Soft Matter

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Interaction of organic ions with proteins

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In this study we have investigated how different proteins interact with big organic ions. Two ions similar in size and chemical structure (Ph_4B^- anion and Ph_4As^+ cation) were studied. The proteins chosen are the two major allergenic proteins of cow's milk, β-lactoglobulin and β-casein, and bovine serum albumin, BSA, as a reference protein. First, a quantitative study to determine the hydrophobic degree of the proteins was performed. Then, electrokinetic and stability measurements on protein-coated polystyrene (PS) microspheres as a function of the tetraphenyl ion concentration were carried out. Our results show that the affinity of the organic ions depends on the hydrophobicity of the interface. Big charge inversions and re-stabilization patterns were observed at very low concentrations of tetraphenyl ions for the most hydrophobic protein interface (with β-casein). Besides, the ionic concentrations needed to destabilize these colloidal systems were roughly one order of magnitude lower for the anion than for the cation. In addition, we studied conformational changes of the adsorbed proteins with a Quartz Crystal Microbalance. Proteins were adsorbed onto hydrophobic flat substrates and then were exposed to the tetraphenyl ions. The protein films swelled or collapsed as a function of the accumulation of tetraphenyl ions. Similarly to the electrokinetic/stability studies, the ionic concentration necessary to trigger structural changes of the proteins films were one order of magnitude larger for the cation than for the anion. All the results evidence that the accumulation of these organic ions onto an interface depends directly on its degree of hydrophobicity. We attribute the different interaction of the anion and the cation with these interfaces to their dissimilar hydration, which makes the anion show a more hydrophobic behaviour than the cation.

1. Introduction

Proteins are made up of amino acids which form complex structures, which are governed and determine the interaction of the proteins with themselves (intramolecular interactions) and their environment. In this regard, the hydrophobic interaction plays a major role in protein folding, adsorption and aggregation.[1] In general, the biological functions of proteins are performed in aqueous solution, where in addition to water they interact with different kind of electrolytes, small organic molecules and other macromolecules.[2] Specific ion-protein interactions can influence the internal structure of proteins and protein-protein interactions, which have important implications on protein folding and stability or denaturation.[3,4] Thus, understanding protein-ion interaction is important from a fundamental point of view as well as for the numerous applications where proteins are involved. For instance, the presence of ions is very relevant in the food industry (formation and stabilization of emulsions) or for biosensor applications, among others.[5,6]

More than 100 years have passed since the pioneering works of Lewitt and Hofmeister, who studied systematically the precipitation of proteins in presence of different salts.[7] They found that ions can be arranged in consistent sequences (now called Hofmeister series) following their influence on the solubility of proteins. However, there is still no conclusive answer about the origin of such effects, which have been reproduced in many colloidal phenomena.[8] In fact, the search of a general theory capable of explaining all the disparity results among ions and interfaces is considered one of the big challenges in Colloid and Interface Science.

It is accepted that water itself plays a fundamental role in water-mediated interactions of ions and surfaces. This is, ionic specific interactions depend on the extension of the hydration of ions and interfaces (their hydrophobic/hydrophilic character). Mechanisms that consider that poorly hydrated (chaotropes) ions accumulate on hydrophobic surfaces but are excluded from hydrophilic ones and, in contrast, highly hydrated (kosmotropes) ions are excluded from hydrophobic surfaces but accumulate on hydrophilic ones have successfully explained many experimental results including those referred to proteins.[9–16] These mechanisms have been partially validated by using big hydrophobic ions that have a large influence over hydrophobic surfaces.[12,17] These big ions have been recently included in the Hofmeister series as super-

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chaotropes.[13] For this kind of ions, like the cation tetraphenylarsonium (Ph_4As^+) or the anion tetraphenylborate (Ph_4B^-), the ion-surface interactions are not always dominated by the electrostatic charge. Often, the most relevant factor is the nature of the surface. In the case of very hydrophobic surfaces, ion accumulation can produce giant charge inversions at low concentration.[12,14] Interestingly, Ph_4B^- usually shows more significant effects at lower concentrations than Ph_4As^+, in spite of their structural similarities: the charge of these relatively big ions is located at their centres, whereas the neutral, hydrophobic rings directly interact with the environment. These differences are related with the different behaviour of anions and cations due to the asymmetrical interactions with water molecules.[15,18–20] Thus, the hydration of Ph_4B^- and Ph_4As^+ is different, as has been shown by previous experimental and simulation studies.[21–23] This difference has important consequences on the ion-surface interactions, which are enhanced when the tetraphenyl ions interact with soft matter systems, like PNIPAM microgels.[14]

Although many studies concerning the interaction of proteins and ions belonging to Hofmeister series have been published, systematic studies considering big hydrophobic ions are still lacking. However, due to the high ionic specificity found with protein systems we would expect a remarkable interaction of the proteins with the super-chaotropes ions. In this paper we report on a study of the interaction of three proteins of biotechnological interest with hydrophobic tetraphenyl ions. Proteins tend to accumulate at interfaces modifying the properties of the sorbent, and in particular its electrostatic charge and hydrophobicity.[24] The final properties of the protein-sorbent complex will depend on the nature of the protein and the degree of coverage. Protein adsorption allows the preparation of soft interfaces with different degree of hydrophobicity. In addition, proteins can exhibit positive or negative net charge depending on their isoelectric point (pI). Thus, the surface charge can be controlled by changing the pH of the medium. This makes adsorbed proteins very versatile systems to deepen in the study of ionic specific interactions. In this work we explored to what extent tetraphenyl ions, acting as counter-ions or co-ions, are sensitive to the hydrophobicity and charge of the protein-coated surfaces.

We investigated β-casein and β-lactoglobulin—the major allergenic proteins of cow's milk and bovine serum albumin, BSA, a well-known globular protein used as a reference. As sorbent, we have used hydrophobic anionic and cationic latex particles; a detailed characterization of the adsorption of the proteins on different substrates is presented elsewhere.[25] The affinity of the tetraphenyl ions to the protein-latex complexes has been investigated by electrophoresis and colloidal stability. In addition, the adsorption of the proteins on a flat hydrophobic surface and their interaction with the tetraphenyl ions has been studied by using a Quartz Crystal Microbalance (QCM-D).

This study aims to inquire into the following aspects. First, we explore the role of the hydrophobic nature of a surface in the ion-surface interactions, in particular for big hydrophobic ions. Second, we study the effects caused by these ions in soft protein interfaces. Finally, we seek to confirm the different behaviour of Ph_4B^- and Ph_4As^+ when interacting with this kind of systems.

2. Materials and methods

2.1 Reagents

All the products were of analytical grade and were used as received. Salts were purchased from Scharlau and Sigma Aldrich. Sodium tetraphenylborate (ref T25402) and tetraphenylarsonium chloride (ref T25305) were obtained from Sigma Aldrich. Water used in all experiments was double distilled and deionized (DDI) with a Milli-Q Water Purification System (Millipore).

2.2 Proteins and buffered solutions

Bovine serum albumin (BSA) (ref A7030 fatty acid and globulin free), β-lactoglobulin (ref L3908 chromatographically purified by the supplier) and β-casein (ref C6905 salt-free, lyophilized by the supplier) were supplied by Sigma Aldrich. Several buffers of ionic strength lower than 2 mM were used: pH 4 was buffered with acetic acid; pH 10 with boric acid. In each case, the pH was adjusted by adding NaOH. In addition, we used a buffered solution at pH 7 with Bis-Tris in order to dissolve the β-casein protein.[26] The pH of this solution was adjusted by adding HCl; the ionic strength of the solution was 2.4 mM. Non-buffered solution at pH 3 was prepared by adding dilute HCl to DDI water.

2.3 Polystyrene latex microspheres

Two kind of polystyrene (PS) microspheres (negatively and positively charged) were used. The anionic latex was synthesized in our laboratories. Sulfonate groups, with a surface charge density of -9.6 $\mu C/cm^2$, provide the negative charges. This latex has a mean diameter of 138±7 nm with high monodispersity.[9,20] The positive polystyrene latex (IKERLAT polymers) has a surface charge density of 17.4 $\mu C/cm^2$ and mean diameter of 475±4 nm. Its positive charge is given by amine groups. Surface charge densities and sizes were determined by direct titration and transmission electron microscopy (TEM), respectively.

2.4 Adsorption of proteins onto Polystyrene latex microspheres

Protein-coated PS microspheres were prepared by physical adsorption. First, the protein was dissolved in a buffered solution at ca. 1 mg/ml. BSA and β-lactoglobulin were prepared in pH 6 buffer (monosodium phosphate), whereas β-casein was dissolved in pH 7 buffer (Bis-Tris).[26] The β-casein concentration employed was always lower than the critical micelle concentration (CMC).[27,28] The solutions were stirred during 1 hour to ensure the complete protein solubilisation. The real concentration of the solutions was determined by UV absorption. The pH was chosen close to the pI of the proteins to achieve a high degree of coverage onto the PS microspheres.[29] At this close-to-neutrality condition, the repulsion

between the neighbouring adsorbed proteins is reduced, while the protein solution remains stable. Physical adsorption of the proteins on the latex particles was carried out as follows: 2.4 ml of each protein solution was added to a final volume of 8 ml of aqueous solution buffered at the same pH of the protein solution. Then, an amount of latex microparticles corresponding to a total area 0.3 m^2 was added to the final solution. This ratio corresponds to 8 mg of protein for each m^2 of latex surface. Incubation was performed in a shaking water bath at 25ºC during 21 hours, as in a previous study.[30] After that, the solution with the complex (latex-protein) was centrifugated at 14000 rpm and 20ºC during 20 minutes (Hettich Mikro 220R) to separate the non-adsorbed protein molecules. The supernatant was discarded and the pellet was redispersed in the same buffer of adsorption. No desorption was found one week after incubation. Latex-protein complexes were discarded after that period.

2.5 Electrophoretic Mobility Measurements

The measurements of electrophoretic mobility were carried out using a Zetasizer Nano Z device (Malvern Instruments). The particles were diluted to a concentration of around 10^{10} particles/cm^3. The reported data were the average of three measurements; the standard deviation was always lower than 5%.

2.6 Colloidal Stability

The balance between the interparticle electrostatic repulsion and the attractive van der Waals interaction determines the stability of the protein-coated latex. When two particles undergo a collision, the probability of forming a dimer depends on the resulting interaction. A larger ionic strength in the solution implies more effective screening of the electrostatic repulsion, facilitating the aggregation of the particles.[11] Tetraphenyl salts were used to modify the stability of protein-coated PS microspheres.

As the colloidal system aggregates, the turbidity of the sample increases. Thus, the absorbance of the sample can be used to study the aggregation process. The aggregation was monitored as a function of time using a spectrophotometer (Spectronic Genesys 5). The optical absorbance of the samples was measured in a rectangular cell (1 cm path length) at 570 nm at 2-s intervals, during 120 seconds. Equal volumes (3 ml) of colloidal dispersion and salt solution were mixed into the cell at the time of measuring. At the first stages of the aggregation process, the absorbance increases linearly with time until it reaches a plateau. The rate of aggregation, reflected in the absorbance/time rate, speeds up with increasing ionic strengths. The ratio of these rates (at different ionic concentrations) are used to calculate the stability factor (W), also called the Fuchs factor, as:[11]

$$W = \frac{k_f}{k_s} = \frac{(\frac{dAbs}{dt})_f}{(\frac{dAbs}{dt})_s} \quad (1)$$

where k_f represents the fastest aggregation-kinetics constant (where each collision between the colloidal particles results in aggregate formation) and k_s is referred to the slower aggregation rate at other ionic concentrations. W is related to the average number of collisions between two particles before they stick irreversibly. The limit values of the parameter W take place when the system is stable (k_s=0 and $W\rightarrow\infty$); or when the system is completely unstable ($k_s=k_f$ and W=1). W is commonly plotted against the ionic strength in double logarithmic scale. In this kind of representation, log(W) typically decreases linearly with the logarithm of the ionic strength when the colloidal particles are aggregating (see Fig. 3c and 3d). The critical coagulation concentration (CCC), the minimum ionic concentration needed to cause the complete destabilization of the system (W=1), can be obtained from a linear fit of the data.

Under certain experimental conditions, the re-stabilization of the colloidal particles is observed at high ionic strengths. Experimentally, this process is reflected in an increasing of W with salt concentration. The minimum concentration needed to re-stabilize the system is known as the critical stabilization concentration (CSC).

2.7 Quartz Crystal Microbalance (QCM-D)

The Quartz Crystal Microbalance with Dissipation monitoring device (QCM-D; Q-sense E1 by Biolin Scientific) is a very sensitive tool commonly used to determine the mass and viscoelastic properties of thin films adsorbed over flat substrates.[31] It is based on a piezoelectric system; an alternating voltage is applied over a quartz crystal, producing the mechanical deformation of the material. The frequency is chosen as the resonance frequency (f) of the crystal. Any small mass deposited over the crystal results in a change in the resonance frequency of the coated crystal, detectable by the device. In addition, in the QCM-D the applied voltage is intermittently switched on and off and the decay in time of the oscillation is monitored. In this way the ''energy dissipation factor'' D is obtained (D is the inverse of the quality factor of the resonance peak, Q, $D=Q^{-1}=2\Gamma/f$, where Γ is the half-band-half-width of the resonance peak). D quantifies the damping in the system and provides information about the viscoelastic properties of the adsorbed film.

For thin, rigidly attached films (small values of ΔD_n upon adsorption) the adsorbed mass can be calculated by using the Sauerbrey equation.[32] This equation relates the change in resonance frequency (Δf_n) with the mass adsorbed onto the substrate:

$$\Delta f_n = -\frac{n}{C} m_{eff} = -\frac{n}{C} \rho_f h_f \quad (2)$$

where n is the overtone order (odd), C is a constant depending on the fundamental resonance frequency of the quartz crystal (in our case C=18 ng $cm^{-2}Hz^{-1}$, with a fundamental frequency of f_F=5 MHz), m_{eff} is the effective areal mass density of the adsorbed film and ρ_f and h_f are the density and the thickness of the adsorbed film, respectively. The values of m_{eff} calculated from eq. 2 may include

solvent associated to the film. The Sauerbrey equation is strictly valid only when the film is homogeneously distributed and rigidly attached to the substrate. This kind of film is characterized by small values of the dissipation ($\Delta D_n \approx 0$) and low dispersion in $\Delta f_n/n$. To obtain h_f, an assumption for the value of ρ_f is necessary; a typical value for soft systems is $\rho_f \approx 1$ g/cm^3.[31] When ΔD is substantially larger than zero, more complex models involving the viscoelastic properties of the film needs to be considered. Commonly the film is described as a homogenous viscoelastic layer with a complex shear modulus G^* in contact with a viscous solvent. To analyze the measured QCM-D data, we have fitted Δf and ΔD for the odd harmonics (n=3 to n=13) using the QTM software, written by D. Johannsmann,[33] to determine m_{eff} taking into account the viscoelastic properties of the protein films.

The protein adsorption procedure for the QCM-D experiments is described in detail elsewhere.[25] Briefly, quartz crystals with gold electrodes were coated with CH_3–terminated self-assembled monolayers (SAM) by 4 hours exposure to a 1mM solution of 1-octadecanothiol in ethanol, to obtain a hydrophobic substrate. Once the quartz crystal was mounted in the cell of the QCM-D, it was exposed to the desired buffer solution for enough time to reach a stable signal and to set the reference values of f_n and D_n ($\Delta f_n/n$=0 and ΔD_n=0). Then, the protein solution was injected in the same conditions that for the adsorption on latex: concentration 1 mg/ml at 25ºC, and pH 6 buffer (monosodium phosphate) for BSA and β-lactoglobulin, or pH 7 buffer (Bis-Tris) for the β-casein. After 30 minutes of adsorption, the cell was rinsed with the protein-free buffer solution to remove the non-adsorbed proteins, and then with buffer solutions at pH 3, 4 or 10 depending on the experience. Then the protein film was exposed to different ionic concentrations of tetraphenyl salts.

3. Results and discussion

3.1 Hydrophobic degree of the β-casein, BSA and β-lactoglobulin proteins

In Fig. 1, we show the structure of the three proteins considered in this work, indicating the hydrophilic or hydrophobic character of each amino acid. The atomic coordinates were obtained from our previous Molecular Dynamics (MD) simulations of BSA, β-lactoglobulin and β-casein performed at 25˚C and neutral pH. As can be seen in the figure, the three proteins have a few hydrophobic residues at their surface, typically surrounded by hydrophilic amino acids. As we will see later, the hydrophobic/hydrophilic balance of each protein is a relevant variable to rationalize the experimental results. In order to make a quantitative evaluation of the hydrophobic/hydrophilic balance of each protein, we have employed the semi-empirical thermodynamic methodology described elsewhere[34], as implemented in the online calculation tool OPM.[35] The employed

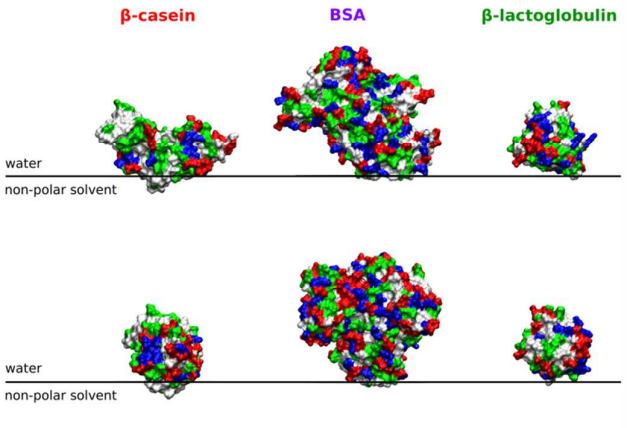

Fig. 1: Results from the OPM calculation (see text) for the preferred location of β-casein, BSA and β-lactoglobulin proteins at a water/non-polar solvent interface (black line). The protein residues are coloured depending on their nature: hydrophobic (white), hydrophilic (green), positively charged (blue) and negatively charged (red). The most hydrophobic part of the protein is immersed in the non polar solvent, under the black line.

scale is based on the thermodynamics of transfer of proteins from water to a non-polar solvent of reference (decadiene). The method requires as a sole input the atomic coordinates of the proteins (which are known from our previous simulations mentioned above) and provides the free energy of transfer from water and the preferred positioning of the protein (which region of the protein prefers to be in water and which in the non-polar solvent).The results of this calculation are shown in Fig. 1. As seen in that figure, only a very small region of the proteins prefers to be solvated by the non-polar solvent. This region is largest for β-casein (containing 12 aminoacids) and it is smallest for β-lactoglobulin (containing only 1 aminoacid), which has a thermodynamic preference to be in a non-polar solvent instead of water. The different size of these regions is also reflected in the solvation thermodynamic quantities, such as the Gibbs free energy ΔG of transfer from water. The calculation gives for β-casein the higher absolute value for the transfer energy (-9.5 kcal/mol), followed by the BSA (-5.1 kcal/mol) and finally the β-lactoglobulin (-1.7 kcal/mol). This means that the three proteins studied in this work can be ranked as follows, with the more hydrophobic/less hydrophilic at the left:

β-casein > BSA > β-lactoglobulin

We know from previous work that tetraphenyl ions (which are big hydrophobic ions) have a strong affinity for hydrophobic interfaces and are repelled by hydrophilic interfaces.[12,14,17] Thus, we expect that the interactions of the ions with the proteins will take place at the hydrophobic regions indicated in Fig. 1 and we can anticipate a different strength of the ion-interaction with each protein, as suggested by the ranking proposed above.

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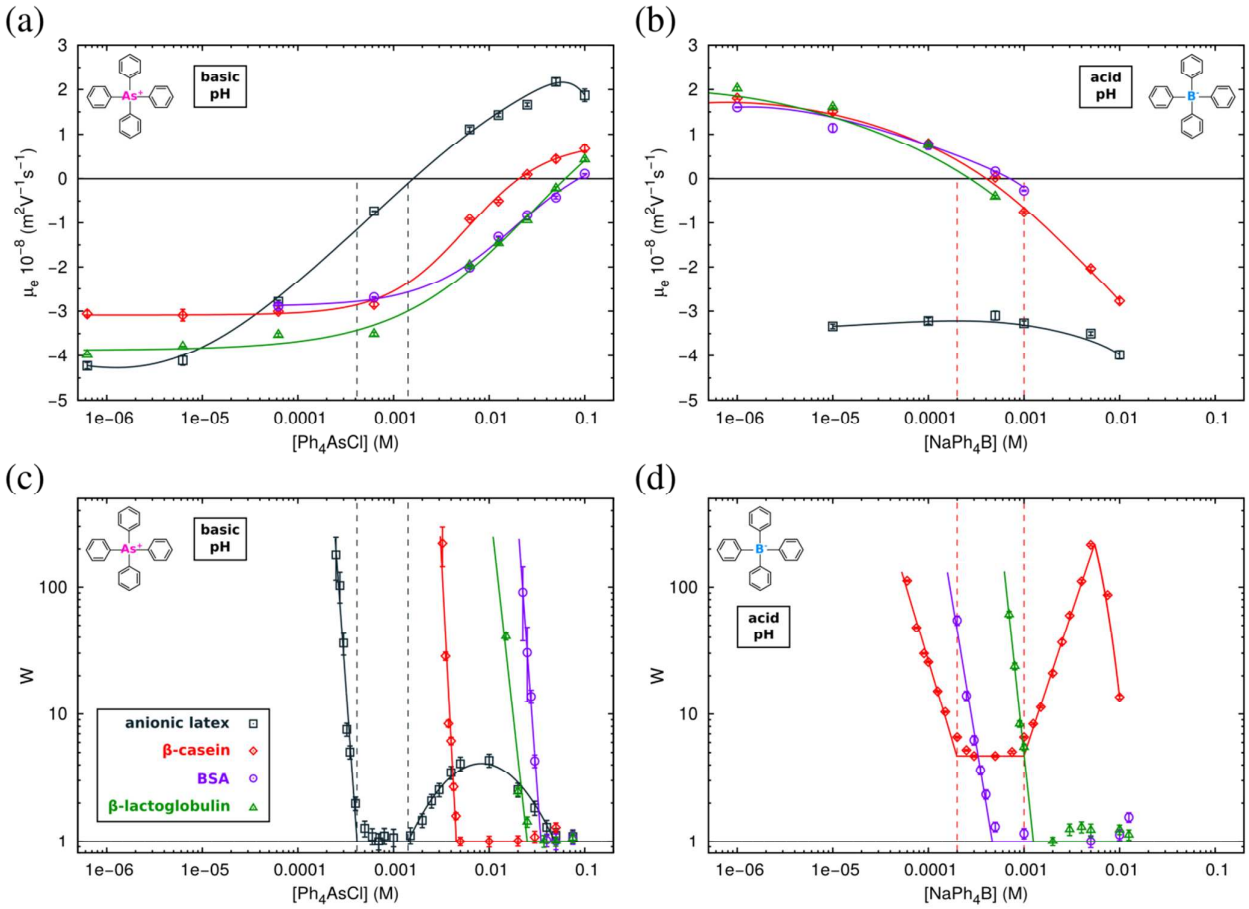

Fig. 2: Electrophoretic mobility (a),(b) and stability (c),(d) measurements of the anionic PS latex (black squares) and the latex-protein complexes: β-casein (red rhombi), BSA (violet circles) and β-lactoglobulin (green triangles) vs. tetraphenyl ion concentration. (a), (c) $Ph_4As^+Cl^-$ at pH 10, and (b), (d) $Na^+Ph_4B^-$ at pH 4 (except for the β-lactoglobulin complex that was measured at pH 3). The solid lines of μ_e curves are a guide to the eye. Solid lines in the stability plots are linear fits to obtain the CCC values. The vertical dashed lines indicate the instability zone for the anionic latex in presence of $Ph_4As^+Cl^-$ and for the β-casein complex in presence of $Na^+Ph_4B^-$.

3.2 Electrokinetic and stability studies of latex-protein complexes in presence of the tetraphenyl salts

We measured the μ_e of the colloids as a function of tetraphenyl ion concentration. The experiments were carried out at basic pH (negatively charged complexes) for the Ph_4AsCl salt and at acid pH (positively charged complexes) for the $NaPh_4B$. Thus, both Ph_4As^+ and Ph_4B^- ions acted as counter-ions for the protein-coated particles. For the study under acidic conditions, pH 4 was used with BSA and β-casein, while pH 3 was used with β-lactoglobulin (μ_e of the complex formed with this protein was close to zero at pH 4).[25] Table 1 shows μ_e results for the bare latex and latex-protein complexes. The effect of the tetraphenyl salts on μ_e for the protein-coated anionic latex is shown in Fig. 2. It is important to remark that the maximum concentration of Ph_4As^+ investigated was ten times larger than that for the case of Ph_4B^-, due to the lower solubility of the anion.

Mobility curves show that for both ions μ_e decreases (in absolute value) as the concentration of tetraphenyl increases. This is partially due to the progressive shrinking of the ion double layer, which reduces the zeta potential. However, the important changes in μ_e observed at low tetraphenyl ion concentrations and the significant differences in the effect of $Na^+Ph_4B^-$ and $Ph_4As^+Cl^-$ clearly

show that screening-induced zeta potential changes is just a (minor) reason of the observed μ_e reduction. Hence, measurements show that tetraphenyl ions adsorb on PS latex and protein covered PS latex particles. The strength of the interaction of ions with particles can be quantified in electrokinetic measurements by the salt concentration required to neutralize the particle (i.e. to obtain the point of zero electrokinetic charge, μ_e=0).[37] In the case of tetraphenyl salts, these concentrations are usually very low due to the strong interaction of these ions with hydrophobic interfaces.[12,14,17]

For the bare anionic PS latex particles (Fig. 2a and 2b), the μ_e differences between Ph_4As^+ and Ph_4B^- can be ascribed to the different role that the anion and the cation play on the anionic latex. Ph_4As^+ acts as counter-ion and the μ_e showed a strong dependence on the salt concentration decreasing from very low concentrations. In contrast, when Ph_4B^- acts as co-ion (Fig. 2b) μ_e remained relatively constant until 10^{-3} M and increased slightly (in absolute value) as the concentration was further increased. When the behaviour of protein-coated latexes is examined, striking differences in the μ_e behaviour in the presence of both ions (which act as counter-ions) are observed. For the Ph_4As^+ cation (Fig. 2a) μ_e values remained constant and equal to those of pH without salt (table 1) until $5{\cdot}10^{-4}$ M. At larger concentrations the mobility progressively decreased. In contrast, in presence of the Ph_4B^- anion μ_e began to decrease from very low concentrations; at $5{\cdot}10^{-4}$ M the mobility for the three complexes latex-protein was very close to zero. In Fig. 2a and 2b we observe that for the latex-protein complexes, the concentrations of the Ph_4B^- anion at μ_e=0 are much smaller than those required to obtain μ_e=0 with the Ph_4As^+ cation. Fig. 2a shows that μ_e is close to zero for the anionic PS latex at 1 mM of Ph_4As^+, at 10 mM for the β-casein complex and almost at 100 mM for the globular protein complexes. Interestingly, this order matches the hydrophobicity sequence calculated before. Hence, Ph_4As^+ has increasing affinity for more hydrophobic protein-latex complexes. For the studies at acid pH (Fig. 2b, and 2d), we did not observe any appreciable difference in the Ph_4B^- concentrations to get μ_e=0 for the different proteins (Fig. 2b), being of the order of 0.2-0.4 mM. Since the complexes have similar bare electrokinetic charge densities (in absolute value; around 0.02 e/nm^2)[25], this result indicates that the interaction of the Ph_4B^- anion with the latex-protein complexes is much stronger than that of the Ph_4As^+ cation. These results are in line with those previously obtained with other colloidal systems (PNIPAM microgels).[14]

Further addition of salt at concentrations higher than those required to obtain μ_e=0 induces charge inversion, that is, the reversal in sign of μ_e. Charge inversion or reversal refers to the attraction of counter-ions to an interface in excess of its own bare charge. In the case of our protein-latex complexes this effect can be clearly seen in presence of Ph_4B^- and Ph_4As^+, for the β-casein complex, which contains the most hydrophobic protein (Fig. 2a and 2b). In addition, the bare latex showed the highest μ_e inversion at the lowest Ph_4As^+ concentration (acted as counter-ion) which reinforces the importance of the interface hydrophobicity in this interaction.

Table 1: Electrophoretic mobility (μ_e) in salt free conditions and critical coagulation concentration (CCC) in the presence of tetraphenyl salts for anionic latex and the latex-protein complexes at different pH

	Mobility $\mu_e{\cdot}10^{-8}$ ($m^2V^{-1}s^{-1}$)		CCC	
Coating protein	salt free, pH=4	salt free, pH=10	$[Ph_4AsCl]$ (mM) pH=10	$[NaPh_4B]$ (mM) pH=4
β-lactoglobulin	2.35 ± 0.08*	-3.69± 0.01	25 ± 3	1.25 ± 0.05*
BSA	2.16± 0.01	-3.44± 0.02	35 ± 3	0.47 ± 0.02
β-casein	1.78 ± 0.02	-3.01± 0.04	4.5 ± 0.1	0.20 ± 0.04
Bare latex	-3.02± 0.06	-4.28± 0.08	0.42 ± 0.01	Stable

(* in this case pH=3)

In order to better understand the interaction of the tetraphenyl ions with the proteins, we also carried out stability studies of the complexes as a function of the concentration of tetraphenyl salts. The results are shown in Figs. 2c, 2d. The values of critical coagulation concentration (CCC) obtained from Figs. 2c, 2d are compiled in Table 1.

The first important thing to note is that the CCC values obtained with Ph_4B^- for the latex-protein complexes are typically 20 to 100 times lower than those obtained with Ph_4As^+. This is consistent with the view, arising from electrokinetic measurements, that the interaction of the Ph_4B^- anion with the protein-latex complexes is stronger than that of the Ph_4As^+ cation.

As seen in Table 1, the complex containing β-casein (the most hydrophobic one) has always the smaller CCC value, both in the case of Ph_4As^+ and Ph_4B^- salts. The CCC values in presence of Ph_4As^+ (Table 1) are very similar for BSA and β-lactoglobulin, being much smaller than that corresponding to the latex-β-casein complex. The CCC values in presence of Ph_4B^- (Table 1) are all very low, and they follow the hydrophobic order established by the thermodynamic calculation: β-casein > BSA > β-lactoglobulin (Fig. 1). In any case, these CCC values are very similar, reflecting the high affinity of the Ph_4B^- anion for all protein complexes. In the case of the bare anionic latex, the organic anion acts as co-ion, consequently the ion-adsorption increases the net charge of the colloid and it stays stable in all the ion concentration range.

The stability results in the presence of tetraphenyl salts have another distinctive feature, which is the existence of a re-stabilization phenomenon, that is, an increase of the stability ratio W with the addition of salt (Fig. 2c and 2d). Re-stabilization occurs when particles are stable at concentrations higher than the CCC. The concentration at which particles begin to show re-stabilization is called Critical Stabilization Concentration (CSC). This is observed

for the bare anionic latex in presence of $Ph_4As^+Cl^-$ and for the latex-β-casein complex in presence of the Ph_4B^- counter-ion (Fig. 2 b). Comparing in Fig. 2 the mobility measurements with the stability measurements, we see that the re-stabilization (increase of *W* with ion concentration in Fig. 2c and 2d)) is observed only for the systems with substantial charge inversion (see Figs. 2a and 2b). At concentrations where the mobility values were close to zero, the colloidal suspension became unstable (*W*=1; aggregate state). However, when the mobility sign changed, the particles were stable again due to the electrostatic repulsion between charged particles (in this case, charged by the super-equivalent adsorption of ions). As the concentration of $Ph_4As^+Cl^-$ is further increased the latex aggregated again due to the screening of the electrostatic repulsion. Charge reversal by Ph_4As^+ adsorption was also observed for the β-casein complex (Fig. 2a), although the charge reversal was not large enough to re-stabilize the system. On the contrary, when the β-casein complex was in presence of the Ph_4B^- counter-ion (Fig. 2b) a great charge reversal occurred at low salt concentration. This inversion brought a re-stabilization of the complex, followed by aggregation at higher concentrations (Fig. 2d). To the best of our knowledge, this is the first time that a re-stabilization generated by charge inversion due to the adsorption of hydrophobic ions on hydrophobic systems has been reported. Re-stabilization processes have been previously reported on hydrophilic interfaces but in those cases the origin of such re-stabilization has been ascribed to hydration forces.[9,15,38–43] Experimentally, re-stabilization on hydrophilic systems does not involve charge inversion and once the re-stabilization occurs the particles remain stable at any higher salt concentrations.

To investigate to what extent the sign of the charge of the substrate influences the observed results, we also carried out several experiments with the proteins adsorbed on cationic PS latex. Results of μ_e with the adsorbed proteins show similar trends than the ones observed for the anionic particles (Fig. 3), suggesting that the interfaces of the complexes are similar in both cases. Moreover, the cationic latex shows the same behaviour than anionic latex, but changing the counter-ion/co-ion role for the tetraphenyl ions. This is, μ_e remained positive in the range of concentrations studied for the Ph_4As^+ acting as co-ion, while a great charge reversal was observed at $5{\cdot}10^{-4}$ M when the Ph_4B^- acted as counter-ion. As previously mentioned, for the anionic PS latex the charge reversal (with the Ph_4As^+ acting as counter-ion) appeared ca. 10^{-3} M (Fig. 2a). This data confirms once again that the adsorption of the anion on hydrophobic surfaces is much stronger than that of the cation.

From the results presented so far we can conclude that there is a strong interaction between the tetraphenyl ions (acting as counter-ions) and the different proteins investigated. Previous studies reported by us have shown that this type of ions interacts strongly with charged hydrophobic species, even when acting as co-ions.[12,14] In order to investigate the influence of this interaction on protein conformation we performed a study of protein-hydrophobic ion interaction using a QCM-D.

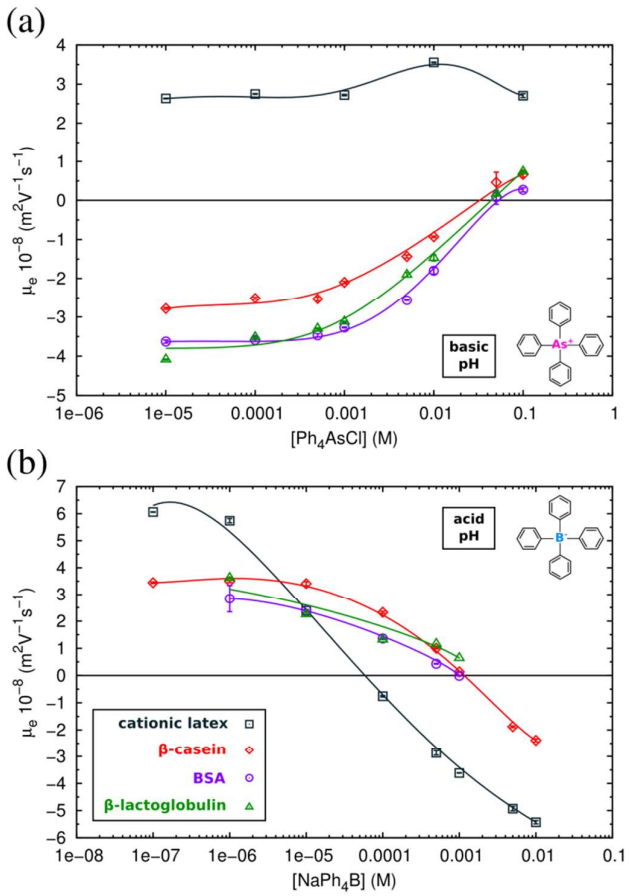

Fig. 3: Electrophoretic mobility measurements of the cationic PS latex (black squares) and latex-protein complexes: β-casein (red rhombi), BSA (violet circles) and β-lactoglobulin (green triangles) vs. concentration of tetraphenyl ions. (a) $Ph_4As^+Cl^-$, at pH 10; (b) $Na^+Ph_4B^-$ at pH 4.

3.3 Conformational changes in adsorbed protein films induced by tetraphenyl salts (QCM-D study)

We have used the QCM-D to explore in more detail the interaction of the different proteins with the hydrophobic tetraphenyl ions. The adsorption was performed onto the QCM-D gold-electrodes coated with methyl-terminated SAMs ($-CH_3$). After 30 min of protein adsorption the solution in the QCM-D cell was exchanged by protein-free buffer solutions at the same pH. Subsequently, the adsorbed protein layers were exposed to basic or acid buffers, solutions of NaCl 10 mM, and sequentially increasing concentrations of the tetraphenyl salts. Finally, the protein layers were exposed again to buffer solutions to verify the reversibility of the process. Two scenarios were explored, with the hydrophobic ions acting either as counter-ions (Figs. 4, 5 and Fig. 1 in supporting information; Ph_4B^- at acid pH or Ph_4As^+ at basic pH) or as co-ion (Figs. 6 and 7 and Fig. 2 in SI; Ph_4As^+ at acid pH or Ph_4B^- at basic pH). Several general trends can be highlighted:

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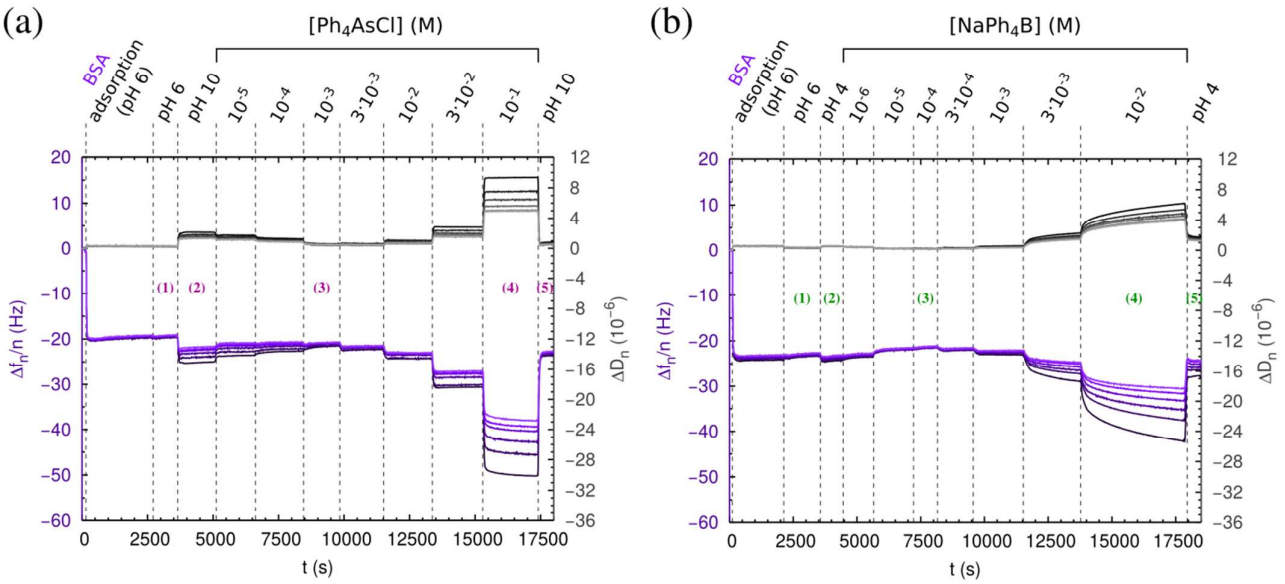

Fig. 4: QCM-D measurements of BSA film in presence of (a) $Ph_4As^+Cl^-$ at pH 10 and (b) $Na^+Ph_4B^-$ at pH 4 (organic ions as counter-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale colour.

i) Influence of pH: For the BSA and β-lactoglobulin, substantial protein swelling was observed when rinsing at pH 10, as evidenced by the increment in Δf_n and ΔD_n (Figs. 4a, 6b, and SI Figs. 1a and 2b). Rinsing with acid buffer also induced some swelling, but to a lesser extent (Figs. 4b, 6a, and SI Figs. 1b and 2a). On the contrary, for the case of β-casein an important reduction in absolute value of $\Delta f_n/n$ upon rinsing was observed in all cases (Figs. 5 and 7) which is probably related to partial protein desorption, as we have discussed elsewhere.[25] The out-of-equilibrium process of desorption of loosely adsorbed β-casein molecules is likely to be responsible for the variability and signal drift observed with this protein upon buffer rinsing.

ii) Tetraphenyl counter-ions (Figs. 4, 5 and SI Fig. 1): Progressively increasing salt concentration induces first the diminution and then the increase of the absolute values of ΔD_n and Δf_n, and of $\Delta f_n/n$ dispersion. As discussed below, we associate this non-monotonic behaviour to increasing association of the hydrophobic ions to the adsorbed protein films. The first stage points to protein dehydration (due to decreasing the effective charge on the proteins) and film densification due to greater screening of intermolecular repulsive electrostatic interaction at larger salt concentrations. The second stage suggests the re-swelling of the films, probably due to film charge inversion.

iii) Tetraphenyl co-ions (cf. Figs. 6, 7 and SI Fig. 2): When the hydrophobic ions act as co-ions, a progressive but less marked protein layer swelling was observed with increasing salt concentration. It is remarkable that the hydrophobic co-ions appear to get associated to the protein films overcoming the electrostatic repulsion, evidencing the determinant role of the hydrophobic interaction (Figs. 6 and 7).

iv) Kinetics: a much slower temporal evolution of f and D was observed for the proteins in contact with Ph_4B^- as counter-ion, compared with the case of Ph_4As^+ (Figs. 4 and 5). On the contrary, no marked difference on the effect of both ions was observed when they acted as co-ions of the adsorbed protein.

v) Protein adsorption and reversibility: Initial (after buffer rinsing) and final (after exposure to tetraphenyl salts) m_{eff} of the adsorbed protein films are presented in Table 2. In all cases, the measured adsorption after buffer rinsing (columns 2 and 5) show a satisfactory agreement with previous QCM studies[44–48] but are clearly in excess of values reported by other methods (e.g. ellipsometry). As has been discussed several times before, this difference is due to the fact that by QCM-D the effective hydrated mass of the film is detected, while ellipsometry methods are rather related to the dry protein mass adsorbed. No significant differences in m_{eff} were observed before and after exposure to the hydrophobic ions when they acted as co-ions. On the contrary, significant changes are observed when they played the role of counter-ions of the protein layer.

Interestingly, *lower* final m_{eff} values were observed after exposure to Ph_4As^+ at basic pH, while substantially *larger* m_{eff} values were found after exposure to Ph_4B^- at acidic pH. These changes indicate a certain degree of irreversibility of ionic co-adsorption on the protein layers, further enhanced by the electrostatic attraction. However, the different trend observed for both ions (swelling for

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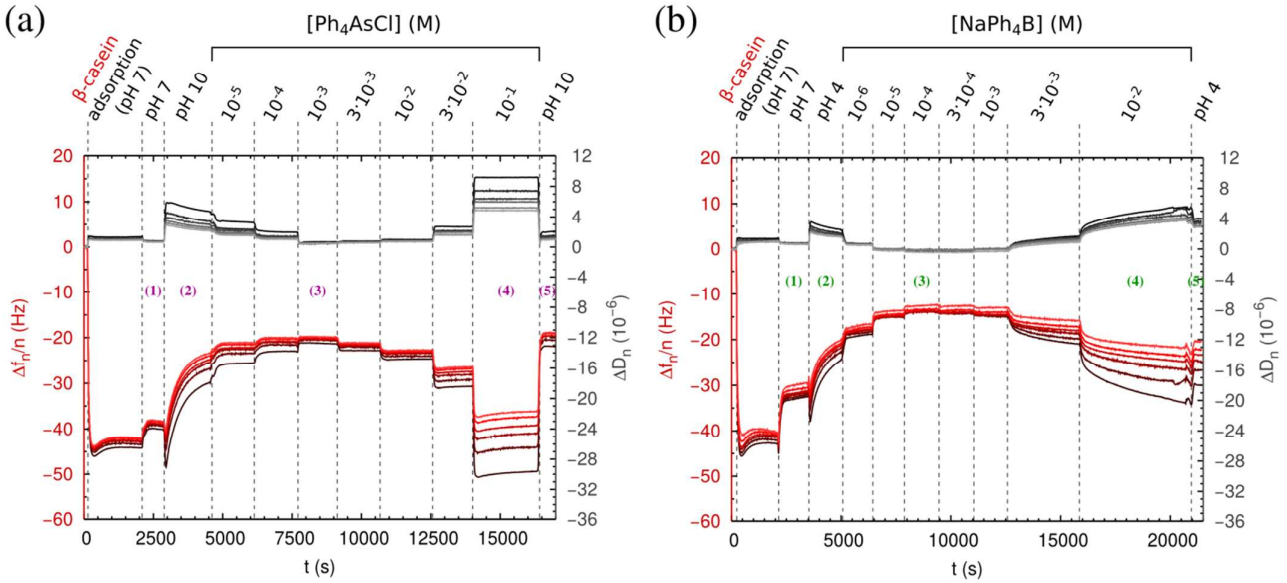

Fig. 5: QCM-D measurements of β-casein film in presence of (a) $Ph_4As^+Cl^-$ at pH 10 and (b) $Na^+Ph_4B^-$ at pH 4 (organic ions as counter-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale colour.

Ph_4B^-, shrinking for Ph_4As^+) points to a larger retention of the hydrophobic anion (Ph_4B^-) after rinsing with salt-free solution. It is also remarkable that upon salt rinsing, the steady-state was always quickly reached for the Ph_4As^+ as the concentration increased while for the Ph_4B^- (as counter-ion) this variation was much slower. Both facts suggest that the hydrophobic anions are being adsorbed deeper in the protein layers.

A compilation of the measured m_{eff} values is presented in Fig. 8. It is interesting to compare the effect of the two tetraphenyl salts (in all cases, the experiments with the Ph_4B^- anion were limited to lower salt concentrations due to its low solubility). When acting as counter-ions of the proteins, it is apparent that Ph_4B^- induces similar effects than Ph_4As^+ at much lower concentrations (Figs. 8a and c), in agreement with electrokinetic and stability experiments (cf. Fig. 2). On the contrary, no marked difference on the effect of both salts (in *f* and *D* shifts or their temporal evolution) was observed when the hydrophobic ions were acting as co-ions of the adsorbed protein (Figs. 8b,d). We can compare the relative m_{eff} variation for the different proteins in the presence of tetraphenyl counter-ions (Fig. 8c).

The relative m_{eff} reduction at intermediate salt concentrations follows the sequence β-casein > BSA > β-lactoglobulin. The opposite sequence is observed for the growth of m_{eff} at high salt concentrations. These results seem to agree with the degree of hydrophobicity calculated before, suggesting that configurational changes due to counter-ion adsorption (collapse and swelling) are directly related to protein hydrophobicity.

Schematic illustrations of the different regimes of protein adsorption and protein-hydrophobic counter-ion interaction are presented in Fig. 9 for Ph_4As^+ (Fig. 9a) and Ph_4B^- (Fig. 9b). Initially, the proteins are densely adsorbed at pH close to pI; the formation of a compact layer is favoured due to the reduced intermolecular electrostatic interaction at this close-to-neutrality condition (panel 1). Changing the pH to basic or acid conditions increases the net molecular charge, increasing intermolecular repulsion and favouring protein swelling (slightly for acid pH and more significant for basic pH; panel 2). For the case of β-casein this promotes desorption of loosely adsorbed macromolecules. Addition of salts has several effects. On one hand, the extension of the electrostatic repulsion is reduced (screened) by adding NaCl or tetraphenyl salts. On the other hand, adsorption of hydrophobic ions has important consequences for the properties of the adsorbed protein layers.

Table 2: Hydrated mass of the adsorbed protein film m_{eff} (±0.2 mg/m^2) at pH 4 and 10, before and after exposing the films to the tetraphenyl salt solutions at the same pH of rinsing

System	adsorption neutral pH/ rinsing pH 4	$NaPh_4B$ (counter-ion)/ rinsing pH 4	Ph_4AsCl (co-ion)/ rinsing pH 4	adsorption neutral pH/ rinsing pH 10	$NaPh_4B$ (co-ion)/rinsing pH 10	Ph_4AsCl (counter-ion)/ rinsing pH 10
β-lactoglobulin	2.4	6.6	2.0	2.2	2.0	2.4
BSA	4.8	6.8	4.9	7.7	7.0	5.8
β-casein	6.5	11.3	5.8	10.4	8.9	6.2

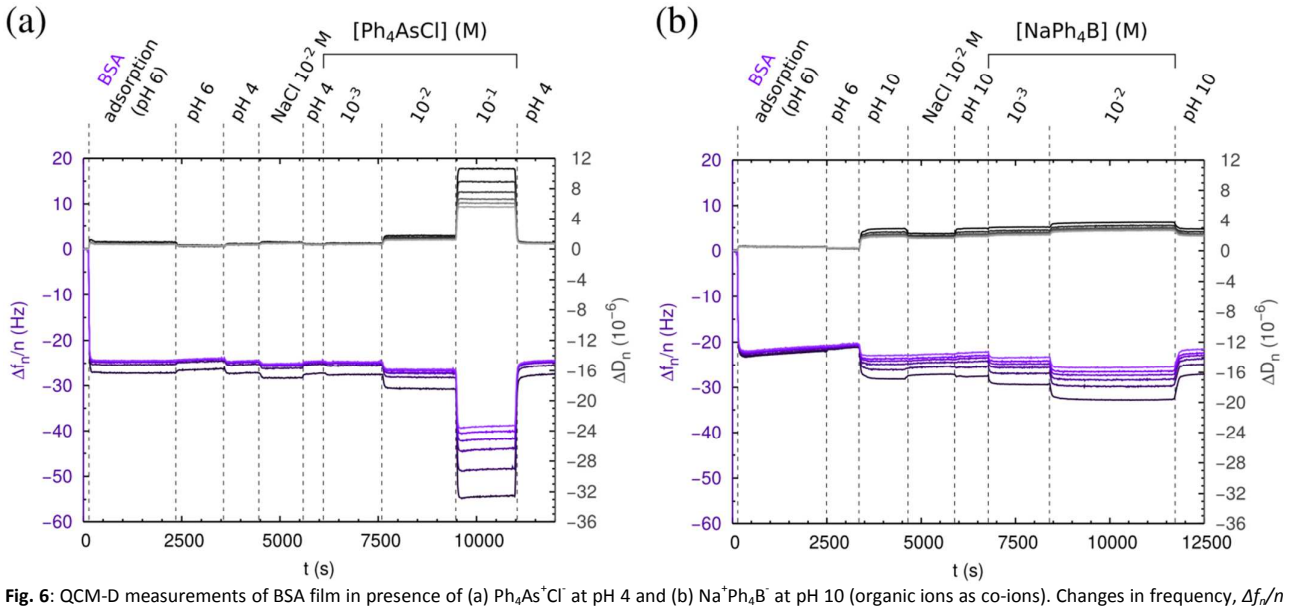

Fig. 6: QCM-D measurements of BSA film in presence of (a) $Ph_4As^+Cl^-$ at pH 4 and (b) $Na^+Ph_4B^-$ at pH 10 (organic ions as co-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale colour.

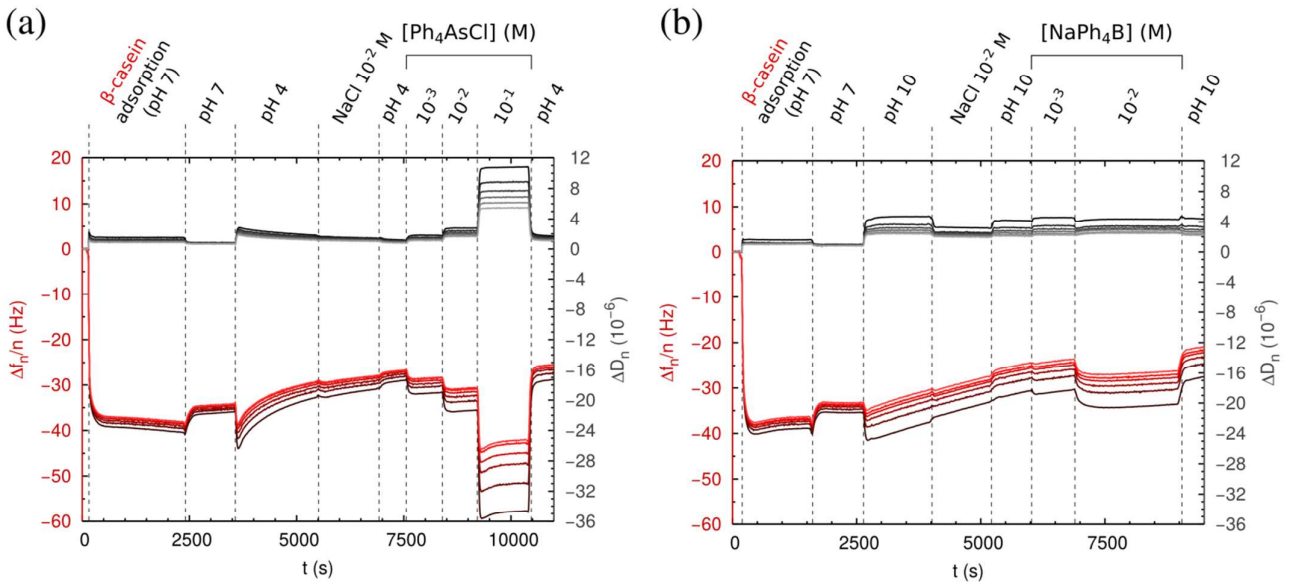

Fig. 7: QCM-D measurements of β-casein film in presence of (a) $Ph_4As^+Cl^-$ at pH 4 and (b) $Na^+Ph_4B^-$ at pH 10 (organic ions as co-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale colour.

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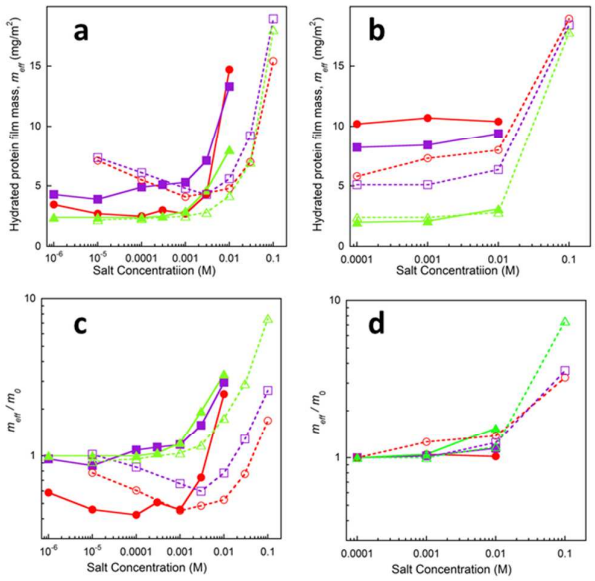

Fig. 8: Protein films m_{eff} measured at different salt concentrations. The tetraphenyl ions act as counter-ions (left; a,c) and co-ions (right; b,d). Red circles: β-casein; Green triangles: β-lactoglobulin; Violet squares: BSA. Open symbols: Ph_4AsCl. Closed symbols: $NaPh_4B$. (a), (b) Measured m_{eff}. (c), (d) m_{eff} normalized by the value measured in absence of salt, m_{eff}/m_0, in log-log representation. A 10-fold reduction in the concentration required to obtain similar changes is observed for Ph_4B^- in comparison with Ph_4As^+, when acting as counter-ions.

The different experimental techniques used in this work reveal the complex behaviour of the proteins in presence of hydrophobic ions; several aspects —which are not observed in presence of simpler salts (e.g. NaCl) — deserve to be highlighted. First, the different behaviour observed in presence of hydrophobic ions acting as co-ions or counter-ions is noteworthy. When acting as co-ions, the effect of the hydrophobic ions seems to be fairly reversible. The opposite is true when they act as counter-ion, suggesting partially irreversible co-adsorption. In addition, there is a more important but slower influence of the hydrophobic anion acting as counter-ion. We have reported in the past that this anion is able to strongly disrupt soft-matter systems, a behaviour which we fail to observe for the case of the hydrophobic cation.[14] A similar scenario could explain the differences between both counter-ions observed in the present study. Finally, the charge reversal of the proteins in presence of these monovalent hydrophobic ions is remarkable. Charge inversion, associated to particular phase transitions in biological or colloidal systems, has often been reported to be a consequence of correlated adsorption of multivalent counter-ions or polyions, if the concentration of adsorbed ions is large enough to overcompensate the original charge. On the contrary, the charge reversal observed in this work is due to different (''chemical'') reasons, related to the combined effect of electrostatic and hydrophobic interaction; it appears at very low concentrations of adsorbing hydrophobic ions.

Progressively increasing concentration of hydrophobic counter-ions first neutralizes (panel 3) and then reverses the charge promoting re-swelling (panel 4) of the adsorbed layer. These results can be related with mobility and stability measurements (Fig. 3). Upon counter-ion adsorption the protein layer is compacted, μ_e goes to zero reducing the stability of the system. The regime of charge reversal was not accessible in electrokinetic/stability measurements (except for the case of β-casein) due to the aggregation of the protein-coated latexes. However, it is clearly evidenced in the QCM-D results. After rinsing with salt-free buffer solutions some degree of irreversibility in the conformational change of protein films is apparent, suggesting incomplete removal of the adsorbed hydrophobic ions. This effect is more marked for the Ph_4B^- anion than for the Ph_4As^+ cation (panel 5).

4. Conclusions

We have studied how three proteins of different hydrophobicity, β-lactoglobulin, β-casein and bovine serum albumin, interact with big hydrophobic ions. By combining studies of electrokinetic and colloidal stability of protein-coated latex with Quartz Crystal Microbalance studies of protein-coated hydrophobic surfaces, we have shown that there is a strong interaction between hydrophobic tetraphenyl ions and protein-coated surfaces. Substantial charge reversal of the surfaces in presence of these monovalent hydrophobic ions was observed. This interaction is substantially stronger for the Ph_4B^- anion in comparison with the Ph_4As^+ cation when they act as counter-ions, and depends on the degree of hydrophobicity of the protein. Salt-induced re-stabilization was also evidenced for the most hydrophobic colloids studied (bare and β-casein coated latex). This strong interaction is further reflected in the conformational changes underwent for the proteins in presence of the tetraphenyl ions. All these results reinforce the idea that

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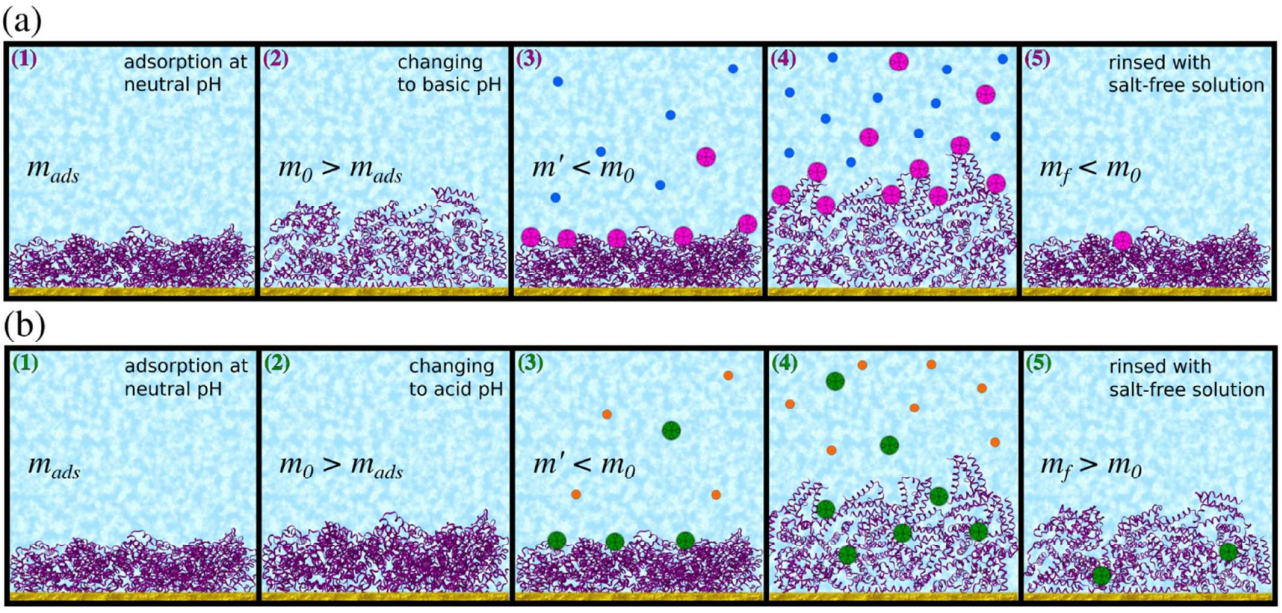

Fig. 9: Schematic cartoon of the conformational changes underwent by the protein films adsorbed onto a flat substrate in presence of $Ph_4As^+Cl^-$ (a) and $Na^+Ph_4B^-$ (b), tetraphenyl ions acting as counter-ions. (1) Proteins are densely adsorbed at pH closed to pI. (2) Changing the pH to basic or acid conditions increases the net molecular charge, increasing intermolecular repulsion and favouring protein swelling (more significantly for basic pH). (3) The tetraphenyl ions, Ph_4As^+ (fuchsia circles) or Ph_4B^- (green circles), are adsorbed onto the protein layer reducing the charge density when they act as counter-ions. The protein film is compacted again. (4) At higher ionic concentrations, the ion adsorption reverses the charge of the protein interface. The protein layer is increasingly swollen as the ionic concentration is increased. (5) Partial ionic removal is observed upon rinsing with salt-free buffer solutions. Blue and orange circles represent Cl^- and Na^+ ions, respectively.

interactions of the tetraphenyl ions with completely/partially hydrophobic interfaces are dominated by the hydrophobic interaction.

Many ions of biological interests are hydrophobic. The results presented in this work illustrate how this hydrophobicity may lead to an unexpected behaviour from purely electrostatic considerations. This knowledge may open avenues to better understanding process involving proteins interacting with hydrophobic ions, e.g. transport phenomena involving ion adsorption and membrane translocation, or for designing novel hydrophobic ion pairing strategies in drug delivery systems.

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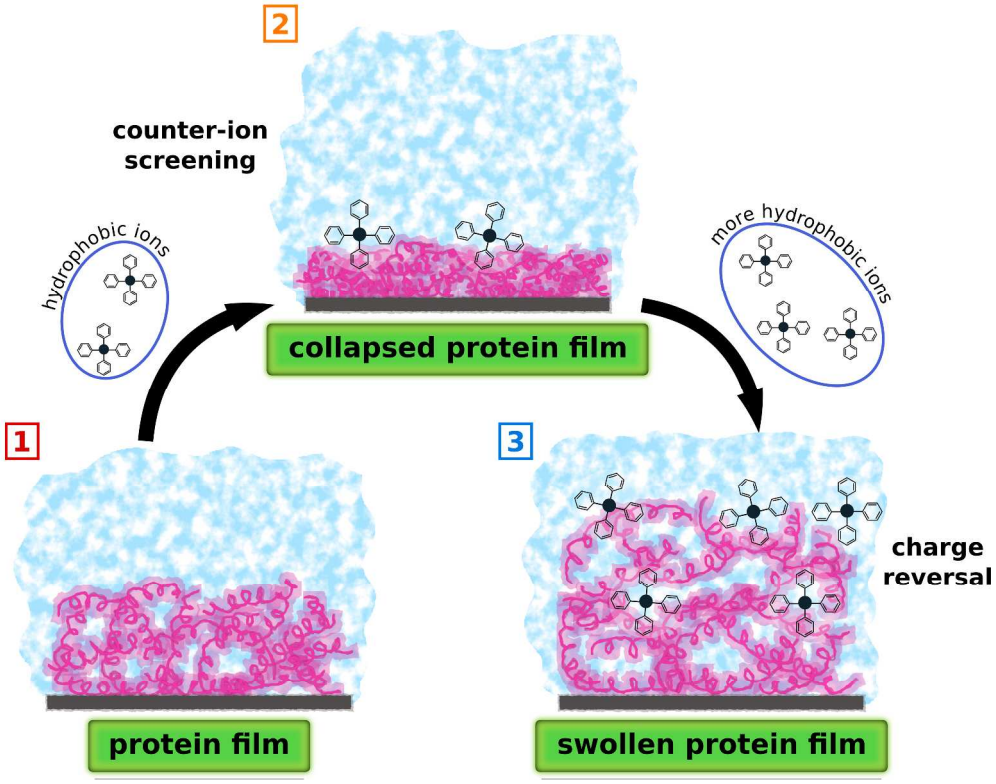
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counter-ion
screening
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swollen protein film

Supporting Information for publication

Interaction of organic ions with proteins

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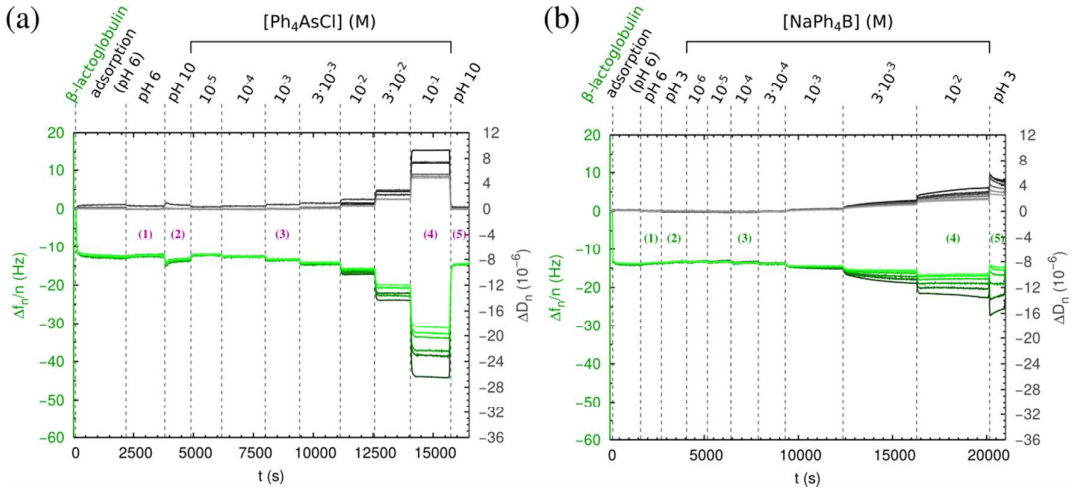

Fig. 1: QCM-D measurements of β-lactoglobulin film in presence of (a) $Ph_4As^+Cl^-$ at pH 10 and (b) $Na^+Ph_4B^-$ at pH 3 (organic ions as counter-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale color.

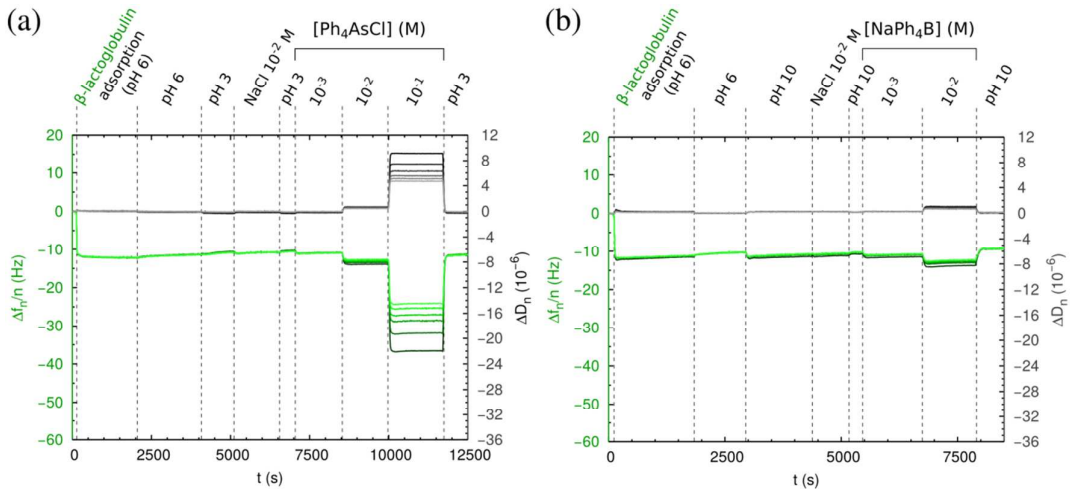

Fig. 2: QCM-D measurements of β-lactoglobulin film in the presence of (a) $Ph_4As^+Cl^-$ at pH 3 and (b) $Na^+Ph_4B^-$ at pH 10 (organic ions as co-ions). Changes in frequency, $\Delta f_n/n$ (negative values) and changes in energy dissipation, ΔD_n (positive values) are represented. Each overtone from 3 to 13 is shown in a different tonality, from dark to pale color.