Variations in Coupled Water, Viscoelastic Properties, and Film Thickness of a Mefp-1 Protein Film during Adsorption and Cross-Linking: A Quartz Crystal Microbalance with Dissipation Monitoring, Ellipsometry, and Surface Plasmon Resonance Study

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We have measured the time-resolved adsorption kinetics of the mussel adhesive protein (Mefp-1) on a nonpolar, methyl-terminated (thiolated) gold surface, using three independent techniques: quartz crystal microbalance with dissipation monitoring (QCM-D), surface plasmon resonance, and ellipsometry. The QCM-D and ellipsometry data shows that, after adsorption to saturation of Mefp-1, there are significant variations in coupled water, viscoelastic properties, and film thickness of the protein film during adsorption and cross-linking behavior. These results provide new insight and understanding about the adsorption kinetics and cross-linking behavior of Mefp-1. They also demonstrate how the three techniques complement each other for biomolecule adsorption studies.

The quartz crystal microbalance (QCM) technique has so far primarily been used for real-time measurements of macromolecule adsorption in liquid-phase research applications.1-3 The two most common applications are in electrochemistry (EQCM) (see refs 1-4 and references therein), and biotechnology,5-8 to measure for example protein adsorption kinetics,9-11 antibody–antigen interactions,12-15 nucleotide hybridization and nucleotide–protein interactions,16-18 and lipid vesicle adsorption19-21 including spontaneous formation of supported bilayers,22-24 as well as bacteria and cell adsorption.25-27

The merit of the QCM technique has so far primarily been the simplicity and sensitivity (in the ng cm⁻² range) by which an adsorbed mass, Δm, can be deduced from a linear relation (the so-called Sauerbrey relation23) between adsorbed mass and measured changes in the resonant frequency, Δf:

$$\Delta m_{QCM} = \left( C_{QCM} / n \right) \Delta f$$

(1)

where $C_{QCM} (=17.7 \text{ ng-cm}^{-2}\text{-Hz}^{-1} \text{ at } f = 5 \text{ MHz})$ is the mass sensitivity constant and $n (=1, 3, ...)$ is the overtone number. (The subscript $s$ reminds us in the following that we are within the Sauerbrey model.)

For rigid, evenly distributed, and sufficiently thin adsorbed layers, eq 1 has indeed been shown to hold to a good approxima-
The second source of “failure” of the Sauerbrey relation is not a
the piezoelectric oscillator \( \propto m_u \), while a viscoelastic or
thicker film constitutes a coupled oscillator for which \( \Delta f \) is not
directly proportional to \( m_u \). In other words, the effectively
coupled mass depends on how the oscillatory motion of the crystal
propagates into and through an adsorbed viscoelastic film.5,28–30
The typical amount of coupled water has in different
systems been shown to vary significantly depending on the nature
of the film, with mass-uptake estimations between a factor of 1.5
and 4 times larger than the molar mass.9,12,13,17 The effects of
coupled water on the QCM response have been theoretically
modeled for stiff, rough,31 and textured QCM—sensor surfaces
and successfully applied to interpret experimental results.

These factors, which at first sight may appear as an undesirable
complications of the QCM technique, compared to, for example,
optical methods such as ellipsometry or SPR, actually provide a
platform for new information about adsorbed films, not obtainable
by, for example, optical techniques, when the conventional QCM
measurements are complemented with Q-factor or dissipation (D)
measurements (QCM-D). With a theoretical platform that can treat
the elastic and inelastic components of the shear-wave propagation
through an adsorbed film, new information can be obtained
through QCM-D measurements.5,28–30

In the present work, this is demonstrated for the first time for
adsorbed biomolecules probed in an aqueous environment using
a model system where the viscoelastic properties can be varied in
situ, by (bio)chemical means; namely, the adsorption and
subsequent polymerization of the mussel adhesive protein, Mytilus
edulis foot protein, Mefp-1 (M_m ~ 120 kD). Mefp-1, which is
composed of 75–85 repeats of the decameric unit, NH_2-Ala-Lys-
Pro-Ser-Tyr-Hyp-Hyp-Thr-I-DOPA-Lys-COOH,35 is especially at-
ttractive for such a study since it, as composed of repeating units
of identical decapetides, is chemically an unusually simple
protein. Moreover, it has an open flexible conformation in
solution16,37 that can be easily changed by cross-linking via
chemical or enzymatic oxidation using NaIO_4 and catechol oxidase
(M_w ~ 110,000), respectively, or via chelating using, for example,
Cu ions.38–40 Specifically, NaIO_4 (which was used as the cross-
linking agent in the present work) initiates the formation of highly
reactive o-quinoines, the oxidation products of diphenols of the
DOPA residues, including formation of di-DOPA cross-links within
and probably also between Mefp-1, i.e., the same reaction as
performed by the naturally occurring enzyme catechol oxidase.39,40
In the experiments described here, Mefp-1 was first adsorbed in
a natively and flexible state on a nonpolar methy-terminated
surface and then cross-linked by NaIO_4.41,42

The adsorption and cross-linking of Mefp-1 were probed using
a recently introduced QCM technique which simultaneously
measures changes in frequency, \( \Delta f \) (related to mass uptake), and
changes in the energy dissipation, \( \Delta D \) (cf. viscoelastic properties),
named QCM-D (see refs 10 and 43 for technical details). A Voigt-
based viscoelastic film model,30,44,45 describing the propagation and
the damping of shear-bulk acoustic waves in a single uniform
viscoelastic adsorbed film in contact with a semi-infinite bulk
Newtonian liquid under no-slip conditions, was used to model the
QCM-D response. The QCM-D data were compared with SPR and
eellipsometry data (i) to obtain independent thickness determina-

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tion, (ii) to compare the structural information contained in the QCM-D energy dissipation measurements with changes in the refractive index, n, of the protein films, and (iii) to derive the amount of coupled water sensed via Δf. The latter is possible for the following reason: The mass-uptake estimation from SPR and ellipsometry is based on the difference in refractive index between the adsorbed protein molecules and water displaced by the proteins upon adsorption. Water associated with the protein film, for example, the hydration shell, is therefore essentially not included in the mass determination by these techniques. In contrast, it is an inherent mass detected by the QCM technique. The amount of coupled water sensed via the QCM-D technique was also verified using D2O substitution measurements.

THEORY

QCM-D Technique. The QCM-D technique (Q-Sense AB), described in detail elsewhere, allowing simultaneous measurements of Δf and ΔD at the first, third, etc.; overtone (n = 1, 3, ...; i.e., f = 5 MHz, 15 MHz, ...) up to n = 7, to obtain the resonant frequencies, f₁, f₃, etc., and the corresponding dissipation values, D₁, D₃, etc., with a repetition rate of ~1 Hz. Since the linear relation between the adsorbed mass and the change in frequency (eq 1) is not necessarily valid for viscoelastic films, inducing additional energy dissipation and exhibiting a frequency (overtone)-dependent response, this type of information is critical. This is because if the system under investigation exhibits a frequency dependence in the measured interval (5–25 MHz in our case), measurements at several harmonics will allow the set of data (at n = 1, 3, ...) to be compared with the theoretical representations (with several unknown parameters) that must be applied in such situations. We have in this work used the so-called Voight-based representation of a viscoelastic solid, in which the adsorbed film is represented by a frequency-dependent complex shear modulus according to

\[
\frac{G}{(\rho_f, \eta_f)} = \frac{G'}{2} + i\frac{G''}{2} = \mu_f + i2\pi f\eta_f = \mu_f(1 + i2\pi f\tau_f)
\]  

where \(\mu_f\) is the elastic shear (storage) modulus, \(\eta_f\) the shear viscosity (loss modulus), f the oscillation frequency, and \(\tau_f\) the characteristic relaxation time of the film. The adsorbed film is further represented by a uniform thickness, \(d_{\text{film}}\) (the subscript -v reminds us that we are within the Voight model) and a uniform density, \(\rho_f\). The adsorbed film is situated between the QCM electrode and a semi-infinite Newtonian liquid. The film is represented by an elastic modulus, \(\mu_f\), a viscosity, \(\eta_f\), and a density, \(\rho_f\). The bulk liquid is represented by a density, \(\rho\), and a viscosity, \(\eta\).

\[
\Delta D = -\frac{\text{Re}(\beta)}{\pi f t_q \rho_q}
\]

where

\[
\beta = 2\pi f\eta_f - i\mu_f - \alpha \exp(2\xi_1 d_{\text{film}})
\]

\[
\alpha = \frac{\frac{\xi_1^2 2\pi f\eta_f - i\mu_f}{\xi_2^2 2\pi f\eta_f} + 1}{\frac{\xi_1^2 2\pi f\eta_f - i\mu_f}{\xi_2^2 2\pi f\eta_f} - 1}, \quad \xi_1 = \sqrt{\frac{(2\pi f)^2 \rho_f}{\mu_f + i2\pi f\eta_f}}, \quad \xi_2 = \sqrt{\frac{2\pi f\rho_f}{\eta_f}}
\]

and where \(\rho_f\) and \(\eta_f\) are the bulk-liquid density and viscosity, respectively.

SPR Technique. The surface plasmon resonance (SPR) technique is based on a collective electromagnetic motion that propagates along a metal surface, associated with which there is a localized evanescent wave with a decay length of ~200 nm. For surface-sensing purposes, advantage is made of the fact that the excitation of the surface plasmon is very sensitive to changes in the refractive index of the medium sensed by the evanescent wave in close proximity to the gold surface. The SPR is excited using monochromatic and plane-polarized light that under total internal refraction conditions is directed through a quartz prism at the interface between the quartz and a thin (~50 nm) layer of gold. At a certain angle, \(\Theta\), of incidence, SPR is excited, resulting in a sharp minimum in the intensity of the reflected light. If the refractive index (n) of the medium outside (decay length ~200 nm) the gold surface changes, by, for example, protein adsorption, there is a proportional change in the angle, \(\Delta\Theta\), at which SPR is excited.
created. The SPR technique thus allows real-time measurements of the mass uptake of proteins, \( \Delta m_{\text{SPR}} \), via the relation

\[
\Delta m_{\text{SPR}} = C_{\text{SPR}} \cdot \text{ARU}
\]

(5)

C_{\text{SPR}} has been calibrated (using a large amount of different proteins) to be 6.5 \( \times 10^{-2} \) ng-cm\(^{-2} \) for adsorption on flat surfaces.\(^{51-53} \) ARU is the measured change in response units (a dimensionless quantity that is proportional to the change in refractive index, \( \Delta n \), at the interfacial region)\(^{53} \). More advanced analysis of the SPR response curve, using more than one wavelength\(^{54} \) of the incident light or detailed line-shape analysis,\(^{55} \) has recently been shown to potentially make an estimation of both the refractive index and the optical thickness of thin films possible. However, to achieve the latter type of information, we have instead complemented our measurements using ellipsometry, which is more established for this purpose.

**Ellipsometry Technique.** Ellipsometry is an optical method recording the change in polarization of elliptically polarized light, when it reflects on a sample surface. If the surface is optically modified, e.g., by protein adsorption, the associated change in polarization is detected. From the changes in the ellipsometric angles (\( \Delta \), \( \psi \)), the refractive index, \( n \), and the optical thickness, \( d_e \) (the subscript e means ellipsometry) of the film can be deduced. In the analyses of the ellipsometry data, the systems were treated as composed of four homogeneous and optically isotropic layers, located between the substrate and the surrounding solution as described elsewhere.\(^{56,57} \) Knowing the substrate properties from the measurements in different ambient media, the average refractive index, \( n_\text{e} \), and the mean optical thickness, \( d_\text{e} \), of the adsorbed layer were calculated numerically from the \( \psi \) and \( \Delta \), with an equation derived for the optical four-layer model used to describe the system under study (see ref 56). The \( n_\text{e} \) and \( d_\text{e} \) values were also used to calculate the amount adsorbed (\( \Delta m \)) according to the formula derived by Cuypers et al., \(^{58} \) based on a ratio between molecular weight and molar refractivity and a partial specific volume of the protein of 4.01 g/mL and 0.742 mL/g, respectively. At a coverage above 100 ng-cm\(^{-2} \), the relative error in the measured thickness and refractive index of the protein films are less than 5%.\(^{56,57} \)

**MATERIALS AND METHODS**

**Measurement Techniques.** The SPR measurements were done using a BIAcore 2000 system in a flow cell providing laminar flow (BIAcore AB), using a flow rate of 30 \( \mu \)L/min. The QCM-D measurements (Q-Sense AB) were done from a static solution, in a cell designed to provide a rapid (<1 s) nonperturbing exchange of different buffer solutions, as described in ref 10. The ellipsometry measurements were done using an automated Rudolph Research thin-film ellipsometer, type 43603-200E, equipped with computer-controlled, high-precision stepper motors and a cuvette system for in situ measurements as described in refs 56 and 57. The angle of incidence was set at 67°, and light of a wavelength of 401.5 nm was used.

**Preparation of Surfaces.** For the QCM measurements, we used polished, AT-cut piezoelectric quartz crystals with gold electrodes deposited on its two faces (i.d. 25 mm) with a fundamental resonant frequency of ~5 MHz (M axtek Inc.). Prior to the surface modifications, the QCM-D sensors and the BIAcore sensor chips (BIAcore AB) dismounted from their holders were cleaned in an UV/ozone chamber for 10 min. This was followed by immersion in a 1:1:6 mixture of \( \text{H}_2\text{O}_2 \) (J. T. Baker) (30%), \( \text{NH}_3 \) (Merck) (25%), and Milli-Q water (Millipore) for 5 min at 70 °C. To obtain a chemically well-defined, electrically inert, nonpolar surface, the crystals and the sensor chips were immersed for more than 12 h in a 1 mM solution of an 18-carbon alkanethiol with a \( \text{CH}_3 \) end group (HS(CH\(_2\))\(_{17}\)CH\(_3\)) (Aldrich Chemicals) dissolved in hexane (Merck). Polished silicon wafers (p-type, boron-doped, resistivity 1–20 \( \Omega\)-cm) for the ellipsometry measurements were purchased from Okmetic Ltd. The wafers were oxidized thermally in a saturated oxygen atmosphere at 920 °C for ~1 h, followed by annealing and cooling in an argon flow. The oxidized wafers (thickness 0.37 mm) were cut into square slides with a width of 12.5 mm and cleaned according to the procedure described in ref 56. The methyl-terminated hydrophobic wafers were obtained by gas-phase silanization of bare wafers according to the following procedure: Plasma treated bare wafers were placed in a reactor, which was evacuated from air by a water suction pump. Dimethyl-octylchlorosilane (1 mL) was injected into the reactor through a septum, and the reaction was continued for ~18 h at 38 °C. The wafers were then rinsed with ethanol followed by water and dried in \( \text{N}_2 \).

**Preparation of Protein Solutions.** The mussel adhesive protein Mefp-1 (Bioscience AB, Stenkullen, Sweden) was prepared in a degassed, 0.1 M acetate buffer (0.75 M NaCl, pH/pD 5.5) prepared from Milli-Q \( \text{H}_2\text{O} \) (Millipore) and \( \text{D}_2\text{O} \) (Larodan Fine Chemicals AB), at a concentration of 25 \( \mu \)g/mL. The pH of this buffer is below the upper limit at which Mefp-1 undergoes spontaneous oxidation and subsequent cross-linking/ aggregation in solution. Prior to its use, NaIO\(_4\) (1 mM) was diluted in the same buffer as used for Mefp-1.

All experiments were repeated at least three times, with a standard deviation of less than 2% for the protein and surface preparations used in the QCM-D adsorption and \( \text{D}_2\text{O} \) substitutions experiments and 10–20% for the ellipsometry and SPR measurements (see Table 1).

**RESULTS AND DISCUSSION**

The adsorption kinetics of Mefp-1 during its adsorption on a methyl-terminated, nonpolar surface was measured by QCM-D, ellipsometry and SPR. The information obtained from QCM-D, by modeling the simultaneous measurement of \( \Delta f \) and \( \Delta D \) at different harmonics, using the Voight-based model is compared with data analyzed using the Sauerbrey relation (eq 1). These data are also compared with the film thickness, \( d_e \), the refractive index, \( n_\text{e} \), and the mass-uptake, \( \Delta m \), estimations obtained from the...
ellipsometry data and with Dn,me, obtained from SPR. After deposition of the Mefp-1 film, its structural transformation upon chemical cross-linking, induced by NaIO4, was recorded with the methyl-terminated surface to a buffer solution containing 25 mg/mL Mefp-1 in 0.1 M acetate buffer (0.75 M NaCl, pH 5.5) followed by exchange of the protein solution for a pure buffer solution, which interrupts the exposure of the surface to the protein solution. The adsorption of Mefp-1 was subsequently followed by addition of the same buffer solution containing 1 mM NaIO4. Changes in the simultaneously obtained D values vs time for the experiment shown in (a), Δf at n = 3 and n = 5 has in this plot been divided by n, to make a comparison between the response at different harmonics easier.

Figure 3. Ellipsometry data for Mefp-1 adsorption and subsequent rinsing and cross-linking using NaIO4 plotted as adsorbed mass (Δmₐ) vs time estimated using the expression derived by Cuypers et al. 58 (see text).

respectively (Figure 2a). The mass uptake is accompanied by an increase in D, with a D−t trace similar to that for Δf, reaching at saturation ~15.8 × 10⁻⁶, ~11.9 × 10⁻⁶, and 10.4 × 10⁻⁶ for n = 1, 3, and 5, respectively (Figure 2b). The negative frequency shift, i.e., increase in coupled mass, is in qualitative agreement with the ellipsometry data (Figure 3), also displaying a monotonic increase in mass uptake, which saturates at ~135 ng·cm⁻².

If the total Δf shifts are converted to mass by eq 1, the resulting mass uptakes are for n = 1, ~1557 ng·cm⁻²; for n = 3, ~1068 ng·cm⁻²; and for n = 5: ~1027 ng·cm⁻². This means that the

### Table 1. Results from the QCM-D (Δmₑ₋ₙ, ΔDₑ, deₙ₋ₙ, deₙ₋ₙ, ηₑ, µₑ) and Ellipsometry (ELM) (Δmₑ, dₑ, deₑ) Data at Saturation Prior to and after Cross-Linking of Mefp-1 Adsorbed on a Methyl-Terminated Surface

<table>
<thead>
<tr>
<th>Adsorption of Mefp-1 on a CH₃-terminated surface</th>
<th>Prior to cross-linking</th>
<th>After cross-linking</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCM Δmₑ₋ₙ (ng·cm⁻²)</td>
<td>n</td>
<td>1557 ± 25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1168 ± 20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1027 ± 20</td>
</tr>
<tr>
<td>ΔDₑ</td>
<td>n</td>
<td>15.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.4 ± 0.2</td>
</tr>
<tr>
<td>deₑ (nm)</td>
<td>n</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Δmₑ₋ₙ in the QCM measurements was estimated using eq 1 and the thickness from either eq 6 (deₑ) or the Voight model (deₑ). The error bar indicates the variation from between two and four measurements.
mass ratio $\Delta m_{s-QCM}/\Delta m_e$ (for $n = 1$) at this point is $\sim 10.7$; i.e., QCM-D appears to measure a 10 times larger mass at $n = 1$ compared to ellipsometry and somewhat lower at the higher harmonics. Being aware that we are using the Sauerbrey relation in a regime where it is questionable (see below), the reason for the much higher mass uptake detected by QCM-D is still attributed to coupled water and not to the viscoelastic nature of the film (see below). The difference in QCM-D response at the three harmonics is, however, caused by a frequency dependence in the viscoelastic properties of the film (cf. the Voight-based model).

Upon rinsing, there is only a slow and small increase and decrease in $\Delta f$ and $\Delta D$, respectively, at all harmonics, a small decrease in $\Delta m_0$, and no detectable change in $\Delta m_{\text{SPO}}$ (not shown). Upon addition of NaIO4, there is a large increase and decrease in $\Delta f$ and $\Delta D$, respectively. As the cross-linking reaction saturates at $t > 70$ min, $\Delta f$ and $\Delta D$ for $n = 1$ have increased and decreased, respectively, to values being about 50 ($\sim 45$ Hz) and 9% ($1.5 \times 10^{-6}$), respectively, of their values prior to rinsing and cross-linking. In contrast, there is only a slight decrease ($< 5\%$) in $\Delta m_0$ (Figure 3) and a 15% increase in $\Delta m_{\text{SPO}}$ (Table 1). The corresponding QCM-D values at $n = 3$ are 65 ($\sim 43$ Hz) and 13.7% ($1.5 \times 10^{-9}$), and at $n = 5$, they are 71 ($\sim 41$ Hz) and 13.7% ($1.5 \times 10^{-9}$). The corresponding final mass uptake according to the QCM data and eq 1 is $\sim 740$ ng cm$^{-2}$, yielding in this case the mass ratio $\Delta m_{s-QCM}/\Delta m_e \approx 4.4$ for the cross-linked film. The fact that there is essentially no or very small detectable changes in the molar mass sensed by the optical techniques suggests that NaIO4 does not bind significantly to the protein film, and negligible protein material is desorbed during the cross-linking reaction. Thus, within the Sauerbrey relation (eq 1) and using the ellipsometry data, $\sim 91\%$ of the coupled mass of the non-cross-linked protein film, sensed via the frequency shift, is made up of coupled water (primarily water), whereas afterward this number is reduced to $\sim 82\%$ This reduction in water content, which is qualitatively correct after correction of the Sauerbrey model, is attributed to release of a large fraction of the water coupled to the protein film prior to the cross-linking reaction.

The large decrease in energy dissipation upon cross-linking (Figure 2b) is typical for a structural transformation from a dissipative nonrigid structure to a stiffer and most likely more compact structure, as observed in several earlier model systems.\textsuperscript{10,11,23} Note that there is, as expected, significantly less difference between $\Delta f_0$ and $\Delta D_0$ at the three harmonics after completed cross-linking, i.e., for the more stiff film. It is thus expected that the Sauerbrey model is more correct to use in this regime (see below).

The coupled water sensed by the QCM-D must be contained within the adsorbed protein film. At high coverage, i.e., at or close to saturation where most water in the protein film is coupled, it is reasonable to represent the protein film with an effective hydrodynamic thickness, $d_{\text{QCM}}$, and an effective density, $\rho_{\text{effective}}$. The effective thickness can be directly expressed by using the mass uptake, $\Delta m_{\text{QCM}}$, estimated from $\Delta f$ (according to eq 1) and the mass uptake from the optical methods:

$$d_{\text{QCM}} = \frac{\Delta m_{s-QCM}}{\rho_{\text{effective}}} = \frac{\Delta m_{s-QCM}}{\rho_{\text{protein}} \Delta m_{s-QCM} + \rho_{\text{water}} (1 - \frac{\Delta m_e}{\Delta m_{s-QCM}})}$$

Taking $\rho_{\text{water}}$ and $\rho_{\text{protein}}$ to be 1000 and $\sim 1400$ kg m$^{-3}$, respectively, the effective thickness of the Mefp-1 film obtained from the QCM data prior to and after cross-linking are $\sim 15.2$ and $\sim 7.0$ nm. If the frequency shift at $n = 3$ for the non-cross-linked film is used, the thickness becomes only $11.3$ nm, thus yielding a significantly lower thickness compared with the fundamental frequency. In contrast, there is essentially no frequency dependence in the thickness determination for the rigid film after cross-linking (Table 1) using eq 6.

This observed frequency dependence of the QCM response before cross-linking originates from the elastic and inelastic components of the shear-wave propagation through the film. Another factor that is important in the present context is the difference in how the bulk water outside the adsorbed film is sensed. If the adsorbed film is rigid, the bulk water exerts a mass load and a viscous (damping) effect as described in the literature (see, e.g., refs 1 and 3). For a dissipative film, the effects on $\Delta f_0$ and $\Delta D_0$ from the bulk water are different. Both the viscoelastic behavior of the adsorbed film and the effects from the bulk liquid are taken into account in the Voight-based model.\textsuperscript{30,44,45} Regarding the richness of information that can be extracted from $\Delta f_0$ and $\Delta D_0$ measurements using these models, we will now see that the apparent complications noted above are actually an advantage.

### Data Analysis Using the Voight-Based Model

To go beyond the Sauerbrey modeling, we use a Voight-based viscoelastic model.\textsuperscript{30,44,45} The protein film is represented with a homogeneous film on the sensor surface using four unknown parameters ($\rho_0, d_0, \mu, \eta$). The protein film is on its other side in contact with a semi-infinite bulk liquid ($\rho, \eta$), as illustrated in Figure 1. Four unknown parameters in the model means that the system is nonuniquely determined as long as we have only two measured quantities ($\Delta f$ and $\Delta D$ at a single harmonic). Under the assumption that the system (the adsorbed film in contact with the bulk liquid) exhibits no other frequency dependence (in the investigated frequency interval, 5–25 MHz) than that introduced via the complex shear modulus in the Voight model, it is possible to obtain a unique determination by making use of measurements at two or more harmonics. This is made use of in the following analysis.

The experimental data (filled and dashed lines, cf. Figure 2) and the best fit (open symbols) using the viscoelastic model are displayed as $\Delta f$ versus time and $\Delta D$ versus time in Figure 4a for $n = 3$ and $n = 5$. Considering the complex nature of these protein films, the agreement between the measured QCM-D data and the model must be regarded as very good. Panels b and c of Figure 4 show the temporal variations in the thickness (Figure 4b) and shear viscosity and shear modulus (Figure 4c) corresponding to the best-fit curves in Figure 4a. Also shown as insets in Figure 4b and c are the optical thickness, $d_o$, and refractive index, $n_o$, respectively, obtained from the ellipsometry data (cf. Figure 3).

At saturated coverage prior to cross-linking, the best fit was obtained for a film represented by an effective hydrodynamic thickness, $d_{\text{QCM}}$, of $22.4$ nm, $\mu = 6.6 \times 10^4$ N m$^{-2}$, $\eta = 1.8 \times 10^{-3}$
N-s-m⁻² using ρ₁ = 1.04 × 10³ kg-m⁻³. After saturated cross-linking, the thickness deduced from the modeling decreases to 7.3 nm with μ₁ = 3 × 10⁵ N·m⁻² and η₁ ~ 6 × 10⁻³ using ρ₁ = 1.18 × 10³ kg-m⁻³. The observed increase in shear modulus and shear viscosity, respectively, and the decrease in thickness upon cross-linking is physically realistic for a transformation from a hydrogel-like to a compact protein film. This picture is further supported by the observed increase (by ellipsometry) in refractive index from ~1.35 to 1.4 upon cross-linking (inset in Figure 4c and Table 1) and the decrease in optical thickness from 21 ± 2 to 5 ± 1 nm (inset in Figure 4b and Table 1), which show that the adsorbed proteins are transformed from an elongated and strongly hydrated state to a contracted and less hydrated state. The hydrodynamic (acoustic) thickness of 22.4 nm prior to cross-linking obtained from the QCM-D data by solving the Voight-based model is in significantly better agreement with the thickness determination from the ellipsometry data (21 ± 2 nm) than if the Sauerbrey value is used (15.2 nm at n = 1 and ~11 nm at n = 1 and 3). This shows that the effective hydrodynamic thickness of the dissipative layer (high ΔD/Δf value) of Mefp-1 is underestimated by a factor of ~1.5 at 5 MHz (and a factor of ~2 at 15 and 25 MHz) with the Sauerbrey approximation. This underestimation of the coupled mass of course also applies to the mass-uptake estimations done using eq 1 (see above), which means that the real amount of coupled water is actually somewhat higher (~94%) than previously estimated (~91%). The effect from this on the effective density and the thicknesses determined from eq 6 and the Voight model is, however, not significant.

After cross-linking, the hydrodynamic thickness of 7.3 nm obtained from the Voight model is essentially identical to that obtained from eq 6, as expected for a compact nondissipative film that exhibits a weak frequency dependence. However, the agreement between this thickness estimation and that obtained from ellipsometry is not perfect, which is attributed to an increase in acoustic contrast compared to optical (refractive index) contrast between the protein film and the bulk water upon cross-linking and to the larger scatter in the ellipsometry data.

**D₂O Substitution.** To further analyze the QCM response and the validity of the parameters determined using the model, H₂O was substituted for D₂O in a measurement otherwise identical to that shown in Figure 2. The obtained data (filled and dashed lines) and the best fit (open symbols) from the measurements conducted in D₂O are displayed as Δf versus time and ΔD versus time in Figure 5a for n = 3 and n = 5. D₂O substitution increases the density and shear viscosity of the bulk liquid and coupled water by ~10% and ~25% respectively. The measured QCM-D responses in the two cases are quite similar. The main difference is a slightly larger frequency shift at saturation prior to and after cross-linking in D₂O, as expected because of the higher density of the coupled liquid in this case. The very similar time evolution of Δf and ΔD in D₂O and H₂O (Figures 4a and 5a), indicates further, as expected, that the D₂O substitution does not significantly influence either the actual protein–surface interaction or the structure of the protein in the adsorbed state. In the modeling of the data obtained with D₂O, it is thus assumed that the thickness of the film formed in D₂O is identical with that obtained in H₂O.

The input parameters used to obtain the best fit in Figure 5a are shown in Figure 5b and c, where Figure 5b shows changes in thickness and shear viscosity versus time and Figure 5c shows the changes in the shear modulus versus time. Also shown for comparison are the results obtained in H₂O (cf. Figure 4c). In D₂O at saturated coverage prior to cross-linking, the best fit for a film locked at a thickness of 22.4 nm (from the H₂O data) was obtained for a film represented by μ₁ = 8.5 × 10⁴ N·m⁻², η₁ = 2.3 × 10⁻³ N-s-m⁻² using ρ₁ = 1.11 × 10³ kg-m⁻³. After saturated cross-linking, the corresponding numbers for a film with a thickness of 7.3 nm was obtained for a film represented by μ₁ ~ 3 × 10⁵ N·m⁻², η₁ = 7 × 10⁻³ using ρ₁ = 1.25 × 10³ kg-m⁻³. As seen, the obtained density of the water-rich film (~94% water) prior to cross-linking is ~8% higher in D₂O compared to H₂O, whereas the corresponding number is only 6% for the dehydrated film (~82% water) after cross-linking, which is in accord with the

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10% higher bulk density of D$_2$O compared to H$_2$O. This analysis thus further supports the above given interpretation that a significant amount of the coupled mass in QCM-D is water, both prior to and after cross-linking. This interpretation is also supported by the fact that both the effective shear modulus and the viscosity are higher (~30%) in D$_2$O compared to H$_2$O prior to cross-linking, as expected for a water-rich film in a more viscous (~25%) medium. The larger scatter in the estimations after cross-linking, which are due to the low absolute $\Delta f$ values, makes it hard to draw similar conclusions regarding the compact state. Note that the results obtained from the modeling of the QCM-D data demonstrate a larger difference between the parameters describing the protein film than is actually expected from the raw data (Figure 5a). The reason for this is that the QCM response is a complex nonlinear function (eqs 3 and 4) of both the film properties and the surrounding medium, which are both changed upon D$_2$O substitution.

CONCLUDING REMARKS
The picture emerging from, and being consistent with, all these data (summarized in Table 1) is the following: Mefp-1 adsorption occurs with a large fraction (~94%) of internally trapped water (hydrogel-like protein film). This causes a much larger mass uptake measured by QCM-D compared to that measured by ellipsometry and SPR, because QCM-D measures hydration water and trapped water. This influence of trapped water is consistent with a number of previous measurements on proteins, antibody–antigen systems, and lipid–vesicle adsorption.\cite{9-15,22-24} The cross-linking reaction causes a significant release of water and a resulting more compact and stiff film (thickness reduction by a factor of ~3), causing an increase in shear viscosity, shear elastic modulus, and refractive index (ellipsometry) due to decrease in hydration.

The results also illustrate the applicability and nonapplicability regimes for the Sauerbrey relation. One important message is that direct conversion of the frequency shift to mass uptake using the Sauerbrey relation for a viscoelastic layer in a bulk liquid leads to an underestimation of the adsorbed mass (or thickness), even for thin films (far from resonance or antiresonance) when there is considerable energy dissipation or a frequency dependence in the $\Delta f$ and $\Delta D$ response measured at multiple harmonics. All these effects are general and may be important to consider for other flexible macromolecules in the adsorbed state, such as, for example, nucleotides, which have recently attracted an increased interest in QCM-based biosensor applications.\cite{6,14,18,19} The apparent mass increase (15%) measured by SPR upon cross-linking (Table 1) is qualitatively consistent with a more compact (thinner) film but quantitatively somewhat larger than expected.\cite{60}

In the context of this work, it is also appropriate to make a comment on one aspect of our results that at first sight seems to contradict previous theoretical modeling of the QCM response for thin viscoelastic films.\cite{28} In the latter cases, it was shown that the thickness (or mass) of thin films inducing increased dissipation can in fact be overestimated by direct use of eq 1. This occurs if the elastic component of the film dominates over the viscous component, i.e., if $2\pi f(\eta_i/\mu_i) < -1$, whereas the mass is underestimated if the viscous component dominates. Physically this can be understood as follows: The dynamic mass, i.e., the mass actually sensed during periodic oscillation, can become either larger or smaller than the static mass, depending on the combination of thickness, elastic modulus, and viscosity.
case, $2\pi f (\eta/\mu)$ is close to 1, signaling that the Sauerbrey relation should hold essentially true. However, this general rule holds true only when the viscoelastic films are probed in air, while in the present case, they are probed in a liquid environment. The difference between these two situations is that, with a liquid bulk phase, the oscillatory motion of the crystal penetrates through the viscoelastic layer and then couples to the liquid phase, the mass load of which differ from the mass load from the bulk liquid prior to the adlayer formation. Using theoretical simulations of “artificial” viscoelastic films, we have found no situations for which the mass of a thin ($<100$ nm) viscoelastic film, making contact between the sensor surface and a bulk liquid, becomes overestimated by direct use of the Sauerbrey relation, although the mass of the same film would in several cases have been overestimated in air (in which the mass load from the bulk is negligible). Thus, there is no contradiction between our results and those in ref 28 when the different situations (air and liquid) are taken into account.

It should finally be pointed out that $\eta$ and $\mu$ are assumed to be frequency independent in the 15–25-MHz regime. This may, however, not be the case, since the estimated relaxation times of $\sim 30$ and $\sim 20$ ns prior to and after cross-linking, respectively, are close to the response time associated with shear oscillation at 15 and 25 M Hz. However, the predicted changes in $\Delta f$ and $\Delta D$ at $n = 1$ (5 M Hz) from the estimations of the film parameters at 15 and 25 M Hz agree within 5% with those measured (not shown), indicating that such contributions are small in the present case. In addition to a possible frequency dependence of the shear viscosity or shear elastic modulus, the nonperfect agreement between the experimental data and the model may have contributions from the nonperfect smoothness of the film(s) and from thickness and lateral variations in the parameters describing the film. In future work, we intend to carefully analyze how the introduction of even further harmonics to the analysis of these more homogeneous types of adsorbed films contribute to a more detailed description of the physical state of the adsorbed films.

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