Investigation of protein adsorption with simultaneous measurements of atomic force microscope and quartz crystal microbalance

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We have combined the tapping-mode atomic force microscope (AFM) and quartz crystal microbalance (QCM) for simultaneous investigation and characterization of protein adsorption on various metallic surfaces using these two instruments. The adsorption of proteins such as human plasma fibrinogen and anti-human immunoglobulin onto the metal or oxide/QCM surface were monitored using both methods at the same time when varying the concentration of proteins. The combination of AFM with QCM allowing the simultaneous measurements with two techniques working at very different scales and probing different properties of the adsorbed layer provides quantitative and qualitative information that can distinguish different protein adsorption behavior. [DOI: 10.1116/1.1593056]

I. INTRODUCTION

The quartz crystal microbalance (QCM) is made of AT-cut quartz crystal that sandwiched between two metal electrodes vibrating when a sinusoidal electric field is applied. Theoretically, when a mass loads its surface, the QCM changes its resonant frequency. The mass, Δm, to frequency shift, Δf, relationship has been modelled by the Sauerbrey to lead to a relationship,

$$\Delta f_{(n)} = \frac{-2 f_{(n)}^2}{n \times A \sqrt{\rho \mu}} \Delta m = \frac{-n \times 2 f_{(1)}^2}{A \sqrt{\rho \mu}} \Delta m,$$

where $f_{(n)}$ is the frequency of nth overtone, ρ is the density of quartz, μ is the shear modulus of AT-cut quartz, A is the macroscopic sensing area, and n being the overtone number.[1] It has been extensively used when investigating the adsorption of proteins on metal surfaces, assuming the biological species forms a rigid layer.[2,3] Because the adsorption of proteins on inorganic surfaces is an important topic in biomaterials research and for the development of biosensors, the QCM technique is widely used for such developments.[4,5] It shows a decrease of its resonance frequency as a function of the change of deposited mass.[6–8] However, the detailed physical adsorption mechanisms cannot be identified with such a crude technique whose sensing area is in the cm^2 range. Somewhat a number of contributions have been published on the resonance vibration behavior of the QCM when a contact probe approaches its sensing electrode.[9–12]

The atomic force microscopy (AFM) is one of the commonly used techniques to provide information about adsorbed proteins with molecular resolution.[13] The observation of biomolecules adsorbed on various kinds of surfaces by AFM has been widely studied and reviewed.[14–17] It is therefore of interest to combine tapping-mode AFM with QCM and to observe the adsorption of proteins in liquid phase with these two complementary techniques at once. The merits of this combination include simplicity, simultaneous observation of the adsorption of proteins at different scales such as μm^2 and cm^2, and most importantly the possibility to understand the adsorption kinetics of biomolecules through the real time comparison of the resonance frequency shifts of QCM with the obtained molecular resolution images of AFM.

II. EXPERIMENT

Human plasma fibrinogen (HPF) and antihuman immunoglobulin (anti-IgG) were chosen as model proteins since they are often used as references for biocompatibility analysis and are large enough to be resolved by AFM imaging.[15,16,18] The experimental setup including the tapping-mode AFM (Molecular Imaging, Phoenix, AZ) and QCM-D (Q-Sense AB, Göteborg, Sweden) combination is represented in Fig. 1. The QCM resonator is made of an AT-cut quartz wafer coated on one side with conductive electrodes obtained by evaporating 5 nm Ti and 50 nm Au. The top electrode, called sensing electrode, formed with 2–10 nm Ti and 50 nm Au or 50 nm of Ta by the *e*-beam evaporation. The typical root mean square (rms) surface roughness of the Au sensing electrode is ~8.5 Å and peak to valley height is ~19 Å. That of the native Ta_2O_5 surface of Ta sensing electrode is ~6.4 Å and it shows a ~20 Å peak to valley height. This surface is thus flat enough to achieve molecular resolution.

The gold surface is modified with 10 mM CH_3 terminated dodecanethiol (Fluka Chem. Co.) in ethanol in order to enhance the hydrophobic interactions between the proteins and its surface. The Ta_2O_5 surface of Ta/QCM surface is silanized with 1% (v/v) n-decyltrichlorosilane (Gelest, USA) in toluene for the same reason as above. Other details of the AFM and QCM combination are described elsewhere.[19] Various concentrations ranging from 250 ng/ml to 100 μg/ml of human plasma fibrinogen (HPF, Sigma, F3897) were prepared by dissolving HPF in phosphate buffer saline solution (PBS) (10 mM KH_2PO_4/Na_2HPO_4, 150 mM NaCl, *p*H 7.4). For the desorption of HPF from Au/QCM surface, 10 mM glycine HCl (*p*H 2.2) solution was used. The antihuman immunoglobulin (anti-IgG, Sigma) was prepared in PBS solu-

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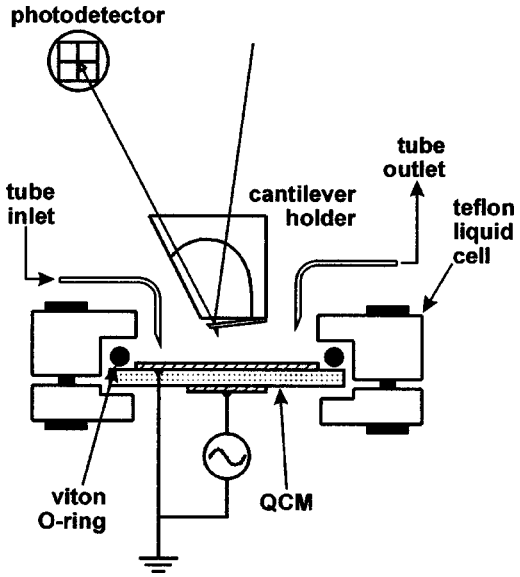

FIG. 1. Schematic diagram of the apparatus combining atomic force microscopy (AFM) and a quartz crystal microbalance (QCM).

tion (6.44 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, *p*H 6.7) with anti-IgG concentrations of 1, 10, and 100 μg/ml. A sodium dodecyl sulphate (SDS) solution (0.5% w/v in H_2O) was used to desorb the anti-IgG from native Ta_2O_5 surface of Ta/QCM. All buffer solutions were prepared with sterile, autoclaved materials and filtered with 0.2 μm pores filter (cellulose membrane, Schleicher & Schuell, Belgium) right before use.

The resonance frequency of QCM in its fundamental mode is ~4.7 MHz, and our instrumentation allows simultaneous tracking of the resonance frequencies of the first, third and fifth overtones. Since the fundamental mode has been shown to be too sensitive to environmental fluctuations,[19] such as flow and viscosity of the liquid, we have monitored the frequency shifts of the third overtone (at 14.1 MHz) or the fifth overtone (at 23.5 MHz) of QCM during the adsorption and desorption of proteins on its surface. After stabilization of the resonance frequency of the QCM, the protein–PBS solutions were introduced from lowest to highest concentration by running a flow for 3 min (flow rate around 1.5 ml/min) and then stopping the peristatic pump for low noise measurements. In order to guarantee that the same area of the electrode was always observed by the AFM, we kept on scanning the surface and simultaneously monitoring the frequency shift of the QCM during introduction of a protein solution. However, the liquid flow induced by the pump degraded the data quality during the solution exchange.

III. RESULTS

A. Adsorption of human plasma fibrinogen

The resonance frequency shifts of the QCM and the AFM images were observed at the same location while increasing the fibrinogen concentration with a scan size of 1×1 μm^2. The shift of the resonance frequency divided by the overtone number was more than 15 Hz when the 250 ng/ml fibrinogen solution was introduced into the liquid cell and 80 Hz when inserting 25 μg/ml fibrinogen solutions. When the fibrinogen with concentration of 250 ng/ml was introduced, the gold surface was smoothened since the fibrinogen filled the intergrain granular spaces.[20] The smoothening of surfaces in the AFM images was quantitatively analyzed with the rms roughness of surface and it is shown in Table I when increasing the concentration to 25 μg/ml HPF. The thiol modified gold surface showed the rms surface roughness of ~8.5 Å and it is decreased to ~7.7 Å when 25 μg/ml HPF solutions were introduced. The entire surface was completely covered with proteins and the granular surface of Au/QCM disappeared in the AFM image when the fibrinogen concentration of 100 μg/ml was introduced. In this state, the QCM shows a distinctive resonance frequency shift of 330 Hz on its third overtone and the AFM image was observed with a rms roughness of ~10.8 Å. This fibrinogen adsorption was mostly reversible with glycine solution.

When the scan size was decreased, the AFM images showed the adsorbed HPF molecules on Au/QCM surface.[20] After introduction of the 25 μg/ml fibrinogen solutions, we can clearly observe the structure on the surface matching the HPF that described in the literature.[21] At this concentration, it can be observed that the fibrinogen molecules have adsorbed preferentially between each Au granule (data not shown).[20]

TABLE I. Analyzed rms surface roughness of obtained AFM images according to the adsorbed proteins and their concentrations.

	Concentration (μg/ml)	rms roughness (Å)
Human plasma fibrinogen (HPF)	0 (in PBS buffer)	8.5
	0.25	7.8
	25	7.7
	100	10.8
Antihuman immuno globulin (Anti-IgG)	0 (in PBS buffer)	6.4
	10	4.7
	100	8.5
	0 (in PBS buffer after the SDS reflux)	10.4

B. Adsorption of antihuman immunoglobulin

The adsorption of antihuman immunoglobulin (anti-IgG) onto the native Ta_2O_5 surface was observed with Ta coated QCM and tapping mode AFM as shown in Fig. 2. When the 1 μg/ml anti-IgG solution was introduced into the liquid cell, the resonance frequency shift of Ta/QCM normalized by the overtone number was less than 5 Hz and was increased to 22 and 47 Hz when the concentration of introduced anti-IgG solution were increased to 10 and 100 μg/ml. The bare 1×1 μm^2 Ta_2O_5 surface observed with AFM in PBS solution showed a typical granular morphology of evaporated metal

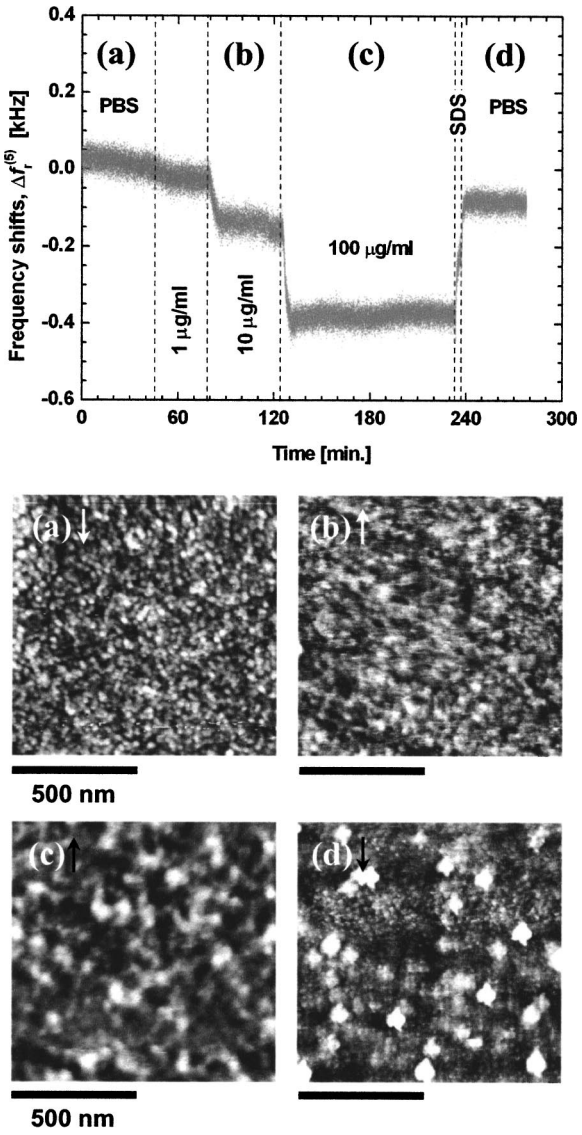

FIG. 2. Resonance frequency shift of the Ta/QCM depending on the different concentrations of anti-human immunoglobulin (anti-IgG) in PBS solution and the $1\times1\ \mu m^2$ AFM topographic images of native Ta_2O_5 surface of Ta/QCM and their scan directions in: (a) PBS solution, (b) 10 μg/ml, (c) 100 μg/ml anti-IgG solution, and (d) PBS solution after the desorption of protein with SDS rinsing.

surface [Fig. 2(a)]. It was difficult to recognize any changes of Ta_2O_5 surface with 1 μg/ml anti-IgG solution in AFM images (data not shown). The Ta_2O_5 surface that showed smoothened granular morphology and decreased rms roughness to ~4.7 Å is displayed in Fig. 2(b) obtained after introducing 10 μg/ml anti-IgG solution into the liquid cell. Figure 2(c) shows the Ta/QCM surface after introducing the 100 μg/ml anti-IgG solution. The granular structured Ta_2O_5 surface was no longer visible and the entire surface was completely covered with proteins at this anti-IgG concentration. As shown in Table I, the analyzed rms roughness of Ta/QCM surface at the 100 μg/ml anti-IgG solution is increased to ~8.5 Å. And this was same as in the case of 100 μg/ml HPF adsorption on Au/QCM surface. When the surface was covered with proteins and the resonance frequency was stabilized in 100 μg/ml anti-IgG solution, SDS solution was introduced to remove the adsorbed proteins from Ta_2O_5 surface. After the reflux of SDS solution for 3 min, the liquid cell was replaced with PBS solution. Even after rinsing the surface with SDS, the observed resonant frequency shift of Ta/QCM did not recover its initial value and showed a 100 Hz shift on its fifth overtone. The AFM image in Fig. 2(d) shows irregular and bulky features with granular Ta_2O_5 background.

IV. DISCUSSION

The finding in the combination of AFM and QCM is the relationship between the resonance frequency shift of the QCM and the obtained AFM images during protein adsorption. Assuming the adsorbed proteins form a rigidly bound layer on the QCM sensing electrode, the Sauerbrey proportionality relationship, $\Delta m \propto \Delta f_{(n)}/n$, can be applied between the resonance frequency divided by the overtone number and the mass bound to the surface:[11] in the adsorption of HPF onto Au/QCM, the sensitivity of a 4.7 MHz resonator is theoretically 20.1 ng/Hz and an 80 Hz frequency shift in 25 μg/ml HPF solution corresponds to an adsorbed mass of 1.6 μg over the 1 cm^2 sensing area of the QCM. HPF being a large molecule with a molar weight of 340 kD,[16] we expect to observe 280 molecules in a 100×100 nm^2 area. This value being obviously greater than that observed on the AFM images, we can speculate that the tapping-mode AFM actually monitors only the topmost layer and the surface is totally covered by proteins when the solution concentration is 25 μg/ml. However, one should be cautious in applying the proportionality relationship when using the QCM in a liquid medium and adsorbing proteins and including a viscous interaction contribution to the frequency shifts which scales with the overtone numbers as $\Delta f = \Delta f_{(n)}/\sqrt{n}$.[22] Unexpectedly high frequency shifts have previously been reported when using QCMs in viscous liquid media.[23] Since the AFM images we obtained show that the fibrinogen does not cover the whole surface of the QCM, we conclude that either the fibrinogen is preferentially adsorbed in the valley region of the Au granular structure and the QCM frequency shift is exaggerated because of viscous interactions with the surrounding fluid, or the surface is totally covered but the very low force tapping-mode AFM imaging only displays distinctive features of the layer in the intergranular areas. The entire Au/QCM surface was visibly coated with a much higher concentration, 100 μg/ml, of fibrinogen. Under this concentration, the Au granular structures disappeared and only the thick biopolymer layer surface was observed in the AFM images.

An anti-IgG molecule has dimensions of $5.9\times13.1\times14.3$ nm with a molar weight of 150 kD.[24] Additional mass due to the adsorption of a monolayer of anti-IgG molecules on the 1 cm^2 surface area of Ta/QCM calculated from these reported dimension and molar weights can be predicted to theoretically lead to a mass increase of ~0.13 to ~0.30 $\mu g/cm^2$ depending on the orientation of the antibodies. The observed total mass adsorbed on the 1 cm^2 sensing

area of the crystal assuming a rigid layer for which the Sauerbrey equation can be used, after 10 and 100 μg/ml anti-IgG adsorption steps together ($\Delta f_{(5)}/5 \approx 69$ Hz), would be ~ 1.4 μg. The mass adsorbed onto the Ta/QCM surface after the introduction of a 10 μg/ml anti-IgG solution only ($\Delta f_{(5)}/5 \approx 22$ Hz), would be ~ 0.44 μg/cm^2. This calculated adsorbed mass is only three times less than the absorbed mass from both 10 and 100 μg/ml anti-IgG solutions, which is still roughly 1.5–3 times higher than theoretically predicted for a monolayer of anti-IgG molecules. However, the adsorption of antibodies at a concentration of 10 μg/ml concentrations could result in a detectable frequency shift of the QCM device (1×1 cm^2), while the 1×1 μm^2 AFM image in Fig. 2(b) would not display distinctive changes. We conclude that the tapping-mode AFM possibly scanned only the topmost layer of adsorbed anti-IgG and the surface is already covered by proteins after running the 10 μg/ml solution while the adsorbed mass derived from the Sauerbrey equation might overestimate the actual mass of protein adsorbed on a Ta/QCM surface because of the interactions of the adsorbed proteins with the viscous liquid environment. These differences between adsorbed protein mass and high frequency shifts in QCM were reported in other studies.[23] The reason for which the resonant frequency doesn't come back to its original value and the AFM image in Fig. 2(d) does not show the original Ta/QCM surface even after the reflux of SDS solution to desorb the anti-IgG from Ta/QCM surface might be due to the leftover adsorbed and denatured antibodies or to crystallized SDS molecules, which are not rinsed away by the final buffer flow.[25] Yet the Ta_2O_5 grains are again visible on the background of images scanned after SDS rinsing, so the significant different appearance of the surface in Fig. 2(c) is attributed to adsorbed anti-IgG on a Ta/QCM surface.

V. CONCLUSION

We have demonstrated the useful combination of tapping-mode AFM and QCM by observing the adsorption of human plasma fibrinogen on a thiolated Au-coated QCM and the adsorption of antihuman immunoglobulin on a silanized Ta-coated QCM with both techniques simultaneously. The complementarity of the information acquired using the combined AFM and QCM shows that this technique is a useful probe for the study of the binding of biomolecules on a surface. More specifically, the frequency decrease that relates to a mass increase upon adsorption of antibodies on a cm^2 scale can be related to the topography of the surface on a μm^2 scale. The informations gathered inform us both on the adsorbed mass of the proteins on the surfaces and the adsorption schemes.

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