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Combined surface acoustic wave and surface plasmon resonance measurement of collagen and fibrinogen layer physical properties

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ABSTRACT

We use an instrument combining optical (surface plasmon resonance) and acoustic (Love mode surface acoustic wave device) real-time measurements on a same surface for the identification of water content in collagen and fibrinogen protein layers. After calibration of the surface acoustic wave device sensitivity by copper electrodeposition and surfactant adsorption, the bound mass and its physical properties – density and optical index – are extracted from the complementary measurement techniques and lead to thickness and water ratio values compatible with the observed signal shifts. Such results are especially usefully for protein layers with a high water content as shown here for collagen on an hydrophobic surface. We obtain the following results: collagen layers include $70 \pm 20\%$ water and are 16 ± 3 to 19 ± 3 nm thick for bulk concentrations ranging from 30 to 300 μg/ml. Fibrinogen layers include $50 \pm 10\%$ water for layer thicknesses in the 6 ± 1.5 to 13 ± 2 nm range when the bulk concentration is in the 46 to 460 μg/ml range.

1. Introduction

Sorption processes at the solid/liquid interface by which (bio)molecules bind to material surfaces are of interest for biosensors, biomaterials, material and surface science. Understanding the three-dimensional organization (including density, solvent content and thickness) of the resulting sorbed film and its evolution during the adsorption process is crucial for many applications in these domains. For biosensors, more specifically, there is a need to monitor the response in real-time, in order to assess the adsorption kinetics, and to be able to distinguish contributions coming from the dry sorbed mass, which is the physical criterion for estimating sensitivity, and those that should be attributed to effects intimately associated to the layer organization, like sorbent-bound water and hydrodynamic effects for example. While a wide variety of methods can qualitatively detect the formation of the sorbed film, almost none of them, taken alone, is quantitative and able to reveal the film organization, and only few can monitor the process in real-time. Scanning probe microscopies might fulfill all these requirements, mainly for submonolayers sorbed on smooth surfaces [1]. Neutron reflectivity [2], X-ray Photoelectron Spectroscopy (XPS) [3], mass spectroscopy and radiolabeling are quantitative techniques able to directly measure the dry sorbed amount. Of all the direct detection (*i.e.* label-less) techniques, we have identified acoustic and optical methods as being the only ones fulfilling two fundamental criteria of our measurements: time resolved and *in-situ* (liquid phase) measurement of the physical properties of the adsorbed layer.

Various methods of direct detection of biochemical layers have been developed, either based on the disturbance of an acoustic wave [4] (quartz crystal microbalance [5] – QCM – and surface acoustic wave devices [6] – SAW) or of an evanescent electromagnetic wave (optical waveguide sensors [7,8], surface plasmon resonance [9,10] – SPR). While each one of these transducers individually provides reliable qualitative curves during protein adsorption on their functionalized surfaces, extraction of quantitative physical parameters such as optical index, density, viscosity or water content requires modeling of the adsorbed layers [11]. The modeling includes multiple parameters which must be identified simultaneously: hence the need for the combination of (acoustic and optical) detection methods in a single instrument [12,13,14,15] to separate contributions as a same layer is reacting with the surface under investigation. Multiple investigators have identified such a combination of measurement methods as fruitful means of extracting independent physical properties of adsorbed layers, including the challenging combination of QCM and SPR [16,17,18,19,20] or comparing the results of successive experiments using different instruments [21,22,23,24], combining QCM and reflectometry [25] or measuring separately using the two techniques [26], or SAW and SPR [27].

We here use a combination of Love mode SAW device and SPR to identify values of density, water content and thickness of surfactant films and protein layers (collagen and fibrinogen) adsorbed on methyl-terminated surfaces. This combined measurement is necessary

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when attempting to convert a raw signal as observed at the output of a transducer (angle shift for SPR, phase and magnitude shift for SAW or frequency and damping for a QCM) to the actual protein mass bound to the surface, which is the physical criterion for estimating the expected highest possible sensitivity of a biosensor since it provides an estimate of the density of active sites on the surface. We furthermore compare the signals obtained from quartz crystal microbalance with dissipation monitoring (QCM-D [28,29]) measurements to that of the SAW and, based on the results obtained from the analysis of the SAW/SPR combination, show how SAW and QCM interact differently with the layer. The QCM displays a strong sensitivity to viscous interactions with adsorbed layers as was shown previously [30,31]. SAW devices are sensitive to mass loading, visco-elastic interactions and electrical charge accumulation on the sensing area [32], but with different influences due to the different frequencies and hence penetration ratio of the shear acoustic wave with respect to the layer thickness.

Love mode surface acoustic waves were chosen for their high mass sensitivity and their compatibility with measurements in liquid media [33,34,35,36]. Being based on the propagation of a shear horizontal acoustic wave, their interaction with the surrounding liquid is restricted to an evanescent coupling with the viscous liquid. Although bulk liquid viscosity properties affect the acoustic wave propagation [37,38], including the phase shift of Love mode SAW [39], we will throughout this investigation consider that the phase shift affecting the SAW device is solely related to adsorbed mass and not to viscous effects of the adsorbed layer, in order to reduce the number of unknowns. Such a crude assumption could be removed by exploiting the SAW insertion losses, for introducing the adsorbed layer viscosity. Throughout the analysis proposed here, we consider hydrodynamic interactions of the acoustic wave with the solvent filled adsorbed layer, as well as the equivalent optical index of the protein-solvent mixture, without focusing on the dynamic viscosity of this adsorbed layer but only on the viscosity of the fluid yielding shear wave evanscent coupling with the solvent.

The chosen protein layers consist of collagen and fibrinogen, selected as references both for their interest in engineering biocompatible surfaces [40,41] but most significantly for their strong solvent content and hence acoustic properties challenging to analyze. Collagen is a fibrillar protein of the extracellular matrix possessing self-assembly properties, involved in biorecognition processes. The collagen macromolecule consists of a triple helix with dimensions about 300 nm in length and about 1.5 nm in diameter, and weights about 300 kilodaltons [42]. Organization of collagen films adsorbed on hydrophobic surfaces - CH_3 – terminated self-assembled monolayers (SAMs) and polystyrene – from 30 to 40 μg/ml – were fully characterized under water or after drying using AFM [43,44], X-ray photoelectron spectroscopy [45] and radioassays. By AFM scratching experiments, thicknesses of the film adsorbed on methyl-terminated surfaces was estimated to be about 20 nm under water and 7–8 nm after drying. Furthermore, it was found that the measurement was strongly influenced at weak applied forces (<0.5 nN), in relation with the long-range repulsion (~50 to ~250 nm) observed by AFM force-distance curves, strongly suggesting that at least some molecules of the film must protrude into the solution [44]. Adsorbed amount of (dry) collagen on methyl-terminated surfaces were estimated to be between 0.4 and 0.8 μg/cm^2 by combining AFM and XPS measurements. Values near 0.5 μg/cm^2 were determined to be adsorbed on polystyrene by radiolabeling.

Fibrinogen, on the other hand, is a blood protein that presents three globular domains linked together by fibrilar segments. Similarly to collagen, its molecular weight is about 340 kilodaltons. Fibrinogen adsorbed on various surfaces has been imaged by AFM as well [46], down to molecular resolution [47,48,49,50].

In this presentation, similar films will be investigated, but under the new perspective of combined acoustic and optical measurements for extracting the thickness, mass, and solvent density in a buffer medium. While the SAW/SPR technique (Fig. 1) and data processing with our

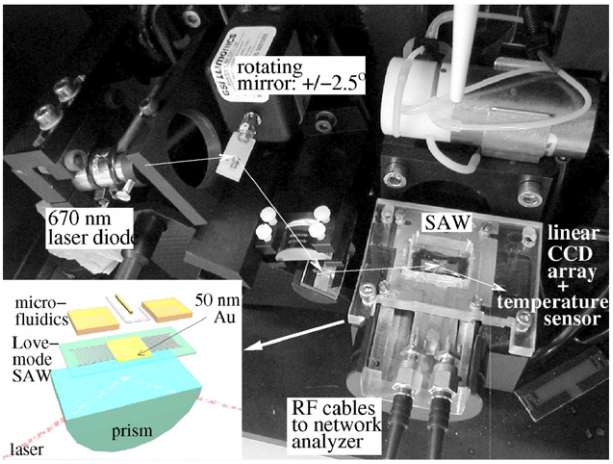

Fig. 1. Picture of the experimental setup combinging a SPR instrument (670 nm-wavelength laser with mechanical sweep of the illumination angle) with a piezoelectric (ST-cut quartz) substrate acting as both SPR sensing area since coated with a 50-nm Au layer and propagating a Love-mode SAW. Bottom-left inset: schematic view of he measurement head, emphasizing the need for integrated microfluidics to prevent liquid from reaching the interdigitated transducers. In this schematic chart, an electrochemical application is considered by showing the (bent) counter electrode and (straight) reference electrode located over the gold-coated sensing area acting as working electrode.

proposed formalism was already shown [51] to be appropriate in the identification of some physical properties of a rigid adsorbed layer (*S*-layer) [52], we here quantitatively analyze the organization of collagen and fibrinogen adsorbed layers which are expected to possess a substantial water content. This condition is expected to lead to the largest differences between acoustic and optical signals since both techniques respond differently to a viscous (solvent containing) layer: acoustic methods tend to overestimate the bound mass due to hydrodynamic interactions, while optical methods provide an estimate of the dry bound mass after appropriate modeling of the response but cannot resolve both parameters, thickness and optical index of the layer which SPR is sensitive to. This optical index can be assumed to be the weighted value of the index of water and that of proteins to the volumic part that these components occupy in the film. SPR response is thus dependent on the film organization and on the dry adsorbed amount.

Unlike the model used in other studies [53], we here assume that the SAW device signal shift is predominantly due to added rigidly bound mass on the electrode. Indeed we have shown that, while the QCM is sensitive *via* hydrodynamic interactions with the topography induced by rough copper electrodeposition [54], SAW is much less sensitive [55]. The contribution of the wave coupling with the viscous fluid to the phase shift [39] will be neglected throughout this investigation. Indeed, collagen films were analyzed using SAW/SPR after different conditionings, inducing a different film organization and related viscoelastic behavior (as probed by AFM and QCM respectively) for a similar adsorbed amount. These differences in the film properties were found to result in only minor changes in the SAW response. For all these reasons, the SAW phase response is considered in this article to be only mass dependent during adsorption phenomena. Hence, we use a proportionality relationship between the mass of the layer Δm per unit area $A = 5 \times 5.5$ mm^2 here ($\Delta m/A$) – including the rigidly bound water – and the frequency shift: $\frac{\Delta m}{A} = \frac{\Delta f}{S \times f_0}$ where f_0 is the frequency at which the phase is monitored in an open-loop configuration, Δf is the frequency shift obtained after conversion from phase to frequency shift through the experimentally measured phase to frequency linear relationship, and S is the mass sensitivity calibrated by copper electrodeposition. Since the mass sensitivity calibration is a central part in extracting quantitative results from the experimental data, we confirm the results obtained with copper electrodeposition by measuring the SAW signal change during adsorption of a surfactant, cetyltrimethylammonium bromide