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Secondary Ion Mass Spectrometry of Proteins

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***Abstract*—**The adsorption of bovine serum albumin (BSA), immunoglobulin G (IgG) and fibrinogen (Fgn) on fluorinated self-assembled monolayers have been studied using time of flight secondary ion mass spectrometry (ToF-SIMS) and Spectroscopic Ellipsometry (SE). The objective of the work has to establish the utility of ToF-SIMS for the determination of the amount of protein adsorbed on the surface. Quantification of surface adsorbed proteins was carried out using SE and a good correlation between ToF-SIMS results and SE was achieved. The surface distribution of proteins were also analysed using Atomic Force Microscopy (AFM). We show that the surface distribution of proteins strongly affect the ToF-SIMS results.

***Keywords*—**ToF-SIMS, Spectroscopic Ellipsometry, Protein, Atomic Force Microscopy.

I. Introduction

BIOMOLECULAR adsorption at solid surfaces is an important and central phenomenon to many medical and technological applications. When a foreign implant is introduced inside the body, a layer of biomolecules, primarily proteins, adsorbs onto the surface of the implant and this influences cell adhesion and behaviour. The rapid adsorption of proteins to almost all surfaces in contact with a biological fluid underlines the importance of controlling protein attachment and this remains a pressing issue in the development of advanced medical and diagnostic devices. The determination of the amount of protein attached to the surface is a critical measurement performed by using a wide range of methods[1].

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is a promising technique for protein adsorption analysis because of its chemical specificity, sensitivity and the potential to obtain information on the orientation of proteins on surfaces[2]. The analysis of surface adsorbed proteins using SIMS includes three objectives: (i) measurement of the surface density of adsorbed proteins, (ii) the detection of low amounts of proteins at surface and (iii) the determination of the identity and or orientation of adsorbed proteins[3].

Because SIMS is sensitive to a wide range of parameters, it is important to develop analytical strategies that are able to distinguish, for example, the effect of density from that of composition and orientation. In ToF-SIMS, an energetic primary ion impacts the surface of interest, causing the ejection of secondary species, some of which are ions and these are mass analysed to produce a mass spectrum of atomic and cluster ions originating from the surface. Because of the energetic nature of this process, the spectrum is typically of highly fragmented species and organic fragments with mass < 1000 u are detached. Therefore, small amino acid fragments are used to study proteins in ToF-SIMS. The advantage of ToF-SIMS is that it is very surface sensitive, which means that it is sensitive to the orientation of proteins. Because of the complexity of ToF-SIMS spectra, advanced data analysis techniques are often required to interpret the resulting data. Wagner *et. al.*[4] showed that combination of ToF-SIMS and Principle Component Analysis (PCA) provides new insights into the composition of adsorbed protein films on biomaterial surfaces. In their work, they used single component adsorbed protein films on three model substrates and multivariate analysis of the ToF-SIMS data to distinguish different protein films. From the work of Wagner *et. al.*[5] and others[6-8], it is established that the distribution of proteins on the adsorbed surface from a mixed protein solution is better explored by ToF-SIMS because of its extreme surface sensitivity and good spatial resolution[9]. However, quantitative analysis of surface adsorbed proteins using ToF-SIMS is still far from being routine and secondary ion intensities may be strongly affected by their local environment, changes in concentration, dewetting and changes in the vacuum environment. In this present work, we have used ToF-SIMS and Spectroscopic Ellipsometry (SE) to study the adsorption of three different proteins on gold with a perfluorodecanethiol self assembled monolayer (SAM). Quantification of surface adsorbed proteins was carried out using SE and a good correlation between ToF-SIMS results and SE was achieved. The surface distribution of proteins were also analysed using Atomic Force Microscopy (AFM). We discuss the ability of SIMS to quantitatively determine the amount of protein at a surface and the advantages and limitations of the technique.

II. Materials and Methods

A. Materials

Bovine serum albumin (BSA) (99%), immunoglobulin G (IgG) (95%) and fibrinogen (Fgn) (96.3%) were purchased from Sigma (Life Science) and were used without further purification. 1*H*,1*H*,2*H*,2*H*-Perfluorodecanethiol (97%) was acquired from Aldrich (Chemistry) and analytical reagent grade ethanol was purchased from Fisher Scientific. To prepare protein solutions, phosphate buffered saline (PBS) solution (pH 7.4±0.1) was obtained from Fisher BioReagents and was used as received. Gold-coated quartz crystals were

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purchased from Q-Sense, Sweden. High purity water was supplied by an Elga UHQ water system with resistivity = 18.2 MΩ·cm, surface tension = 72.8 mN/m, and pH = 5.5 at 22 °C.

Protein adsorption was carried out in a Quartz Crystal Microbalance (QCM) instrument and the details of the adsorption procedure could be found elsewhere[10]. In short, gold-coated quartz crystals were hydrophobized-using perfluorodecanethiol in ethanol following the protocol mentioned in the ref.[11] and were used in QCM instrument to adsorb different proteins from different concentrations.

B. Methods

Quartz Crystal Microbalance (QCM)

The samples were prepared using a QCM. The QCM instrument used in this study was a Q-Sense E1 (Q-sense, Sweden) with a temperature controlled fluid cell. For all the protein solutions, the time of protein adsorption was two hours and flow rate was kept constant at 200 μL/min. Five steps were used in the process of liquid flow through the flow cell for protein adsorption, the sequence was: water; PBS buffer; protein in PBS buffer; PBS buffer and water. In all cases, other than protein with buffer, the flow continued until the frequency shows no further change. The last step water flow was included to prevent salt from the buffer drying on the surface. An example of the IgG adsorption from the protein solution using QCM is shown in Figure 1.

As received gold-coated quartz crystals were hydrophobized by immersing in 1 mM perfluorodecanethiol in ethanol solution for 18 hours and were rinsed with copious amount of ethanol and blow dried afterwards. Freshly thiolated crystals were used in QCM to adsorb three different proteins from three different concentrations. The rate of protein solution flow and time of protein adsorption were kept constant. The quantified values of adsorbed protein amounts and thickness by QCM technique are not included in this paper and can be found in ref.[10]. After protein adsorption, the samples were blow-dried and were taken for spectroscopic ellipsometry and ToF-SIMS measurements.

Spectroscopic ellipsometry (SE)

Spectroscopic ellipsometry was carried out on a Woollam M2000DI instrument (JA Woollam, NE, USA) with three incidence angles of 65°, 70° and 75° to the surface normal. Ellipsometric data were fitted to a two-layer model using Complete Ease Software (JA Woollam, NE, USA). The first layer of the model consists of a gold substrate with optical constants determined experimentally. The second layer represents the thiol with or without protein. As the optical constants for thiol and protein are very close to each other, for the purpose of the calculation we consider them as one layer. The second layer was represented by two parameters Cauchy model,

$$n = A + B/\lambda^2 \quad (1)$$

with A=1.42 and B=0.01 μm^{-2}[12]. The only free parameter in the fittings is the thickness of the protein layer d_{SE}. The thickness of the thiol layer determined to be 1.10nm thick from measurements on freshly prepared thiolated gold.

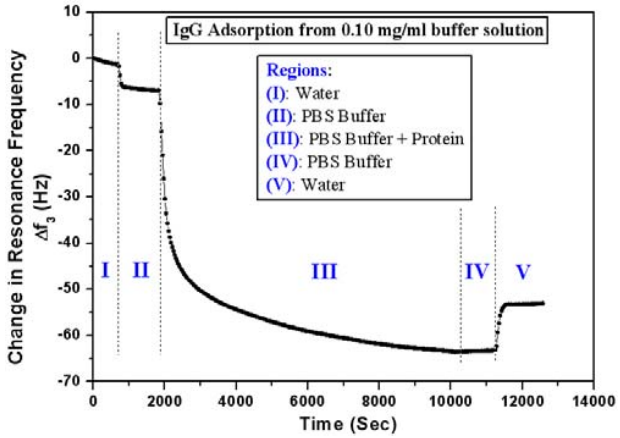

Fig. 1 Typical QCM experiment showing the real time acquisition of frequency shift induced by IgG adsorption from 0.10mg/ml protein solution on hydrophobized gold-coated quartz crystal sensor chip. The data show changes in the third overtone frequency (Δf_3)

Time of flight secondary ion mass spectrometry (ToF-SIMS)

Static SIMS analyses were carried out using an TOF-SIMS IV instrument from ION-TOF GmbH, Germany with single-stage reflectron design[13] and an extractor voltage of 2000 V. Positive and negative ion spectra were obtained using a focused Bi_3^+ liquid metal ion gun at 25 keV energy, incident at 45° to the surface normal. The ion gun is operated in high current bunched mode with two crossovers for high mass resolution with spatial resolution of 5 µm. Raw data containing the secondary ions was recorded at 128×128 pixel with a field view of 500µm × 500µm. After the acquisition, the data was calibrated and analysed using instrument software.

Atomic force microscopy (AFM)

AFM was used to study the homogeneity and topographic features of the adsorbed proteins. The imaging was performed in air by non-contact (NC-AFM) tapping mode using an MFP-3D Asylum AFM instrument at room temperature. Super sharp silicon probes (Nanosensors, Switzerland) with resonance frequency 330 kHz, 10nm nominal radius and force constant 42 N/m were used to image the QCM-D adsorbed dried proteins. AFM imaging of the protein surfaces was performed before and after SIMS analysis to establish the effect of ultra high vacuum (UHV) experiments. Image processing was carried out using WSxM 4.0 Beta 1.2 software[14].

III. RESULTS AND DISCUSSION

As shown in fig.2, it is found that with increasing protein concentration, there is an increase in thickness of the protein layer, measured by ellipsometry.

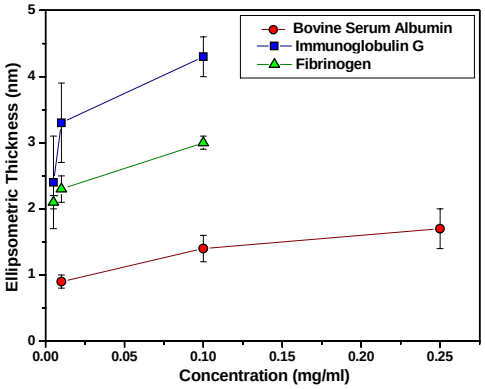

Fig. 2 Adsorbed protein layer thickness (d_{SE}) measured by spectroscopic ellipsometry, as a function of protein solution concentration for (○) BSA, (□) IgG and (Δ) Fgn. Lines are intended only to guide the eye

ToF-SIMS measurements were carried out with a view to obtain quantitative analysis of the protein films. In Figure 3, the attenuation of secondary positive ion arising from the SAM ($C_8F_{17}^+$) with increasing protein layer thickness measured by SE is shown. The data is fitted with an exponential decay function with decay constant 1.8 nm. However, the thinner IgG layers do not follow this trend. Such behaviour may be expected in the case of a patchy overlayer of proteins.

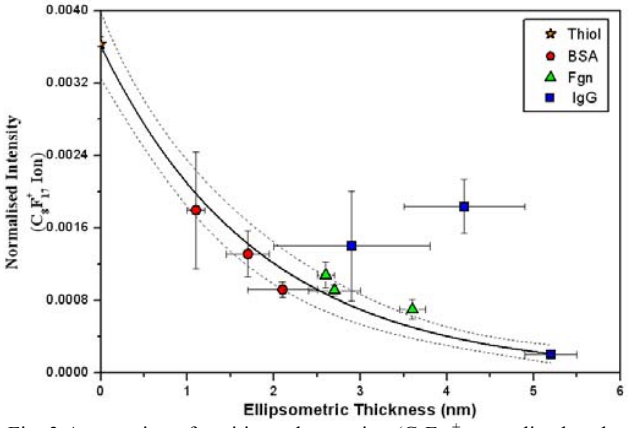

Fig. 3 Attenuation of positive substrate ion ($C_8F_{17}^+$ normalized to the total count) in ToF-SIMS spectra due to the adsorption of different proteins with increasing layer thickness measured by SE is shown. The solid line represents the exponential decay equation fitting [$y = A_0 \exp(-x/t) + y_0$] with decay constant (t) 1.8nm. The dotted lines represent the 95% confidence interval.

The negative secondary ions, coming from protein layers in the ToF-SIMS data shows gradual increase with increasing thickness of protein layers, except for one IgG film.

In fig.4, normalized intensity of the CN^- ions is plotted against the protein layer thickness measured by SE. Similar trends

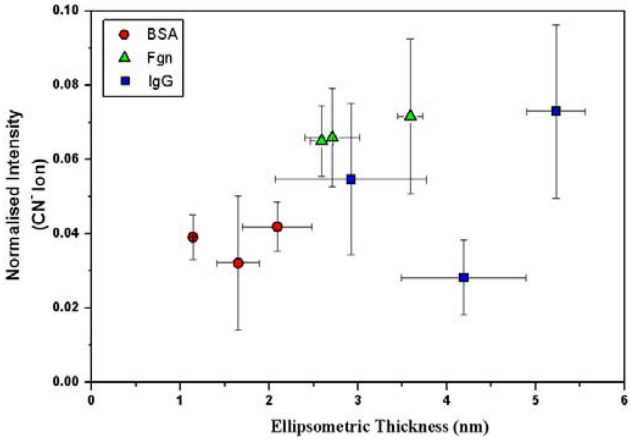

Fig. 4 Plot of normalized (to the total count) negative SIMS ion species (CN^-) from proteins in negative ToF-SIMS spectra with increasing protein layer thickness.

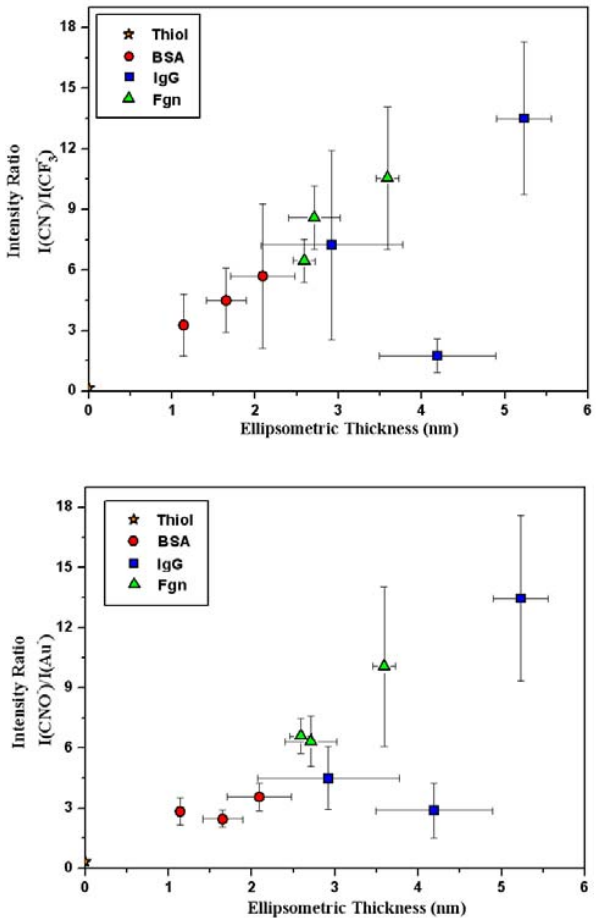

Fig. 5 Relative secondary ion intensity of ions arising from proteins (CN^- and CNO^-) and those from the substrate (CF_3^- and Au^-)

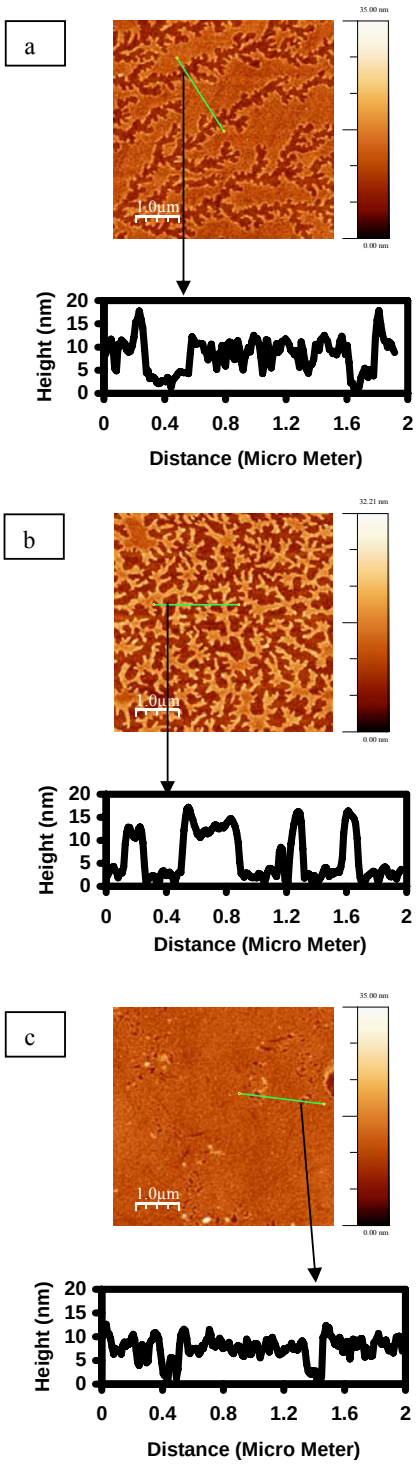

Fig. 6 TM-AFM topographic images of IgG adsorbed layer [from (a) 0.005 mg/ml, (b) 0.01mg/ml and (c) 0.10 mg/ml protein solution] on hydrophobized gold-coated quartz crystal sensor chip after XPS data acquisition. The topographic cross-sections profiles of the images are shown below the actual images and are from left to right direction

were found for other ion fragments coming from proteins. As expected, for thicker films of proteins, the cyanide secondary ion intensity increases as the amount of protein (presumably lysine and amide groups in the protein) on the surface increases. The data here are more scattered, since the films are already close to the thickness that can be probed by SIMS. It is evident that at least one of the IgG films does not follow the general trend, consistent with the $C_8F_{17}^+$ attenuation results.

Figure 5, shows the relative secondary ion intensity of ions arising from proteins and those from the substrate. There is a clear correlation between secondary ion intensities from proteins and substrates with the thickness of the protein layer with the exception of at least one IgG film, for reasons outlined above.

AFM imaging was carried out on the protein films before and after SIMS analysis. The results for IgG films after analysis are shown in figure 6. It was found that the thinner IgG films appeared to de-wet after UHV analysis. The thicker films adsorbed from 0.10 mg/ml protein solutions, showed a lower level of dewetting (fig. 6c). However, for thinner films (fig. 6 a & b) the effects were very pronounced and the topography changed drastically. In all the figures, the cross-sections are shown and the heights of the dewetted layers are measured. This observation is fully in accord with the disparity between the SIMS and SE results for IgG films. The IgG film of intermediate thickness showed the largest disparity and the AFM confirms that this film has the most uneven distribution of proteins. On all other samples, no topographic features were observed, indicating that the protein films were uniform.

IV. Conclusion

In this work, we have studied the three different proteins with different layer thickness using ellipsometry and SIMS. We have shown that there is a very good correlation between the relative intensities of secondary ions arising from proteins and substrates with the thickness or the amount of the protein adsorbed on the substrate. However, the surface coverage of the proteins and inhomogeneities in the surface layers can be detected by comparing the SE and SIMS data. In our case, two of the IgG layers dewetted under UHV condition of SIMS instrument. This highlights the need for a proper understanding of the surface topography, the distribution of over layer material and the effect of instrumental conditions on the sample while any instrument is employed to analyse biological samples.

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