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ORIGINAL CONTRIBUTION

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Interactions of hydrophobically modified poly(sodium acrylate) with globular proteins

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Abstract The association of a series of hydrophobically modified poly(sodium acrylate) (HMPA) with lysozyme, a cationic globular protein, or with bovine serum albumin (BSA), an anionic globular protein, was investigated at pH = 9 by rheology and to a lesser extent by steady-state fluorescence spectroscopy. Under suitable concentration conditions, this association leads to a drastic viscosity enhancement which is improved when the polymer hydrophobicity is increased.
A mechanism is proposed: the hydrophobic regions of the globular proteins interact strongly with the alkyl groups of one or more polymer chains. In the later case, the macromolecules are crosslinked via the proteins, which leads to viscosity enhancement and even gelation. Analogies and differences between these systems and surfactant/HMPA systems previously studied in our laboratory are emphasized and discussed.

Key words Poly(acrylic acid) – hydrophobically modified polymer – globular protein – interaction – rheology

Introduction

Over the last few years, many studies have been devoted to hydrophobically modified water soluble polymers, i.e., water soluble polymers with low levels of pendent alkyl chains [1–13]. Upon a certain polymer concentration, these materials exhibit much higher viscosities in aqueous solutions than the corresponding unmodified polymers because of the association of the hydrophobic moieties leading to a reversible crosslinking of the system.

Upon addition of surfactant, a more complex behavior is found. Mixtures with a constant amount of hydrophobically modified polymer and increasing surfactant concentrations display viscosities which rise to a maximum and then decrease [10, 14–19]. This behavior has been attributed to the interactions of the surfactant micelles with the polymer alkyl chains. For some optimum surfactant concentration, micelles solubilize alkyl chains belonging to different macromolecules and crosslinking of the system via these mixed micelles thus occurs (Fig. 1). At high surfactant concentrations the micelles are in large excess. In a borderline case, each mixed micelle only contains one alkyl chain attached to a macromolecule. Consequently, the connectivity of the system vanished and the viscosity decreases [14, 17].

In this work, we report on the association of hydrophobically modified poly(sodium acrylate) (HMPA) with globular proteins and we try to draw a comparison with HMPA/surfactant systems. The two systems could actually display some similarities as both micelles and globular proteins are amphiphilic objects of nanometric dimensions with a liquid hydrophobic core for the former and a more structured core for the later. This first study has mainly been based on rheological measurements and to a lesser extent on fluorescence spectroscopy.

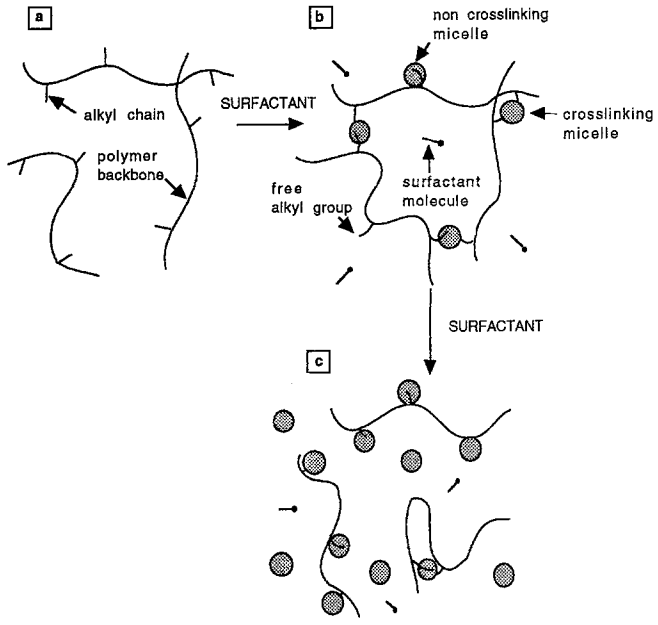


Fig. 1 Schematic view of the association mechanism between HMPA and surfactant micelles: a: HMPA in the absence of surfactant, b: The same solution as in (a) with intermediate amount of surfactant. Mixed micelles are formed leading to the crosslinking of the polymer chains, c: Further addition of surfactant leads to the formation of mixed micelles with low content of polymer alkyl chains. The connectivity of the system is lost

Materials and methods

Our experiments were carried out with two types of globular proteins: lysozyme and bovine serum albumin (BSA). At the pH of the experiment lysozyme is a cationic protein with an isoelectric point of 11, a molecular weight of 14 300 and an apparent Stokes radius of 2 nm [20]. It catalyzes the cell wall hydrolysis of most Gram-positive bacteria. BSA is an anionic protein with an isoelectric point of 4.9, a molecular weight of 69 000 and an apparent Stokes radius of 4 nm up to pH = 10 [20]. A major physiological function of BSA is the transportation of fatty acids and other amphiphiles in the plasma and it is now well established [21] that BSA has high affinity adsorption sites for lipids and surfactants. Chicken-egg lysozyme and fatty acid free bovine serum albumin (fraction V) were obtained from Sigma Chemicals as respectively 95% and 99% pure crystallised and freeze-dried proteins and were used without any further purification. Proteins were solubilized in deionised water 2 h before use to minimize denaturation. pH of the stock solutions was adjusted to 9 by addition of a HCl or NaOH solution.

Poly(acrylic acid) (PA) of average molecular weight 150 000 was purchased from Polysciences. The hydrophobically modified poly(acrylic acids) (HMPA) were synthesized according to a reaction described previously [11]. They are random copolymers with the following structure:

$$-(CH_2-CH)_{100-x}-(CH_2-CH)_{x}-$$

$$COO^-Na^+ \qquad CONHR,$$

where x is the molar modification ratio and R is an alkyl chain. The following designation has been adopted: 3C18 is a sample containing 3 mol% of octadecyl chains. Both precursor (PA) and HMPA were used in their sodium salt form.

Stock solutions of polymer were prepared under magnetic stirring at least 18 h before use and pH was adjusted to 9 by addition of a proper amount of a HCl or NaOH solution.

Mixtures containing a given polymer concentration, C_p, and protein concentrations ranging from 0.01 to 5% (by weight) were prepared from the stock solutions of polymer and protein. After vigorous shaking, samples were allowed to stand at room temperature 2 h at least before study.

Viscometric measurements were performed at 25 °C with a Contraves Low-Shear 30 viscometer. Steady-state fluorescence spectra were recorded on an Aminco 500 SPF spectrometer thermostated at 25 °C. The excitation wavelength was set at 334 nm and band passes were set at 5 and 0.5 nm for excitation and emission respectively. Pyrene (6×10^{-7} M in the samples) was used as a probe and the ratio I_1/I_3 of the intensities of the first to the third peak of the emission spectrum was used to monitor the hydrophobicity of its microenvironment.

Results and discussion

Lysozyme/HMPA

Figure 2 displays the variation of viscosity as a function of lysozyme concentration for aqueous solutions of PA, 1C18 and 3C18. The precursor exhibits constant and rather low viscosities. For a lysozyme concentration higher than a critical value referred to as critical precipitation concentration (cpc), phase separation occurs. This precipitation is due to the binding of the cationic protein to the oppositely charged polyelectrolyte [20, 22]. Similar phenomena have been observed in mixtures of anionic and cationic polyelectrolytes [23] and in mixtures of anionic polyelectrolytes and cationic surfactants [17, 24, 25].

A very different behavior is seen when 3C18 is used. A pronounced maximum in the viscosity as a function

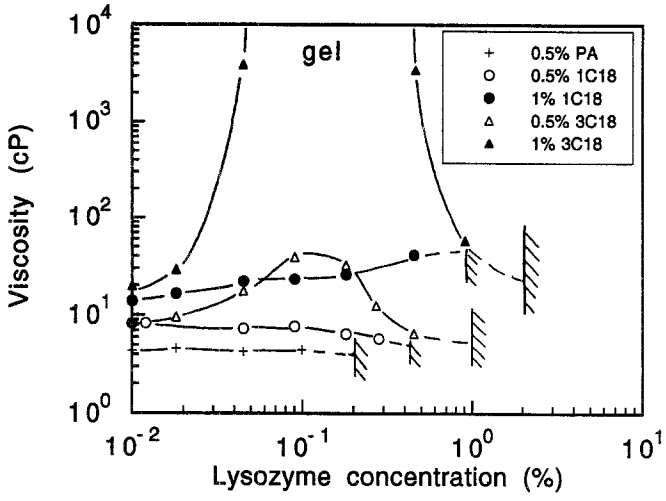


Fig. 2 Variation of the viscosity as a function of lysozyme concentration for several polymers at various concentrations (shear rate = 0.06 s^{-1}). The hatch marks represent the limit of precipitation

of lysozyme concentration is found (with 1% 3C18 gelation is observed) and is followed by precipitation. The lysozyme/1C18(1%) system exhibits only a slight increase of viscosity. One can notice that the higher the modification ratio, x, or the higher the polymer concentration, C_p, the more pronounced the maximum. Another important feature is also that the critical precipitation concentration is much higher with HMPA than with PA and increases with C_p and x.

All these results are very similar to those observed when cationic surfactants, for instance DTAB (dodecyltrimethylammonium bromide), are added to HMPA solutions [17, 26]. They can easily be explained assuming that a crosslinking mechanism similar to the one depicted in Fig. 1 is involved. Upon addition of lysozyme, the alkyl grafts begin to bind the proteins. Each protein molecule is thus associated through several alkyl moieties to one or more macromolecular chains. The number of such mixed clusters is however small and the connectivity of the system is low. When increasing the lysozyme concentration the number of mixed clusters belonging to two or more macromolecular chains raises. These mixed aggregates play the role of crosslinks and a strong increase in viscosity or even a gelation may occur. However, a large addition of lysozyme decreases the mean number of alkyl units bound per protein. Finally, the probability to find alkyl grafts belonging to two different HMPA chains involved in the same cluster becomes low and therefore the viscosity of the system decreases. At a final step precipitation occurs, due to the electrostatically driven binding of the cationic protein onto the anionic polyelectrolyte as in the case of the unmodified poly(sodium acrylate).

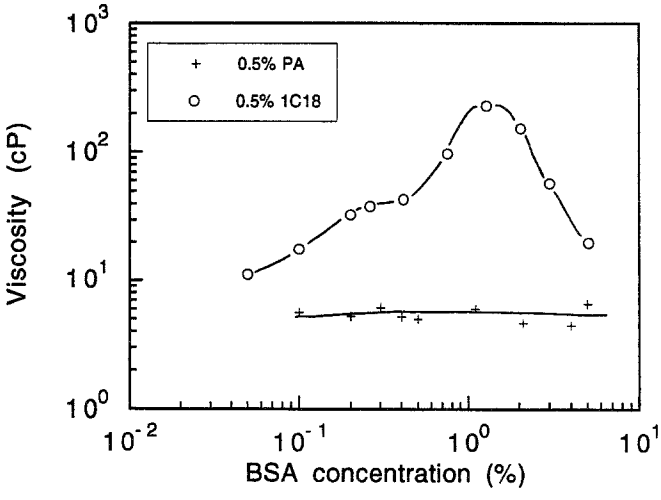
At this stage, many similarities between the DTAB/HMPA and the lysozyme/HMPA systems have been emphasized. Moreover, molecular data also appear to be in reasonable agreement. For the DTAB/3C18(1%) system, the maximum of viscosity occurs for a surfactant concentration of approximately 5×10^{-3} mol/l. Assuming that the hydrophobic microdomains involved in the macromolecular crosslinks (Fig. 1) are similar to the DTAB micelles which contain approximately 50 surfactant molecules [27], the maximum of viscosity corresponds to 10^{-4} mol/l of hydrophobic microdomains. With lysozyme/3C18(1%) the maximum of viscosity is found for a lysozyme concentration of 7×10^{-5} mol/l. Besides, at the cpc, we have respectively 7×10^{-3} mol/l of mixed micelles and 1.4×10^{-3} mol/l (2%) of lysozyme. However the above comparison emphasizing the similarities between the lysozyme/HMPA system and the DTAB/HMPA system should not be regarded as conclusive about the details of the association mechanism but rather as a qualitative guide for interpretation.

BSA/HMPA

Figure 3 shows the variation of viscosity as a function of BSA concentration for aqueous solutions of 0.5% PA or 1C18. Mixtures of BSA and 0.5% or 1% 3C18 as well as mixtures of BSA and 1% 1C18 lead to very viscous solutions and gels that could not be studied with the Low-Shear 30 rheometer.

No precipitation was observed over the range of BSA concentrations investigated, neither with PA nor with HMPA. Once again, with HMPA, a viscosity enhancement was first obtained then a maximum and a decrease

Fig. 3 Variation of the viscosity as a function of BSA concentration for 0.5% PA and 0.5% 1C18 solutions (shear rate = 0.06 s^{-1})



occurred whereas no significant variation of viscosity was found with PA. Similar results are observed when mixing an anionic surfactant, sodium dodecyl sulfate (SDS) and HMPA [16].

However, the viscosity evolution as a function of BSA concentration is not as steady as observed for lysozyme. It displays a maximum at 1.2% (i.e., 1.7×10^{-4} mol/l) BSA preceded by a kind of secondary maximum at about 0.3% (i.e., 4×10^{-5} mol/l) BSA. This secondary maximum can be related to the behavior of sodium dodecylsulfate (SDS)/1C18(1%) mixtures [16]. This later system presents a viscosity maximum at 3×10^{-3} mol/l SDS corresponding to a mixed aggregate concentration of about 4×10^{-5} mol/l (assuming approximately 80 SDS molecules per aggregate [28]). However, even if similarities with the case of the anionic surfactant micelles are obvious the behavior of the BSA/HMPA appears to be more complex as evidenced by the existence of the two maxima in the viscosity curve. Moreover, another significant difference with the case of surfactants can be underlined. The viscosity enhancement is more important with cationic than with anionic surfactants as for the former the electrostatic attractions favor the interactions of the surfactant with the polyelectrolyte, whereas in the later case the electrostatic repulsions counteract the interaction of the micelles with the polymer. Here, we observed viscosities that are much higher with BSA (anionic) than with lysozyme (cationic). Several explanations might be suggested:

- Proteins do not have an even surface charge repartition. Although the global charge is negative, the protein bears patches of the opposite charge [29] so that anionic surfactants may be locally strongly attracted.
- Furthermore, BSA has specific adsorption sites for amphiphiles and may thus interact with the alkyl groups of the polymer more strongly than lysozyme does. Note that in vivo BSA acts as a carrier of fatty acids and related amphiphiles in the plasma.

Figure 4 displays the variation of the pyrene fluorescence intensity ratio, I_1/I_3, as a function of BSA concentration for 0.5% solutions of PA and 1C18 and for a blank without polymer. The I_1/I_3 values are comparable to those reported with surfactants under similar conditions [30]. BSA would thus constitute for pyrene a hydrophobic microenvironment as micelles do. A plateau is observed for BSA concentrations ranging between 0.04 and 0.2%. The values are roughly the same for both polymers ($I_1/I_3 = 1.25$–1.30), but they are much higher for the protein in the absence of polymer ($I_1/I_3 = 1.45$–1.50). However, upon addition of sodium chloride the plateau for the BSA solutions is shifted down to the level observed with the polymers. The difference between experimental

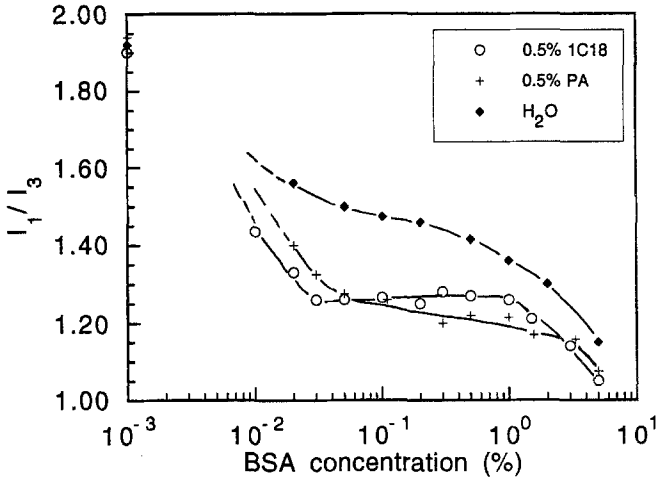


Fig. 4 Variation of the ratio I_1/I_3 as a function of BSA concentration in the absence of polymer and in the presence of 0.5% PA or 0.5% 1C18

data in the presence and in the absence of polymer thus appears to be solely due to the intrinsic ionic strength of the polyelectrolytes.

For BSA concentrations in the range 0.2 to 2% (where the strong rheological effect is observed), the values of the ratio I_1/I_3 for the BSA/1C18 system are slightly, but significantly above the values observed for the BSA/PA system. This difference could be explained by a competition between the polymer alkyl chains and pyrene for the access to the hydrophobic sites of BSA. Another hypothesis might also be that the formation of protein/polyelectrolyte mixed aggregates would make up a more hydrophilic environment than BSA itself because of the presence in the aggregates of carboxylate groups from the polymer backbone.

The three systems (even the blank) display a final decrease of the ratio I_1/I_3. One can think that at pH = 9 and at high BSA concentrations an unfolding of the protein might occur inducing the exposure of SH groups and then the formation of BSA aggregates because of an SH-SS intermolecular exchange reaction. Such denaturation phenomena of BSA have already been described with cationic detergents at pH = 9 by Aoki et al. [31, 32].

On the basis of a BSA auto-aggregation, the rheological behavior of the BSA/1C18 system (with the secondary and the primary maxima) would then be very similar to the behavior of the pentaethylene glycol dodecyl ether (C_{12} E_5)/HMPA systems [18]. $C_{12}E_5$ is a nonionic surfactant that forms micelles which grow with increasing surfactant concentrations. $C_{12}E_5$/HMPA systems exhibit viscosity curves with two maxima instead of the single maximum observed when spherical micelles of constant size are involved. However, further experiments are needed

in order to elucidate the more complex behavior of BSA/HMPA systems and to check the hypothesis of a BSA auto-aggregation.

Concluding remarks

Hydrophobically modified derivatives of poly(sodium acrylate) interact strongly with globular proteins as evidenced by rheological studies. It is likely that the hydrophobic parts of the polymer bind onto the hydrophobic sites of the protein contributing to the stabilization of the polymer/protein aggregate. Under suitable concentration conditions the protein molecules act as crosslinkers between the polymer chains and highly viscous solutions or gels are formed. Under the same conditions the unmodified parent polymer does not induce any viscosity enhancement. This rheological behavior is similar to the one observed with surfactant/hydrophobically modified polymer systems. This similarity is presumably due to the fact that in both systems hydrophobic interactions play an important role and to the qualitative similarities between micelles and globular proteins as both are amphiphilic objects of nanometric dimensions. It is however obvious that the two systems also display important differences and further studies are needed in order to make conclusions on the molecular basis of the protein/HMPA association. Nevertheless, we believe that this rheological behavior is general and can be obtained with various polymer/protein systems provided that the protein displays a hydrophobic character and that the polymer has a small fraction of very hydrophobic groups. Studies dealing with the effect of the protein nature and the importance of the protein denaturation during its association with the hydrophobically modified polymer are now in progress in our group. Finally, it is noteworthy that Akiyoshi et al. have recently reported evidence of association between hydrophobically modified pullulan and various globular proteins [33]. However, their study was performed in very dilute solutions and they did not provide information on the rheological behavior of the systems studied.

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