - \{[H^+]_{HA} - [H^+]_{Control}\} \\ &= [H^+]_{SOD\text{-}HA} - [H^+]_{SOD} - [H^+]_{HA} + [H^+]_{Control} \qquad (1)\end{aligned}$$

where $[H^+]_{SOD\text{-}HA}$, $[H^+]_{SOD}$, $[H^+]_{HA}$, and $[H^+]_{Control}$ indicate the residual H^+ concentrations of SOD-HA mixtures, SOD solutions, HA solutions, and 0.05*M* NaCl. Given the existence of electrostatic interactions between SOD and HA, $-NH_2$ (SOD) binds to one H^+ to form $bond;NH_3^+$ (SOD) which may attract $-COO^-$ (HA) to build up a "salt bridge." Hence, $\Delta[H^+]_{net}$ is larger than zero. As shown in Figure 5, $\Delta[H^+]_{net}$ approximates zero at the volume of 0.1*M* HCl (V_a) less than 0.90 mL (pH > 9.2). It indicates no obvious electrostatic interactions occur between SOD and HA. In the range of $V_c - V_\phi$, $\Delta[H^+]_{net}$ is bigger than zero, and increases with V_a, suggesting that electrostatic interactions are continuously enhanced. The enhanced interactions result in the formation of the SOD-HA soluble complex. In the range of $V_\phi - V_d$, $\Delta[H^+]_{net}$ further increases to a maximum at $V_a \approx 1.25$ mL (pH ≈ 2.9). Accordingly, the interactions are enhanced enough to promote formation of SOD-HA aggregates. After $V_a > V_d$, $\Delta[H^+]_{net}$ is still greater than zero, but decreases with the increase of V_a. At the time the interactions are reduced to the extent that SOD-HA aggregates begin to redissolve. Interestingly, a variance of turbidity with the HCl adding amount is similar to that of $\Delta[H^+]_{net}$ (data not shown). It further confirms that electrostatic interactions control formation of SOD-HA complexes and aggregates.

It is assumed that the titratable groups in the SOD-HA system are as follows: m $-NH_2$ (To be simplified, $-NH_2$ is representative of all basic groups of SOD including $-NH_2$ and imidazolyl, etc.), n $-COO^-$ (SOD), and q $-COO^-$ (HA). f_m, f_n, and f_q are supposed to indicate respectively the percent of the individual groups bound to H^+ in the total of individual ones at a given pH and ionic strength under noninteracting conditions. Given the existence of interactions, the percent of mf_m enhanced by HA is indicated as α (≥ 0), and that of nf_n reduced by HA is β (≥ 0). At the same time, that of qf_q reduced by SOD is γ (≥ 0). Therefore, the H^+ binding sum of the isolated SOD and HA (i.e., noninteracting) is $N_H = mf_m + nf_n + qf_q$, and that of the SOD-HA system is $N'_H = (1 + \alpha)mf_m + (1 - \beta)nf_n + (1 - \gamma)qf_q$. As shown in Figure 5, $\Delta[H^+]_{net}$ ($=N_H - N'_H$) is always larger than or equal to zero in the range of experimental errors. Hence, the following relation can be obtained:

$$N'_H \leq N_H \Rightarrow \alpha mf_m - \beta nf_n \leq \gamma qf_q \qquad (2)$$

The left of eq. (2) is representative of an increment of H^+ binding of SOD arising from HA, and the right is a decrement of H^+ binding of HA arising from SOD. Hence, the prerequisite to the SOD-HA binding is that the H^+ binding increment of SOD is lower than the H^+ binding decrement of HA. A difference between them increases with lowering pH

before the aggregate redissolution according to Figure 5. It suggests that SOD-HA complexation has a greater effect on the H^+ binding capacity of HA at lower pH. Theoretically, a proper increase of $\alpha m f_m - \beta n f_n$ is favorable to the SOD-HA binding. However, its excessive increase will result in a mutual repulsion between SOD molecules upon bearing excess positive charges. Once $\gamma q f_q$ is lower than $\alpha m f_m - \beta n f_n$, the repulsion between SOD molecules will exceed the attraction between SOD and HA. It will lead to a decrease of the number of SOD bound to HA and even the aggregate redissolution. Therefore, a suitable pH should be adjusted to prepare SOD-HA soluble complexes or aggregates.

In fact, eq. (2) can be applied to most of protein-acidic polyelectrolyte systems. Also, it can be used in protein-basic polyelectrolyte systems after a proper modification (to give the modified equation is beyond the scope of this work). If polymer residues have the same concentration in protein-acidic polyelectrolyte systems, (1) for strong polyelectrolytes such as poly(styrene sulfonate) and dextran sulfate, their anions are sufficient to obtain a great $\gamma q f_q$, and thus the protein-polymer binding occurs in the larger range of pH; (2) for weak polyelectrolytes including poly(malic acid), HA, and so on, their anions are partially neutralized and $\gamma q f_q$ is moderate, so proteins are bound to polymers only in the narrower pH range; (3) for those polymers with weaker titratable groups, for instance poly(vinyl alcohol), their rare anions results in a small $\gamma q f_q$, so the electrostatic binding is difficult between proteins and polymers. It is noticed especially that the protein species is also an essential factor influencing the pH range of the protein-polymer electrostatic binding. This influence is similar to that of polymer species.

CONCLUSIONS

Complexation between SOD and HA occur in the aqueous solution at a suitable pH. SOD-HA mixtures go through four stages including noninteraction, soluble complexes, aggregates, and their redissolution, with decreasing pH. Electrostatic interactions are the main factor that forces the SOD-HA binding. This binding occurs initially above the protein pI because of the charge heterogeneity on protein surfaces. Electrostatic interactions exist between local positive charges on the protein surface and anionic pendant groups on the polymeric chain of HA. Owing to screening effects of salt ions, the ionic strength affects pH_c and pH_ϕ. HA concentrations do not influence pH_c but pH_ϕ.

HA makes the H^+ binding capacity of $-NH_2$ (SOD) enhanced, and consequently the formed $bond; NH_3^+$ (SOD) interacts with $-COO^-$ (HA) to build "salt bridges." At pH 2.9, the quantity of "salt bridges" is enough to form stable SOD-HA complexes and even aggregates. When the pH is lower than 2.9, more $-COO^-$ groups on HA are neutralized so that SOD-HA aggregates begin to redissolve. The net increment of H^+ ($\Delta[H^+]_{net}$) is always larger than zero at $V_c - V_d$, suggesting the existence of electrostatic attractions between SOD and HA. The necessary condition of protein-polyelectrolyte binding is that an increment of H^+ binding of proteins is less than a decrement of H^+ binding of polyanions. This work can help researchers select suitable proteins, polymers, and process conditions to prepare protein-polymer complexes and aggregates, which is important for the drug delivery system.

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