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ORIGINAL CONTRIBUTION

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Electrostatic interactions between amphoteric latex particles and proteins

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Abstract The electrostatic interactions between amphoteric polymethyl methacrylate latex particles and proteins with different pI values were investigated. These latex particles possess a net positive charge at low pH, but they become negatively charged at high pH. The nature and degree of interactions between these polymer particles and proteins are primarily controlled by the electrostatic characteristics of the particles and proteins under the experimental conditions. The self-promoting adsorption process from the charge neutralization of latex particles by the proteins, which have the opposite net charge to that of the particles, leads to a rapid reduction in the zeta potential of the particles (in other words colloidal stability), and so strong flocculation occurs. On the other hand, the electrostatic repulsion forces between similarly charged latex particles and the proteins retard the adsorption of protein molecules onto the surfaces of the particles. Therefore, latex particles exhibit excellent colloidal stability over a wide range of protein concentrations. A transition from net negative charge to net positive charge, and vice versa (charge reversal), was observed when the particle surface charge density was not high enough to be predominant in the protein adsorption process.

Keywords Amphoteric latex particles · Polymethyl methacrylate · Adsorption of proteins · Electrostatic interactions · Colloidal stability

Introduction

A polymeric support comprising numerous submicron latex particles, which has an extremely large interfacial area, represents an attractive candidate for the purification of proteins from a crude biological mixture. Binding proteins onto the particle surface via the electrostatic interaction is the most widely used recovery method. Various positively charged or negatively charged latex particles have been prepared and used to purify proteins in this way [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. Homola and James [13] carried out surfactant-free emulsion polymerizations of styrene, *N*,*N*-diethylaminoethyl methacrylate, and MAA in a batch reactor in order to prepare and characterize amphoteric polystyrene latexes. However, the colloid stability of these polymerizations was not good, as shown by the very high amounts of coagulum produced (4.2–38.1%).

In our previous work [14], stable monodisperse amphoteric latexes were synthesized by the semibatch surfactant-free emulsion copolymerization of methyl methacrylate (MMA) and methacrylic acid (MAA), initiated by 2,2′-azobis(2-amidinopropane) dihydrochloride (V-50). The radical chain polymerization comprises a sequence of initiation, propagation, and termination, and results in a polymethyl methacrylate

chain terminated with the initiator radical species (HCl - - - $HN-C(NH_2)_2-C(CH_3)-$). Furthermore, the hydrophilic end group tends to be distributed within the latex particle surface layer. At low pH, these particles have a net positive charge due to the ionized amino group originating from V-50. At high pH, on the other hand, they become negatively charged owing to the ionized carboxyl group originating from MAA. This means that there is a pH at which these particles exhibit a net charge of zero (pI). As expected, at constant V-50, the pI values of these latexes decrease as the concentration of MAA used in the polymerization recipe is increased. The objective of this paper was to study electrostatic interactions between these amphoteric latex particles and various proteins with different pI values. The proteins chosen for this work include lysozyme (pI = 11), hemoglobin (pI = 6.8), and pepsin (pI = 1). The results obtained from this work should provide a better understanding of adsorption of proteins onto these amphoteric latex particles at different pH values.

Experimental

Materials

Four monodisperse amphoteric polymethyl methacrylate latexes were obtained from [14]; some of their chemical and physical properties are summarized in Table 1. Other chemicals used in this study include lysozyme (Sigma), hemoglobin (Sigma), pepsin (Sigma), hydrochloric acid (Nacalai Tesque), sodium hydroxide (Riedel-de Haen), sodium chloride (Riedel-de Haen), and deionized water (Barnsted, Nanopure Ultrapure Water System, specific conductance $< 0.057\ \mu S/cm$). Before the protein adsorption experiments, amphoteric latexes were subjected to centrifugation at 8,000 rpm for 30 min (Beckman, J2–21) and dispersed in deionized water by sonication (Delta DG-1). This procedure was repeated five times in order to remove the residual monomer, the initiator, the water-soluble oligomer, and other residues in the aqueous phase. Particle size data, obtained via dynamic light scattering (DLS, Otsuka, Photal LPA-3000/3100), showed that these latexes were very stable toward at least five cycles of centrifugation-dispersion [14]. All other chemicals were used as received.

Table 1 Some chemical and physical properties of amphoteric polymethyl methacrylate latexes, obtained from [14]

Property	SB3	SSB1	SSB2	SSB3
[MAA] (%)[a]	1	0.48	1.5	2.5
[V-50] (%)[b]	0.11	0.64	0.63	0.63
d_v(nm)[c]	219	255	250	259
d_w/d_n[d]	1.04	1.05	1.05	1.05
$C_{V\text{-}50}$ (%)[e]	0.25	1.21	1.08	1.02
pI	4.5	6.6	5.9	4.7

[a] Weight percentage of MAA, based on total MMA in the polymerization recipe;
[b] Weight percentage of V-50, based on total water in the polymerization recipe;
[c] Volume-average particle diameter of the latex product;
[d] Polydispersity index of particle size distribution of the latex product;
[e] Weight percentage of V-50 ultimately incorporated into latex particles

Electrostatic interactions between amphoteric latex particles and proteins

First, the pH of the aqueous solution of protein with prescribed concentration (lysozyme, hemoglobin, or pepsin A) and the amphoteric latex (SB3, SSB1, SSB2, or SSB3) was adjusted to 3, 7, or 9.5 using 0.1 N HCl and 0.1 N NaOH. The total solids content of the latex particles was kept constant at 0.01%, and the ionic strength of the mixture of latex particles and protein was kept constant at 2 mM NaCl throughout this work. The latex sample, with a volume of 3 ml, was then mixed with an equal volume of the protein solution. The latex particles and protein were mixed using a magnetic stirrer at 25 °C for 6 h. The primary parameters investigated in this work are the amphoteric latexes and proteins with different values of pI, the protein concentration, and the pH of the mixture of latex particles and protein. The zeta potential (ζ) and average particle diameter (d_p) of latex particles with adsorbed protein molecules were determined by a zeta potential meter (Malvern, Zetamaster) and DLS (Otsuka, Photal LPA-3000/3100), respectively. The dilution water used for the measurements of ζ and d_p had the same ionic strength and pH as the latex sample. The ζ and d_p data reported in this work are the average of at least ten and three measurements, respectively.

Results and discussion

Interactions between latex particles and lysozyme

For reference, the zeta potentials (ζ) of the amphoteric latex particles SB3, SSB1, SSB2, and SSB3 in the absence of protein are shown in Fig. 1 [14]. Figure 2 shows the influence of lysozyme concentration on the ζ of these latex particles at pH 3, 7, and 9.5. At pH 3, which is well below the pI values of these latexes (Table 1), all of the colloidal systems in the absence of protein carry a net positive charge (Fig. 1). Therefore, the ζ data obtained from a particular colloidal system comprising latex particles and lysozyme do not change very much when the lysozyme concentration increases (Fig. 2a). This is simply because, at pH 3, lysozyme with pI = 11 is also

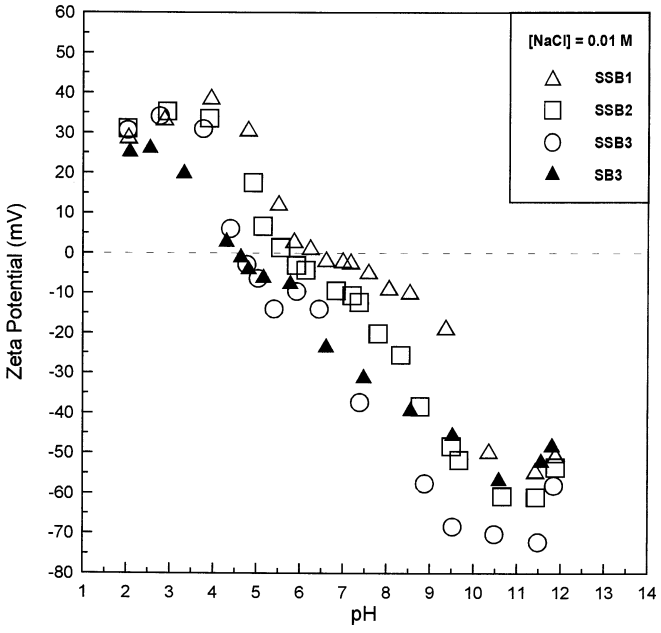


Fig. 1 Effect of pH on the zeta potential of amphoteric PMMA particles at constant ionic strength (0.01 M NaCl). Filled triangles: [V-50] = 0.11% and [MAA] = 1% (SB3); unfilled triangles: [V-50] = 0.64% and [MAA] = 0.48% (SSB1); squares: [V-50] = 0.63% and [MAA] = 1.5% (SSB2); circles: [V-50] = 0.63% and [MAA] = 2.5% (SSB3) [14]

positively charged and so a strong electrostatic repulsion force exists between the latex particle and lysozyme molecule. Under the circumstances, the adsorption of lysozyme onto the latex particles is severely retarded and these particles are quite stable toward the addition of lysozyme. This is also supported by the fact that the average particle diameter (d_p) remains relatively constant when the lysozyme concentration increases, as shown in Fig. 3a. Furthermore, at constant lysozyme concentration, the ζ of latex particles (in decreasing order) is SSB1 > SSB2~SSB3 > SB3. This trend is consistent with the pI values of amphoteric latexes (Table 1). The larger the pI of the polymeric support, the higher the ζ of the colloidal system. The comparable ζ versus lysozyme concentration curves for SSB2 and SSB3 are most likely due to the different volume-average particle diameters (d_v) (Table 1). At constant polymer weight, the total particle surface area is inversely proportional to d_v. Therefore, the particle surface charge density, which is linearly proportional to $C_{V\text{-}50}/(1/d_v)$, in decreasing order, is SSB1 (3.09) > SSB2 (2.70)~SSB3 (2.64) > SB3 (0.55). $C_{V\text{-}50}$ represents the weight percentage of V-50 ultimately incorporated into latex particles, and the numeric values in parentheses are the ratios of $C_{V\text{-}50}/(1/d_v)$. As expected, the larger the particle surface charge density, the higher the ζ.

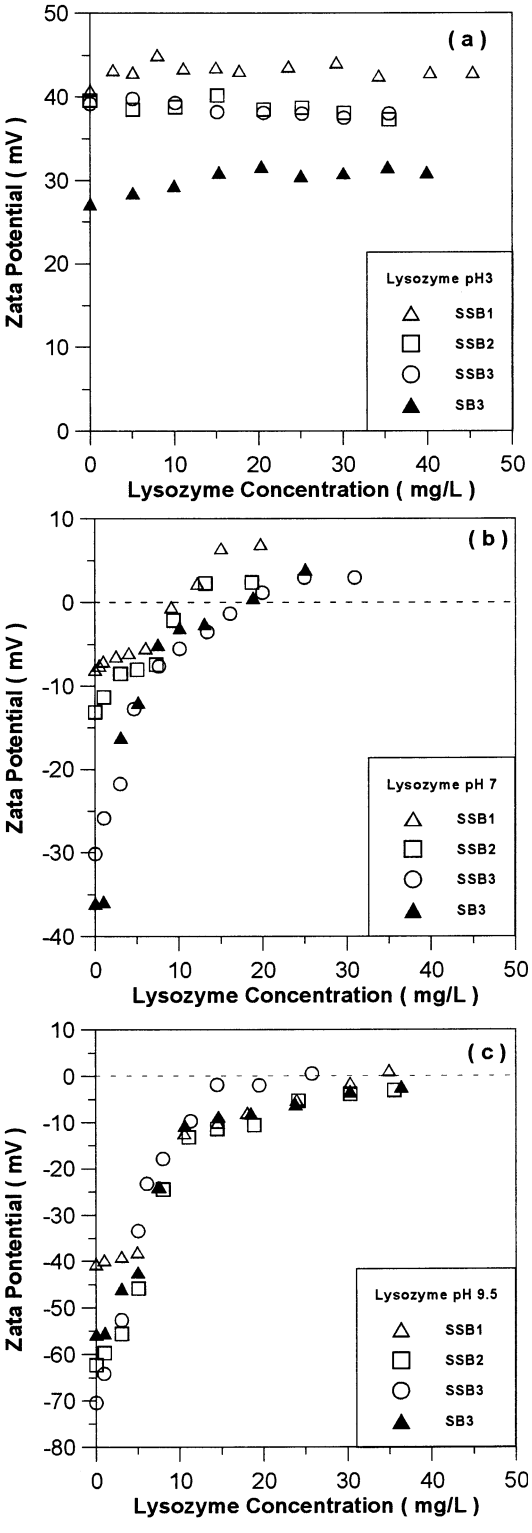


Fig. 2 Zeta potential of amphoteric PMMA particles as a function of the lysozyme concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

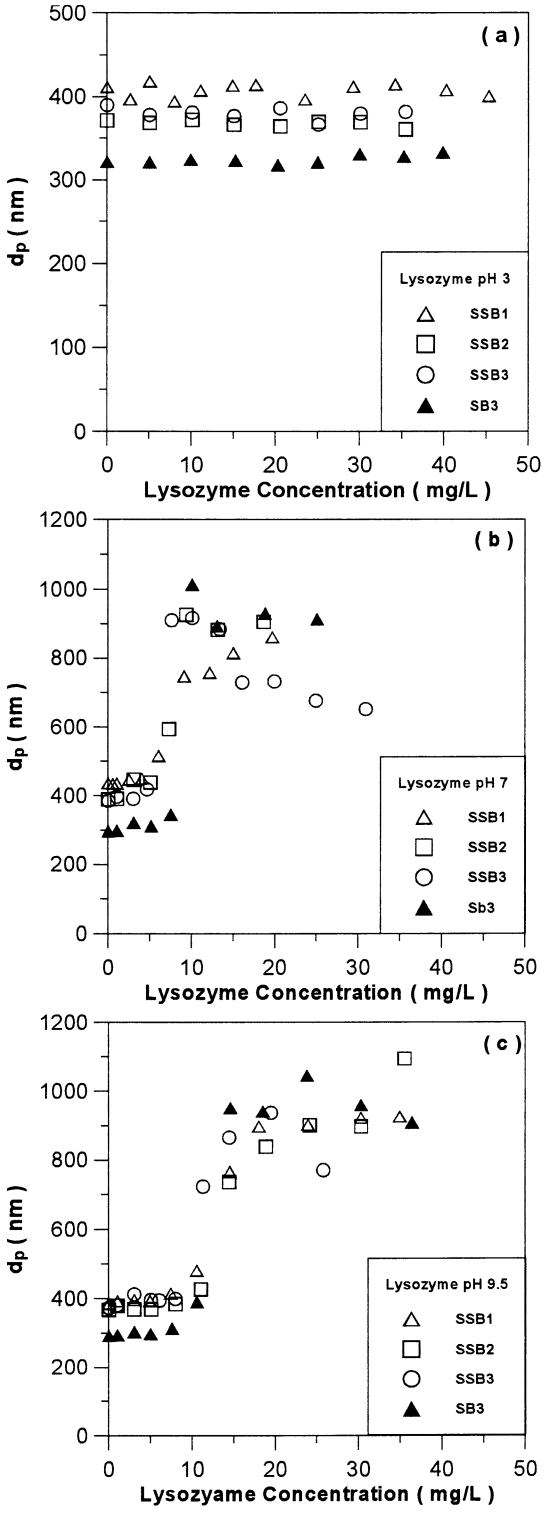


Fig. 3 Average amphoteric PMMA particle diameter as a function of the lysozyme concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

Figure 2c shows the ζ versus lysozyme concentration profiles for amphoteric latexes at pH 9.5. In this case, all of the latexes are negatively charged, because pH 9.5 is well above their pI values (Table 1). A common feature of these ζ versus lysozyme concentration profiles is that ζ decreases rapidly toward zero when the lysozyme concentration increases. The self-promoting adsorption process [15, 16] provided by the charge neutralization of latex particles by lysozyme is primarily responsible for this behavior. The corresponding d_p versus lysozyme concentration data for the latexes at pH 9.5 are shown in Fig. 3c. At first, the value of d_p remains relatively constant, then it increases rapidly, and finally levels off when the lysozyme concentration increases from 0 to 35 mg/L. The colloidal stability is satisfactory for all of the latexes when the lysozyme concentration is below ~10 mg/L. Beyond 10 mg/L lysozyme, the $|\zeta|$of the latex particles (< 10 mV) is not high enough to prevent particles from flocculation, as evidenced by the rapidly increased d_p with lysozyme concentration. In this series of experiments, no charge reversal was observed, even when the lysozyme concentration approached 35 mg/L (Fig. 2c) because the negatively charged latex particles predominate in the colloidal system at pH 9, as shown by the very large absolute values of ζ (in the range ca. −45 to −70 mV) in Fig. 1. It is also interesting to note that both the ζ and d_p versus lysozyme concentration data become indistinguishable for the latexes at pH 9.5 compared to their counterparts at pH 3. The insignificant effect of particle surface charge density on the colloidal stability is attributed to the very strong electrostatic interaction between latex particles and lysozyme, which exhibit opposite charges at pH 9.5.

The ζ and d_p versus lysozyme concentration data for amphoteric latexes at neutral pH are shown in Fig. 2b and Fig. 3b, respectively. At pH 7, lysozyme carries a net positive charge, and all of the latexes carry a net negative charge since their pI values are smaller than 7 (Table 1). The absolute values of ζ (in the range ca. 0 to −25 mV) in the absence of protein are not very high (Fig. 1). As would be expected, these latexes at neutral pH are less stable toward the adsorption of lysozyme compared to those at pH 9.5. This is supported by the ζ and d_p data shown in Figs. 2 and 3;ζ reaches zero and d_p starts to rise steeply from a constant d_p region at lower levels of lysozyme for the experiments conducted at pH 7. Beyond the point of zero charge, charge reversal ($- \rightarrow +$) occurs in the lysozyme concentration range ~10–20 mg/L due to further adsorption of positively charged lysozyme molecules onto latex particles (Fig. 2b). In addition, enhanced colloidal stability is experienced on charge reversal, as shown by the slightly decreased d_p with lysozyme concentration in Fig. 3b. Nevertheless, this restabilization phenomenon at high lysozyme concentration is not significant because of the relatively low ζ ($0 < \zeta < 10$ mV) (Fig. 2b).

Interactions between latex particles and pepsin

Figures 4 and 5 show the influence of pepsin concentration on the ζ and d_p of latexes SB3, SSB1, SSB2 and SSB3, at different pH values, respectively. At pH 3, the negatively charged pepsin (pI = 1) shows a tendency to adsorb onto all the latex particles carrying a net positive charge. Consequently, ζ decreases rapidly, and at first d_p remains relatively constant and then it increases rapidly with increasing pepsin concentration due to charge neutralization (Figs. 4a and 5a). In addition, SB3 shows the worst colloidal stability toward the addition of pepsin. A transition from net positive charge to negative charge is also observed for all of the latexes investigated in this work. This is attributed to the intermediate ζ ($\sim$25–35 mV) obtained from these colloidal systems in the absence of protein (Fig. 1), which cannot be the predominant factor in determining the electrostatic properties of latex particles during the protein adsorption process. However, the particle surface charge density provided by adsorbed pepsin molecules is not high enough to impart excellent stability to latex particles. This is due to the fact that pepsin (pI = 1) would carry a rather weak net negative charge at pH 3.

By contrast, at pH 9.5, both the latex particles and pepsin exhibit net negative charges. As expected, all of the latexes are very stable toward the addition of pepsin, as shown by the relatively constant ζ and d_p data in the pepsin concentration range studied (Figs. 4c and 5c). Furthermore, the contribution of adsorbed pepsin (if any) to the particle surface charge density is insignificant because of the very strong electrostatic repulsion force between the latex particle and pepsin. In the absence of protein, the absolute values of ζ are reduced significantly when the pH decreases from 9.5 to 7 (Fig. 1). This would then enhance the probability of adsorption of pepsin onto latex particles via hydrophobic interaction. This postulation is supported by the increased $|\zeta|$ and relatively constant d_p when the pepsin concentration increases (Figs. 4b and 5b).

Interactions between latex particles and hemoglobin

Finally, hemoglobin with an intermediate pI value (6.8) was used to study the electrostatic interactions between the amphoteric latex and hemoglobin. Figures 6 and 7 show the influence of hemoglobin concentration on the ζ and d_p of latexes SB3, SSB1, SSB2, and SSB3, at different pH values, respectively. At pH 3, both the latex particle and hemoglobin have a net positive charge and so the colloidal stability of all the latexes is satisfactory. When the hemoglobin concentration increases, both ζ and d_p remain relatively constant, as shown in Figs. 6a

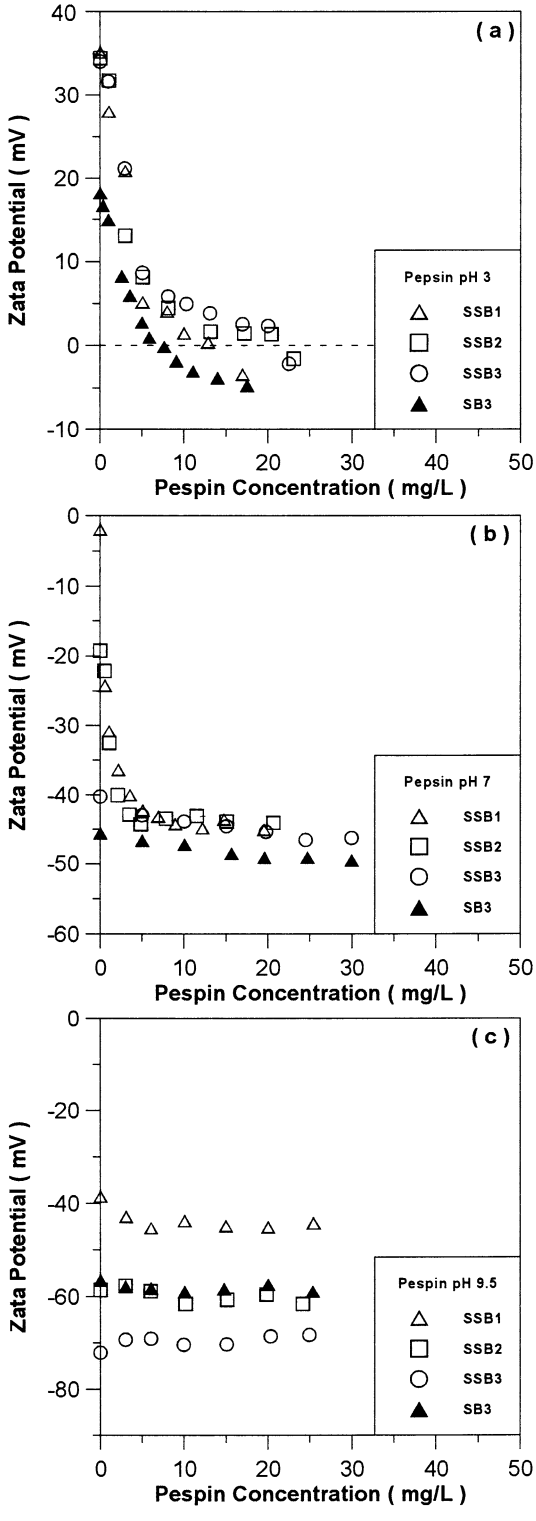


Fig. 4 Zeta potential of amphoteric PMMA particles as a function of the pepsin concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

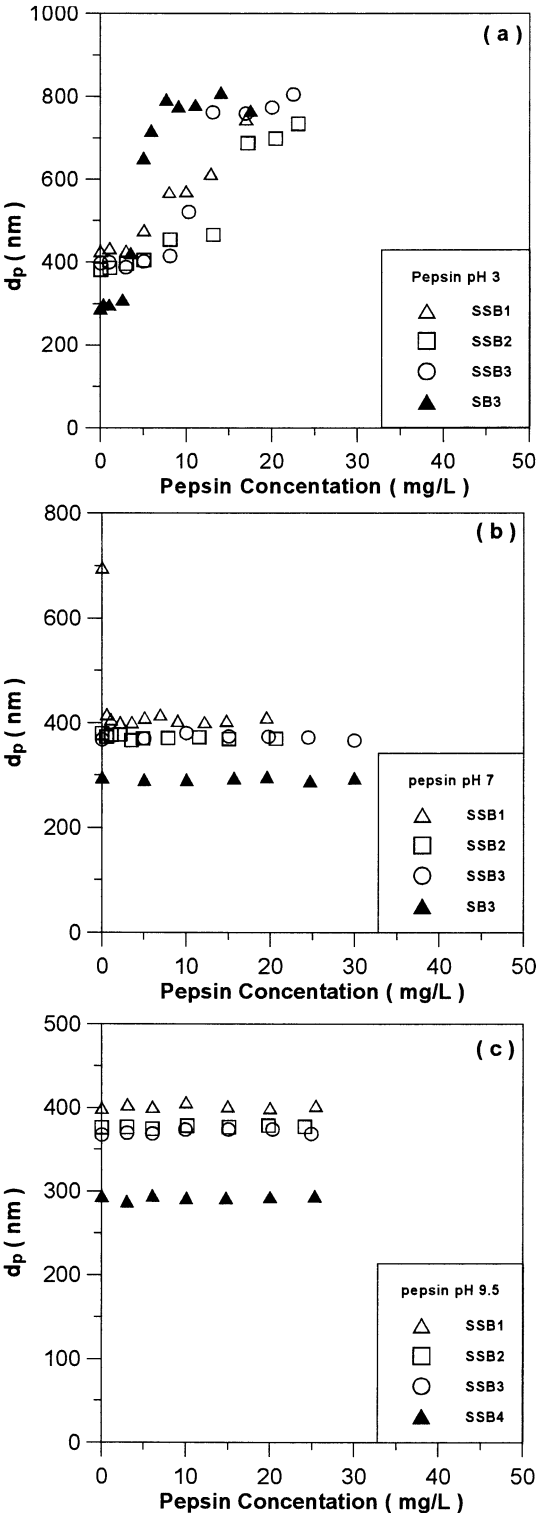


Fig. 5 Average amphoteric PMMA particle diameter as a function of the pepsin concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

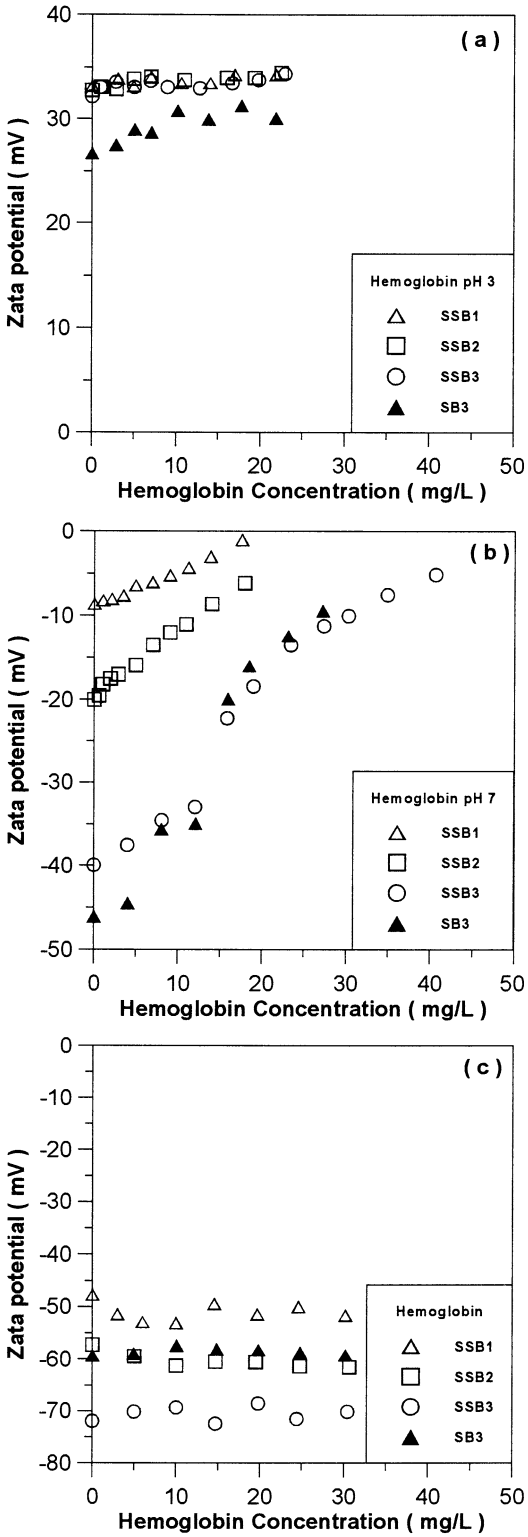


Fig. 6 Zeta potential of amphoteric PMMA particles as a function of the hemoglobin concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

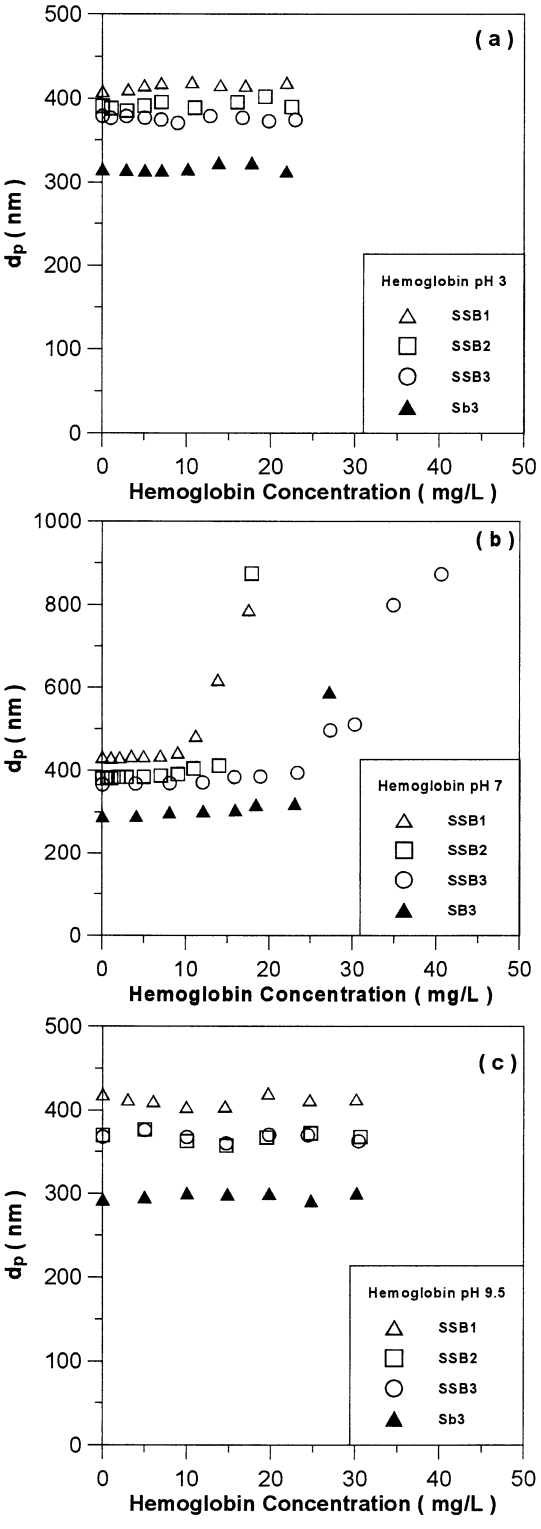


Fig. 7 Average amphoteric PMMA particle diameter as a function of the hemoglobin concentration at different pH values. Filled triangles: SB3; unfilled triangles: SSB1; squares: SSB2; circles: SSB3. **a** pH 3; **b** pH 7; **c** pH 9.5

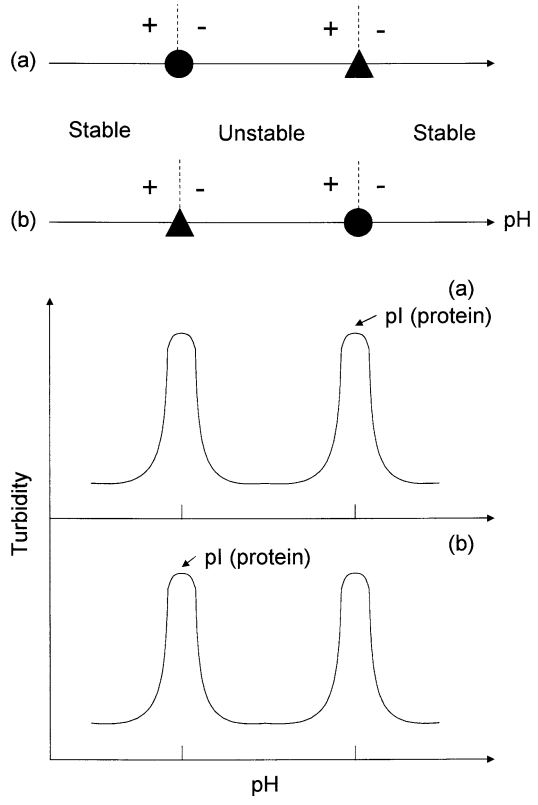
and 7a. Similar experimental results are obtained from colloidal systems comprising latexes and hemoglobin at pH 9.5, except that both the latex particle and hemoglobin are negatively charged in this case. All of these results imply that adsorption of hemoglobin onto latex particles at pH 3 and 9.5 is severely retarded due to the mutual electrostatic repulsion mechanism.

As mentioned above, at pH 7, all of the latexes carry a net negative charge, but the absolute values of ζ (in the range ca. 0 to −25 mV) of these colloidal systems in the absence of protein are not very high. Hemoglobin should possess a net charge of about zero, since its pI is very close to neutral pH. The ζ and d_p data in Figs. 6b and 7b show that all of the latexes become unstable when the hemoglobin concentration increases from 0 to 40 mg/L. It is also interesting to note that the colloidal stability, in decreasing order, is SB3∼SSB3 > SSB2 > SSB1, which is quite consistent with the ζ of the latexes in the absence of protein at pH 7 (Fig. 1). The gradually decreasing ζ with hemoglobin concentration shown in Fig. 6b suggests that hemoglobin should be slightly positively charged, and it could be adsorbed onto latex particles via the electrostatic interaction under our experimental conditions.

Based on the colloidal instability phenomenon caused by the electrostatic attraction between the amphoteric latex particles and protein molecules, the isoelectric point of a protein sample can be determined by the following approach. For example, one can select an amphoteric latex with pI = 5 to interact with an unknown protein sample. If the colloidal system is stable at pH 4 but unstable at pH 6, the pI of the protein should be greater than 6. On the other hand, the pI of the protein should be less than 4 provided that the colloidal system is unstable at pH 4 but stable at pH 6. If satisfactory colloidal stability is achieved at both pH 4 and 6, the pI of the protein should lie between pH 4 and 6. Once the approximate value of pI is identified in this manner, the pI of the protein sample can be further narrowed down to a more accurate range simply by using additional latexes of different pI. Alternatively, a single amphoteric latex of known pI can be used to interact with a protein sample at various pH values. Then, the point at which the transition from an unstable state to a stable state (a) or from a stable state to an unstable state (b) occurs represents the pI of the protein, as illustrated in Scheme 1.

Conclusions

The electrostatic interactions between amphoteric polymethyl methacrylate latex particles and proteins with different pI values were investigated. These latex particles possess a net positive charge at low pH, but



Scheme 1 A schematic representation of the method that uses a single amphoteric latex of known pI to interact with a protein sample at various pH values in order to determine the pI of the protein. Triangles: pI(protein); circles: pI(latex). **a** pI(protein) > pI(latex); **b** pI(protein) < pI(latex)

they become negatively charged at high pH. The nature and degree of interactions between these polymer particles and proteins are primarily controlled by the electrostatic characteristics of the particles and proteins under the experimental conditions. At pH 3, pepsin (pI = 1) is negatively charged and this promotes the self-promoting adsorption process from the charge neutralization of latex particles by pepsin. Accordingly, colloidal stability decreases rapidly with increasing pepsin concentration. A transition from net positive charge to net negative charge (charge reversal) is observed for all of the latexes investigated in this work. This is attributed to the adsorption of sufficient negatively charged pepsin molecules onto latex particles. On the other hand, both lysozyme (pI = 11) and hemoglobin (pI = 6.8) carry a net positive charge at pH 3. An electrostatic repulsion force exists between the latex particle and protein, thereby leading to satisfactory colloidal stability.

At pH 9.5, lysozyme carries the opposite charge to that of the latex particles and, therefore, it shows a tendency to adsorb onto latex particles, accompanied by strong flocculation. By contrast, excellent stability is achieved for the colloidal system comprising negatively charged latex particles and hemoglobin (or pepsin) at pH 9.5. Finally, amphoteric latexes at neutral pH become unstable toward the addition of lysozyme (or hemoglobin) due to charge neutralization. Charge reversal also takes place in the lysozyme concentration range ~10–20 mg/L. On the other hand, the probability of adsorption of pepsin onto latex particles is enhanced, probably due to the hydrophobic interaction. Consequently, the colloidal stability of latexes is quite satisfactory during the protein adsorption process.

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