Existence of Hydration Forces in the Interaction between Apoferritin Molecules Adsorbed on Silica Surfaces

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The atomic force microscope, together with the colloid probe technique, has become a very useful instrument to measure interaction forces between two surfaces. Its potential has been exploited in this work to study the interaction between protein (apoferitin) layers adsorbed on silica surfaces and to analyze the effect of the medium conditions (pH, salt concentration, salt type) on such interactions. It has been observed that the interaction at low salt concentrations is dominated by electrical double layer (at large distances) and steric forces (at short distances), the latter being due to compression of the protein layers. The DLVO theory fits these experimental data quite well. However, a non-DLVO repulsive interaction, prior to contact of the protein layers, is observed at high salt concentration above the isoelectric point of the protein. This behavior could be explained if the presence of hydration forces in the system is assumed. The inclusion of a hydration term in the DLVO theory (extended DLVO theory) gives rise to a better agreement between the theoretical fits and the experimental results. These results seem to suggest that the hydration forces play a very important role in the stability of the proteins in the physiological media.

I. Introduction

The interaction between protein molecules underlies many biological, chemical, and technological processes. Protein interactions play a role in several diseases, such as cataracts,1–3 sickle cell anemia,4 cryoimmunoglobulinemia,5 and plaque formation.6 Different studies indicate that a deeper understanding of protein interactions may suggest strategies for treating or preventing certain diseases. From a technological perspective, association of protein molecules facilitates numerous separation processes; crystallization of protein is one example, and it is also important in characterizing the macromolecule itself. In some instances, however, this association is detrimental: for example eye lens transparency depends on the aggregation of γ-cristallin proteins in physiological conditions.7 The knowledge of the protein interactions is an essential step in understanding numerous cellular and technologically relevant processes.

The growth of protein crystals proceeds by mechanisms that are still not well understood. At present protein crystallization is still mostly achieved experimentally by “trial and error”, and on the basis of a number of empirical rules.8 There is clearly a need for a more fundamental understanding of the mechanisms controlling protein crystallization, and this obviously requires a good knowledge of the forces between protein molecules in solution and of their dependence on solution conditions, including pH and salt concentration.8–10 The design of a crystallization protocol requires knowledge of the thermodynamics properties of protein solutions. Protein solution thermodynamics is governed by temperature, pressure, protein concentration, and the strength of protein−protein forces. These forces, which result in an effective (repulsive or attractive) interaction potential, can be tracked with a variety of techniques such as X-ray scattering, light scattering, and osmotic pressure.11,12 Some evidence suggests that there is a direct correlation between the pair interactions and crystallization behavior of proteins.13,14 mediated by a correlation between the solubility and the strength of these pair interactions.15

Investigations of protein solution behavior have relied on the strong similarities of the molecular properties of

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protein solutions to those of colloidal dispersions. These macromolecules in solution are charged Brownian objects with sizes between a few nanometers and a few tens of nanometers. Hence the macroscopic properties, dynamics, and stability of protein solutions have been described within the framework of the methods and approaches relevant to colloid science. The interaction between two protein molecules can be considered by taking into account different contributions to the interaction free energy. The currently used models in the colloid field are derived from the DLVO theory (Derjaguin, Landau, Verwey, Overbeek), where two types of interactions play a major role: van der Waals attraction and electric double layer repulsion. Generally, in this theory, the colloidal particles are assumed to be at infinite dilution. Thus, the analysis can be confined to the study of the interaction of a pair of colloidal particles. On the other hand, the solvent is treated as a continuous dielectric medium and the ions are taken to be point ions.

Different experimentally observed features of stability behavior, such as the stability of some colloids at high electrolyte concentrations,\textsuperscript{14,15} contradict the DLVO theory. These experiments have stimulated a number of papers dedicated to the problem of the description of the interaction between colloidal particles or surfaces such as smooth mica,\textsuperscript{20,21} glass fiber,\textsuperscript{22} rutile,\textsuperscript{23} lipid\textsuperscript{24} and nonionic surfactant bilayers,\textsuperscript{25} proteins,\textsuperscript{26,27} etc., immersed in an electrolyte solution. It follows, and at present it is well recognized, that in many cases DLVO theory breaks down for the treatment of the short-range interaction between colloidal particles. The experiments led to the conjecture that another repulsion (a non-DLVO one), due to the organization of the solvent around particles, should also be present. This force has been attributed to a modified water structure at the surface and this water is commonly referred to as “structured”. This repulsion is called the “structural” or “hydration” force. Different works in the literature, like those of Parsegian et al.,\textsuperscript{30–32} point out the importance of hydration effects in systems such as proteins (collagen). It is important to underline that with proteins (and also with mica and with amphoterics latices) the hydration repulsion, in contrast with the case of the electric double layer repulsion, is usually greater as the electrolyte concentration increases.\textsuperscript{33,34}

In the past few years, different devices have been developed to directly measure the interaction between two surfaces. The two most important ones are the surface force apparatus (SFA)\textsuperscript{35} and the atomic force microscope (AFM).\textsuperscript{36} Usually, systematic studies on protein interaction have been restricted to small molecular weight model proteins such as lysozyme, where a combination of indirect techniques has been employed (light scattering, osmotic pressure, small-angle X-ray scattering, etc.).\textsuperscript{37–39} However, recently, a few works have appeared in the literature in which the interactions of different proteins have been studied by means of the SFA.\textsuperscript{40,41} The present paper reports experimental force–distance data obtained by the AFM technique for the approach of silica surfaces covered with the apoferritin protein.

Apoferritin protein represents an unique globular model system for studying the solution structure and dynamics of colloid-like biopolymers because the form factor of apoferritin is well-known: it is a very symmetric and nearly spherical molecule with approximately 12 nm in outer diameter.\textsuperscript{42} Apoferritin is an iron storage protein consisting of 24 peptide subunits joined through noncovalent linkages. One of these subunits forms a cavity for iron ions inside the molecule. This molecule carries a net negative charge at neutral pH that ensures its excellent solubility in water due to electrostatic interparticle interactions. Fewer studies have been devoted to the interaction between the apoferritin molecules. The aggregation of apoferritin in solution leading to the creation of dimers and multimers has been investigated recently.\textsuperscript{13,43} A few years ago, the influence of the salt concentration on the intermolecular distances in adsorbed layers of apoferritin molecules was studied.\textsuperscript{44,45} When iron is present in the core (the protein is then referred to as ferrit instead of apoferritin), the protein is easily observable by electron microscopy. Moreover, its magnetic properties allow detection of this protein by means of magnetic imaging methods, which is used in medical examinations.\textsuperscript{46,47} The adsorption of ferritin on different substrates has been also studied by imaging with an AFM.\textsuperscript{48}

The goal of the current investigation was to quantitatively demonstrate the significance of hydration forces in protein–protein interactions. It was assessed through comparisons of DLVO and extended DLVO (with a hydration force term) theory predictions with AFM direct measurements of colloid interparticle interactions.
force measurements. The forces between protein layers were investigated in a range of electrolyte concentrations, type of electrolyte, and solution pH values.

II. Materials and Methods

The apoferritin (obtained from equine spleen) used in the experiments was obtained from Sigma. All chemicals used were of analytical grade quality. The pH was controlled using different buffers (acetate at pH 5 and phosphate at pH 7, at constant ionic strength 2 mM); a dilute HCl solution was used to keep the medium at pH 3. Double distilled water (Milli-Q System) was utilized to prepare these buffers. The solutions of higher electrolyte concentrations used in the interaction experiments were prepared by the addition of NaCl or CaCl₂ to these buffers.

The apoferritin used in the experiments is a protein with a molecular weight around 480 000 g/mol and with an isoelectric point around 4.0 from a silica wafer (CSIC, Spain).

The AFM (Digital Instruments, U.S.A.) and the colloid probe were prepared to measure the interactions between apoferritin layers. With this aim, 1 mg/mL apoferritin solution was prepared. With double distilled water.

Silica spheres, with a diameter of 5 μm, were supplied by Bangs Lab. (U.S.A.). A colloidal silica particle was glued to the end of the cantilever using an optical microscope and a micromanipulator arm. Silica planes, with an area around 1 cm², were obtained from silica wafer (CSIC, Spain).

The force measurements were performed using a Nanoscope III AFM (Digital Instruments, U.S.A.) and the colloid probe technique. The technique used in this study is well described in the literature. In brief, the colloidal particle is mounted on a cantilever, and the flat surface is displaced in a controlled manner toward and away from the probe in an aqueous medium. The interacting forces between the probe and the flat surface can be obtained from the deflection of the cantilever, which is monitored by a laser beam reflecting off the back of the cantilever to a split photodiode. Deflection of the cantilever versus displacement of the flat surface data is supplied directly by the AFM. These raw data can be converted into a force versus separation curve by assuming a zero point for both the separation and the force. The zero point of force is obtained from the baseline of the raw data, where the cantilever deflection is constant (the plane and the sphere are far apart). The zero point for separation distance is obtained from the “compliance region” of the raw data, where the cantilever deflection varies linearly as a function of the plane displacement (the plane and the sphere move jointly).

To scale the force measurements correctly, the spring constant of the AFM cantilevers (which were standard V-shaped tipless cantilevers) must be known accurately. A value of 0.12 N/m was achieved using the resonance method proposed by Cleveland et al.

Prior to injection of the protein, the interactions between the silica surfaces were measured, and it was confirmed that characteristic silica type interactions were observed (results not shown). After the protein adsorption, different solutions (free of proteins) were injected in the fluid cell of the AFM to study the effect of pH and salt concentration on the interaction between silica surfaces covered with apoferritin. These interaction curves were clearly different from the interaction curves between bare silica surfaces: the presence of a compressible region in the approach curves and adhesions in the separation curves is unmistakable evidence of the existence of a protein layer adsorbed on the silica surfaces. It is important to remark that the curvature of the colloidal probe, the dimensions of the protein molecule, and the adsorption conditions used, maximum protein adsorption, guarantee that the interaction takes place between numerous apoferritin molecules adsorbed on the probe and numerous apoferritin molecules adsorbed on the plane substrate.

When studying protein interactions via AFM, it is important to determine how readily conformational changes in the adsorbed layer occur. In all cases reported herein, the interaction force curves were very reproducible over many consecutive compression cycles at the same conditions of pH and salt concentration (this means that all of the force curves obtained at the same medium conditions overlap each other). This fact evidences that the protein adsorbed strongly to silica surfaces and that the pressure generated between the surfaces did not seem to induce any irreversible structural changes in the adsorbed layers. The force curves obtained at certain conditions, even after changing the medium pH and salt concentration and going back to the original conditions (changes in pH from pH 7 until pH 3 and vice versa at fixed salt concentration; changes in salt concentration from high value until low value and vice versa at fixed pH), were also very reproducible, which suggests that protein did not desorb nor underwent irreversible conformational changes. Measurements with different spheres and substrates were also highly reproducible.

III. Theoretical Background

In the classical DLVO theory, the total potential energy \( V_T \) between two interacting particles is given by

\[
V_T = V_E + V_A
\]

(1)

The attractive London-van der Waals energy \( V_A \) between a sphere and a plane can be expressed as

\[
V_A = -\frac{AR}{6\epsilon}
\]

(2)

where \( R \) is the particle radius, \( x \) is the distance between the surfaces of the sphere and the plane, and \( A \) is the Hamaker constant of the system. Equation 2 does not take the electromagnetic delay into account. [The van der Waals interaction force should be calculated taking into account that the system is constituted by five layers: silica—apoferritin—aqueous medium—apoferritin—silica. However, when the interaction is of short range like in our case, the van der Waals force can be approximated to the case of a simplified system of three layers: apoferritin—aqueous medium—apoferritin. In fact, the van der Waals interaction between bare silica surfaces (the Hamaker constant in aqueous medium is \( 8.3 \times 10^{-21} \) J²) was negligible beyond 5 nm of separation (result not shown). Since the thickness of an adsorbed layer of apoferritin is negligible beyond 5 nm of separation (result not shown), it is important to remark that the curvature of the colloidal probe, the dimensions of the protein molecule, and the adsorption conditions used, maximum protein adsorption, guarantee that the interaction takes place between numerous apoferritin molecules adsorbed on the probe and numerous apoferritin molecules adsorbed on the plane substrate.] The electrostatic energy \( V_E \) between a sphere and a plane under boundary conditions of constant surface potential (in the case of low surface potentials) is given by

\[
V_E = \frac{2}{3} \pi R^3 \epsilon \kappa^2
\]

where \( \kappa \) is the Debye length, and \( \epsilon \) is the permittivity of the medium.


(53) Giesbers, M.; Bijsterbosch, C. J. Colloid Interface Sci. 1976, 52, 251.


(56) Giesbers, M.; Bijsterbosch, C. J. Colloid Interface Sci. 1976, 52, 251.


Apoferritin Molecules Adsorbed on Silica Surfaces

The simplest expression for the hydration forces is the following empirical relation:

\[
\frac{F_{\text{HYD}}}{R} = C_H \exp\left(-\frac{x}{\lambda}\right)
\]

where \(C_H\) is a hydration constant and \(\lambda\) is the decay length. Both parameters have been also considered as fitting parameters.

A parameter that is needed is the Hamaker constant, a material property that characterizes the strength of the van der Waals interactions. To our knowledge, the Hamaker constant for apoferritin has not been reported in the literature. In this paper, this constant has been determined by two different ways. First, it can be calculated via Lifshitz theory, where spectroscopic data are needed. In this case, the Hamaker constant is equal to\(^{(29)}\)

\[
A = \frac{3}{4} \frac{kT}{\varepsilon P} \left(\varepsilon_P - \varepsilon_S\right)^2 + \frac{3h\nu}{16\sqrt{2}} \left(n_P^2 - n_S^2\right)^2
\]

where the subscripts P and S refer to protein (apoferritin) and solvent (water), respectively. \(\nu\) is the refractive index, \(\epsilon\) is the dielectric constant, and \(\nu\) is the ionization potential of the protein. The Hamaker constant has been estimated from the values \(\epsilon_P = 80, n_S = 1.33, \epsilon = 1.97,\) and \(\nu = 12\) eV.\(^{(81)}\) The result is \(4.30 \times 10^{-21}\) J.

On the other hand, an alternative way to determine the value of the Hamaker constant for the interaction apoferritin–water–apoferritin (\(A_{\text{PWP}}\)) is the surface thermodynamic approach of van Oss.\(^{(62)}\) \(A_{\text{PWP}}\) can be expressed as

\[
A_{\text{PWP}} = (A_p^{1/2} - A_w^{1/2})^2
\]

being \(A_w\) and \(A_p\) the Hamaker constants of water and protein, respectively, in a vacuum. The literature value for \(A_w\) is \(3.7 \times 10^{-20}\) J.\(^{(29,63)}\) According to the approach of van Oss, the surface energy of a solid, as well as the solid–liquid interfacial energy, may be separated in two components. The first is the apolar (Lifshitz–van der Waals) component \(\gamma_L^{\text{LW}}\) and the second is the polar (Lewis acid–base) component \(\gamma_A^{\text{AB}}.\) It can be shown that there is a reliable proportionality between the Hamaker constant of a material (\(A\)) and the apolar surface tension component \(\gamma_L^{\text{LW}}\)

\[
\gamma_L^{\text{LW}} = \frac{A}{24\pi l_0^2}
\]

where \(l_0\) is a minimum equilibrium distance whose value is \(1.568\) Å.\(^{(62)}\)

Contact angles \(\alpha\) measured with pure Lifshitz–van der Waals liquids yield the \(\gamma_S^{\text{LW}}\) of the solid, once the \(\gamma_L^{\text{LW}}\) of the liquid is known, according to the Young–Dupré equation:\(^{(62)}\)

\[
1 + \cos \alpha = 2\left(\gamma_S^{\text{LW}}/\gamma_L^{\text{LW}}\right)^{1/2}
\]

We have used this approach to obtain the Hamaker constant of the apoferritin. Glass microscope slides were immersed in a solution of apoferritin (1.2 mg/mL, 0.1 M NaCl, pH 4.9) for a few hours. After the protein adsorption took place, the microscope slides were left to dry for several
The contact angle was measured by means of the axisymmetric drop shape analysis-profile (ADSA-P) technique. The apolar liquid used was diiodomethane \((\gamma_{\text{LW}} = 50.8 \text{ mJ/m}^2)\). The measurements carried out on the same and on different slides were very reproducible. An averaged value of \((47.5 \pm 1.9)^\circ\) was obtained for the contact angle of diiodomethane on apoferritin layer. Using eqs 11–13 (where the solid is the apoferritin layer and the liquid is diiodomethane), the estimated value of the Hamaker constant for apoferritin across water \((A_{\text{HWP}})\) is \(4.24 \times 10^{-21} \text{ J}\). As can be seen, the Hamaker constant value obtained from Lifshitz theory is similar to that obtained from the approach of van Oss.

**IV. Results and Discussion**

**Interaction in the Presence of NaCl.** The interaction force profiles for an apoferritin-coated colloid probe and an apoferritin-coated planar surface as a function of pH at low NaCl concentration \((0.01 \text{ M NaCl})\) are presented in Figure 1. These curves (and all of the curves shown in this paper) correspond to the interaction when surfaces approach each other. The forces measured on separation show adhesive minima that are characteristic of adsorbed polymer or protein layers. However, only the forces on approach are of interest for the current work.

The forces between apoferritin adsorbed silica surfaces shown in Figure 1 are monotonically repulsive at pHs 3 and 7. Proteins are amphoteric molecules whose surface charge depends on the pH of the medium. When the latter is away from the isoelectric point \((\text{iep})\) of the protein \((\text{pHs} 3 \text{ and } 7)\), the charges on both protein layers get higher and the interaction at large separations is dominated by an electrical double layer repulsion. Although the apoferritin in solution has a higher charge at pH 3 (positive charge) than at pH 7 (negative charge) according to titration works,\(^{50}\) it can be seen in Figure 1 that the magnitude of the repulsion at pH 7 is higher than at pH 3. This may point out that the apoferritin exposes more acid (negatively charged) groups than basic (positively charged) groups toward the solution when it is adsorbed on silica surfaces. This makes sense since, in the adsorption conditions, silica is negatively charged; the protein could orientate most of the basic (positively charged) groups toward the silica surface in the adsorption process.

At the time to fit the experimental results eqs 7 and 9 must be modified. The reason of such modification is the uncertainty in the origin of separation distances \((x = 0 \text{ nm})\). One of the main disadvantages of the AFM is the impossibility of directly determining the separation distances between the interacting surfaces. This separation is inferred by considering the surfaces to be in contact (null separation, \(x = 0 \text{ nm}\)) when they move jointly (“compliance region”). This is true when the surfaces are hard. However, if there is a compressible layer on the surface, the situation of the plane \(x = 0 \text{ nm}\) corresponds to the plane of maximum compression of the layer (see Figure 2); the thickness of that layer is experimentally inaccessible including the AFM. Therefore, eqs 7 and 9 must be modified including the displacement \(2d\)

\[
\frac{F_{\text{DLVO}}}{R} = 2\pi\kappa \times \exp(-\kappa(x - 2d) + \exp(-\kappa(x - 2d)(\Psi_s^2 + \Psi_p^2) - \frac{1}{1 - \exp(-2\kappa(x - 2d))}) - \frac{A}{6(x - 2d)^2}) \tag{14}
\]

\[
\frac{F_{\text{HYD}}}{R} = C_H \exp\left(-\frac{(x - 2d)}{\lambda}\right) \tag{15}
\]

One difficulty associated with the evaluation of the van der Waals force between protein-coated surfaces is that protein layer/water interface is not smooth on a molecular scale. Thus, the origin plane of the London—van der Waals force is therefore also not known with precision. In our analysis, therefore, the position of this plane with respect

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to that of the maximum protein layer compression ($x = 0$ nm) is determined by the parameter $s$ (see Figure 2), which is obtained directly from the fitting of the experimental results.

Just as is the case for the London–van der Waals force, the presence of the protein layers makes it impossible to accurately ascribe a location of the origin plane of the double layer forces. Ohshima et al. have proposed a potential distribution model for hard particles coated by an ion-penetrable charged surface layer, which has been used to describe the electrophoretic behavior of protein-coated particles. According to this model, when the surface-charged layer is thicker than the inverse of the Debye–Hückel parameter, the potential within the surface-charged layer is in practice equal to the surface potential at the boundary between the protein layer and the surrounding solution. As the electric double-layer interaction is governed by the interfacial potential, we have assumed that the origin plane of the electrostatic repulsion corresponds to the limit of the protein layer.

With these assumptions, eq 14 is modified again to introduce a displacement between the planes of origin for both interactions (electrostatic and van der Waals, see Figure 2):

$$F_{\text{DLVO}} = \frac{2\pi \varepsilon \kappa}{R} \times \exp(-\kappa(x - 2d)) \frac{2W_s\Psi_P + \exp(-\kappa(x - 2d))(\Psi_S^2 + \Psi_P^2) - 1 - \exp(-2\kappa(x - 2d))}{6(x - 2s)^2}$$ (16)

In all figures corresponding to protein–protein force profiles, a separation distance of zero ($x = 0$ nm) is defined as the point at which the protein layers are completely compressed. It is important to emphasize that the experimental data have been fitted until the point of initial contact of the apoferritin layers ($x = 2d$). This distance depends on the experimental conditions, as can be seen in the different figures shown in this work (a vertical dotted line is introduced in all the figures to indicate it). Below that position the steric interaction comes into play. Complete modeling of the compression of an adsorbed protein film is well beyond the scope of this experimental article and provides a significant challenge to the theoretical biophysicists which has yet to be solved. Therefore, theoretical predictions to take the steric repulsion into account are not included in the fits (that is why the experimental points have been fitted until the point of initial contact of the apoferritin layers).

The best fits were obtained considering electrostatic interaction under constant surface potential boundary conditions and with $s = 0$ nm. This value of $s$ means that the origin plane of the van der Waals interaction coincides with the plane of maximum compression of the protein layer, in concordance with the assumption proposed by different authors in their SFA analysis. It should be noticed that, considering a constant value for the Hamaker constant, we have implicitly assumed that the protein layer is a homogeneous phase with an uniform density. However, this is strictly not true, especially in the outer parts of the protein layer. However, it could be acceptable to think that the contribution of the most external part of the protein layer to the London–van der Waals interaction should be somewhat smaller than the contribution of the rest of the protein layer due to a lower protein density in that region.

Figure 3 shows the effect of NaCl concentration on the interaction force between apoferritin layers at pH 3. The force curve at 0.01 M NaCl is repulsive at large separation distances and decays exponentially as the separation distance increases. The addition of electrolyte suppresses the strength and reduces the range of the electrostatic interactions. At high salt concentration (1 M), the electrostatic contribution is completely screened and the force becomes attractive under the influence of the London–van der Waals interaction between the coated surfaces.

Using the above approach, predictions based on DLVO theory (eq 16) are also shown in Figure 3. The fitting parameters are summarized in Table 1. The parameter $d$ was obtained from the interaction curve at 1 M NaCl, where a minimum (attraction) followed by the steric repulsion is observed. We have considered that the protein layers come into contact at the minimum position ($2d$ around 5 nm for pH 3). In accordance with this assumption, each protein layer is compressed in 2.5 nm. It is important to notice that, in most of the globular proteins, the protein layer is compressed by 1–2 nm before a hard wall is attained.

It can be seen in Figure 3 that overall there is an acceptable agreement between the experiments and calculated forces. This agreement clearly demonstrates that the long-range forces observed in this system have an electrostatic origin, which corresponds to the overlap of the electrical double layers emanating from the adsorbed apoferritin. In fact, as the concentration of electrolyte increases the electrostatic repulsion between protein molecules is screened. (Since the buffer counterions were Na$^+$ ions in all cases (with the exception of pH 3, where HCl was used), they should be taken into account in the final electrolyte concentration. However, the ionic strength

![Figure 3. Normalized force versus separation distance $x$ for apoferritin-apoferritin interaction at pH 3 in different NaCl concentrations: □ 0.01 M; ○ 0.1 M; ▲ 1 M. The inset is a zoom of the graph. The solid lines represent the fits according to DLVO theory (eq 16). The fitting parameters are shown in Table 1.](attachment:figure3.png)
of the buffer is so low (2 mM) that it can be neglected by comparison with the ionic strength of the NaCl solutions. Only for the most dilute solution might the Na" ions from buffer be taken into account. On the other hand, the Na" ions from the apoferritin solution for adsorption do not represent any controversial point. As has been explained in the Materials and Methods section, after the protein adsorption on silica surfaces from a 1 mg/mL apoferritin solution (0.1 M NaCl, pH 5) the AFM fluid cell was rinsed by injecting large volumes of double distilled water. In this way, the nonadsorbed protein and the Na" ions of the original solution were removed from the system before to inject the aqueous solutions (free of protein) in which interaction forces were measured.\[Equations 16 and 15 were used to fit the experimental data. In every case \( s = 0 \) nm. Values of \( \pm 1 \) mV, \( \pm 0.2 \) nm, \( \pm 0.02 \) mN/m, and \( 0.02 \) nm can be considered as error estimates for surface potentials, \( d \), \( \gamma \), and \( \lambda \), respectively.

The interaction force data obtained between apoferritin-coated surfaces immersed in solutions at pH 5 containing different background electrolyte concentrations are presented in Figure 4a. Since this pH is close to the iep of the protein, the electrostatic repulsion is negligible and, therefore, no effect of salt concentration should be expected. However, examining all of the force curves, we note that the interaction force is more repulsive with increasing the ionic strength, contrary to the predictions of the commonly applied DLVO theory. It should be expected that the addition of electrolyte both suppresses the strength and reduces the range of the electrostatic interaction, with the van der Waals force becoming the dominant interaction at high salt concentration. We deduce that different types of interactions dominate the low and high salt concentration regions. At 0.01 M, a minimum, due to the attractive forces, is observed at a surface separation around 3 nm followed by a steric repulsion arising from the compression of the adsorbed protein molecules. Above this distance, there is practically no interaction. In the case of 1 M NaCl, the force profile does not present any minimum and the repulsion is more long-ranged. It cannot be explained in terms of electrostatic arguments.

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<th>pH 3</th>
<th>pH 5</th>
<th>pH 7</th>
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<tr>
<td>NaCl</td>
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<tr>
<td>0.01 M</td>
<td>( \psi_s = \psi_p = 14 ) mV ( \psi_s = \psi_p = -7 ) mV ( \psi_s = \psi_p = -20 ) mV</td>
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<tr>
<td>0.1 M</td>
<td>( \psi_s = \psi_p = 4 ) mV ( \psi_s = \psi_p = -2 ) mV ( \psi_s = \psi_p = -9 ) mV</td>
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<td>1 M</td>
<td>( \psi_s = \psi_p = 0 ) mV ( \psi_s = \psi_p = 0 ) mV ( \psi_s = \psi_p = 0 ) mV</td>
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<tr>
<td>CaCl₂</td>
<td>0.01 M</td>
<td>( \psi_s = \psi_p = 13 ) mV ( \psi_s = \psi_p = -1 ) mV ( \psi_s = \psi_p = -5 ) mV</td>
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<tr>
<td>0.05 M</td>
<td>( \psi_s = \psi_p = 4 ) mV ( \psi_s = \psi_p = 0 ) mV ( \psi_s = \psi_p = 0 ) mV</td>
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Equations 16 and 15 were used to fit the experimental data. In every case \( s = 0 \) nm. Values of \( \pm 1 \) mV, \( \pm 0.2 \) nm, \( \pm 0.02 \) mN/m, and \( 0.02 \) nm can be considered as error estimates for surface potentials, \( d \), \( \gamma \), and \( \lambda \), respectively.
appears, and the experimental value of the force is greater than that calculated by DLVO theory (Figure 4b). These results suggest that other types of forces are stabilizing the apoferritin molecules at high electrolyte concentration. This non-DLVO repulsion appears at a distance prior to the overlapping of the protein layers.

The interaction forces between apoferritin-coated surfaces at pH 7 as a function of the electrolyte concentration are plotted in Figure 5a. As shown, under these conditions, the interaction forces are purely repulsive; no attractive forces are observed at any electrolyte concentration. The electrostatic repulsions observed at pH 7 are much stronger than those observed at pH 3, with a surface potential of 14 mV for pH 3 compared with −20 mV for pH 7 in 0.01 M NaCl. This result could indicate that the surface of the protein is more charged at pH 7 than at pH 3.

The most striking aspect of the results shown in Figure 5a is that the increase in the electrolyte concentration from 0.1 to 1 M NaCl does not give rise to a decrease in the interaction force. The forces at 0.01 and 0.1 M NaCl are in agreement with DLVO predictions (solid lines in Figure 5a, fitting parameters in Table 1); however, the interaction profile for 1 M NaCl cannot be fitted to this theory, and it presents an additional non-DLVO force before the protein layers overlap (as in the case of high electrolyte concentration at pH 5), as can be seen in Figure 5b.

Figure 5. (a) Normalized force versus separation distance \( x \) for apoferritin-apoferritin interaction at pH 3 in different NaCl concentrations: (\( \square \)) 0.01 M; (\( \odot \)) 0.1 M; (\( \triangle \)) 1 M. The inset is a zoom of the graph. The solid lines in the inset represent the DLVO fits (eq 16) corresponding to 0.01 M NaCl and 0.1 M NaCl. (b) Theoretical fits of the interaction curve obtained at 1 M NaCl: the dashed line is the DLVO fit (eq 16), whereas the solid line corresponds to the extended DLVO fit incorporating a hydration force term (eq 15). The fitting parameters are shown in Table 1.

The key to explain the observed deviation from DLVO behavior at high electrolyte concentration and pH above

the iep of apoferritin may be related to the hydrophilic character of the protein surface (we must take into account that adsorbed proteins expose a hydrophilic surface toward the solution). The lower free energy of water molecules near hydrophilic surfaces leads to the formation of a structured water shell around those surfaces.68,75–77 The overlapping of hydration layers of different surfaces causes an increase in the free energy of the system, and therefore, a repulsion appears (the so-called hydration forces). The formation of that structured water layer may be favored by the presence of hydrated ions near the protein surface.68,78 In our case, hydration forces have been detected at pH above the iep (when the apoferritin is negatively charged) and at high NaCl concentration. This indicates that the presence of a large quantity of Na\(^+\) counterions (adsorbed or not) near the protein surface is crucial to the formation of the structured water layer. Na\(^+\) are very hydrated cations,49 which presumably retain some of their water of hydration when they accumulate in the vicinity of the protein surface. The negative charge of apoferritin increases as the pH value increases, so it is comprehensible that the hydration forces are more pronounced at high pH (more Na\(^+\) counterions near the protein surface). However, when the protein is positively charged (at pH below the iep) hydration forces are absent because the Cl\(^-\) anions (the counterions in this case) are practically not hydrated29 and, therefore, do not favor the formation of a structured water layer around the apoferritin surface. (The absence of hydration forces below the iep has previously been inferred from flocculation studies of amphoteric latices and ascribed to this mechanism.49)

The measured forces at 1 M NaCl and pHs 5 and 7 have been fitted to the extended DLVO predictions (solid lines in Figures 4b and 5b) which include the hydration force term given by eq 15. The hydration force parameters are shown in Table 1. Notice that the inclusion of the hydration forces improves the agreement between the theoretical fits and the experimental data.

**Figure 6.** Normalized force versus separation distance \( x \) for apoferritin-apoferritin interaction at pH 3 in different CaCl\(_2\) concentrations: (\( \square \)) 0.01 M; (\( \odot \)) 0.05 M; (\( \triangle \)) 0.5 M. The inset is a zoom of the graph. The solid lines represent the fits according to DLVO theory (eq 16). The fitting parameters are shown in Table 1.
solutions. It should be noted here that the effect of salt concentration corroborates the electrostatic nature of the protein interaction at pH 3, i.e., the presence of high concentration of CaCl₂ in the medium causes a larger screening of the protein-protein interaction. As can be seen, the force curves conform quite well to DLVO theory (the fitting parameters are shown in Table 1).

Figure 7a shows the results of the force measurements conducted at pH 5 in 0.01, 0.05, and 0.5 M CaCl₂ solutions. It is clear that the interaction curves for 0.01 and 0.05 M are very similar in the sense that the interaction is attractive before the protein-protein contact. Both curves can be satisfactorily fitted by the DLVO theory (solid lines, which overlap each other, in Figure 7a). However, the interaction curve for 0.5 M does not correspond to the conventional DLVO theory (Figure 7b). Since the electrostatic component of the DLVO interaction should be negligible at pH 5, it might be expected that the attractive van der Waals component should dominate at this concentration also and that the force curve should be similar to those obtained at the other electrolyte concentrations. However, similarly to the case of Na⁺ counterions, the high concentration of hydrated Ca²⁺ present near and/or adsorbed on the interface provides a repulsive hydration interaction preventing the protein-protein attraction predicted by the DLVO theory. This weak force appears at a separation below 7 nm. The force curve at 0.5 M CaCl₂ was also fitted using the extended DLVO theory (solid line in Figure 7b) incorporating a hydration force term (eq 15). We can observe a good correlation between this fit and the experimental values. Once again, this type of interaction is not observed in the case of the same electrolyte concentration but at pH 3, showing a clear difference between pH values above or below the isoelectric point (IEP) of the apoferritin. Hydration forces have been also detected at pH 7, as can be seen in Figure 8, panels a and b, where the interaction curves obtained at different CaCl₂ concentrations are shown.

The parameter values that lead to the best theoretical fits in each condition are listed in Table 1. Analysis of these data provides useful information, such as the relatively low surface potential of apoferritin layers. In addition it can be observed how the surface potential of the apoferritin layer at low salt concentration increases (in absolute value) away from the IEP of the protein. This surface potential is reduced as the concentration of the background electrolyte is increased, in line with expectations based on the Gouy-Chapman theory for the behavior of the electrical double layer. The orders of magnitude of our surface potential seem reasonable for protein systems.

At high salt concentration and at pH above the IEP of the apoferritin, hydration forces appear. Table 1 also includes the hydration force parameters $C_H$ and $\delta$. As has been commented previously, the explanation of the existence of this non-DLVO repulsion observed in apoferritin-coated surfaces may be that the water structure in the vicinity of the protein layer changes significantly in the presence of high electrolyte concentrations. It is possible that hydrated cations such as Na⁺ and Ca²⁺ (specifically adsorbed or not on the surface) favor the formation of a structured water layer strongly bound to...
the protein surface. The hydration forces result from the overlapping of the hydration layers of different protein surfaces. It might be expected that the parameter $\lambda$, related to the size of the hydrated cations and the thickness of the water layer around the hydrophilic surfaces, should be higher in the case of CaCl$_2$ ($\text{Ca}^{2+}$ is a more hydrated and bigger cation than Na$^+$). However, the $\lambda$ values obtained for both NaCl and CaCl$_2$ are very similar. Besides, the intensity of the hydration forces increases ($C_d$ grows) as the pH increases (at least in the case of NaCl): the more negatively charged the apoferritin surface (the higher the pH), the greater the adsorption of cations. For both the monovalent cation Na$^+$ and the divalent cation Ca$^{2+}$, the effect of hydration forces is evident, but it is actually more pronounced in the case of Na$^+$ (higher value of $C_d$). This might be due to the fact that the number of Ca$^{2+}$ ions at 0.5 M is smaller than the number of Na$^+$ ions at 1 M. It is noteworthy that the values of $\lambda$ match within the ranges found in the literature (between 0.1 and 2.2 nm). The values found in the literature for $C_d$, corresponding to very hydrophilic surfaces, range from 1.2 mN/m for quartz to 40 mN/m for mica. In our case, the value of $C_d$ is quite low presumably because a protein layer is not as charged and hydrophilic as silica or mica.

Table 1 also includes the values of the compression distance $d$ of the protein layers. At 0.01 M NaCl, $d$ increases away from the iep of the apoferritin. This behavior might be related to the net charge of the protein. At pH values far from the iep, the globular proteins can undergo expansions due to internal electrostatic repulsions between their charged groups. This phenomenon may be especially important in the case of big proteins such as apoferritin. Those internal electrostatic repulsions are screened as the NaCl concentration increases; the protein layer is then less expanded, and therefore, a decrease in the $d$ value is observed. In this situation, the protein layer can be compressed 2.4 nm (independently of pH). The tendency observed at pH 5 is opposite of that observed at other pHs: $d$ is smaller at 0.01 M NaCl than at 0.1 M NaCl. This pH is very close to the iep of the apoferritin. This means that the protein has almost the same number of positively and negatively charged groups. The internal electrostatic attractions between these groups could favor a further compression of the protein layer at low NaCl concentrations. These internal electrostatic attractions are screened as the NaCl concentration increases; this leads to the increase of $d$ from 1.6 nm at 0.01 M NaCl to 2.4 nm at higher NaCl concentrations. The “natural value” of $d$ observed with NaCl is slightly reduced with CaCl$_2$. The presence of Ca$^{2+}$ ions seems to compress the protein layer in such a way that $d$ reaches the value of 1.9 nm (more or less independent of pH and CaCl$_2$ concentration).

Nevertheless, the possible conformational changes of the protein are reversible processes, as can be inferred from the reproducibility of the measurements (see the Methods section). The values of $d$ presented in Table 1 are reasonable values taking into account the dimensions (see the Introduction) and crystallographic data of cubic structure with unit cell of 18.29 nm $\times$ 18.29 nm $\times$ 18.29 nm, or tetragonal structure with unit cell of 14.72 nm $\times$ 14.72 nm $\times$ 15.26 nm$^{31}$ of the horse-spleen apoferritin.

Now it may be opportune to make some comments about the fitting parameters. First of all, it can be observed in Table 1 that $\Psi_S$ and $\Psi_P$ coincide in every case. These two potentials have been treated as independent parameters in order to gain generality. [What happens if the adsorbed protein quantity is different on the plane and on the sphere, i.e., if a silica surface were to be replaced by a ceramic or metal surface, or if the two silica surfaces had different charges in water (this is not unusual because both silica surfaces could be of different nature and, consequently, they could have a different surface density of silanol groups?) In that case both surface potentials on the sphere and on the plane might be different.] The fact that the fitting procedure returns equal values of $\Psi_S$ and $\Psi_P$ is the expected result and gives support both to the quality of the data but also the model and fitting. Therefore, the two parameters $\Psi_S$ and $\Psi_P$ can be considered as effectively one. On the other hand, it is necessary to emphasize that eqs 7 and 16 are valid only at low surface potential $\Psi < (kT/ze)$, a condition that is fulfilled in all cases.

We must also make other observation: the zero-frequency term of the Hamaker constant $A$ in eq 10 is 65% of the total value, and it is strongly screened by the electrolyte at high salt concentration. However, this decrease in the van der Waals attraction cannot explain the observed experimental behavior at high electrolyte concentrations. In those conditions, the double layer repulsion is negligible. If there is not any other repulsive mechanism, the only interaction present would be the van der Waals attraction. Although the Hamaker constant $A$ is screened at high electrolyte concentrations, it is not null (at least it is equal to 35% of the original value). Therefore, at very low separation distances an attractive interaction should appear in any case (whenever the salt concentration is high enough as to neglect the double layer repulsion). Effectively, that attractive interaction can be observed at pH 3 and 1 M NaCl (Figure 3) and at pH 3 and 0.5 M CaCl$_2$ (Figure 6). However, that attractive interaction is not observed at pH above the iep of apoferritin. If we are taking into account only the screening of the van der Waals attraction, no effect of the pH should be detected and the same behavior should be observed below and above the iep of apoferritin. On the other hand, even in the case of complete screening of the van der Waals (which, as we have exposed before, cannot occur), the total interaction would be null but not repulsive. In this way, interaction curves such as that shown in Figure 5b (pH 7 and 1 M NaCl), where a clear repulsive interaction is observed, could not be explained. In summary, if the electrolyte screening of the van der Waals interaction is taking into account, probably best theoretical curves could be obtained (notice that, however, good theoretical fits are obtained without considering this correction). However, that screening cannot explain the repulsive interaction observed in Figure 5b; a hydration term is needed to reproduce the experimental behavior.

The application of the model presented in this paper to reproduce the experimental force curves has been very successful. Nevertheless, the hydration model used in this work is an empirical model, which should be considered as a first step toward the elaboration of a more complete theoretical model.

V. Conclusions

The present work, using AFM measurements, investigates the effect of pH and electrolyte concentration on apoferritin—apoferritin interactions and analyses how different counterions may affect these interactions. Electrostatic and steric forces are the dominant interactions between apoferritin layers at low salt concentrations. DLVO theory fits the experimental force curves quite well.
in these conditions. However, the AFM measurements carried out at pH values above the isoelectric point of the apoferritin and at high electrolyte concentration give clear evidence of a non-DLVO contribution that is attributed to hydration forces. This short-range repulsive force appears before the protein layers come into contact. It is observed that the non-DLVO interaction increased with increasing the pH and varied in magnitude with cation type (Na\(^+\) or Ca\(^{2+}\)). It seems that the origin of these forces is associated with the formation of a structured water layer around the protein surface, which is crucially favored by the presence (and/or adsorption) of hydrated cations at the protein—solvent interface at high electrolyte concentration. The measured forces have been satisfactorily fitted to the extended DLVO theory, where a hydration term has been included.

The conclusions of this work are in agreement with experimental results previously obtained about the colloidal stability of protein-coated particles,\(^{26,68,78}\) with AFM data about the interaction between bovine serum albumin (BSA) layers adsorbed on different substrates\(^{82,83}\) and with the results obtained in light scattering experiments of apoferritin solutions.\(^{13,27,43}\) These results are especially interesting in the application of such complexes to immunoassays (tests for disease detection): the stabilization of the antibody-coated particles in the absence of the specific antigen can be achieved by means of hydration forces (simply changing the pH and the salt concentration of the medium).\(^{84,85}\)

This paper shows that the AFM colloid probe technique can provide a useful means of directly quantifying the interaction between biological macromolecules. The AFM technique has not yet been extensively used to directly measure the interaction between protein molecules. In the works that do it,\(^{79,86,87}\) the experimental conditions (pH and electrolyte concentrations) were usually not the appropriated ones to detect hydration forces.

From a colloidal point of view, proteins are weakly charged particles. Since the physiological ionic strength (around 0.15 M) is relatively high, electrostatic stabilization is hardly the most likely candidate to explain stability in living organisms. Hydration repulsion is a more likely option, and its sensitivity toward the nature of the particular ions allows for precise regulation of their biological functions.

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