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Functionalization of silicon tips with HSA for molecular swelling AFM measurements

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The design of biocompatible synthetic surfaces is an important issue for medical applications. Surface modification techniques provide good approaches to control the interactions between living systems and implanted materials [1]. A similar approach can be used to investigate protein unfolding. The aim of our work is study a particular biocompatibility system: we have used chemical deposition of proteins (HSA-human serum albumin) over a silicon surface functionalized with a SAM (self-assemble monolayer). In fact, the microscope was used first in force curve mode for check if the SAM was effectively bonded to the surface, and after to imagine the protein bonded over the silicon sample. Both contact mode and tapping mode were performed during our analysis. In the present communication we report the preliminary results of an investigation aimed at exploiting atomic force microscopy (AFM) to measure the forces involved in the unfolding of proteins adsorbed on functionalized surfaces.

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1. Introduction

The use of biomaterials to interface with living systems, such as fluids, cells, and tissues of the body, has played an increasingly important role in modern day medical applications. In particular, synthetic and natural polymers, metals, ceramics, composites, and tissue-derived materials have been applied in medicine and pharmaceutics [1]. The minimum requirements for biomaterials are: non-toxicity, functionality, sterilizability, and *biocompatibility*. Basically, biocompatibility can be divided into two groups: mechanical and interfacial. Mechanical compatibility is sometimes referred to the bulk compatibility, whereas interfacial compatibility often is referred to biological compatibility. In this work, we use the term biocompatibility referring to interfacial biocompatibility [1], which is more concerned with the physical and chemical interactions between the foreign material and the living body when they are brought into contact. The interfacially biomaterials can be described as those in which the adverse biological responses occur at much reduced rates or in which the required tissue adhesion is promoted, depending on the objective of the biomaterial and whether tissue adhesion is desirable or not. Protein adsorption is known to be the very first stage of the interactions between the foreign surface and the body tissue or fluids. The aim of our work is study a particular biocompatibility system: we have used chemical deposition of proteins (HSA-human serum albumin) over a silicon surface functionalized with a SAM (self-assemble monolayer).

2. Experimental

Theoretical support: Many experimental approaches in biology and biophysics as well as applications in diagnosis and drug discovery require proteins to be immobilized on solid substrates. In fact, the idea of creating protein arrays (arrays of proteins attached to a solid support) has started to attract increasing attention over the last three years due to the completion of several genomes including the human one. Various methods are available for attaching proteins to solid surfaces: most rely on non-specific adsorption or on the reaction of chemical group within proteins (mainly, amino and carboxylic acid groups) with surfaces containing complementary reactive groups. In both cases the protein is attached to the surface in random orientations. In order to be useful for protein attachment the corresponding reaction must happen between a unique chemical groups present or introduced in the protein with a complementary group contained on a surface where the protein will be attached (Fig. 1).

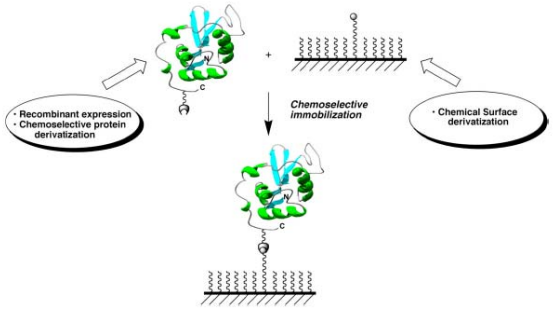


Fig. 1. Concept of chemoselective reaction between a protein and an appropriately chemically modified surface.

This reaction must not be affected by the presence of the other reactive groups in the protein (amino, carboxylic acid, thiol and hydroxyl). In this way the protein is specifically attached to the surface through a unique reacting moiety which provides control of the orientation of the protein on the surface. When trying to attach proteins to surfaces, the most common employed surfaces are silicon based (glass slides or Si/SiO2 wafers) or metals (Au and Ag). The most used agent for chemically functionalization of silicon based surfaces is (γ–aminopropyl)trialkoxylsilane (APS), (Fig. 2) which is relatively cheap and easy to handle.

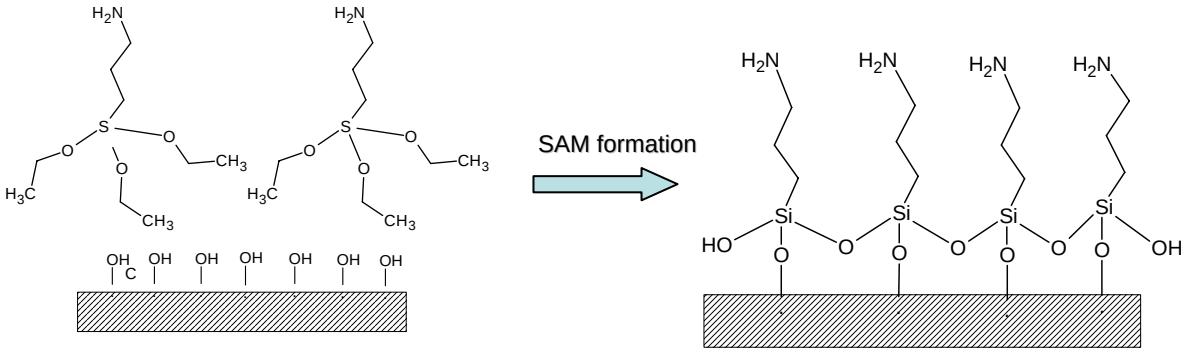


Fig. 2. Hybridization and functionalization of silicon surface.

Moreover, the presence of the amine group allows carrying out a variety of subsequent chemical transformations to produce the desired complementary chemical moieties that will react with the appropriately modified protein. Our aim is the study of a particular biocompatibility system: for modifying surface by covalently immobilizing proteins and to forming stable and homogeneous layer of peptides to favour cell adhesion on this surface, we have used chemical deposition of proteins (HAS-human serum albumin) over silicon surface functionalized with a SAM (self-assembled monolayer). This procedure was used also on silicon tips

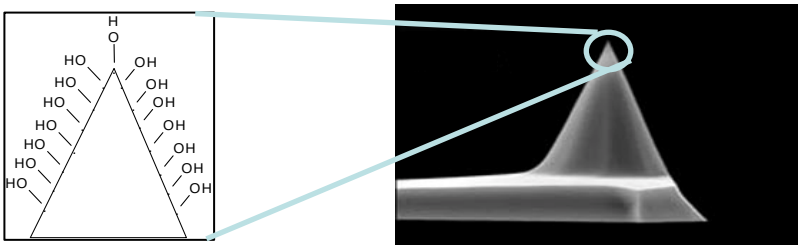


Fig. 3. Functionalization of silicon tip.

Materials: Human serum albumin (HAS) was purchased from Sigma. 3-Aminopropyltriethoxysilane (APTES, Sigma), dichloromethane (Backer), and methanol (Backer) were used as received. Toluene (Backer) was distilled over Na/K alloy under anhydrous nitrogen atmosphere. Silicon (100) wafers were purchased from SilChem. "V shaped" AFM probes (Veeco) with k = 0.7 N/m were used.

Methods: Sample preparation involved two steps: first the silicon surface was prepared with multiple chemical treatments (degrease with sulphuric acid and hydrogen peroxide 3:1, boiling solutions of dichloromethane, methanol and acetone) and activated in boiling water for 48h. Moreover the SAM formation was performed putting the silicon in a toluene diluted solution of APTES (3-aminopropyl-triethoxysilan) under nitrogen atmosphere. Wettability changes were investigated with qualitative measurements of contact angle: it was observed significant difference between untreated silicon, and APTES functionalized silicon. The second step involved the functionalization of the surface with the protein: this was achieved by putting the silicon surface in aqueous solution of human serum albumin (HAS) with a concentration 7×10^{-4} M for 24h. All these steps are made in order to prepare and functionalize the surface, which was investigated with AFM technique later. In fact, the microscope was used first in force curve mode for check if the SAM was effectively bonded to the surface, and after to imagine the protein bonded over the silicon sample. Both contact mode and tapping mode were performed during our analysis.

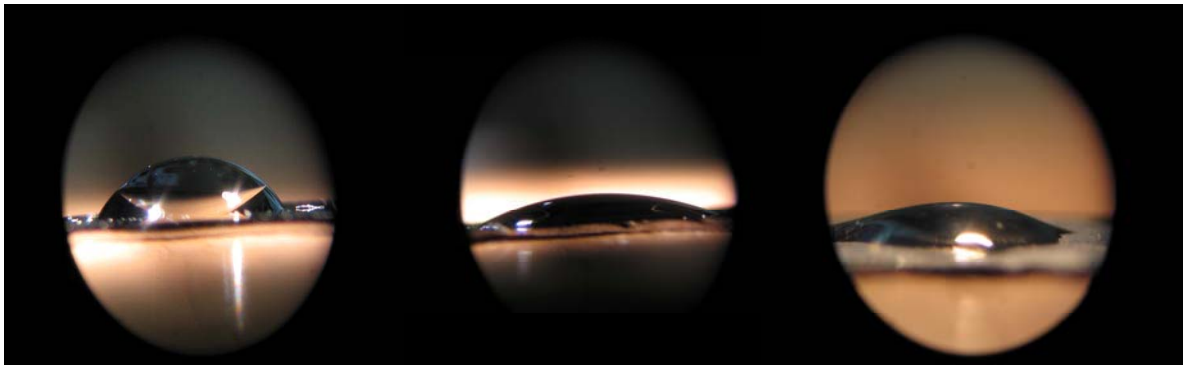
3. Results and discussion

Covalent linking of HAS molecules to the tip of AFM probes was performed in three stages. First, hydroxyl groups were formed on the silicon surface by activation in

piranha solution, followed by treatment in boiling water. Then, hydroxyl groups were converted into amino groups by reaction with APTES. For comparison, the same treatment was applied also to the surface of silicon wafers. Finally, activated tips were reacted with HAS in water solution. The characterization of the sample was reported by contact angle for wetting measurements and atomic force microscopy (AFM).

The quality of stable monolayer can be estimated from wetting measurements. This is due to the fact that the shape of a liquid droplet on a plane, homogeneous surface (which is the result of the free energy of this droplet) is affected by the free energy of this surface. The contact angle is related to the surface free energy by the Young's equation:

$$\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL} \tag{1}$$



Static contact angle measurements confirmed the presence of an increasingly hydrophilic surface layer on hydroxylized, silanized, and HSA-treated samples. To observe and measure the angle of contact, we used ImageJ programme to calculate different angles: $\theta_1 = 45.374^0$, $\theta_2 = 21.681^0$, $\theta_3 = 22.479^0$

Different AFM techniques were also exploited to check the functionalization of the silicon surface. Force-distance curves substantiated the presence of APTES on the silicon surface. To observe the functionalization of the silicon surface with APTES solution and HSA bonding, we have used this technique.

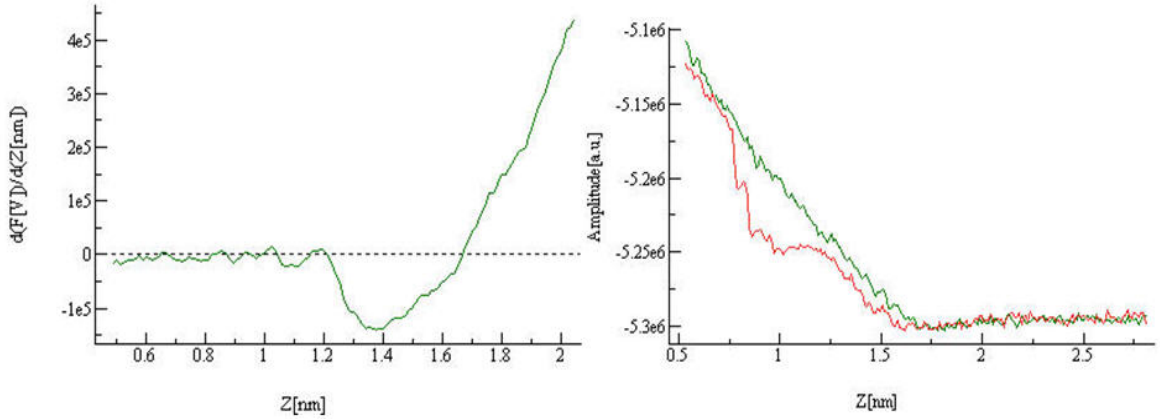


Fig. 3. Force distance curve (left) and force amplitude curve (right) in tip-surface functionalized with APTES, interaction.

In order to evidence the HSA bonding on the functionalized silicon surface, we have calculated the middle energy of the cantilever from force-distance curve.

The HSA functionalized tip was approached to APTES-modified wafer surface and a force distance curve was recorded in air at 20 nm/s rate. Under these conditions, meniscus forces caused by the moisture present on the sample surface affected the force – distance curves, but only in the jump-off region (above 30 nm). In this case, the calculus for the force of swilling between two gradients for force-distance curve: for the first step (little) deflection is 0.071 V, the force F_1=160 pN and energy $E_1 = 8.71 \times 10^{-6}$ kcal, the second step (high) is 0.127 V, the force F_2=270 pN and energy $E_2 = 1.24 \times 10^{-4}$ kcal and the third is jump off contact.

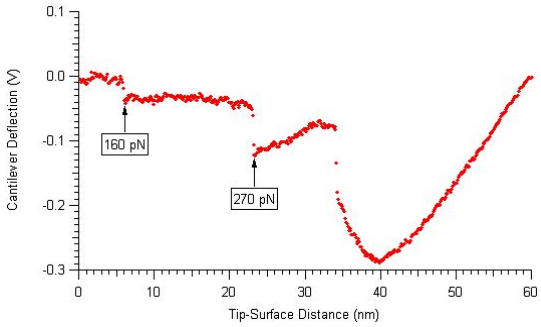


Fig. 4. Force-distance curve for HSA bonding on functionalized surface.

For observing functionalized silicon surface with APTES, we have used contact and tapping mode. To put in evidence the atomic profile we have comparative measurements with mica for avoiding the roughness of surface. Since the atomic resolution measurements was been observed a difference between pure silicon surface and functionalized silicon surface.

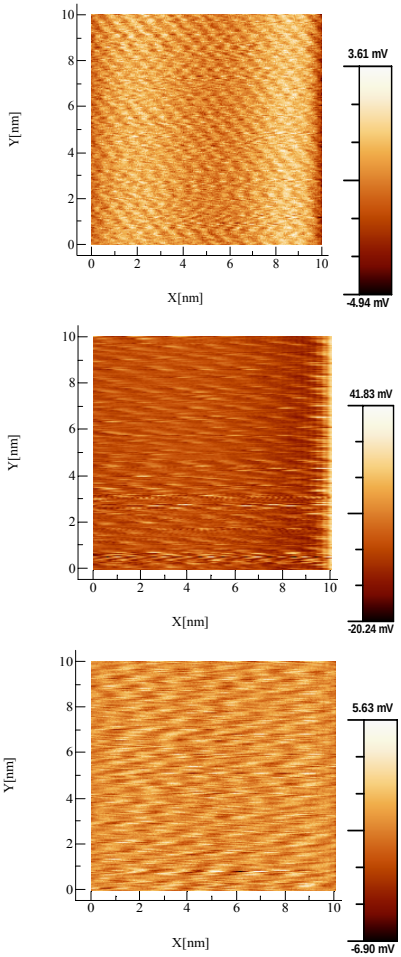


Fig. 5. Atomic resolution (deflection) of: mica surface (1), pure silicon (2) and functionalized silicon with APTES (3).

Also, we made topographical measurements for functionalized silicon surface and for the HSA bonded on silicon surface functionalized.

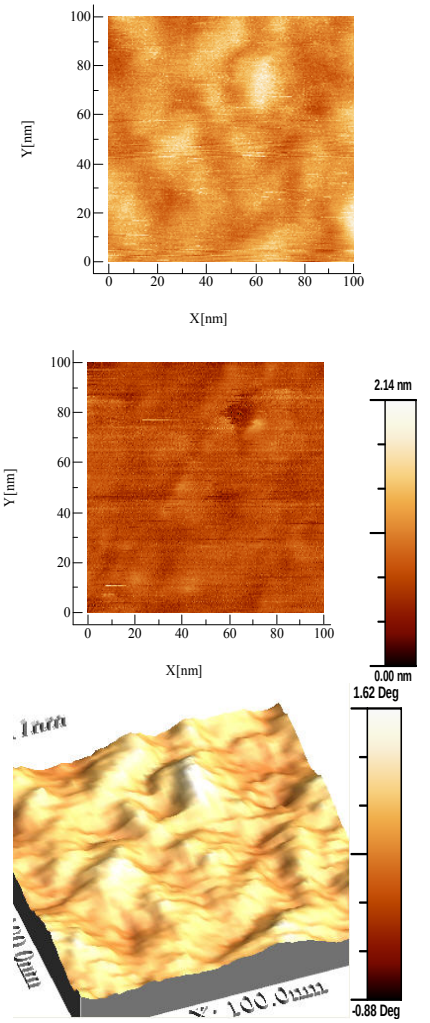


Fig. 6. Topography, Phase and 3D image for HSA on silicon surface letting in APTES solution

For evidence of HSA molecules, we have made a surface profile for interesting parts.

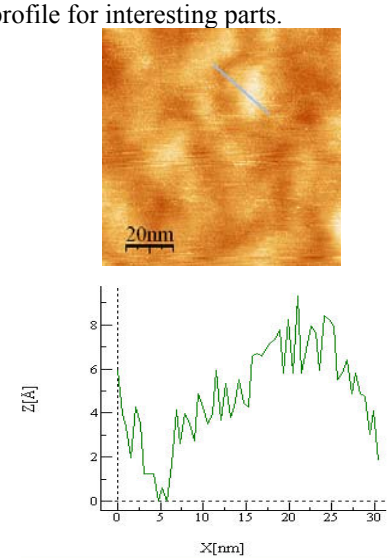


Fig. 7. Profile for HSA molecules for functionalized silicon surface.

4. Conclusions

Measurements over functionalized surface show that SAMs (self assemble monolayer) was obtained with APTES solution on silicon surface. We have verified the formation of the APTES monolayer through force-distance curves. We also observed the albumin over surface by nanoidentification method. AFM measurements: topography and phase imaging show a good contrast and give us information about the shape, morphology and dimensions of protein aggregates. AFM probe functionalization with proteins allows for measuring the swelling forces involved in protein unfolding during the elongation process. Silicon surfaces and tip can be easily functionalized with reactive SAMs. Protein can be covalently bonded between the tip and the surface. Swelling experiments showed good reproducibility even for several approaching - retracting cycles.

Two distinct force values have been found showing a two-steps unfolding of HSA.

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