Adhesion Forces between Protein Layers Studied by Means of Atomic Force Microscopy

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Adhesion forces between different protein layers adsorbed on different substrates in aqueous media have been measured by means of an atomic force microscope using the colloid probe technique. The effects of the loading force, the salt concentration and pH of the medium, and the electrolyte type on the strength, the pull-off distance, and the separation energy of such adhesion forces have been analyzed in depth. Two very different proteins (bovine serum albumin and apoferritin) and two dissimilar substrates (silica and polystyrene) were used in the experiments. The results clearly point out a very important contribution of the electrostatic interactions in the adhesion between protein layers.

I. Introduction

The study of material adhesion poses scientific challenges but is of a great interest for a range of industrial, technological, and medical applications. Especially interesting is bacterial and/or protein adhesion, which can affect negatively the operation of industrial equipment and wastewater plants, for example. Bacterial and/or protein adhesion also plays a critical role in the failure of dental biomaterials and biomedical implants.[1−4]

Despite its interest, the fundamental mechanisms governing bacterial adhesion are still poorly understood. However, the use of modern and sophisticated techniques in surface force detection has allowed important advances in this issue. Among these techniques, atomic force microscopy has reached a leading position.

Since its invention,[5] the atomic force microscope (AFM) has become a more and more versatile and powerful device. Initially designed to image surfaces, it was also applied to measure surface forces very soon, especially since the introduction of the colloid probe technique.[6] In this technique, the interaction between a spherical colloid probe and a plane is measured as the surfaces approach (approach curve) and separate (retraction curve). The AFM has also turned out to be very useful to probe biomolecules and biological systems (bacteria, proteins, DNA, etc.),[7] to measure the binding force between ligand−receptor or antigen−receptor pairs,[8,9] to study the mechanical properties of proteins,[8,10] or even to quantify the force needed to extract proteins from cellular membranes.[9]

A number of works published recently in the literature have taken advantage of the high sensitivity of the AFM to measure and analyze the adhesion of some bacteria to different surfaces. Thus, for example, Razatos et al.[11] studied the affinity of different strains of *Escherichia coli* to different surfaces from the interaction force curves obtained with an AFM when monolayers of such bacteria were approached to silicon nitride tips or glass coated with poly(methyl methacrylate) (PMMA). But adhesion can be measured directly from the retraction curves obtained when two surfaces are separated. In this way, Fang et al.[12] analyzed the adhesion between an AFM tip and different regions of sulfate-reducing bacteria. Other extensive and very systematic work was carried out by Vadillo-Rodríguez et al., who studied the adhesion between AFM tips and different bacteria from approach and retraction force curves.[13−16]

Since proteins are important components in cellular membranes, it is also attractive to relate the protein adhesion to cellular or bacterial adhesion, as recently done by Xu and Logan.[17] These authors have measured the adhesion forces between different protein layers and glass or latex colloid probes using an AFM. The results were analyzed according to a gradient force method.[18] In general, Xu and Logan found a correlation between the adhesion

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forces measured and the relative charges of the proteins. Studying the adhesions between two protein layers, they also concluded that electrosteric forces dominate the interactions between protein-coated materials in comparison to hydrophobic forces.[17]

In this paper (which can be considered as a part of a more extensive work devoted to the interactions between protein layers carried out by the authors), we present a systematic study of the adhesion forces that appear between protein layers adsorbed on different substrates. The measurements were carried out in aqueous media by means of an AFM using the colloid probe technique. The effects of changing the loading force and the pH of the medium on the strength, the pull-off distance, and the separation energy of the adhesion forces are presented in this paper. In addition, the effects of ionic strength and electrolyte type on such adhesion forces have been explored in this work.

II. Materials and Methods

The force measurements were performed using a Nanoscope III AFM (Digital Instruments, USA) and the colloid probe technique. In this technique, which is well described in the literature,[6,19] the interaction between a sphere joined to the end of a flexible cantilever and a plane put on the AFM piezo-scanner is measured as a function of their separation. When the sphere and the plane approach or separate each other, the interaction between them causes a deflection of the cantilever that can be measured by means of an optic system. With an appropriate calibration and knowing the spring constant of the cantilever, the interaction force as a function of the separation distance between the surfaces can be obtained. Following the resonance method proposed by Cleveland et al.,[20] spring constants between 0.07 N/m and 0.12 N/m were obtained for the different V-shaped tipless cantilevers employed in this research. The measured surface forces have been normalized by the radius of the colloid probe in order to facilitate the comparison with the results obtained with other colloid probes of different sizes.

Two very different proteins have been used in the experiments: bovine serum albumin (BSA) and apoferritin. The BSA, supplied by Biokit (Spain), is a monomeric protein with a molecular weight of 67 000 g/mol and an isoelectric point (iep) around 4.7.[21] It has been so extensively studied that it can be considered as a model protein. The apoferritin (obtained from equine spleen) used in the experiments were supplied by SIGMA. Apoferritin is a bigger protein than BSA, with a molecular weight around 480 000 g/mol and an iep in the range 4.0−4.7.[22−24] Its nearly spherical shape (12 nm in diameter[25]) is also different from the ellipsoidal shape of BSA (11.6 nm × 2.7 nm × 2.7 nm in dry;[26] 14 nm × 3.8 nm × 3.8 nm in aqueous medium[27]).

All chemicals used were of analytical grade quality. The pH was controlled using different buffers (acetate at pH 5, phosphate at pH 7, borate at pH 9, all at a constant ionic strength of 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 $CaCl_2$ to these buffers.

To obtain the interaction force between protein layers, the proteins were adsorbed on both the colloid probe and the plane placed inside the AFM fluid cell. Substrates of different nature were used as colloid probe and plane: silica and polystyrene. Silica spheres, with a diameter of 5 μm, were purchased from Bangs Lab. (USA). Polystyrene spheres, with a diameter around 4 μm, were supplied by Ikerlat Polymers (Spain). The colloid probes were prepared by gluing a colloidal particle (made of silica or polystyrene) to the end of the cantilevers using an optical microscope and a micromanipulator arm. Silica and polystyrene planes, with an area around 1 cm^2, were obtained from a silica wafer (CSIC, Spain) and from a polystyrene Petri dish, respectively.

The protocols of adsorption were the following: BSA was adsorbed on silica or on polystyrene surfaces (sphere and plane) by the injection of 1 mg/mL BSA solution (0.01 M NaCl, pH 4.8)[28−32] in the AFM fluid cell, where the surfaces were immersed; apoferritin was adsorbed on silica surfaces (sphere and plane) by the injection of 1 mg/mL apoferritin solution (0.1 M NaCl, pH 4.8) in the AFM fluid cell, where the surfaces were immersed.[33] [Note that, in solution, apoferritin is unstable and splits in different subunits at low ionic strengths; that is why 0.1 M NaCl is used in the adsorption protocol of apoferritin instead of 0.01 M NaCl.] The adsorption took place at room temperature (22 °C) during 3 h in the case of BSA and during 1 h in the case of apoferritin; after that, the nonadsorbed protein was removed by flushing the AFM fluid cell with double distilled water in each case.

Prior to injection of the protein, the interactions between the bare surfaces (silica−silica and polystyrene-polystyrene) were measured (results not shown; the experimental force curves between silica surfaces are presented and analyzed in ref 34). 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 the surfaces covered with protein. These interaction curves were clearly different from the interaction curves between the bare surfaces: the presence of a compressible region in the approach curves and adhesions in the separation curves are unmistakable evidences of the existence of a protein layer adsorbed on the surfaces. The approach curves were very reproducible in each case, which indicates that the proteins did not desorb from the surfaces during the experiments; these approach curves have been presented and analyzed elsewhere.[35−38] In this paper, we focus on the study of the retraction curves which, unlike the approach curves, are not so reproducible. At least 20 force curves were obtained at each pH and electrolyte concentration. The retraction curves shown in this paper are the average of those force curves.

The adhesion between the surfaces covered by protein can be characterized by three parameters: the adhesion force, the pull-off distance and the separation energy.[17] The adhesion force F_a is the maximum negative (attractive) force measured during the separation of the surfaces. The pull-off distance L_p is defined as the minimum separation at which all of the bonds between the surfaces are broken (null force). The pull-off distance represents the maximum length that proteins can be stretched during the separation of the surfaces. The separation energy E_s is the work needed to separate the surfaces, which is given by the area contained between the retraction curve and the baseline (line of null force).

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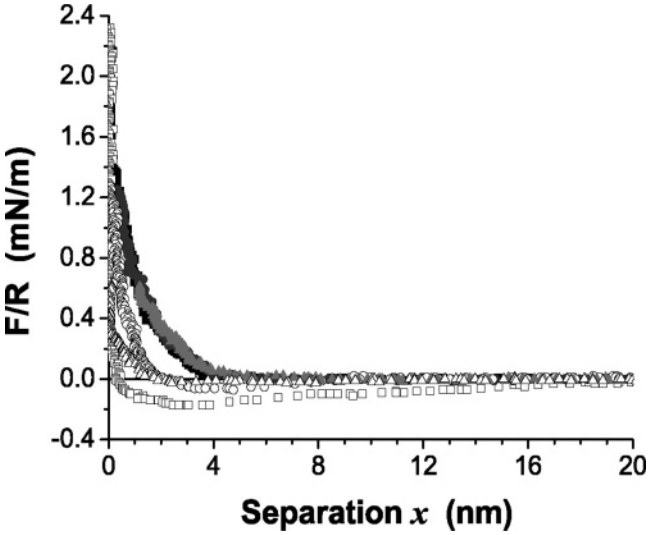

Figure 1. Effect of the intensity of compression on the interaction force curves between BSA layers (adsorbed on silica) in a solution at pH 7 and 0.01 M $CaCl_2$. Close symbols represent the approach curves, whereas open symbols correspond to retraction curves: (□) high compression (2.4 mN/m); (○) medium compression (1.3 mN/m); (△) low compression (0.6 mN/m).

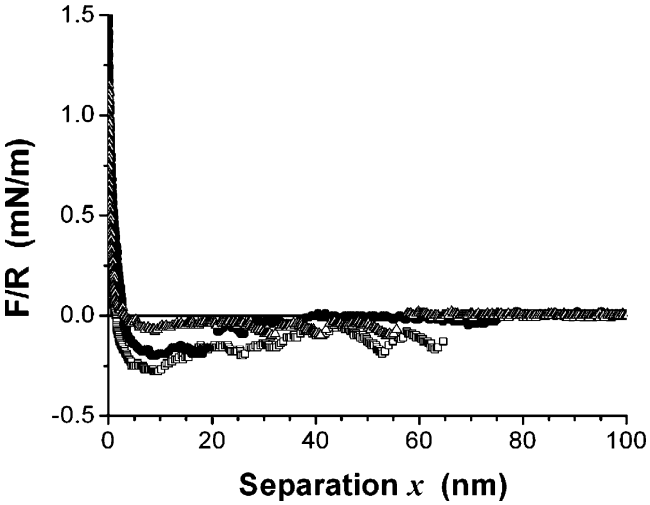

Figure 2. Effect of the intensity of compression on the retraction force curves between apoferritin layers (adsorbed on silica) in a solution at pH 3 and 0.01 M $CaCl_2$: (□) high compression (3.8 mN/m); (●) medium compression (2.2 mN/m); (△) low compression (1.1 mN/m).

III. Results and Discussion

Effect of the Compression. Figures 1 and 2 are two examples that reflect the influence of the intensity of compression of the protein layers on the adhesion forces between them. It can be observed, as a general trend, that an increase in the strength of compression leads to an increase in the adhesion force between the protein-covered silica surfaces. This occurs with both proteins BSA (Figure 1) and apoferritin (Figure 2). The corresponding approach force curves are also presented in Figure 1 to show their high reproducibility, independent of the intensity of the compression.

The increase of the strength of compression seems to favor the formation of a larger number of bonds (hydrogen bonds, electrostatic attraction between groups of opposite charge, etc.) between the proteins of both surfaces, which is reflected in the increase of the adhesion forces.

The same behavior shown in Figures 1 and 2 is observed, in general, at any pH, at any salt concentration and with both substrates silica and polystyrene. All of the force curves presented in the following correspond to high compression conditions.

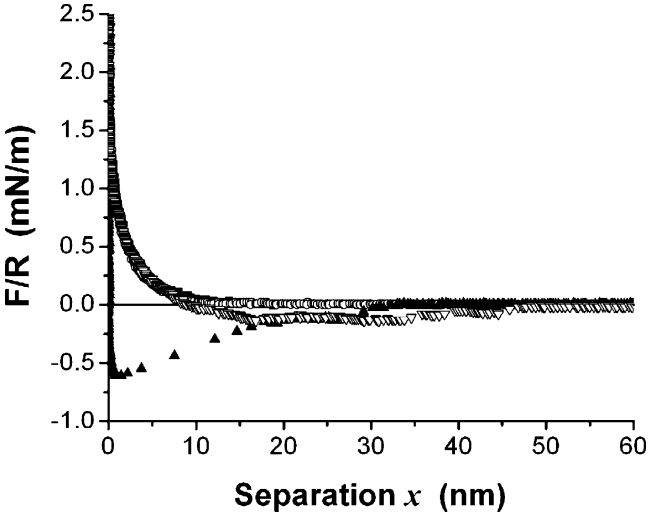

Figure 3. Effect of the pH on the adhesion between BSA layers (adsorbed on silica) in a NaCl 0.01 M solution: (■) pH 9; (○) pH 7; (▲) pH 5; (▽) pH 3.

Table 1. Adhesion Forces (Normalized by the Colloid Probe Radius), Minimum and Maximum Pull-Off Distances, and Separation Energies (Normalized by the Colloid Probe Radius) for the Adhesion between BSA Layers (Adsorbed on Silica) in Aqueous Media at Different pHs and at Different Concentrations of Different Salts (NaCl or $CaCl_2$)

		F_a/R (mN/m)	L_p (nm)	E_s/R (10^{-12} J/m)
0.01 M NaCl	pH 9	-0.01 ± 0.04	0−25	0 ± 0.2
	pH 7	-0.02 ± 0.06	0−40	-0.1 ± 0.3
	pH 5	-0.60 ± 0.10	35−40	-8.4 ± 1.7
	pH 3	-0.20 ± 0.10	60−100	-3.7 ± 1.5
0.1 M NaCl	pH 9	-0.15 ± 0.10	22−47	-1.9 ± 0.7
	pH 7	-0.08 ± 0.02	21−28	-0.7 ± 0.1
	pH 5	-0.36 ± 0.05	30−50	-3.4 ± 1.5
	pH 3	-0.14 ± 0.02	35−100	-2.0 ± 1.0
1 M NaCl	pH 9	-0.22 ± 0.10	40−70	-2.8 ± 1.2
	pH 7	-0.07 ± 0.03	31−75	-1.4 ± 0.5
	pH 5	-0.30 ± 0.05	30−60	-3.2 ± 0.7
	pH 3	-0.50 ± 0.04	40−135	-12.0 ± 1.0
0.01 M $CaCl_2$	pH 9	-0.25 ± 0.10	27−120	-2.9 ± 1.0
	pH 7	-0.17 ± 0.05	12−46	-1.8 ± 0.8
	pH 5	-0.45 ± 0.06	26−67	-9.1 ± 1.0
	pH 3	-0.24 ± 0.10	56−124	-5.5 ± 1.5
0.05 M $CaCl_2$	pH 9	-0.20 ± 0.06	20−91	-4.0 ± 2.0
	pH 7	-0.29 ± 0.05	20−31	-2.8 ± 0.6
	pH 5	-0.44 ± 0.04	28−40	-7.5 ± 1.5
	pH 3	-0.22 ± 0.08	56−102	-4.7 ± 2.0
0.5 M $CaCl_2$	pH 9	-0.30 ± 0.06	50−90	-8.5 ± 2.0
	pH 7	-0.25 ± 0.04	32−47	-3.2 ± 0.8
	pH 3	-0.37 ± 0.03	40−68	-6.5 ± 1.0

Effect of the pH. Figure 3 shows the effect of the pH on the retraction force curves obtained between BSA layers (adsorbed on silica) in a NaCl 0.01 M solution. The values of adhesion forces (normalized by the sphere radius), the minimum and maximum pull-off distances, and separation energies (normalized by the sphere radius) are presented in Table 1. A strong adhesion can be observed at pH 5 (near the iep of BSA). However, at pHs 7 and 9, the adhesion is very low or even negligible. At pH 3, the adhesion is not as strong as at pH 5, but nonetheless some BSA molecules seem to remain bound and are stretched until distances as long as 100 nm. This surprisingly high elongation of the proteins does not affect the reproducibility of the approach

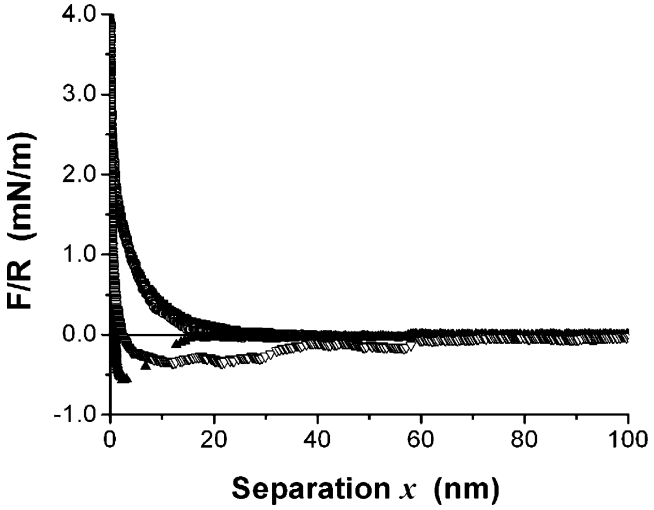

Figure 4. Effect of the pH on the adhesion between apoferritin layers (adsorbed on silica) in a NaCl 0.01 M solution: (■) pH 9; (○) pH 7; (▲) pH 5; (▽) pH 3.

Table 2. Adhesion Forces (Normalized by the Colloid Probe Radius), Minimum and Maximum Pull-Off Distances and Separation Energies (Normalized by the Colloid Probe Radius) for the Adhesion between Apoferritin Layers (Adsorbed on Silica) in Aqueous Media at Different pHs and at Different Concentrations of Different Salts (NaCl or $CaCl_2$)

		F_a/R (mN/m)	L_p (nm)	E_s/R (10^{-12} J/m)
0.01 M NaCl	pH 9	−0.10 ± 0.05	87−133	−2.5 ± 1.0
	pH 7	−0.10 ± 0.06	0−93	−2.0 ± 1.2
	pH 5	−0.65 ± 0.08	28−109	−10 ± 3
	pH 3	−0.35 ± 0.07	87−145	−15 ± 4
0.1 M NaCl	pH 9	−0.24 ± 0.10	70−125	−10 ± 3
	pH 7	−0.15 ± 0.04	52−107	−4.6 ± 1.5
	pH 5	−0.46 ± 0.10	52−98	−6.6 ± 1.8
	pH 3	−0.63 ± 0.05	50−143	−12.3 ± 2.0
1 M NaCl	pH 9	−0.31 ± 0.10	74−150	−12 ± 4
	pH 7	−0.10 ± 0.06	12−87	−1.2 ± 2.0
	pH 5	−0.16 ± 0.02	27−66	−1.4 ± 0.5
	pH 3	−0.62 ± 0.13	20−109	−18 ± 4
0.01 M $CaCl_2$	pH 9	−0.55 ± 0.10	53−130	−14 ± 5
	pH 7	−0.45 ± 0.05	34−78	−5.4 ± 2.0
	pH 5	−0.70 ± 0.10	32−110	−9.7 ± 1.5
	pH 3	−0.28 ± 0.05	46−100	−9.7 ± 2.0
0.05 M $CaCl_2$	pH 9	−0.76 ± 0.10	54−110	−25 ± 8
	pH 7	−0.68 ± 0.09	25−66	−8 ± 3
	pH 5	−0.87 ± 0.10	26−97	−11 ± 3
	pH 3	−0.38 ± 0.05	26−105	−4.9 ± 1.5
0.5 M $CaCl_2$	pH 9	−0.60 ± 0.10	62−136	−24 ± 6
	pH 7	−0.45 ± 0.10	47−82	−10 ± 4
	pH 5	−0.33 ± 0.10	23−65	−3.5 ± 1.5
	pH 3	−0.56 ± 0.08	38−60	−5.5 ± 1.7

curves (results not shown), which indicates that proteins quickly recover their initial conformation.

Similar behaviors to that observed with BSA are also detected in the case of apoferritin. This can be seen in Figure 4, in which the influence of pH on the retraction curves between apoferritin layers (adsorbed on silica) in a NaCl 0.01 M solution is presented. Again, the strongest adhesions appear at pHs 3 and 5, whereas the lowest adhesions correspond to pHs 7 and 9. The parameters that characterize the adhesions in the case of apoferritin are displayed in Table 2. The highest adhesion force is obtained at pH 5 (near the iep of apoferritin), as in the case of BSA. The pull-off distances are longer than in the case of BSA, which is understandable if we take into account the larger dimensions of the apoferritin molecule.

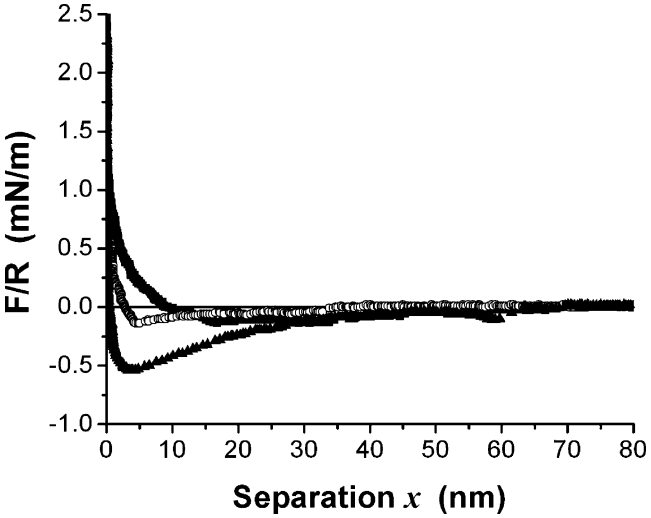

Figure 5. Effect of the NaCl concentration on the adhesion between BSA layers (adsorbed on silica) in a solution at pH 3: (■) 0.01 M; (○) 0.1 M; (▲) 1 M.

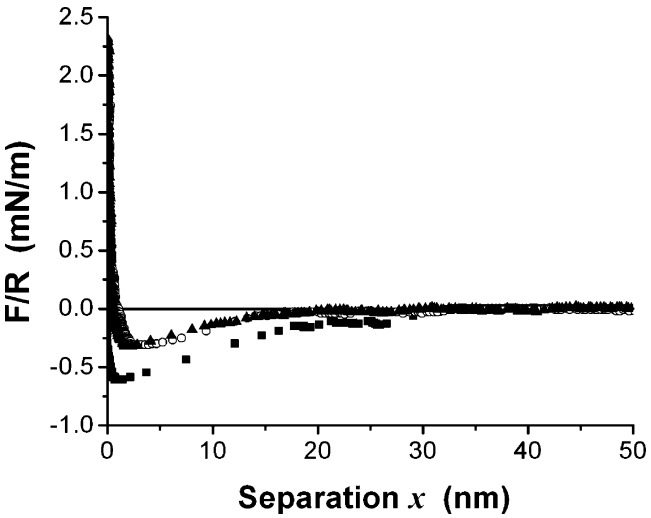

Figure 6. Effect of the NaCl concentration on the adhesion between BSA layers (adsorbed on silica) in a solution at pH 5: (■) 0.01 M; (○) 0.1 M; (▲) 1 M.

Effect of the Salt Concentration. The effect of the NaCl concentration on the adhesion between BSA layers (adsorbed on silica) in a solution at pH 3 is presented in Figure 5. The corresponding adhesion parameters are shown in Table 1. As can be seen, the increase in the NaCl concentration favors the adhesion between the protein layers (increase in the adhesion force and in the separation energy). The same occurs at pHs 7 and 9 (see Table 1). A general observation of this work is that the lower the repulsion of electrostatic origin the higher the adhesion between the proteins is. Thus, in general, the adhesion is more important when the net charge of the proteins is low (at pH 5, near iep; see Figure 3) or when the electrostatic repulsions are screened at high salt concentration (see Figure 5). In these cases, the bond formations between protein molecules when both layers are compressed seem to be more important than in the other cases.

In contrast with the behavior at pHs 3, 7, and 9, the adhesion between BSA layers decreases as the NaCl concentration increases at pH 5. This can be observed in Figure 6 and in Table 1. This could be explained in the following way: pH 5 is very near the

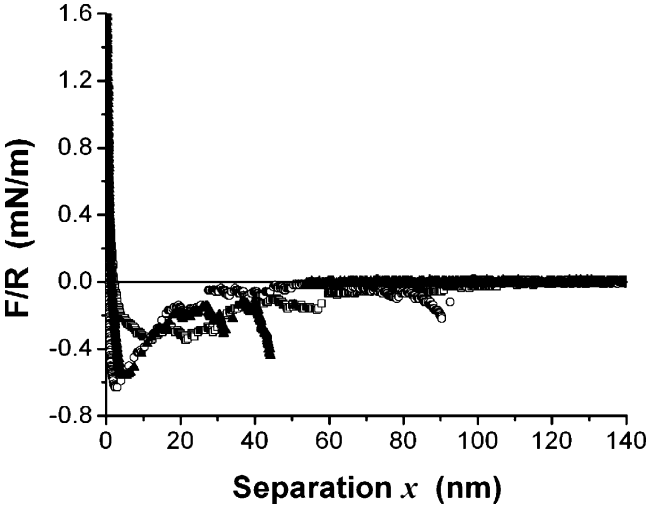

Figure 7. Effect of the NaCl concentration on the adhesion between apoferritin layers (adsorbed on silica) in a solution at pH 3: (□) 0.01 M; (○) 0.1 M; (▲) 1 M.

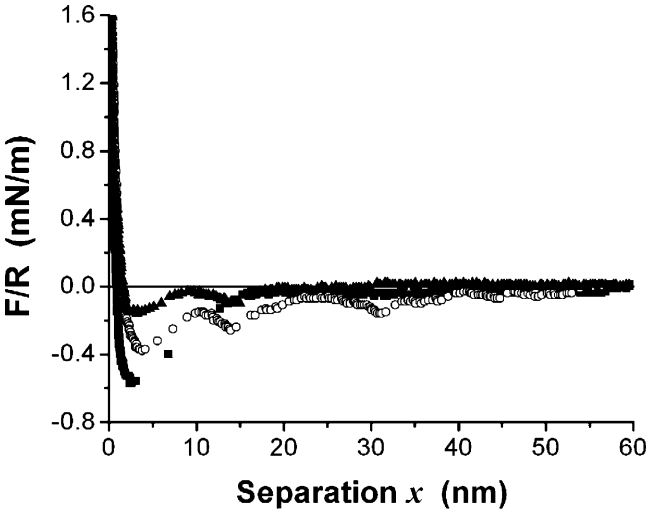

Figure 8. Effect of the NaCl concentration on the adhesion between apoferritin layers (adsorbed on silica) in a solution at pH 5: (■) 0.01 M; (○) 0.1 M; (▲) 1 M.

iep of BSA and, therefore, the proteins possess almost the same number of positively and negatively charged groups. When the protein layers are approached and compressed, charge correlations lead to electrostatic attractions that increase the adhesion between proteins in the different layers. Such a mechanism is consistent with the highest adhesion measured at pH 5 in Figures 3 and 4. The screening of those electrostatic attractions when the salt concentration is high leads to a decrease in the measured adhesion.

Similar tendencies have been found in the case of apoferritin. Figures 7 and 8 show the effect of the NaCl concentration on the adhesion between apoferritin layers (adsorbed on silica) in solutions at pH 3 and 5, respectively, and Table 2 summarizes the values of the adhesion parameters. In general, analogously to the case of BSA, the increase in NaCl concentration gives rise to an increase in the adhesion between the apoferritin layers (increase in the adhesion force and in the separation energy) at pHs 3, 7, and 9, but the opposite occurs at pH 5.

Effect of the Salt Type. As mentioned above, the strength of the adhesion between the protein layers seems to be very influenced by interactions of electrostatic origin. To test this point a 2:1 electrolyte was used ($CaCl_2$) and the results were compared to those obtained with a 1:1 electrolyte (NaCl). Figures 9 and 10 and Table 1 allow such comparison for the case of BSA

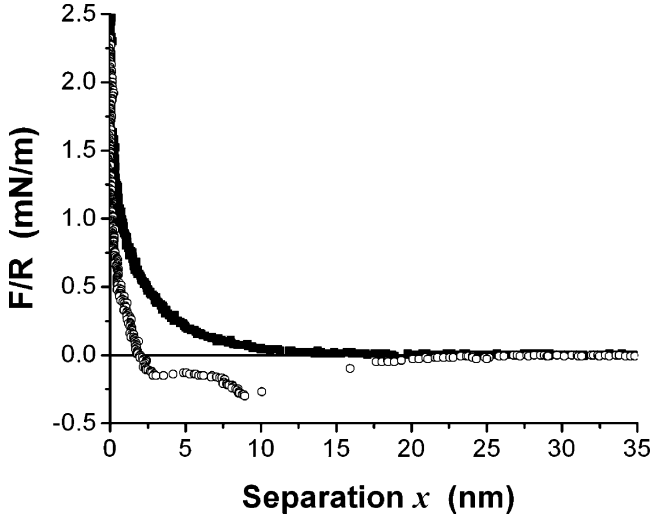

Figure 9. Effect of the salt type on the adhesion between BSA layers (adsorbed on silica) in a solution at pH 9: (■) NaCl 0.01 M; (○) $CaCl_2$ 0.01 M.

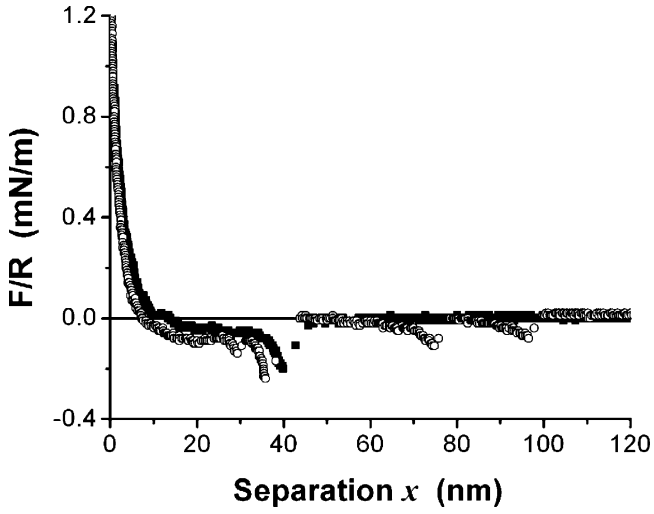

Figure 10. Effect of the salt type on the adhesion between BSA layers (adsorbed on silica) in a solution at pH 3: (■) NaCl 0.01 M; (○) $CaCl_2$ 0.01 M.

layers adsorbed on silica. In Figure 9, it is possible to observe a clear increase of the adhesion when $CaCl_2$ was used instead of NaCl at pH 9. The same occurs at pH 7 (Table 1). At these pHs (above the iep) the BSA is negatively charged. The electrostatic repulsion between the negative groups of the BSA molecules is more effectively screened by divalent counterions (Ca^{2+}) than by monovalent ones (Na^+), which favors the bond formation when the protein layers are compressed in the former case. Ca^{2+} ions can also form bridges between negative charges of different proteins, which leads to an increase in adhesion too.

On the other hand, the adhesion between BSA layers is practically unaffected by the use of NaCl or $CaCl_2$ at pH 3 (see Figure 10). This is because at this pH (below the iep) the BSA is positively charged and the counterions are in both cases the Cl^- anions. The Ca^{2+} bridging mechanism obviously cannot apply in this case.

Similar results are obtained in the case of apoferritin layers (Table 2). This clearly points out the generality of electrostatic interactions in the adhesion between protein layers.

Effect of the Adsorption Substrate. So far we have only presented the adhesion studies between protein layers adsorbed on silica. To analyze the influence of the substrate, adhesion

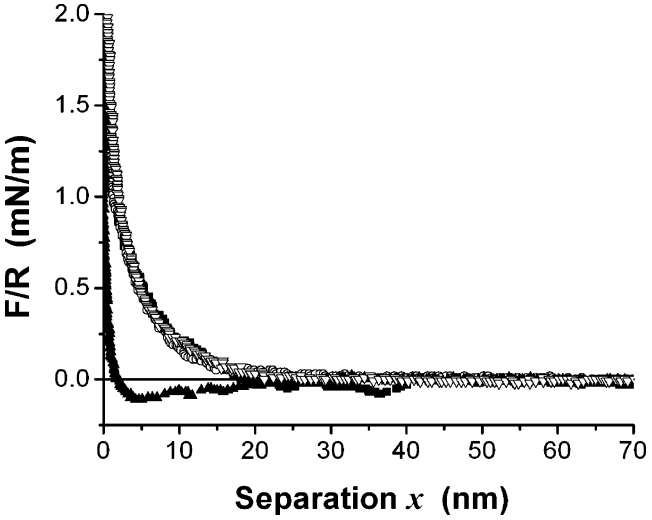

Figure 11. Effect of the pH on the adhesion between BSA layers (adsorbed on polystyrene) in a NaCl 0.01 M solution: (■) pH 9; (○) pH 7; (▲) pH 5; (▽) pH 3.

Table 3. Adhesion Forces (Normalized by the Colloid Probe Radius), Minimum and Maximum Pull-Off Distances and Separation Energies (Normalized by the Colloid Probe Radius) for the Adhesion between BSA Layers (Adsorbed on Polystyrene) in Aqueous Media at Different pHs and at Different Concentrations of Different Salts (NaCl or $CaCl_2$)

		F_a/R (mN/m)	L_p (nm)	E_s/R (10^{-12} J/m)
0.01 M NaCl	pH 9	-0.10 ± 0.08	52−79	-1.0 ± 1.5
	pH 7	-0.05 ± 0.05	0−65	-0.5 ± 0.5
	pH 5	-0.11 ± 0.04	29−173	-2.5 ± 1.5
	pH 3	-0.04 ± 0.03	0−76	-0.6 ± 0.5
0.1 M NaCl	pH 9	-0.30 ± 0.10	103−163	-16 ± 3
	pH 7	-0.13 ± 0.06	32−102	-3.5 ± 2.0
	pH 5	-0.09 ± 0.02	9−68	-2.0 ± 1.0
	pH 3	-0.04 ± 0.04	0−104	-1.0 ± 0.7
1 M NaCl	pH 9	-0.08 ± 0.04	41−99	-1.7 ± 1.0
	pH 7	-0.02 ± 0.03	0−40	-1.0 ± 0.7
	pH 5	-0.02 ± 0.02	0−15	-0.1 ± 0.2
	pH 3	-0.31 ± 0.03	42−65	-5.9 ± 0.9
0.01 M $CaCl_2$	pH 9	-0.14 ± 0.04	80−100	-4.8 ± 2.0
	pH 7	-0.17 ± 0.06	40−90	-5.0 ± 2.0
	pH 5	-0.22 ± 0.05	50−77	-4.8 ± 2.0
	pH 3	-0.02 ± 0.02	0−73	-0.4 ± 0.4
0.5 M $CaCl_2$	pH 9	-0.40 ± 0.04	80−194	-19 ± 5
	pH 7	-0.19 ± 0.08	20−100	-9 ± 5
	pH 5	-0.06 ± 0.03	0−40	-0.6 ± 0.4
	pH 3	-0.14 ± 0.04	12−105	-3.0 ± 1.5

measurements were also carried out between BSA layers adsorbed on a very different substrate: polystyrene.

Figure 11 shows the effect of the pH on the retraction force curves between BSA layers (adsorbed on polystyrene) in a NaCl 0.01 M solution. The adhesion parameters are present in Table 3. As in the case of silica substrate, the higher adhesion occurs at pH 5 (near the iep of the protein); at other pHs, the adhesion is almost negligible.

The effect of the salt concentration on the adhesion can be also analyzed in Table 3. Again, in general the adhesion between the BSA layers increases as the salt concentration increases at pHs 3, 7, and 9, whereas the opposite occurs at pH 5. This phenomenon can be interpreted in terms of the electrical charge of the protein and the screening role of the electrolyte, as for the case on silica. As an example, Figure 12 presents the retraction force curves between BSA layers (adsorbed on polystyrene) in a solution at pH 3 and different NaCl concentrations.

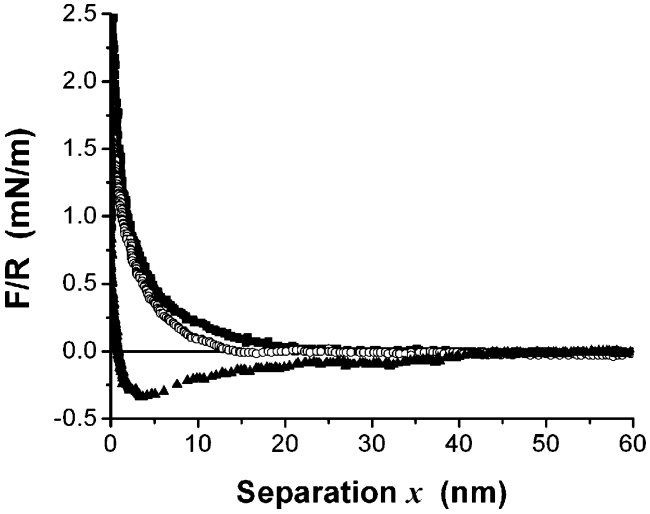

Figure 12. Effect of the NaCl concentration on the adhesion between BSA layers (adsorbed on polystyrene) in a solution at pH 3: (■) 0.01 M; (○) 0.1 M; (▲) 1 M.

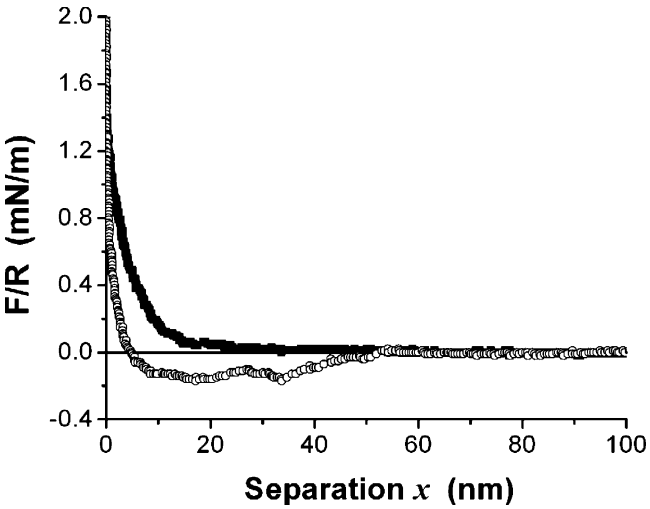

Figure 13. Effect of the salt type on the adhesion between BSA layers (adsorbed on polystyrene) in a solution at pH 7: (■) NaCl 0.01 M; (○) $CaCl_2$ 0.01 M.

An increase of the adhesion when $CaCl_2$ is used as electrolyte instead of NaCl at pHs 5, 7, and 9 can be also noted in Table 3. Figure 13 shows the comparison between the retraction force curves obtained in a solution at pH 7 and 0.01 M of each salt. This increase in the adhesion seems to be related to the greater screening effect of the electrostatic repulsion between the negative charge groups of the BSA by the Ca^{2+} counterions (see the comments in the previous section). At pH 3, however, the adhesion is less affected by the change of NaCl by $CaCl_2$ (see Table 3), since in this case the counterions (Cl^-) are the same in both cases. This behavior was also found in the case of BSA adsorbed on silica.

If Tables 1 and 3 are compared, it is possible to observe that, in general, there is a decrease in the adhesion when polystyrene substrates are used instead of silica substrates: both the adhesion force and the separation energy are, in general, smaller in the case of BSA adsorbed on polystyrene. However, the maximum pull-off distance is very often higher in this case. This could be due to the fact that a higher BSA quantity adsorbs on polystyrene than on silica. According to different works in the literature, the maximum BSA quantity adsorbed on silica (in our conditions of maximum adsorption) is around 2.2 mg/m^2,[28] whereas in the case of adsorption on polystyrene this value increases until 3.5

mg/m^2, approximately.[30,32] A higher quantity of BSA on the polystyrene surfaces has two effects: on one hand, there is a higher number of charged groups which, at pHs away from the iep, increase the electrostatic repulsions when the protein layers are compressed; on the other hand, the protein molecules may be in a more compact conformation, which reduces the interpenetration when the protein layers are compressed. Both effects give rise to a decrease in the measured adhesion.

General Discussions

Although the error intervals are very important, some tendencies can be extracted from the values of the parameters presented in the tables shown in this work. It is possible to note that at low salt concentration (0.01 M) the adhesion is higher (higher adhesion force and higher separation energy) at pH 5, near the iep of the proteins. This adhesion, in general, increases as the salt concentration increases, with the exception of the case at pH 5, where the opposite occurs. The observed increase of the adhesion with the salt concentration is in contrast with the results of Xu and Logan,[17] who detected a decrease of the adhesion between several proteins (BSA−BSA, BSA−protein A, BSA−lysozyme, BSA−polylysine) when the ionic strength rose. However, our results are in agreement with the well-known increase of the bacterial adhesion with the ionic strength.[39,40] In this way, it seems to be a correlation between the adhesion results of bacteria and the adhesion results of proteins, which is not so surprising if we take into account that proteins are important components in bacterial membranes. Nevertheless, the effect of other bacterial components such as (phospho)lipids, lipopolysaccharides, flagella, extracellular polymeric substances, and other biopolymers on the bacterial adhesion should be also analyzed in detail.

An increase in the adhesion between the protein layers is also observed in general at pHs 5, 7 and 9 when $CaCl_2$ is used instead of NaCl (compare the results corresponding to 0.01 M of both salts); at pH 3, however, no effect of electrolyte type seems to exist. Thus, the valence of the counterions (Na^+ or Ca^{2+} at pH above the iep; Cl^- at pH below the iep) has influence on the adhesion between the protein layers. All of these tendencies suggest that the electrostatic interactions between the charged groups of the compressed proteins are very important in the adhesion between the surfaces covered by such proteins.

The pull-off distances observed (Tables 1−3) are surprisingly large in some cases. In general, the maximum pull-off distances are longer for apoferritin than for BSA, which is in agreement with the higher size of the former protein. It is usual to observe in some cases a series of spikes when the proteins are being stretched in the retraction force curve (see for example Figures 2, 7, 8, and 10). These spikes clearly reflect the different extensions and bond breaks between the proteins of both layers.

IV. Conclusions

This paper studies and analyzes the effect of pH, electrolyte concentration, type of electrolyte and substrate on the adhesion between protein layers (BSA and apoferritin) by means of an AFM and the colloid probe technique.

The influence of the pH and the concentration and type of electrolyte on our adhesion results clearly points out a very important contribution of the electrostatic interactions in the adhesion between protein layers. This is in agreement with Xu and Logan's conclusion:[17] the electrosteric forces dominate the interaction between the protein-coated materials in comparison to hydrophobic forces.

The reduction in the adhesion observed when silica substrates are replaced by polystyrene substrates could be ascribed to a higher quantity of protein adsorbed in the last case, which increases the electrostatic (more charged groups) and steric (more compact conformation) repulsions between the protein layers.

This paper shows that the AFM together with the colloid probe technique can provide a very useful means of directly quantifying the adhesion between surfaces covered by biological macromolecules.

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