How Proteins Stabilize Colloidal Particles by Means of Hydration Forces

J. A. Molina-Bolívar* and J. L. Ortega-Vinuesa

Grupo de Física de Fluidos y Biocoloides, Departamento de Física Aplicada, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

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The classical theory of colloidal stability, the well-known Derjaguin−Landau−Verwey−Overbeek (DLVO) theory, predicts a loss of stabilization with increasing salt concentration. An anomalous stability of the system is found at high salt concentrations when proteins cover colloidal particles. It has been demonstrated that this "non-DLVO" stability only takes place on hydrophilic systems and hydration forces are responsible for this phenomenon. In this work, different proteins (IgG, fibrinogen, myoglobin, and serum albumin) have been adsorbed onto a chloromethylstyrene (CMS) latex. The influence of the protein nature on the non-DLVO stabilization has been studied. This anomalous stabilization mechanism caused by hydration forces has been observed for all the studied proteins, although some experimental results (e.g., the critical stabilization concentration) vary for different proteins and degrees of coverage. In addition, we have shown that the orientation of the protein molecules immobilized on the CMS surface is different depending on the adsorption pH. At low pH values, when protein and polymer surfaces have different signs of charge, the macromolecules are adsorbed with a preferential orientation which differs from that obtained at higher pH values. This has been corroborated by both stability and immunoreactivity studies. Finally, we have also observed that proteins are in a dynamic state when they are adsorbed on the CMS surface. After a long time, the macromolecules tend to expose their hydrophilic areas to the aqueous medium and to hide their hydrophobic zones from solution.

1. Introduction

In general, in the dispersion of particles in a liquid, frequent encounters between particles occur because of Brownian movement. Whether such encounters result in permanent contact or whether the particles rebound and remain free is determined by the forces between them. A dispersion is colloidally stable when its particles remain permanently free.

The stability of colloidal dispersions is related to various phenomena in many industrial processes. The theory of colloidal stability and the conditions under which particulate materials can be separated from the liquid phase through coagulation play a central role in water science and technology.

According to present theories, colloidal stability depends on the interaction of the various attractive and repulsive forces acting between particles. The attractive forces are similar in all dispersions: London van der Waals interaction. However, the repulsive forces may be electrostatic or steric in nature. It is customary to distinguish the two classes of colloids whose general behavior is entirely different. These classes are generally called lyophobic and lyophilic colloids, respectively; it is assumed that in the lyophilic colloids a strong affinity exists between the particles and the molecules of the dispersion medium, whereas in the lyophobic colloids the affinity is either weak or absent. The stability of lyophobic colloids is determined by only one factor (the electric charge of the particles), and that of lyophilic colloids by two factors (charge and hydration).

The classical Derjaguin−Landau−Verwey−Overbeek (DLVO)[1,2] theory was developed to explain the role of electric effects on the colloidal stability. This theory generally fails to predict the stabilities of very hydrophilic and very hydrophobic particle suspensions. It is now known that interaction energies other than London van der Waals and electrostatic repulsion exist for both cases.

For hydrophobic solids, Israelachvili and Pashley[3] were the first to directly measure the extraneous forces on surfactant-coated mica surfaces. They and other investigators[4,5] showed that the non-DLVO force is attractive and can be 10−100 times larger than the dispersion force. For hydrophilic surfaces, the existence of non-DLVO forces was recognized much earlier.[6] More recently, many investigators conducted surface force measurements with mica[7] and glass[8] and confirmed the existence of the repulsive hydration force. It is a much shorter-range force than the attractive hydrophobic force.

The repulsive hydration force strongly depends on the type and concentration of electrolytes present in solution. The hydration force becomes stronger with increasing hydration energy of the cations involved.[9,10]

Although a lot of work has been done on stabilization of colloidal systems by nonionic macromolecules,[11] cationic and anionic polyelectrolytes,[12] only a little attention has been paid to stabilization by amphoteric macromolecules such as proteins.

* To whom correspondence should be addressed

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Table 1. Main Characteristics of the Latex Particles[a]

D (nm)	PDI	σ (μC/cm^2)	[Cl$^-$] (μmol/g latex)
204 ± 8	1.0045	−3.1 ± 0.4	121 ± 10

[a] D = diameter; PDI = polydispersity index; σ = surface charge density; [Cl$^-$] = concentration of the chloromethyl groups in the latex.

This article deals with the influence of adsorbed proteins on the stability against aggregation of hydrophobic colloids, which here is a polychloromethylstyrene latex such as the colloids used in immunology where protein-coated latexes are used as test material.[13,14] The use of a chloromethystyrene (CMS) latex allows protein molecules to covalently bind onto the polymer surface, and thus desorption of molecules caused by changes in pH or ionic strength of the medium is prevented. The latex particles, which are initially hydrophobic, are converted by the adsorption of protein into hydrophilic particles that retain their stability in the absence of electrostatic repulsive forces when the classical DLVO theory predicts aggregation.

2. Experimental Section

2.1. Materials. All chemicals used were of analytical 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 8−9, constant ionic strength 2 mM).

The latex was synthesized by means of a core−shell emulsion polymerization in a batch reactor. In the first step, the core of the polymer particles was prepared at 90 °C by batch emulsion polymerization. After polymerization, the seed was kept overnight at 90 °C to decompose the initiator. The seeded batch emulsion polymerization of CMS was performed to put the shell onto the polystyrene core synthesized in the first step at 60 °C. This latex was kindly provided by Joxe Sarobe (Chemical Engineering Group, University of Basque Country, Spain).

Latex was cleaned by "serum replacement technique" until the electrical conductivity of liquid was less than 2 μS/cm. The particle size distribution of the latex was obtained by transmission electron microscopy (TEM) using a H-7000 FA Hitachi microscope on representative samples of more than 500 particles (automatically analyzed with Bolero software, AQ Systems). Surface charged groups were determined by conductometric titration. Determination of chloromethyl surface groups of the latex particles was based on the nucleophilic attack of the deprotoned glycine on the chloromethyl groups.[15] The latex was reacted with 0.24 M glycine and 0.182 M NaOH. The reaction was carried out at 35 °C. To include the possible hydrolysis of chloromethyl groups by temperature, a blank without glycine/NaOH was determined for each data point. The chloride ions released were determined, as a function of time, after acidification with concentrated HNO_3 by using a chloride-selective electrode to locate the end point of the automatic titration with $AgNO_3$. Particle concentration of the stock latex solution was 5×10^{11} particles/mL. The main characteristics of the latexes are shown in Table 1.

This latex presents chloromethyl groups on the surfaces. These groups are capable of binding protein molecules covalently. The extent of covalent protein binding to the surface is approximately 60% of the initial coverage, according to a previous publication.[15]

2.2. Proteins. C-reactive protein (CRP), nonreactive polyclonal IgG, and polyclonal anti-CRP−IgG (everything from rabbit), were obtained, purified, and kindly donated by Biokit S. A. (Spain). Fibrinogen (Fib), myoglobin (Myo), and bovine serum albumin (BSA) were purchased from Sigma (Reference numbers F8630, M8007, and A4503, respectively). Proteins were dialyzed just before use. The main characteristics of the proteins have been summarized in Table 2. It is worthwhile to comment about the hydrophobic character of the protein surfaces, because protein adsorption depends not only on sorbent surface hydrophobicity but also on the nature of the macromolecules. Advancing contact angle (θ_a) measurements allow a rough estimation of the hydrophobicity of the external part of the protein molecules (more details can be found in ref 16). From the θ_a data (Table 2) we see that albumin surfaces are less hydrophobic than IgG, which in turn is less hydrophobic than fibrinogen. We have not found θ_a data for myoglobin in the literature. Nevertheless, as Norde[17] stated, the smaller the protein molecule and the larger the deviation from sphericity, the larger the area-to-volume ratio. As a result, small protein molecules tend to have a relatively more hydrophobic surface than larger molecules. In fact, for myoglobin, 50% of the water-accessible surface area is made up of apolar atoms. So it is plausible to think that myoglobin presents the most hydrophobic surface compared with the other three proteins. Therefore, the degree of hydrophobicity of the surface of our proteins would qualitatively be:

Table 2. Main Characteristics of the Proteins

protein	molecular weight	isoelectric point	advancing contact angle [16]
IgG	150 000	6.0−8.0	71°
fibrinogen	340 000	5.8	82°
myoglobin	17 800	7.0	
BSA	66 000	4.8	56°

(less hydrophobic) BSA < IgG < Fib < Myo (More hydrophobic)

All proteins were stored at −20 °C until required.

2.3. Protein Adsorption. Protein concentrations before and after adsorption were determined by direct UV spectrophotometry at 280 nm (Σ_{IgG} = 1.40 mL mg^{-1} cm^{-1}, Σ_{Fib} = 1.36 mL mg^{-1} cm^{-1}, Σ_{Myo} = 1.75 mL mg^{-1} cm^{-1}, Σ_{BSA} = 0.66 mL mg^{-1} cm^{-1}). Five experimental variables remained constant in every adsorption experiment: the total polymer area added (0.3 m^2), the final volume (8 mL), the ionic strength (I = 0.002 M), the incubation temperature (T = 25 °C), and the incubation time (15 h). The adsorption experiments were carried out in a thermostatic bath in which samples were gently agitated in a rotatory plate. Stock latex suspension (200 μL) was added to different buffers containing the desired protein (from 0 to 350 μg/mL). After incubation, samples were spun, the pellets redispersed in 5 mL of the desired buffered solution, and supernatants were filtered using a poly(vinylidene difluoride) filter (Millipore, pore diameter = 0.1 μm) just before measuring the protein concentration. This last filter presents an extremely low affinity for protein adsorption, so the filtration step does not interfere negatively with the calculation of the adsorbed protein amount.

2.4. Electrophoretic Mobility. Electrophoretic mobility measurements were performed with a Zeta-Sizer IV (Malvern Instruments). The sensitized polymer particles were diluted in the desired medium for 10 min, just before measuring. Final particle concentration was equal to 3×10^9 particles/mL. Then mobility data were taken from the average of four measurements at the stationary level in a cylindrical cell. Two samples were assayed in this way. The standard deviation of such values was always less than 5%.

2.5. Colloidal Stability. All particle aggregation studies were carried out using a low-angle light-scattering technique (nephelometry). Scattered light intensity was observed at 10° for 100 s.

The scattering cell shape was rectangular, with a 2-mm path length. The cell was thoroughly cleaned with chromic acid, rinsed with distilled water, and then dried using an infrared lamp. Equal quantities (1 mL) of salt and sensitized particle solutions were

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mixed and introduced into the cell by an automatic mixing device. Dead time was quite short.

The latex dispersions used for such coagulation experiments had to be sufficiently dilute to minimize multiple scattering effects, while still having an experimentally convenient coagulation time. For the complexes used a concentration of 2×10^{10} particles/mL was determined to be satisfactory. Before experiments, fresh suspensions of complexes were sonicated for 2 min to break up 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. In this work, the stability ratio was obtained experimentally from the rate constant of coagulation of the colloidal particles measured with the low-angle light-scattering technique developed by Lips and Willis,[18] where the total scattering intensity for a dispersion of identical primary particles with a time varying distribution sizes is:

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

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

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.

2.6. Immunoreaction Experiments. Immunoagglutination reactions were performed at pH 8 (borate 13 mM), NaCl 800 mM, BSA 0.8 mg/mL, glycine 0.1 M, and N_3Na 1 mg/mL used as a preservative. The aggregation process of the IgG−latex system induced by the presence of antigen was monitored in a spectrophotometer for 5 min at 570 nm. On 3 mL of CRP solution (at different concentrations) 65 μL of anti-CRP−IgG−latex suspension was added and rapidly mixed. The initial particle concentration in the cuvette before aggregation was 10^{10} particles/mL. The experiments were carried out at least twice.

3. Theoretical Treatment

The total potential energy (V_T) of the interaction, which determines the colloid stability, between two particles is defined as:[1,2]

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

The term V_A, the energy of interaction between the particles caused by van der Waals dispersion forces, is expressed as:[19]

$$V_A = -\frac{A}{6}\left[\frac{2a^2}{H(4a+H)} + \frac{2a^2}{(2a+H)^2} + \ln\frac{H(4a+H)}{(2a+H)^2}\right] \tag{4}$$

where A is the Hamaker constant for particles interacting in the medium (water), a is the particle radius, and H is the distance between the surfaces of the particles.

The quantity V_E represents the repulsive interaction between the electrical double layers of the particles. According to the constant potential model, a reasonable expression to V_E [for moderate potential in the Stern layer ($\Psi_\delta < 50$ mV)] is:[20,21]

$$V_E = 2\pi(a+\Delta)\epsilon\epsilon_0\left(\frac{4k_BT}{z_i e}\gamma\right)^2 e^{-\kappa(H-2\Delta)} \tag{5}$$

where κ is the Debye parameter

$$\kappa = \left(\sum_i \rho_i e^2 z_i^2/\epsilon\epsilon_0 k_B T\right)^{1/2} \tag{6}$$

and γ is given by the following equation:

$$\gamma = \tanh\left(\frac{z_i e\Psi_\delta}{4k_BT}\right) \tag{7}$$

Ψ_δ is the Stern potential, z_i is the valence of the ion, ρ_i is the ionic concentration of ions i in the bulk, and Δ is the thickness of the Stern layer. In fact, eq 5 is valid for $\kappa a \gg 1$.

The stability ratio (W) previously introduced as the ratio between rapid and slow aggregation rates depends on the total interaction energy. With use of the modified Fuchs treatment [22,23] we can write:

$$W = \frac{\int_0^\infty \frac{\beta(u)}{(u+2)^2}\exp\left(\frac{V_T}{k_BT}\right)du}{\int_0^\infty \frac{\beta(u)}{(u+2)^2}\exp\left(\frac{V_A}{k_BT}\right)du} \tag{8}$$

where β is the hydrodynamic correction factor, and u is $(H - 2a)/a$. When this correction is unnecessary, $\beta = 1$.

The stability factor can be computed by numerical integration using eq 8. In this way, it is necessary to fit a theoretical curve to the experimental points of W versus electrolyte concentration, using a pair of values of A and Ψ_δ as fitting parameters with our own computer program. The most relevant aspect of this fitting method is the fact that A and Ψ_δ have different effects. Ψ_δ affects the slope of the theoretical curve, whereas the Hamaker constant influences the plot intercept. To fit the experimental values of W it is necessary to know the ionic size, Δ, for the adsorbed ions of the Stern layer. Different situations of total or partial hydration of the counterion used in stability studies can be found in the literature.[24,25] In this work, we have used the diameter of dehydrated cation (0.095 nm for Na^+ and 0.099 nm for Ca^{2+}).[26]

4. Results and Discussion

4.1. Protein Adsorption. A study about the maximum amount of adsorbed protein on the CMS surface as a function of pH was first carried out. Results are shown in Figure 1. Maximum Γ_{pl} values were obtained near the pI of the proteins (except for myoglobin), as expected. In fact, these values coincide with the pI of the latex−protein complexes, which were calculated by means of electrophoretic measurements (shown below). At pH values lower than pI (which are not far from the protein pI) protein

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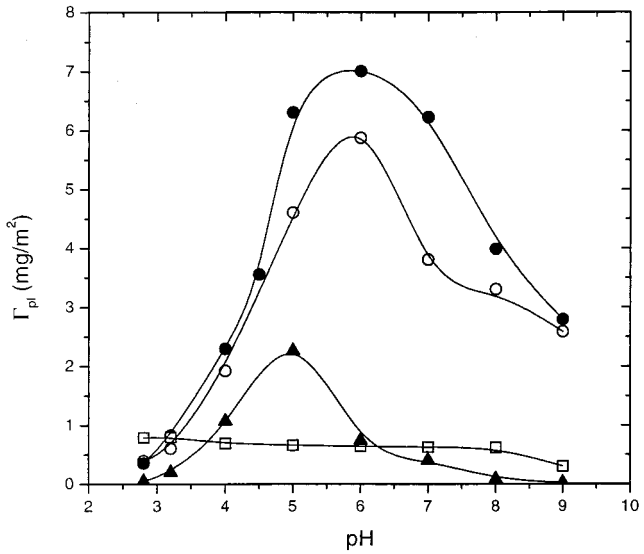

Figure 1. Maximum amount of adsorbed protein as a function of adsorption pH. Symbols: ○, IgG; ●, fibrinogen; □, myoglobin; and ▲, BSA. Ionic strength of the medium = 0.002 M; T = 25 °C; t = 15 h; total area of latex added = 0.3 m^2.

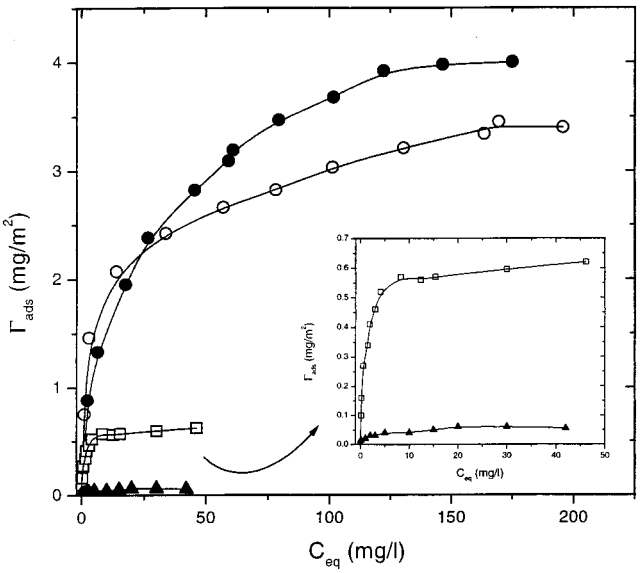

Figure 2. Adsorption isotherms at pH 8 (I = 0.002): ○, IgG; ●, fibrinogen; □, myoglobin; ▲, BSA. T = 25 °C; t = 15 h; total area of latex added = 0.3 m^2.

molecules have a compact structure; and an additional electrostatic attraction force also exists that favors the protein (with positive charge) surface union. Results shown in Figure 1 agree with a well-known pattern that numerous authors have found when working with different "soft" proteins.[27−29] The explanation can easily be found in the literature.[30,31] The adsorption of proteins having a high structure stability, "hard" proteins (e.g., myoglobin), is governed mainly by both electrostatic interaction and changes in the hydration layer of the hydrophobic patches of both the sorbent and the protein. In contrast, proteins of low structural stability, "soft" proteins (e.g., IgG, fibrinogen, and BSA), contain an additional driving force for adsorption, which is related with structural alterations. Macromolecules in solution possess a compact structure when the medium pH coincides with the pI of the protein; this structural organization is partially lost when proteins spread on the sorbent surface, leading to a net increase of the entropy of the system.[32] This is why maximum Γ_{pl} values are usually achieved near the pI of soft proteins. Besides, outside of the pI, lateral intermolecular interactions become important and they tend to reduce the adsorbed amounts.[33] A different pattern is observed for the myoglobin or other hard protein. The dehydration of hydrophobic patches of both latex and myoglobin surfaces mainly drives the adsorption in the whole range of studied pH. In addition, attractive electrostatic interactions take place at pH 3−5, and repulsive forces appear for pH 8−9. This is why Γ_{pl} is higher for the former pH values and lower for the latter. Norde et al.[34,35] have also shown the important role played by the electrostatic forces in the adsorption of myoglobin at different pH values. Lateral protein−protein interactions do not seem to be relevant for this protein.

An adsorption isotherm at pH 8 (I = 0.002 M) was carried out for each protein. Results are depicted in Figure 2. This pH was chosen because both stability and immunoreactivity experiments were performed at such a pH. In this case the CMS surface and all the proteins are negatively charged. The affinity of the proteins for the sorbent can be qualitatively defined merging the initial part of the isotherm with the total adsorption line[36] (y-axis in Figure 2). The affinity of BSA for the latex surface is extremely poor. This must be because this protein is the most charged and hydrophilic protein with which we have worked. The highest affinity is shown by the myoglobin, although the maximum amount of adsorbed protein is low because of its low molecular weight. Both latex and myoglobin are negatively charged, as mentioned above; thus the adsorption process must be led by hydrophobic forces. This "high" affinity would be justified by the hydrophobic character of this protein (see comments about the proteins given in the Experimental section). It should be noted that the major driven force for adsorption of "hard" proteins on polymer surfaces is the dehydration of superficial hydrophobic groups.[17,30] Finally, IgG and fibrinogen present similar affinities for the latex surface, which are not high.

4.2. Electrophoretic Mobility. The electrokinetic behavior of protein-latex complexes depends on (a) the nature of both latex and protein, (b) the amount of adsorbed protein, (c) the pH, and (d) the ionic strength of the resuspension medium.[29] We have only measured the electrophoretic mobility (μ_e) of some complexes as a function of pH. These complexes were: IgG−latex (3.80 mg/m^2) and Fib−latex (3.98 mg/m^2), both presenting similar coverages; Myo−latex (0.69 mg/m^2) and BSA−latex (2.26 mg/m^2 complex sensitized at pH 5), which were the highest coverages obtained for these two proteins. The μ_e of the bare latex also was measured as a blank. Results are shown in Figure 3. The most important feature that can be extracted from this experiment is the pI (pH at

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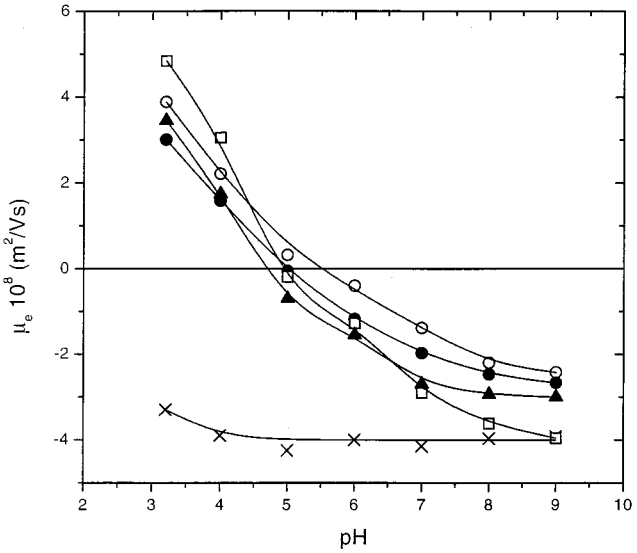

Figure 3. Electrophoretic mobility versus pH: ×, bare latex; ○, IgG 3.80 mg/m^2; ●, fibrinogen 3.98 mg/m^2; □, myoglobin 0.69 mg/m^2; ▲, BSA 2.26 mg/m^2. Particle concentration in cell = 3 × 10^9 particles/mL.

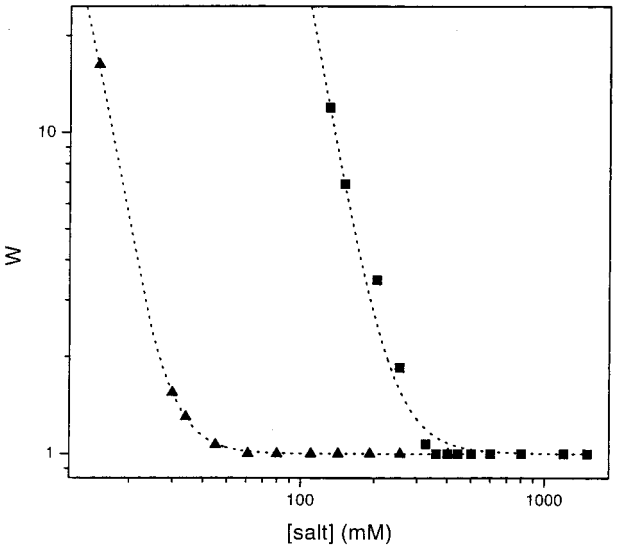

Figure 4. Dependence of the stability factor (W) on the electrolyte concentration for CMS latex: ■, NaCl; ▲, $CaCl_2$. Theoretical dependence (- - -) according to eq 8.

which $\mu_e = 0$) of the complexes. In all cases the pI is shifted toward lower pH values compared with the pI of the pure proteins (see Table 2). This behavior is explained by the influence of the polymer surface charge. The pI of the IgG and Fib complexes (having similar degrees of coverage) is 5.5 and 5.0, respectively. For the Myo and BSA complexes the pI is 4.9 and 4.6, respectively. There was a loss of colloidal stability of our systems at their corresponding pI values, as expected.

At pH 8−9 the μ_e values of IgG, Fib, and BSA complexes differ from the μ_e of the bare latex and the Myo complex (which almost coincide). Electrophoretic mobility reflects the ζ-potential of the colloidal particles but not the superficial potential Ψ_o. As a smooth polymer surface is being covered by macromolecules, it becomes more ridged and irregular. This shifts the slipping plane of the complex outward causing a decrease in the ζ-potential, and thus in μ_e. This is observed for our protein−latex complexes except Myo−latex. In this case myoglobin is the smallest protein (see Table 2) and the above-mentioned explanation would not be applicable. In addition, the coverage of this complex is also low, and one could think that the protein almost would not affect the μ_e value of the bare latex. However this reasoning is erroneous, because the presence of myoglobin completely changes the electrokinetic behavior of the latex at pH values less than 8.

4.3. Colloidal Stability. *4.3.1. Colloidal Stability of Bare Particles.* First, we evaluate the influence of the counterion valence on the stability of the bare latex, and compare our experimental results with the DLVO theory.

Figure 4 shows the dependence of W with the electrolyte concentrations of NaCl and $CaCl_2$ for the bare latex. W decreases gradually with increasing salt concentration until a certain value is reached (critical coagulation concentration, ccc) and the curve remains parallel to the concentration axis. The latter part of the curve corresponds to the region of rapid aggregation, whereas the former part corresponds to the slow aggregation regime. Adding salt dispersion initiates aggregation by suppressing the double-layer repulsion between particles. According to the DLVO, below the ccc, the thickness of the electrical double-layer repulsion decreases with increasing salt concentration. Double layer is entirely suppressed, above the ccc, and the aggregation rate is independent of the salt concentration (rapid aggregation). Reerink and Overbeek[37] have shown, considering several approximations, a linear relationship between log W and log electrolyte concentration. From this linear dependence an approximated value of the ccc can be obtained for both counterions, which is 340 ± 40 mM and 40 ± 8 mM for Na^+ and Ca^{2+}, respectively. It is clearly the highest screening effect for divalent cation. Shulze[38] indicated that the coagulating power of salts increased very strongly with the charge number of the cations.

The Hamaker constant and the diffuse potential of the latex were obtained by fitting the theoretical expression of W (eq 8) to the experimental values (Figure 4). The obtained values were $A = 0.37 \times 10^{-20}$ J, $\Psi_\delta = -16.4$ mV for Na^+ and $A = 0.60 \times 10^{-20}$ J, $\Psi_\delta = -17.0$ mV for Ca^{2+}.

With use of the Stern potential and Hamaker constant values obtained this way, estimations of the total potential energy of interaction curves were computed for both counterions (Figure 5). It can be observed that the increase in electrolyte concentration provokes a decrease in the height of the potential maximum. The latter, that prevents aggregation, finally disappears when the electrolyte concentration is similar to the experimental ccc in agreement with the theoretical prediction.

When we compare the Hamaker values obtained for the two counterions, the A value increases with decreasing ccc. Similar results are found in the literature.[39−41] Because the Hamaker constant is considered to be an intrinsic property of the material, the electrolyte should not affect its value. Apparently the counterion can react with the particles to change the calculated value of the Hamaker constant measured by the coagulation experiment, thereby revealing the incompleteness of the theory. The general explanation for this discrepancy is the dependence on ionic concentration of the zero-frequency contribution of the van der Waals force. This zero-frequency part is reduced by a factor roughly proportional to $\exp(-2\kappa H)$.

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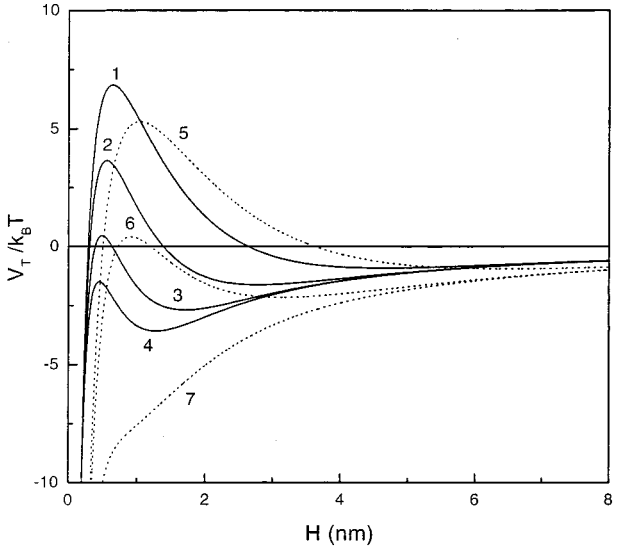

Figure 5. Calculated total interaction potential (V_T in k_BT units) versus distance for CMS latex: (1) 95 mM NaCl; (2) 200 mM NaCl; (3) 360 mM NaCl; (4) 600 mM NaCl; (5) 15 mM $CaCl_2$; (6) 40 mM $CaCl_2$; (7) 200 mM $CaCl_2$.

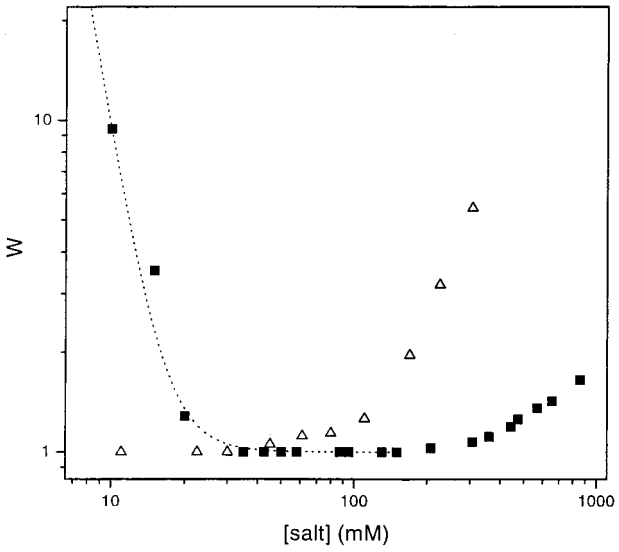

Figure 6. Dependence of the stability factor (W) on the electrolyte concentration for CMS−IgG complex with 3.45 mg/m^2: ■, NaCl; △, $CaCl_2$. Theoretical dependence (- - -) according to eq 8.

4.3.2. Colloidal Stability of IgG-Covered Particles. In this section we study the influence of IgG on the stability of the CMS latex. Figure 6 shows the stability curve for a IgG−latex complex with a protein coverage of 3.45 mg/m^2 at pH 8 for NaCl and $CaCl_2$ as aggregating salt. (The net charge of the complex is negative and the cation is the counterion.) This diagram shows two regions: a DLVO and a nonclassical DLVO region. In the first region, aggregation occurs as expected, increasing gradually with increasing concentration until the rapid regime is reached. It is striking that it may be necessary to add electrolyte amounts for aggregation smaller than those needed for coagulation of the bare latex. The ccc value for the IgG−latex complex aggregated by Na^+ is 30 ± 8 mM, whereas the value for the bare latex system is 340 ± 40 mM.

Within the DLVO theory the colloidal stability should be minimal and independent of the salt concentration for values higher than the ccc. However, the colloidal stability of the IgG−latex system increases when the salt concentration is further increased (nonclassical DLVO region). The minimum electrolyte concentration provoking this effect is referred to as the critical stabilization concentration (csc)[42,43]. The csc values for Na^+ and Ca^{2+} are 170 ± 30 mM and 32 ± 6 mM, respectively. The value found for the csc in our system is in line with those described for bubble coalescent and mica surfaces, ranging from 0.1 to 0.2 M for 1:1 electrolytes.[7,44]

The effect of counterion valence in the non-DLVO region and in the classical DLVO region is opposite. In the non-DLVO region the coagulating power of cation increases with the decreasing charge number (not an expected trend).

This anomalous stability has been explained previously by the so-called hydration forces.[43,45] Experimental investigations of the coagulation properties of a wide range of colloidal dispersions suggest that not all systems can be explained by use of the classical DLVO theory. The structure of water near an interface differs from that in the bulk, and this hydration might influence the interaction between particles at short distances. Consequently, the classical DLVO theory generally fails to predict the stabilities of very hydrophobic or very hydrophilic particle suspension. For example, the colloidal stability of silica at its isoelectric point,[46] prevention of bubble coalescent at high ionic strength,[44] deposition of polystyrene latex on glass surfaces,[47] swelling of clays,[48] and many hydrophilic colloidal particles, and most biological surfaces and macromolecules, remain separated in aqueous solution even in high salt or in the absence of any net surface charge.[49,50] Reported results with amphoteric charged latex also point to a deviation in the behavior with respect to the classical DLVO theory.[42]

It is well established that water molecules strongly bind to protein surfaces.[51] As ionic strength increases above the csc, hydrated cations adsorb to hydrophilic proteins and they presumably retain some of their water of hydration.[10] An overlap of the hydrated cations near the two mutually approaching particles creates a repulsive hydration force which could be described thermodynamically as a decrease in entropy.

Other different possibilities could justify this anomalous behavior of protein−latex complexes with high salt concentrations. The first possibility is steric stabilization due to the macromolecule nature of the protein. This effect could be overriden by electrostatic forces at low ionic strength, but it could predominate with high ionic strength. Second, electrostatic attraction between amphoteric complex surfaces can be a major force in aggregation, diminishing the strength with increasing salt concentration.

With any of these possibilities, however, a more or less symmetric behavior should be expected for similar opposite net charge in the surface complex. But, in a previous

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publication,[43] we observed that the anomalous stabilization mechanism only appears when the pH is above the pI of the complex (negative net charge) suggesting more of a relationship with the nature of ions. In fact, we observed the dependence of the anomalous stabilization mechanism with cation type in Figure 6, increasing their effect with the size of the hydrated cation. This result is in line with the suggested stabilization by hydration forces. On the other hand, the particle−particle van der Waals interaction may be reduced by the adsorbed layer water/cation, thus reducing this attraction with increasing ionic strength.

To justify this anomalous behavior, an extension to DLVO theory including hydration forces, and its dependence on salt concentration, is intended. 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:

$$V_T = V_A + V_E + V_h \tag{9}$$

where V_h is the repulsive hydration interaction energy.

The development of the theory of structural forces is in its initial stage so far. Theoretical work was initiated by Marcelja and Radic.[52] Immediately after the first report on measurements of exponentially decaying repulsive hydration forces between lipid bilayers, they formulated an elegant mesoscopic theory. Upon this initial approach, various molecular mechanisms where proposed. In the first approximation, the experimental data demonstrate that these forces (expressed by area unit), as a rule, depend exponentially on the interlayer thickness:[10,26,52]

$$P(H) = P_0\, e^{-H/\lambda} \tag{10}$$

the decay length (λ) is in the range 0.2−1.1 nm for 1:1 electrolytes, and it does not vary significantly with the ionic strength.[53] The hydration force constant P_o, determined experimentally for several different hydrophilic solid materials, lies within the range $10^6-5\times10^8$ Nm^{-2}.[54]

The question concerning the way by which a change in the electrolyte concentration influences hydration forces still remains to be solved. Some investigations show that they increase with increasing electrolyte concentration for rutile[55] and mica,[9] whereas the dependence in silica is opposite.[56]

Starting from the empirical exponential function to describe the hydration forces (eq 10) and using the Derjaguin approximation[56] for spheres of radius a, we can propose:

$$V_h(H) = \int_H^\infty \pi a P_0 \lambda\, e^{-H/\lambda}\, dH = \pi a P_0 \lambda^2\, e^{-H/\lambda} \tag{11}$$

Because we have observed a dependence of the non-DLVO 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):

$$V_h = \pi a (N_A C_h c_e) \lambda^2\, e^{-H/\lambda} \tag{12}$$

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

Including eq 12 on eq 9, estimations of the total potential energy of interaction as a function of the separation distance were computed and presented in Figure 7 for the IgG−latex complex. In the classical DLVO region, the total potential energy of interaction is obtained from eq 3 using the Hamaker constant (1.03×10^{-20} J) and the diffuse potential (−15.9 mV) obtained by fitting the theoretical expression of W (eq 8) to the experimental values (see Figure 6). For the non-DLVO region, the total potential is obtained from eq 9. Parameters λ and C_h have been adjusted to match experimental results. The obtained values ($\lambda = 0.6$ nm and $C_h = 1.6\times10^{-20}$ J) are similar to those found in the literature. For the decay length, our value agrees with the 0.2−1.1 nm range described for hydrophilic systems. On the other hand, the estimated C_h gives rise to values of P_o between 1.6×10^6 and 3×10^6 Nm^{-2} for 170 and 300 mM NaCl, respectively. They match within the range $10^6-5\times10^8$ Nm^{-2} previously cited and are close to the lower limit (as expected for a system not so hydrophilic as mica, for example).

In Figure 7, interaction potential maximum (energy barrier) decreases initially with increasing salt concentration, and so does stability. The electrolyte concentration at which V_{max} becomes zero is similar to the experimental ccc in agreement with the theoretical prediction. However, if this concentration is increased further, the barrier achieves a minimum and then starts to increase because of the hydration forces, becoming equal to 0 once again for an electrolyte concentration equal to the experimental csc. The appearance of a potential barrier could account for the anomalous stability at these high electrolyte concentrations.

4.3.3. Influence of the Protein Nature on Colloidal Stability. Hydration forces depend not only on the properties of the intervening medium (pH, ionic strength, counterion valence), but also on the chemical and physical properties of the surfaces, for example, whether they are hydrophilic or hydrophobic, whether amorphous or crystalline.

This stabilization mechanism was first observed with IgG adsorbed on the CMS latex. To generalize this mechanism, the study was extended to other proteins. Figure 8 shows the stability diagrams for IgG−latex (3.45 mg/m^2), Fib−latex (3.92 mg/m^2), and Myo−latex (0.57 mg/m^2) complexes at pH 8 using $CaCl_2$ as electrolyte. For the Myo−latex sample the NaCl was also used as aggregating salt.

As can be seen stabilization by hydration forces appears for the proteins when working with Ca^{2+}. The stabilization in the Myo−latex complex is rather low. This might be caused by two independent factors: on one hand, the protein coverage is too low for the myoglobin (0.6 mg/m^2 compared with 3.5 or 3.9 mg/m^2 for IgG or Fib, respectively).

This could explain why some characteristics of the Myo−latex complex are similar to those of the bare latex, as can be the μ_e at pH 8−9 or the ccc obtained with NaCl: 340 mM for the bare particles and 300 mM for the Myo−latex complex (see Figure 8). On the other hand, myoglobin is the most hydrophobic protein as shown in the Experimental Section. However, the hydration forces are relevant for hydrophilic surfaces. For the more "hydrophilic" proteins (IgG and Fib) the stabilization by hydration forces

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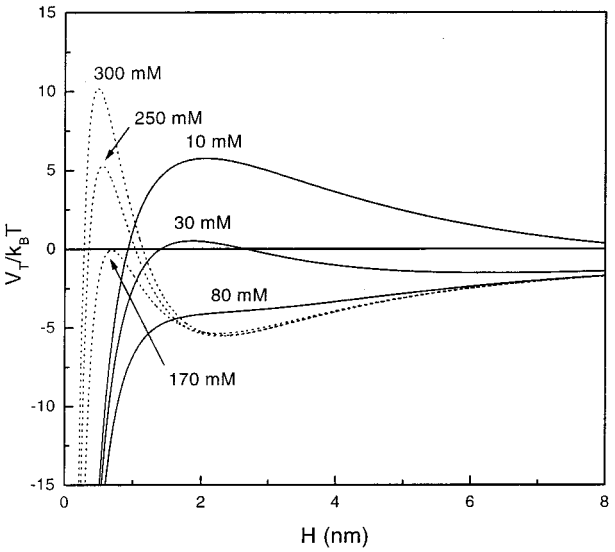

Figure 7. Calculated total interaction potential (V_T in k_BT units) versus distance for a IgG−latex complex with 3.45 mg/m^2 using the DLVO extended by inclusion of hydration forces. Hamaker constant (A), 1.03×10^{-20} J; radius (a), 102 nm; Stern potential (Ψ_δ), −15.93 mV; decay length (λ), 0.6 nm; hydration constant (C_h), 1.6×10^{-20} J.

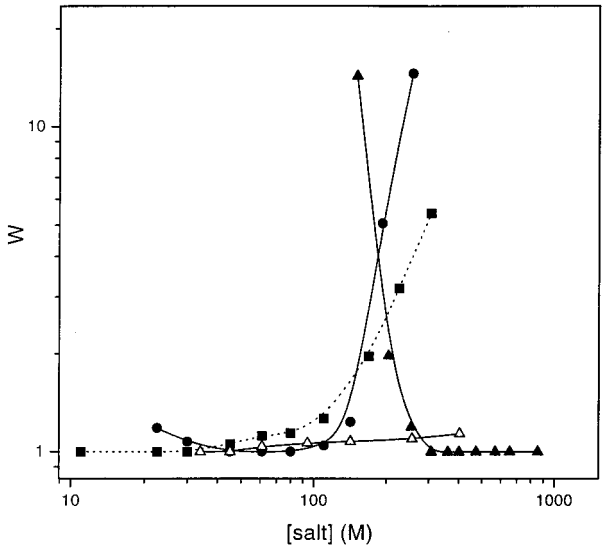

Figure 8. Dependence of the stability factor (W) on the $CaCl_2$ concentration for: ■, CMS−IgG 3.45 mg/m^2; ●, CMS−Fib 3.92 mg/m^2; △, CMS-Mio 0.57 mg/m^2; ▲, CMS-Mio 0.57 mg/m^2 with NaCl.

is significant for both electrolytes $CaCl_2$ and NaCl (data not shown). This stabilization phenomenon must also take place for the most hydrophilic protein, BSA. However it was experimentally impossible to corroborate this statement. The BSA−latex complex with 2.26 mg/m^2 was completely stable at this pH for all the electrolyte concentrations tested, and thus it is not possible to analyze the stabilization process by hydration forces if the system is not previously aggregated.

4.3.4. Influence of the Protein Conformation. The stabilization by hydration forces in latex−protein systems appears as a consequence of the hydrophilic character of the interface. This character depends on the nature of the protein layer, as shown previously, and probably on the conformation and orientation of the adsorbed protein.

To test this hypothesis we have studied the colloidal stability of two IgG-latex complexes at pH 8. They have

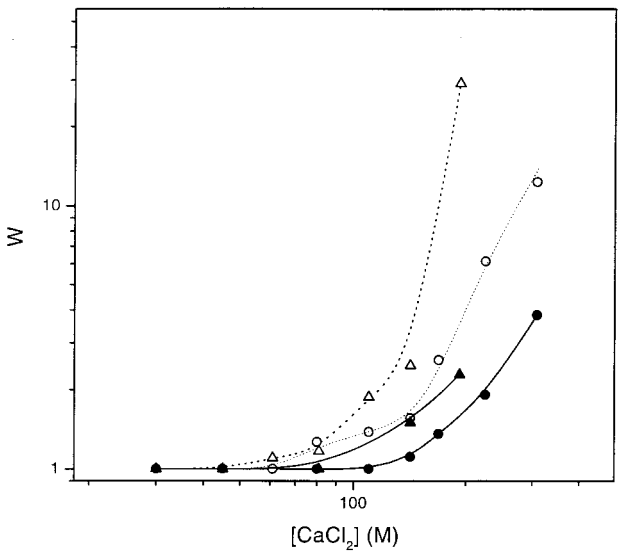

Figure 9. Dependence of the stability factor (W) on the $CaCl_2$ concentration for: ■, CMS−IgG; ▲, CMS−Fib. Open symbols correspond to complexes sensitized at pH 8. Close symbols correspond to complexes sensitized at pH 4.

identical coverage (2.9 mg/m^2) but one of them was sensitized at pH 8 (when the net charge of IgG is negative) and the other at pH 4 (the net charge of the protein and the polymer surface is opposite). A similar study was performed for two Fib−latex complexes obtained at pH 4 and pH 8, both with a protein-coating level of 2.8 mg/m^2. The data are plotted in Figure 9. Desorption of protein from the surface was tested after redispersion in pH 8 before the stability experiments. No desorption was observed. Similar patterns are observed for both proteins (Figure 9). The colloidal stability significantly depends on the adsorption pH.

The role played by the electrostatic forces during the adsorption process is considerable,[58] and these interactions can provoke preferential orientations when proteins are absorbed on the surface. It is plausible that the protein conformation in the adsorbed state depends on the adsorption pH. At pH 4 an electrostatic attraction exists between the positive protein and the negative CMS surface. The protein molecule would approach to the polymer surface facing it with the most hydrophilic (charged) patches or regions. On the other hand, when adsorbing at pH 8, the proteins exhibit their charged hydrophilic areas to be the solution. Therefore, when adsorption pH is above the isoelectric point of the protein, a higher hydrophilic orientation of the macromolecule is expected. The most hydrophilic is the surface, the larger is the stabilization by hydration forces. This is why the complexes sensitized at pH 8 are more stable in the non-DLVO region than those obtained at pH 4, which is observed for the both proteins.

Another strategy to study the influence of protein conformation on the colloidal stability is to follow colloidal aggregation of a IgG−latex complex (3.45 mg m^{-2}) at pH 8 at different times after sensitization (Figure 10). Stabilization can be seen with increasing time. Several authors[59,60] conclude that adsorbed proteins are in a slow dynamic state. They change their conformation with time,

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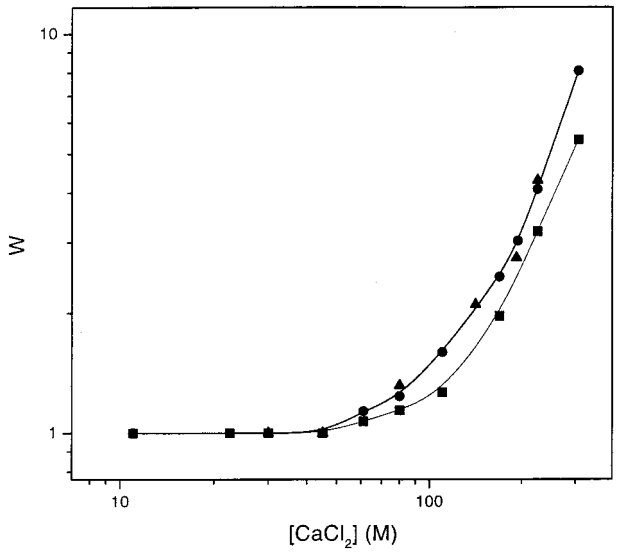

Figure 10. Dependence of the stability factor (W) on the $CaCl_2$ concentration for a CMS−IgG complex with 3.45 mg m^{-2} at pH 8 and different times after sensitization: ■, 1 day; ●, 9 days; ▲, 20 days.

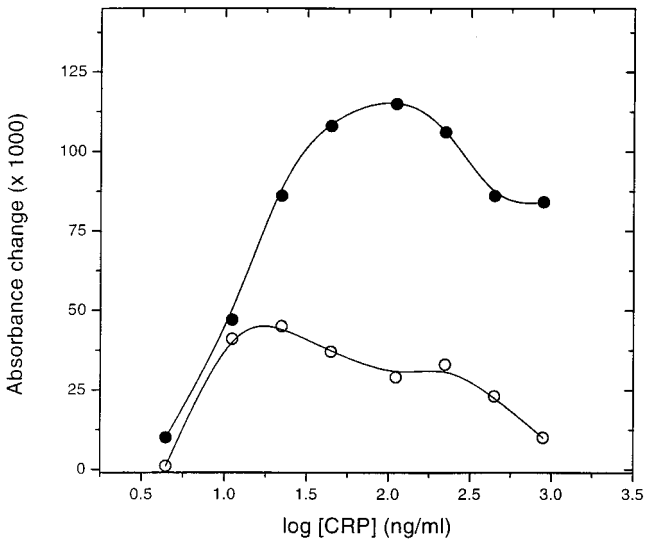

Figure 11. Absorbance changes during a 5 min reaction versus CRP concentration. ●, IgG adsorbed at pH 8 (2.91 mg/m^2); ○, IgG adsorbed at pH 4 (2.88 mg/m^2).

suggesting a tendency of the system to expose more hydrophilic sites to the protein−water interface, whereas the more apolar sites are oriented to the hydrophobic polymer surface.[61,62] Those changes 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 observed the experimental results shown in Figure 10 to be in line with a dependence of the stabilization mechanism on the hydrophilic nature of the interface.

It is worthwhile to highlight how the analysis of the colloidal stability in the non-DLVO region is sensitive enough to detect these conformational changes.

4.3.5. Immunoreactivity. To complete the study of preferential orientations of the adsorbed protein molecules as a function of the adsorption pH, immunoreactivity assays were performed. To make results comparable between different antibody−latex complexes, it is necessary to work with identical IgG coverages. Two complexes were obtained: one of them was sensitized at pH 8, where both latex and protein were negatively charged, and its coverage was equal to 2.91 mg/m^2; the other complex was sensitized at pH 4, and its coverage was similar to the former (2.88 mg/m^2). The stability results shown above demonstrate that differences exist between complexes obtained at pH values higher and lower than the protein pI. The immunoassays tried to corroborate such results. If the IgG molecules are adsorbed with different orientation at pH 8 and pH 4, the accessibility of the antigen for the binding sites of the antibody will be different and, thus, both complexes will differ in reactivity.

A first difference was obtained in the absence of CRP. The complex sensitized at pH 4 was not completely stable in the reaction medium, which has a salt concentration of 800 mM NaCl (data not show). This result agrees with those shown in Figure 9.

The reactivity of both complexes against CRP is shown in Figure 11. A typical precipitine (bell-shape) curve was obtained in both cases. However, significant differences can be appreciated. As can be seen, if IgG molecules are adsorbed at pH 8, the binding sites for the antigen are prominantly placed facing toward the solution. That is, the Fc moiety of the antibody tends to be facing toward the polymer surface. This antibody portion is more hydrophobic than the $F(ab')_2$ fragment, which is more hydrophilic. This is why, under electrostatic repulsion conditions (pH 8), IgG molecules tend to approach to the CMS surface by its less charged portion: the Fc one. In this case, the most hydrophilic (and charged) part, namely $F(ab')_2$, would be exposed to the aqueous medium. The explanation is similar to that stated in the stability results. At pH 4 the IgG molecules are adsorbed with the $F(ab')_2$ fragment near to the surface particle, maintaining the Fc portion exposed to the aqueous medium. This would explain why the complex sensitized at pH 4 possesses both low reactivity and low stability at high salt concentrations. It should be noted that the hydration forces do stabilize the complex sensitized at pH 8 in the same physicochemical conditions.

Finally, it is worth mentioning that, for a given IgG−latex complex, the aggregation process caused by the presence of antigen goes faster in low-stability conditions, that is when repulsion energies between particles are low.[63] So, if the binding sites were equally orientated for the complexes sensitized at pH 8 and pH 4, then the "pH 4 complex" would be more reactive, as it is more unstable in the reaction medium. Because this does not happen we can conclude that the IgG molecules adsorbed at pH values higher than the protein pI (i.e., pH 8) have a preferential orientation which differs from that of the ones sensitized at pH values lower than the protein pI (i.e., pH 4). It should be noted that we talk about a *preferential* orientation; we do not say that *all* molecules are adsorbed in the same way.

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5. Summary

An exhaustive study about a non-DLVO stabilization mechanism of colloidal particles covered by different proteins has been performed. This stabilization appears at high salt concentrations and it is caused by hydration forces. It has been considered not only qualitatively but also quantitatively. A new hydration repulsion potential (V_h) has been included in the classical DLVO theory. Four proteins have been tested. It has been shown that the efficiency on the stabilization highly depends on the hydrophobic/hydrophilic character of the adsorbed protein, observing low stability for the most hydrophobic protein (myoglobin).

In addition, the stability study on the non-DLVO region allows obtaining relevant information about the orientation of the adsorbed protein molecules and about conformational changes that take place slowly in the adsorbed state. The results given by the stability studies concerning the orientation of the protein molecules have been corroborated by immunoreactivity work. It has been shown that when protein and surface possess the same sign of charge, protein molecules tend to adsorb on the surface exposing its most hydrophilic (charged) areas facing toward the bulk.

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Nomenclature

a = particle radius
A = Hamaker constant
D = particle diameter
ccc = critical coagulation concentration
csc = critical stabilization concentration
c_e = salt concentration (in mM)
C_h = hydration constant
CMS = chloromethylstyrene
H = distance between the surfaces of two particles
I = ionic strength
I_θ = intensity of light dispersed to an angle θ.
k = rate constant
k_r = rate constant for rapid coagulation
k_s = rate constant for slow coagulation
N_A = Avogadro number
n_s = number of primary particles
$P(0)$ = hydration force per unit area for $H = 0$
$P(H)$ = hydration force per unit area as a function of H
PDI = polydispersity index
pI = isoelectric point
$u = (H - 2a)/a$
V_A = attractive interaction energy caused by van der Waals dispersion forces
V_E = repulsive interaction energy caused by electrical forces
V_h = repulsive interaction energy caused by hydration
V_T = total interaction energy
W = Fuchs' stability ratio
z_i = ion valence

Greek Letters

β = hydrodynamic correction factor
γ = hyperbolic tangent of the dimensionless Stern potential divided by 4
Γ = amount of adsorbed protein
Γ_{pl} = amount of adsorbed protein in the adsorption "plateau"
Δ = thickness of the Stern layer
ζ = zeta potential
θ_a = advancing contact angle
κ = Debye parameter
λ = decay length
μ_e = electrophoretic mobility
ρ_i = concentration of ion "i"
σ = surface charge density
$\Sigma_{protein}$ = extinction coefficient of a protein
Ψ_0 = surface potential
$\Psi\delta$ = Stern potential

Proteins

IgG = Immunogamma globulin G
Fib = Fibrinogen
Myo = Myoglobin
BSA = Bovine serum albumin
CRP = C-reactive protein

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