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Probing fundamental film parameters of immobilized enzymes—Towards enhanced biosensor performance. Part I—QCM-D mass and rheological measurements

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ABSTRACT

Enzyme immobilization is an ever-growing research-area for both analytical and industrial applications. Of critical importance in this area are the effects of immobilization procedures upon the functionality of the immobilized biomolecules. Both beneficial and detrimental effects can be conferred through the selection and tuning of the immobilization procedure. Quartz-crystal microbalance with dissipation (QCM-D) has been previously used to great effect in tracking alterations to thin films of biomolecules immobilized onto quartz transducers.

In this study, we investigate the ability of QCM-D to track and monitor film parameters of a monolayer of laccase immobilized on a series of self-assembled monolayers (SAMs), differing in lateral density of binding residues on the SAM and height of the SAM from the quartz surface. Both mass gains and rheological parameters for these varying surfaces were measured and trends later compared to the apparent enzyme kinetics of the immobilized laccase films, assessed electroanalytically (Paper II in this two part study). For covalent attachment of proteins, both shear and viscosity were increased relative to physically adsorbed proteins. An increase in lateral density of protein-binding surface of the SAM components was shown to increase the shear/viscosity of the resultant film while an increase in distance from the electrode (through incorporation of lysine linkers) was shown to decrease the shear/viscosity while simultaneously increasing the wet mass gain of the films. Shear and viscosity may be indicative of both enzyme denaturation and increased lateral protein packing within the film structure hence it is assumed that less distortion occurs with the inclusion of linkers which allow for more optimal protein immobilization.

1. Introduction

The majority of protein immobilization techniques have two often-conflicting factors to balance, namely finding a means to attach proteins in as firm a manner as desired for the application, while ensuring that the active conformation/s of that protein are retained in the finished product. While many immobilization techniques have been investigated in biosensor-application research, much of this research is not addressing a key factor in this—an understanding of the interplaying factors that affect the final conformations present in the attached biomolecule, and, further, their effects on the functioning of the attached biomolecule. A greater understanding of the biomolecules participating in biosensing applications is, hence, of high desirability [1]. Furthermore, a deeper appreciation of the changes undergone by these biomolecules during their immobilization, and the subsequent effects that these alterations have on the functionality of that biomolecule is of utmost importance in the design and construction of immobilized-biomolecule structures, regardless of their intended use. Due to the alteration in protein conformation upon binding and catalysis of a substrate, an overly reticulated protein molecule for example, may undergo additional decrease in efficiency based on the lack of protein mobility.

A number of papers have examined the immobilized film-structures of biomolecules using quartz crystal microbalance (QCM), and QCM-D technology (QCM with dissipation) through measurement of frequency changes (Δf) and in the case of QCM-D, the dissipation of molecules immobilized on quartz crystal surfaces [2–4]. These provide information on amount of mass bound, film thickness, viscoelasticity and assessment of conformational changes which can occur upon biomolecule immobilization. However, no such studies have attempted to relate these physical parameters identified to the performance of an amperometric

Abbreviations: QCM-D, quartz-crystal microbalance with dissipation; SAM, self-assembled monolayer; SLB, succinic-lactic acid buffer pH 4.5; V.I., viscoelasticity index; *f*, frequency; *D*, dissipation; SPR, surface plasmon resonance; AFM, atomic force microscopy.

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Table 1
Compositions of the various surfaces investigated.

SAM number	Cysteamine content (%)	Cysteamine modification[a]
1	1	None
1.1	1	glut
1.2	1	glut–lys–glut
1.3	1	glut–lys–glut–lys–glut
2.1	25	glut
2.2	25	glut–lys–glut
2.3	25	glut–lys–glut–lys–glut
Au	25	None

[a] Layer-by-layer, starting at the basal SAM. "glut" designates glutaraldehyde and "lys" indicates the addition of a lysine residue.

sensor at similarly formed films, nor to the immobilized-enzyme kinetics thereof determined via electroanalytical techniques.

The aim of this work was twofold: to examine (i) whether QCM-D could be used to monitor fundamental film parameters that may impact on the operational properties of an amperometric sensor manufactured in the same manner and (ii) whether the protein-film characteristics explored through the use of QCM-D as a fundamental technique show any correlation with the kinetic parameters and functioning of the laccase when used as a biorecognition element in a biosensor for the electrochemical detection of hydroquinone. This study does not aim to construct a desirable biosensor. With reference to this paper, the aim is rather to determine if QCM-D can be of use in the fabrication of biorecognition layers by examining the fundamental physical constraints involved in the immobilization of biorecognition elements to transducers. While QCM [5,6] and QCM-D [7] has been used as a gravimetric measurement of immobilized laccases at various surfaces, similar studies have not been reported in the literature for QCM-D analyses.

To this end Paper I in this two-part paper examines the physical film characteristics observed following the immobilization of laccase onto gold quartz crystal surfaces, either through physical adsorption or through covalent attachment to modified mixed thiol monolayers in different configurations, as in Table 1. Herein we examine the effect of differing proportions of active vs. non-active components of self assembled monolayers for protein attachment and the effect of the inclusion of lysine linkers on the rheological properties (rigidity, shear and viscosity) of the modified surfaces as determined by QCM-D. Paper II in this two part series examines whether the information on the protein film characteristics obtained can be related to the observed kinetic functioning of the enzyme when attached to gold electrodes for amperometric detection of hydroquinone.

2. Methods and materials

2.1. Apparatus

Crystals used were AT-cut quartz crystals, QSX-301, surfaced with gold. These were mounted in titanium QCM-D flow chambers, housed in a Q-Sense E4 QCM-D sensor system (Q-Sense®, Sweden).

Flow of 50 μl min^{-1} was regulated by an Ismatec® peristaltic pump with the chamber temperature set at 20 °C 10 min prior to the start of QCM-D analysis.

2.2. Reagents

All reagents used were of analytical purity, (Sigma–Aldrich), and used without further purification. Stated purities of all reagents used in this research follow: 99% succinic acid, >99% cysteamine chloride, 98% β-mercaptoethanol, 99.9% sodium dodecyl sulphate (SDS) and 99+% L-lysine. Lactic acid, 85% (w/w), and 25% (w/v) glutaraldehyde (Grade 1, high purity) were obtained in solution form at the stated concentrations and laccase purified from a culture of *Trametes versicolor*, was obtained as a lyophilized powder.

QCM-D analysis and protein modification of the electrode surface took place using 0.025 M succinic and 0.025 M lactic acid buffer (SLB), adjusted with NaOH to a pH of 4.5. All non-proteinaceous solutions were degassed by ultrasonication to remove bubbles for at least 10 min prior to QCM-D analysis.

Laccase solutions were prepared in SLB to a final concentration of 3.67 mg ml^{-1}. These were prepared >1 h before use and stored at 4 °C. Laccase solutions were allowed to stabilize at room temperature for 15 min prior to QCM-D analysis.

2.3. Methodology

2.3.1. Self-assembled monolayer (SAM) formation

100 mM stock solutions of cysteamine and β-mercaptoethanol were prepared in de-aerated water and stored at 4 °C in the dark until used (within 36 h of preparation).

Gold-surfaced crystals were cleaned as outlined previously [8]. Mixed solutions of cysteamine and β-mercaptoethanol were prepared in de-aerated 20% (v/v) ethanol, 80% (v/v) water solution to a final concentration of 0.1 mM and the gold surfaces exposed to this solution for at least 8 h at 4 °C in the dark. Two different deposition solutions of different molar fractions of cysteamine and β-mercaptoethanol were prepared: a solution of 1:99 cysteamine:β-mercaptoethanol (henceforth referred to as "SAM 1") and 1:4 cysteamine:β-mercaptoethanol ("SAM 2") were used to modify the gold surface.

2.3.2. Self-assembled monolayer modification to produce a multi-layered SAM

1 mM solutions of glutaraldehyde or L-lysine were prepared in either: milliQ water (for ex vitro crystal modification) or 0.025 M SLB (for QCM-D analysis). Surfaces were exposed to alternating solutions of the two for 5–10 min for each solution, followed by an ethanol/water rinse (ex situ) or a 0.025 M SLB rinse for 10 min (in situ). Table 1 indicates the various completed surfaces prior to the addition of laccase where, for example, surface SAM 2.3 would be exposed to sequential alternations of glut–lys–glut–lys–glut. (See Supplementary material).

2.3.3. Attachment of laccase molecules: QCM-D analysis and biosensor fabrication

Prior to exposure to the laccase solution, SAM-covered crystals loaded into the QCM-D chamber were exposed to 1 mM glutaraldehyde for 5 min and then rinsed with SLB for 5–10 min in order to activate them in a similar manner as described elsewhere [9]. Laccase solution was flowed over the QCM-D crystals until such a time as a stable mass response was recorded (typically 10–15 min), followed by a 10-min rinse with SLB, and a short exposure (2 min) of the crystal to 0.3% (w/v) SDS solution and a final extensive wash step using SLB. Flow rate was maintained at 50 μl min^{-1}.

2.3.4. Raw data treatment of QCM-D response curves

The principles of QCM-D are outlined in the literature [2,10]. Upon attachment/detachment of bound mass to the surface of an oscillating piezoelectric material, the shift in resonant frequency, Δf, is related to the change in mass through the Sauerbrey equation [11], Eq. (2). This relation is only valid for thin, rigid films firmly attached onto the piezoelectric surface that couple with the surface's oscillation [2,3]. Viscoelastic films (such as protein) or those with coupled water generally result in a dampening of the frequency to occur, and an overestimation of the mass of the attached layer [2,3] when solely interpreting mass gain using the Sauerbrey formalism. To compensate for this, data regarding the viscoelasticity and strength of the attached layer can then be obtained from measurements of the so-called dissipation factor, D (dimensionless units, $\times 10^{-6}$)—measured by the speed at which the oscillating material returns to rest in the absence of applying a potential difference to the system. D is essentially the ratio of energy dissipated from the surface of the crystal vs. that stored during one period of oscillation. During operation of the QCM-D, measurements of Δf and ΔD are taken sequentially at multiple overtones i.e. integer multiples of the resonant frequency. This analytical method can provide further information of thickness, shear and viscosity of the layer [12].

For QCM-D analysis, the same solvent (0.025 M SLB, pH 4.5) was used throughout to minimize the perturbation of the QCM-D response by a large alteration of the solvent composition. For similar reasons, low concentrations of glutaraldehyde and L-lysine were used, additionally to decrease non-specific binding at the electrode surface and the reversible polymerization of the glutaraldehyde [9]. Frequency and dissipation data were normalised to possessing values of 0 after glutaraldehyde activation of the SAM surface prior to the attachment of the protein film, when surfaces requiring glutaraldehyde activation was used. This was performed in order to account for very slight drifts in the Δf and ΔD values following glutaraldehyde exposure.

Two major parameters of the protein binding were used to determine the efficacy thereof. The first is the viscoelasticity index (V.I.). The viscoelastic index of the protein determined in these studies is the inverse of the conventional $\Delta D/(\Delta f/n)$ relation [13] and calculated as in Eq. (1) in order to display increases in rigidity more intuitively).

$$\text{V.I.} = \frac{\Delta f_{\text{tot}}}{\Delta D_{\text{tot}}} \quad (1)$$

where Δf_{tot} is the total change in frequency, normalised after the stable frequency of response of the glutaraldehyde activation (Hz), with $\Delta f_{\text{tot}} = f - f_{\text{o}}$ being the difference between the recorded frequency value (f) assessed after reaching a stable frequency and the initial frequency recorded at a stable baseline prior to sample adsorption (f_{o}). Similarly, ΔD_{tot} is the total change in dissipation for the same ($\times 10^{-6}$ dimensionless units).

Secondly, from Δf_{tot}, the Sauerbrey equation was used to determine the mass gain on the crystal surface. The Sauerbrey equation was only held to be valid if the V.I. was $\geq 10 \times 10^6$ Hz. The Sauerbrey equation [11] is solved as follows:

$$\Delta m = \frac{(\Delta f) \times C}{n} \quad (2)$$

where Δm is the mass gain at the electrode surface (in ng cm^{-2}), C is the mass sensitivity constant (17.7 ng Hz^{-1} cm^{-2}), and n is the overtone number used in the determination (n = 1, 3, 5...13).

The V.I. and the mass gain were determined at the three major stages of the protein attachment: (1) at the height of protein attachment, (2) at the steady-state of the subsequent rinse of loosely adsorbed proteins and (3) following a short SDS rinse in order to remove all protein that was not covalently bonded to the electrode surface. Experiments were performed in at least triplicate, and the mean values with standard deviation presented henceforth.

2.3.5. Rheological monitoring

Film thickness, elastic shear modulus and viscosity determinations of the attached protein films were performed using Q-Tools viscoelastic determination functions using the raw Δf and ΔD values drawn from a minimum of 3 overtones and a maximum of 5. The Voigt-based model of coupled motion for viscoelastic films attached to a quartz piezoelectric sensor developed by Voinova et al. [14] was used for the determination of these parameters during the laccase attachment/detachment stages. The film density, fluid viscosity and fluid density were assumed to be 1000 kg m^{-3}, 0.001 kg ms and 1100 kg m^{-3}, respectively for all protein films examined. An abbreviated summary of the modeling system and relevant calculations used in these determinations is available [13], as is a more detailed investigation into the mathematics and assumptions underlying this system [15].

The Voigt mass gains of the film at different states of rinsing were determined from the film thickness, and effective film density. In order to maintain parity between replicants, laccase films adsorbed to surfaces (Au and SAM 1) were modeled as possessing low shear (>10^5 N m^{-3}) in order to satisfactorily fit the experimental data of all the replicants to the model data. All other surfaces were modeled under the assumption of a high-shear (<10^5 N m^{-3}), in order to provide a satisfactory fit to the data and a consistent grouping of rheological parameters between replicants.

3. Results and discussion

3.1. Self-assembled monolayer formation and modification

Table 1 outlines the various surfaces and surface modifications examined for the immobilization of laccase onto a self-assembled monolayer consisting of (a) different ratios of cysteamine and β-mercaptoethanol and (b) differing lengths of linkers (or spacers) for attachment of the laccase enzyme. β-mercaptoethanol was selected as a non-active SAM filler [16–18] (i.e. in order to tailor the space between cysteamine SAM components). A combination of L-lysine and glutaraldehyde were selected as the linkers between proteins and the electrode SAM.

3.2. Characterisation of typical protein film attachment and protein wash-off

Fig. 1 shows a typical QCM-D response for the attachment of laccase to a sensor surface, in this instance, SAM 2.2. Δf and ΔD values are normalised relative to the glutaraldehyde activation of the SAM, with the phase shifts indicated by labeled arrows.

Typically, the attachment process of laccase consisted of the following phases in all surfaces examined: a rapid increase of bound mass to the sensor, as evidenced by a decrease in frequency, Δf, (concomitant with a rise in dissipation, D, which serves to indicate an increase in film viscoelasticity), followed by slow stabilization of that response. Wash-off of the protein occurred in two similar stages, with final phase being that of a very slow and steady loss of mass from the sensor surface. The inclusion of a dilute SDS wash was used to induce a more rapid removal of unbound protein from the electrode surface [19] and the post-SDS phase exhibits a more rapidly stabilized removal of mass from the electrode surface. The differences between the Δf vs. time and ΔD vs. time gradients of the buffer rinse stages before and after exposure to SDS confirm this.

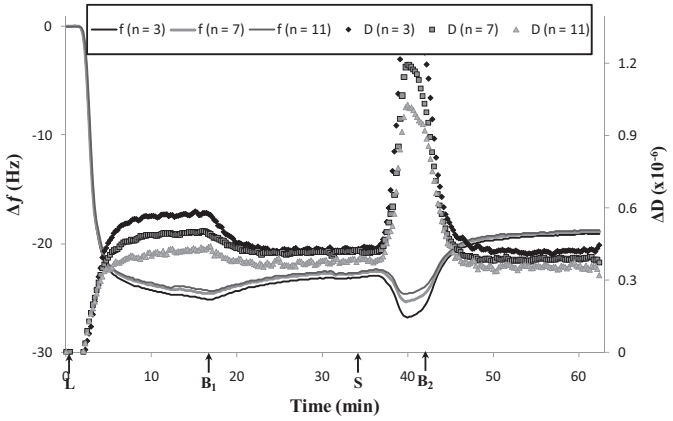

Fig. 1. ($\Delta f/n$) vs. time and ΔD vs. time plots (overtone number, n = 3, 7, 11) of the attachment of laccase to the surface of SAM 2.2. Values are normalised relative to the QCM-D response following glutaraldehyde activation of the SAM and rinse-off of unbound aldehyde. Arrows indicate alteration of the solvent flowing over the crystal. Circles represent the dissipation response and triangles depict the frequency shift. Legend: L = 80 U ml^{-1} laccase solution, B_1 and B_2 = buffer wash, S = 0.3% SDS solution.

3.3. Mass and V.I. variations between laccase-adsorption phases and binding surfaces investigated

Due to the better resolution of the phases, Δf and ΔD values drawn from overtone n = 9 were used for calculating V.I. and mass gain as displayed in Fig. 2(A) and (B).

In Fig. 2, mass gain displayed was calculated using the Sauerbrey equation for all treatments, except for analyses following the SDS

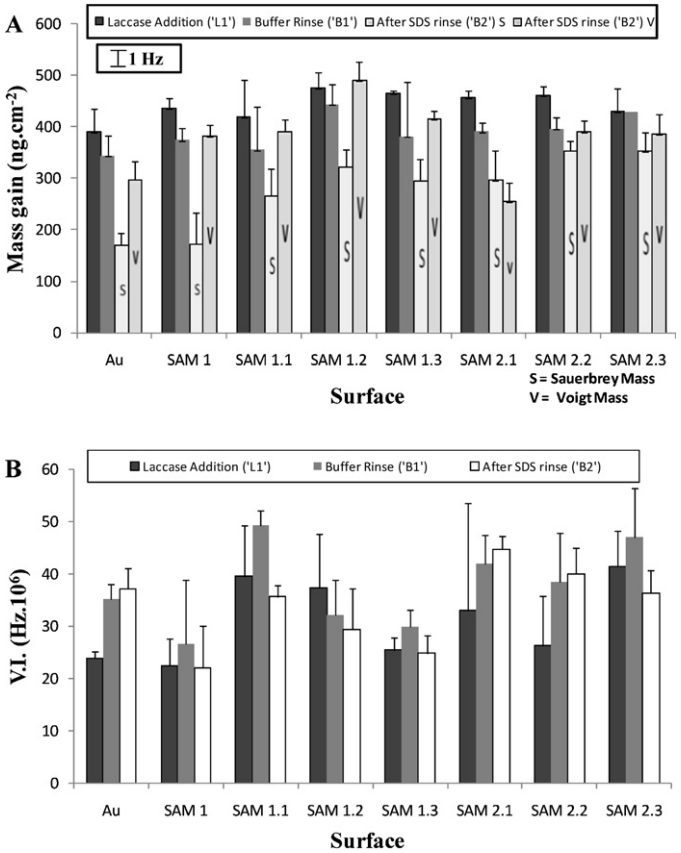

Fig. 2. (A) Protein film mass gain for the configurations of protein attachment. Values are drawn from stable areas of the laccase adsorption-desorption phases indicated in Fig. 1. (B) Viscoelastic Indices of same protein films. Uncertainties represent standard deviation from the mean of ≥ 3 independent measurements. Inset box represents a standard deviation equivalent to ±1 Hz of frequency shift.

rinse step where these are reported using both the Sauerbrey equation (after SDS rinse, S) and Voigt viscoelastic modeling (after SDS rinse, V). Of the four categories displayed in Fig. 2, only the last three ("Buffer rinse" and "After SDS-rinse, S and V") should be considered important for elucidating the efficacy of any electrochemical biosensor fabricated in the same manner as was modeled on the QCM-D. It must be considered that the initial measurement (that of the protein film after a buffer rinse) is indicative of an adsorbed protein multilayer, as opposed to the washed enzyme monolayer that was used for electrochemical determination. SDS was selected as a denaturation agent in order to remove loosely adsorbed proteins, in order to quantify the mass of proteins that were covalently attached, or otherwise strongly bound, to the electrode surface [19].

Considering here only the Sauerbrey determined mass gains in this section, comparable amounts of protein was retained after the SDS wash on both basal films and between controls. Taking into account uncertainties, average bound masses of ~280 ng cm^{-2} were recorded for both SAM 1.1 and SAM 2.1 and ~173 ng cm^{-2} for Au and SAM 1). This is indicative of a bound monolayer (or sub-monolayer in the cases of controls) with a greater cohesiveness than a multilayer would possess. The use of dilute SDS would ensure that only loosely bound proteins would be removed from the transducer surface, leaving behind the sub- to full-monolayer of bound protein.

The two controls used for this study, namely, laccase adsorbed onto a plain gold surface (designated Au in Table 1) and onto 1:99 cysteamine: β-mercaptoethanol surface (designated SAM 1) show that the addition of dilute SDS removes the majority of the protein film formed by the adsorption of laccase. Laccase adsorbed onto gold displayed a greater averaged V.I. (film rigidity) than SAM 1, probably due to the adoption of a non-optimal surface-bound conformation of the bottom-most layer on contact with the gold electrode. Since SAM 1 possesses both hydrophilic and basic SAM head-groups, this increased hydrophilicity may aid the proteins in retaining a solvated conformation when interacting with this surface. The low V.I. value of SAM 1 (Fig. 2(B)) and larger proportional mass loss after rinsing (relative to other surfaces) are indicative of a less rigidifying interaction between protein and surface. The low average V.I. value, coupled with the large degree of uncertainty present in the determination of V.I. and bound mass itself may indicate the presence of a sub-monolayer of protein of variable coverage retained after SDS exposure, possessing a substantial mass contribution from coupled water trapped in-between adsorbed protein molecules on the surface contributing to the dissipation of the film.

A comparison between the two basal SAM (SAM 1.1 and SAM 2.1) layers shows immediate differences in their rheological properties. Consistently, protein films attached onto SAM 2-based surfaces showed greater rigidity than their SAM 1 counterparts. An increased surface concentration of cysteamine provides a greater number of attachment points between the enzyme and the electrode. Increased reticulation of the enzyme will have the effect of increasing the rigidity of the film, by decreasing the possible conformations and orientations that the enzyme can adopt following attachment. This increase in rigidity with increase in protein concentration conforms with previous studies [7]. This also helps to explain why a consistently higher level of protein binding was also observed for SAM 2 surfaces when compared to their SAM 1 counterparts. The initial surfaces themselves bound a comparable amount (~266 ng cm^{-2} in SAM 1.1 compared to 296 ng cm^{-2} for SAM 2.1 Fig. 2(A)), but a much greater average V.I. value was recorded for SAM 2.1 (44.1×10^6 Hz Fig. 2(B) compared to SAM 1.1 (35.6×10^6 Hz). Film rigidity has previously been used as a parameter to investigate inter-protein cross-linking of adsorbed films onto QCM-D crystals [13] and it is logical to assume that a greater degree of surface–protein bonds would increase the degree of rigidity.

An increase in the amount of bound mass (following SDS desorption) was found to be the general trend with the addition of lysine linkers connecting the surface to the protein (Fig. 2(A)). Similar behaviour has been previously observed with the inclusion of ionic spacers between the surface and laccase using QCM, with surface plasmon resonance indicating a significant increase in bound mass [5]. With a greater degree of linker length and mobility [20] the number of protein-binding attachment points was increased, resulting in greater mass binding, as supported by other research [21]. Additionally, the presence of carboxyl and amine groups present on lysine, and the carbonyl groups present on glutaraldehyde are expected to increase the hydrophilicity of the attachment surface, decreasing denaturation of the enzyme when interacting with this linker [20].

The addition of lysine linkers to the SAMs was proposed to decrease rigidity (Fig. 2(B)) in the two methods: firstly, as stated above, by allowing for enzymes to be linked in a less denaturing conformation through the increased linker mobility, and secondly by increasing the space between the attached protein and the electrode surface. Since proteins have been shown to alter their conformation from a solvated to a bound conformation when contacting a solid surface [22] an increase in bond length between the bound protein film and the electrode would enable a better retention of the solvated conformation [21]. A larger degree of protein solvation would increase the amount of coupled water occurring within the film, effectively increasing the amount of bound mass of the protein film and increasing the dissipative effect of the film. Thus, an increase in spacer length was expected to simultaneously lower the V.I. and increase the bound mass gain, respectively.

The possibility of cross-linking occurring between linker molecules must also be considered. As linker mobility increases with linker length, there occurs an increasing probability of linkers interacting with one-another, decreasing available covalent linkages to protein. This is a potential explanation behind the lowered final mass gain that appeared between SAM 1.2 and SAM 1.3 (323 ng cm^{-2} and 296 ng cm^{-2}, respectively, while possessing similar standard deviations of ±30 ng cm^{-2}). A similar effect between SAM 2.3 and SAM 2.2 does not occur, possibly since the inter-cysteamine space was greatly decreased in SAM 2, resulting in less overall linker mobility. As stated above, the inclusion of coupled water would produce a large wet-weight of the protein film, but very little actual bound protein contributing to the mass of this film [14] while the low rigidity (and larger height) of this film may decrease the amount of apparent mass monitored by the Sauerbrey formalism [4] accounting for discrepancies in mass between SAM 1.2 and SAM 1.3.

By itself, the V.I. is a fairly data-poor indicator of the effects undergone by the layer. The reliance on a single harmonic overtone to investigate the film properties produced a higher degree of uncertainty in the determination of bound mass by the Sauerbrey formalism, compared to the mass calculated from the Voigt-modeled film height (Fig. 2(A)) and relatively high uncertainties in the determination of V.I. values, making comparisons between closely related sub-protein films (e.g. SAM 1.1 and 1.2) problematic. Frequency and dissipation measurements are useful, but not considered reliable indicators of film properties and several important assumptions are ignored when merely using these two results as an indicator of film properties [15]. The following section highlights this phenomenon.

3.4. Rheological parameters of the attached film

Table 2 displays the viscoelastic parameters elucidated from the pseudo-stable wash-off phase of the laccase attachment utilizing

Table 2
Shear modulus, film viscosity and film thickness of the surfactant-washed film determined using Voigt modeling using data drawn from the QCM-D measurements.

Surface[a]		Mass gain[b] (ng cm^{-2})	Film thickness (nm)	Shear modulus (10^5 N m^{-3})	Viscosity (10^{-3} N s m^{-3})
Gold	- Prior	569 ± 58	5.17	0.42 ± 0.23	2.9 ± 0.7
	- Post	298 ± 35	2.71	0.26 ± 0.09	2.6 ± 0.1
SAM 1 (phys)	- Prior	611 ± 34	5.56	0.33 ± 0.06	2.7 ± 0.3
	- Post	382 ± 20	3.48	0.16 ± 0.05	2.5 ± 0.1
SAM 1.1	- Prior	571 ± 88	5.19	7.31 ± 1.78	8.3 ± 1.2
	- Post	392 ± 22	3.56	6.69 ± 0.76	6.6 ± 0.8
SAM 1.2	- Prior	545 ± 35	4.96	3.44 ± 1.35	4.7 ± 0.5
	- Post	490 ± 35	4.46	5.21 ± 0.84	3.3 ± 0.4
SAM 1.3	- Prior	691 ± 76	6.29	0.30 ± 0.06	2.3 ± 0.4
	- Post	415 ± 14	3.78	1.42 ± 0.29	3.1 ± 0.2
SAM 2.1	- Prior	413 ± 59	3.76	10.40 ± 3.80	7.6 ± 2.2
	- Post	255 ± 37	2.32	11.50 ± 3.29	8.33 ± 1.30
SAM 2.2	- Prior	458 ± 48	4.17	11.25 ± 1.92	7.25 ± 1.49
	- Post	391 ± 31	3.56	12.60 ± 0.09	7.20 ± 1.48
SAM 2.3	- Prior	438 ± 22	3.99	5.19 ± 2.90	6.43 ± 2.09
	- Post	386 ± 39	3.51	9.50 ± 2.50	6.56 ± 0.46

[a] "Prior" refers to values drawn from the end of the wash-off stage prior to treatment of the film with SDS ("B_1" phase in Fig. 1 of this paper) and "post" to values drawn from a stable response during buffer rinse after SDS exposure ("B_2").
[b] As calculated by multiplying the film thickness by the modeled film density (1100 kg m^{-3}).

Voigt modeled mass gains. The modeling system used was unable to accurately determine the parameters of the post-SDS wash phase for some of the films presented here without a high degree of uncertainty, hence the inclusion of the pre-SDS, buffer-rinsed protein layer. This also presents valuable information regarding the alteration of the film after exposure to SDS (and thus, a sub- to full monolayer of protein, as opposed to the semi-multilayer of protein that a simple buffer rinse leaves on the crystal).

QCM-D has been previously coupled with rheological-modeling software to describe both the mechanical properties of viscoelastic proteinaceous thin-films and alterations undergone by said films [12–14,23–26]. A good correlation with validative techniques, such as AFM and SPR has been noted once corrections have been made to accommodate the effects of bound and/or coupled water [25,27].

Mass gains determined by Voigt modeling were consistently higher than those estimated by the use of the Sauerbrey equation [3] as shown in Fig. 2(A). The Voigt modeling takes into account viscous energy loss arising from a non-rigid film [3] where the dissipative effect of a viscoelastic film (such as a protein layer) causes an underestimation of the mass/film thickness in the Sauerbrey relation. Additionally, the Sauerbrey equation does not take into account film swelling through solvent intercalation [4]. Mass and film thickness are interrelated as described in the footnote of Table 2. This value is assumed to be relatively independent of the estimated film density as determined by Larsson et al. [28]. Since the film density was used as a constant between the surfaces examined, it is expected that the greatest areas of alteration (i.e. film viscosity and elastic shear modulus) be substantially different between surfaces, and thus may not be truly representative of these actual values, but still remain useful as a means of comparison.

In Table 2 the film thicknesses themselves seem very low relative to expected values from a packed protein surface, however, flattening of protein has been noted previously in QCM research [29] and can be attributed to either flattening under sub-monolayer coverage or to conformational changes extant between crystalline protein and immobile protein. Teichroeb et al. [30] provide evidence to suggest that a monolayer of BSA protein adsorbed onto support deforms dimensions indicated in the crystallographic data.

The two controls used highlight the detail of the information that may be gained from the use of the modeling system used herein (Table 2). Even though comparable mass gains/film thicknesses were observed in both phases (569 and 611 ng cm^{-2} prior to the SDS rinse and 298 and 382 ng cm^{-2} after the SDS rinse for laccase physically adsorbed onto the gold surface (Au and SAM 1, respectively) between the controls, higher viscosity and shear modulus values were observed for the gold surface than the SAM-coated gold surface. This indicates either a greater degree of proteinaceous packing, and/or an increased denaturation of the protein at the surface–solvent interface, due to the decreased hydrophilicity of the unmodified gold surface [22]. A greater degree of distortion/denaturation occurring at the laccase molecule as it interacts with a surface should decrease the amount of surface-facing hydrophilic/ionizable residues, but allowing predominantly protein–protein interactions through hydrophobic interactions, but decreasing protein–solvent interactions [31]. Logically, as the amount of coupled or bound water in the protein film decreases,

Table 3
Rheological parameters calculated relative to the final mass of the protein films following an SDS rinse.

Surface	Shear modulus (10^3 N cm^2 ng^{-1} m^{-3})	Viscosity (10^{-6} N s cm^2 ng^{-1} m^{-3})	V.I.$_{n=9}$
Gold	0.08 ± 0.01	8.9 ± 1.1	37.2 ± 3.9
SAM 1 (phys)	0.04 ± 0.01	8.2 ± 0.5	22.0 ± 8.3
SAM 1.1	2.07 ± 0.23	16.8 ± 2.3	35.6 ± 2.1
SAM 1.2	0.92 ± 0.18	6.8 ± 0.9	32.2 ± 6.6
SAM 1.3	0.34 ± 0.07	7.5 ± 0.6	29.9 ± 3.3
SAM 2.1	4.51 ± 1.67	32.7 ± 6.9	44.1 ± 2.3
SAM 2.2	3.21 ± 0.18	18.4 ± 3.9	40.1 ± 4. 9
SAM 2.3	2.46 ± 0.70	17.0 ± 2.1	36.3 ± 4.4

the viscosity and elastic shear modulus of the film would increase as the film increases its cohesiveness, a similar phenomenon to that noted with the binding of streptavidin to a biotin layer [27]. Therefore, a viscosity or shear increase could indicate that either (1) a greater degree of protein packing (as a function of mass) relative to the bound water in the film, (2) an increased strength of attachment between surface and protein or (3) an increased average level of denaturation/conformational alteration undergone by the attached protein or a fusion of the aforementioned factors [27].

The activation of SAM 1 with glutaraldehyde greatly increased both the viscosity and shear of the protein film (Table 2), even though a mere 10 ng cm^{-2} difference in modeled mass was observed. The viscosity increased from an anticipated 2.5–6.6 $\times$ 10^{-3} N s m^{-3} and the shear from 0.16 to 6.69 $\times$ 10^{5} N m^{-3} for physically adsorbed laccase and covalently bound laccase after the SDS rinse. This change was expected and attributed to the covalent linkages formed between the protein film and the sensor surface, causing increased protein film rigidity. The increase of protein-binding cysteamine residues between SAM 1 and SAM 2 causes a similar increase in viscosity and shear modulus. This phenomenon is further highlighted in Table 3, where the rheological parameters are normalised to the bound mass of the protein film.

As shown in Table 2, the covalent addition of L-lysine to the SAM components as a linker caused an increase in the mass/film thickness among surfaces studied compared to their basal SAMs i.e. SAMs 1.1 and 2.1. Generally, the shear and viscosity of the final protein film initially decreases concomitantly with an increase in the number of L-lysine between the SAM and the protein. This function may also be attributable to the further inclusion of water at the protein film (especially when monitoring differences in film thickness and mass). However, the statistical significance of the trends of these decreases is questionable.

Similar uncertainties in the rheological properties have been noted for QCM-D viscoelastic modeling [32] due to the analytical problems associated with viscoelastic modeling in the absence of external information regarding film thickness [15], while the determination of film thickness itself is less affected. Hence, in Table 3, shear and viscosity of the protein films (determined at the post-SDS rinse phase) are divided by the film mass in order to further explore differences extant between the surfaces.

Table 3 satisfactorily shows that these parameters tend to decrease when related to the average film mass following SDS desorption. This indicates that the use of L-lysine to separate the attached protein from the surface tends to decrease the shear and viscosity of the protein film (i.e. increase the degree of solvation of the attached protein molecules) *per unit mass added*. This was the anticipated effect of the addition of the linkers to the surfaces examined. Hence, this indicates that, not only does the amount of proteinaceous mass increase with increasing length of linkers, but that the addition of linkers tends to decrease the extent of denaturation exhibited by the attached protein films. In other studies, retention of enzyme activity has been reported for laccase bound through zirconium phopshonate functional groups [5].

It is important to note at this juncture that a higher protein loading does not necessarily entail a better biorecognition element for a protein-based biosensor. When considering the effects of a packed-protein layer, certain issues such as the decreased protein flexibility (due to steric hindrance caused by the presence of other bound biomolecules and/or electrostatic repulsions caused by same), the covalent method of attachment and the resultant non-optimal catalytic conformations that the proteins adopt for the aforementioned reasons could impede the function of the biosensor [33]. For the same reasons, a decreased accessibility of the substrate/s to the enzymes (internal diffusional resistance) and (by inference) decreased accessibility of the products to the electrode surface and the slower diffusional rates that these cause may all influence the efficacy of a protein film when considering its application to a biosensor. This shows that one cannot rely on a single parameter (such as mass gain) in order to determine which the best protein immobilization method is. In this case, viscosity and shear data derived from QCM-D data may be invaluable indicators of, not only (1) the level of distortion and potential denaturation arising from both the immobilization method and subsequent protein–surface interactions but also of (2) film properties arising from the lateral interactions of the biomolecules with one another.

While this represents the first such study and is worthy of further investigation given the potential of such tools in biosensor design, a limitation which should be borne in mind is the inability to accurately determine both film-thickness and rheological data simultaneously. In this regard, QCM-D shares this problem with other methods of establishing film thickness in liquid mediums such as surface plasmon resonance spectroscopy and ellipsometry [15]. Independent measurement of the film thickness (such as liquid-phase AFM) would substantially decrease uncertainty of the produced rheological data.

4. Conclusions

QCM-D technology, coupled with rheological modeling of the results, showed consistent alterations in the physical properties of attached laccase films occurring between the surfaces examined in this study. Film rigidity (V.I.), bound mass, viscosity and shear modulus values increased when protein was attached to the surface via glutaraldehyde–cysteamine covalent bonds (SAM 1.1), compared to laccase attached by physical adsorption (SAM 1 and Au).

Rigidity, viscosity and shear modulus values further increased when the density of cysteamine in the SAM surface was increased (SAM 2.1). Differences between SAM 2.1 and SAM 1.1 surfaces show that the presence of further reactive binding sites on the SAM surface as for SAM 2 do not, by themselves, increase the amount of mass gained at the electrode, but greatly influence the rigidity of the attached protein film (due to the number of increased bonds occurring between the attaching proteins and the electrode surface). The addition of lysine linkers between the piezoelectric sensor and the protein film was shown to decrease the rigidity of the attached protein film and increase the mass gain at the electrode surface above that found at electrodes without lysine. This is more evident in the rheological parameters once they are related to the mass gain at the electrode surface, which show a consistent decrease in the modeled viscosity and shear moduli with increasing lysine-glutaraldehyde linkers for SAMs containing low surface density of cysteamine (SAM 1.1–1.3) and for those containing higher surface densities of cysteamine (SAM 2.1–2.3). This evidence strongly suggests that the extent of distortion undergone by the proteins during attachment to the surface decreases as a function of linker addition.

When combined with the concomitant decrease in film rigidity that was observed with the addition of lysine the increase in bound mass with increasing linker length indicated in this instance, that the increase is caused by the desirable addition of both additional protein and film-associated water.

Data obtained from this phase of study thus agrees with those inferred from previous research regarding the use of spacer-arms in protein (enzyme) immobilization. When constructing and optimising thin-film biosensors, the QCM-D may prove to be a valuable apparatus for monitoring fundamental film parameters that will impact on the operational properties of the said biosensor/s.

It was of great interest to examine whether differences in the film rigidity showed any correlation with the kinetic functioning of the laccase molecules when attached (in the manners outlined above) to an electrochemical transducer. This is explored in Paper II of this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.enzmictec.2011.05.011.

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