Colloidal stability of protein-polymer systems: A possible explanation by hydration forces

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In this paper the stability domains of immunoglobulin (IgG fragment) $F(ab')_2$-polymer systems have been examined using a low-angle scattering technique. The rates of aggregate formation are expressed in terms of a stability ratio as a function of electrolyte concentration. After the usual rapid aggregation achieved at a certain ionic strength (critical coagulation concentration), an abnormal stabilization is observed with increasing ionic strength. This exceptional stability at high electrolyte concentration cannot be explained by the Derjaguin, Landau, Verwey, and Overbeek [B. V. Derjaguin and L. Landau, Acta Physicochim. USSR **14**, 633 (1941); E. J. W. Verwey and J. Th. G. Overbeek, *Theory of the Stability of Lyophobic Colloids* (Elsevier, Amsterdam, 1952), Vols. 1 and 2] theory, which attributes the colloidal stability to the London–van der Waals attraction and the electrostatic repulsion. Effects of electrolyte concentration, counterion valence, pH, protein coverage, and time on the experimental stability are investigated. A possible explanation based on the so-called ''hydration forces'' is proposed. [S1063-651X(97)05804-2]

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I. INTRODUCTION

The stability of a colloidal dispersion is determined by the total interaction potential close to the surface. According to the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory [1,2], this total interaction potential is the sum of the repulsive electrostatic interaction energy (V_E) and the attractive London–van der Waals (dispersion) energy (V_A). Electrostatic repulsion decays approximately exponential with the distance of separation H, whereas the van der Waals forces are proportional to H^{-1}. As a consequence, the total interaction energy, as a function of the distance, normally presents two minima and one maximum. The maximum represents the energy barrier ($V_{\max}$) opposing coagulation. If particles approach each other with sufficient kinetic energies to overcome $V_{\max}$, coagulation will occur and the suspension will be destabilized. As electrostatic repulsion depends on the electrolyte content, so does the energy barrier. The stability of colloidal suspensions can then be controlled by changing the ionic strength of the solution, the attraction being assumed as constant.

The application of the classical DLVO theory, however, has its limitations. Some hydrophilic systems remain stable in the presence of high salt concentrations, where the DLVO theory predicts aggregation. Lipid bilayers, and other model membranes and an aqueous solution, experience a strong repulsion at close proximity [3]. Although previously observed in several systems [4], these additional repulsive forces were first measured by Israelachvili and Adams [5] in the interaction of mica surfaces immersed in an aqueous KNO_3 solutions. They found an exponential decay of these repulsion forces with distance, and referred to them as ''hydration'' (or ''structural'') forces. They are of crucial importance in the stability of colloids, and were invoked to rationalize these phenomena which could not be explained by the classical DLVO theory, e.g., adhesion, wetting, flotation, and in biomembrane systems [6]. For colloids and interfaces in an aqueous media the hydration forces are attributed to the hydration of adsorbed counterions and ionic functional groups in the surface [7–10].

The origin and nature of this force has long been controversial, especially in colloidal and biological literature. A well-known interpretation of this force is that a polar surface induces an ordering of the solvent which exponentially decays away from the surface. An overlap of the ordered-solvent layers near the two mutually approaching surfaces creates a force. Whatever the reason for hydration of the surface (electrostatic polarization of water or hydrogen bonding), it significantly reduces the free energy of the system. Partial dehydration of the ions adsorbed and/or of the surface groups due to the mutual approach of two surfaces, will lead to an increase in the system energy. This results in a repulsive force [11].

The protein-covered colloidal system presented in this paper can be considered as another exception to the DLVO theory. It can be stable at high salt concentrations where the theory predicts aggregation.

The aim of the present study is to investigate the influence of several parameters in this anomalous behavior. This stability mechanism is of potential interest in the development of immunoassays based on colloidal aggregation (latex agglutination tests).

II. EXPERIMENTAL METHODS

All chemicals used were of analytical grade quality. Water was purified by reverse osmosis, followed by percolation through charcoal and a mixed bed of ion-exchange resins. In protein experiments, pH was controlled using different buffers (acetate at pH 3–5, phosphate at pH 6–7, borate pH 8–9, constant ionic strength 2 mM).

The latex was synthesized by means of a core-shell emulsion polymerization in a batch reactor. The core was a seed

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of polystyrene and the shell was obtained by copolymerization of styrene and chloromethylstyrene. Details are described elsewhere [12,13]. The diameter of the polystyrene beads was 201 ± 5 nm and the polydispersity index 1.003, which indicates monodispersity. Surface charge, as determined by conductimetric titration, was -3.7 ± 0.2 μC cm^{-2}, strong acid. Chloromethyl groups, capable of attached protein molecules covalently, were determined to be 2.11 ± 0.14 mequiv m^{-2}.

$F(ab')_2$ antibody fragments from a rabbit polyclonal immunoglobulin (IgG) were kindly donated by Biokit S. A. (Spain). They were obtained by pepsin digestion of IgG, and purified by gel filtration followed by protein A chromatography to remove undigested IgG. Purity was checked by sodium dodecil sulfate poliacrilamide gel electrophoresis (SDSPAGE), and the molecular weight was found to be 102 kilodaltons (kDa). No IgG contamination was detected. The isoelectric points (IEP) of the $F(ab')_2$ preparations, determined by isoelectric focusing, were in the range 4.7–6.0.

$F(ab')_2$ was attached to the latex particles by incubating the latex (0.4 m^2) and protein solution in phosphate buffer saline (pH 7.4) at 35 °C and 5 h. The samples were then centrifuged and resuspended in deionized water. The amount of protein attached to the latex particles was determined from the difference of protein concentration before and after adsorption by spectrophotometry at 280 nm. The adsorption isotherm [12] shows a plateau value of 3.2 mg m^{-2}, in agreement with a homogeneous [14–17] monolayer of flat $F(ab')_2$ molecules with dimensions $142\times38\times38$ Å^3 [18].

Desorption of protein from the surface was tested in the pH range 3–9, with ionic strengths up to 3*M* during one week, for the complexes with maximum coverage. No desorption was observed. In order to measure covalently bound protein to chloromethyl groups, a severe treatment with 1% SDS, 0.2*M* Tris, pH 11 at 50 °C was undertaken. 50% of the protein (that was physically adsorbed) was removed from the surface.

All particle aggregation studies were carried out using a low angle light scattering technique (nephelometry) for measurement of the coagulation rates in conjunction with a computer. Scattered light intensity was followed at 10° during 100 s.

The scattering cell shape is rectangular, with a 2 mm path length. The cell is thoroughly cleaned with chromic acid, rinsed with distilled water, and then dried using an infrared lamp. Equal quantities (1 ml) of salt and complex solutions were mixed and introduced into the cell by an automatic mixing device. Dead time is quite short.

The latex dispersions used for such coagulation experiments have to be sufficiently dilute to minimize multiple scattering effects, whilst still having an experimentally convenient coagulation time. For the complexes used here, a concentration of 2×10^{10} particles per milliliter was determined to be satisfactory. Prior to the experiments, fresh suspensions of complexes were sonicated for 2 min to breakup any initial clusters. The stability ratio (W) is a criterion for the stability of the colloidal system

$$W=\frac{k_r}{k_s}, \tag{1}$$

in which the rate constant k_r describes rapid coagulation, and k_s is the rate constant for the slow coagulation regime. Thus the inverse of the stability ratio provides a measure of the effectiveness of collisions leading to coagulation.

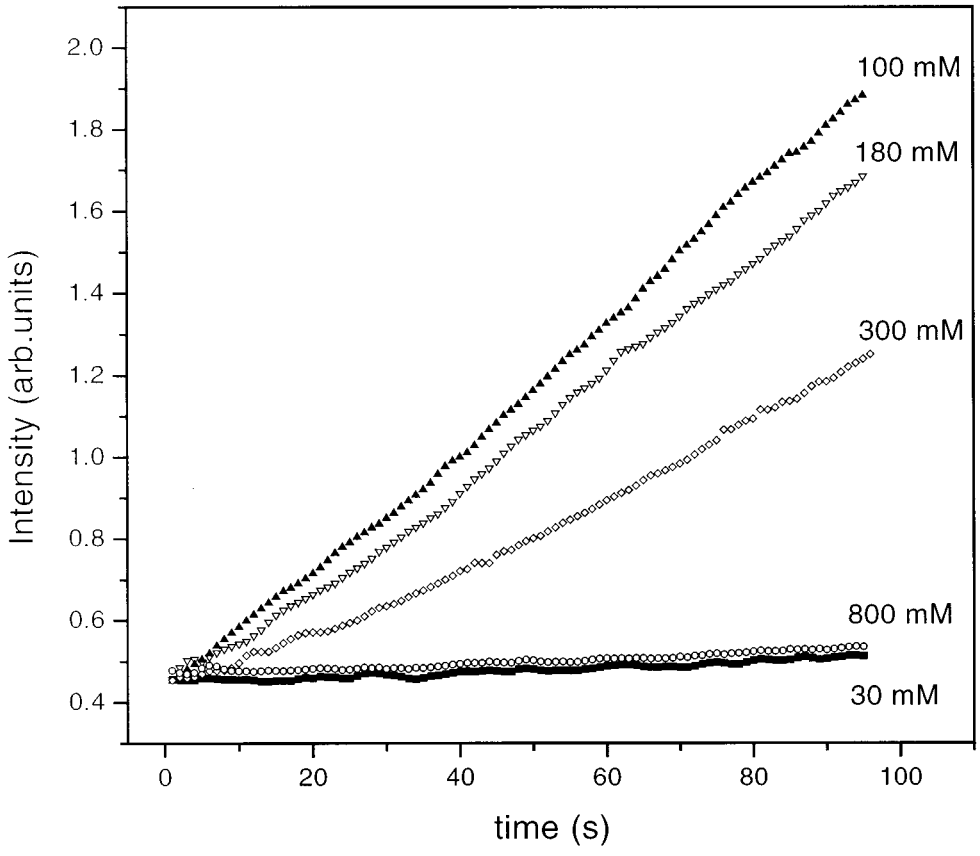

FIG. 1. Scattered light intensity (arbitrary units) vs time for a typical coagulation experiment with a $F(ab')_2$–PS-PCMS complex at different electrolyte concentrations (NaCl).

In this work, the stability ratio was obtained experimentally from the rate constant of coagulation of the colloidal particles measured using the low angle light scattering technique developed by Lips and Willis [19], where the total scattering intensity for a dispersion of identical primary particles with a time varying distribution size is [20]

$$\frac{I(t,\theta)}{I_\theta(0)} = 1 + 2kn_s t, \tag{2}$$

where $I_\theta(0)$ is the initial intensity of light scattered at angle θ, n_s the number of primary particles, and k the rate constant.

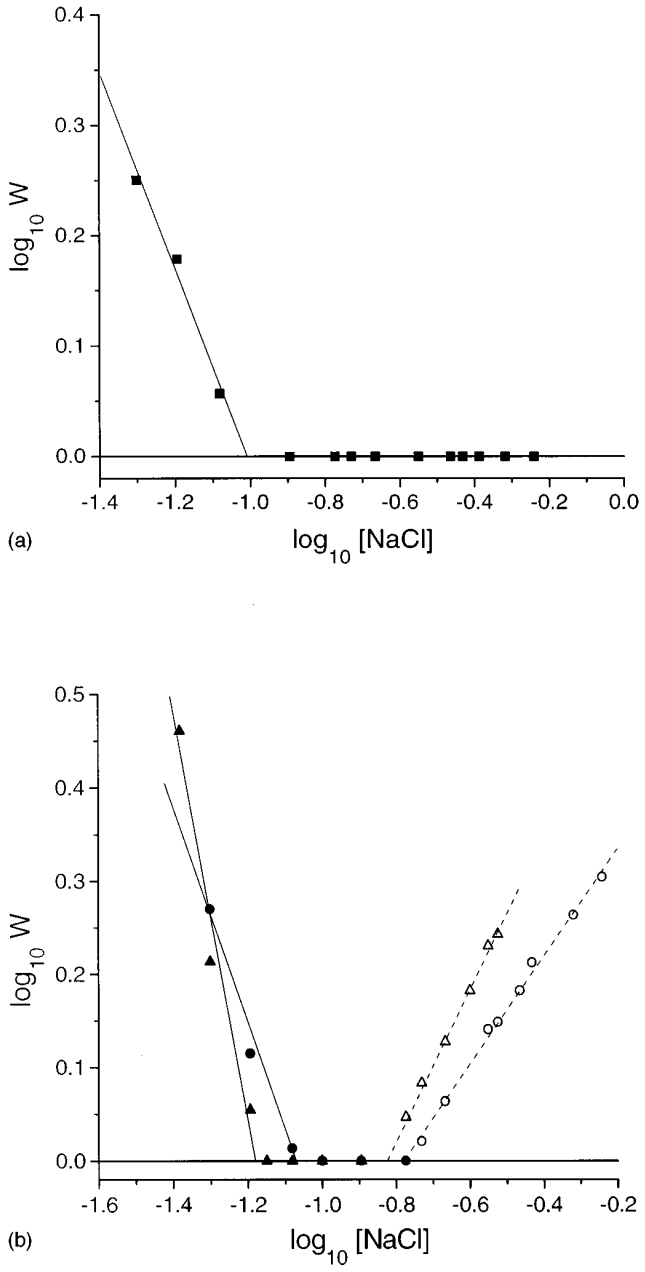

FIG. 2. $\log_{10}W$ vs $\log_{10}$ [NaCl (M)] for a F(ab′)$_2$–PS-PCMS complex at pH 7.3 with three different surface coverages: (a) ■, 1.3 mg m^{-2} (CCC of 98±8 mM). (b) ○, 2.4 mg m^{-2} (CCC of 84±6 mM, CSC of 166±12 mM); △, 3.0 mg m^{-2} (CCC of 66±4 mM, CSC of 133±10 mM). Closed symbol, DLVO zone, open symbol, non-DLVO zone.

The scattered light intensity at low angles increases linearly with time, and then an absolute coagulation rate can be obtained from the slope if the number of primary particles is known.

A typical experiment is presented in Fig. 1, where scattered intensity at 10° is recorded as a function of time for five different electrolyte concentrations. Linear dependence is well accomplished, with correlation coefficients generally better than 0.99. The slopes increase with increasing electrolyte concentration until a maximum is reached. The maximum slope (100 mM in this case) represents rapid coagulation (k_r). The stability factor (W), calculated for each coagulation experiment, is the ratio of the maximum coagulation rate (k_r) to the particular coagulation rate (k_s). Anomalous non-DLVO behavior is apparent from the observation of a slower aggregation with electrolyte concentrations larger than 100 mM.

$\text{Log}_{10}W$ values were then plotted versus $\log_{10}$ [salt] to determine the experimental stability domains of the colloid. As Reerink and Overbeek [21] have shown with several approximations, a linear relationship exists between $\log_{10}W$ and $\log_{10}c_e$. Their treatment is based upon the assumption that the value of the potential maximum in the interaction curve of two approaching spheres is approximately constant and neglecting possible contributions from hydrodynamic interaction.

III. RESULTS

The main result of this work is the anomalous colloidal stability of a protein-polymer system at high ionic strength, where DLVO theory predicts aggregation. This behavior was observed by Healy *et al.* [22] for the aggregation of amphoteric latex, which showed stability at high salt over the critical coagulation concentration (CCC). The minimum concentration provoking this effect was described as the critical stabilization concentration (CSC). The present study deals with the influence of different experimental variables on this behavior: surface protein coverage, pH, counterion nature, and time.

A. Influence of protein coverage

An example of this behavior in our system is shown in Fig. 2 for a protein-polymer complex with three different surface coverages. At a low salt concentration, in all cases $\log_{10}W$ decreases linearly with $\log_{10}$[salt], reaching a limit aggregation state (CCC). For high coverages [Fig. 2(b)], nevertheless, a non-DLVO region appears when salt concentration is further increased. A new linear dependence of increasing stability develops over the so called CSC. Since the stabilization mechanism depends on protein coverage (CSC decreases with increasing coverage), it could be ascribed to the nature of this layer.

B. Influence of pH

For a sensitized polymer with 3.2 mg m^{-2} of F(ab′)$_2$, it is possible to observe (Fig. 3) that the stabilization mechanism does not appear when pH is below the IEP of the protein. Since the protein is positively charged at this pH, it suggests that only cations can provoke the anomalous stability (as

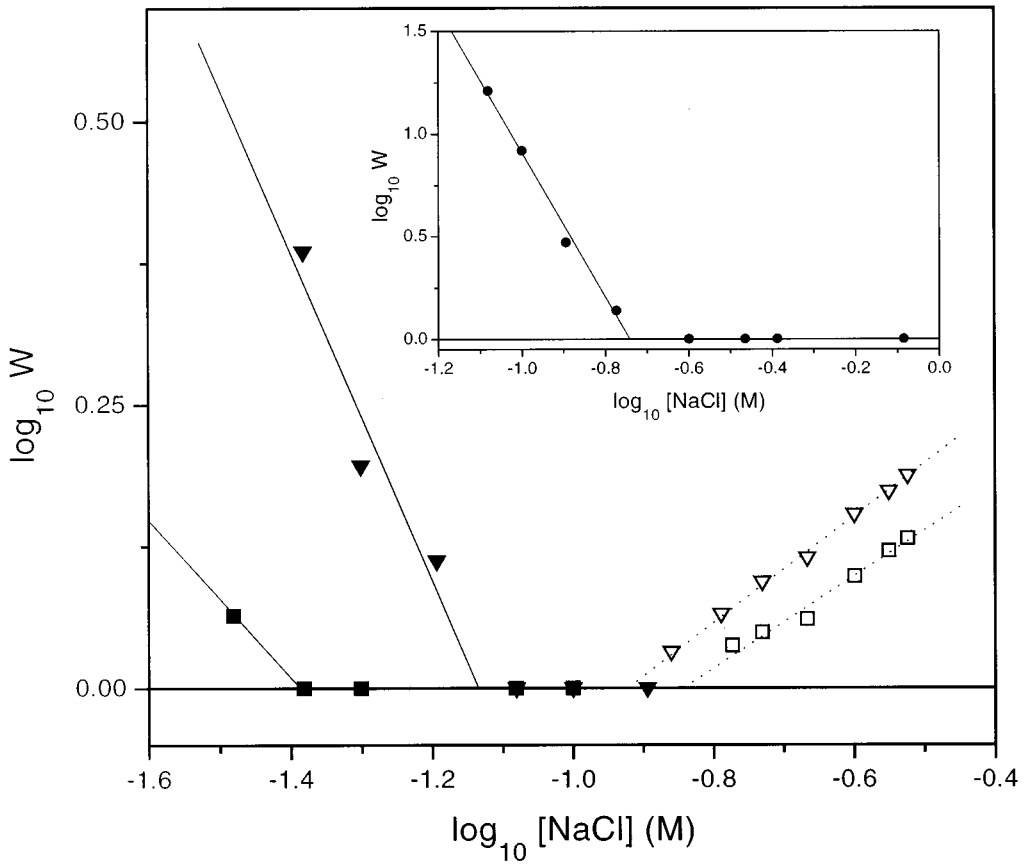

FIG. 3. $\log_{10}W$ vs $\log_{10}$ [NaCl] (M) for a F(ab′)$_2$–PS-PCMS complex with 3.2 mg m^{-2} at three different pHs: ●, pH 3.6 (CCC of 180±10 mM); □, pH 5.4 (CCC of 41±4 mM, CSC of 140±10 mM); △, pH 7.2 (CCC of 74±7 mM, CSC of 119±9 mM). Closed symbol, DLVO zone; open symbol, non-DLVO zone.

previously reasoned for amphoteric latex [22]), bubble coalescence [23], mica [24], and deposition of latex on glass surfaces [6]). For higher pH's (around and over IEP), the anomalous behavior is clearly visible: the higher the pH, the smaller the salt concentration difference between CCC and CSC.

C. Influence of counterion

Figure 4 shows the dependence of aggregation on salt concentration for two different monovalent counterions (Na^+, Cs^+) in a system with a coverage of 2.6 mg m^{-2}. The expected behavior in the DLVO region, i.e., coincidence of aggregation lines in the left of the figure, is not completely accomplished, although differences to not have much significance (CCC: Na^+ 97±8 mM, Cs^+ 93±8 mM). At this point, it should be noted that the DLVO treatment does not take ion adsorption inside the Stern layer into account, while several authors [25–27] have shown the influence of the ion type in the double layer structure and properties.

In the high salt region, non-DLVO stabilization is clearly seen for both cations. For a given salt concentration, however, an improved stability is observed with Na^+. The electrolyte concentration from which complex stabilization reappears (CSC) is lower for Na^+ (138±10 mM) than for Cs^+ (160±12 mM), suggesting a dependence of the stabilization mechanism with the ion type.

When divalent cations, instead of monovalents, are used to induce aggregation (Fig. 5), a similar dependence with ion is seen. Mg^{2+} shows a lower CSC (49±3 mM) than Ca^{2+} (64±4 mM). With divalent cations such as Ca^{2+} and Mg^{2+}, however, conclusions are not straightforward, since the effect of these ions on biological structures are rather complex. From a practical point of view, however, it is noteworthy that divalent cations provoke full unstability of the system, even with the lower salt concentration studied (15 mM), but they can again stabilize the suspension with an intermediate salt concentration (at ca. 0.4M Na^+ the system is colloidally unstable, while at the same concentration Mg^{2+} is stable).

D. Influence of time

When stability is studied for the same latex-protein complexes, but at different times after sensitization, a curious affect is observed (Fig. 6). The first interesting remark in this figure is the null influence of time in the DLVO region, suggesting that, effectively, parameters like surface potential and coverage have not changed (i.e., no desorption is occurring). In the non-DLVO region, nevertheless, time is affecting CSC, decreasing until a constant value is achieved after ten days.

IV. DISCUSSION

Figure 2 shows that the anomalous stabilization mechanism on this system can be attributed to the presence of a

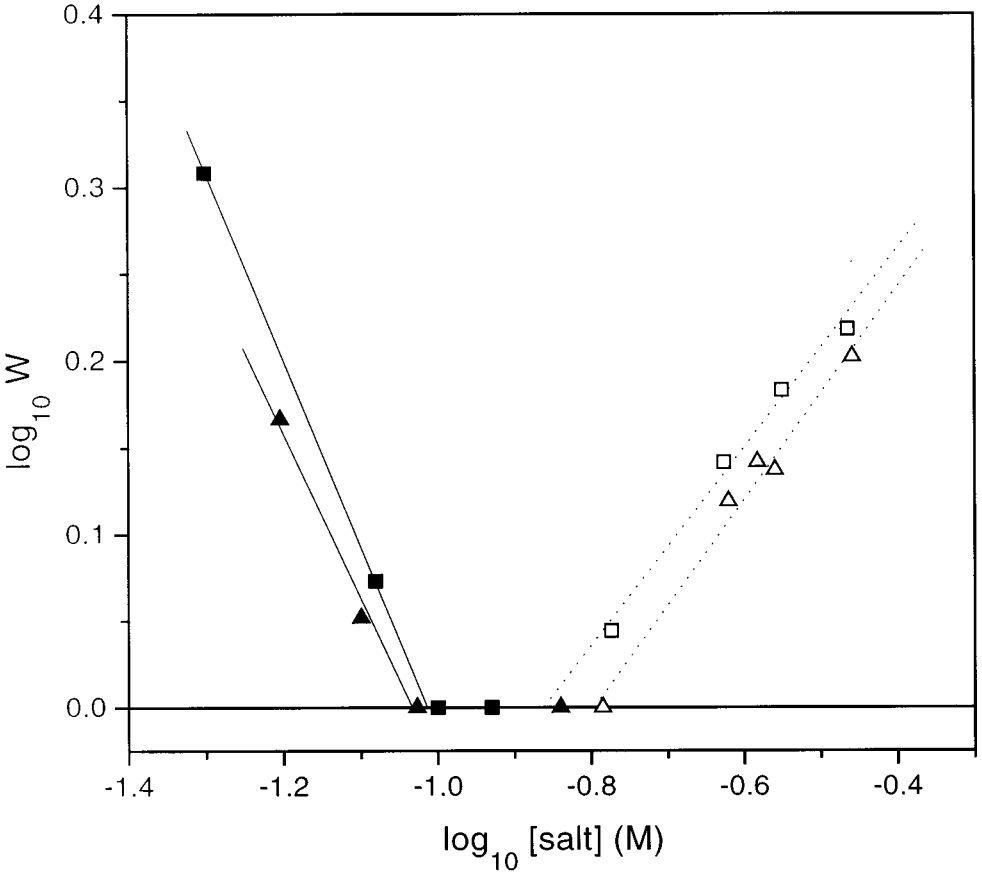

FIG. 4. $\log_{10}W$ vs $\log_{10}$ [salt (M)] for a $F(ab')_2$–PS-PCMS complex with 2.6 mg m^{-2} at pH 7.1: □ Na^+ (CCC of 97±8 mM, CSC of 138±10 mM); △, Cs^+ (CCC of 93±8 mM, CSC of 160±12 mM). Closed symbol, DLVO zone; open symbol, non-DLVO zone.

protein layer surrounding the particle. Due to the macromolecular nature (amphoteric and amphiphilic) of the protein, different possibilities can be suspected as responsible for this behavior [12]. Steric effects could account for the anomalous stabilization at a high ionic strength, where electrostatic interactions are severely diminished. In another way, electrostatic attraction between amphoteric complex surfaces could also be a major force in aggregation, this effect being decreased with increasing salt concentrations.

With any of these possibilities, however, a more or less symmetric behavior should be expected for a similar opposite net charge in the surface complex. The results shown in Fig. 3 reveal that this is not the case. At pH 3.6, below the IEP, the anomalous stabilization does not appear, suggesting a relationship with ion nature. It occurs only when the net complex charge is negative, and an excess of cations is present. Since cations exist in aqueous media highly hydrated, while anions are practically not hydrated [25,28], a relation of the stabilization mechanism with hydration of adsorbed ions seems plausible. In fact, we can observe in Figs. 4 and 5 a dependence with ion type, increasing their effect with the size of the hydrated cation.

For a similar interface (amphoteric latex with COO^- and NH_2^+ groups at the surface), Healy *et al.* [22] described the same phenomena, attributing responsibility to a ''hydration barrier at the interface.'' Hydration forces, as mentioned in the Introduction, are widely recognized for hydrophilic surfaces as strongly stabilizers. Polypeptide outer structure in a polymer-protein complex can present both domains, hydrophilic and hydrophobic. If we assume a main hydrophobic force for adsorption, enrichment of hydrophilic patches is expected for the new interface [29]. An increase in bulk salt concentration should lead to specific hydrated ions adsorption into this interface. The more hydrophilic the microscopic region, the larger the decrease in the free energy of the system, setting then a barrier for interparticle aggregation (which needs dehydration of the surface to occur).

Proteins adsorbed to a surface remain in a slow dynamic state. Several authors [30–33] point out in their conclusions to conformational changes of adsorbed proteins with time, suggesting a tendency of the system to expose more hydrophilic sites to the protein-water interface, while the more apolar sites are oriented to the hydrophobic polymer surface [29,34,35]. Those changes should probably tend to minimize free energy by increasing hydrophobic contacts with surface and hydrophilic exposure to the aqueous environment.

Assuming that alterations in the structure will tend to increase the hydrophilic character of the protein water interface, we observe the experimental results [Fig. 6] to be in line with a dependence of the anomalous stabilization with the hydrophilic nature of the interface.

The relation between solubility in nature proteins and stability in protein-covered particles is something difficult to understand. Their behavior in respect to the salt concentration is opposite, whereas they present a similar aqueous interface. Proteins increase the solubility reaching a maximum, and then they turn more insoluble with increasing salt concentration. Protein-covered colloids are originally stable, and become unstable with salt in the medium. At higher salt concentrations, however, they are colloidally stable again. The salt range for these two opposite behaviors is more or less the same (ca. 0–1.5M). The main argument to resolve this

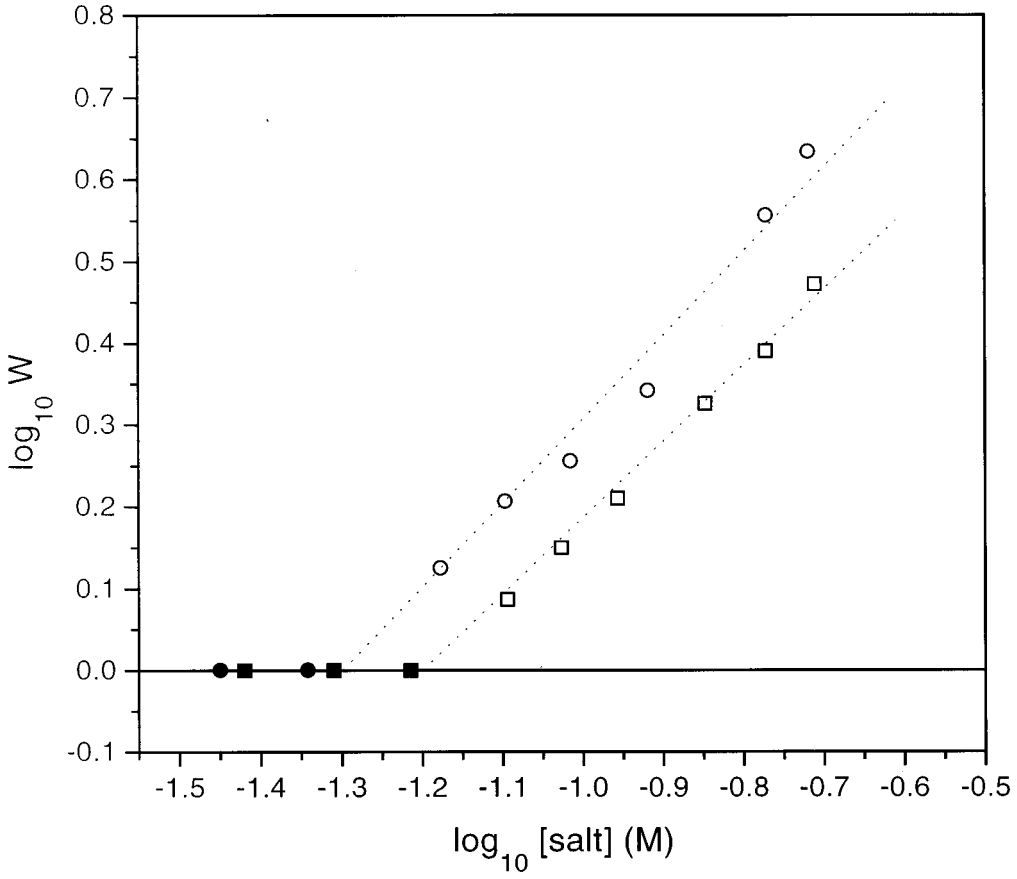

FIG. 5. $\log_{10}W$ vs $\log_{10}$ [salt (M)] for a F(ab′)$_2$–PS-PCMS complex with 2.6 mg m^{-2} at pH 7.1: ○, Mg^{++} (CSC 49±3 mM); □, Ca^{++} (CSC of 64±4 mM). Closed symbol, DLVO zone, open symbol, non-DLVO zone.

apparent controversy should erase from the difference in the ''colloidal'' size (5–10 nm the protein, 200 nm the particles), which can provoke big differences in the involved forces for solubility and/or stability.

In an attempt to justify this anomalous behavior, an extension to the DLVO theory including hydration forces, and its dependence with salt concentration, can be intended. Churaev and Derjaguin [36] made a first approximation of this problem including a (nonionic strength dependent) ''structural term'' to the classical theory.

As in their proposal, if hydration forces are to be included in the DLVO theory, the net potential energy for the interaction between two colloidal particles must be described by the algebraic sum of three potentials:

$$V_T = V_A + V_E + V_h, \tag{3}$$

where V_A is the London–van der Waals dispersion energy, V_E represents the term for the repulsive electrostatic interaction, and V_h is the repulsive hydration interaction energy.

Starting from an empirical exponential function to describe this structural force $P(H)$, in the form first described by Marcelja and Radic [5,8,37–39]

$$P(H) = P_0 e^{-H/\lambda} \tag{4}$$

and using the Derjaguin approximation for spheres of radius a [40]

$$V_h(H) = \int_H^\infty \pi a P_0 \lambda e^{-H/\lambda} dH = \pi a P_0 \lambda^2 e^{-H/\lambda}. \tag{5}$$

Since we have observed a dependence of the abnormal stabilization mechanism on the salt concentration, the hydration force should depend on it in the same way. If we assume, as a first approximation, that the hydration interaction energy is directly proportional to salt concentration (c_e)

$$V_h = \pi a (N_A C_h c_e) \lambda^2 e^{-H/\lambda}, \tag{6}$$

where N_A is the Avogadro number, C_h is the proportionality constant that we have defined as ''hydration constant,'' and the concentration is expressed in mM.

Including Eq. (6) on Eq. (3), estimates of the total potential energy of interaction, as a function of the separation distance, were computed, and presented in Fig. 7 for a model system similar to that used in the experimental section. In Fig. 7(a) standard DLVO calculations are plotted for different salt concentrations using the parameters specified in the caption (Hamaker constant and Ψ_δ values have been acquired from a specific stability studio involving a similar F(ab′)$_2$-latex system [41]. Figure 7(b) shows the addition of estimated hydration forces contribution. Parameters λ (decay length) and C_h (a new proportionality parameter is introduced here) have been adjusted (λ between literature values) to match experimental results.

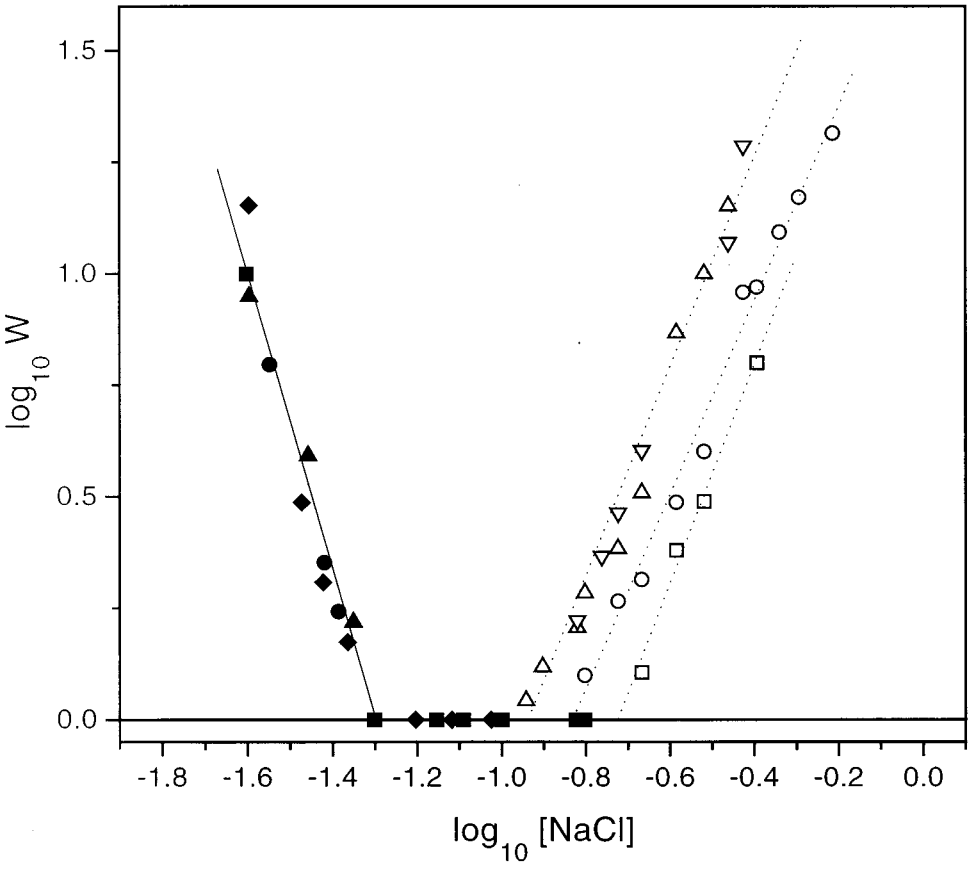

FIG. 6. $\log_{10}W$ vs $\log_{10}$ [NaCl (M)] for a F(ab′)$_2$–PS-PCMS complex with 3.2 mg m^{-2} at pH 6.3 and different times after sensitization: □, 1 day (CCC of 47±4 mM, CSC of 190±12 mM); ○, 5 days (CCC of 47±4 mM, CSC of 147±10 mM); △, 10 days (CCC of 47±4 mM, CSC of 118±8 mM); ▽, 24 days (CCC of 47±4 mM, CSC of 118±10 mM); Closed symbol, DLVO zone; open symbol, non-DLVO zone.

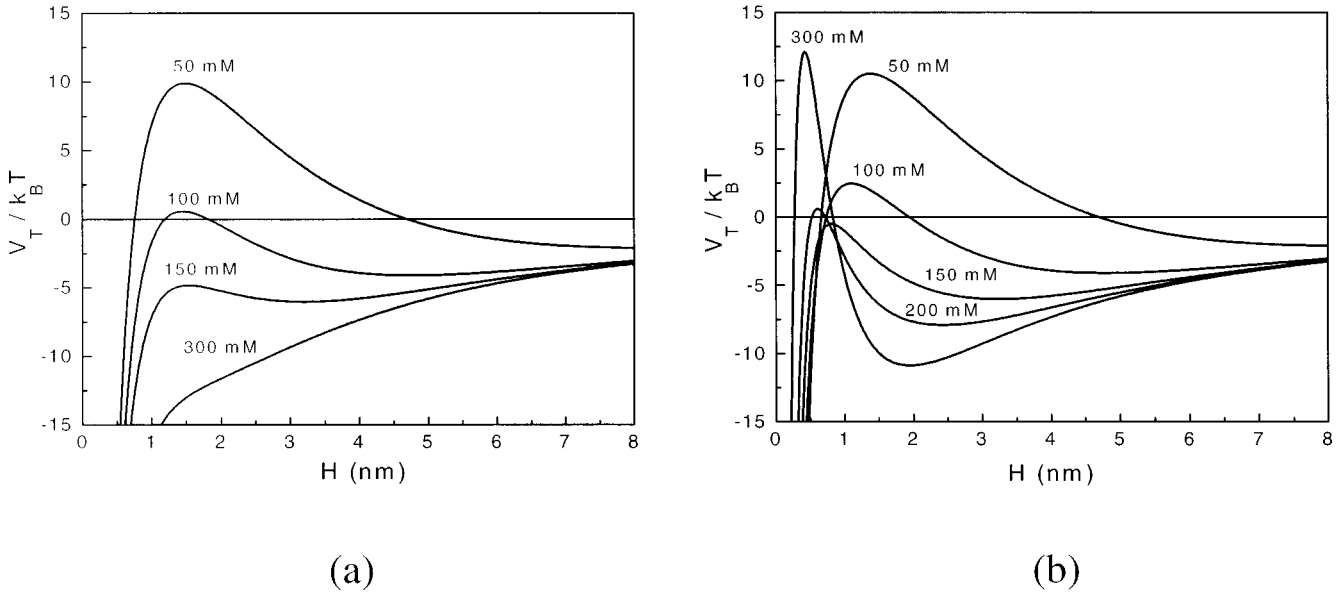

FIG. 7. Calculated total interaction potential (V_T in k_BT units) vs distance for different 1:1 electrolyte concentrations. (a) DLVO theory. (b) DLVO extended by hydration forces inclusion. Hamaker constant (A), 2×10^{-20} J; radius (a), 100 nm; Stern potential (Ψ_δ), 27 mV; decay length (λ) 0.36 nm; hydration constant (C_h), 1.1×10^{-19} J.

In both figures, the interaction potential maximum (energy barrier) decreases with increasing salt concentration, and so does stability. The electrolyte concentration at which V_{max} becomes equal to 0 is called the critical coagulation concentration (CCC). However, if this concentration is further increased [see Fig. 7(b)] the barrier achieves a minimum and then starts to increase, becoming equal to 0 once again (CSC). The appearance of the potential barrier could account for the anomalous stability at these high electrolyte concentrations.

Figure 2(b) shows, nevertheless, a striking point in the inclusion of this term to the DLVO theory: the existence of a secondary minimum which could provoke reversible coagulation. Dilution experiments carried out with our polymer-protein system do not show this phenomenon, otherwise present in hydrophilic systems like silica.

V. CONCLUSIONS

Low-angle scattering measurements conducted with aqueous suspensions of the $F(ab')_2$–PS-PCMS system show that the particles strongly aggregate with increasing salt concentration as predicted by the DLVO theory. The aggregation efficiency was maximal at the critical coagulation concentration (CCC). However, at the so-called critical stabilization concentration (CSC) and above, the suspensions were more stable than predicted by the theory.

We attribute this observed aggregation change to the existence of a hydration repulsion resulting from the hydrated counterions adsorbed on the protein. This repulsive force should dominate the interaction at short range, when the double layer is compressed. An extension to the DLVO theory, including hydration forces and its dependence with salt concentration, has been proposed. The hydration force decays in a simple exponential manner with increasing distance, as described in the classical models. As a first approximation, we have assumed that hydration forces are directly proportional to the electrolyte concentration.

The total interaction energy as a function of separation distance curves, calculated by the extended DLVO theory, indicates the possible existence of two electrolyte concentration where the energy barrier disappears (CCC and CSC) in concordance with the experimental results. The appearance of a deep secondary minimum in these calculations, however, suggests the possible existence of reversible coagulation.

ACKNOWLEDGMENTS

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