

AFM AND XPS STUDIES OF PROTEIN ADSORPTION ONTO GRAPHITE SURFACES

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Introduction

The literature of Carbon Science contains very little information about the behaviour of carbon surfaces when exposed to physiological environments, such as those which support cell adhesion, even though carbon based materials have been investigated for use in prosthetic devices such as heart valves, ligament repair and vascular stents. The concentration and conformation of adsorbed proteins is critical in determining specific interactions between cell adhesion species (integrins) and biomaterial surfaces in a wide range of situations.

In this study, highly orientated pyrolytic graphite (HOPG) has been used as a model surface for the study of the behaviour of two human plasma proteins which is of high biological importance in contact with biomaterials surfaces.

Experimental

Human serum albumin (HSA) adsorption onto freshly cleaved HOPG surface (basal plane) was studied using atomic force spectroscopy (AFM) and X-ray photoelectron spectroscopy (XPS). Preliminary studies upon human plasma fibronectin (Fn) adsorption onto HOPG were performed using the AFM.

Protein solutions of concentration 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2 and 4mg/ml were prepared using HSA (essentially fatty acid and globulin lyophilized powder, purity 99%) in Dulbecco's phosphate buffered saline (pH=7.2). Fibronectin solutions of concentration 0.1, 0.5, 1, 5 and 10 µg/ml were prepared using human plasma fibronectin (Sigma) and trisbuffer saline, pH=7.5, 0.05M. Droplets (200µl) of the protein solution were incubated at 37°C for 1h in contact with the HOPG surfaces, rinsed and then washed in MilliQ water for 4 hours under agitation. The samples were dried slowly at room temperature overnight (12-14h). In order to study the influence of drying conditions on the AFM imaged topography of the adsorbed HSA layer a second set of HSA adsorbed samples was dried relatively quickly under a gentle flow of warm air (37°C) for approximately two minutes.

The AFM analyses were performed using a Digital Instruments Nanoscope III, under ambient conditions in tapping mode, immediately after drying of the samples. The surface chemical composition was analyzed using a Kratos X-ray photoelectron

spectrometer. Contact angle measurements were performed using a 20 μ l static drop of distilled water.

Results and Discussion

The substrate used is essentially atomically smooth and the AFM images indicate a RMS roughness of 0.19-0.26nm and contain some small steps of height of approximately 0.3-0.7nm which probably correspond to dislocations of 1 or 2 graphite planes [1].

Individual features have been identified when imaging the basal planes after adsorption from an HSA solution of concentration 1 μ g/ml. These features present two different shapes: one triangular/circular with a diameter of 25 \pm 3nm and the other oblong of lengths 35 \pm 5nm width 24 \pm 2nm and height about 3nm (Figure 1). Due to the tip radius of curvature the true width of the imaged features is extended by 10-20nm. Human serum albumin is known to present different conformations in aqueous solutions as a function of pH. The normal form which is essentially heart shaped with dimensions of 8x8x3nm and the F form, which is oblong and 4x12.9nm [2] could be attributed to the molecular features detected in these experiments (Figures 1a and 1b).

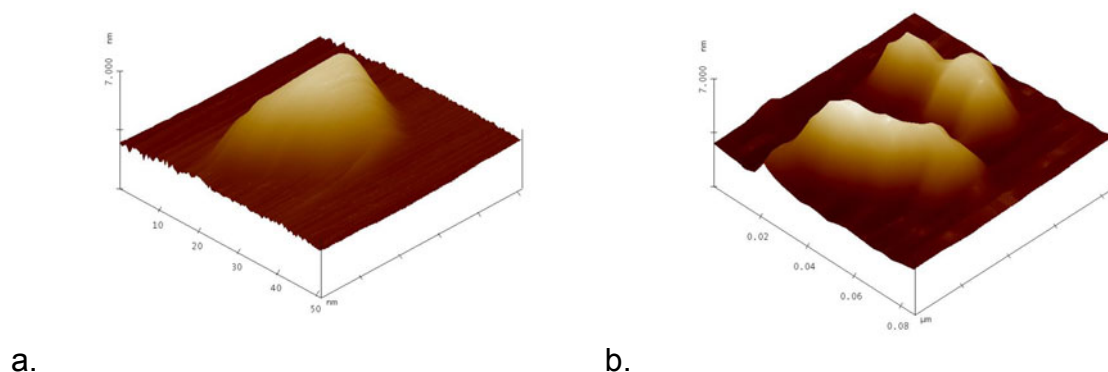


Figure 1. TM AFM topographic images of probably individual HSA molecules showing two shapes: triangular(a), probably N form and oblong (b) possibly F form. The size of the image is: 55X55X7nm(a) and 90X90X7nm(b)

In both of the drying conditions studied preferred adsorption of the protein molecules along the steps was noticed (Figure 2a) which agrees with data reported by Quist for albumin adsorbed on mica [3] and by Berrie for fibrinogen on graphite [4]. At neutral pH HSA is reported to be 8nm wide and 3 nm height i.e. about 10 to 20 times higher than the step features evident on these surfaces. Interestingly, when the images of the protein adsorbed along the graphite-steps are studied under higher magnification, the protein molecules appear to bridge the step, with one part of the molecule 'sitting' on the lower plane and one part on the upper plane. The overall apparent preference for the steps could be due to the presence of oxygen species adsorbed at valence electrons on

the edge of the plane, effectively forming a line of polar sites along the step. There is also likely to be an enhanced dispersion potential along the step which results from the overlapping of the individual potential wells associated with the plane and the adjacent edge.

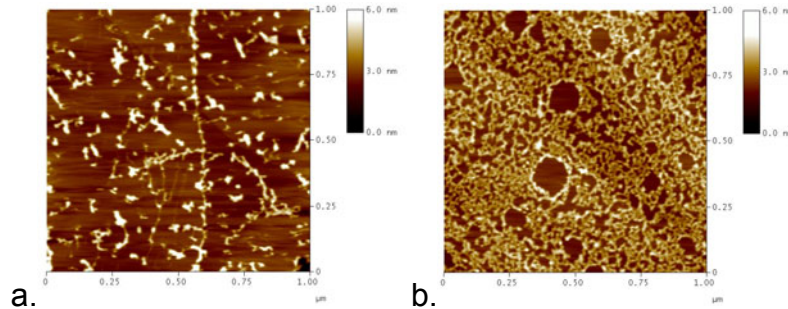


Figure 2. Topographic AFM images of individual features of proteins adsorbed on HOPG: a.-adsorption from an HSA solution(1 μ g/ml) imaged after a short time of drying, the surface was washed in PBS prior to water washing in order to remove the weaker bonded proteins and achieve a better image of individual features; b.-adsorption of a Fn solution (0.1 μ g/ml) (overnight dried)

In the case of fibronectin adsorption from the weakest solution studied (0.1 μ g/ml) individual features which were either looped, folded or extended have been identified in the AFM images. The extended features are 60-80nm long and 1.2 \pm 2nm high as shown in Figure 2b and are very similar in size and shape to those reported by Lin [5] on mica surfaces. Fibronectin is a dimer, formed by two very similar monomers which are joined near their C-termini by two disulfide bonds. Electron microscopy studies reported the Fn molecule to be a long, thin, highly flexible strand with the length of the dimer molecule varying from 120 up to 160nm with a width of 2nm [6]. Therefore, the extended features measured in our study could be assigned to a monomer/half molecule of the fibronectin. Previous biophysical studies suggested that Fn molecules are folded in low salt solutions (2 mM) and extended in higher salt (200mM) and high pH buffers [7,8].

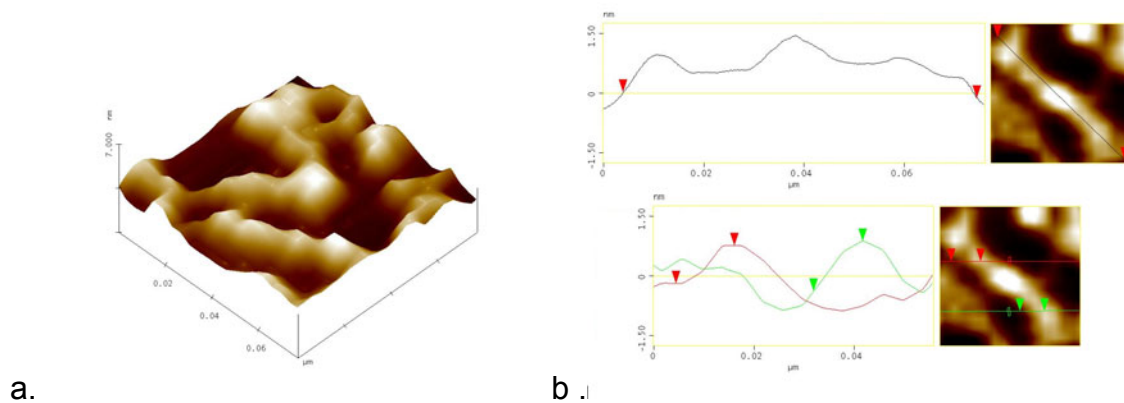


Figure 3.a. Topographic image of a possible dimer of FN with an extended arm and a folded one; b. Top view and cross sections image of an extended monomer (the length is 70nm and the height from 1 to 1.2 nm)

Most of the imaged features seem to be sharply curved and bent, probably crossing each other to produce a tangled and compact structure. They appear to be similar with the model proposed by Rocco [7] which can be explained by the surface binding process or the fact that the samples were washed with water prior to imaging which will induce the more folded form [7,8].

When protein was adsorbed on HOPG surfaces at higher concentrations and dried overnight the formation of a network, with peak and ridges was observed. As the protein solution concentration increases, the protein has a tendency to aggregate into a structure with narrower valleys and wider ridges, thus the network appearing more compact. Similar behaviour was identified in the case of the both HSA and Fn as showed in Figure 4a and 4c.

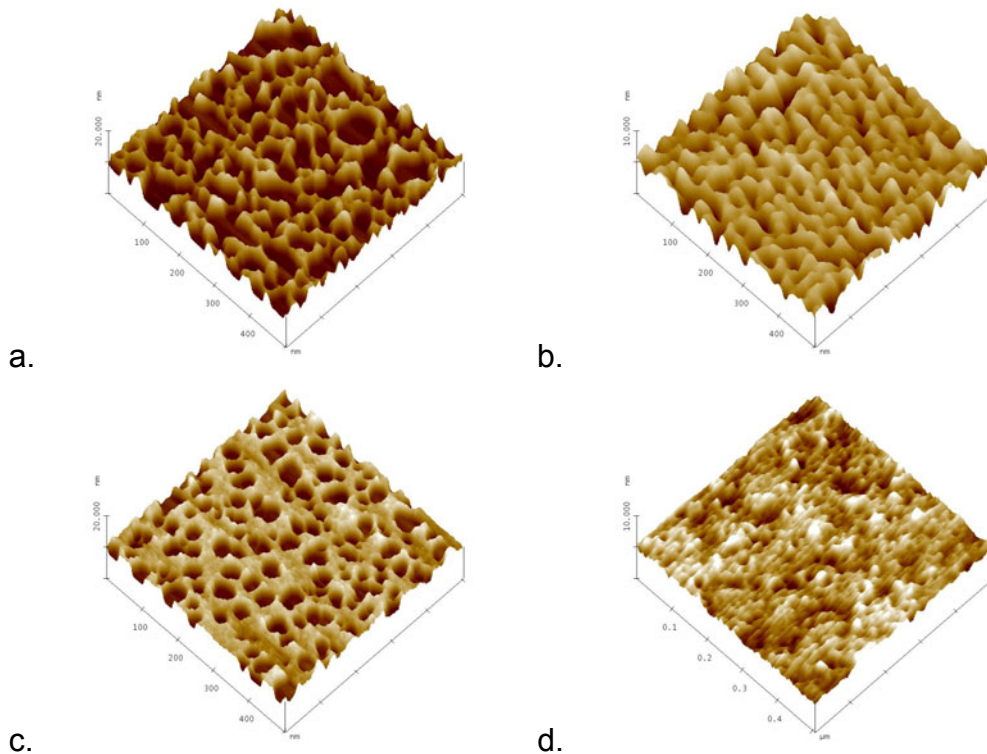


Figure 4. TM-AFM images of HSA (a, d) and Fn (b, c) solution adsorbed on HOPG surfaces of concentration 0.01mg/ml(a, b, c) and 2mg/ml(d). The samples were dried overnight(a, c) or for only few minutes(b, d)

For the samples dried quickly, a very different type of structure was imaged with no network-type structure apparent and with a grainy type topography (Figure 4b and 4d). The images are very similar at different concentration, the average height being around 1nm, and the RMS roughness in the range 0.5-0.7nm. At higher concentrations some grains of bigger size are apparent which may be due to aggregates of HSA formed in solution prior to adsorption or may be impurities such as globulin present in the HSA.

These very different images of the protein film in different drying conditions led us to try another experiment. The protein adsorbed from the weakest HSA solution was imaged in situ, while drying. The initial images show individual features which are randomly arranged on the basal planes. After one hour of drying at room temperature in the AFM apparatus the same surface gave an image which showed a more organized structure with the proteins showing the tendency to arrange into the network structure with circular gaps. After drying overnight the sample was imaged again and now showed a well formed network circular shaped holes. The depth of the holes appears to correspond with the thickness of the film and therefore reveals the substrate. These features are thought to be formed by the de-wetting of the HAS from the hydrophobic HSA surface, there may also be an associated change of protein conformation.

The XPS survey spectra of freshly cleaved HOPG show only one single C1s peak at a binding energy of 284.6 eV. After protein adsorption a second peak appears at 400 eV which is the proteinaceous N1s peak. When a blank sample was analyzed (0 mgHSA/mlPBS) an O1s peak appears at 530 eV, the oxygen being thought to originate from adsorption of water or oxygen onto the graphite surface or from impurities from the tape used to peel the graphite. The small silicon peaks that appear in the washed samples are also probably due to exposure of the tape (Figure 5). This oxygen and silicon was considered irrelevant in the semi-quantitative assay of the protein adsorption onto the graphite surface.

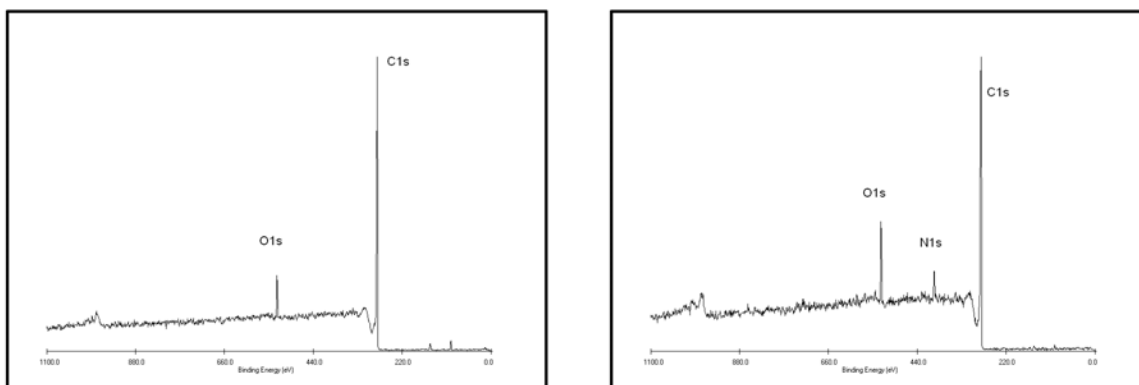


Figure 5. Survey spectra of the samples after the HSA adsorption (left-0mg/ml and right-4mg/ml)

The nitrogen atomic concentration has been used as a measure of the irreversibly bound albumin and, as shown in Figure 6, was found to increase with increasing HSA solution concentration. Also shown are average water contact angles (measured after protein was adsorbed) which shows that θ increases slightly as the level of surface protein increases. When the AFM images presented above are analysed to derive roughness data it was found that RMS roughness also increases with adsorbed protein concentration.

The XPS, AFM and water contact angle measurements indicate that at lower protein solution concentrations (up to 0.001mg/ml) there is a direct correlation between the amount of adsorbed protein and the protein solution concentration which suggests that the adsorption process is diffusion controlled. After 0.1mg/ml the N1s signal becomes constant at between 3.5 and 3.8 atom % and the contact angle levels out at about 70° which suggests that surface is fully covered with layer of protein. After the point when the layer is complete its surface properties do not change significantly but of course the layer may become thicker as adsorption proceeds.

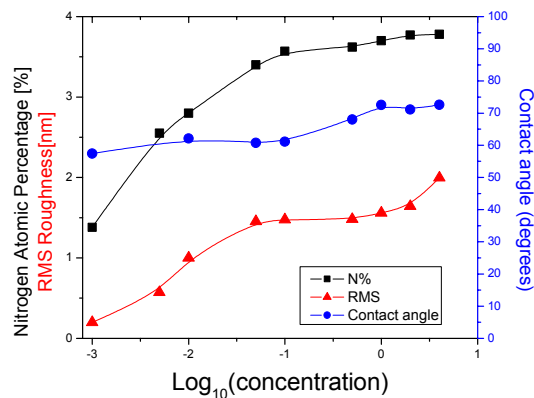


Figure 6. Nitrogen atomic percentage, RMS roughness and average contact angle variation with the \log_{10} (concentration) of the HSA solution

As all the data presented in Figure 6 were obtained from the overnight/slow dried samples a further discussion about these results should be considered. According to the theories of protein adsorption onto solid surfaces [9], as contact or residence time increases, the protein tends to orientationally and conformationally adjust with respect to the interface. In the initial moments after the removal of the solution, protein might still have the same conformation as in the solid-liquid system, i.e. hydrophilic region oriented towards the solution and hydrophobic region towards the hydrophobic substrate [10]. When the samples are drying it is expected that the hydrophobic portion of the protein is exposed to air while the hydrophilic portion will be orientated towards aqueous phase. The protein would be expected to adjust its orientation and conformation to the air-solid interface, this being a possible explanation of the very different images obtained for the different drying conditions. Therefore, further AFM studies under a liquid environment would give a better understanding of protein adsorption on HOPG. This, and quantitative measurements of HSA adsorption are being attempted at present.

Conclusions/Summary

HSA and Fn adsorption onto HOPG was studied. AFM topographic analysis showed the formation of a network or a grainy structure, dependent upon drying conditions.

For adsorption from the HSA solution of concentration 1 µg/ml and Fn solution of concentration 0.1 µg/ml individual molecules have been imaged. HSA appear to take one of two forms depending upon the adsorption energy and site. Fn appears to be adsorbed into the coiled, bent form.

XPS results show an increase of the nitrogen atomic percent of the surface with increasing initial protein solution concentration. Contact angles decrease drastically after protein adsorption then increases slightly up to ~70°.

References

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