Reversible Gelation in Hydrophobic Polyelectrolyte/Protein Mixtures: An Example of Cross-Links between Soft and Hard Colloids

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ABSTRACT: Measurements were conducted on the linear viscoelastic properties of hydrophobically modified poly(sodium acrylate) reversibly cross-linked with proteins to form gels. The native structure of proteins was not markedly affected upon association with the amphiphilic polyelectrolyte. Hydrophobic interaction was shown to overcome Coulombic effects on interpolymer complexation. Because of their relative hydrophobicity, neutral papain and positively charged lysozyme were found to be less efficient cross-linking agents than negatively charged bovine serum albumin. These reversible gels exhibited similarities with chemically cross-linked macromolecules such as a plateau modulus at high frequency and characteristic scaling at the transition point between sol and gel. The sol−gel transition diagrams were determined. A semiquantitative approach, based on scaling laws, was able to capture the essential rheological properties of these mixtures, namely sol−gel transition and variations of storage modulus.

Introduction

Macromolecules have found a large number of applications in solutions as thickening agents and rheological modifiers controlling the flow of soft materials. The formation of transient associations between macromolecules results in dramatic modifications of the mechanical properties such as thickening, shear-thinning, or gelation. In water-based formulas, hydrophobically modified polymers (HMP) have been recognized for their striking thickening efficiency and the formation of reversible gels due to their self-association.[1,2] Reversible gels have also been obtained by addition of molecules or particles that can reversibly bind the macromolecules, such as borate ions and guar[3] or poly(vinyl alcohol),[4] chromate ions and polyacrylamide,[5] and ionomers and multivalent counterions.[6] Similarly, a supplementation of the HMP solutions with additives or particles that strengthen the hydrophobic association between the polymers has been reported as a means to induce gelation at constant polymer concentration. For instance, strong gels have been obtained, below the self-association concentration of HMP, in the presence of a small amount of surfactants, micelles, vesicles or lipid lamellae,[7,8] or proteins.[9]

As compared to other systems, the protein/HMP was expected to reflect generic behaviors of mixtures containing rigid colloids (the proteins) and associative polymers. Globular proteins, playing the role of "connectors" for the HMPs, offered indeed interesting opportunities to work with a variety of monodisperse colloids, of various charge densities, and a high surface asymmetry (possibly depicted at angstrom resolution from crystallographic studies), without being markedly subject to large reorganizations. Some mixtures of a protein and a poly(acrylic acid) modified with C18 dangling groups, such as HMP, have already been studied using viscometry.[9] At pH 9 and low ionic strength, it has been shown that the supplementation of an HMP solution with either a positively charged (lysozyme), or a negatively charged (BSA) protein results in variations of viscosity, possibly over several decades. Typical bell-shaped variations of viscosity as a function of protein concentration, at constant polymer concentration, have been reported and compared to thickening effects in mixtures of similar HMP and surfactant.[7] On the basis of a qualitative description, widely recognized now in the case of micelle/HMP mixtures,[7] a scheme of gelation in protein/HMPA solutions has been suggested.[9] Namely, several hydrophobic dangling groups could be bound to each protein (or to an oligomer of proteins). At low concentration, proteins would be able to form clusters containing several HMP chains. Provided that at least two HMP chains can share a common protein, the cross-links would be formed. Increasing the concentration of protein would first increase the number and size of such clusters and consequently the viscosity. However, upon addition of large amount of protein, when all the chains of HMP would be saturated by an excess of protein, polymer chains would not share proteins anymore: the connectivity and the thickening effect should be completely lost. Before reaching the latter saturation condition, the viscosity should diminish upon addition of the protein.

From viscometry, however, the gelation of such mixtures cannot be studied unambiguously because of the sensitivity of this property to the shear rate and the possible flow of reversible gels at low shear rates. Aiming at a more accurate characterization of cross-links formation, the present work reports measurements of the frequency dependence of the elastic modulus G' and the loss modulus G'', in mixtures of proteins and hydrophobically modified poly(acrylic acids) (HMPAs) soluble in water in their sodium acrylate form. A set of synthetic HMPAs was studied, varying their hydrophobicity at fixed molecular weight. The rheological properties of mixtures containing polyanionic HMPA and either positively charged, negatively charged, or neutral

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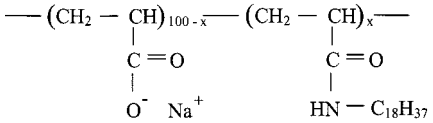
proteins were compared. Special attention was brought to determine accurately the sol−gel transition points, using a scaling criterion of the elastic and loss moduli as evidenced by H. Winter and colleagues on chemical gels. Quantitative interpretation of the results was derived from a model based on the scaling behavior of macromolecules and on the assumption of site-association of proteins along the backbones.

Material and Methods.

Sample Preparation. Bovine serum albumin (BSA), papain, and lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO) as 95−98% pure crystallized and freeze-dried protein. So-called poly(acrylic acid) of average molecular weight 150 000 was supplied by Polysciences Inc. (Warrington, PA). Gel permeation chromatography measurements, performed in $NaNO_3$ solution, gave actually a number-average M_w of 130 000 and a polydispersity index of 4 for this sodium polyacrylate precursor. The hydrophobically modified poly(acrylic acid)s (HMPA) were synthesized according to a reaction described previously[10] by grafting octadecylamine at random along the backbone of the precursor. The HMPAs have the same polymerization degree as their precursor and they were obtained in the sodium salt form with the following structure:



Here, x is the molar modification ratio and $C_{18}H_{37}$ is a linear alkyl chain. Dispersity in composition was low, and x was defined within 1%, as revealed by a separation and analysis of HMPA mixtures.[11] A polymer sample containing x mol % of alkyl dangling groups was denoted 150-xC18. In the present work, the polymer modification ratio ranged from 3 to 7 mol %.

High-purity water obtained from a Millipore milli-Q water system was used to make all the solutions. A stock solution of sodium dihydrogenophosphate−NH_3 buffer, at a final phosphorus concentration of 100 mM, was prepared with analytical reagent-grade sodium dihydrogenophosphate (Prolabo, Paris, France) adjusting the pH to 9.0 by the addition of ammonia. In the final samples, diluted to 20 mM in the presence of protein and polymer, the pH decreased to 8.7. Polymer stock solutions (2 wt % in water) were prepared under magnetic stirring at least 24 h before use. The protein was dissolved in water to minimize the formation of aggregates. The daily preparation of protein stock solutions, and supplementation with NaN_3 (400 ppm) prevented bacterial growth.

Mixtures containing protein and polymer at different concentrations (0.02−5.00% and 0.05−1.3%, respectively) were made from the stock solutions at a final buffer concentration of 20 mM. After vigorous shaking, samples were incubated at room-temperature overnight before being centrifuged for 10−30 min at 2000g to get rid of bubbles. Incubation for only 2 h was found to result in the same rheological behavior as 1-day old samples. Both loss and storage moduli were also kept unchanged during several days upon storage at 4 °C.

Rheology. Rheological measurements were performed on a Rheometrics Fluids spectrometer II (Rheometrics, Inc., Piscataway, NJ) using cone-plate geometries (25 mm or 50 mm in diameter). Samples were slowly loaded onto the plate and 10−15 min were allowed for the stresses to relax and for thermal equilibration (25 °C). The elastic modulus G' and the loss modulus G'' were obtained by subjecting samples to dynamic oscillatory tests during which a sinusoidal strain was applied and the resulting stress was recorded. From strain sweep tests, the linear viscoelastic region was determined to extent up to a strain of 100%. Frequency sweep experiments were performed at low amplitude of strain (<10%), covering the range 0.1−100 rd/s. Regularly, two successive measurements were performed on the same sample resulting in two identical spectra. However, at lower frequencies, the time required for measurements was long and difficulties arose due to evaporation.

Polarimetry. Measurements of optical rotation, ϑ, were conducted at 25 °C in a Perkin-Elmer model 241MC polarimeter, using a cell of 10 cm path length. The mean residual rotation $[m]_\lambda$, at a wavelength λ, was calculated by

$$[m] = \vartheta M_0/100lc$$

where M_0 is the mean residual molecular weight, l the path length in dm, and c the protein concentration in g/100 mL. M_0 is equal to 111 g/mol for lysozyme and 114 g/mol for BSA. The change of optical rotation with wavelength was analyzed in terms of the Moffit−Yang equation:[12]

$$[m]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

Here a_0, b_0, and λ_0 are constants. In the case of proteins, the value of λ_0 is assumed to be 212 nm when λ is above 350 nm.[12] The Moffit−Yang equation has been used extensively for the estimation of the α-helix content of polypeptides and proteins. On a semiempirical basis, it was shown that $b_0/630$ gives an excellent estimate of the average fraction of residues involved in α-helices. The value of a_0 reflects more complex effects of asymmetric environments.

Results and Discussion

Thickening Effect. Measurements of G' and G'' as a function of the frequency were performed at low amplitude of strain (<10%), being thus virtually independent of the strain. Different samples were initially compared at a constant shear frequency of 10 rd/s (arbitrarily chosen although high enough to stand on the plateau of G' when gels were obtained). The elastic modulus at 10 rd/s, denoted G'_{10}, was measured at constant polymer concentration as a function of protein concentration (Figure 1). The modulus G'_{10} exhibited a bell-shaped variation similar to the variations of the viscosity reported by Petit and colleagues.[9] At high protein concentrations, the elastic character of the medium returned to a value close to its initial value measured without protein.

The thickening effect, probably due to association between HMPA and water-soluble proteins, was found to be mainly of hydrophobic origin. The amplitude of the effect, as estimated by G'_{10}, correlated with the hydrophobicities of the partners rather than with their charges. The higher the modification rate of the HMPA, the higher were the values of G'_{10} at given polymer and protein concentrations (Figure 1a). As reported in surfactant/HMPA systems[1], the thickening power of polymers ranked as 150-3C18 < 150-5C18 < 150-7C18. The different systems containing positively charged lysozyme, or neutral papain, or negatively charged BSA were all found to exhibit qualitatively similar curves. However, the mixtures containing the more hydrophobic protein (i.e., the BSA) were found to reach higher values of G'_{10} up to 1 decade above those with lysozyme or papain (Figure 1b) at the same polymer concentration, at pH 8.7, and at the same ionic strength (NaH_2PO_4−ammonia buffer 20 mM). Despite the presence of Coulombic repulsion between the two partners, negatively charged at that pH, the BSA/HMPA mixtures gave stronger gels than mixtures with positive or neutral proteins. A grafting density along the HMPA as low as 3 mol % with C18 groups was enough to overcome the

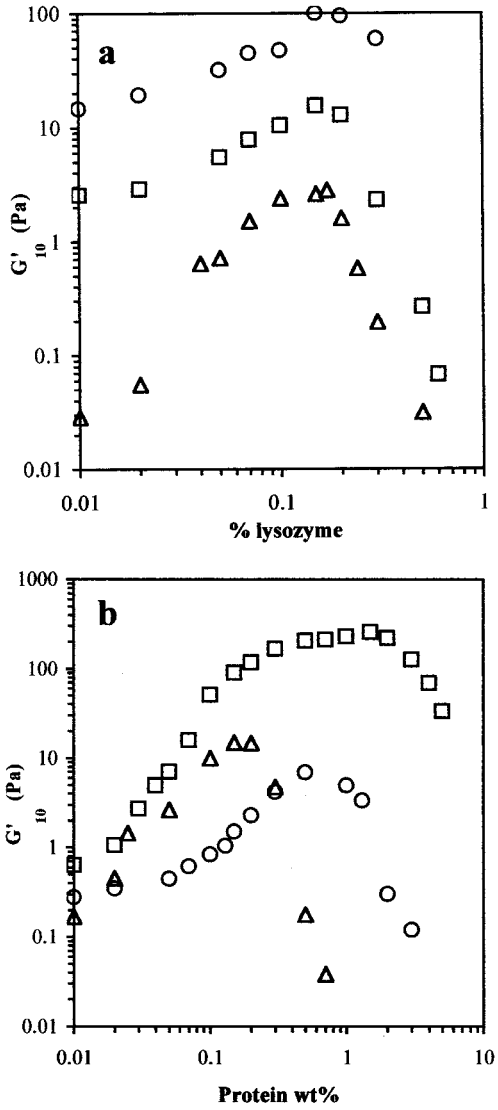


Figure 1. Storage modulus G'_{10}, measured at 10 rd/s, as a function of protein concentration, in various protein/HMPA samples. $NaH_2PO_4-NH_3$ buffer 20 mM pH 8.7, except for HMPA 150-7C18 (because this polymer was insoluble in the presence of the buffer, pH was adjusted at 9 using NaOH only). (a) HMPA/lysozyme samples, at constant polymer concentration. Key: (△) 150-3C18 0.58 wt %; (□) 150-5C18 0.53 wt % (○) 150-7C18 0.59 wt %. (b) 150-3C18/protein samples at constant polymer concentration. Key: (△) polymer 0.86 wt % + lysozyme. (○) polymer 0.94 wt % + papain. (□) polymer 0.94 wt % + BSA.

repulsion between the charge carried by the BSA (about −20 at pH 9) and the 97 mol % of sodium acrylate units remaining along the backbone of the HMPA.

Dynamic Behavior. Liquidlike and gellike behaviors were more accurately distinguished from the frequency dependence of G' and G''. In the absence of proteins, a pure HMPA solution can exhibit viscoelastic properties of a gel or of a liquid.[10] The mixtures of protein and polymer were first characterized at polymer concentrations below the self-association threshold of the HMPA (i.e. $G' \sim \omega^2$ and $G'' \sim \omega$, Figure 2 , case "no protein"). The behavior of mixtures in the presence of a low protein concentration was also found to be Maxwellian. Increasing the protein content, at constant polymer concentration, changed the dynamics drastically. In a wide range of protein concentration, the dependence of moduli G' and G'' became typical of a gel: beyond some threshold frequency, G' was found to be higher than G''

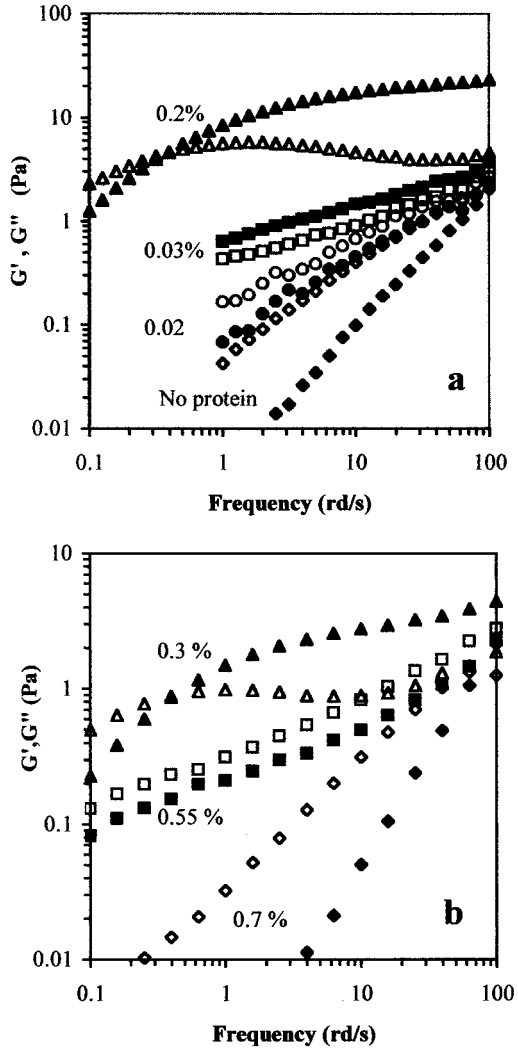


Figure 2. A log−log plot of dynamic moduli vs frequency of HMPA 150-3C18/lysozyme solutions. Open symbols G''; Filled symbols G'. The gel points, corresponding to parallel variations of G' and G'', are close to [lysozyme] = 0.025% or 0.55% on this plots. (a) Polymer 0.9 wt %, $NaH_2PO_4-NH_3$ buffer 20 mM pH 8.7. Key: (▲, △) lysozyme 0.2 wt %; (■, □) lysozyme 0.03 wt %; (●, ○) lysozyme 0.02 wt %; (◆, ◇) no lysozyme. (b) Polymer 0.9 wt %, boric acid-NaOH buffer 20 mM pH 8.7. Key: (▲, △) lysozyme 0.3 wt %; (◆, ◇) lysozyme 0.55 wt %; (◆, ◇) lysozyme 0.7 wt %.

and almost independent of the frequency (Figures 2 and 3). Although the threshold frequency varied with the composition of the mixtures, it was low enough to enable the observation of a plateau value of G' over 2 decades (more than 3 decades in the case of BSA, Figure 3). At still higher protein concentrations, mixtures recovered the viscoelastic characters of a liquid when the HMPA chains were disconnected again. Therefore, at each fixed low polymer concentration, we expect to find two critical protein concentrations corresponding to two boundaries between liquid and gelified samples.

At HMPA concentrations higher than the self-association threshold, the dynamic behavior corresponding to a gel was found from zero protein up to a maximum concentration above which liquidlike flow was observed. High protein concentrations were thus able to disconnect direct polymer−polymer clusters. In addition, all these samples having a high concentration of proteins exhibited a drop of G' at low frequencies, indicating that the associations between macromolecules were always reversible.

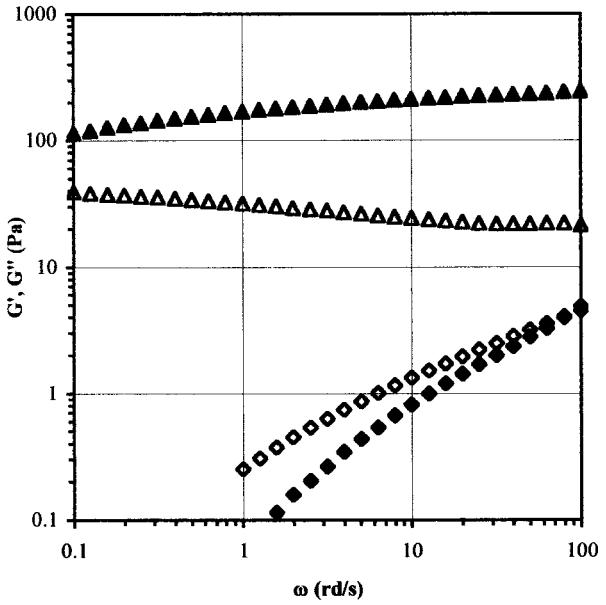


Figure 3. A log−log plot of dynamic moduli vs frequency of HMPA 150-3C18/BSA mixtures. Polymer 1.1 wt %, NaH_2PO_4−NH_3 buffer 20 mM pH 8.7. Key: (◆,◇) reference curve without protein; (▲,△) curve in the presence of BSA 0.7 wt %. Open symbols = G''; filled symbols = G'.

Sol−Gel Transitions. The boundaries between sol state and gel state were determined more accurately, using the criterion provided by H. Winter and colleagues in the case of chemical gels.[14] Namely, at the so-called "gel point", rheograms are expected to exhibit a typical scaling behavior as a function of the frequency ω:

$$G' \sim G'' \sim \omega^n \quad (0 < n < 1)$$
$$\tan \delta = G''/G' = \tan(2\pi/n) \qquad (1)$$

In the case of chemical gels, theory and simulations predict values of n equal to $^2/_3$ or 0.72 ± 0.02, in excellent agreement with experiments.[14] Experiments performed on physical gels have however given values of n in the range 0.5−0.7.[15] This particular scaling (eq 1) was observed in our mixtures. Close to some critical compositions, a log−log plot of the moduli as a function of frequency gave two parallel lines (e.g., around lysozyme 0.025 wt % on Figure 2a and 0.55 wt % on Figure 2b). The common slopes, n, of these two lines varied in the range 0.4−0.8. This value of the scaling exponent was found to be markedly sensitive not only on the type of protein and HMPA but also on the concentration of samples.

As expected two such critical protein concentrations were found in mixtures containing a fixed HMPA concentration below the polymer self-association threshold (Figure 2a,b). They were called first gel point (lowest protein content) and second gel point. Only one critical protein concentration was obtained for samples above the HMPA self-association concentration. As it corresponded to the limit between a gel domain and mixtures containing protein-saturated HMPA chains, it was considered to be similar to the latter second gel point. At protein concentrations much lower or higher than a critical gel-point value, the crossover between the curves of G' and G'' in a very limited range of frequency (Figure 2 with lysozyme 0.2%, 0.3%, or 0.7%) showed that the parallel variation, as observed at the gel points, completely vanished far from the sol−gel transition. The variations of G'_{10} were found to be significantly higher around the gel points than in the sol or in the gel domains. The first gel point was located in the region of rapid increase of G'_{10} as a function of protein concentration (Figure 1). The vicinity of the second gel point corresponded to the strong decrease of G'_{10} with protein.

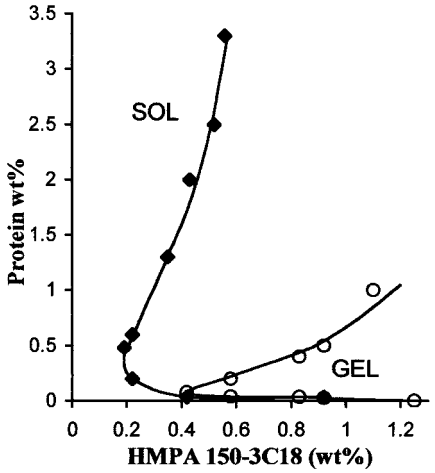


Figure 4. Sol−gel transition diagrams of HMPA 150-3C18/protein in NaH_2PO_4−NH_3 buffer, 20 mM pH 8.7. Gelation thresholds were determined as the compositions exhibiting the scaling of the dynamic moduli given by eq 1. Key: (◆) HMPA/BSA; (○) HMPA/lysozyme. Lines are just a guide for the eye.

In the gel domain, G' does not vary markedly with the frequency beyond typically 1 rd/s. Between the two gel points, G'_{10} was therefore a good estimation of the plateau modulus of the gel.

Variations of Characteristic Points. For each fixed polymer concentration, the description above enabled us to extract three characteristic values of the protein concentration, namely those at the first and second gel points and that corresponding to the maximum of gel strength (i.e. maximum of G'_{10}). Sol−gel transition diagrams were based on the variation of these two gel points with polymer concentration (Figure 4). They exhibited a complex nose-shaped variation. As a rule, the protein concentration at the first gel point (lower boundary) appeared to decrease slowly with polymer concentration. On the contrary, the protein concentration at the second gel point (upper boundary) was found to increase rapidly with the polymer content. An extrapolation of both the upper and lower boundaries toward low polymer concentrations defined a crossover polymer concentration that should be the minimum value required to form a gel. This threshold depended on the hydrophobicity of the partners. The lower value extrapolated for the BSA/150-7C18 system was about 0.1 wt % (not shown) while it was about 0.2 wt % for BSA/150-3C18 system and 0.4 wt % for lysozyme/150-3C18 (Figure 4). Below this threshold the scaling behavior (eq 1) was never reached, although a maximum in both G'_{10} and low-shear viscosity was still found at an optimal protein concentration. In Figure 4, the gel domain appeared to be more extended in the presence of BSA than with lysozyme. However, owing to the large difference in molecular weight between the two proteins (66 500 and 14 500 g/mol, respectively), the diagrams were also compared using the molar concentration of the partners (Figure 5). In both cases, molar or weight concentrations, the gel domain was found significantly broader in BSA-containing gels. A higher hydrophobicity

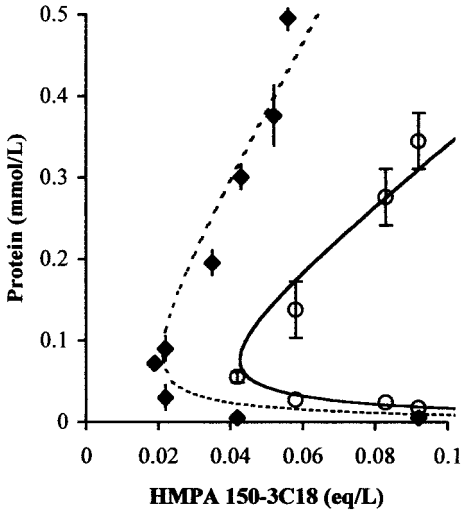


Figure 5. Fit of sol−gel transition diagrams of HMPA 150-3C18/protein using eq 3 and neglecting the concentration of free protein. Full line: $A = 280$; $KM_n = 2200$; $m = 3$. Dotted line: $A = 125$; $KM_n = 9000$ and $m = 3$. Key: (◆, ○) experimental data obtained below the polymer self-association concentration, and redrawn from Figure 4 using the same symbols. "Error" bars correspond to the minimum concentration difference between two successive samples whose composition was in the vicinity of the gel point.

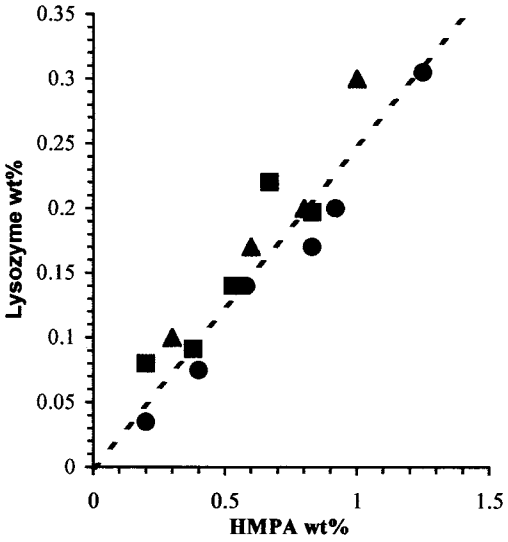


Figure 6. Lysozyme concentration giving the maximum of the storage modulus G'_{10} as a function of polymer concentration (i.e. maxima of bell-shaped curves such as in Figure 1). Key: (●) HMPA 150-3C18; (■) 150-5C18; (▲) 150-7C18. Dotted line: Variation calculated using $\alpha = 0.5$ and $A = 290$ in eq 4.

of either the protein or HMPA made both the gel stronger (higher values of G' and G'') and the gel region more extended on the diagram of sol−gel transition.

In the gel domain, the maximum of G'_{10} at constant polymer concentration showed the conditions of highest connectivity in the sample. Determinations of this optimal composition were carried out over a range of polymer concentrations. Summarized on a diagram, the locus of the corresponding ([HMPA], [lysozyme]) couples of concentrations matched with a straight line passing through the origin (Figure 6). The highest density of cross-links along the chains was reached at a constant ratio between lysozyme and polymer. Various HMPAs gave the same rule: thus the optimal lysozyme/polymer ratio was not markedly dependent on the hydrophobicity of HMPA. In terms of number-average composition, it

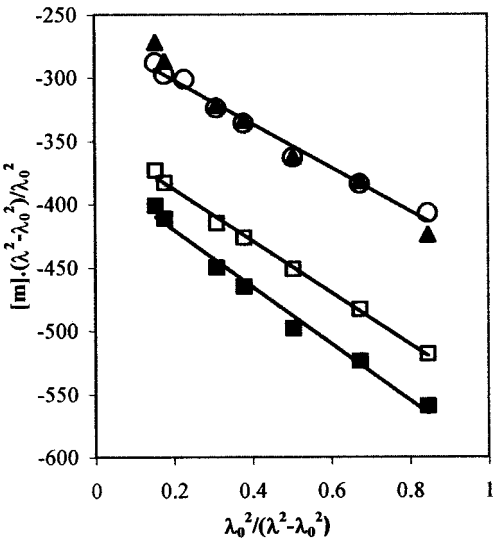


Figure 7. Moffit−Yang plot of variations of the mean residue rotation [m'] with wavelength λ in the case of lysozyme, with the same buffer as in Figure 2. Key: (○) native lysozyme in buffer; (▲) lysozyme 0.5 wt % + 150-3C18 0.9 wt %; (□) denatured lysozyme 0.5 wt % in sodium dodecyl sulfate 0.28 wt %; (■) denatured lysozyme in sodium dodecyl sulfate 0.47 wt %. Lines: linear regressions corresponding to α-helix fractions of 27.6% (native), 33%, and 35.7%, respectively.

corresponded to a mean value of 630 acrylic units per protein (average over the three HMPAs tested) and from 19 to 45 C18 groups per lysozyme molecule depending on the HMPA grafting rate. Although the region of maximum of G'_{10} was broader, a similar behavior with BSA/HMPA 150-3C18 mixtures led to an estimate of the optimal protein-to-polymer ratio of 0.75−1 g/g (890−670 acrylates per protein). In both cases, lysozyme and BSA, the charge of the polymer greatly exceeded that of the proteins. For instance, at pH 8.7, the global charge of lysozyme is +6, one protein carrying 19 weak basic groups and 10 weak acid groups.[16] At the maximum of G'_{10}, the charge of the protein was therefore likely to be completely shielded by the polymer backbone. A ratio of more than 20 dangling C18 groups per protein was also much higher than the number of putative hydrophobic patches per protein (1 or 2 per lysozyme, 3−6 per BSA). In the conditions of gelation it appeared therefore impossible that all the acrylic units and the C18 groups of HMPA are interacting with specific sites of the native proteins.

Preservation of the Globular Structure of Proteins. Among various origins of the differences between BSA and lysozyme, a possible sensitivity of the initial structure of the protein was investigated. The native structures of these two proteins correspond to a folding of one single polypeptide chain in a compact and rigid globule containing substructures such as α-helices. Accurate details on the relative positions of atoms in these structures have been reported from X-ray studies of protein crystals.[16,17] Denaturation of the proteins, for instance in the presence of surfactants, usually results in a modification of the fraction of α-helices or an aggregation between the globules.[18] The fraction of α-helices was measured by polarimetry, using the Moffit−Yang plot.[12] The curve was found to be essentially unchanged upon addition of HMPA 150-3C18 in either native lysozyme (Figure 7) or native BSA solutions (all the measurements were carried out either close to a sol−gel transition or in the sol domain because

of difficulties arising from the loading of a gel in the cells). In the same conditions, the presence of sodium dodecyl sulfate (SDS) at a similar concentration completely denatures the proteins.[18] As a result, the fraction of α-helices increased from 28% to 36% upon addition of SDS and the Moffit−Yang plots were significantly shifted as compared to the native protein (Figure 7). On the other hand, mixtures of BSA and HMPA were transparent at any composition. Quasi-elastic light scattering studies of BSA/HMPA dilute solutions have shown the absence of large aggregates.[19] Moreover, SDS−PAGE electrophoresis analyses of BSA, incubated in various HMPA, have shown the absence of irreversible oligomers.[20] Denaturation of BSA can indeed be irreversible due to the formation of disulfide bridges between aggregated proteins. Such aggregation was not observed in our systems.

Altogether, our measurements showed that neither the secondary structure nor the state of aggregation of the proteins were markedly affected by the presence of HMPA. In addition, the strong association between protein and HMPA resulted in an almost complete binding of proteins in the range of concentration studied in this work. In the case of BSA, binding isotherms of the protein onto various HMPA were obtained by capillary electrophoresis.[20] The gel domain and the composition of mixtures studied by polarimetry were found to correspond to a concentration of free BSA lower than 0.05 wt %. In the case of lysozyme, the fraction of free protein was estimated from the demixing properties of polyacrylate/lysozyme mixtures. In the presence of a large excess of lysozyme, well beyond the gel domain, precipitation of protein/polymer complexes occurred. This phenomenon was certainly related to a demixing process of polyanion/polycation mixtures rather than protein/protein aggregation. It has also been observed in the presence of unmodified poly(sodium acrylate).[21,22] Such precipitation of protein/polyelectrolyte complexes has been attributed to Coulombic effects and charge cancellation.[23] In our systems, titrations of the supernatants showed that more than 95% of the lysozyme coprecipitated with the polymer (not shown). In the gels, i.e., at a lower amount of protein, it was therefore expected that lysozyme would be totally bound to HMPA. It was thus ascertained that most of the proteins were bound to HMPA both in the conditions of gelation and in the samples used for polarimetry measurements.

Model of Gelation. Similarities between protein/HMPA systems and chemical gels were expected as a generic feature of particle/HMPA networks, provided that both several association sites per particles and a minimum value of the cross-link lifetime(s) exist. At frequencies higher than the inverse of this minimum lifetime, the protein/HMPA network behaves like a permanent structure with a defined mean functionality of the cross-links. Recent models of transient networks of associating polymers have now established, on a quantitative basis, relationships between the properties of reversible cross-links and values of storage modulus.[24,25] For instance, Pezron and colleagues have successfully fitted phase diagrams and G' in the case of guar/Borax mixtures.[26] Provided that the separation between cross-links is small as compared to chain length, it was likely that these theoretical approaches should hold for our systems. Additional effects should however be suspected, in the case of associative polymers able to form so-called "superchains"[27] or when the functionality of the cross-links can vary with dilution.[28] The approach proposed here involves a slight modification of the usual model, as used by Pezron, neglecting the possible additional complexity of associative polymer. According to Pezron, the number of cross-links in a semidilute solution of uncharged polymer is essentially proportional to the number of monomer−monomer contacts in the absence of cross-linker. The concentration of contacts C scales as [polymer]^m (m being a constant expressed as a function of the swelling exponent ν as $m = 3\nu/(3\nu - 1)$). Recent theoretical and experimental studies have been devoted to scaling theory and predictions of storage modulus in the special case of polyelectrolyte chemical gels.[29,30] Similar scaling of C is expected in polyelectrolyte, being modulated however by the charge density of the chain and the salt concentration.[31] At low salt, the scaling should correspond to the behavior of rigid rods and $m = {}^3/_2$. In the high salt limit, the behavior of uncharged polymer in good solvent is recovered[31] and $m = {}^9/_4$. The case of Gaussian chain, giving $m = 3$, has also been described for polyelectrolyte gels.[29]

Among the polymer/polymer contacts, only those involving also proteins must be counted as cross-links. For simplicity, one HMPA chain was compared to a string of successive binding sites for proteins. This assumption was consistent with measurements of binding isotherms showing that the formation of BSA/HMPA complexes is similar to an adsorption of protein onto a surface.[20] Two states of the site—namely free or bound—were considered, assuming that a bound site contains only one protein. As a result, the concentration of cross-links was calculated as the concentration of interpolymer contacts C weighted by the probability of choice, at random, of one free site and one bound site (it was assumed for simplicity that one cross-link contains only one protein globule and two HMPA sites, but the calculation can be easily generalized for higher functionalities). Alternatively, the other possibilities of contact between (i) two sites of chains already bound to two proteins or (ii) two free sites were considered as sterile. Note that the possible self-association of HMPA and the possible presence of protein oligomers limit the validity of this assumption. For instance, the model cannot be used without modifications beyond the critical polymer self-association concentration, i.e. about 1 wt % for the 150-3C18 in our conditions. To sum up, the concentration of cross-links X in the semidilute regime scaled as

$$X \sim K\alpha(1 - \alpha){C_p}^m \qquad (2)$$

denoting $(1 - \alpha)$ as the mean fraction of empty binding sites along the polymer, C_p the polymer concentration, and K an association constant related to the energy of cross-link formation. To reach the gelation threshold, about two intermolecular cross-links per HMPA chain were required. More precisely, the functionality of cross-links should influence the critical number of cross-links per chains that would remain however in the order of one. Consequently, a generic equation of the sol−gel boundary was written as

$$K\alpha(1 - \alpha) \sim {C_p}^{1-m}/M_n \qquad (3)$$

where M_n is the number-average molecular weight of HMPA.

In general, α should depend on the equilibrium between free sites along HMPA and free proteins. Owing to the strong association between the partners, the concentration of free proteins was neglected (see the previous chapter). α was thus almost proportional to the protein-to-polymer ratio. Denoting A as the mean number of acrylic monomer per site, it gave:

$$\alpha = A[\text{protein}]_{\text{total}}/C_{\text{p}} \quad (4)$$

Equations 3 and 4 were found to capture the essential features of sol−gel transitions, namely the minimum threshold polymer concentration, the slow variation of protein concentration at the first gel point, and the rapid increase of protein concentration at the second gel point. Provided that m is higher than 1, a minimum in the polymer concentration to obtain a gel struture of the mixture is indeed predicted from eq 3 when the term $K\alpha(1-\alpha)$ reaches its maximum. Recalling that α varies between 0 and 1, the term $\alpha(1-\alpha)$ varies in the range 0−0.25. To our knowledge, all the values of m proposed for flexible polyelectrolytes in the presence of salt were larger than 1.5 and lower than 3.

Above this concentration threshold, $\alpha.(1-\alpha)$ decreases rapidly with polymer concentration. At high enough polymer concentration, a value close to zero of this term gave two limiting cases, namely α about 1 and α close to 0, that were expected to correspond to the two gel points. At the so-called first gel point, when the concentration of protein is low as compared to the polymer ones, α should be small. Equations 3 and 4 led to

$$[\text{protein}]_{\text{total}} \sim C_{\text{p}}{}^{2-m}/KAM_{\text{n}} \quad (5)$$

The concentration of protein at the first gel point was thus expected to vary slowly with the polymer concentration. Measurements were consistent with $m = 3$ (Figure 5). The range of polymer concentration being however limited (due to the absence of gelation below 0.2 wt % and polymer self-association beyond 1 wt %), only a rough estimate of m was possible. In addition, the model neglected possible cross-links that would not involve protein. When self-association takes place—even to a small extent—the formation of a network is likely to occur at a lower protein concentration: the fits on Figure 5 presumably overestimated the importance of protein, and thus the value of m, especially at high polymer concentration.

The second solution of eq 3, corresponding to the second gel point, is reached when α is approaching 1. The total protein concentration should thus be proportional to the polymer ones. The linear variation of the upper sol−gel boundary was indeed consistent with the data at high enough polymer (Figure 5 and results on lysozyme/150-5C18 and lysozyme/150-7C18 systems not shown). The assumption of high polymer concentration being not completely valid here, deviations from proportionality were not surprising. Equation 3 was shown nevertheless to hold very well to the data points, even at low polymer concentrations, substituting the scaling symbol ∼ by an equality, and floating K and A to fit the complete curve (Figure 5). It was possible to fit both BSA/150-3C18 and lysozyme/150-3C18 diagrams. The values of A obtained from the fitting procedure were found to be similar to the inverse of the slope obtained assuming simply a linear variation of the second gel point. Fitted values of A (125 in the case of BSA and 280 with lysozyme) indicated that one to three hundred acrylic units were involved in one "site". In the present model, the formation of one cross-link corresponds to the association between one protein and two such sites. The model led therefore to the presence of a large number of both acrylic monomers (250/BSA and 560/lysozyme) and C18 groups per protein in a cross-link (18 octadecyl groups per lysozyme with the 150-3C18). The number of C18 groups per cross-link can be compared to the number of recognized hydrophobic pockets per BSA (three major pockets and up to six hydrophobic domains) but overcame markedly the one or two pockets per lysozyme. Similarly, the number of acrylic units per cross-link ($2A$) was found to be significantly higher than the total charge per protein (about +6 per lysozyme and −20 per BSA). Even the number of cationic residues per lysozyme, 19, was too small to balance the high negative charge of two sites. In this model, one site should therefore be extended over region markedly larger than the length of HMPA in close contact with the surface of the protein. A site might correspond to a domain in which the bound protein influences the conformation of HMPA chains. With respect to K values, it might be argued that BSA was found to associate more strongly HMPA ($KM_{\text{n}} = 9000$ mol^2 L^{-2}) as compared to lysozyme ($KM_{\text{n}} = 2200$ mol^2 L^{-2}). Owing to the small difference between the values, the possible influence of the protein charge and size on prefactors in the scaling laws should however limit the validity of such a conclusion.

The model of site-bound proteins was also applied to predict variations of G' in the gel domain. In the case of the present reversible gels, the plateau value of G', above 1 rd/s was expected to give information about the density of cross-links. When a gel of polyelectrolyte is kept in the conditions of its formation, it is now recognized that this plateau value is proportional to the mean density of cross-links along the connected macromolecules.[29] However, variation of salt concentration and swelling induce a more complex behavior possibly related to modification of a prefactor in the scaling law.[29] Assuming only that G'_{10} was an increasing function of the concentration of cross-links, the bell-shape variation of G'_{10} at constant polymer concentration (Figure 1) was in good qualitative agreement with variation of $X \sim \alpha(1-\alpha)$ (eq 2). Moreover, the maximum of these curves should correspond to a maximum in X. The eq 2 predicted $\alpha = 0.5$ at these maxima. Neglecting the fraction of free proteins, as discussed above, gave using eq 4: $[\text{protein}]_{\text{total}} = C_{\text{p}}/2A$, in excellent agreement with the proportionality observed on Figure 6. The slope of the line corresponding to the 150-3C18 on Figure 6 led to a mean value of A equal to 290 acrylate units/lysozyme (i.e., $2A = 580$ monomers per cross-links), close to the value of A deduced from the fit of the second gel point on Figure 5. Note that the difference between this value and the average value of the ratio (630 monomer/protein), given in the paragraph "variation of characteristic points", for the mean slope of the three curves on Figure 6 is only due to minor differences between 150-3C18 and the other two polymers. The model of site-bound proteins applied thus successfully both at the sol−gel threshold and in the gel domain, using the same value of A, the "length of a site". The mean number of monomers in one a site might however seem rather high as compared to both the charge and the number of hydrophobic patches on one protein. Both the am-

Table 1. Plateau Value, G_∞, of the Elastic Modulus and Its Ratio to NkT[a] Close to the Optimal Protein/HMPA Ratios in the Gel Domain

polymer	protein	polymer/protein, mol/mol	C_p,[b] wt %	G_∞, Pa	G_∞/NkT
150-3C18	lysozyme	2.2 ± 0.2	0.4	3	0.04
			0.56	5	0.05
			0.9	22	0.14
			1.3	100	0.43
			2.0	230	0.65
150-3C18	BSA	1.6	0.40	10	0.14
			0.60	49	0.46
			0.80	122	0.86
			1.0	200	1.1
			1.3	400	1.7
150-5C18	lysozyme	2.2	0.42	3.0	0.04
			0.59	16	0.16
			0.93	81	0.51
150-5C18	BSA	2.4	0.93	440	2.8
150-7C18	lysozyme	2.7	0.45	30	0.42
			0.55	43	0.48
			0.80	133	1.0

[a] k being the Boltzmann constant, T the temperature, and N the number-average concentration of HMPA chains determined using the value of 130 000 for the average molar mass of the precursor under its sodium form). [b] C_p: polymer concentration.

phiphilic and polyelectrolyte characters of the HMPAs help with the interpretation of such a large value. At low salt concentration, the repulsion between the charges along a polyelectrolyte chain (and also between two chains) makes it difficult for the monomers to come close to each other. At a length scale of the order of magnitude of the debye length (1 nm in the present systems) a polyelectrolyte should exhibit a rodlike behavior and strongly repel other chains. The value of A should therefore indicates the number of monomer inside such an electrostatic "forbidden" volume of the order of a few cubic nanometers. Within this framework, the absence of variation of A upon increase of the density of C18 grafts is not surprising. Alternatively, the HMPAs were shown to self-associate above a critical association concentration not far from the concentrations used in this study. The proteins might play the role of a nucleation point for the self-association of C18 groups, lowering the energy of micelle formation due to the adsorption of the monomers onto the protein surface. Similar phenomena, resulting in a decrease of the critical micellar concentration, have been reported in the case of association between polymer and surfactants. In addition, the association of up to 16 octadecyl groups per BSA has been measured by equilibrium dialysis in the case of short relatives of the present HMPAs[19] (same structure but a M_w of 5000 instead of 130 000). Thus, a value of 20−40 C18 groups per site might reflect the aggregation number of hydrophobic clusters that are formed at the contact of a protein.

For a more quantitative insight into the topology of the gels, the fraction of elastically active chains was estimated using the values of G_∞/NkT (where G_∞ is the plateau modulus of G', N the number of HMPA chains per unit volume, and kT the product of the Boltzmann constant and the temperature). In the gel domain and close to the sol−gel transition boundary, however, a slight drift of G' hampered the accurate determination of G_∞. For that reason, only the compositions surrounding the maxima of G'_{10}, as shown in Figure 1, were regarded as reliable. Different polymer/protein systems of varying the polymer concentrations were thus compared at a fixed polymer/protein ratio (Table 1). At a high enough polymer concentration, G_∞/NkT was found to be higher than 0.5 and possibly higher than 1. These high values were consistent with the expected topology of the gel assuming several cross-links per chain. In addition, this ratio always remained of the same order of magnitude as the number of bound proteins per chain (Table 1). Assuming a functionality of 4 and a value of α close to 0.5 (i.e., half of the proteins involved in a cross-link for the highest association constant K), it was indeed expected that the number of elastically active chains is at best equal to the total number of bound proteins.

At lower polymer concentrations, or with the partners of low hydrophobicity, G_∞/NkT appeared to be significantly lower than 1. It varied typically by a factor of 10 upon an increase of the polymer concentration by a factor of 2 or 3. Although the extrapolation of G' to high frequencies became less accurate close to the tip of the diagrams on Figure 4, such a high sensitivity to the concentration strongly suggests that the simple model proposed here does not match the structure of the gel in this region. Two possible origins for this discrepancy might explain that the number of active strands in the network are much less than the HMPA chains. (i) So-called superbridges, in the language of Annable,[27] could be formed involving several HMPA within a single elastically active strand. From the ratio G_∞/NkT, these superbridges should connect up to 10 HMPA molecules between two active cross-links. In this model, the concentration-dependent balance between intramolecular association (loops of an HMPA around a single protein) and intermolecular superbridges dominates the rheological properties. (ii) Alternatively, the importance of polydispersity might also be revealed at low polymer concentration. Owing to the high polydispersity of the precursor (polydispersity index of 4), it is likely that only the long HMPA chains remained in a semidilute regime when the samples were diluted. The low fraction of active chains would then reflect the fraction of the longest HMPA in the mixture. It should be possible to settle which structure correspond to these gels by a study of the characteristic longest relaxation time. Annable observed that the disengagement rate of a superbridge must be several time faster than a single chain. In case i, a relaxation time, such as the inverse of the crossover frequency of G' and G'', is therefore expected to increase significantly with the polymer concentration (by a factor of 10 typically). On the contrary, case ii can correspond to a constant relaxation time, provided that the composition of the cross-links is not modified by the concentration. In practice, the crossover frequency ω_x of G' and G'' was found to decrease rapidly from about 60−100 rd/s in the sol region (both at low or high protein concentrations) down to a value usually lower than 0.1 rd/s in the gel domain. Owing to the drying of the samples in the cell and the high sensitivity of the modulus to the concentration, it was unfortunately not possible to characterize the samples at very low frequencies (typically lower than 0.05 rd/s). Only the 150-3C18/lysozyme mixtures enabled us to make accurate measurements. In this system, ω_x reached a minimum at 0.2−0.3 rd/s that was not found to be markedly dependent on the polymer concentration in the range 0.4−0.93%. In the same concentration range, G_∞/NkT increased by a factor of 3 (Table 1). The minimum of ω_x decreased down to 0.1 rd/s in a solution of 1.1% 150-3C18. Beyond a polymer

concentration of 1.3%, it was too low to be measured. Although this first value indicated the possible absence of superbridges, further characterizations are clearly required. To this aim, the improvement of the preservation of samples loaded in the rheometer cells, and the synthesis of less polydisperse precursor will deserve further efforts in order to check the importance of superbridged chains at low polymer concentration.

Conclusion

Since the previous report on thickening effects in HMPA/protein mixtures,[9] the characterization of viscoelastic properties of these systems confirmed the formation of connected structures exhibiting common properties with reversible gels. The formation of cross-links correlated qualitatively with the hydrophobicity of both HMPA and proteins. In addition, the presence of a plateau modulus of G', the flow at low frequency, and the power-law variation of dynamic moduli at the gelation threshold were altogether consistent with a gelation phenomenon involving cross-links with a narrow distribution of lifetimes. Owing to both the polyelectrolyte nature of HMPA and the presumably complex association between the alkyl groups and the protein,[20] deviation from well-established relationships between G' and cross-link density are highly likely. In particular, the structure and functionality of cross-links might not be unique. To overcome this difficulty, the HMPA/protein association was tentatively schematized as an adsorption of proteins, along the polymer backbone, onto "sites" of fixed mean length. Assuming, in addition, the increase of G' with the concentration of cross-links, and a power-law variation of polymer/polymer contacts in the semidilute regime led to a model of gelation in satisfactory agreement with experiments: Both sol−gel transitions and bell-shaped variation of G' upon supplementation with protein were fitted using one set of three adjustable parameters. According to this model, the typical number of monomer units involved per lysozyme in a cross-link was about 600 whatever the HMPA structure. Understanding the origin of such a high number of acrylate per lysozyme deserves further study. Some questions to be considered include the estimation of a characteristic lifetime of the cross-links, the relationship between protein surface and association, the effect of ionic strength on both the sol−gel transition and the moduli. Taking benefit from specific selectivity of proteins, these systems should be developed toward their use as biosensitive thickeners.

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