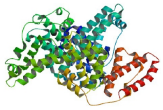
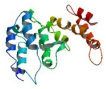
STSM SCIENTIFIC REPORT: Leonor Pérez Fuentes

During my short scientific stay at the Centre de Recherche Paul Pascal (CRPP, CNRS-University of Bordeaux, France) supervised by Dr. Carlos Drummond, we have carried out several experimental studies of protein adsorption over different kind of surfaces. For that, we have used a Quartz Crystal Microbalance with Dissipation monitoring device (QCM-D) [1]. The QCM-D is a very sensitive tool, commonly used to determine the thickness and viscoelastic properties of nanofilms adsorbed over flat substrates. This device is capable of measure a number of resonance frequencies of the quartz crystal, so any small mass adsorbed over the crystal results in a frequency change, detectable by the device. In addition, in the QCM-D the voltage applied is intermittently switched on and off and the decay in time of the oscillation is monitored. This give us information about the energy dissipation in the adsorbed film, which allows us to extract the viscoelastic properties of the sample.

The proteins chosen for this study have been β-casein and β-lactoglobulin (milk proteins) and bovine serum albumin (BSA) as reference protein. The knowledge of the physical properties of these proteins has a biotechnological interest due to their allergenicity for a significant percentage of the population.

BSA and β-lactoglobulin, are globular proteins and exhibit a similar adsorption behavior, whereas β-casein is a disordered protein and more hydrophobic than the other two, showing a different behavior. In the next table are included some size properties of these proteins [2-5]:

Protein	Dimensions (nm)	Molecular weight (Da)
BSA 	14 x 4 x 4	66000
β-lactoglobulin 	3,6 x 3,6 x 3,6	18000
β-casein 	Disordered protein: $R_g \sim 5{,}4$	24000

In all our experiments we have used quartz crystal with gold electrodes and then they have been treated to obtain surfaces with hydrophobic or hydrophilic character. At first, we performed adsorption isotherms for the different proteins over hydrophobic substrates. In these experiments, the gold electrode was covered with thiol molecules with $-CH_3$ termination, which form chemicals bonds with the gold surface. After that, we covered this surface with a 300 nm thick polystyrene layer by spin coating, which can be easily removed after each experiment. The hydrophobicity of the surface was determined by water contact angle. In this case, the contact angle for the polystyrene surface was 90º.

In Figure 1 we can see the adsorption isotherms for the three proteins over the polystyrene surface. We show the thickness of the adsorbed protein film versus the protein concentration injected (The last point in the Figure 1 corresponds to the rinsed at the same pH of the protein solution):

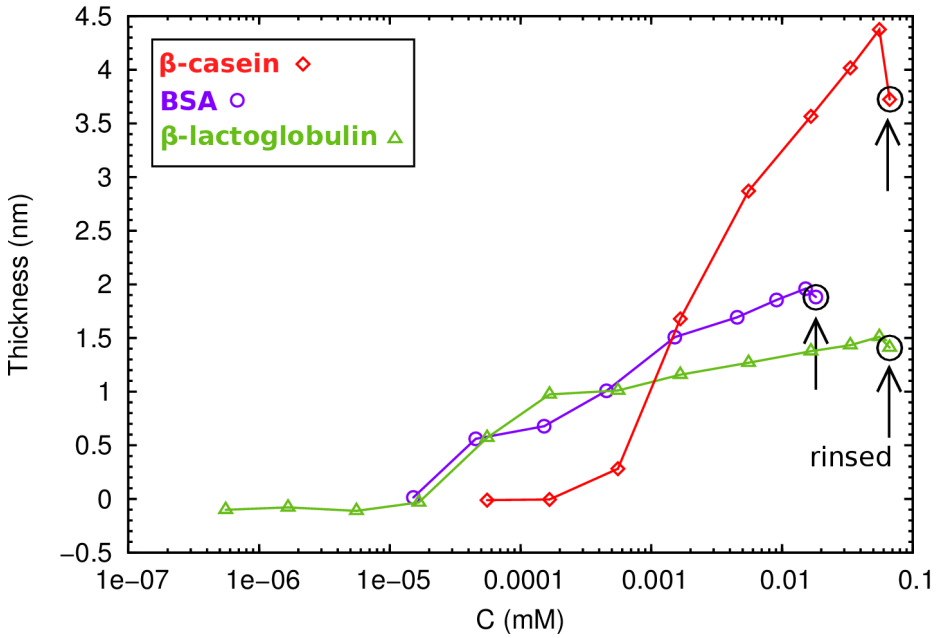


Figure 1: Adsorption isotherm of the different proteins studied.

In each case, the thickness of the protein films has been calculated by using Sauerbrey's equation, which relates the resonance frequencies changes with the mass adsorbed on the crystal:

$$\Delta f_n = -\frac{n}{C} m_f = -\frac{n}{C} \rho_f h_f$$

where n is the overtone order, C is a constant depending on the fundamental resonance frequency of the quartz crystal, m_f is the areal mass density of the adsorbed film and ρ_f and h_f are the density and the thickness of the adsorbed film, respectively.

In Figure 1 we can see that BSA and β-lactoglobulin begin to adsorb at $5 \cdot 10^{5}$ mM approximately and stabilize in a similar thickness value about 2 nm. On the other hand, β-casein begin to adsorb at $5 \cdot 10^{4}$ mM and reaches 4,5 nm of thickness at almost 0,1 mM. We did not investigate larger concentrations because this protein forms aggregates under these conditions. The adsorptions were made close to the isoelectric point of each protein in order to obtain the maximum coverage, pH 6 for BSA and β-lactoglobulin and pH 7 for β-casein while assuring protein solubility.

In order to compare the protein adsorption over hydrophobic surfaces with different chemical composition, we carried out the adsorption of β-casein over the gold-coated surface with -CH_3 thiol molecules, whose water contact angle was 106º. In Figure 2, we show the β-casein thickness adsorbed over both types of hydrophobic surfaces, polystyrene film and gold-CH_3 surface. We can observe that the thickness of β-casein layer is very similar in both cases, about 6 nm.

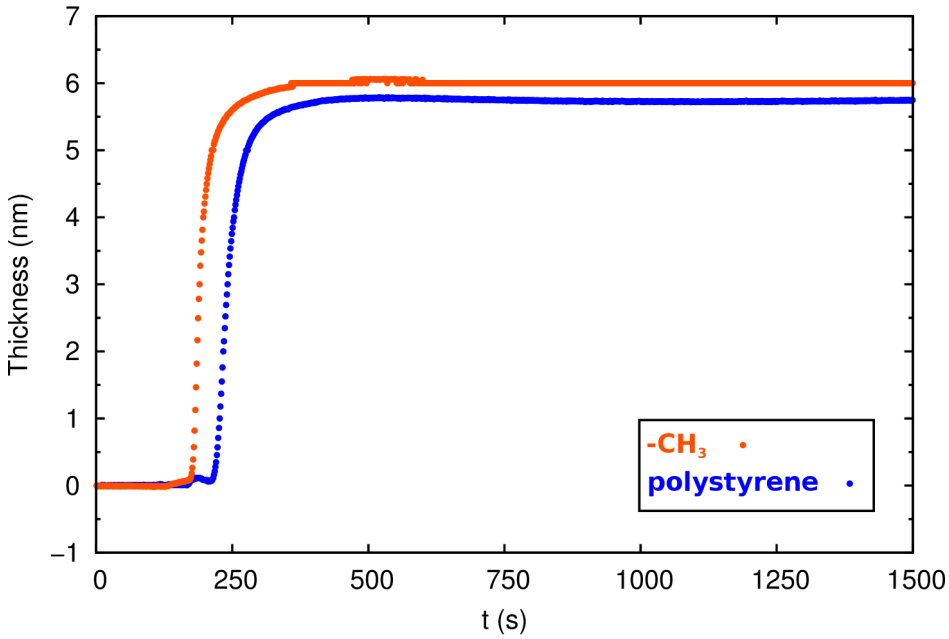


Figure 2: Temporal evolution of β-casein adsorption on different hydrophobically-modified quartz crystal.

We then studied the adsorption of the proteins mentioned over hydrophilic surfaces. For that, the hydrophobicity of polystyrene surface was modified by UV-oxidation [6]. This treatment does not change significantly the thickness or the morphology of the film and reduces the water contact angle with the exposure time. After 10 minutes of exposure, the water contact angle of the surface was 53º. In Figure 3 we can compare the protein adsorption over hydrophobic substrate (-CH_3 termination) and hydrophilic substrate (oxidized polystyrene):

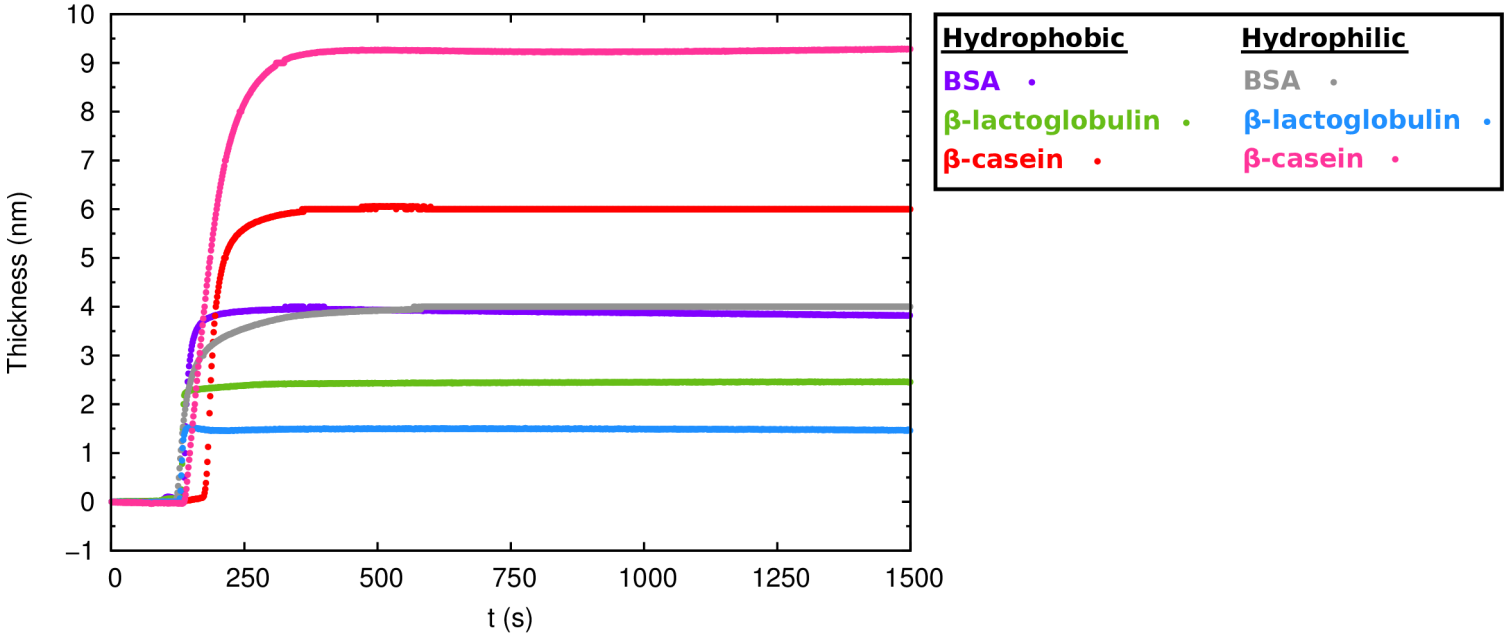


Figure 3: Temporal evolution of protein adsorption on hydrophilic and hydrophobic surfaces.

We can observe that the adsorption behavior over hydrophobic and hydrophilic surfaces is similar for the two globular proteins, BSA and β-lactoglobulin, whereas in the case of β-casein, the film thickness is larger for the hydrophilic surface than for the hydrophobic one. We will need to analyze these data deeply in order to explain the differences observed in the conformation of the proteins over surfaces with dissimilar wettability, since the larger thickness can be caused by more amount of adsorbed protein or by a swelling in the protein structure.

After completing the protein adsorption, we studied how the pH of the environment affects the protein conformation and the protein-surface interactions. As the adsorption pH was close to the isoelectric point the proteins are almost electrically uncharged, reducing electrical repulsion between neighbor proteins and guaranteeing the maximum coverage over the surface. On the contrary, by changing the pH of the environment the proteins acquire electrical charge. In our experiments, after protein adsorption and rinsing with water at the same adsorption pH (close to neutrality), we studied the response of the different systems to both acid and basic pH. When changing to pH 4, these proteins become positively charged; on the contrary, they acquire a negative charge at basic pH values. In both cases, we first noticed a quick increased in the thickness of the protein film (linked to protein swelling) followed by an apparent desorption of proteins over time. Another interest point observed after pH change is the separation of the different overtones in both, frequencies and dissipation, signaling the importance of viscoelastic effects on the observed response. In conclusion, the protein structure depends on its electrical charge that results in the formation of a rigid film or a swollen film because of water absorbing.

Finally, we are very interested in studying the influence of ionic strength on proetin conformation and energy dissipation. We observed that if we increased the ionic concentration of the medium, for example by adding NaCl, the protein electrical charge is screened and the effective thickness of the film decreases. More interesting is the interaction of big hydrophobic ions with the proteins, and its influence on their conformation. We have found before [7] that these ions interact very strongly with soft-matter systems at very low concentrations. Their affinity toward hydrophobic interfaces is so large that they are able to change the electrical charge of nanosystems, to the point of inducing charge reversal (when they are acting as counterions), at small concentrations. These ions, the tetraphenylborate anion (Ph_4B^-) and the tetraphenylarsonium cation (Ph_4As^+), have a similar size and the same chemical structure (one central atom rounded by four phenyl rings), the only significant difference between them is the central atom (B or As) that confers the sign of the electrical charge. However, it seems to be that the anion is more hydrophobic than the cation due to the different hydration capabilities of the anions and the cations [8, 9]. We have been able to use these ions as coions or as counterions, since proteins can be positively or negatively charged depending on the pH medium.

During our experiments we observed that polystyrene was affected by tetraphenyl ions, for this reason in these studies we worked only with the gold-CH_3 surfaces, which have hydrophobic character. The experiments were made following these steps. First, we performed the protein adsorption and rinsed with solution at the same adsorption pH. After that, we carried out the change to the desired pH (acid or basic). Then, we inject the tetraphenyl solutions raising the ionic concentration progressively. Lastly, we rinsed with solution at the same pH that ionic solution and after that with solution at the initial pH in order to check if a significant protein desorption had occurred during the process.

In all cases, we observed firstly a decreasing in the thickness of the protein film because of the screening of the electrical charge, when ions acted as counterions. However, when we reached a certain ionic concentration, proteins began to swell due to the electrical charge inversion and therefore the charge increases in the opposite sign. As an example, in Figure 4 can be observed a complete experiment with β-casein and the $Ph_4As^+Cl^-$ salt, where the Ph_4As^+ cation is acting as counterion, since the protein is negatively charged at pH 10:

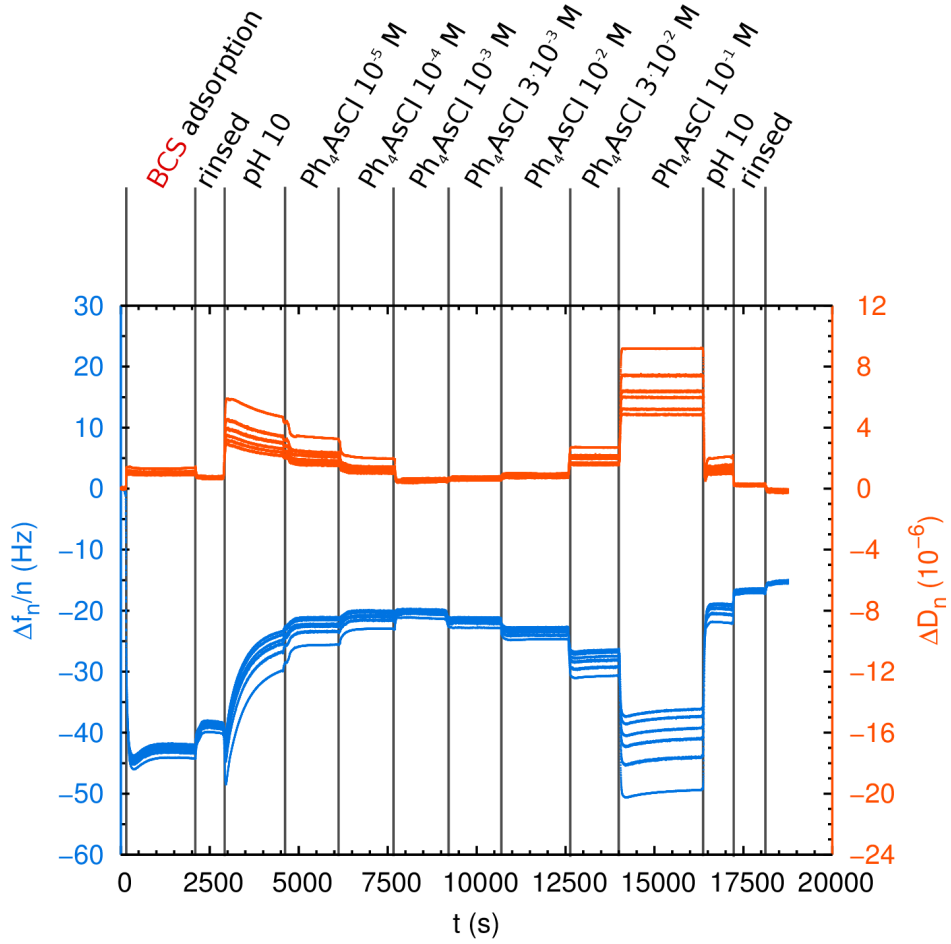


Figure 4: QCM-D: changes in resonance frequency (blue) and dissipation signal (orange) for different harmonics for different stages of adsorption and rinsing, as indicated. The hydrophobic ion Ph_4As^+ acts as counter-ion.

We can see both, frequency (blue) and dissipation (orange) changes and how the different overtones are close when a rigid protein film is formed, and more separated when the proteins swell and absorb water. This occurs at pH change and more remarkably in presence of tetraphenyl ions at 0,1 M, when the protein acquires a great positive electrical charge because of the charge reversal, since tetraphenyl ions feel an important hydrophobic attraction towards the proteins.

On the contrary, when tetraphenyl ions act as coions, the electrical charge of the protein increases. In spite of the electrical repulsion between the protein and the tetraphenyl ions, the hydrophobic interaction prevails and these monovalent ions adsorb onto the protein. Therefore, the protein film swells and increases its thickness with increasing the ion concentration. In Figure 5 we show a complete experiment with β-casein and the $Ph_4As^+Cl^-$ salt where the Ph_4As^+ cation is acting as coion, since the protein is positively charged at pH 4. We represent again the frequency and the dissipation energy changes in blue and orange colors respectively:

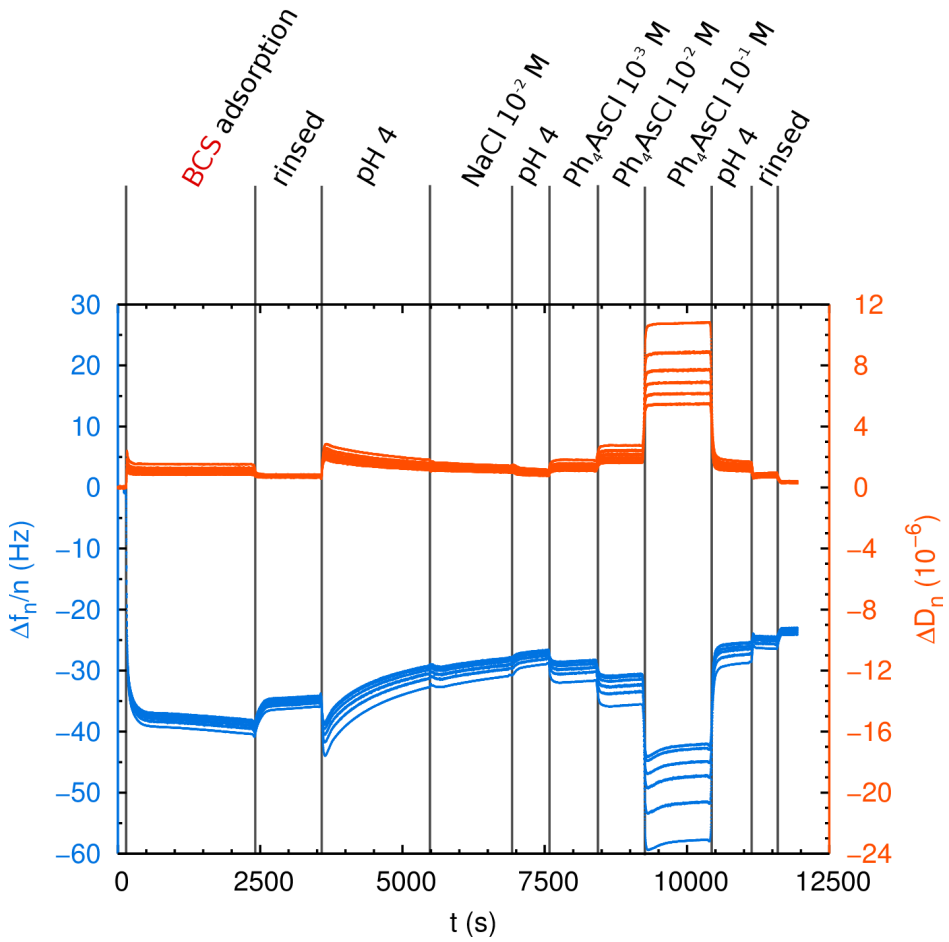


Figure 5: QCM-D: changes in resonance frequency (blue) and dissipation signal (orange) for different harmonics for different stages of adsorption and rinsing, as indicated. The hydrophobic ion Ph_4As^+ acts as co-ion.

This variety of experiments has been performed for the three proteins (BSA, β-lactoglobulin and β-casein) and the two tetraphenyl ions (Ph_4B^- and Ph_4As^+), acting both as counterions and coions. In all cases we have obtained a similar behavior as the one shown in Figures 4 and 5. We observed that the protein is more charged electrically in presence of the Ph_4B^- anion than the Ph_4As^+ cation at the same ionic concentration, the anion feels a greater attraction to the proteins or any other hydrophobic or soft interface than the cation, in agreement with previous observations made by us [7].

In conclusion, during this research stay, we have carried out a comprehensive study of protein adsorption over different kind of surfaces and their behavior under pH changes, as well as their interactions with big hydrophobic ions, all measured and characterized by QCM-D. These results will be analyzed more deeply and will be put altogether with other experimental results obtained at the University of Granada, as protein adsorption over latex nanoparticles, as well as, molecular dynamics simulations of theses proteins in analogous conditions as the experiments, with the final aim to obtain a broad study of the physical properties and interactions of these kind of systems.

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