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Formation and cross-linking of fibrinogen layers monitored with *in situ* spectroscopic ellipsometry

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ABSTRACT

Thick matrices of fibrinogen with incorporation of a matrix metalloproteinase inhibitor were covalently bonded on functionalized silicon surfaces using an ethyl-3-dimethyl-aminopropyl-carbodiimide and N-hydroxy-succinimide affinity ligand coupling chemistry. The growth of the structure was followed *in situ* using dynamic ellipsometry and characterized at steady-state with spectroscopic ellipsometry. The growth was compared with earlier work on *ex situ* growth of fibrinogen layers studied by single wavelength ellipsometry. It is found that *in situ* growth and *ex situ* growth yield different structural properties of the formed protein matrix. Fibrinogen matrices with thicknesses up to 58 nm and surface mass densities of 1.6 μg/cm^2 have been produced.

1. Introduction

The biological acceptance of a foreign material that comes into contact with biological fluids (e.g. as an implant) is a complex procedure but is believed to largely depend on proteins adsorbing to its surface. During the last decades numerous investigations have been performed dealing with adsorption from single protein solutions as well as binary and ternary protein solutions where both steady-state and/or dynamics of adsorption have been monitored by a variety of techniques, e.g. ^{125}I radiolabeling [1], circular dicroism [2], ellipsometry [3], reflectometry [4], AFM [5] and intrinsic fluorescence measurements. Most of the work has been focused on presentation of thickness and/or surface mass density of protein films in steady-state. In many investigations also the dynamics of adsorption are monitored and modeled with objective to understand binding and reaction kinetics. In many cases this is not an easy task as most adsorption phenomena are diffusion limited due to the unstirred layer close to a surface. However, there are relatively few publications presenting investigations of the dynamics as well as steady-state values of thickness and surface mass density with the objective to determine the structure of the layer and possibly also the orientation of the protein molecules. Furthermore, the application of thick layers of proteins are of interest in several areas, e.g. as carrier of drugs for drug delivery, in sensor technology and to prevent proteins to denature on artificial surfaces like implants.

The aim of this work is primarily to find a preparation technique for formation of thick protein layers, i.e. more than a surface-bound monolayer, and to develop an ellipsometric methodology to quantitatively determine its surface mass density, refractive index and thickness. This will aid the understanding how the structure of a protein layer evolve as adsorption proceeds. A minor aim of the project is also to perform a pilot study to incorporate a drug into a protein matrix for drug delivery purposes. Fibrinogen (Fib) was chosen as protein and both single layers and protein matrices (up to ~60 nm thickness) were grown onto functionalized silicon. The drug used is doxycycline which is a matrix metalloproteinase inhibitor (MMPI). A potential use of an MMPI is to inhibit a matrix metalloproteinase, e.g. collagenase which is supposed to be responsible for collagenolysis in wounds after implantation which in turn delays and complicates healing. Layers of Fib and MMPI were covalently cross-linked using the ethyl-3-dimethyl-aminopropyl-carbodiimide and N-hydroxy-succinimide (EDC/NHS) affinity ligand coupling chemistry [6]. Results obtained by *in situ* spectroscopic ellipsometry (SE) were compared with earlier published results from *ex situ* ellipsometry obtained at a single wavelength [7]. Such comparison facilitates the understanding of the difference between growing thick matrices of proteins entirely *in situ* and growing thick protein layers with a rinsing and drying step between each sublayer formation.

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2. Experimental

2.1. Surface preparation

Silicon (1 0 0) wafers cut in 10 mm × 20 mm pieces were used as substrates and were cleaned at 80 °C during 5 min in a solution of high purity distilled water (Milli-Q), hydrogen peroxide (H_2O_2, 30% v/v, Merck, USA), and ammonia (NH_3, 5% v/v, Merck) with volume ratios 5:1:1, followed by a similar treatment in high purity distilled water, hydrogen peroxide, and hydrogen chloride (HCl, 37% v/v, Merck) with volume ratios 6:1:1. After each cleaning step the surfaces were rinsed in Milli-Q water at least 4 times and dried in dry nitrogen gas. Due to this treatment the thin silicon dioxide layer on the surfaces became hydrated, giving the surfaces a hydrophilic character. In order to bind proteins onto the silicon surfaces, the latter were functionalized in 0.2 mL APTES ($H_2N(CH_2)_3Si(OC_2H_5)_3$, Aldrich Chemical Company Inc., USA) at 6 mbar pressure and 60 °C during 10 min. The temperature was increased to 150 °C during 60 min and the samples were rinsed in xylene and dried in nitrogen gas. The surfaces were finally incubated in 6% glutaraldehyde (GA) ($HOC(CH_2)_3CHO$, Sigma Aldrich, Germany) in 0.15 M phosphate buffered saline (PBS) at pH 8.5 in room temperature during 30 min, and then rinsed in Milli-Q water. GA has reactive aldehyde groups in both ends and can therefore act as a linker molecule between two amine groups, where in this case one group is bonded to APTES and one is at the surface. Thus, this final step produces a functionalized surface with –CHO groups. The dried samples were stored in closed Petri dishes and used within a week.

2.2. Materials and protein incubation procedure

Doxycycline (Sigma, USA), an antibiotic commonly used for treatment of various infections, was used as MMPI at a concentration of 1 mg/mL. In the first experiment (named ML1), ZnCl was added to a concentration of 0.5 mg/mL to enhance the binding of MMP to MMPI after release in the body. As it may cause gel forming, the ZnCl was excluded in the following experiments (ML2 and ML3). All measurements were performed in 0.15 M PBS at pH 5.5. A stock solution of human fibrinogen (Hyphen Biomed, France) of 8 mg/mL in 0.15 M PBS at pH 5.5 was prepared and diluted to 1.1 mg/mL at incubation. For each step of EDC/NHS incubation a fresh solution was prepared from 3.75 mg 0.2 M EDC (Sigma, USA) and 5.75 mg 0.05 M NHS (Sigma, USA) per mL PBS at pH 5.5. The EDC/NHS solution was always used fresh since the EDC solution is unstable at room temperature. A "layered" Fib matrix was built up as follows. After two incubations of Fib, one layer of doxycycline was adsorbed followed by two layers of Fib. After adsorption of each layer of Fib as well as doxycycline, the sample was incubated in EDC/NHS for cross-linking and surface activation. In Fig. 1(a), a schematic of the "layered" structure is shown. From now on layer is used in the context of an incubation step and a multilayer (ML) consists of more than one incubation of Fib whereas a single layer (SL) is a single Fib incubation without EDC/NHS activation. It should be pointed out that real multilayers with sharp interfaces are not formed in each incubation step as new adsorbing molecules may penetrate and cross-link into an already formed surface layer. It would be more appropriate to talk about a protein matrix with increasing surface mass density as adsorption and further binding occurs.

2.3. Method

The experiments were performed *in situ* meaning that data during the whole experiment were recorded with the sample in liquid, which differs from earlier work by Tengvall et al. [7] where measurements were performed in air *ex situ* after each incubation step.

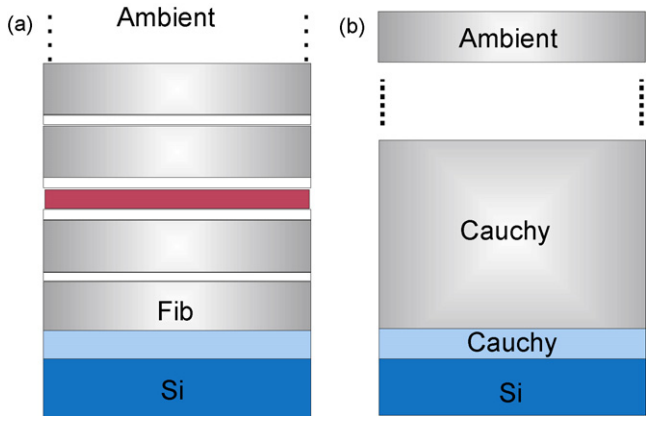

Fig. 1. (a) A schematic of the layered stack of proteins with four layers of cross-linked Fib. The red (darker) layer in the center is doxycycline used as an MMPI, whereas the bright thin layers correspond to EDC/NHS. The layer between the first Fib layer and the Si substrate represents silicon dioxide including APTES and GA. (b) Two-layer model applied to SE data analysis with the bottom Cauchy layer representing the oxide including APTES and GA and the second Cauchy layer representing all cross-linked layers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.3.1. Spectroscopic ellipsometry

The *in situ* measurements were performed using a variable angle spectroscopic ellipsometer (VASE) from J.A. Woollam Co., Inc. at an angle of incidence of 68° in the spectral range 350–1050 nm. A glass cell with a magnetic stirrer and a flow system for rinsing, as shown in Fig. 2, was used. All incubations were performed in PBS at pH 5.5 during 30 min (excluding 15 min for the spectral measurement before rinsing) using a protein concentration and a doxycycline concentration of 1 mg/mL. After each incubation, the cell was rinsed in PBS at a flow of 3.1 mL/min during 30 min using peristaltic pumps, whereby the sample was in liquid during the whole experiment and was never exposed to air. Dynamics at a wavelength of 500 nm were recorded during incubation and rinsing, whereas spectral measurements were done after each incubation and rinsing step. This summed up to a total number of spectral measurements of at least 22 and a total of at least 20 dynamic scans for one ML experiment consisting of four Fib layers. The time for each ML experiment reached a minimum of 15 h. Preparation of and results from three ML's with four or five layers of Fib in total, using this procedure, are presented.

The WVASE (J.A. Woollam Co. Inc.) software [8] was used for data acquisition and modeling of the experimental data. The refractive

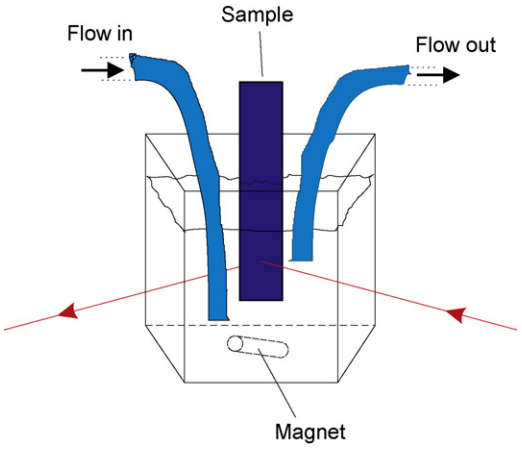

Fig. 2. Schematic of the glass cell with magnetic stirrer and flow system used in ellipsometric *in situ* experiments.

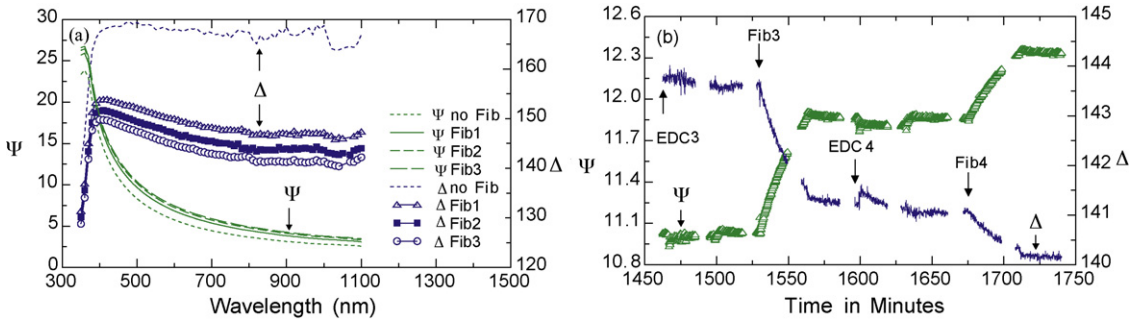

Fig. 3. (a) Spectroscopic scans recorded before the first Fib layer and after formation of the 1st, 2nd and 3rd Fib layers. (b) Dynamics recorded during incubation and rinsing of the 3rd and 4th EDC/NHS and Fib layers. The lack of data between incubation and rinsing steps is due to recording of spectra.

indices of protein layers are wavelength-dependent [9] Arwin, even though this is not always considered in analysis of ellipsometric data on protein adsorption. It is less well known that ions in the liquid and also protein molecules, but to a smaller extent, affect the ambient refractive index, n_a and makes it larger than that of water. This has been taken into account in this work by using spectral PBS refractive index data [10]. For refractive index, n, of layers a Cauchy model was used. In this dispersion model n equals

$$n = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4}$$

where A, B and C are fitting parameters and λ is the wavelength. The optical model used in the analysis consists of two Cauchy layers on top of a silicon substrate, see Fig. 1(b). The thickness, $d_{SiO_2+APTES+GA}$, comprising the layer of SiO_2, including APTES and GA was fitted to spectral Ψ and Δ data recorded before exposure to Fib using a Cauchy model and the parameters for this layer were then fixed and used in the subsequent fittings of Ψ and Δ recorded in presence of the protein matrix layer. The cell windows have a small in-plane birefringence affecting Δ to the order of 0.3°. In principle this could be included in the model as an explicit cell window correction. However, there are several other small imperfections and we have chosen to include them all (with window effects included) by fitting the constants and thickness of the first Cauchy layer using a spectrum recorded immediately before the first Fib layer was adsorbed. In this way we can minimize the effects of systematic errors and it is ensured that differences between subsequently recorded spectra and the initial spectrum are due to layer formation only. SE data recorded for all incubated layers were fitted using a second Cauchy model. At the end of each experiment the second Cauchy layer include layers from all incubation steps.

The mean squared error (MSE) is a quantification of the difference between experimental and model generated data and can be used as a figure of merit of the quality of the fit. The MSE were for all films lower than 0.6 and the mean value was for all experiments 0.39, considered a low value. Modeled thicknesses and refractive index values were used to calculate the adsorbed amount of protein, the surface mass density Γ, according to de Feijter's formula [11]

$$\Gamma = \frac{d(n - n_a)}{dn/dc}$$

where d is the thickness of the protein layer, n the refractive index of the protein layer, and dn/dc the protein refractive index-increment. A dn/dc value of 0.18 cm^3/g was used, a value commonly used for protein adsorption [12]. For the silicon substrate, spectral refractive index data from WVASE were used.

2.3.2. Null ellipsometry

Measurements in the earlier work by Tengvall et al. [7] were performed *ex situ* by null ellipsometry using an Auto-EL III (Rudolph Research, USA) programmed for measurements on silicon in air at $\lambda = 633$ nm. The thicknesses were calculated according to the McCrackin evaluation algorithm [13] with an assumed protein refractive index of 1.465. The samples were rinsed in PBS and blown dry with nitrogen after each incubation followed by measurements performed in air. The films were built up of Fib layers cross-linked with EDC/NHS.

3. Results and discussion

Spectroscopic and dynamic ellipsometric scans from one experiment are presented in Fig. 3. In the spectral scan shown in Fig. 3(a), experimental Ψ and Δ before adsorption and after formation of one, two and three Fib layers can be viewed. The dynamic scan in Fig. 3(b) shows incubation and rinsing of EDC/NHS and Fib layers, during formation of Fib layers three and four. Data from monitoring incubation of layers three and four are selected to highlight the resolution of data. It is important to consider that the layer is a representation of the adsorbed molecules during one incubation step and does not necessarily mean that a monolayer of molecules is formed on the surface. From spectral data of the type in Fig. 3(a), the layer optical properties and Γ were determined. Fig. 4(a) shows d and Γ for each incubated layer of three ML experiments, with the first point representing the substrate with a layer composed of native oxide, APTES and GA (called Si in the figure). The values of d and Γ increase at all Fib incubations. Notice that the thickness of the first Fib layer is in the order of 40 nm which is much larger than the thickness-increase due to the second, third and fourth Fib incubations. The thickness-increase is then in the range 2–12 nm (or 2–17 nm, if Fib 4 in ML1 is included) as can be seen in Fig. 4(b). Fig. 4(c) shows that Γ for the first Fib layer in each experiment ranges from 0.6 to 1.0 $\mu g/cm^2$ and the increase for the second, third, fourth and fifth Fib layers is in the range 0.05–0.25 $\mu g/cm^2$ (excluding Fib 4 in ML1). In Fig. 4(a) it is also seen that neither EDC/NHS treatments nor MMPI incubations do affect Γ which stays constant, whereas the thickness decreases after EDC/NHS treatments indicating that the layer matrix becomes denser, most likely due to cross-linking of the Fib layer. The deviation of Fib 3 of experiment ML1 in Fig. 4 can be explained by less adsorption of Fib due to that the pumping system was running during Fib exposure. Thereby the protein concentration decreased very quickly during this incubation step. The next Fib layer (Fib 4) increased remarkably (17 nm and 0.46 $\mu g/cm^2$), probably due to that the surface had been activated twice due to two incubations of EDC/NHS with only small amounts of Fib molecules adsorbed in-between.

Included in Fig. 4(b) and (c) are results from adsorption of single Fib layers named SL1 to SL5 as well as data from earlier *ex situ* experiments measured with null ellipsometry [7]. For comparison of results from Ref. [7], Fig. 4(b) also shows two measurement points (cross symbols) representing *ex situ* measurements of Fib layer SL5,

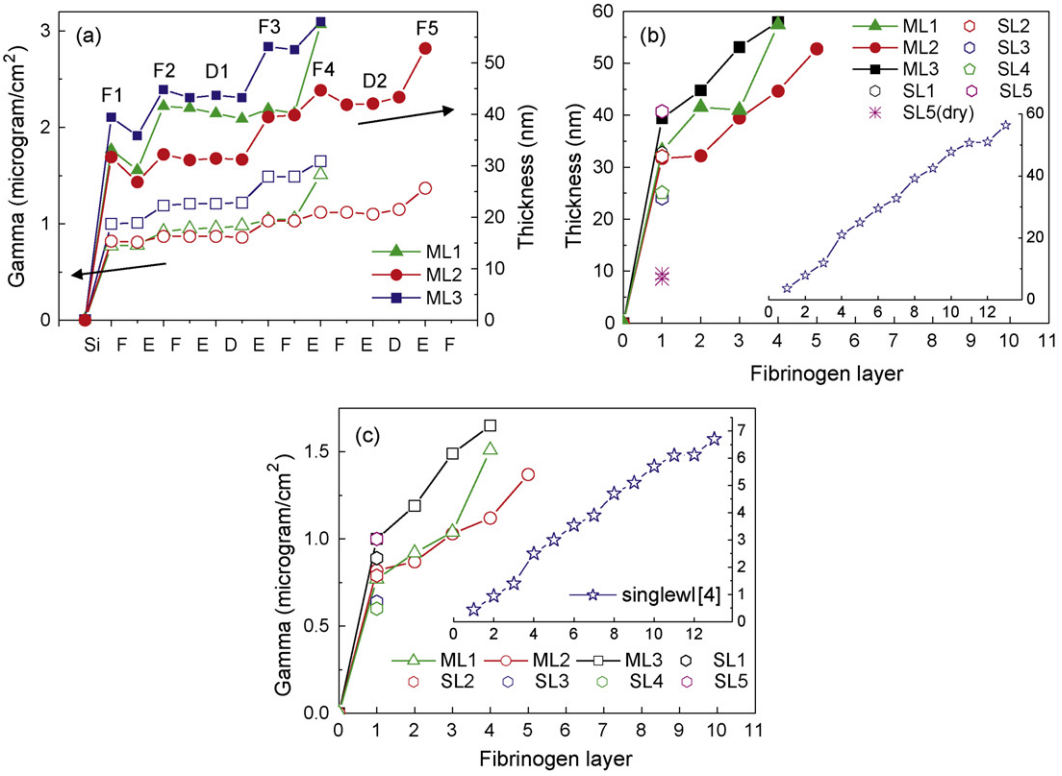

Fig. 4. Surface mass density (open symbols) and thickness (filled symbols) of incubated layers. In (a) results after all incubations are plotted with F, E and D representing Fib, EDC/NHS and doxycycline, respectively, and F1 and D1 representing Fib layer 1 and doxycycline layer 1, and so on. In (b) and (c) only results after Fib incubations are plotted. The insets in (b) and (c) show data from earlier *ex situ* experiments measured with null ellipsometry [7].

which after incubation and measurement *in situ* was dried in flowing nitrogen and measured in air. This would represent the drying step between measurements in the *ex situ* ML experiment in Ref. [7]. The data was modeled with both a fitted n as well as with a fixed $n = 1.465$ (same n as used in [7]), explaining the two thickness values in the graph. The measurement in air gives a dramatic decrease in the thickness value, from almost 41 nm to 8.5 and 9.5 nm, where the latter value is obtained with $n = 1.465$. These thickness values are larger compared to those in Ref. [7] which was in the order of ~4 nm and might be explained by a longer incubation time.

The refractive index after each incubation step and versus Fib layer thickness is plotted in Fig. 5(a) and (b), respectively, and indicates a relatively small variation in n throughout the protein layer growth. The rather low n values can be explained by the presence of more water molecules in the protein layer as compared to measurement of a protein layer in air which most likely collapses and thus densifies leading to a high-index layer. In comparison, the refractive index for PBS is 1.3373 at this wavelength [10]. The lower values of n for the first two Fib layers of ML1 indicate a less dense protein layer structure and thereby a layer containing more water. Notice the index-increase due to EDC/NHS treatments between Fib exposures as will be further discussed below.

In Fig. 6, thickness, refractive index, and Γ are plotted versus time for one of the experiments (ML3) and it is clear that the thickness increases during each incubation of Fib but decreases or stays constant during the EDC/NHS as well as the doxycycline incubation. However, the refractive index increases during the first Fib incubation but decreases for subsequent protein layers. On the contrary, the index due to incubation with EDC/NHS increases for each incubation step, whereas the index decreases during incubation of doxycycline (except for D1 in ML1). The thickness-decrease during the EDC/NHS steps can be explained by cross-linking of the

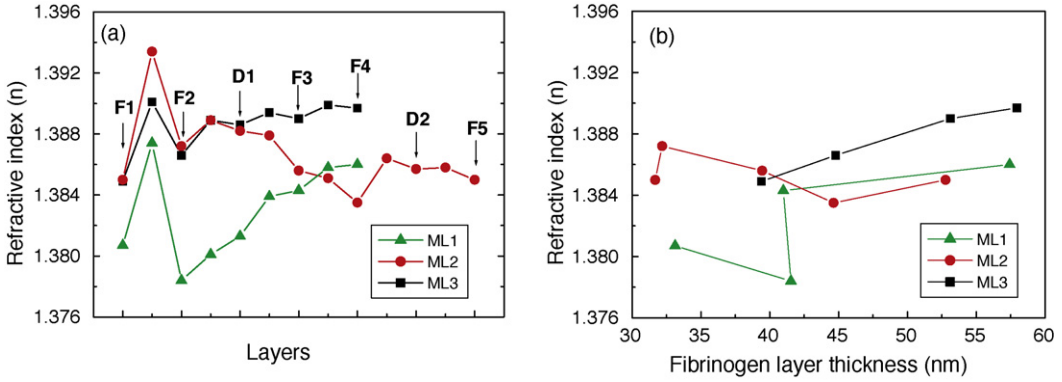

Fig. 5. Variation of refractive index at 500 nm versus (a) layers (F1 and D1 represents the first Fib and doxycycline layer, respectively) and (b) versus Fib layer thickness.

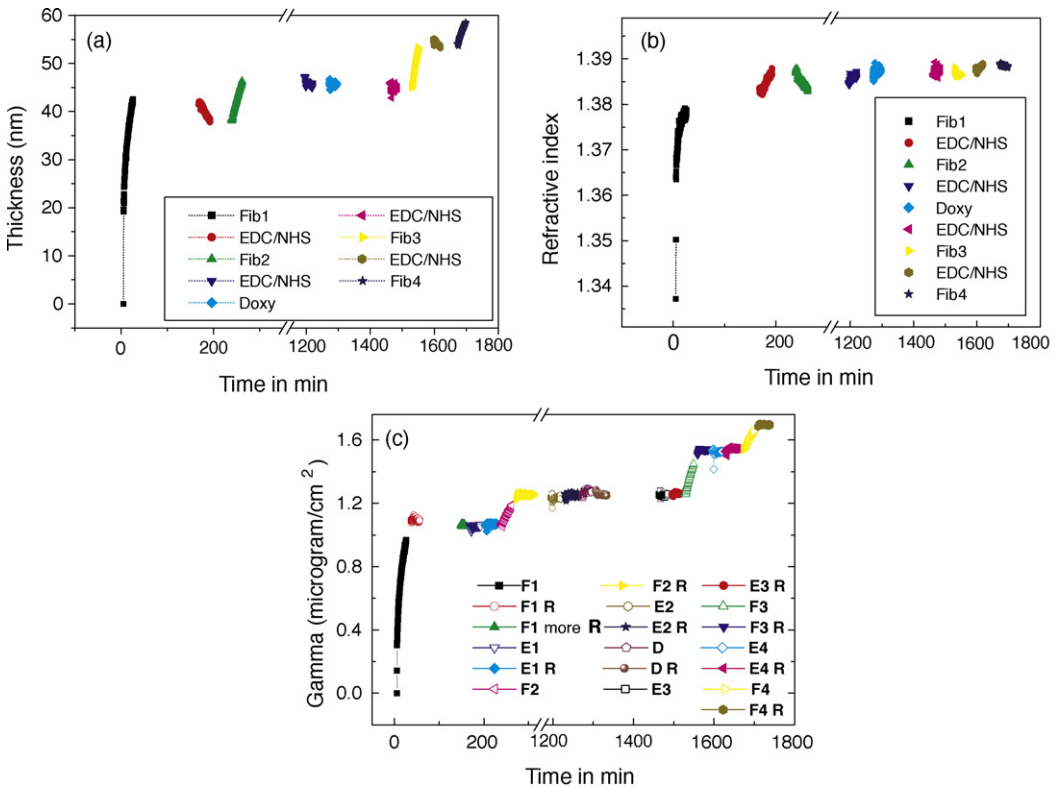

Fig. 6. Dynamics at 500 nm of thickness (a), refractive index (b), and Γ (c) for all subsequently formed layers in experiment ML3 (R represents rinsing).

existent Fib layer leading to shrinking of the layer which then simultaneously becomes denser. The decrease of the refractive index in combination with a thickness-increase during incubation of Fib layer two (and onwards) follows from that the new Fib molecules bind quite sparsely to the activated Fib molecules of layer one giving a very low density layer close to the ambient solution. However, the layer becomes densified during the next EDC/NHS incubation indicated by the thickness-decrease, the refractive index-increase and

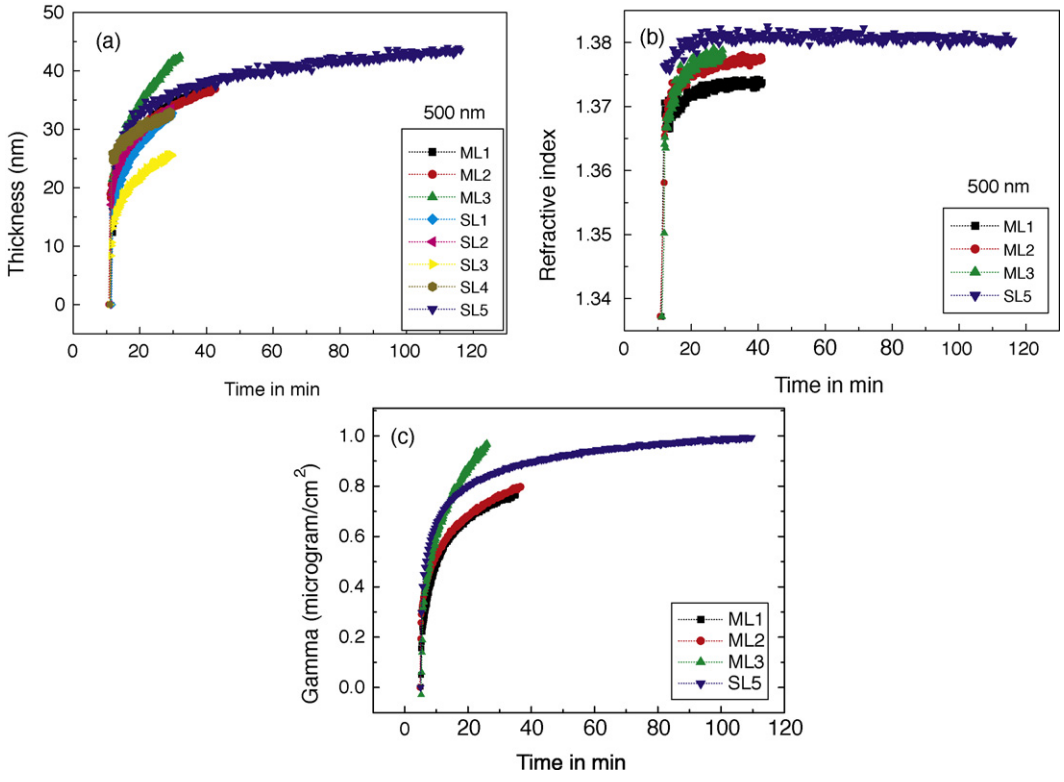

Fig. 7. Thickness (a), refractive index (b), and Γ (c) versus time during adsorption of the first Fib layer in three ML and one (five in (a)) SL experiments.

that the surface mass stays fairly constant. Recall that in the model each new incubation is not modeled as if a new layer is formed but together with the previously formed layer, meaning that we determine the average value of n of the whole protein matrix after each incubation step.

Fig. 7 shows the dynamics at a single wavelength (500 nm) of four experiments (eight in (a)) during incubation of Fib layer one. It is seen that n reaches a stable value after about 30 min of incubation, whereas d and Γ continues to increase even after almost 2 h. Experiment ML3 shows a larger adsorption, in consistence with the full experiment (see Fig. 4), and could be explained by a more efficiently activated substrate surface with more binding sites.

The agreement of our results with other work is satisfying regarding thickness and surface mass density. Thickness values of up to 30 nm and adsorbed amounts of 0.49 $\mu g/cm^2$ of Fib on hydrophobic surfaces (methylated silica) was measured with null ellipsometry by Malmsten [14]. These values are similar to our results as well as the refractive index reaching values of 1.37. Elwing et al. [15] obtained Γ up to 0.9 $\mu g/cm^2$ for an adsorbed Fib layer (using a concentration of 1 mg/mL) on silicon surfaces with wettability gradients, which is slightly lower compared to our results. Neither of the authors discussed the structure of the protein layer. Schaaf and Dejardin [4] have presented n in the range 1.345–1.370 and a mean value of d in the range 7.5–20 nm of Fib adsorbed on silica studied with scanning angle reflectometry assuming a bilayer model.

4. Discussion and proposed molecule-organization

Numerous ellipsometric studies of the dynamics of protein adsorption have been presented [16–18]. However, in most cases the separation of the refractive index n and the thickness d of a layer is not possible due to that only single wavelength data are available. The ellipsometric data are then mainly sensitive to the optical mass nd. An attempt to separate n and d often fails due to their strong correlation and one resorts to assume a value on n whereby a value on d can be determined. The thickness obtained in this way is then a fictitious parameter and its value depends on the index chosen. If an index corresponding to the intrinsic index of the protein is chosen, the thickness obtained is the thickness the layer would have if it collapses and becomes dense. In this work, high precision spectral ellipsometric data are available and a separation of n and d is possible. Due to that a protein layer may exhibit both in-plane and out-of-plane density variations, the obtained value on d neither represents the collapsed thickness nor the true extension but we consider the value to be close to the extension. The value of n represents an effective value of the layer index from which density information is accessible. For a 100% dense layer, n would be the intrinsic protein index. This situation rarely occurs as protein molecules cannot be packed in a perfect homogeneous layer.

Access to the dynamics of layer density and extension during layer formation opens up possibilities to discuss the overall structure of protein layers. There are very few methods that can be used for this purpose. For example, *in situ* AFM can provide some detail although there is always a risk that interactions with the AFM probe may influence the structure. Due to the lack of methods and experimental data carrying information about *in situ* structure of protein layers, there are very few structural models discussed. Here we make an attempt to contribute by proposing a structural model for a fibrinogen matrix on a surface. The model is based on thickness, refractive index and surface mass density evaluated from spectroscopic ellipsometric data.

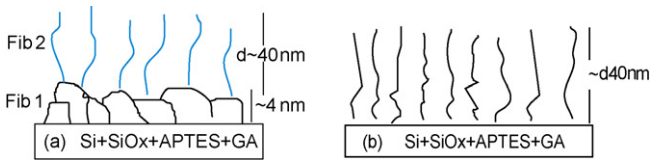

Fig. 8. Comparison of a simplified Fib layer structure (end-on orientation) using *ex situ* and *in situ* growth. (a) Schematic geometry of two layers of cross-linked Fib in the moment of incubation of the second layer. Fib 1 represents the layer structure after the first incubation step including rinsing and drying. Fib 2 corresponds to the layer structure before the second rinsing and drying step. (b) One layer of end-on adsorbed Fib grown *in situ* and measured by spectroscopic ellipsometry.

4.1. Surface interactions and fibrinogen adsorption

Protein adsorption to a surface includes a number of processes, and several parameters of both the surface and the protein molecules are important. For example, the protein net charge and the charge distribution and degree of hydrophobicity of prosthetic groups available at the surface influence the organization of molecules and the protein structure. As shown by Fuss et al. [19] hydrophobic or positively charged surfaces cause fibrinogen molecules to spread out (inducing unfolding) in opposite to hydrophilic and negatively charged surfaces that tend to keep the molecule in its characteristic trinodular shape. The fibrous Fib molecule is rather flexible and also undergoes conformational changes upon adsorption [20].

The GA-treated surfaces in our experiments show an intermediate behavior of hydrophobicity. Surface binding of protein molecules can be facilitated through APTES and GA treatment of a silicon surface and cross-linking through EDC/NHS activation after each adsorbed Fib layer. The possible cross-links between the GA-surface and the Fib molecule is due to bonding between the aldehyde (–COH) and, either the N-terminal of one of the peptide chains in the molecule, or an amine from lysine residues on the exterior of the Fib molecule. The cross-links between fibrinogen molecules after activation of carboxylic acid groups using EDC/NHS are simply peptide bonds between carboxylic groups at C-terminals or from glutamic acid or aspartic acid residues from the activated molecule, and the N-terminal or any amine from the adsorbing molecule.

Fibrinogen molecules also show ability to self-assemble without conversion to fibrin monomers and the subsequent cross-linking process forming a fibrin network. There are two self-association sites of the fibrinogen molecule that participate in the fibrinogen assembly and cross-linking, both situated on the γ chain of the molecule [21,22]. One is the so called D:D-site that can be found at the outermost position of the D-domain and induces a lateral bonding with end-to-end alignment of fibrinogen molecules. The other is the XL-site which is situated in a carboxy-terminal region of the γ chain and facilitates transverse cross-linking of fibrinogen molecules.

4.2. The adsorbed fibrinogen matrices – simplified models

A comparison of *in situ* and *ex situ* growth of protein matrices, shows that *ex situ* growth of Fib [7] render protein thickness-increases of similar values (~4 nm) for all incubations, whereas the first Fib incubation step of experiments monitored *in situ* render a much thicker layer (~40 nm) compared to incubation step two to five where the thickness-increase is in the order of 2–12 nm at each step in the ML experiments. This difference might be explained by a drying step of the surface before measurement in the *ex situ* experiments, leading to denaturation of the proteins as schematically shown as layer Fib 1 in Fig. 8(a). This results in a collapsed layer of denatured proteins which is much thinner than the native protein matrix we have observed in the *in situ* experiments. The remarkably larger thickness after the first Fib incubation in our experiments, up to ~40 nm, is somewhat unexpected and is not self-explanatory,

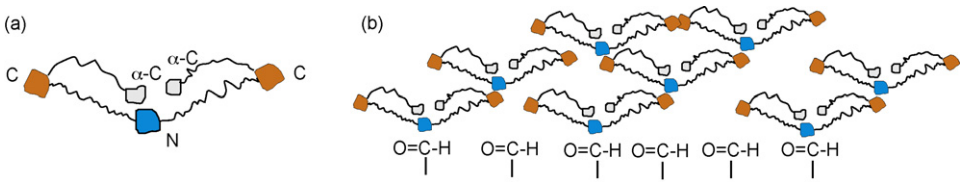

Fig. 9. Possible structure of the first incubated layer of Fib molecules. (a) A single molecule with the N-terminal in the center and C-terminals at the ends with the small globular regions representing the C-termini of γ-chains. (b) A tentative model of eight Fib molecules bonded to a functionalized silicon surface. A Fib molecule measures in this configuration ~10–15 nm in height and the Fib matrix can reach a thickness of 40 nm. After inspiration from Fuss et al. [19].

although it is tempting to think of the adsorbed protein molecules in the first layer as in an end-on orientation, as sketched in Fig. 8(b).

A possible adsorption mechanism, to explain Fig. 8(b), could be cross-linking of an amine group to the surface or hydrophobic interactions as discussed earlier. However, if an end-on orientation of the molecules would be assumed, hydrophobic interactions could not likely explain the adsorption, since the C-terminals at the ends of the oval Fib molecule (measuring 46 × 6 × 6 in nm corresponding to a molecular weight of 340 kDa) are charged. Furthermore, the N-terminals of the Fib molecule, which would be an assumed group to take part in the cross-linking, are situated in the molecule's center in the so called E-domain [23]. Thus it is impossible that the protein is stretched in its full length of ~46 nm if a cross-link between a Fib N-terminal and an aldehyde at the surface is established, as seen in Fig. 9(a). At maximum, the extension of the Fib layer, normal to the surface can be half of the molecule length, i.e. 23 nm. This structure gives a proximal length of the molecule in the range 10–15 nm due to a variable angle between the rod-like regions. The thinnest observed first "layer" of Fib of the eight experiments is 24 nm and the thickest reaches a value of 41 nm. With this approach these thickness values indicate that the first adsorption step must include more than a monolayer, which suggests that several "layers" of the molecule adsorb during one incubation as pictured in Fig. 9(b). However, even though the surface is activated for cross-linking to the N-terminal or other regions with amines (lysine), other parts of the molecule still participate in the adsorption process as discussed earlier.

Hence, more likely, a simplified description of the first incubated layer would instead be a mixture of covalent bonds (both N-terminally and other amines) and hydrophobic interactions. In addition, also Fib assemblies are formed, as schematically shown in Fig. 10. After an initial phase forming a monolayer on the surface the adsorption process is slowed down due to a lower number of available reactive aldehydes on the surface. The monolayer is bound rather tight to the surface giving the Fib molecules low flexibility. Gradually during build-up, however, the additionally adsorbed molecules become more flexible and mobile making it less likely for Fib molecules in solution to adsorb to the growing layer.

After 30 min of incubation the first adsorption step was interrupted and the surface was activated. After activation of the carboxylic groups of the Fib matrix, with an EDC/NHS incubation step, the Fib molecules from the second incubation is cross-linking and partly diffusing into the first layer increasing the surface mass density. However, at the layer/solution interface the newly adsorbed Fib molecules tend to form a more loose structure in the growing layer. It is important to notice that during the EDC/NHS incubation the refractive index increases, the thickness decreases, whereas Γ stays constant which is an indication of a densified shrinked layer. This can be explained by an effective cross-linking taking place also among Fib molecules from the first incubation step. Even though the expression "layer" has been used in the discussion, it is important to stress that there are no well defined layers formed but rather a Fib matrix. It is easier though, to describe the surface assembly of Fib molecules from one incubation step as a layer. The thickness-increase after the second Fib incubation (5.5–12.5 nm), always smaller than the first, is probably also a result of mixed interactions. Incubation three, four and five in the growth of the protein matrix result in a similar behavior.

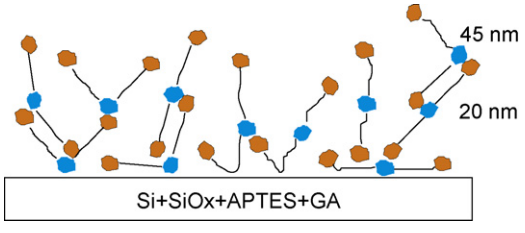

Fig. 10. A possible structure of a Fib matrix on a functionalized Si surface. Mixed events of adsorption of the first Fib layer grown *in situ* are shown.

Schaaf and Dejardin [4] suggested a bilayer model with a mixture of end-on and side-on adsorption of Fib on silica. Mårtensson et al. [24] presented models of molecular organization of lactoperoxidase on silicon dioxide with a refractive index of 1.5 of the protein film, measured by null ellipsometry. They suggest end-on oriented molecules with large repulsive interactions between molecules on hydrophilic silicon surfaces whereas a side-on orientation is suggested for the protein molecules on hydrophobic silicon surfaces. This model includes also the adsorption of proteins with mixed orientations. The authors also present a conformational change model in which the molecules change conformation upon adsorption and binds stronger to the surface and thereby become less mobile. Similarities between this model and our model with Fib might be established.

5. Summary

The difference between protein matrices prepared *in situ* and matrices prepared with a drying step between incubation steps has been demonstrated, as well as a more sophisticated method for analysis of the ellipsometric data. Spectral ellipsometric data provides enhanced resolution and allows a separation of layer thickness and index, compared to measurement at a single wavelength which is more commonly used for protein adsorption.

From the results of this work it is established that thick layers of Fib are produced if the incubation of Fib onto a silicon surface is interrupted by a drying step between the incubation steps. However, if protein layers with biological function are desired in an application, it has been shown that Fib must be incubated subsequently *in situ*. The first Fib incubation step in these experiments produces a layer that is thicker than a monolayer indicating an adsorption process with a complicated molecular organization with a mixed set-up of molecular interactions. Contributions to the development of a model for layered growth of fibrinogen have been provided.

If only single wavelength ellipsometric measurements data are available, the use of Γ instead of d for the presentation of protein growth on surfaces is suggested to avoid the difficulties to with the correlation between n and d. Γ can also easily be compared with results from other methods like radio immunoassays [25].

The effect of surface activation after EDC/NHS incubation compared to a GA-activated surface is smaller according to the results from cross-linking of Fib layers in this work. The smaller amount of Fib cross-linking after EDC/NHS activation can probably be explained by the fact that the Fib molecules from the earlier incubated Fib layer are present during the EDC/NHS incubation and these molecules are cross-linking to one another during this step which leads to a smaller number of activated carboxylic groups available for the next Fib incubation. This generates a thinner but denser Fib layer and matrix.

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