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Measurement of interactions between protein layers adsorbed on silica by atomic force microscopy

J J Valle-Delgado[1], J A Molina-Bolívar[2], F Galisteo-González[1,4], M J Gálvez-Ruiz[1], A Feiler[3] and M W Rutland[3]

[1] Grupo de Física de Fluidos y Biocoloides, Departamento de Física Aplicada, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain
[2] Departamento de Física Aplicada II, Escuela Universitaria Politécnica, Universidad de Málaga, 29013 Málaga, Spain
[3] Department of Chemistry, Surface Chemistry, Royal Institute of Technology and Institute for Surface Chemistry, 100 44 Stockholm, Sweden

E-mail: galisteo@ugr.es

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Abstract

The present work, using an atomic force microscope and the colloid probe technique, investigates the interaction forces between bovine serum albumin (BSA) layers and between apoferritin layers adsorbed on silica surfaces. The measurements have been carried out in an aqueous medium at different pH values and NaCl concentrations. Similar behaviours have been found with both proteins. Electrostatic and steric forces dominate the interactions between the protein layers at low NaCl concentrations. However, a very strange behaviour is found as a function of pH at high NaCl concentrations. The results obtained under these conditions could be explained if the presence of hydration forces in these systems is assumed.

1. Introduction

The knowledge of the interactions between protein molecules in solution is fundamental to understand protein functions in nature and in all practical processes involving proteins, such as treating biological disorders [1], structure-based drug design [2], purifying protein mixtures [3], understanding protein diffusion in concentrated solutions [4] and stabilizing protein therapeutic formulations [5]. In protein condensation diseases the primary initial step in pathogenesis is the loss of solubility of the proteins, resulting in the formation of a condensed phase. Examples of such phases include a dense liquid phase, amorphous aggregates, or crystals that form in cataracts [6, 7], or the formation of fibres that are responsible for sickle cell disease [8] and Alzheimer's disease [9]. Thus the characterization of the interactions between proteins is

[4] Author to whom any correspondence should be addressed.

the first step for building strategies aimed at inhibiting such diseases [10]. One particularly important process is protein crystallization, which depends upon a large fan of experimental parameters, i.e. pH, temperature, nature and concentration of salt, etc. The determination of suitable conditions for growing protein crystals remains largely an empirical art that is usually accomplished in practice by extensive screening experiments. Over the years, many attempts have been made to rationalize crystallization assays. The study of the macromolecular interactions in solution appears more and more as a powerful tool to predict crystallization conditions. Static and dynamic light scattering [11, 12], as well as fluorescence anisotropy [13] and small angle x-ray scattering [14], have been used to monitor interactions in protein solutions and it has been suggested that an interaction parameter could serve as a potential index of crystallization.

Proteins are particles in the colloidal size range, and therefore any attempt to understand and predict protein behaviour would benefit from a description of protein solutions in terms of simple models in interacting colloidal particles. Indeed, applications of ideas from colloidal science to proteins have lately seen a steady growth. In this context the celebrated DLVO (Derjaguin–Landau–Verwey–Overbeek) theory [15, 16] supposes that the total interaction potential between two particles is the sum of two independent interactions: the electrostatic repulsion (that keeps the system stable) and the van der Waals attraction (that tends to provoke the aggregation of the particles). While appropriate for a wide variety of systems, the DLVO approach failed to account for some experimentally observed features of stability behaviour. Hence, other induced (or effective) interactions, such as hydrophobic attraction and hydration forces, have been put forward [17].

The existence of repulsive hydration forces between protein molecules has been often postulated in terms of the osmotic second virial coefficient, especially because it can be measured using traditional colloidal characterization techniques, such as that previously stated. Petsev *et al* [18, 19] have studied the apoferritin–apoferritin interactions in the presence of Na^+ at pH 5.0 using light scattering methods. These authors observed that the second osmotic virial coefficient exhibits a minimum when plotted against the electrolyte concentration. The ascending branch of this dependence was attributed to the existence of hydration forces. On the other hand, Molina-Bolívar *et al* [20, 21] have invoked the hydration forces to explain the high colloidal stability of particles covered with proteins.

In the study of protein–protein interactions there are a number of important aspects which remain open:

(1) as the methods used measure the interaction parameters indirectly, large and sometimes unpredictable errors can occur, therefore the reliability of the data should be checked by at least two independent methods [22];
(2) these methods are generally labour intensive and expensive in terms of both time and protein [23];
(3) systematic studies on protein interaction have been restricted to small molecular weight model proteins like lysozyme;
(4) up to now, hydration forces between proteins have only been inferred indirectly, from measurements by light scattering techniques [24];
(5) more data are required to clarify the correlation between the hydration forces and the net charge of protein, the dependence on the electrolyte concentration and on the kind of protein.

In this paper, in order to get a better insight into the protein–protein interactions, and especially into the hydration forces, we focus on measuring directly these interactions by means of an atomic force microscope (AFM). The AFM is a sophisticated and very sensitive

instrument that allows the direct measurement of forces between surfaces [25–28]. Its applicability is evidenced by the wide variety of surfaces that have been investigated. By the aid of this instrument we have obtained the interactions, in different aqueous media, between bovine serum albumin (BSA) layers and between apoferritin layers adsorbed on silica (two important globular proteins). Such studies have made use of the colloid probe technique in which a colloidal particle is immobilized at the end of an AFM cantilever. This approach allows the direct measurement of colloidal interactions with the advantage of uniquely allowing quantification of local variations in such interactions.

2. Materials

Interactions between protein layers adsorbed on silica were measured in aqueous media at different pH and NaCl concentrations (in this paper only approach interaction curves are shown). All chemicals used were of analytical grade quality. NaCl solutions were prepared with double distilled water (Milli-Q System). The error estimations in the final concentrations were below 1% of the respective value. The pH was controlled using different buffers: an acetate buffer at pH 5 (3.15 mM CH_3COOH plus NaOH until the final pH was achieved); a phosphate buffer at pH 7 (1.13 mM NaH_2PO_4 plus NaOH until the final pH was achieved); a borate buffer at pH 9 (5.14 mM H_3BO_3 plus NaOH until the final pH was achieved); a dilute HCl solution was used to keep the medium at pH 3. The ionic strength of these solutions was below 2 mM.

All interaction measurements were carried out by means of a Nanoscope III AFM (Digital Instruments, USA) using the colloid probe technique. This technique allows the measurement of the interaction between a sphere (connected to the end of a cantilever) and a plane (put on the piezo-scanner of the AFM) as a function of their separation. The colloid probe (sphere connected to the cantilever) is held stationary and the flat surface is displaced in a controlled manner toward and away from the probe in an aqueous media. The interaction 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. More details about this technique and the operation mode of the AFM can be found in the literature [25–28]. In order to scale the force measurements correctly the spring constant of the cantilevers (which where standard V-shaped AFM tipless cantilevers) must be known accurately. This was achieved using the resonance method proposed by Cleveland *et al* [29]. The experimental force data have been normalized by the radius of the sphere attached to the cantilever.

Silica spheres, with a diameter of 5.08 ± 0.05 μm, were supplied by Bangs Laboratory (USA). Silica planes, with an area around 1 cm^2, were obtained from a silica wafer (CSIC, Spain).

Two proteins were used in the experiments: BSA and apoferritin. The BSA, supplied by BIOKIT (Spain), is a monomeric protein with a molecular weight around 67 000 g mol^{-1} and with an isoelectric point (iep) around 4.7 [30]. The apoferritin was supplied by SIGMA, and it has a molecular weight around 480 000 g mol^{-1} and an iep around 4.0 [18]. These proteins were adsorbed on silica surfaces (sphere and plane). The adsorption protocol for the BSA was the following [31]: 1 mg ml^{-1} BSA solution (0.01 M NaCl, pH 5) was injected in the AFM fluid cell, where the silica surfaces were immersed; the adsorption took place during 3 h at room temperature (22 ± 1 °C) and, finally, double distilled water was injected to eliminate the non-adsorbed protein. The adsorption protocol for the apoferritin was [32]: 1 mg ml^{-1} apoferritin solution (0.1 M NaCl, pH 5) was injected into the AFM fluid cell, the adsorption took place during 1 h at room temperature (22 ± 1 °C) and again double distilled water was

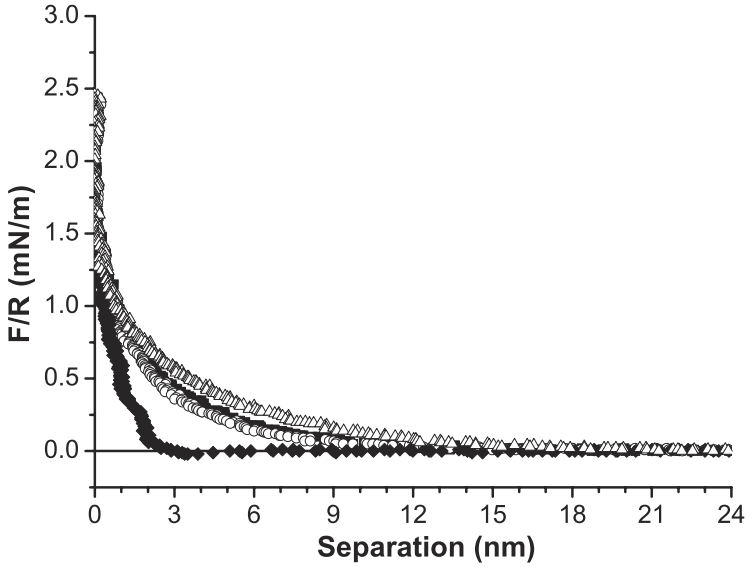

Figure 1. Normalized force versus separation distance for the BSA–BSA interaction at 0.01 M NaCl and at different pHs: ■, pH 9; ○, pH 7; ♦, pH 5; △, pH 3.

injected to eliminate the non-adsorbed protein. These adsorption protocols give rise to protein monolayers strongly bound to the silica surfaces [31–33].

3. Results and discussion

The effects of pH and NaCl concentration on the interactions between protein layers have been analysed. 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 NaCl concentration). This fact evidences that the proteins were strongly adsorbed on the silica surfaces. The pressure generated between the surfaces did not induce any irreversible structural changes in the adsorbed layers. On the other hand, the replacement of the solution did not result in any changes in the force profiles, that is, the reproducibility of the measurements after changes in the medium conditions (changes in pH from pH 9 to pH 3 and vice versa at fixed NaCl concentration; changes in NaCl concentration from high value until low value and vice versa at fixed pH) was highly satisfactory. This indicates that proteins did not desorb or undergo irreversible conformational changes when the medium conditions were changed. Finally, the interaction curves were unaffected after leaving the samples several hours in a 10 mM NaCl and pH 5 medium.

Figures 1 and 2 present the interaction force profiles (approaching curves) for the BSA–BSA interaction and for the apoferritin–apoferritin interaction, respectively, at low salt concentration (0.01 M NaCl) and as a function of pH. Protein molecules interact with each other by a number of mechanisms. The framework usually adopted to model interactions is, as for colloids, composed of an attractive van der Waals force and a repulsive screened Coulombian potential (DLVO theory). The van der Waals interaction between the sphere glued to the tip and the surface is given by [17]:

$$V_{\mathrm{A}} = -\frac{AR}{6x} \tag{1}$$

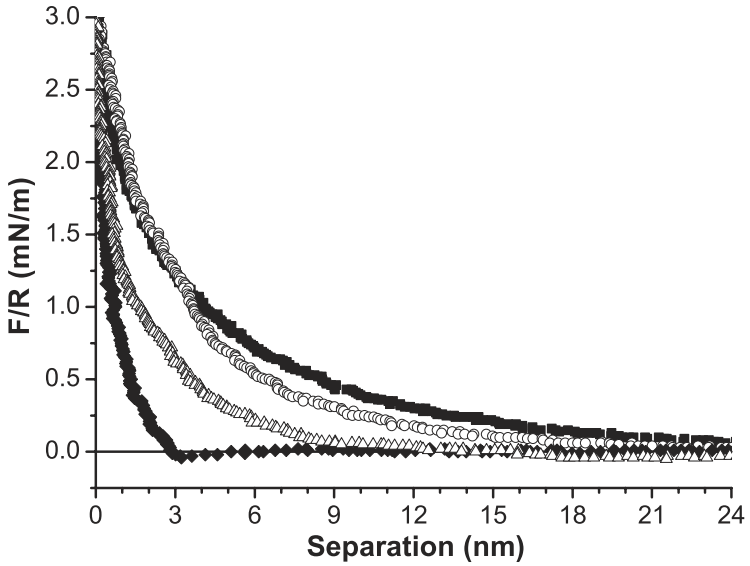

Figure 2. Normalized force versus separation distance for apoferritin–apoferritin interaction at 0.01 M NaCl and at different pHs: ■, pH 9; ○, pH 7; ♦, pH 5; △, pH 3.

where R is the sphere radius, x is the distance between the surfaces of the sphere and the plane, and A is the Hamaker constant of the system. This attractive potential with macromolecules in aqueous buffer is short range. On the other hand, the repulsive component corresponds to a Coulombian potential screened in solution by a diffuse layer of counterions. In the frame of the Debye–Hückel approximation, the Debye length characterizes the electrostatic range, and it depends on the ionic strength of the solutions. The inverse of the Debye length, κ, is given by the following equation:

$$\kappa = \sqrt{\frac{\sum_i n_i e^2 z_i^2}{\varepsilon k_B T}} \tag{2}$$

e being the electron charge, ε the dielectric constant of the medium, k_B the Boltzmann constant, T the absolute temperature, n_i the number concentration in the bulk of ions of type i, and z_i the valence of such ions. The electrostatic energy V_E between a sphere and a plane (under boundary conditions of constant and low surface potentials) can be approximated by [16, 34]:

$$V_E = \varepsilon \pi R \left[(\Psi_S + \Psi_P)^2 \ln (1 + \exp(-\kappa x)) + (\Psi_S - \Psi_P)^2 \ln (1 - \exp(-\kappa x)) \right] \tag{3}$$

where Ψ_S is the surface potential of the sphere and Ψ_P the surface potential of the plane. This repulsive potential is a function of the effective charge of the macromolecule and it is long ranged.

The interplay of these DLVO intermolecular forces can qualitatively explain the trends observed in the interaction force profiles. The behaviour at low ionic concentration is governed by the long-range electrostatic interactions. The protein is strongly charged when the medium pH is away from the iep (pHs 3, 7 and 9), so the interaction between the adsorbed layers at large separations is dominated by electrical double layer repulsion. Whereas at short distance a strong repulsion attributed to steric forces owing to the overlap of the adsorbed protein layers is observed. The interaction force profile at pH 5 for both proteins is clearly different from the other pHs. The curve presents two force regimes, above and below a separation distance

of approximately 3 nm. Above this distance and for larger separations there is practically no interaction because this pH is close to the iep of proteins (the net charge is around zero) and, therefore, the electrostatic repulsion is negligible. Due to the van der Waals attractive forces a primary minimum is observed at a surface separation of 3–4 nm followed by a steric repulsion arising from compression of the adsorbed protein molecules. Besides, at this pH a strong adhesion appears when surfaces are separated (results not shown). The dependence of the electrostatic repulsion on the pH is due to the amphoteric behaviour of proteins: their surface charge depends on pH in such a way that its value is positive at pHs below the iep and negative at pHs above the iep. Thus the surface potential increases as the pH moves away from the iep because the surface charge also increases (in absolute value). An increase in the surface potential provokes an increase in the repulsive electrostatic interaction as inferred from equation (3).

If we compare both figures, it is observed that the interaction between BSA layers adsorbed on silica surfaces is weaker (that is, less repulsive) and less extended than the interaction between apoferritin layers adsorbed on silica surfaces. In the case of BSA the strongest interaction is observed at pH 3 (BSA protein is positively charged) because at this pH the BSA surface charge is the highest [35]. Whereas, in the case of apoferritin the most repulsive interaction occurs at pH 9 (when the protein charge is negative).

The interaction force data obtained between apoferritin-coated surfaces immersed in solutions at pH 3 containing different background electrolyte concentrations are presented in figure 3. The curves indicate a decrease of repulsive interactions as the electrolyte concentration increases from 0.01 to 1 M NaCl. The increase in the salt concentration both causes a screening of the electrostatic interaction and reduces its range, in such a way that at 1 M NaCl the electrostatic interaction becomes negligible. At this electrolyte concentration an attraction is observed before the overlap of protein layers occurs (around 5 nm). This attraction may be due to the attractive van der Waals forces which should be dominant in this range. The curves showed in figure 3 contain the essential features of the DLVO predictions, namely a long-range electrostatic repulsive interaction screened by counterions and a short-range attractive interaction.

In figure 4 the effect of the NaCl concentration on the apoferritin–apoferritin interaction at pH 5 is presented. One difference with regard to figure 3 is that no attraction appears at 1 M NaCl but it is present at 0.01 M NaCl (at a distance of around 3.2 nm). At this pH, close to the iep of the apoferritin, the electrostatic repulsion is practically negligible, and therefore no effect of salt concentration should be expected. However the interaction force obtained at 1 M NaCl is stronger than that obtained at 0.01 and at 0.1 M NaCl. It is surprising that the interaction force increases with increasing the electrolyte concentration. This fact indicates that other repulsive interaction different to the electrostatic appears at this pH. In general, the protein layer thickness depends on the electrolyte concentration and on the pH [36]. Proteins expand when the ionic strength is lowered, or the pH moves away from the iep, due to an increased repulsion between the protein charges [31, 37]. Therefore the non-DLVO repulsion observed at 0.1 and 1 M could not be attributed to steric repulsion because at this salt concentration proteins are less expanded that at lower ionic strength.

One striking aspect of the results is that the distance where the attraction minimum appears is higher at pH 3 (and 1 M NaCl) than at pH 5 (and 0.01 M NaCl). In these experimental conditions any electrostatic contribution to the interaction force is negligible and only the van der Waals interaction is present before the protein layers overlap. At pH 3 and 1 M NaCl the electrostatic repulsions are screened, whereas at pH 5 and 0.01 M NaCl the electrostatic repulsions are absent. The variation of the distance where steric repulsion occurs could stem from conformational changes within the adsorbed apoferritin layers. At pH 5 and 0.01 M

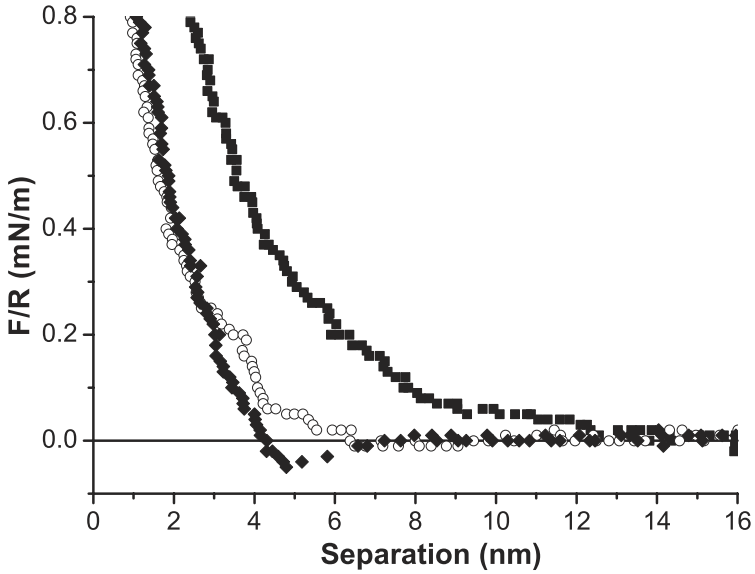

Figure 3. Normalized force versus separation distance for apoferritin–apoferritin interaction at pH 3 as a function of NaCl concentration: ■, 0.01 M; ○, 0.1 M; ♦, 1 M.

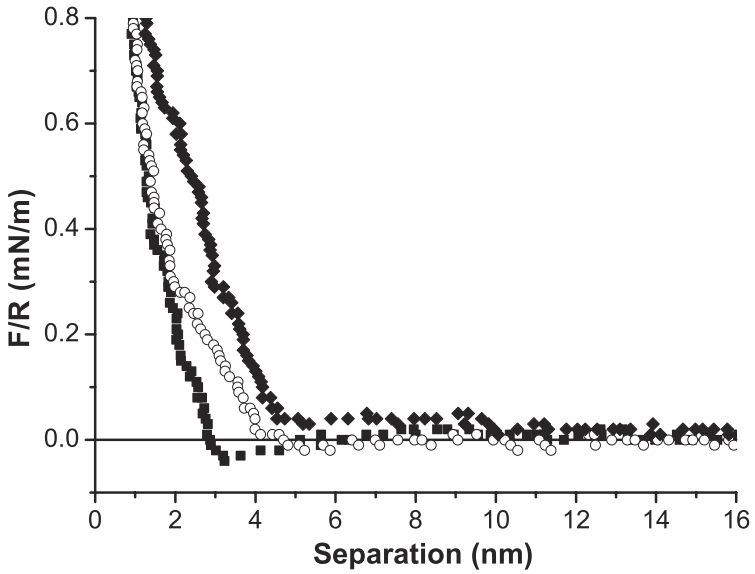

Figure 4. Normalized force versus separation distance for apoferritin–apoferritin interaction at pH 5 as a function of NaCl concentration: ■, 0.01 M; ○, 0.1 M; ♦, 1 M.

NaCl there are both negatively and positively charged amino acids present in the protein molecule. Electrostatic interaction between these residues could influence the conformation of the protein molecules, making them more compact. Whereas at pH 3 and 1 M NaCl some repulsive interactions between positively charged amino acids could remain, leading to more open packing of molecules on the surfaces.

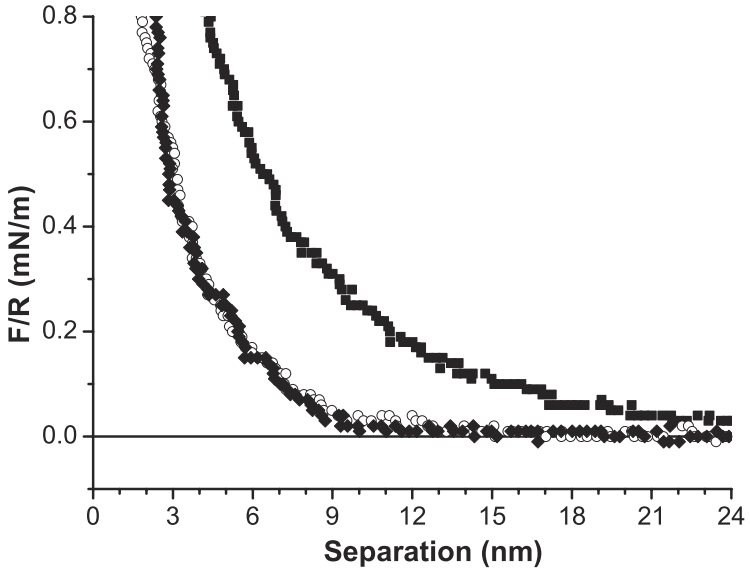

Figure 5. Normalized force versus separation distance for apoferritin–apoferritin interaction at pH 7 as a function of NaCl concentration: ■, 0.01 M; ○, 0.1 M; ♦, 1 M.

The existence of a non-DLVO force can be also achieved when we observe the experimental results at pH 7 (see figure 5). All the force profiles presented in figure 5 are always repulsive and decay exponentially with increasing separation distance. At 1 M NaCl the electrostatic repulsion is negligible. Therefore there should be an additional repulsive interaction which prevents the van der Waals attraction between the apoferritin layers in these conditions. The results with BSA (not shown) are very similar: an attraction between the BSA layers appears at high NaCl concentration when the pH is below the iep due to the existence of only van der Waals forces. But that attraction disappears at pH above the iep and an extraneous repulsion is observed.

Some authors suggest that the strange repulsion observed at high salt concentration and at pHs above the iep of the proteins may be due to the formation of a water layer strongly bound around each protein layer; the overlap of such water layers leads to a repulsion which is known as hydration forces [18–21, 38]. We must take into account that adsorbed proteins expose a hydrophilic surface towards the solution. But in this case the dominant factor to the formation of a strongly bound water layer is the presence of hydrated ions near the protein surface. At pHs above the iep the protein is negatively charged and the counterions near the protein surface are the Na^+ cations, which are very hydrated ions [17]. These ions contribute with their hydration shell to the formation of the strongly bound water layer when their concentration is high enough (at high salt concentration). However at pHs below the iep the protein is positively charged and the counterions near the protein surface are the Cl^- anions, which are practically not hydrated [17]. Therefore in this case the hydration layer around the protein surface is not formed and the hydration forces do not appear. To our knowledge, only a few papers have used the AFM to directly measure the interaction between protein molecules [31, 37, 39]. However, no work has presented a direct measurement of the hydration forces between proteins because the used experimental conditions (pH and electrolyte concentrations) were not appropriate. In a continuing theoretical study, we will interpret quantitatively our experimental results.

4. Conclusions

The interaction forces between BSA layers and between apoferritin layers adsorbed on silica surfaces were directly measured with an AFM in an aqueous media at different pH and NaCl concentrations. Electrostatic and steric forces are the dominant interactions between protein layers at low NaCl concentrations. At high NaCl concentrations the interaction is different depending on whether the pH is below or above the iep of each protein. In both cases (BSA and apoferritin) an attraction was observed between the protein layers at high NaCl concentration and pHs below the iep. This attraction is probably due to van der Waals forces (the electrostatic repulsion is completely screened). However the interaction is always repulsive at pHs above the iep. An additional repulsive interaction prevents the van der Waals attraction between the protein layers in these conditions. We assume the repulsive hydration forces are the responsible for the observed behaviour.

Acknowledgments

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