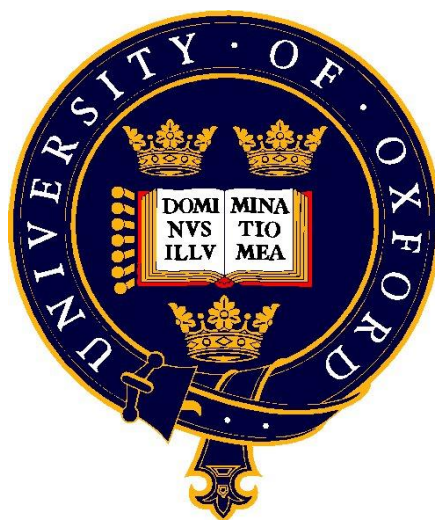


**Structure–function studies of the oxidoreductase
bilirubin oxidase from *Myrothecium verrucaria* using
an electrochemical quartz crystal microbalance with
dissipation**



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Exeter College, University of Oxford
Trinity 2014

A thesis submitted to the Board of the Faculty of Mathematical, Physical and Life

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Abstract

Structure-function studies of the oxidoreductase bilirubin oxidase from *Myrothecium verrucaria* using an electrochemical quartz crystal microbalance with dissipation

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This thesis presents the development and redesign of a commercial electrochemical quartz crystal microbalance with dissipation (E-QCM-D). This was used to study factors affecting the efficiency of the four electron reduction catalysed by the fuel cell enzyme bilirubin oxidase from *Myrothecium verrucaria* immobilised on thiol modified gold surfaces.

Within this thesis, the E-QCM-D was used to show that application of a constant potential to bilirubin oxidase adsorbed to thiol-modified gold surfaces causes activity loss that can be attributed to a change in structural arrangement. Varying the load by potential cycling distorts the enzyme by inducing rapid mass loss and denaturation. Attaching the enzyme covalently reduces the mass loss caused by potential cycling but does not mitigate activity loss. Covalent attachment also changes the orientation of the surface bound enzyme as verified by the position of the catalytic wave (related to the overpotential for catalysis) and reactive labelling followed by mass spectrometry analysis.

The E-QCM-D was used to show how electrostatic interactions affect enzyme conformation where high pH causes a reduction in both mass loading at the electrode and a reduction in activity. At pH lower than the enzyme isoelectric point, there is a build up of multilayers in a clustered adsorption. When enzyme adsorbs to hydrophobic surfaces there is a rapid denaturation which completely inactivates the enzyme. Changing the surface chemistry from carboxyl groups to hydroxyl and acetamido groups shows that catalysis is shifted to more negative potentials as a result of an enzyme misorientation. Further to this, increasing the chain length of the thiol modifier indicates that an increased distance between surface and enzyme reduces activity, enzyme loading and results in a conformational rearrangement that permits electron transfer over longer distances.

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Abbreviations

°C	degrees Celsius
μA	microamps
μg	micrograms
μM	micromolar
nA	nanoamps
ABTS	2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonate)
3MPA	3-mercaptopropionic acid
3MPOL	3-mercaptopropan-1-ol
4MPAA	4-mercaptoacetanilide
4MBA	4-mercaptobutyric acid
4MBZ	4-mercaptobenzoic acid
4MPOH	4-mercaptophenol
6MHA	6-mercaphexanoic acid
8MOA	8-mercaptooctanoic acid
11MUA	11-mercaptoundecanoic acid
BSA	bovine serum albumin
BFC	Biofuel cell

CA	chronoamperometry
CV	cyclic voltammogram
DLS	dynamic light scattering
E_{cat}	potential at the steepest part of the catalytic wave
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
E-QCM-D	electrochemical quartz crystal microbalance with dissipation
E-SPR	electrochemical surface plasmon resonance
i	current
i^{max}	maximum current
i^0	starting current
i^{∞}	resting current
k_{on}	rate of adsorption
k_{off}	rate of desorption
k_{ox}	rate constant for oxidation reaction
k_{red}	rate constant for reduction reaction
M	molar
min	minute
MCO	multicopper oxidase
mV	millivolt

MvBOx	bilirubin oxidase from <i>Myrothecium verrucaria</i>
NHS	N-hydroxysuccinimide ester
nm	nanometer
ORR	oxygen reduction reaction
PDB	protein data bank
PFV	protein film voltammetry
pH	$-\log[\text{H}^+]$
pI	isoelectric point
QCM	quartz crystal microbalance
QCM-D	quartz crystal microbalance with dissipation monitoring
s	second
SAM	self-assembled monolayer
SCE	standard calomel electrode
SHE	standard hydrogen electrode
SPR	Surface plasmon resonance
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TNC	trinuclear copper centre
TvL	laccase from <i>Trametes versicolor</i>
T1	Type 1 copper site

T2	Type 2 copper site
T3	Type 3 copper site
V	volt
vs	versus
Δd	dissipation change
$\Delta d^{\max}$	maximum dissipation change
Δd^0	starting dissipation change
Δd^{∞}	resting dissipation change
$\Delta d/\Delta f$	dissipation-frequency gradient
Δf	frequency change
$\Delta f^{\max}$	maximum frequency change
Δf^0	starting frequency change
Δf^{∞}	resting frequency change
$\Delta i/\Delta f$	adsorbed mass efficiency
$\Delta G, \Delta H, \Delta S$	free energy, enthalpy and entropy of reaction
τ	current decay time constant

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Chapter 1:

Introduction and Literature Review

1 Introduction and Literature Review

1.1 Overview

The work reported in this thesis examines the oxidoreductase, bilirubin oxidase from *Myrothecium verrucaria* (MvBOx), part of the larger family of enzymes known as multicopper oxidases (MCO). These MCOs catalyse the oxygen reduction reaction (ORR), possess signature electrocatalytic behaviour and are of interest for their applicability for use in enzymatic biofuel cells.

The introduction will look at the importance of the ORR for use in enzymatic fuel cells and will also present the electrochemical theory used when monitoring these processes along with enzyme immobilization procedures for both direct and mediated electron transfer at electrode surface.

The theory of the standard and electrochemical quartz crystal microbalance with dissipation (QCM-D and E-QCM-D) will be presented alongside historic developments and the potential for using this technology in redox enzyme catalysis. The thermodynamics and kinetics of protein adsorption will then be considered alongside discussion of current adsorption models and the development of a new model for adsorption incorporating the E-QCM-D. Some of the work presented in the introduction has been used for publication and the accepted manuscript is included in the Appendix.

Chapter 2 presents the materials and methods used and Chapter 3 presents the development of a new E-QCM-D module, with designs and drawings detailing changes that were made to the original commercial module. Chapter 4 is a continuation of published work (Appendix) and presents the results of an investigation into sources of inactivation of MvBOx adsorbed to modified-gold surfaces with emphasis on simulating fuel cell conditions and examining long term performance. Chapter 5 looks at the effects

of pH, thiol chain length, surface chemistry and affinity adsorption on the structure and activity of *MvBOx*.

1.2 Oxygen and the oxygen reduction reaction

Oxygen (O_2) is the most abundant element in the earth's crust and also constitutes approximately 21% of the volume of air. The high electronegativity of oxygen makes it a highly reactive species and it readily takes up electrons to form compounds such as water and hydrogen peroxide and can react with metals such as iron, to form oxides.

The two most common allotropes of oxygen are dioxygen (O_2) and ozone (O_3), both of which are strong oxidizing agents. In animals, dioxygen is essential for aerobic respiration. It is the terminal electron acceptor in the process that converts sugars to energy (via glycolysis, the citric acid cycle and oxidative phosphorylation), where oxygen itself is reduced to water. Dioxygen is also a product of the photosynthetic pathway where plants convert water and carbon dioxide into dioxygen and sugars.

Ozone is thermodynamically unstable towards dioxygen, and it is formed by the reaction of dioxygen with atomic oxygen via the splitting of O_2 by ultraviolet radiation.

A third allotrope of oxygen exists, called superoxide, which comprises a superoxide anion with the chemical formula, $O_2^{\bullet-}$. Superoxide is a product of the one electron reduction of dioxygen. Whereas molecular oxygen is a diradical containing two unpaired electrons, in superoxide, the addition of a second electron fills one of the two degenerate molecular orbitals, leaving the charged ionic species with a net charge of -1 .¹ Superoxide is however biologically toxic and is typically removed from the body by the enzyme superoxide dismutase which spontaneously converts it to O_2 and hydrogen peroxide. Typical dismutases with copper-zinc cofactors have a k_{cat} (turnover frequency) of 1667 s^{-1} .²

1.2.1 Oxygen Latimer and Frost diagrams

Oxygen exists in four common oxidation states where the most stable oxidation states are 0 and -2. The oxygen Latimer diagram (Figure 1.1) details the most highly oxidised form of the species and also includes the forms with successively lower oxidation states going from O_2 on the left to H_2O on the right, and indicates whether the species will disproportionate in solution.

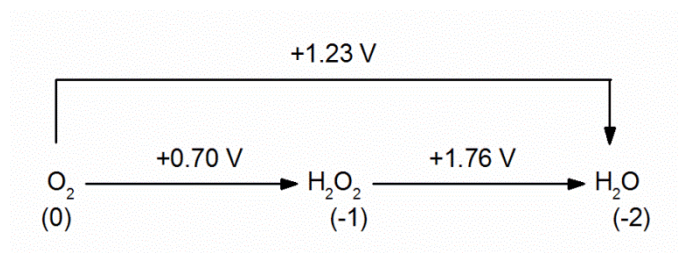
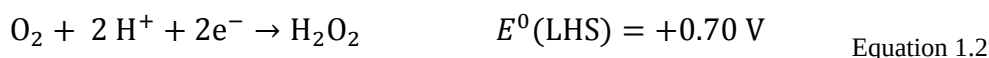
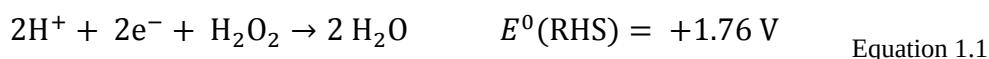


Figure 1.1. The oxygen Latimer diagram for the O_2/H_2O couple with the oxidation states of oxygen in each case labelled in parenthesis.

The process of disproportionation is best understood using the two half reactions for the reduction of hydrogen peroxide to H_2O (Equation 1.1) and the reduction of O_2 to hydrogen peroxide (Equation 1.2)



The standard potential for the cell for this overall reaction is

$$E^0 = E^0(\text{RHS}) - E^0(\text{LHS}) = 1.76 \text{ V} - 0.70 \text{ V} = 1.06 \text{ V} \quad \text{Equation 1.3}$$

A species is unstable and has a tendency to disproportionate in solution if the potential on the right hand side of the Latimer diagram is greater than the potential on the left and hence hydrogen peroxide is the more unstable species. The oxygen Latimer diagram above also includes the standard potential for the two nonadjacent species (the O_2/H_2O

couple). The overall standard potential for this reaction is calculated using the Gibbs Free Energy of reaction which uses E^0 (LHS) and E^0 (RHS) in combination with the number of electrons (n) for each half reaction in the following manner.

$$G^0(\text{O}_2/\text{H}_2\text{O}) = G^0(\text{O}_2/\text{H}_2\text{O}_2) + G^0(\text{H}_2\text{O}_2/\text{H}_2\text{O}) \quad \text{Equation 1.4}$$

$$-nFE^0(\text{O}_2/\text{H}_2\text{O}) = -nFE^0[(\text{O}_2/\text{H}_2\text{O}_2) + (\text{H}_2\text{O}_2/\text{H}_2\text{O})] \quad \text{Equation 1.5}$$

$$4E^0(\text{O}_2/\text{H}_2\text{O}) = 2E^0(\text{O}_2/\text{H}_2\text{O}_2) + 2E^0(\text{H}_2\text{O}_2/\text{H}_2\text{O}) \quad \text{Equation 1.6}$$

$$E^0(\text{O}_2/\text{H}_2\text{O}) = \frac{E^0(\text{O}_2/\text{H}_2\text{O}_2) + E^0(\text{H}_2\text{O}_2/\text{H}_2\text{O})}{2} \quad \text{Equation 1.7}$$

$$E^0(\text{O}_2/\text{H}_2\text{O}) = \frac{0.70 \text{ V} + 1.76 \text{ V}}{2} = 1.23 \text{ V} \quad \text{Equation 1.8}$$

More precisely, the actual thermodynamic standard potential for oxygen reduction to water is 1.229 V vs. the Standard Hydrogen Electrode (SHE).³ The information from the Latimer diagram above can be used in the construction of an oxygen Frost diagram (Figure 1.2) which illustrates the stability of oxidation states of oxygen relative to their standard electrode potential and depicts whether a particular species, $X(N)$, is a strong oxidising or reducing agent.

A Frost diagram is a plot of NE^θ versus oxidation state for the half reaction:



or in the case of oxygen it can be for the half reaction for the conversion of O_2 to H_2O_2 :



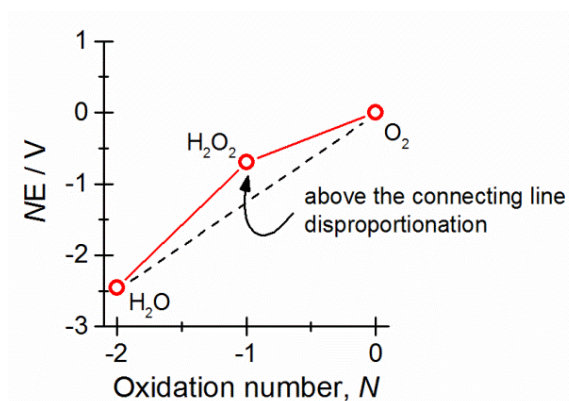


Figure 1.2. Frost diagram for oxygen in acidic solution ($\text{pH} = 0$). The diagram shows a plot of standard potential (NE^θ) versus oxidation number (N) and shows the position of hydrogen peroxide above the line which connects the two adjacent species. This indicates the lack of stability of the species and that it will readily undergo disproportionation.

Standard potential is directly proportional to the standard Gibbs Free Energy (ΔG^0) according to Equation 1.12. The change in oxidation state (N) is defined as the amount of electrons gained or lost by an atom in a compound. Therefore, by definition, the change in oxidation state is the same as the number of electrons, n , and Equation 1.12 is converted to Equation 1.13 (under standard conditions). Note here that Equation 1.11 is the Gibbs Free Energy in non standard conditions.

$$n\Delta E = -\Delta G/F \quad \text{Equation 1.11}$$

$$n\Delta E^0 = -\Delta G^0/F \quad \text{Equation 1.12}$$

$$NE^0 = -\Delta G^0/F \quad \text{Equation 1.13}$$

where F is Faraday's constant ($96485 \times 10^4 \text{ C mol}^{-1}$).

Therefore using the above derivation, the oxygen Frost diagram is actually a plot of Gibbs Free Energy versus the oxidation number for the reactions in Equation 1.1 and Equation 1.2 and illustrates how thermodynamically favourable a half reaction can be on an electrode surface.

1.2.2 The oxygen reduction reaction

Oxygen also plays an important role in bioinorganic catalysis, particularly in fuel cell systems, where it acts as one of the two major fuel sources. Thermodynamically, the standard potential for the reduction of dioxygen to water is 1.229V.³ The reduction of dioxygen or the oxygen reduction reaction (ORR) occurs mainly via two pathways: the 4 electron reduction of O₂ to H₂O and the two electron reduction of O₂ to H₂O₂ (Table 1.1). The one electron reduction of O₂ to superoxide may also occur in non-aqueous aprotic solvents. The ORR is an important reaction not only in biological respiration but also in energy converting systems such as fuel cells. Typical ORRs with their corresponding thermodynamic electrode potentials are listed in Table 1.1.

Table 1.1. Thermodynamic electrode potentials of the ORR. The 4e⁻ reduction and the two 2e⁻ reductions are shown with their corresponding electrode potential values versus the standard hydrogen electrode.³

Electrolyte	ORR	Thermodynamic electrode potential (V vs. SHE)
Acidic aqueous solution	$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow \text{H}_2\text{O}$	1.229
	$\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2$	0.70
	$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow 2 \text{H}_2\text{O}$	1.76

In fuel cell systems, the 4-electron reduction is important in the generation of current at an electrode surface, however due to cathode activation polarisation (caused by limits in the kinetics of the reaction at the electrode surface), this thermodynamic potential is difficult to achieve. This results in an overpotential (difference between the literature thermodynamic value and the actual potential, see section 1.5.5) and an overall energetic loss in this process, which in terms of a fuel cell system equates to a loss of power. This indicates that alternative strategies are needed which are more economically viable and catalyse the ORR at potentials closer, or equal, to the thermodynamic electrode potential.

1.3 Catalysts for the oxygen reduction reaction

1.3.1 *Inorganic catalysts*

A wide array of inorganic catalysts have been assessed for oxygen reduction ranging from porphyrins and transition metal phthalocyanines⁴ and palladium alloys (Pd–Co, Pd–Cr, Pd–Ni)⁵ to more recently developed cobalt oxide nanocrystals⁶ and first-row transition-metal based chalcogenides⁷. However these metal catalysts exhibit slow oxygen reduction and are typically supported on carbon surfaces. Platinum remains an attractive noble-metal catalyst due to its enhanced turnover frequencies.⁸ However, platinum also exhibits an overpotential of ~300 mV for the oxygen reduction reaction and slow reaction kinetics, which leads to high platinum loading and high costs, preventing the widespread use of platinum in fuel cells and limiting the scaling up of new technologies.⁹ The substantial overpotential is also a major source of inefficiency in low temperature fuel cells and as a result attention has now turned to biological electrocatalysts as an alternative.

1.3.2 *Biological catalysts*

Oxygen reduction plays an important role in biological processes such as oxidative phosphorylation, or the electron transport chain, where cytochrome *c* oxidase reduces molecular oxygen to water via two copper atoms (and two haem centres) within its central structure.¹⁰ In a similar fashion, the catalytic centres of numerous dioxygen reducing enzymes are made up of copper and/or iron (haem) centres.¹¹ Enzymes that activate or bind dioxygen have a wide variety of biological functions as described in Table 1.2.

These enzymes have in common a reduction pathway involving the binding of dioxygen to reduced copper(I) species and generate no long lasting and potentially damaging intermediates such as peroxide, unless the enzyme's specific function is to

produce peroxide. Some proteins like tyrosinase, catechol oxidase and laccase exhibit lower overpotentials than platinum¹² and the work in this thesis takes advantage of this important feature using bilirubin oxidase as the enzyme of choice.

Table 1.2. Catalytic copper proteins involved in the binding and/or activation of dioxygen.^{10,13-16}

Protein	Reaction details	Type of copper centre involved	Additional function
amine oxidase	coupled oxidation of amines to aldehydes and reduction of dioxygen to peroxide	mononuclear	regulation of amine levels in higher eukaryotes
galactose oxidase	coupled oxidation of alcohols to aldehydes and reduction of dioxygen to peroxide	mononuclear	hydrogen peroxide generation
dopamine β -monooxygenase	coupled conversion of dopamine to norepinephrine by hydroxylation of benzylic position of ethylamine side chain, oxidation of ascorbate and reduction of dioxygen to water	mononuclear	controls levels of hormones and also dopamine and norepinephrine levels
peptidylglycine α -amidating monooxygenase	coupled hydroxylation of glycine at α -position, oxidation of ascorbate and reduction of dioxygen to water	mononuclear or noncoupled binuclear	regulation of peptide hormones
haemocyanin	reversible binding of O ₂	coupled binuclear	oxygen transport
Tyrosinase	hydroxylation of aromatic ring and reduction of dioxygen to water	coupled binuclear	aromatic ring oxidation
catechol oxidase	coupled oxidation of catechol to o-quinone and reduction of dioxygen to water	coupled binuclear	aromatic ring oxidation
cytochrome c oxidase	coupled oxidation of cytochrome c and reduction of dioxygen to water	binuclear Cu-Fe(haem)	proton pump in electron transport chain
ceruloplasmin	coupled oxidation of Fe(II) to Fe(III) and reduction of dioxygen to water	trinuclear	iron homeostasis
Laccase	coupled oxidation of phenols and reduction of dioxygen to water	trinuclear	
bilirubin oxidase	coupled oxidation of bilirubin to biliverdin and reduction of dioxygen to water	trinuclear	
ascorbate oxidase	coupled oxidation of ascorbate and reduction of dioxygen to water	trinuclear	

In oxygen-activating enzymes, including those mentioned in Table 1.2, the basic role of the enzyme is not specifically the reduction of dioxygen. The presence of dioxygen simply provides a sink for electrons that are produced in a coupled oxidation reaction. The ORR is of particular interest in technological applications, such as fuel cells, and the naturally occurring catalysts for the ORR can give insight into the ORR mechanism and improve existing processes which utilise this system for catalysis.

1.4 Multicopper Oxidases

The multicopper oxidases (MCOs) are a family of enzymes that are typically single-subunit proteins incorporating four redox-active Cu atoms. These copper proteins were originally characterised by the type of copper site they contain. These were named type 1 (T1) copper, type 2 (T2) and type 3 (T3) copper.¹⁷ Mononuclear copper sites observed in native proteins (Table 1.2) either adopt the type 1 (T1) or type 2 (T2) electronic structure.¹⁴ Type 1 (T1) copper sites or blue copper sites exhibit intense charge-transfer absorption which result from the covalent coordination from a thiol ligand donated by a cysteine side chain to the copper centre.

This interaction gives rise to the intense blue colour of the enzyme, but more importantly gives rise to dramatic enhancement of electron transfer activity. More recently, copper-containing proteins have been defined in terms of their spectroscopic properties and coordination geometry (Table 1.3).

Type 2 sites are more closely related to aqueous copper (II) ions, and are found in the catalytic domains of the enzyme rather than electron transfer sites. Both T1 and T2 copper sites are electron paramagnetic resonance active (EPR-active) as they have a d^9 electron configuration resulting in unpaired spins.¹⁸ T1 and T2 denote sites that contain just one copper ion. Enzymes with a T3 centre usually catalyse the oxidation of substrates

with molecular oxygen being the oxidant of choice. Typically for enzymes catalysing the ORR, there will be a T2 copper associated with a T3 copper.¹² This is known as the trinuclear cluster (TNC). Due to strong antiferromagnetic coupling between the two $S=1/2$ metal ions, there is no EPR signal for T3 centres. MCOs contain one T1, one T2 and two T3 copper centres¹⁶ which are important in the dioxygen reduction pathway in bilirubin oxidase.

Table 1.3. Coordination and spectroscopic properties of copper centres^{10,14,17,19-21}

Type	Coordination geometry	Spectroscopic properties	Examples
T1	2 N & 2 S. Distorted tetrahedral	UV-Vis absorbance around 610 nm EPR active	plastocyanin, azurin, stellacyanin
T2	N & O donor ligands. Square planar	EPR active	superoxide dismutase, galactose oxidase
T3	histidine ligands. binuclear	UV-Vis shoulder at 330 nm	haemocyanin, tyrosinase
T2/T3 cluster	as above	as above	ascorbate oxidase, ceruloplasmin, laccase
Cu _A	2 histidine, 2 cysteine, 1 glutamate, 1 methionine. mixed valence binuclear copper binding site	EPR active	cytochrome <i>c</i> oxidase, nitrous-oxide reductase ¹⁹
Cu _B	3 histidine, binuclear with cytochrome haem <i>a</i> ₃	Weak UV-vis absorbance at 655 nm	cytochrome <i>c</i> oxidase
Cu _z	7 histidine & sulphur bridge. Binuclear	UV-vis absorbance at 540 and 650 nm	nitrous-oxide reductase ¹⁰

This structural arrangement is vital for the oxygen reduction reaction and all MCOs couple the oxidation of a substrate to the 4-electron reduction of dioxygen to water (Table 1.1). Laccase and ascorbate oxidase are examples of the better characterised enzymes within this class, with bilirubin oxidase,²²⁻²⁴ CueO,^{25,26} having been more recently characterised.

Bilirubin oxidase and laccase both show smaller overpotentials for ORR than a typical particulate Pt-based catalyst, used in conventional low-temperature polymer-electrolyte membrane fuel cells.^{27,28}

1.4.1 Bilirubin oxidase

The MCO bilirubin oxidase from *Myrothecium verrucaria* (*MvBOx*) utilises the T1, T2 and T3 copper sites for the ORR while catalysing the oxidation of bilirubin to biliverdin,²⁹ with the concomitant reduction of O₂ to water. This is a reaction of interest, where it can be used at the cathode in low-temperature bio-fuel cell applications.^{30,31} Bilirubin oxidase was originally used in the determination of bilirubin in serum and as a diagnostic for jaundice.^{32,33}

In the ORR, for each turnover of O₂ which occurs at the T2/T3 Cu site, four separate electron transfers occur to the T1 Cu site (the primary electron entry site) which is located close to the protein's surface.^{14,34} The T1 Cu mediates electron transfer between the substrate and the TNC where the final electron acceptor O₂ is converted to H₂O.³⁵ The structure of the enzyme reveals an unusual environment around the blue T1 site that includes two non-coordinating hydrophilic amino acids, asparagine and threonine.²⁴

The interaction between the binding pocket (near the T1 site) of *MvBOx* and its highly conjugated natural organic substrate, bilirubin, has been used to stabilise the enzyme on a pyrolytic graphite electrode, more than doubling its electrocatalytic activity compared to simple adsorption on non-modified graphite.²⁴ The important feature of *MvBOx* immobilization is in positioning the enzyme with the T1 site as close to the electrode surface as possible, facilitating electron transfer during the ORR.

1.5 Electrochemical theory

Redox enzyme catalysis is readily studied using electrochemistry. Studies are based on the reversible nature of the redox enzyme to exchange electrons with an electrode surface, resulting in signature changes in the current vs. potential measurement across the electrode.³⁶

1.5.1 Equilibrium electrochemistry

An electrode process involves the transfer of charge across the interface between an electrode and a species in solution, shown by Equation 1.14



The electron in the reaction above is provided by an electrode (elec) which acts as an electron source. As the electron transfer reaction moves towards equilibrium, a net charge separation develops between the electrode and the solution.³⁷ This charge separation causes a potential difference at the interface of electrode and solution. This potential difference is the difference between the electrode potential and solution potential, according to Equation 1.15:

$$\Delta\phi_{elec/sol} = \phi_{elec} - \phi_{sol} \quad \text{Equation 1.15}$$

However, only a potential difference can be measured in the above setup. Therefore in order to measure the applied potential from the electrode, a second electrode with a fixed potential drop is introduced into the solution, called the reference electrode. In this way when measurements of potential difference are made between the two electrodes the overall applied potential (or voltage) is given by

$$E = (\phi_{elec} - \phi_{sol}) + \text{constant}_{ref} \quad \text{Equation 1.16}$$

where the term in brackets refers to the electrode supplying the electrons to the reaction, and ‘constant_{ref}’ is the constant potential difference drop at the reference electrode.³⁷

Typical experimental set ups for equilibrium chemistry are comprised of two half-cells with one cell containing the reference electrode and the other cell containing a chemically inert metal electrode (e.g. gold) or working electrode. The two cells are separated by a porous membrane or salt bridge which maintains a complete electrochemical connection.

1.5.2 Nernst equation

The general charge transfer reaction at any given electrode where n electrons are transferred is given by (Equation 1.17):



The Gibbs Free Energy for the reaction is given by Equation 1.18:

$$\Delta G = \Delta G^0 + RT \ln Q \quad \text{Equation 1.18}$$

where R is the gas constant, T is the temperature in Kelvin and Q is the reaction quotient and is a function of the concentrations of the species in Equation 1.17. The standard potential for the reaction is related to the Gibbs free energy (as described in section 1.2.1) and substituting both Equation 1.11 and Equation 1.12 into Equation 1.18 gives:

$$-nFE = -nFE^0 + RT \ln Q \quad \text{Equation 1.19}$$

This rearranges to:

$$E = E^0 - \frac{RT}{nF} \ln Q \quad \text{Equation 1.20}$$

The reaction quotient Q is expressed as the concentration of reactants over products ($[O]/[R]$). Converting to log base 10 and substituting in T (298 K) and Faraday's constant into Equation 1.20 gives the Nernst equation:

$$E = E^0 - \frac{0.592 \text{ V}}{n} \log \frac{[O]}{[R]} \quad \text{Equation 1.21}$$

The Nernst equation indicates that the electrical potential of a cell depends on the reaction quotient. As the redox reaction in Equation 1.17 proceeds, the concentration of reactants decreases. Conversely the product concentration increases and as equilibrium is reached ΔG approaches zero. The reaction quotient is then linked to Gibbs Free Energy by Equation 1.22.

$$K_{eq} = Q = \exp \left(-\frac{\Delta G^0}{RT} \right) \quad \text{Equation 1.22}$$

where K_{eq} is the equilibrium constant.³⁶

When looking at pH based measurements $\log_{10}Q$ can be expressed in terms of the hydrogen ion concentration within the solution:

$$E = E^0 + \frac{0.592 \text{ V}}{n} \log[H^+] \quad \text{Equation 1.23}$$

which simplifies to:

$$E = E^0 + \frac{0.592 \text{ V}}{n} \text{pH} \quad \text{Equation 1.24}$$

Therefore for every unit change in pH there is a 59.2 mV change in the standard potential. This relationship is utilised in pH meters when testing the pH value of buffer solutions. The Nernst equation shows that the potential at the electrode under equilibrium conditions is dependent on the temperature, concentration and pH of the species-containing solution.

For the purpose of this thesis, unless otherwise stated, the reference electrode is given versus the standard hydrogen electrode or SHE and by convention SHE is defined as possessing an absolute potential of zero.

1.5.3 Dynamic Electrochemistry

1.5.3.1 The 3-electrode cell

In a typical three-electrode cell (Figure 1.3), a counter electrode, also called an auxiliary electrode, is used to make a connection to the electrolyte so that a current can be applied to the working electrode to counter the current passed to the redox reaction.

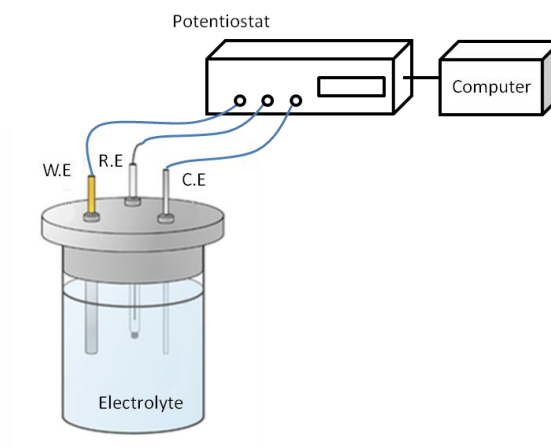


Figure 1.3. Typical 3-electrode cell setup using a stationary working electrode. Common working electrodes can consist of inert metals such as gold, silver or platinum, or carbon based materials such as glassy carbon or pyrolytic graphite electrodes. The applied potential and current passed via the counter and working electrodes is controlled by a potentiostat (see Materials and Methods) connected to a computer.

1.5.3.2 *Mass transport*

The rate of reaction on the electrode surface is influenced by the applied potential as discussed in Section 1.5.2. In addition, the rate of reaction is also affected by the rate of transport of reactant from solution to the electrode surface. Equation 1.17 suggests that for a fixed surface area of electrode, the reaction can be controlled by two factors, the rate constant for the reduction reaction, k_{red} and the surface concentration of the reactant species (O). If the rate constant is large enough that any reactant at the electrode surface is immediately converted into products, the amount of current generated will be controlled by the amount of fresh reactant reaching the electrode from bulk solution.³⁷

1.5.3.3 *Diffusion*

The rate of movement of material by diffusion is predicted by Fick's laws of diffusion, where the rate of movement of material by diffusion (diffusional flux, J) is related to the concentration gradient and the diffusion coefficient, D :

$$J_o = -D_o \left(\frac{\partial C_o}{\partial x} \right) \quad \text{Equation 1.25}$$

where C_o is the concentration of material and the negative sign indicates the material moving down from areas of high concentration to low concentration.

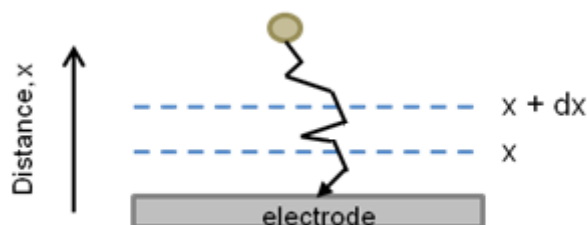


Figure 1.4. Diagram showing how reactant diffuses via Brownian motion. Due to the reaction only occurring at the electrode surface there will be a lower reactant concentration at the electrode compared to bulk solution. This gives rise to a diffusion layer where a small layer of reactant accumulates near the electrode but not quite at the interface (x) compared to the bulk solution further out ($x + dx$).

Using Equation 1.25, the rate of diffusion as a function of time can be predicted:

$$\frac{\partial C_o}{\partial t} = D_o \left(\frac{\partial^2 C_o}{\partial x^2} \right) \quad \text{Equation 1.26}$$

where the rate of change in the concentration over time is related to the change in concentration gradient and the steeper the concentration change (going from bulk to ($x + dx$) to x , Figure 1.4) the greater the diffusion rate.

1.5.3.4 Convection

Convection naturally occurs in a solution and causes the solution to mix in an unpredictable manner. The application of force on the solution by a pump or a flow of gas acts to remove this natural convection. When the force is applied and the flow of solution is slow and controlled, the flow will become stable with no mixing in the lateral direction (opposite or perpendicular to the flow). This is called laminar flow.³⁸ If the solution is pumped through with greater force or higher speed then the flow can become turbulent

and mixing becomes random and uncontrolled (Figure 1.5). For laminar flow conditions, the mass transport equation for convection is predicted by:

$$\frac{\partial C_o}{\partial t} = -v_x \left(\frac{\partial C_o}{\partial x} \right) \quad \text{Equation 1.27}$$

where v is the velocity of the solution.

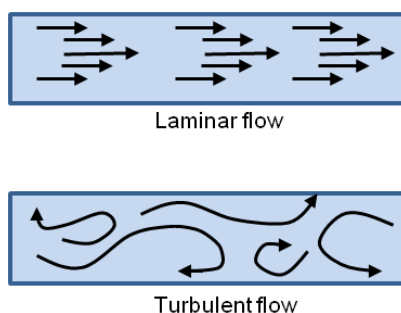


Figure 1.5. The top panel shows the controlled direction of flow or laminar flow through a solution. The bottom panel indicates turbulent flow when convection is uncontrolled.

1.5.3.5 Rotating disc electrode

The rotating disc electrode (RDE) setup is a method for introducing convection into an electrochemical cell where the rate of forced convection is controlled by the rotation rate of the electrode and is therefore predictable (Figure 1.6).

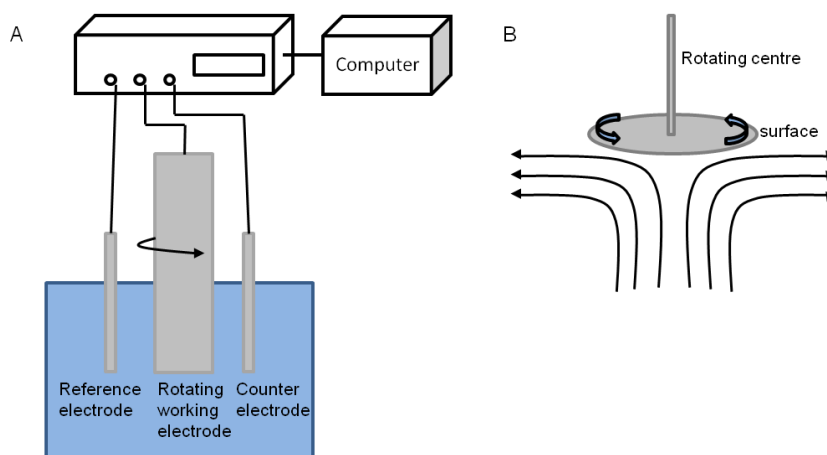


Figure 1.6. Schematic of the rotating disc electrode setup. Panel A shows a typical set up of the rotating disc electrode setup and panel B shows how the laminar flow path at the rotating electrode and how the act of rotation drags material to the electrode surface.

Increasing the rotation speed of the electrode, results in a higher flux of material to the surface. This causes an increase in the current measured by an electrochemical cell and when the rotation rate is kept above a certain value (the minimum rotation rate needed to maintain laminar flow), the diffusion and flow conditions can be modelled using the Levich equation (Equation 1.28) which shows that the current is proportional to the square root of rotation speed:³⁹

$$i = 0.62nFAD^{\frac{2}{3}}\omega^{\frac{1}{2}}\nu^{-\frac{1}{6}}C \quad \text{Equation 1.28}$$

where n is the number of electrons transferred in the half reaction (Equation 1.29), F is the Faraday constant, A is the electrode surface area (cm^2), D is the diffusion coefficient ($\text{cm}^2 \text{ s}^{-1}$), ω is the rotation speed (radian s^{-1}), ν is the kinematic viscosity of the solution ($\text{cm}^2 \text{ s}^{-1}$) and C is the concentration of the electroactive species (mol cm^{-3}). Kinematic viscosity is dependent on the ratio of dynamic viscosity (μ) to density (ρ) of the solution. A mass transport limited current arises from the fact that the system reaches a steady state and so the current reaches a plateau once the equilibrium at the surface is driven to the products side.

In the case of multicopper oxidase electrochemistry the electrode is rotated to control the effect of limited mass transfer between the electrode and the solution (e.g., O_2 depletion) which can mask the intrinsic catalytic ability of the enzyme. Measuring the current at varied rotation rates allows for mass-transport effects to be corrected using the Koutecký-Levich equation.^{3,40}

1.5.4 Kinetics of electron transfer

The current flowing in either the reductive or oxidative steps can be predicted when considering a single electron transfer reaction between two species, O and R:



and the current flowing in either direction is predicted using the following relationships:

$$i_{ox} = nFAk_{ox}[R] \quad \text{Equation 1.30}$$

$$i_{red} = -nFAk_{red}[O] \quad \text{Equation 1.31}$$

where A is the electrode surface area, n is the number of electrons in the process, F is Faraday's constant, k_{red} and k_{ox} are the rate constants for Equation 1.29 and $[R]$ and $[O]$ are the concentrations of product and reactant, respectively. Note, by definition the reductive current is negative and the difference in signs here indicates the flow of current in different directions. The rate constants are directly affected by the applied potential and this can be seen using transition state models.

The net current flowing for the reversible reaction is therefore:

$$i = i_{ox} + i_{red} \quad \text{Equation 1.32}$$

therefore

$$i = nFA(k_{ox}[R] - k_{red}[O]) \quad \text{Equation 1.33}$$

Note that when the rate of reaction (Equation 1.29) is at equilibrium, the Nernst equation (Equation 1.20) holds and the current flow ceases ($i=0$). Therefore the fluxes must be balanced:

$$k_{ox}[R] = k_{red}[O] \quad \text{Equation 1.34}$$

Simple transition state models show that when a reactant, A is converted to its product, B (Figure 1.7 and Equation 1.35) there is an associated rate constant and the rate of reaction is predicted by Equation 1.36.

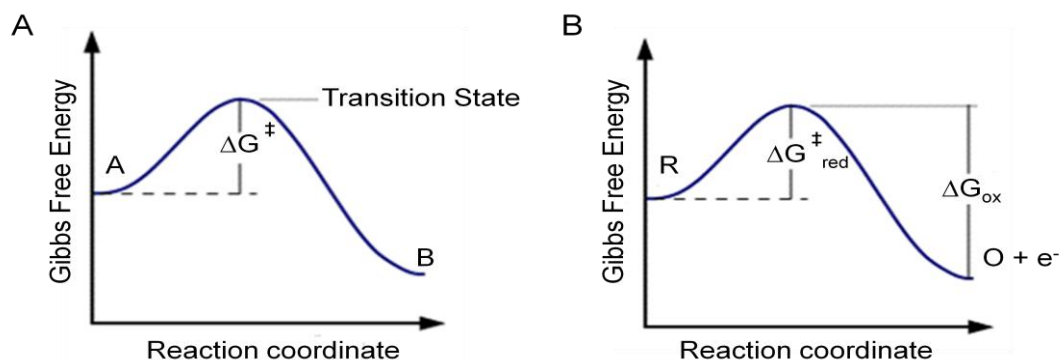


Figure 1.7. Transition state theory for converting reactants into products. Panel A shows the conversion of reactant A into product B via a transition state requiring a thermodynamic push equal to the ΔG . Panel B shows the same principle applied to the reaction in Equation 1.29.



$$k = Z \exp - \left(\frac{\Delta G^\ddagger}{RT} \right) \quad \text{Equation 1.36}$$

The term in brackets is the Free Energy change needed in taking the reactant from its initial value to the transition state shown in Figure 1.7A.

If the same principle is applied to the reaction in Equation 1.29, for a single applied voltage the free energy profile is qualitatively the same as the chemical process that occurs. In agreement with the relationship between Gibbs Free Energy and applied potential (Equation 1.11, 1.12 & Equation 1.13), the Free Energy plots vary as a function of applied potential:

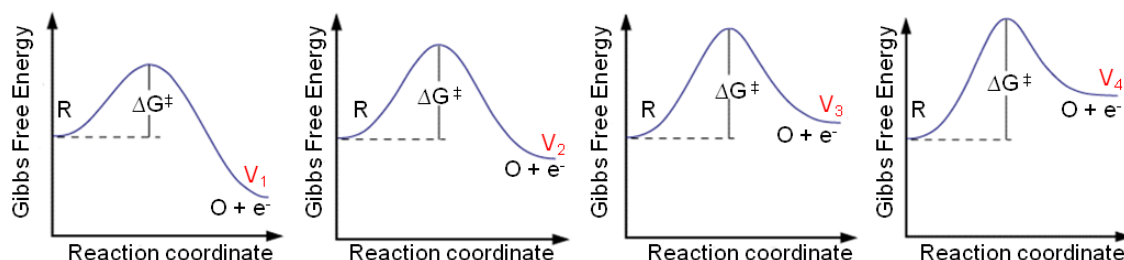


Figure 1.8. As the applied voltage changes from left to right, the overall barrier height (i.e. activation energy) is seen to alter as a function of the applied voltage. Note that the left hand side of each figure corresponding to the Free Energy of R is not dependent on the applied potential, whereas the right hand side ($O + e^-$) shows a strong dependence.

In Figure 1.8, with an applied potential, V_1 , the formation of the reactant (O) in Equation 1.29 is thermodynamically favoured. As the potential varies, the formation of product (R) is favoured. In the case of enzyme electrochemistry this would involve the potential being shifted in the negative direction.³⁷ If the effect of applied potential on the Gibbs Free Energy is assumed to follow a linear relationship then the change in Gibbs Free Energy for both reduction and oxidation reactions will follow Equation 1.37 and Equation 1.38.⁴¹

$$\Delta G_{\text{red}}^{\ddagger} = \Delta G_{\text{act}}^{\ddagger} + \alpha FV \quad \text{Equation 1.37}$$

$$\Delta G_{\text{ox}}^{\ddagger} = \Delta G_{\text{act}}^{\ddagger} - (1 - \alpha)FV \quad \text{Equation 1.38}$$

The parameter α is called the charge transfer coefficient and provides an insight into the way the transition state is influenced by the voltage. A value of 0.5 means that the transition state occurs mid-way between the reactants and products response to applied voltage. In operating fuel cells, the charge transfer coefficient is the parameter that signifies the fraction of overpotential that affects the current density.

Looking specifically at the reduction reaction, the rate of reaction is given as:

$$k_{\text{red}} = Z \exp \left\{ \left(\frac{-\Delta G_{\text{act}}^{\ddagger}}{RT} \right) \left(\frac{-\alpha FV}{RT} \right) \right\} \quad \text{Equation 1.39}$$

where $\Delta G_{\text{act}}^{\ddagger}$ is the activation Free Energy change and is only dependant on the chemical species changing and not the applied potential.

Equation 1.39 indicates that the rate constant for the electron transfer step (in the reductive direction) is proportional to the exponential of the applied voltage. Therefore the rate of electrolysis can be changed simply by varying the applied voltage. This relationship between rate of reaction and voltage is important in protein film voltammetry and the value of V is related to the overpotential applied for the onset of catalysis in the ORR (Section 1.5.5).

1.5.5 Overpotential

Equilibrium electrochemistry measures the electrode potential under equilibrium and therefore as the rates of oxidation and reduction become equal, one method for detecting the oxidation and/or reduction is to force the electrode potential to take a value that is different from the equilibrium potential. When the applied potential at the electrode (E) is equal to the equilibrium potential, no current flows. For any other value of E , Equation 1.17 (the reaction at the electrode) will no longer be at equilibrium with the electrode potential. Therefore to ‘drive’ the electrode reaction, a potential that is different to the equilibrium potential must be applied, and this deviation from the equilibrium potential is known as the ‘overpotential’.³⁷

$$\eta = E - E_e \quad \text{Equation 1.40}$$

While the system may still move towards equilibrium, by taking electrons from the electrode, any oxidation or reduction is detected as a current which measures the amount of electric charge passing the electrode per unit time. Therefore the current magnitude, at this overpotential, is proportional to the rate of reduction or oxidation. At low currents, the rate of reaction is related to overpotential according to the Tafel equation:

$$\eta = a \ln \left(\frac{i}{i_0} \right) \quad \text{Equation 1.41}$$

where a is the Tafel slop (V), i is the current density (A m^{-2}) and i_0 is the exchange current density (A m^{-2}) which is the current density in the absence of net electrolysis at zero overpotential.

1.5.6 Butler–Volmer equation

The dependence of k_{red} and k_{ox} on overpotential is similar to Equation 1.39 with overpotential, η , substituted for the voltage:

$$k_{red} = Z \exp \left\{ \left(\frac{-\Delta G^\ddagger}{RT} \right) \left(\frac{-\alpha F \eta}{RT} \right) \right\} \quad \text{Equation 1.42}$$

$$k_{ox} = Z \exp \left\{ \left(\frac{-\Delta G^\ddagger}{RT} \right) \left(\frac{(1 - \alpha) F \eta}{RT} \right) \right\} \quad \text{Equation 1.43}$$

These both simplify to Equation 1.44 and Equation 1.45

$$k_{red} = k_{red}^\phi \exp \left(\frac{-\alpha F \eta}{RT} \right) \quad \text{Equation 1.44}$$

$$k_{ox} = k_{ox}^\phi \exp \left(\frac{(1 - \alpha) F \eta}{RT} \right) \quad \text{Equation 1.45}$$

Substituting Equation 1.44 and Equation 1.45 into Equation 1.33 gives the relationship for the net current, i , flowing at the working electrode:

$$i = i_0 \left(\frac{[R]_0}{[R]_{bulk}} \exp \left\{ \frac{(1 - \alpha) F \eta}{RT} \right\} - \frac{[O]_0}{[O]_{bulk}} \exp \left\{ \frac{-\alpha F \eta}{RT} \right\} \right) \quad \text{Equation 1.46}$$

and this simplifies to the Butler-Volmer equation:

$$i = i_0 \left(\exp \left\{ \frac{(1 - \alpha) F \eta}{RT} \right\} - \exp \left\{ \frac{-\alpha F \eta}{RT} \right\} \right) \quad \text{Equation 1.47}$$

when the solution is well stirred and the surface concentration of both reactant and product will be equal to their bulk concentrations.

1.5.7 Cyclic voltammetry

The technique of cyclic voltammetry (CV) is a type of measurement where the electrode potential is ramped linearly between two potentials over time. This ramping is known as the scan rate and can be defined as the rate at which the potential of the electrode is changed, i.e. $v = (dE/dt)$. The main purpose of using this technique is to obtain detailed information about any reversible electron transfer process occurring like that seen in Equation 1.29.³⁷

Figure 1.9 is a typical response of a thermodynamically reversible process in electrochemistry.

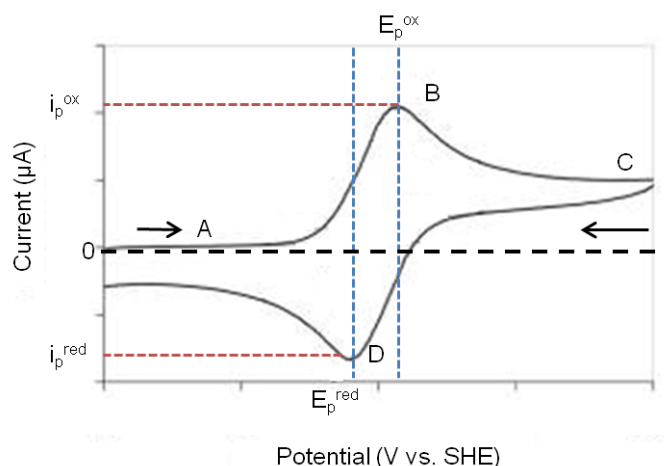


Figure 1.9. An example of a cyclic voltammogram for a reversible process in a liquid for a freely diffusing species

At point A, only the reduced species (R) of the redox couple is present and no current is observed because the initial potential applied is lower than the standard potential of the redox couple. At point B, the redox potential (E_p^{ox}) is sufficiently positive that any R that reaches the electrode surface is instantaneously oxidised ($R \rightarrow O + ne^-$). The current is now diffusion limited. Due to this diffusion-controlled mass transport, rapid replenishment of new, fresh reactant to the electrode surface cannot occur; therefore, the current begins to decrease and reaches point C. As the scan is reversed, the opposite process happens. At point D, the redox potential is sufficiently negative that any reactant (O) reaching the electrode surface is instantaneously reduced to product i.e. $O + ne^- \rightarrow R$.

A reversible redox coupled with fast kinetics shows significant current flows at small overpotentials and the magnitudes of the two peaks formed on the forward and backward scans are identical.

A one electron transfer, reversible redox couple should have a ratio of the peak current, i_p , for both the oxidative and reductive scans of one:⁴²

$$\frac{i_p^{\text{ox}}}{i_p^{\text{red}}} = 1 \quad \text{Equation 1.48}$$

1.5.8 EC and EC' mechanism

Cyclic voltammetry can be used to investigate the reactivity of chemical species. When a reactant undergoes an electron transfer which is immediately followed by a chemical reaction (similar to that in Equation 1.29), the rate of electron transfer is governed by Fick's 2nd law for diffusion (Equation 1.26).

However as the product (R) of the reaction is also being used up during this reversible process, according to Equation 1.49 the rate of production for R is also needed (Equation 1.50)



$$\frac{\partial[R]}{\partial t} = D_o \left(\frac{\partial^2[R]}{\partial x^2} \right) - k_{EC}[R] \quad \text{Equation 1.50}$$

where k_{EC} is the chemical rate constant and can be studied by varying the scan rate during cyclic voltammetry. This is known as an EC mechanism.

The EC' mechanism is an important variation of the EC mechanism where there is a new catalytic chemical reaction step which drives the electron transfer. In this situation one of the products of R is the original starting material (Equation 1.51) and therefore the system is autocatalytic. The faster the chemical reaction the more starting material is replenished.



The EC' reaction forms the basis of the reaction that occurs during redox enzyme catalysis such as when MCOs catalyse the reduction of oxygen to water, in the process oxidising their natural substrate. This process is readily studied using protein film voltammetry.

1.5.9 Protein film voltammetry

Protein film voltammetry (PFV) provides thermodynamic and kinetic descriptions of electron transfer and the coupled chemistry occurring within redox proteins at the surface of electrodes. A small amount of redox protein (typically $\sim 1 \text{ pmol cm}^{-2}$) is immobilized as a sub-monolayer film onto the surface of an electrode,⁴³ such that it remains catalytically active. The reductive current generated in a PFV experiment is measured using cyclic voltammetry and is proportional to the activity of the enzyme according to Equation 1.52:

$$i = -nFA\Gamma k_{\text{cat}} \quad \text{Equation 1.52}$$

where i is the catalytic current (negative for reductive processes by convention), n is the number of electrons involved in the reaction (4 for the ORR), F is Faraday's constant, A is the surface area of the electrode, Γ is the electroactive coverage of the enzyme, and k_{cat} is the enzyme's turnover frequency (about 300 s^{-1} for MCOs). After immobilisation of an enzyme on an electrode surface and measurement of changes in the reductive current generated, the resulting catalytic wave is similar to that seen in Figure 1.10.

By varying the applied potential (*i.e.*, the thermodynamic driving force for the reaction) and looking at the subtle changes in the catalytic current, PFV can provide a reliable method for studying the behaviour of redox-active catalytic enzymes, including MCOs. At high potential, there is insufficient driving force to overcome the sluggish kinetics of the oxygen reduction reaction.

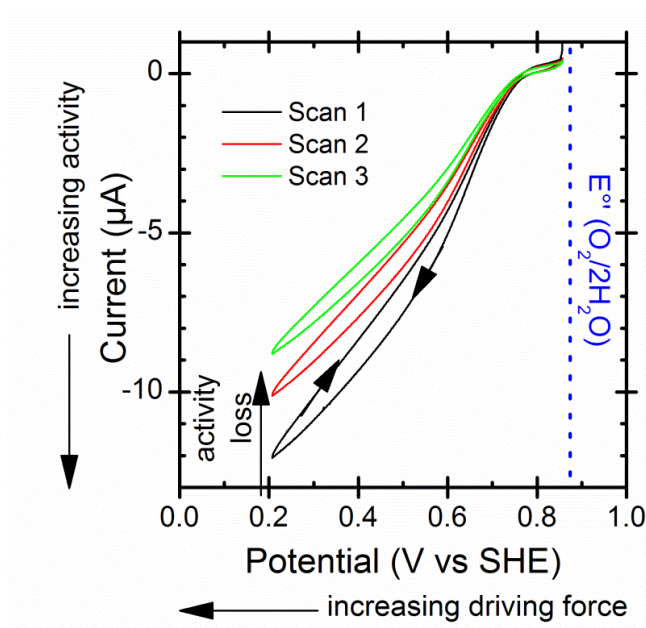


Figure 1.10. Typical electrocatalytic wave of an MCO on a pyrolytic graphite rotating disc electrode. The arrows indicate the direction of the potential sweep. The change in the magnitude of the catalytic wave at low potential between scans is indicative of the stability of the enzyme on the electrode surface.

As the potential is swept lower, there is an onset of catalysis at the point where electrons are being moved through the enzyme and begin catalysing the ORR (Table 1.1). The rate of reaction increases approximately exponentially with the limiting factor being the limited rate of electron transfer in the system. Eventually at very low potential, a mass transport limited current arises from the fact that the system reaches a steady state and so the current reaches a plateau. At low potentials, the rate of reaction may also become limited by something intrinsic to the enzyme, most probably k_{cat} . (The slope at low potentials is believed to be due to heterogeneous enzyme orientations on the surface and consequent heterogeneity in interfacial electron-transfer rates).⁴⁴ The E_{cat} describes the potential at the steepest part of the catalytic wave and can be used to understand the changes in catalysis with respect to the standard potential for dioxygen reduction.

1.5.10 Chronoamperometry

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is changed and the resulting current is monitored as a function of time. Within this thesis, chronoamperometry is almost entirely used without potential stepping where the working electrode is kept at a constant potential and the current generated by redox active enzyme is monitored over time.

1.5.11 Catalytic Voltammetry of MCOs

Catalytic voltammetry of MCOs has most commonly been observed on graphite electrodes or gold electrodes modified with self-assembled monolayers (SAMs). For high-potential MCOs, voltammetry is typically observed in the region pH 3–5, where activity is optimum, and for low-potential MCOs (bilirubin oxidase), the voltammetry was demonstrated at more natural pH nearer pH 7, closer to their optimum for activity.

Table 1.4 summarises the catalytic electrochemistry carried out on bilirubin oxidase as well as other closely related plant and fungal laccases. Tsujimura *et al.* reported the first example of the oxygen-reducing capabilities of a bilirubin oxidase enzyme in chloride-containing phosphate buffer at neutral pH (7) using MvBOx coated on a carbon felt electrode.⁴⁵ Direct electron transfer (DET) between MCOs including bilirubin oxidase has been studied extensively^{28,46,47} with gold electrochemistry now being a suitable and attractive target for enzyme immobilization. Modification of the gold electrode with thiol monolayers allows for controlled enzyme orientation and surface dependant catalytic activity⁴⁸⁻⁵⁰ (see section 1.7.2).

Table 1.4. Catalytic copper proteins which are involved in dioxygen binding and activation.

Enzyme	Electrode material	pH	Voltammetry	Catalysis	Conditions
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵¹	highly ordered pyrolytic graphite edge (HOPGE)	4,7	650–200 mV vs Ag/AgCl/sat. KCl 20 mV s ⁻¹	sigmoidal wave E_{cat} : 460 vs Ag/AgCl/sat. KCl	Stirred O ₂ saturated 0.1M phosphate buffer 25°C
Bilirubin oxidase from <i>Myrothecium verrucaria</i> M4670 mutant⁵¹	HOPGE	4,7	650–200 mV vs Ag/AgCl/sat. KCl 20 mV s ⁻¹	sigmoidal wave E_{cat} : 230 vs Ag/AgCl/sat. KCl	Stirred O ₂ saturated 0.1M phosphate buffer 25°C
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵²	spectroscopic PGE (SPGE)	4	1000–0 mV, 10 mV s ⁻¹	sigmoidal wave E_{cat} approx 700 mV	0.26 mM O ₂ Rotation not mentioned 0.1 M citrate phosphate buffer
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵³	HOPGE modified with naphthyl-2-carboxylate	6		sigmoidal wave E_{cat} approx 750 mV	Rotating disc electrode O ₂ saturated 0.1M phosphate buffer 25°C
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵⁴	Polycrystalline Au-disk electrodes modified with 20 nm Au nanoparticles	7.4	Linear sweep voltammetry 575–150 mV vs. SCE 2 mV s ⁻¹	sigmoidal wave E_{cat} approx 450-500 mV	Air saturated solution 50 mM phosphate buffer Room temperature
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵⁵	Au electrode with 15 nm Au nanoparticles	7	700–0 mV 10 mV s ⁻¹	sigmoidal wave E_{cat} approx 500-525 mV	Rotating disc electrode O ₂ saturated 0.1 M phosphate buffer 25°C
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁴⁶	Nanoporous Au electrodes	7	700–100 mV vs Ag/AgCl/sat. KCl 5 mV s ⁻¹	sigmoidal wave E_{cat} approx 500 mV	Stationary gold electrode 0.1 M citrate-phosphate buffer
Bilirubin oxidase from <i>Myrothecium verucaria</i>⁵⁶	Low density graphite electrodes modified with 5 nm Au nanoparticles	7.4	800–0 mV vs Ag/AgCl/sat. KCl 10 mV s ⁻¹	sigmoidal wave E_{cat} approx 500 mV	500 rpm, 1 atm O ₂ 0.1 M phosphate buffer 27°C
Bilirubin oxidase from <i>Trachyderma tsunodae</i>⁴⁷	SPGE	7	1000–0 mV 10 mV s ⁻¹	sigmoidal wave E_{cat} approx 700 mV	1 atm O ₂ Rotation not mentioned Buffer: 0.1 M citrate phosphate
Bilirubin oxidase from <i>Trachyderma tsunodae</i>⁴⁷	3mercaptoproionic (3MPA) acid modified gold	7	650–150 mV 1 mV s ⁻¹	sigmoidal wave E_{cat} approx 500 mV	O ₂ saturated 0.1M phosphate buffer 25°C

Bilirubin oxidase from <i>Trachyderma tsunodae</i>⁵⁷	Vitreous (glassy) carbon complexed with [Os-(4,4'-dichloro-2,2'-bipyridine) ₂ Cl] and cross linked with poly(ethylene glycol) diglycidyl ether (PEGDGE))	7.2	0–(–400) mV 5 mV s ^{–1}	sigmoidal curve E_{cat} approx -100 mV	Rotating disc electrode, 4000 rpm 1 atm O ₂ saturated, 20 mM PBS 37°C
Laccase from <i>Rhus vernicifera</i>⁵⁸	Gold/SAM modified covalently attached	7	700–100 mV 20 mV s ^{–1}	sigmoidal curve E_{cat} approx -410 mV	Air saturated buffer 21°C
Lacase from <i>Trametes hirsutus</i>⁵⁹	carbon cloth with osmium based redox polymer	5	925–525 mV 1 mV s ^{–1}	sigmoidal curve E_{cat} 820 mV	1 atm O ₂ rotation: 500 rpm and 4000 rpm 200 mM citrate buffer 37.5°C
Lacase from <i>Trametes hirsuta</i>⁶⁰	SAM modified gold: cysteine, cystamine, aminothiophenol	5	400–(–600) mV vs Ag/AgCl 20 mV s ^{–1}	O ₂ reduction begins at 100 mV vs. for Aminothiophenol but others show no difference from blank	O ₂ conc. or rotation not Mentioned 0.1 M phosphate buffer

In this table E_{cat} is used in order to describe the position of the catalytic wave and corresponds approximately to the halfway point of the steepest part of the wave. Standard calomel electrode reference electrode, $E_{\text{SHE}} = E_{\text{SCE}} + 0.241 \text{ V}$ at 25 °C. Silver/silver chloride reference electrode, $E_{\text{SHE}} = E_{\text{Ag/AgCl}} + 0.209 \text{ V}$ at 25 °C.

1.6 Fuel Cells and the ORR

The fuel cell concept was first derived by William R. Grove where the process of hydrogen and oxygen extraction via electrolysis was reversed. In the presence of platinum electrodes (also used as catalysts), this process allowed a current to be passed through the same circuit and the system was originally termed a ‘gas battery’.⁶¹ The first fuel cell was developed by Francis Bacon which ran on pure O₂ and H₂ using nickel electrodes and an alkaline catalyst.⁶²

Biofuel cells (BFCs) employ biocatalysts instead of the typical inorganic catalysts to generate currents using different fuels and oxidants. In the case of enzymatic BFCs, the chemical energy of the biofuel is converted into electrical energy (i.e. oxygen in the

ORR). The very first BFC was conceived in the early 20th century, when M.C. Potter used microbial cells on platinum electrodes to produce electricity.⁶³ In the early 1960s, Clark and Lyons then introduced the term ‘enzyme electrode’ using glucose oxidase (GOx) to oxidize glucose (using a Clarke oxygen electrode).⁶⁴ This was followed by the first report on an enzymatic fuel cell, where the electron-transferring process in GOx was utilised as a potential anodic reaction in a biochemical fuel cell, constructed of plexiglass, platinum-foil electrodes, and an ion-exchange membrane for conduction between the two anodic and cathodic compartments.⁶⁵

In a typical biofuel cell, the organism or enzyme on the anode oxidises substrates, hydrogen for example, and the electrons pass through the external circuit to the cathode where the electrons transfer to preferably a high-potential electron acceptor such as oxygen. Enzymes and micro-organisms are renewable resources which can conceivably be produced in large amounts, employ greater selectivity than noble metals and operate at low temperatures (this may be an advantage for producing implantable devices). However, there are problems with attachment of the enzyme/microorganisms to the electrode as well as the need for monitoring pH and temperature and further probing changes in enzyme/protein conformation on attachment.

A key issue in the development of enzymatic electrodes for biofuel cell applications is the efficient electrical communication between the enzyme and the electrode. If an enzyme is capable of transferring electrons to and from the electrode, the process is termed direct electron transfer (DET). However, when the redox unit responsible for electron transfer through the enzyme is buried deep in the enzyme, a redox active species or mediator is incorporated in order to overcome the kinetic barrier for electron transfer (i.e. the overpotential barrier) and facilitate electron transfer between enzyme and

electrode. This process is termed mediated electron transfer (MET). This thesis however specifically looks at the DET of enzymes attached to electrode surfaces.

1.6.1 Cathodic redox enzymes

As mentioned earlier, the catalytic site of MCOs such as *MvBOx* and laccases consists of four copper atoms including the T1 primary electron entry site,⁵² which can electrically couple to and receive electrons from an electrode. High potential laccases exhibit T1 redox potentials of up to 0.78 V vs. NHE⁶⁶. Bilirubin oxidase exhibits lower T1 redox potentials and therefore requires a greater thermodynamic push to initiate catalysis (the reported T1 reduction potential for *MvBOx* is 0.48–0.49 V vs. NHE at pH 5.3).^{23,51} However due to the attractive properties of working at neutral pH and displaying important biochemical properties such as high activity, stability, and low extent of glycosylation,^{23,67} bilirubin oxidase has been used in a variety of biotechnological applications such as biosensor construction,^{56,68} dioxygen biocathodes operating in a H_2/O_2 biofuel cell⁶⁹⁻⁷¹ and glucose/ O_2 biofuel cells.⁵⁴

1.7 Enzyme immobilisation for DET

Studies of DET between proteins and electrodes yield important information on the thermodynamics and kinetics of biological redox processes and DET reactions. The first reports on DET with a redox active protein were published in 1977 where Eddows and Hill investigated the reversible electrochemistry of cytochrome c immobilized on modified gold electrodes. Later, Yeh and Kuwana showed the same reversible electrochemistry (through the use of cyclic voltammetry) of cytochrome c immobilized on tin-doped indium oxide electrodes.⁷²⁻⁷⁴ Berezin *et al.* later showed that DET existed for larger redox proteins with enzymatic activity and that in the presence of oxygen, laccase-modified carbon electrodes exhibited DET. However, under anaerobic conditions

the laccase-modified graphite electrode gave a response indistinguishable from the background voltammograms obtained in the absence of the enzyme.^{75,76} DET between carbon electrodes and different laccases under anaerobic conditions was confirmed with cyclic voltammetry and square wave voltammetry in work by Thuesen *et al.* and Christensen *et al.* respectively^{77,78}

For biosensors based on DET, the advantages are the superior selectivity of operating in a potential window closer to the enzyme's redox potential and tuning the electrode surfaces for targeted attachment of the redox enzyme, taking into account the electron tunnelling distance between enzyme and electrode.⁷⁹⁻⁸¹

1.7.1 Choice of surface

The first example of DET for the ORR with *MvBOx* was reported by Tsujimura *et al.* on carbon electrode surfaces with high crystal graphite edge density. It was found that enzyme activity depended on the functional groups present on the graphite surface.⁸²

The same group reported the electrostatic immobilization of *MvBOx* into a cationic poly-lysine matrix generating a current density of up to 1 mA cm^{-2} ,⁸³ and they later reported the use of carbon cathodes in a fructose/ O_2 biofuel cell.^{51,84} Kokoh *et al.* adsorbed *MvBOx* onto carbon black electrodes⁸⁵ and further to this, Hanashi *et al.* developed a self powered glucose sensing system, cross-linking *MvBOx* with glutaraldehyde on a ketjen black cathode.⁸⁶ More recently Flexer *et al.* have used carbonaceous foams in the development of enzymatic BFCs.⁸⁷

In PFV, typically, unmodified pyrolytic graphite edge plane (PGE) electrodes have been used.^{88,89} In these studies, the spontaneous adsorption of enzyme to the surface of these graphite electrodes can be attributed to the variety of oxygen-containing surface groups on PGE surfaces and the ability of these to act as sites for non-specific adsorption.⁹⁰ These surfaces are freshly generated by polishing or sanding, and thus the

surface of the electrode (and consequently the amount of enzyme adsorbed and its orientation distribution) can be heterogeneous and variable. This variability in adsorbed enzyme coverage (Γ in Equation 1.52) results in a variability in current response and difficulty in quantitative analysis of enzyme activity.

To address the issue of reproducibility, gold films can be formed on substrates with near atomic flatness. Unmodified gold surfaces, however, cause most proteins to behave unpredictably and therefore to prevent this, self-assembled monolayers (SAMs) are formed using short, homogenous thiol-terminated layers.

1.7.2 Thiol chemistry

Self-assembled monolayers involve the self-assembly of thiol terminated alkyl chains on gold (in the context of this thesis), forming structurally well-defined and stable chemical interfaces with tuneable functionality and film thickness.⁹¹⁻⁹³ These structures are made up of three distinct parts (Figure 1.11): (1) a head group which interacts strongly with the solid support, (2) an alkyl chain spacer which gives stability to the assembly via van der Waals forces and (3) a functional group which couples to the biological molecule being targeted (however coupling is not always directly).

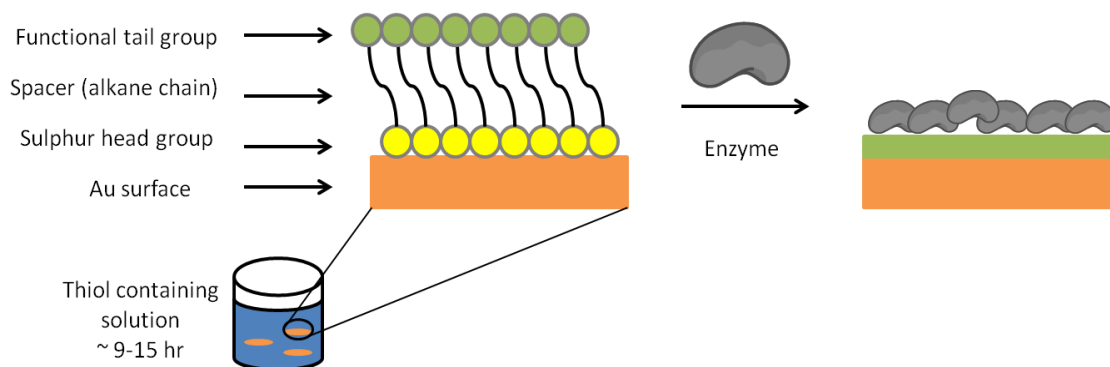


Figure 1.11. Diagrammatic representation of an alkanethiol self-assembled monolayer used for enzyme immobilization and surface specific attachment. Typical preparation times were between 9 and 15 hrs and involved placing gold substrates in 10 mM ethanolic solutions of the thiol and grown under a nitrogen atmosphere.

The work in this thesis uses gold-coated quartz crystals with self-assembled monolayers (SAMs) of thiol-terminated molecules to provide a flat, well-ordered, chemically homogenous surface for enzyme adsorption.⁹⁴

1.7.2.1 Immobilization via non-covalent interactions

Direct electron transfer between immobilised MCOs, including *MvBOx*, on modified gold electrodes has been intensively studied.^{28,47,95} Immobilization of biomolecules on SAMs on surfaces can be achieved through electrostatic, hydrophobic or polar interactions.⁹⁶ Caglayan and others reported the attachment of bovine serum albumin (BSA) on a carboxyl terminated SAM using electrostatic interactions between the terminal CO₂ group of the thiol layer and the NH₂ groups on the surface of the BSA molecule.⁹⁷ Similarly, de Groot *et al.* looked at the electrostatic immobilization of cytochrome *c* on a carboxylic acid terminated thiol on gold. They found that immobilization could only be achieved for a pH in the range 3.5-5.5 reflecting the fact that the protein is only sufficiently positively charged below pH=5.5.⁹⁸

Salaj-Kosla reported the adsorption of *MvBOx* on nanoporous gold electrodes which generated up to 0.8 mA cm⁻² (after an epoxy cap was added) and later used this cathode in a BFC.⁹⁹ Wang *et al.* reported a mediator-less enzymatic biofuel cell with a cathode made with 20 nm gold particles modified with *MvBOx*. They presented its use in a BFC as a power source for contact lenses.^{54,100}

More targeted immobilization was seen in a study by Yamamoto *et al.* where they immobilized cytochrome *c* through interaction of a pyridine-terminated thiol group and the heme of the cytochrome. The pyridine unit associated directly with the redox-center of the enzyme therefore giving a better defined orientation between protein and electrode.¹⁰¹ More recent studies have shown this to be true when targeting MCOs for

electrocatalysis. In a study by Blanford *et al.*, graphite electrodes were functionalised with aminoanthracene residues that targeted immobilization of the enzyme near to the electron entry point (T1 site) of laccase from *Trametes versicolor* and showed greater catalytic activity compared to graphitic surfaces with no modification.¹⁰²

Ohno *et al.* covalently bound MvBOx on electrodes covered with thiol-modified gold nanoparticles producing 5.2 mA cm^{-2} at 4000 rpm rotation rate.⁵⁵ More recently, Gutierrez-Sanchez *et al.* reported the immobilization of MvBOx covalently bound onto gold nanoparticles. The biocathode generated $140 \text{ } \mu\text{A cm}^{-2}$ under serum-mimic conditions and was stable for at least 6 days.⁵⁶

1.7.2.2 Orientation and electron tunnelling distance

When considering the DET mechanism for enzyme adsorption, it requires the catalytic centre of the enzyme to be within the correct tunnelling distance to the electrode surface. In an early study by Shleev *et al.*, DET was studied on spectrographic graphite electrodes (SPGE) and it was also shown that the orientation (i.e. the positioning of the T1 site) of bilirubin oxidase from *Trachyderma tsunodae* was dependant on the nature of the electrode material. On SPGE, the enzyme was oriented with the T1 close to the electrode surface while on gold surfaces it was found to orient with the T2/T3 centres closer to the surface.⁴⁷

In another study by Tominaga *et al.*, it was shown that bilirubin oxidase showed electrochemical activity when adsorbed onto carboxylic acid-modified gold surface compared to a complete lack of activity on alkyl, amine and alcohol-modified surfaces. In this study, it was also shown that the electron tunnelling distance from the T1 copper site to the electrode interface, based on the carbon chain length of a carboxylic acid terminated thiol ($\text{C}_n\text{-COOH}$ where $n = 2, 5, 7$), was approximately 17Å .¹⁰³ Therefore to

facilitate DET in enzymatic biofuel cell conditions, not only is the surface chemistry, but tailoring the surface to control electron transfer distance is also important. DET between enzyme and electrode has been achieved using different immobilization strategies in attempts to appropriately orient the enzymes for efficient electron transfer.^{28,104,105}

1.7.2.3 Immobilization via covalent interactions

Proteins can be anchored through covalent bond formation via the reaction with exposed functional groups on the surface of functionalized thiol-modified surface.

In early studies Dong *et al.* reported the covalent immobilization of cytochrome *c*, cytochrome *c* oxidase, and horseradish peroxidase onto gold surfaces modified with 3-mercaptopropionic acid using EDC chemistry.¹⁰⁶ Herrworth *et al.* later immobilized antibodies onto a thiol-modified gold surface via the activation of terminal carboxylic acid groups using a similar protocol.¹⁰⁷ This method of immobilization is widely used because it requires a single step with no external coupling agent and also keeps the protein molecule permanently fixed and reduces enzyme leeching. EDC-NHS chemistry was also used to functionalise 3-mercaptopropionic acid-modified gold, for covalently binding bilirubin oxidase from *Trachyderma tsunodae*, however this covalent attachment positioned the enzyme in an orientation with the T2/T3 copper sites closer to the electrode surface than the T1 site.⁴⁷ This resulted in lower catalytic competency compared to the same enzyme immobilized on a graphite electrode and indicates that a balance between stable immobilization and correct orientation is needed.

1.8 Structured electrodes for MET

Mediated electron transfer is dependent on the nature of the electrical connection between the electrode and the redox enzyme, where electrons are shuttled from the electrode to the enzyme via a third chemical species, called a redox mediator. Different

types of mediators such as ABTS have been used. Tsujimura *et al.* reported the first example of oxygen reduction in phosphate buffer containing ABTS using *MvBOx* coated onto a carbon felt electrode.¹⁰⁸ The electrode was held at -0.17 V vs. Ag|AgCl and generated a current density of 0.5 mA cm^{-2} over 2 hr. This system was then incorporated into a H_2/O_2 fuel cell setup and operated (at a potential close to the standard electromotive force for the reaction of H_2 and O_2 to water) at neutral pH and ambient temperature.¹⁰⁹

Kamitaka *et al.* used ABTS in their study on the effects of axial ligand mutations on the electrocatalytic activity of *MvBOx* and another study by Habrioux *et al.* showed that *MvBOx* adsorbed onto carbon black (Vulcan XC72R) incorporating ABTS (albeit immobilized within a Nafion[®] matrix) produced an increase in the half wave potential (E_{cat}) from 0.43 to 0.46 V vs. SCE (i.e. the thermodynamic ‘drive’ required for catalysis was reduced).⁸⁵

Another approach for enzyme immobilization is to entrap the enzymes in redox polymers, originally proposed by Heller. These redox polymers, where the permeation of biological species and electrons is rapid, ensure an efficient electrical connection between electrode and enzyme, regardless of enzyme orientation.¹¹⁰

Mano and Heller reported that *MvBOx* cathodes could be operated under physiological conditions using the osmium based redox polymer $[\text{Os} (4,4'\text{-dichloro-2,2'\text{-bipyridine)}_2\text{Cl}]^{+/2+}$. The first example involved immobilization of *MvBOx* onto the Os redox polymer operating at a current density of 5 mA cm^{-2} when held at -0.18 V vs. the reversible $\text{O}_2/\text{H}_2\text{O}$ electrode potential (1.229 V vs. SHE).¹¹¹ The second example involved the immobilization of bilirubin oxidase from *Trachyderma tsunodae* and this cathode generated a current density of 3 mA cm^{-2} whilst holding at a potential of -140 mV, a potential much closer to the reversible $\text{O}_2/\text{H}_2\text{O}$ potential.⁶⁹ Both these cathodes were demonstrated to be more efficient than platinum for the ORR.¹¹²

Protein–surface interactions play a major role in the amount of catalytic activity seen within a typical redox-active MCO. Functionalising surfaces to affect protein adsorption is common and therefore the thermodynamic and kinetic properties of enzyme adsorption are important factors when considering protein behaviour. Enzyme orientation is also an important factor when deciding which immobilization procedure to use and the strength of attachment (i.e. whether permanent or not) and position of attachment will ultimately affect how well the enzyme performs. The well-established surface-modification routes for gold electrodes provide an attractive platform for tailoring a surface for enzyme immobilization for both DET and this can be further probed using a quartz crystal microbalance.

1.9 Quartz crystal microbalance

The quartz crystal microbalance is a technique for measuring the adsorption of small amounts of material (ng cm^{-2}) onto the surface of a quartz crystal in solution. This method uses the piezoelectric effect of quartz to measure changes in the mass attached to the crystal. Typically, thin layers of gold (~ 100 nm) are evaporated or sputtered onto both sides of an AT-cut α -quartz crystal.

1.9.1 Piezoelectricity

Piezoelectricity is the ability of certain materials, such as quartz, Rochelle salt and tourmaline, to convert mechanical energy into electrical energy and vice versa.

The *direct piezoelectric effect* occurs when these materials are subjected to mechanical stress which generates an electric charge proportional to that stress. The physical basis of operation of the QCM originates in the *inverse piezoelectric effect* in that the same materials become strained when an electric field is applied, the strain/stress again being

proportional to the applied field. This has been exploited in the development of oscillator circuits used in printing equipment and ultrasound imaging.^{113,114}

1.9.1.1 Quartz crystal

Quartz is the most stable form of silica (or silicon dioxide, SiO_2) and the cut-angle of quartz, with respect to the quartz crystal orientation, determines the mode of oscillation. An AT-cut crystal, which is most commonly used for QCM applications, is fabricated by slicing through a quartz rod with a cut angle of $35^\circ 15'$ with respect to the crystallographic optical z-axis, as shown in Figure 1.12.

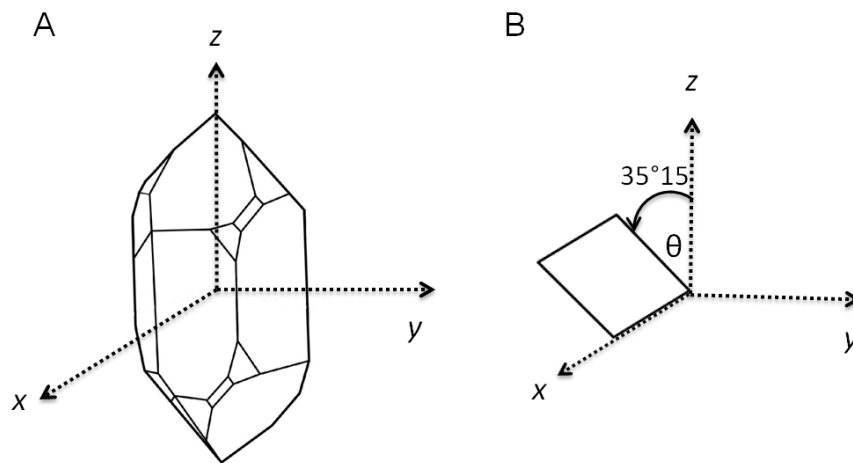


Figure 1.12. A typical AT-cut crystal. AT-cut crystals can be cut at $35^\circ 15'$ to the crystallographic z-axis.

AT-cut crystals operate using a thickness shear mode of vibration (Figure 1.13A) where application of an alternating field gives rise to vibrations in two directions: the first runs parallel to the applied field (normal to the crystal surface), and the second propagates as a transverse shear wave through the crystal in the thickness direction (Figure 1.13B).

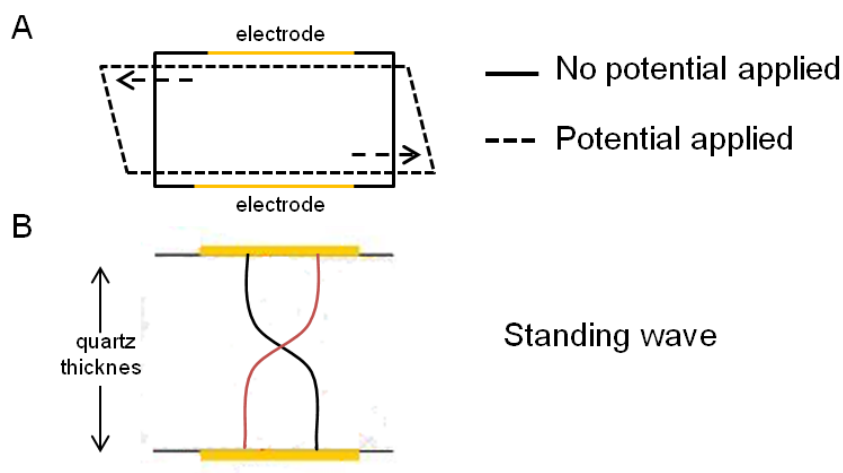


Figure 1.13. Thickness shear mode of vibration. Panel A shows the thickness shear mode of vibration when a potential is applied across the crystal and Panel B shows the propagation of a transverse wave through the thickness of the crystal (black line is the forward wave and red line is the reflected wave)

Standing shear waves are generated when the thickness of the crystal (t_q) is twice the acoustical wavelength:¹¹⁵

$$t_q = \frac{v_q}{2f_1} = \frac{\lambda_q}{2} \quad \text{Equation 1.53}$$

where v_q is the velocity of the acoustic wave generated in the quartz (3336 ms^{-1}), λ_q is the wavelength of the acoustic wave in quartz and f_1 is the fundamental frequency.

In QCM studies a thin disk sliced from single crystals of the α -quartz is used. The disk is sandwiched between two metal electrodes that are vapour deposited on either side of the crystal. Gold electrodes have been the most commonly used in QCM studies, because of the ease with which Au is evaporated. However, Cu, Ni, Pt and other metals have also been employed.¹¹⁶

1.9.2 Gas-phase QCM

Some of the early chemical applications of QCM were to measure mass binding from gas-phase species to quartz crystal surfaces which lead to the development of some of the

earliest chemical sensors for moisture and volatile organic compounds,^{117,118} environmental pollutants,¹¹⁹ and gas-phase chromatography detectors.¹²⁰

The idea of using a gas phase piezoelectric sensor was first suggested by King.¹¹⁷ Sauerbrey was then responsible for developing a gravimetric method for analysing mechanically coupled mass in the gas phase.¹²¹ In later work by Tsionsky, gas-phase adsorption on the QCM was studied under constant pressure, whilst only the partial pressures of the inert gas and substance-containing gas were changed.¹²²

Two methods were developed by which resonance frequency changes of a quartz crystal were recorded from the gas phase.¹²² In the ‘stationary method’ a stable frequency was established whilst holding the crystal in a chamber filled with an inert gas at a given pressure. The substance being studied was then mixed with the inert gas and fed into the chamber. This was carried out at different partial pressures in order to maintain the total pressure constant.

In the ‘flow method’ the inert gas was passed through a saturator containing the substance of interest. The partial pressure of the gas now containing the substance was controlled by adjusting the temperature. The down side of both the above methods was that the recorded frequency change within the QCM crystal was a result of both substance gas adsorption and inert gas adsorption. Stockbridge derived an equation for how the frequency of a quartz crystal linearly increases with pressure:¹²³

$$\frac{\Delta f}{f_0} = C \cdot p \quad \text{Equation 1.54}$$

where p is the pressure and C , the pressure constant ($1.045 \times 10^{-5} \text{ MPa}^{-1}$).

1.9.3 Mass sensitivity in the liquid phase

In the 1980s, Kanazawa and Gordon showed that a QCM could be used to study adsorption processes in liquids.¹²⁴ Nomura and Hattori then showed that measurements

can be taken with the crystal immersed in a liquid to detect micromolar concentrations of cyanide in solution.¹²⁵ Caruso *et al.* showed the advantages of using the QCM alongside surface plasmon resonance (SPR) and atomic force microscopy (AFM) to identify hydrated and dry ferritin mass coverage, thickness and density on gold substrates for potential use in immunosensors.¹²⁶

1.9.3.1 Mass measurements

Equation 1.53 shows that increasing the thickness of the crystal (t_q) will cause an increase in the wavelength of the acoustic wave in the quartz crystal ($2t_q$) and an overall decrease in the fundamental frequency. On this basis, it can be assumed if the overall mass (thickness) on the crystal changes this will result in a negative change in the frequency.¹²¹

The quartz crystal microbalance (QCM) utilizes this mass sensing based on the assumption that the deposition of material onto a crystal will exist at the antinodes of the standing waves that propagate across the thickness of the crystal. In this way the frequency change is calculated as if it were due to an increase in the crystal thickness itself.¹²¹

The shift in resonant frequency caused by a very small change in crystal thickness is given by the differential of Equation 1.55 (Equation 1.55 is a rearranged form of Equation 1.53).

$$f = \frac{1}{2}vt^{-1} \quad \text{Equation 1.55}$$

$$df = -\frac{1}{2}vt^{-2}dt \quad \text{Equation 1.56}$$

Dividing Equation 1.56 by Equation 1.55 gives the negative relationship between Δf and Δt ;

$$\frac{df}{f} = -t^{-1}dt \quad \text{Equation 1.57}$$

The increase in mass on the crystal (i.e. the formation of a thin layer (m_{ads}) on the crystal surface) is given by Equation 1.59:

$$m_{ads} = \rho_{ads}t \quad \text{Equation 1.58}$$

$$dm_{ads} = \rho_{ads}dt \quad \text{Equation 1.59}$$

and therefore the change in thickness of a crystal with an adsorbed mass of m_{ads} is

$$dt_{ads} = \frac{1}{\rho_{ads}} dm_{ads} \quad \text{Equation 1.60}$$

Assuming that the additional mass and the crystal have the same density combining Equation 1.60 with Equation 1.57 gives

$$df = -f\rho_{ads}^{-1}t^{-1}dm_{ads} \quad \text{Equation 1.61}$$

Making t the subject of Equation 1.55 and combining it with Equation 1.61 gives the relationship between the change in frequency of the crystal and the change in mass:

$$df = -2\rho_{ads}^{-1}f^2v^{-1}dm_{ads} \left(\text{units of } \frac{\text{m}^3}{\text{kg}} \cdot \frac{1}{\text{s}^2} \cdot \frac{\text{s}}{\text{m}} \cdot \frac{\text{kg}}{\text{m}^2} \right) \quad \text{Equation 1.62}$$

This rearranges to give the Sauerbrey equation

$$\Delta f = -n \frac{2f_1^2}{\rho_{ads}v_{ads}} \cdot \Delta m_{ads} = -nC \cdot \Delta m \quad \text{Equation 1.63}$$

where n is the harmonic number and C is the mass response (Sauerbrey constant) of the crystal. The crystals used in this thesis have a fundamental frequency, f_1 , of 4.95 ($\pm$ 0.05) MHz and utilise an AT-cut quartz crystal where the density of quartz, $\rho = 2648 \text{ kg m}^{-3}$ and the shear velocity (v) = 3336 m s^{-1} , yielding a mass response that equates to $1.77 \times 10^{-7} \text{ kg m}^{-2} \text{ Hz}^{-1}$.

Quartz crystals may be operated at the fundamental or higher odd harmonics. The fundamental frequency of ‘AT-cut’ crystals is related to the thickness of the crystal

(Equation 1.56) and so for a crystal resonating at a fundamental frequency of 5 MHz the crystal thickness is approximately 330 μm , while a 15 MHz crystal is three times thinner.

When a potential is applied to a quartz crystal operating in thickness shear mode, the crystals resonate at multiple frequencies (harmonics). These harmonics represent the mechanical shear modes in the crystal and as a result, the change in frequency can be monitored at any odd harmonic as well as the fundamental (resonance) frequency. At lower harmonics, acoustic energy trapping is inefficient and leads to higher sensitivity to bulk changes, crystal mounting and mechanical vibrations. Energy trapping refers to the idea that the energy contained in the mechanical oscillations of the crystal is confined to the area of the crystal used as the electrode. The energy trapping is less confined at lower harmonics and is therefore more sensitive to mounting and small vibrations during setup.¹²⁷ Due to the linear relationship between mass response and harmonic number (Equation 1.63), operating the crystals at higher frequencies is desirable.

Operating the crystals at higher frequencies is desirable for increased mass sensitivity due to the linear relationship between harmonic number and frequency change (Equation 1.63). However, thinner crystals, which have a higher fundamental frequency, are more fragile, so a compromise is needed where the crystals operating at lower fundamental frequencies are driven at higher harmonics. Using higher-frequency harmonics for analysis rather than a thinner crystal with high fundamental frequency gives the same mass sensitivity without the accompanying fragility. However, the higher harmonics experience overlap between anharmonic sidebands (those nodes which are perpendicular to the plane of the frequency resonator) which cause interference between actual signals and spurious responses, ultimately resulting in low signal-to-noise.¹²⁸ When adsorbate reaches the crystal surface, the increase in the mass of the crystal is measured as a

decrease in frequency Figure 1.14. If evenly distributed, added mass can be seen as a direct increase in thickness of the crystal itself.

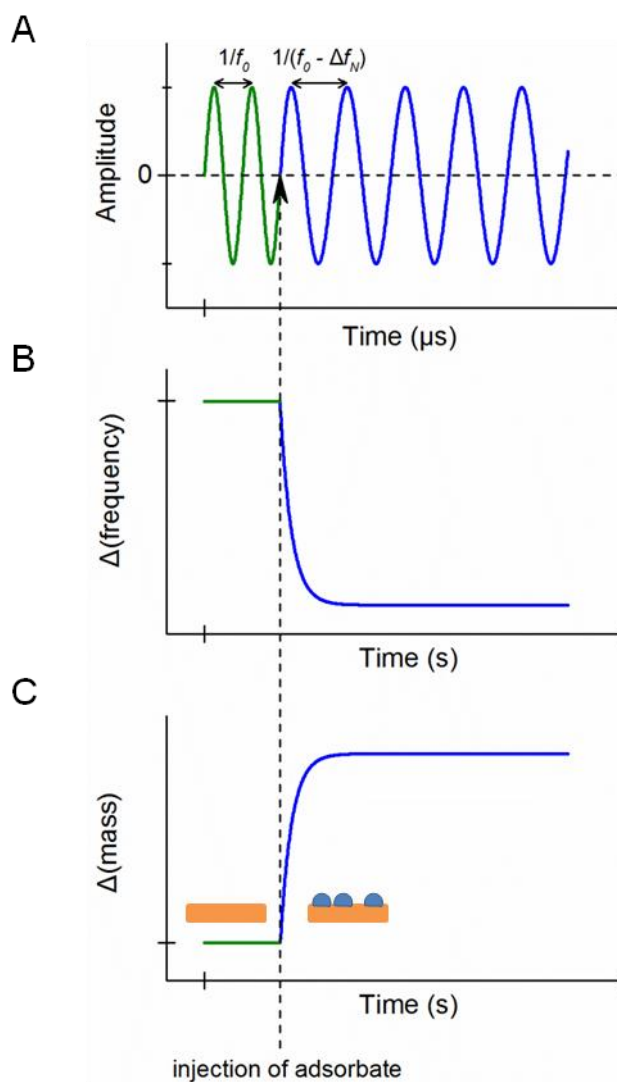


Figure 1.14. Mass sensing diagram. Panel A shows the decrease in frequency of the resonant harmonic when mass is adsorbed to the surface of the quartz crystal. Panel B shows the measured frequency change using the Q-sense measurement apparatus. Panel C shows the associated increase in mass upon injection. The time scale resolution is different for Panel A compared to Panels B and C.

QCM analysis has limitations dependant on the viscosity and density of the substance in question. When a vibrating crystal is exposed to a viscous medium there is a decrease in frequency caused by an additional shear drag of the solution, according to Equation 1.64.

$$\Delta f = -f_o^{3/2} [(\rho_l \eta_l)(\pi \rho_q \mu_q)]^{1/2} \quad \text{Equation 1.64}$$

The relaxation time needed for gas molecules to equilibrate thermally is dependent on the loss or gain in kinetic energy once contact with the vibrating surface has been made and only becomes small enough when the pressure is above 300 torr.¹²² In fluids, this relaxation time is much shorter.

1.9.3.2 *Limitations of the Sauerbrey equation*

The added mass on the crystal can be treated as an equivalent thickness change of the crystal itself. However this only holds true if the additional mass meets three conditions:¹²⁹

1. The additional mass is relatively small compared to the overall weight of the crystal.
2. It is rigidly adsorbed.
3. The additional mass is evenly distributed across the active area of the crystal.

However when the adsorbed mass is sufficiently viscous and soft in nature, the adsorbed layer undergoes deformation. The larger and more irregular the molecule, the more the crystal oscillation will cause the adsorbate to deform. The adsorption of large proteins such as multicopper oxidases creates a scenario where the assumptions above break down; the protein is large (6-8 nm diameter, 60-64 kDa)^{17,24} and easily deforms once adsorbed to a crystal surface.^{115,130} Also, proteins are often heavily hydrated and hence the QCM is, in fact, a detection technique for the dynamic (hydrated) mass of biological molecules. As the size and the structural flexibility of the biological molecules increases, a second parameter is introduced to account for a soft adlayer's resistance to vibration and its ability to store mechanical energy, that is, its viscoelasticity.¹³¹

1.9.4 The dissipation factor

1.9.4.1 The quality factor

The Q factor is defined as the ratio of energy stored in the resonator to the amount of energy dissipated by the generator, per oscillation cycle, at the resonant frequency;

$$Q = 2\pi \cdot \frac{\text{Energy stored}}{\text{Energy dissipated per cycle}} \quad \text{Equation 1.65}$$

The Q-factor is dimensionless and is a measure of the strength of the damping of resonator oscillations and is an indication of whether an oscillator is under-damped or over-damped. It also characterizes a resonator's bandwidth relative to its resonant frequency. High Q values, typically >0.5 , have very little damping with a lower rate of energy loss compared to the stored energy, meaning resonance frequencies have long decay times.

1.9.4.2 The dissipation factor

‘Dissipation’ quantifies the vibrational energy an oscillator loses per cycle and is a measure of the viscoelastic dampening from an adsorbate on a QCM sensor. Dissipation is the energy lost to inelastic collisions that occur at the surface of the crystal. Soft films increase collisions with surrounding molecules resulting in higher dissipation. Rigid films, having fewer collisions, give a lower dissipation. This parameter is used to assess the rigidity and arrangement of an adlayer immediately after adsorption and to monitor how that layer changes over time, for example, through structural rearrangements or denaturation.¹³²

The dissipation factor, D , is now taken into account and is the inverse of the Q factor;

$$D = \frac{\text{Energy dissipated per cycle}}{\text{Energy stored} \cdot 2\pi} = \frac{1}{Q} \quad \text{Equation 1.66}$$

When an adsorbate is rigidly coupled to the surface of a crystal, the changes in viscoelasticity are minimal as the layer dissipates energy slowly. The idea is similar to that of a tuning fork. If the fork is struck and held in air, it will resonate at a given frequency until all vibrational energy is lost to the surrounding air molecules. If at some point while it resonates, the fork were to be submerged into liquid, the vibrational energy would be damped more heavily and the resonance would decay more rapidly. In a similar way, a softer layer deposited on the crystal dampens the oscillation of the resonator, more energy is dissipated and the resonance decays more quickly than for a more rigidly adsorbed layer (Figure 1.15C and D).

When considering adsorbed protein, the layer is a composite of the adsorbed species together with trapped water molecules.¹²⁹ To this end, the dissipation can be used to look at how water associated with the protein can affect the stability of different surface-bound species and what this means for structure–function relationships.

One method for measuring the dissipation is by monitoring the decay in the amplitude of a crystal's oscillation after the drive potential has been turned off (Figure 1.15A&B).

The rate of decay depends on the environment of the crystal, especially the adsorbate. The amplitude of the oscillation (A) is fitted to an exponentially damped sinusoid from the point at which the resonator drive circuit is switched off ($t = 0$):

$$A(t) = A_0 e^{-\frac{t}{\tau}} \sin(2\pi f t), t \geq 0$$
Equation 1.67

where τ is the decay time constant and is related to dissipation by:

$$D = \frac{1}{\pi f \tau}$$
Equation 1.68

The dissipation response is typically plotted as a *change* in energy dissipation between baseline conditions (e.g., a clean crystal in aqueous buffer) and the loaded crystal in the same medium.

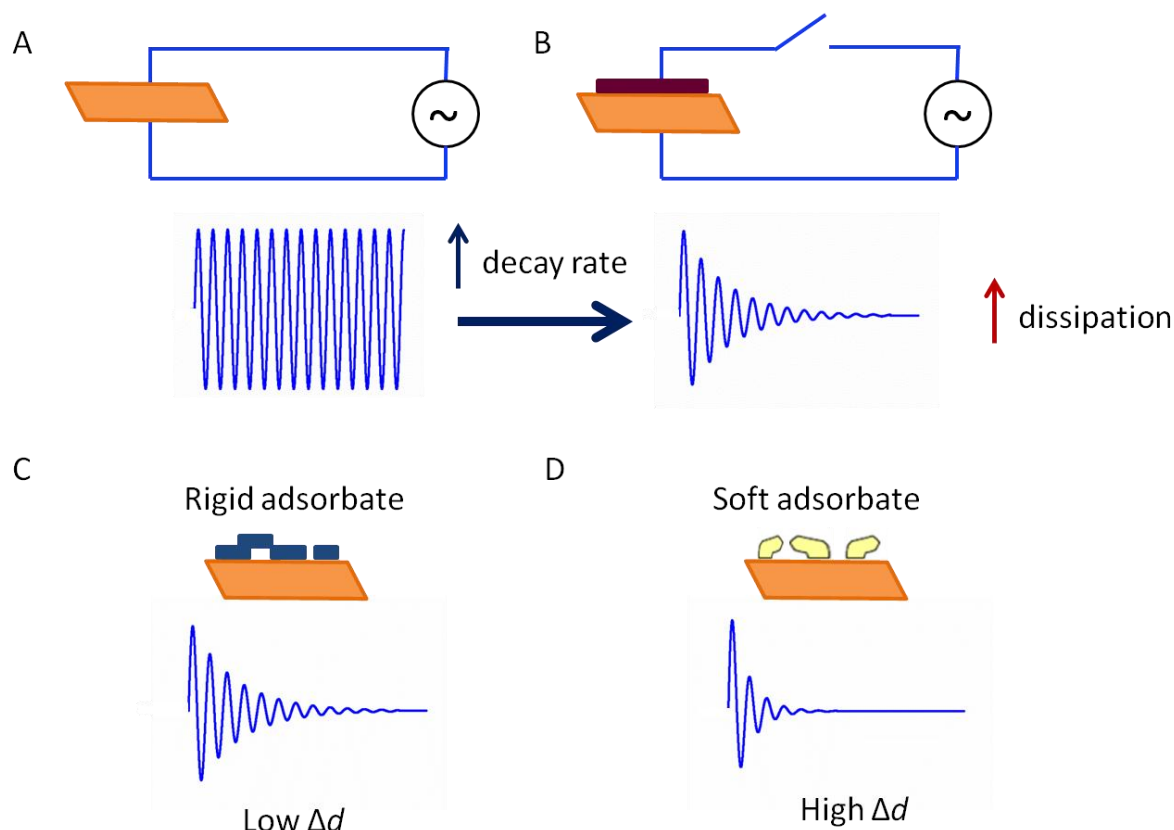


Figure 1.15. Dissipation sensing. Schematic illustrating one method of quantifying dissipation between rigid and soft adlayers. While an alternating potential is applied across the crystal there is an associated sinusoidal electrical current response (A). If the circuit is then switched off, the amplitude will decay exponentially with time (B), and this decay time constant is inversely proportional to dissipation. Rigid layers experience fewer inelastic collisions, so experience a relatively low decay rate and lower dissipation (C). Soft layers experience more inelastic collisions and higher decay rates and therefore higher dissipation (D).

1.9.5 Motional resistance

Measuring motional resistance, the energy lost within the vibrating area of a resonating crystal, is an alternative method to quantify energy dissipation from an adlayer.¹³³ Oscillating mechanical systems can be represented as an equivalent Butterworth–van Dyke electrical circuit, consisting of a ‘static’ arm that represents the resonator’s electrical characteristics and a ‘motional’ arm that represents its mechanical properties.¹³⁴

The static arm consists of a capacitance, C_0 , which arises from the electronic capacitance of the sensor. The motional arm is modelled by a motional resistance, R_m ,

which relates to the energy losses of the sensor; a motional capacitance, C_m , which is attributed to the stored energy during oscillation which in turn is related to the nature of adsorbate; and a motional inductance, L_m , which models the kinetic energy of the resonator system and represents the inertial component of displaced mass during oscillation (Figure 1.16, Panel A). Provided there is a sufficient amplification gain, the voltage returned on a load resistor (R_L) positioned outside the series circuit (Figure 1.16B) will sustain the oscillation of the crystal. This load resistance is a combination of the motional resistance of quartz and the resistance due to energy losses on mass loading.

The static capacitance generates a lead current that is driven into the load resistor with a known resistance and this must be cancelled. To this end, an amplifier (Amp) is introduced into the circuit which drives a transformer (Trf) with two secondary windings, with one of the windings inverting the amplified voltage. This then generates a current via a variable capacitor, C_v , and cancels out C_o ($C_v = C_o$). In a series resonance, the reactance of L_m and C_m then cancels and leaves just R_m and R_L , which needs a gain, A_v , to maintain oscillation:

$$A_v = \frac{R_m + R_L}{R_L} \quad \text{Equation 1.69}$$

In this way, if the gain is known, the motional resistance of the adsorbed species can be measured as:

$$R_m = R_L \cdot (A_v - 1) \quad \text{Equation 1.70}$$

Geelhood *et al.* have shown that as long as the static quartz capacitance is properly cancelled, typical dissipation measurements are in good agreement with measurements of motional resistance and are related to each other by:¹³⁵

$$D = \frac{R_m}{\omega L_m} \quad \text{Equation 1.71}$$

where ω is the radial frequency.

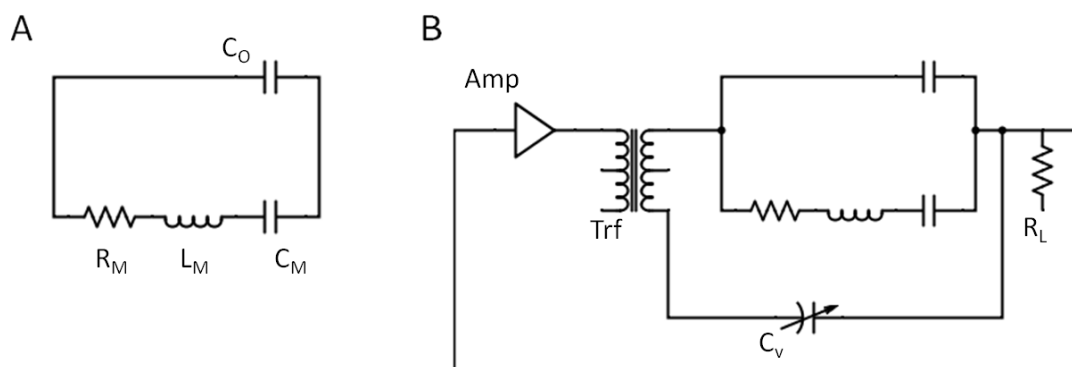


Figure 1.16. Butterworth–van Dyke motional resistance circuit. Panel A shows the Butterworth–van Dyke model for a quartz crystal resonator consisting of static and motional arms. Panel B shows the introduction of a voltage amplifier (Amp) and transformer (Trf) that inverts the applied voltage driven into a variable capacitor (C_v) to cancel out the static capacitance, C_O .¹³⁶

1.9.6 QCM and enzyme adsorption

Plots of frequency change (Δf) versus time can be used to identify the adsorption mechanism (e.g., Langmuir/random sequential adsorption, multilayer adsorption). Plots of dissipation change (Δd) versus time can highlight dehydration and structural evolution.

In Figure 1.17, panel A illustrates how QCM-D can be sensitive to protein orientation by comparing similar masses of protein in two different orientations. The vertically aligned molecules (a) are more flexible than those lying on the surface because they have fewer surface attachment sites (b), so have a higher dissipation for a similar adsorbed mass (Panel B).

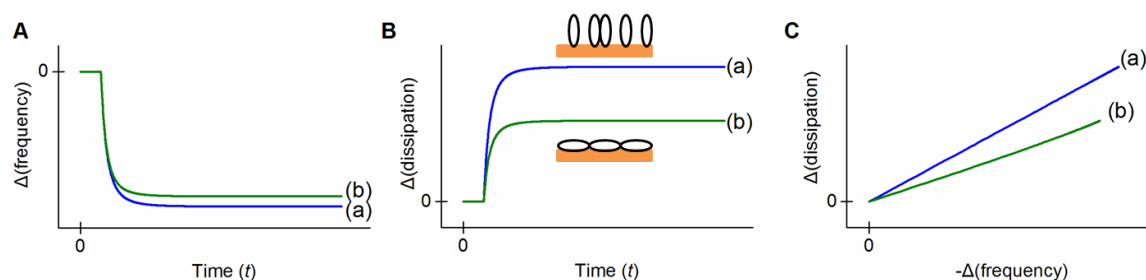


Figure 1.17. Model frequency, dissipation and Δd vs. Δf plots for protein adsorption and orientation change. Panel A indicates the differences between mass loading and dissipation when protein alignments change on adsorption. Panel B indicates the step-by-step processes of reversible binding and subsequent changes in gradient in the Δd vs. Δf plots.

The dissipation versus frequency plot shows the difference in gradient between the two orientations (Panel C). The orientation of the adsorbed species is therefore a necessary factor to consider when looking at biomolecular attachment with the QCM.¹³⁷

Figure 1.18 shows the reversible binding of antibody to antigen. Step 1 shows the relative frequency (Panel A) and dissipation changes (Panel B) on antigen adsorption. Step 2 shows antibody adsorption, which results in a large increase in dissipation, due to the elongated and more ‘wobbly’ structure of the antibodies. Step 3 shows that the antibody binding is fully reversible, because the dissipation returns to its original value. This appears in the dissipation versus frequency plot as a reversible change in the gradient (Panel C).

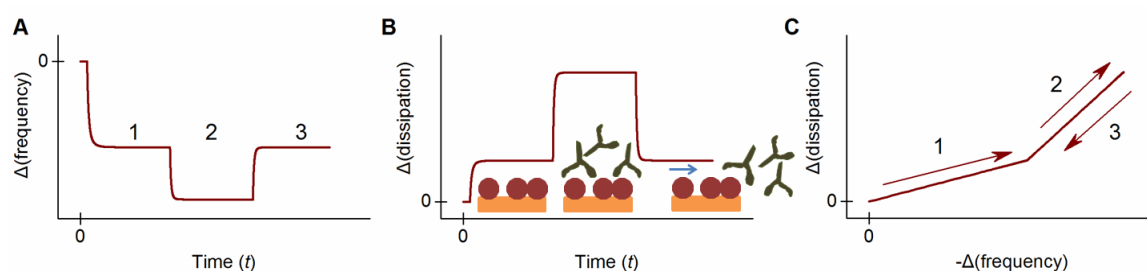


Figure 1.18. Model frequency, dissipation and Δd vs. Δf plots for reversible protein adsorption. Panel A shows the frequency changes during the reversible binding of antibody to antigen. Panel B shows the changes in dissipation changes on antigen adsorption. Panel C shows the reversible change in the gradient during adsorption and desorption. An additional coating to prevent non-specific binding is not shown.

An example of a typical frequency and dissipation measurement is shown in Figure 1.19. In this example plots of Δd vs. Δf for an adsorption typically show regions with different gradients. The gradient normalises the energy dissipation of the adlayer to its mass, so changes in slope indicate changes in adsorbate–surface interactions during the adsorption and desorption process. This is illustrated in Figure 1.19, which shows typical data from the adsorption of bilirubin oxidase from *Myrothecium verrucaria* (MvBOx) on a layer of 3-mercaptopropionic acid on a gold-coated quartz sensor.

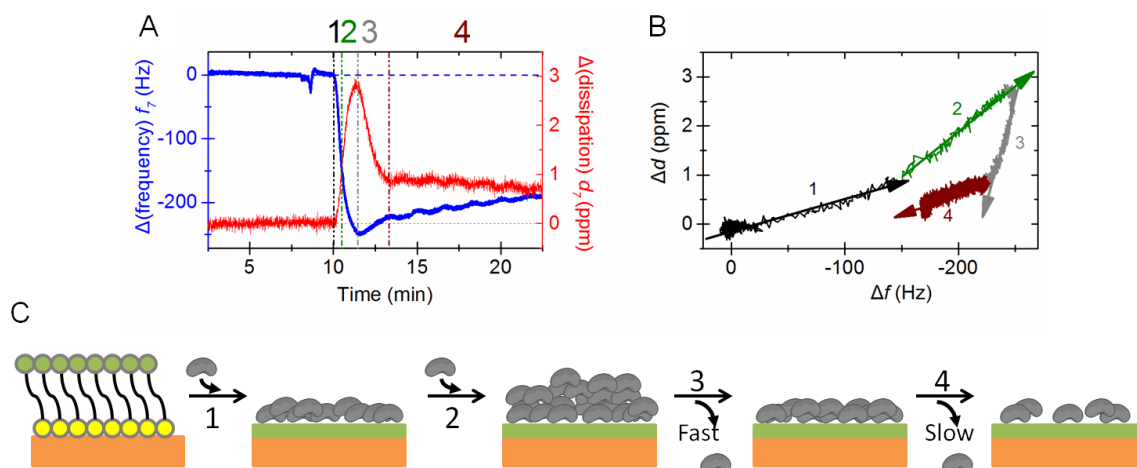


Figure 1.19. Actual representation of protein adsorption with frequency, dissipation and Δd vs. Δf plots. Panel A shows a typical frequency–dissipation response trace for enzyme adsorption. Panel B shows the Δd vs. Δf relationship with different binding regimes (numbered 1–4), which are illustrated as a cartoon in Panel C. The general pattern can sometimes take that of a two-stage adsorption. The first stage of adsorption indicates a primary layer formation (1) followed by a secondary layer which is more loosely adsorbed as it dissipates more energy for a given mass (2). This is followed by a combination of rapid mass loss from the loosely adsorbed secondary layer (3) and a slower desorption (with some stiffening and dehydration) of the more strongly adsorbed primary layer (4).

Protein adsorption,¹³⁸ and cell adsorption¹³⁹ dissipation studies suggest that any adlayer deposited onto the surface of an oscillating crystal will cause an increase in the dissipated energy and overall, an increase in the slope of the Δd vs. Δf relationship. As a result enzyme adsorption to a surface can be broken down into separate phases or regimes and complex binding patterns and subsequent structural mechanisms can be understood.

1.9.7 Electrochemical quartz crystal microbalance

The first combination of QCM with electrochemistry was in a report by Jones and Mieure. They used Δf measurements to establish concentration–mass relationships for cadmium layers on QCM electrode surfaces that were electrodeposited in an external electrochemical cell.¹⁴⁰ They later used this method to analyse a mixture of metals and establish sensitivity factors for each metal at each associated deposition voltage.¹⁴¹ The first reported application of E-QCM was from Nomura and Iijima for the determination of

Ag(I) concentrations by electrodeposition.¹⁴² A true electrochemical quartz crystal microbalance (E-QCM) combines the QCM with an electrochemistry module *in situ* to allow mass changes at electrode surfaces to be directly correlated to redox processes.^{131,143} More recently, the E-QCM has been applied to a variety of industrial applications ranging from probing structural changes in DNA for clinical diagnostics,¹⁴⁴ investigating inhibition of copper corrosion¹⁴⁵ and in refrigeration and portable power generation.¹⁴⁶

1.9.8 E-QCM-D: Uses and applications

Redox-active proteins play an important role in heterogeneous biocatalysis,^{147,148} biosensors^{149,150} and enzyme-catalysed biofuel cells.^{151,152} The E-QCM-D is well suited to such applications because of the technique's ability to relate electron-transfer processes, surface adsorption and structural rearrangements.^{49,153,154}

Jensen *et al.* highlighted differences between the mass adsorption of enzyme and resulting enzyme activity on flat and nanostructured gold electrodes.¹⁵³ The adsorption of enzyme to nanostructured interfaces was found to be significantly larger compared to flat surfaces, and no substantial desorption was observed within 24 h. A decreased enzymatic activity was observed over the same period of time and this indicated a slow conformational change of the adsorbed enzyme induced by the surface topology, which has implications in biosensor development and surface structure design.

Olejník *et al.* have shown the difference in electroactivity between laccase immobilized on charged surfaces. Gold electrodes were functionalized with phenyl groups ending with aminoethyl or carboxyethyl entities via the electrochemical reduction of 4-aminoethylbenzene- or 4-carboxyethylbenzene-diazonium salts. The ORR current on the positively charged layers had an onset potential close to the T1 reduction potential,

however for negatively charged layers, the onset of reduction was shifted by 0.04V to a more negative potential. The electrocatalytic current value was also significantly smaller on the negatively charged surface although the amount of adsorbed enzyme was similar.⁴⁹ These results suggest a direct relationship between enzyme orientation, surface topology and catalytic current.

In a study by Li *et al.* they showed that the amount of conformational flexibility, or the amount of protein unfolding, of laccase from *Trametes versicolor* increased quickly at low concentration of a guanidine hydrochloride denaturant. They suggested that the multicopper active sites in laccase are formed by relatively weak interactions and hence may be more flexible than the intact laccase molecule as a whole.¹⁵⁴ This sets precedence for using the E-QCM-D to study conformational flexibility of similar MCO active sites.

Hodak *et al.* have used the E-QCM-D to monitor thiol adsorption followed by layer-by-layer assembly of ferrocene-modified poly-(allylamine) and glucose oxidase to gold. They reported increasing catalytic glucose oxidation with increasing layer build up.¹⁵⁵ These findings are important as they highlight the advantage of using structured electrodes for enzyme immobilization and this has importance in biofuel cell design.

An E-QCM has previously been used to follow the adsorption and electrocatalysis of bilirubin oxidase from *Myrothecium verrucaria* (MvBOx) on bare gold sensors.¹⁵⁶ They suggested that the lower catalytic current density on gold, compared to that on carbon electrodes, was a result of enzyme denaturation. Further to these findings our group published a study on the combination of chronoamperometric control, potential cycling and dissipation measurements of MvBOx on modified gold electrodes detailing the non-desorptive elements contributing to activity loss.⁵⁰

1.9.9 Applying the E–QCM–D to redox enzyme catalysis

The E–QCM–D has a significant advantage over other surface techniques as it allows simultaneous monitoring of the electroactive current generated by redox enzyme catalysis with concurrent mass and viscoelastic monitoring. The real benefit of this technique comes from the ability to normalise biocatalytic activity to adsorbed mass.

To a first approximation, the change in frequency is proportional to the change in mass according to the Sauerbrey equation and the adsorbed mass in turn is proportional to the surface coverage of enzyme (Γ_{enz}):

$$\Delta m = -\frac{C}{n} \cdot \Delta f_n = m_h \cdot \Gamma_{\text{enz}} \quad \text{Equation 1.72}$$

where n is the harmonic number, m_h , is the ratio of the enzyme's hydrated mass to its dry mass and C is the Sauerbrey constant (section 1.9.3, Equation 1.63)

The magnitude of catalytic current (i_{cat}) generated by the enzyme is also proportional to surface coverage and the turnover frequency (see section 1.5.8, Equation 1.52)

Therefore, combining Equation 1.52 and Equation 1.72 shows how the changes in current and mass can be used to estimate the relative turnover frequency of the enzyme during catalysis:

$$\frac{i_{\text{cat}}}{\Delta f_n} = \frac{CnFA}{nm_h} \cdot k_{\text{cat}} \quad \text{Equation 1.73}$$

For oxidoreductase adlayers, plots that combine frequency change, dissipation change, time and catalytic current data can be used to distinguish various post-adsorption processes. An idealised adsorption is shown in Figure 1.24 which is discussed further in Section 1.10.

1.10 Physics of protein adsorption

Proteins tend to be large amphiphatic molecules and this characteristic means that large proteins are intrinsically surface-active and will adsorb to solid surfaces. The ensuing protein-surface interactions determine the fate of surface immobilized materials due to protein binding and unfolding having an important role in protein-surface interactions. When globular protein molecules exist within an aqueous environment, protein adsorption proceeds when the ΔS of the folded protein (relative to that of the unfolded structure) is outweighed by the net ΔH and ΔS of the intra- and intermolecular interactions i.e. electrostatic, hydrophobic, hydrogen bonding and van der Waals (dispersion) interactions¹⁵⁷ and under most conditions the three-dimensional structure of a protein molecule is only marginally stable.¹⁵⁷

1.10.1 *Thermodynamics of protein adsorption*

When an aqueous protein solution is exposed to a surface, in this case an electrode surface; spontaneous accumulation of protein molecules at the electrode-solution interface is observed. Protein adsorption can only occur if the Gibbs Free Energy of the system decreases. Protein adsorbs according to Equation 1.74:

$$\Delta G_{\text{ads}}^{\theta} = \Delta H_{\text{ads}}^{\theta} - T\Delta S_{\text{ads}}^{\theta} < 0$$
Equation 1.74

where ΔH , ΔS and ΔT are the changes in enthalpy, entropy and temperature of the system during adsorption, respectively.¹⁵⁸

Most proteins do not simply attach to or detach from an interface with certain adsorption and desorption probabilities. Instead, structural re-arrangements, changing surface affinities during adsorption (change of surface chemistry) and external parameters such as temperature, pH, ionic strength, and buffer composition may come into effect.

1.10.1.1 *Conformational entropy*

Proteins have different regions on their surface depending on the local composition of amino acid residues and for this reason the protein's surface can be divided into different domains that can be hydrophobic, hydrophilic, positively, or negatively charged nature.^{159,160} On hydrophilic surfaces proteins expose their hydrophilic patches toward the surface and on hydrophobic surfaces proteins direct their hydrophobic patches to the surface. In the most qualitative picture of adsorption, if there exists a domain on the enzyme surface that is hydrophobic in nature and the surface is also hydrophobic, once adsorption begins conformation changes will cause it to unravel and expose its hydrophobic core to the hydrophobic surface.¹⁶¹ This will result in a substantial increase in the conformational entropy during adsorption.¹⁵⁷ Further to this, dehydration of both the surface and enzyme will provide an entropic driving force for adsorption.¹⁶¹

This overall gain in entropy as a result of the net change between folding and unfolding state of the enzyme is the major driving force for protein adsorption.

1.10.1.2 *Hydration effect*

The hydration layer directly surrounding an enzyme in solution is highly ordered and therefore has low entropy. As the temperature increases this low-entropy hydration layer is no longer favoured and the ordered water molecules will become more disordered by moving back into the bulk solution. The major driving force for protein adsorption in this case would then be an entropy gain arising from the release of surface adsorbed water molecules and subsequent structural rearrangements inside the protein. The amount of surface adsorbed proteins therefore generally increases at elevated temperatures according to Equation 1.74.^{157,162-164} As a result, the 'TΔS' in Equation 1.74 becomes proportionally more positive with increasing temperature and the native globular structure

becomes less stable.¹⁵⁷ The process gives rise to a large heat capacity when going from ordered to disordered phases and this increase implies that both the enthalpy and entropy of the hydration layer will increase with increasing temperature.¹⁵⁷

1.10.1.3 *pH and electrostatic interactions*

When the pH and the isoelectric point (pI) of a protein are equal, the protein exists as a net neutral entity. When $\text{pH} < \text{pI}$, proteins exhibit net positive charge whereas at higher pH ($\text{pH} > \text{pI}$) proteins exhibit a net negative charge. If the charges on the protein and the charges on the adsorbate surface are opposite, the protein will be attracted to the surface. However, if one entity holds a greater net charge than the other this would result in a net charge in the contact region between the protein and the adsorbate surface. This contact region has a low dielectric permittivity and therefore the accumulation of net charge in this region would be energetically unfavourable.¹⁵⁷

Adsorption rates are high when the protein and substrate both have different charges and are direct results of the opposing electrostatic attractions accelerating the migration of the protein towards the surface. However, the total mass load is generally observed to be maximized at (or near) the isoelectric point.^{137,165,166} Proteins adsorbing at positively or negatively charged interfaces, expose regions of their surface harbouring opposite charges to facilitate binding. However, this concept means that in some cases, a net positively or net negatively charged protein may adsorb to surfaces with similar charge, where the smaller regions of opposite charge encourage binding.¹⁶⁰

1.10.1.4 *Surface effects – surface energy*

The surface energy is a measure of the tendency of a surface to repel an adhesive, in this case a protein molecule. Low surface energy refers to a surface's repelling tendency, while high surface energy refers to a surface's attracting tendency. The contact angle

(θ) is the angle, conventionally measured through the liquid, where the protein meets the solid surface and it quantifies the surface energy as the 'wettability' of a solid surface. A study by Whitesides *et al.* on the effect of wettability on protein adsorption highlighted that proteins, irrespective of size show an increased tendency to bind to hydrophobic surfaces compared to hydrophilic ones.¹⁶⁷

Surfaces with low surface energy tend to have higher contact angles and are hydrophobic. The interactions between protein and hydrophobic surfaces (those with high contact angle) are favourable as the entropy gain from liberated water molecules (from protein and/or surface) and changes in conformation, cause a more negative Gibbs energy (Equation 1.74).

1.10.1.5 Orientation

Proteins commonly take on elliptical or rod-like shapes although there are special cases of where proteins taken on more irregular shapes such as the heart-like (BSA) and y-shaped (antibody) proteins. In solution, proteins move freely whereas on a surface each protein will adapt a certain orientation which determines which part of the molecule interacts with the surface and which part is exposed to the bulk solution.⁴⁷ This is an important issue when adsorbed proteins are receptors, enzymes, or have any other specific bioactivity such is the case with MCO enzymes.

1.10.2 Kinetics models for protein adsorption

The adsorption of proteins such as MCOs on surfaces is a complex process. The adsorbing particles are large, and, thus, the surface-protein interactions are usually long range and the strength is many times the thermal energy. Further to this, early studies of protein adsorption showed that the interactions between protein molecules, ranging in size and shape from large anisotropic proteins like BSA to smaller proteins such as lysozyme,

and adsorbate surface can be strongly influenced by conformational changes upon adsorption.¹⁶⁸⁻¹⁷¹

Simple models can provide valuable insight into the adsorption process, and it is important to keep in mind that each model has limitations, and a given model may not be appropriate to describe the adsorption of a particular protein under certain experimental conditions. In this section different models will be described. A detailed review of various adsorption models and their applications to macromolecular adsorption was given by Schaaf *et al.*¹⁷²

1.10.2.1 Diffusion limited model

The simplest model for protein adsorption is diffusion limited adsorption. In this model the movement of protein molecules towards the surface is based on a purely diffusive process where the rate of diffusion is the limiting factor. If a particle reaches the surface it will adsorb irreversibly where the surface density of protein will change with time according to Equation 1.75:¹⁷³

$$\Gamma = 2c \sqrt{\frac{Dt}{\pi}} \quad \text{Equation 1.75}$$

where Γ is surface density, c is the bulk concentration of protein and D is the diffusion constant for the protein (see section 1.5.3.3). As protein concentration increases, so does the rate of adsorption.

1.10.2.2 The Langmuir model

The Langmuir adsorption model is another very simple adsorption model. Protein molecules are assumed to be non-interacting and only a single layer of protein is permitted to form on the adsorbate surface where one protein will bind to one surface ‘site’. The process is reversible based on Equation 1.76.



where P is the protein, S is a surface ‘site’ and k_{on} and k_{off} are the rate constants for adsorption and desorption of protein to and from surface sites, respectively.

$$\text{rate of adsorption} = k_{\text{on}}[P][S] \quad \text{Equation 1.77}$$

$$\text{rate of desorption} = k_{\text{off}}[PS] \quad \text{Equation 1.78}$$

where at equilibrium both rates are equal so that the rate of association to the surface, K_a , is given by Equation 1.79:

$$K_a = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{[PS]}{[P][S]} \quad \text{Equation 1.79}$$

To obtain K_a experimentally, the fractional coverage of the surface, that is, the ratio of filled to total surface ‘sites’, can be measured:

$$\Gamma_{\text{frac}} = \frac{[PS]}{([S] + [PS])} = \frac{K_a[P]}{1 + K_a[P]} \quad \text{Equation 1.80}$$

where the amount of bound protein plateaus at $\Gamma_{\text{frac}}=1$ and the surface has a monolayer coverage and plotting $1/\Gamma$ vs. $1/[P]$ gives a plot whose gradient is $1/K_a$ (Figure 1.20).

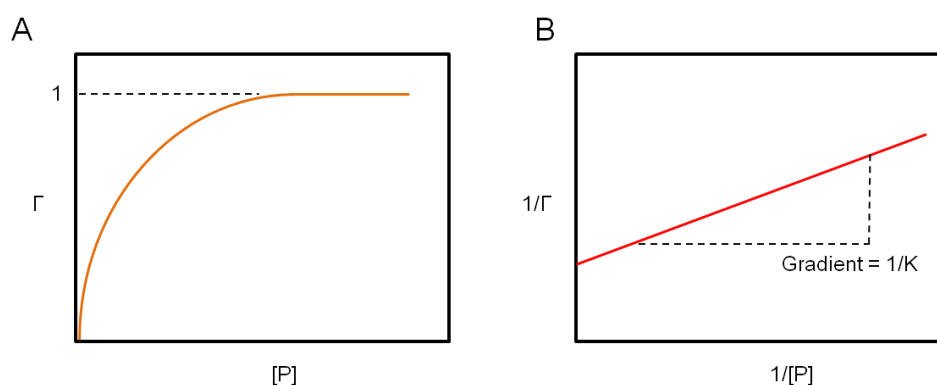


Figure 1.20. Langmuir model for protein adsorption. Panel A shows the relationship between surface coverage and protein concentration where $\Gamma = 1$ is achieved when all surface sites are occupied. From this plot the equilibrium constant can be calculated using a plot of $1/\Gamma$ vs. $1/[P]$ where the gradient is $1/K_a$.

The overall equation for change in protein coverage versus time is given by:

$$\frac{d\theta}{dt} = k_{\text{on}} \cdot c_s \cdot \left(1 - \frac{\theta}{\theta_{\text{max}}}\right) - k_{\text{off}} \cdot \theta \quad \text{Equation 1.81}$$

where θ is the protein coverage, θ_{max} is the maximum coverage when no more binding sites are available and c_s is the protein concentration directly above the surface.

The Langmuir model describes reversible adsorption, and it is often used in the case of the adsorption of small molecules. It should be used with care when large molecules are concerned, since these often adsorb irreversibly. Large protein molecules are also anisotropic, so they will take up a larger area when they adsorb and therefore in reality the surface saturation density will always be <1 .

1.10.2.3 *Random Sequential adsorption model*

As an improvement over the Langmuir model the random sequential adsorption (RSA) model interprets protein adsorption based on geometrical effects.¹⁷⁴⁻¹⁷⁶ In this model, proteins are assumed to be rigid particles and interact with the surface in the following way: (i) a protein molecule with adsorbs to a surface site provided there is sufficient space around this position, (ii) if there is not enough space, a new adsorption attempt is made at an alternative site where sufficient space is available. The protein molecules adsorb at random such that they do not overlap with any molecules that have already adsorbed successfully. Once on the surface, the position of the protein molecule remains permanently fixed meaning adsorption is irreversible. Initially, the chance of random adsorption is high and the surface fills rapidly. As the surface coverage increases, the adsorption probability decreases until it is impossible to place another object without overlap. When this condition is met, the surface is said to have reached a ‘jamming limit’ which is described by Feder’s law:¹⁷⁷

$$\Gamma_{\text{frac}\infty} - \Gamma_{\text{frac}}(t) \cong t^{-1/d} \quad \text{Equation 1.82}$$

where d specifies the number of coordinates necessary to describe the position of the adsorbing molecules on the surface e.g. $d = 2$ for a sphere (the centre point). However as a result of d , this adsorption model breaks down when anisotropic molecules adsorb to the surface in different orientations and with multiple contact points. This model has been very useful in understanding why the kinetics of protein adsorption do not follow the Langmuir predictions, however further conformational changes need to be considered as well as changes in molecular footprint due to relaxation/denaturation and/or reorientation processes.¹⁷⁸⁻¹⁸⁰

1.10.2.4 Overshooting

An ‘overshoot’ during the adsorption process relates to a situation where the adsorption profile passes a local maximum before surface saturation (Figure 1.21A). Ohshima *et al.* described the idea of overshooting using a ‘time delay’ model. According to this model, an overshoot is when the adsorbate surface is temporarily oversaturated with protein and equilibration is only seen after a net desorption of molecules despite a further supply of the molecule in solution.^{181,182}

Vroman *et al.* expanded on this and investigated the adsorption of proteins from blood plasma to a solid interface. They found that the protein fibrinogen rapidly adsorbs but after passing a maximum coverage, finally settles at a much lower coverage. They showed that when using differing protein compositions this overshoot is seen as a displacement effect where fibrinogen is replaced by other proteins of higher surface affinity (Figure 1.21).^{183,184} This effect was called the ‘Vroman effect’ (Figure 1.19B).¹⁸⁵

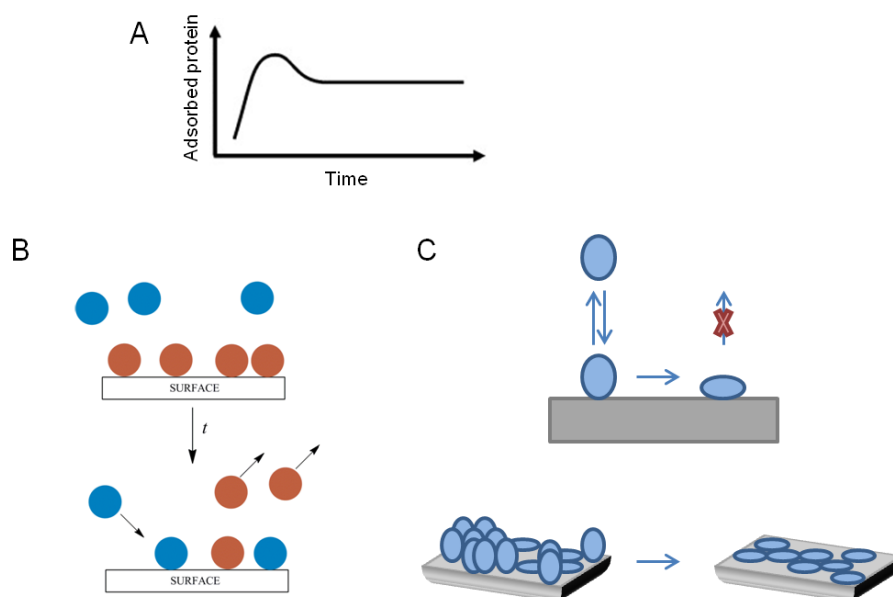


Figure 1.21. Protein adsorption overshoot. Diagram showing the overshoot mechanisms in protein adsorption. Panel A shows the change in adsorbed protein over time with a clear overshoot before a stable response. Panel B indicates the Vroman effect where enzyme with higher affinity displaces pre-adsorbed enzyme. Panel C indicates the post-adsorption change in conformation of the protein and how the protein becomes irreversibly bound.

1.10.2.5 Multiple states model

When considering the DET mechanism for enzyme adsorption, DET requires the catalytic centre of the enzyme to be within the correct tunnelling distance to the electrode surface. With the MCOs and bilirubin oxidase, this requires the primary electron entry site to be closely positioned to the electrode surface, however this can be difficult if the T1 site is buried within the structure of the enzyme. One of the more basic adsorption models is to treat the protein as a colloidal particle and treat adsorption as four distinct processes (Figure 1.22)^{130,164,186}

1. Migration. Movement of the protein towards the surface via Brownian motion and diffusion down a concentration gradient (step 1).
2. Attachment. Attachment of protein at the solid surface (conformation A).

3. Conformation change. Changes in protein conformation (2 & 3) may affect the strength and duration of binding. This can also lead to structural deformation of the protein (B) and further to complete deformation/denaturation (C).
4. Desorption. Protein desorption occurs if binding is weak or binding is reversible (1) where each change in conformation is partnered with a subsequent desorption step (2' and 3').

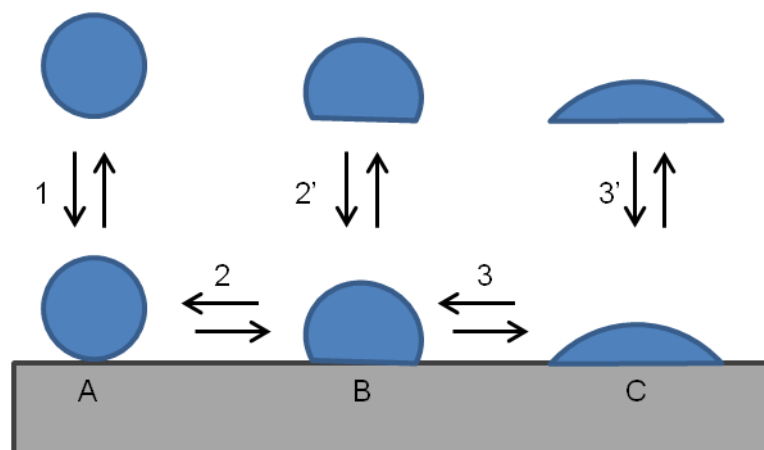


Figure 1.22. A schematic showing a model for protein adsorption and relaxation. The protein is first transported via Brownian motion to the surface (1). The protein adsorbs in a particular conformation (A) and undergoes structural rearrangement depending on the nature of the surface (B and C). This can also alter the protein function. Desorptive processes take place depending on binding strength and/or surface chemistry effects (3 and 3'). Based on a figure and model proposed by Lundstrom *et al.*^{130,186}

In Figure 1.22, protein approaches the surface in random orientations and due to the anisotropic nature of the protein, adsorption may occur in different orientations.¹⁸⁷ This irregular shape suggests that upon adsorption, even if the adsorbed molecules are closely matched in terms of orientation on the electrode surface, there will be areas on the surface that remain untouched.^{164,188} In some cases the protein footprint increases, by increasing the number of bonds made to the surface, thus the actual area occupied by a protein (footprint) also increases. This indicates that there is an energy barrier present where the protein needs to be in the correct spatial orientation for interaction. To help overcome this energy barrier, the protein needs to overcome the electrostatic repulsion between surface and molecule which may cause dehydration.¹⁸⁹

1.10.2.6 Overview of kinetic models

In addition to the models mentioned, an overview of some of the important kinetic models, is shown in Figure 1.23.

McGuire *et al.* have proposed a ‘two-state’ model where proteins adsorb either reversibly or irreversibly (Figure 1.23C). Two possible mechanisms for this model were suggested. The first one is a transition pathway through which all proteins must initially adsorb in a reversible state and then may undergo a transition into an irreversible state. The second possible mechanism is a parallel adsorption of the protein where it either adsorbs in a reversible state or adsorption is directly into the irreversible state.¹⁹⁰

Rabe *et al.* developed the model in Figure 1.23D where the model contains three different adsorbed states: an initial irreversible state, a reversible intermediate state, and a final irreversible state. Initially as long as the surface is empty or only partially covered, bulk proteins adsorb on the surface via the initial state (square). A transition occurs between the initial and the intermediate state (diamond) due to lateral protein-protein interactions. This transition proceeds rapidly suggesting an orientation change rather than a conformational rearrangement. The final transition into an irreversible state is much slower and therefore is more attributed to a conformational change.¹⁹¹

Rabe *et al.* also suggest that protein molecules approaching pre-adsorbed molecules on the surface are attracted vertically to the surface due to the overlap of the electrostatic forces between the protein and surface. In the lateral direction, the distribution of the charges of the pre-adsorbed protein and of the incoming protein results in a net repulsive effect. The result is that the incoming protein molecules are guided towards areas of other pre-adsorbed proteins (Figure 1.23E). This mechanism can lead to the build up of patches of low and high surface coverage.¹⁹²

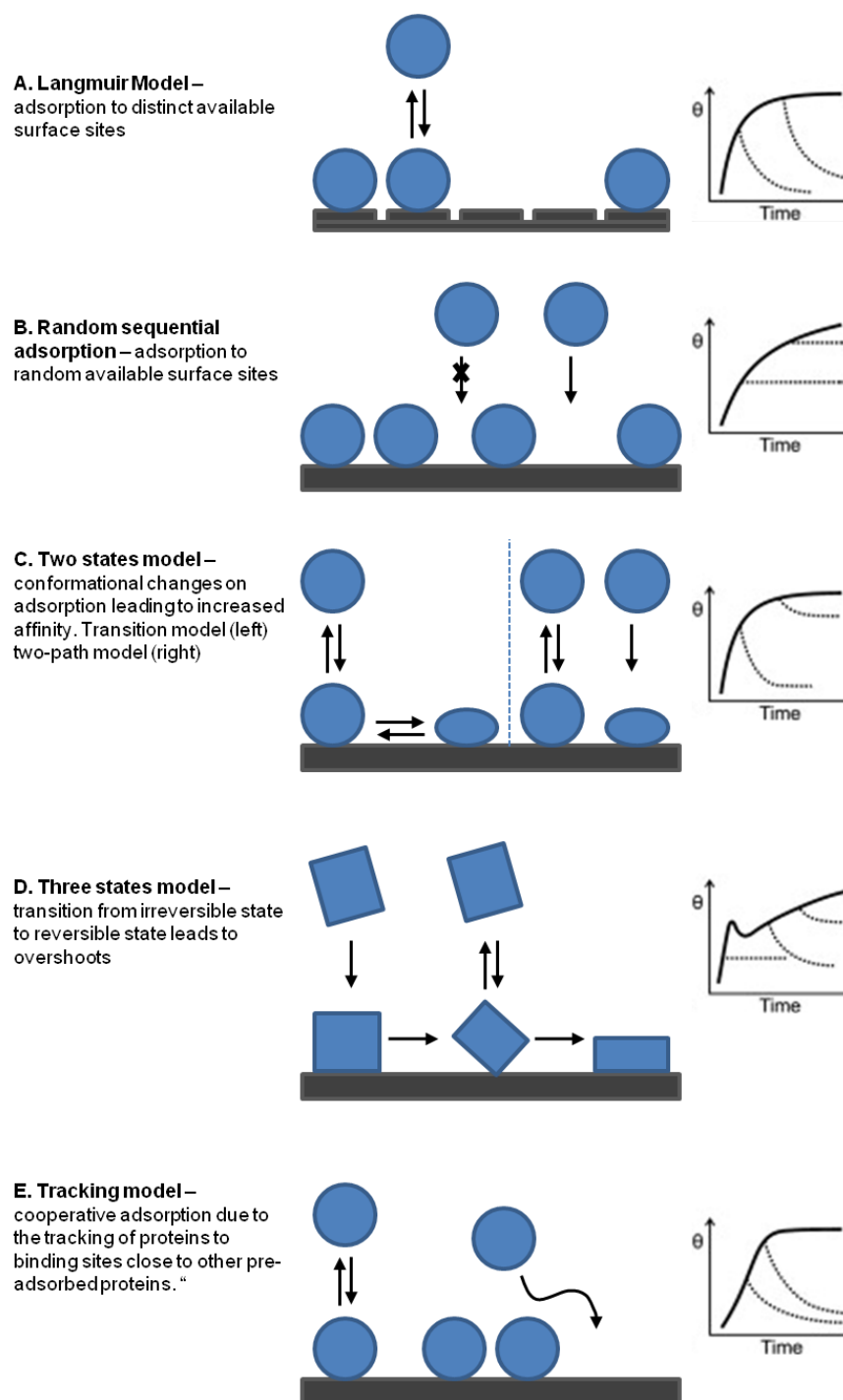


Figure 1.23 Overview of some of the important kinetic models for protein adsorption.¹⁹⁰⁻¹⁹⁵ Image adapted from reference¹⁹⁶

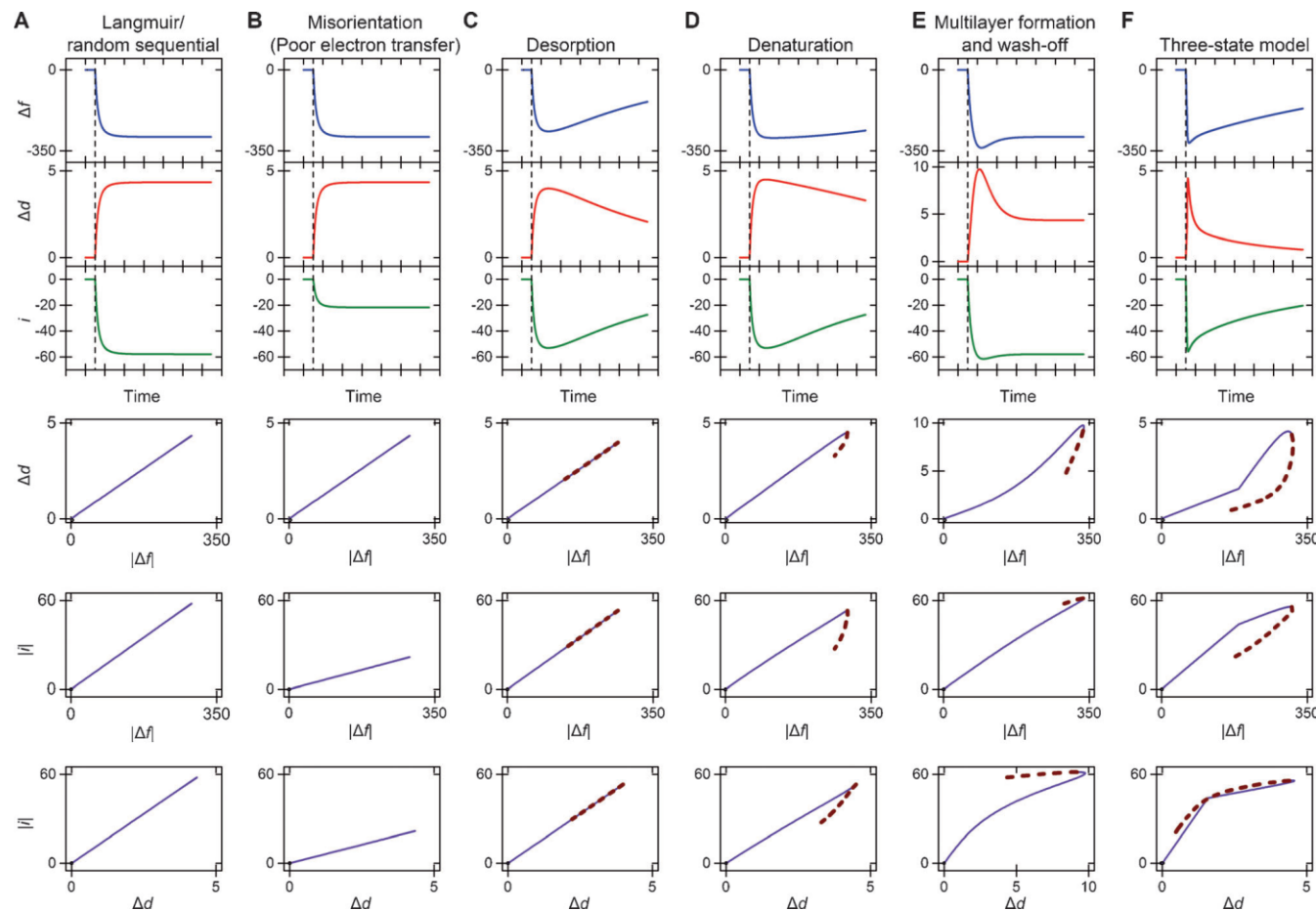


Figure 1.24. Model E-QCM-D responses for the adsorption of an oxidoreductase and common post-adsorption phenomena. Each vertical panel shows, from the top down, the frequency response (Δf), the dissipation response (Δd) and the catalytic current (i) followed by dissipation versus frequency, activity versus frequency, and activity versus dissipation plots. A) Idealised adsorption of a monolayer with no subsequent desorption or denaturation. B) A similar case, but in which the enzyme is orientated in a way that does not promote efficient electron transfer, which leads to a smaller current response or a given mass of adsorbed enzyme. C) Simulation of enzyme desorption from the electrode surface. No structural changes are included so the current response is proportional to the adsorbed mass and the adsorption (Δf) and desorption (Δd) lines overlap in the other plots. D) Effects of denaturation accompanied by some dehydration. E) Effect of multilayer formation with a pronounced peak in the dissipation as the surface forms multilayers of enzyme. This layer contributes little to the overall activity then desorbs rapidly. F) Adsorption based on a three-state model from Rabe and co-workers.¹⁹¹ It shows a superficially similar response for frequency, dissipation and activity, but there are clear differences in the plots that compare activity to the frequency and dissipation responses

1.10.2.7 Enzyme adsorption model for the E–QCM–D

The models presented here are based on a numerical model with discrete time steps of 0.1 seconds and takes into account changes in frequency, dissipation, electroactivity and subsequent changes in gradient of the Δd vs. Δf relationship during enzyme adsorption of $MvBOx$.

In an idealised Langmuir adsorption (Figure 1.24A) the oxidoreductase adsorbs irreversibly to form a single, stable layer in an orientation for efficient electron transfer according to Equation 1.83.



where BOx refers to $MvBOx$ and S represents the surface site on the quartz crystal electrode where adsorption occurs. The crystal frequency is defined to be proportional to the fractional surface coverage:

$$f = F\phi \quad \text{Equation 1.84}$$

By the proportional constant F with unit of frequency and the fractional surface coverage, ϕ , is

$$\phi = \frac{\text{actual mass}_{ads}}{\text{maximum mass}_{ads}} = \frac{1 - S}{S_{max}} \quad \text{Equation 1.85}$$

The change in frequency over time is given by:

$$\frac{d\Delta f}{dt} = ([BOx](1 - \phi)k_{on} - [BOx]_{ads}k_{off})F \quad \text{Equation 1.86}$$

and the amount of adsorbed $MvBOx$ is calculated using Equation 1.87

$$[BOx] = \begin{cases} 0, & t < t_{inj} \\ [BOx]_0 \exp\left[-\frac{t - t_{inj}}{\tau}\right] & \end{cases} \quad \text{Equation 1.87}$$

where t_{inj} is the time at which enzyme is injected into solution and τ is the ‘mixing time’ (the time taken from injection for the enzyme to reach the surface of the crystal) and is set at 10 seconds.

The dissipation is given by:

$$d = Df \quad \text{Equation 1.88}$$

and normalises viscoelasticity to the adsorbing mass by using the proportionality constant, D , which is given by

$$D = \frac{\partial \Delta d}{\partial \Delta f} \quad \text{Equation 1.89}$$

For all models D is set to $-0.015 \text{ ppm Hz}^{-1}$.

The current is given by:

$$i = If \quad \text{Equation 1.90}$$

and is similar to a turnover frequency (k_{cat}) normalised to adsorbed mass:

$$I = \frac{\partial \Delta i}{\partial \Delta f} = \frac{\text{mol of electrons produced}}{\text{adsorbed mass per unit area}} \quad \text{Equation 1.91}$$

For the idealised Langmuir model, I is given the value of $0.2 \text{ } \mu\text{A Hz}^{-1}$.

The blue lines show the expected frequency change and red lines show the expected dissipation changes. Both the dissipation change and catalytic current response (green lines) are directly proportional to the adsorbed mass. Thus, the associated plots of current versus Δf and Δd (purple lines) are linear and monotonic. If the protein is not in an orientation that permits efficient electron transfer (Figure 1.24B), the plots appear superficially identical to those in Figure 1.24A, but the current generated per unit mass adsorbed is lower where I is set to a lower value ($0.075 \text{ } \mu\text{A Hz}^{-1}$) compared to that in the Langmuir adsorption.

If loss in catalytic activity is due solely to desorptive processes (Figure 1.24C), dissipation and current will still be proportional to adsorbed mass during the desorptive process however the value of k_{off} is changed to 0.0075. The activity-frequency and activity-dissipation after the

point of maximum activity (dashed brown lines) will exactly overlap the trace from the initial adsorption (purple lines).

Denaturation of the protein (Figure 1.24D) can produce a similar trend in activity loss to enzyme desorption however due to the process of denaturation occurring over a longer time period than desorption, τ is set at 30 seconds. Denaturation often leads to layer stiffening (a decrease in Δd) and some loss of mechanically associated water (an increase in Δf) therefore the brown and purple traces do not overlap. The drop in activity per unit of adsorbed mass means that the average turnover frequency of the enzyme has dropped (Equation 1.73).

Finally, Figure 1.24E shows the expected E-QCM-D response for the adsorption mechanism for the build up enzyme layers (see section 1.9.6, Figure 1.19). The first adlayer is relatively stiff, tightly associated with the surface, and oriented for efficient electron transfer with the electrode. This first layer follows idealised Langmuir adsorption and k_{off} is set to 0. Subsequent layers are less rigid, more weakly attached, and have less efficient interfacial electron transfer.

This secondary layer dissipates a disproportionate amount of energy for its mass and contributes little to the catalytic current. Thus the dissipation curve shows a pronounced peak, whereas the frequency and current traces show relatively small dips. The current versus dissipation plot shows two adsorption regimes, then deviates in the opposite direction from the identical plot in Figure 8D. For this extra contribution a secondary set of constants are employed. Table 1.5 shows the values used in this model alongside the secondary set of constants used for the overshoot or ‘multilayer and wash-off’ process.

Table 1.5. Kinetic parameters used for the modelling described by Figure 1.24

Constant	Langmuir	Mis-orientation	Desorption	Denaturation	Overshoot
k_{on}	100	100	100	100	100
k_{off}	0	0	0.0075	0.003	0
$k_{\text{on-extra}}$					50
$k_{\text{off-extra}}$					0.2
D (ppm Hz ⁻¹)	-0.015	-0.015	-0.015	-0.015	-0.015
D _{extra}					-0.09
I (μA Hz ⁻¹)	0.2	0.075	0.2	0.2	0.2
I _{extra}					0.1
Δt (s)	0.1	0.1	0.1	0.1	0.1

In the example of ‘overshoot’ $k_{\text{on-extra}}$ is set to half as much as k_{on} and k_{off} is set to 0.2 indicating desorption of excess enzyme. The secondary layer dissipates a proportionate amount of energy for its mass, and contributes little to the catalytic current. The constant D is set to 6 times higher to show the build up of a softer layer and thus the dissipation curve shows a pronounced peak, while the frequency and current traces show relatively small dips. The value of I_{extra} is set to half as much as I and this highlights a fall in current suggesting poorer electron connectivity between the enzyme layers and the surface. The dissipation versus current plot shows two adsorption regimes, then deviates in the opposite direction from the identical plot in Figure 1.24D.

The three-state model proposed by Rabe and co-workers is simulated in Figure 1.24F.¹⁹¹ The dissipation versus frequency plot shows four distinct regions, with a clear inflection point on the purple adsorption trace at a critical surface concentration. The current versus frequency desorption trace (dashed) deviates in the opposite direction from that in the multilayer desorption model, whereas the current versus dissipation desorption trace follows a multi-stage path that depends on the mass and viscoelastic parameters associated with the three enzyme states.

1.11 Other techniques for studying protein adsorption

There are several techniques for detection and quantification of surface protein adsorption and some of the commonly used techniques are highlighted in Table 1.6.

Table 1.6 This table shows some of the important techniques used for protein adsorption. Adapted from¹⁹⁷

Technique	Principle	Information depth	Sensitivity	Notes/information
Ellipsometry ^{223,224}	Detection of polarization change of light reflected on a sample	100-300 nm	0.1-1 nm	Amount adsorbed, kinetics and isotherms. conformation information of adsorbed layer
AFM ^{198,199}	Atomic interaction between surface and tip are used to generate an atom-scale, electron density image of a surface.	Uppermost atomic level	Single atoms	Protein visualization; dimensions of protein, adhesion, friction forces, conformation, topography
ToF-SIMS ²⁰⁰	Ion bombardment sputters secondary ions from the surface which are characterized according to their m/z ratio using a time-of-flight mass spectrometer	<1 nm in 'static mode' and 1 μ m in 'dynamic Mode'	10^{11} – 10^{17} atoms cm^{-2}	Map of protein distribution; identification of different proteins; conformation; degree of denaturation, surface coverage
ATR-FTIR ^{201,202}	IR spectroscopy using evanescent field above the surface of a specially cut crystal in which the light propagates between the crystal surface	1-5 μ m	1-10 mol%	Identification of the typical chemical functional groups, of proteins. Conformation information of the adsorbed protein layer
SPR ²⁰³	Influence of adsorbed molecules on electron charge density waves (surface plasmons) in the surface of a thin metal film (Au or Ag)	50-300 nm	0.1-1 ng cm^{-2}	Identification of chemical functional groups of proteins. Conformation of the adsorbed protein layer

ATR-FTIR - attenuated total reflection Fourier transform infrared spectroscopy, ToF-SIMS-time-of-flight secondary ion mass spectrometry, AFM-atomic force microscopy.

Many additional techniques to those detailed in Table 1.6 have been employed to look at surface protein interactions including grazing incidence infrared spectroscopy,^{204,205} and dual polarization interferometry (DPI).^{206,207} Although there are a high number of techniques available, electrochemical surface plasmon resonance proves an attractive technique to be used alongside QCM-D and E-QCM-D (appendix).

1.12 Conclusion

This thesis describes the use of an E-QCM-D to probe changes in the electrocatalytic activity of *MvBOx* adsorbed to organothiol-coated gold quartz crystals. This introduction presented important considerations for quantifying enzyme catalysis alongside a review of the historical and current literature. This includes understanding the role of the ORR, and the use of the QCM-D and E-QCM-D for analysing mass, structural and electrocatalytic changes in enzyme immobilization. The thermodynamic and kinetics of protein adsorption were summarised and a model which incorporates the E-QCM-D has been presented.

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Chapter 2: Materials and Methods

2 Materials and Methods

2.1 E-QCM-D experimental setup

QCM-D data were acquired using a Q-Sense E1 system with accompanying Q-Soft data acquisition software. QCM-D measurements without concurrent electrochemical analysis used a Q-Sense Flow module 401 (total volume $\sim 140\ \mu\text{l}$, volume above sensor surface $\sim 40\ \mu\text{l}$, non-sensor surfaces Ti or Viton). E-QCM-D measurements used a modified Q-Sense QEM401 module (volume above sensor surface $\sim 100\ \mu\text{l}$). The PTFE insert on the electrochemistry module was replaced by a custom-made insert made of poly(oxomethylene) (Delrin) for better wettability, to accommodate a standard 5.7 mm OD reference electrode, and to provide a more robust seal when liquid was pulled through the cell. The peristaltic pump (Ismatech) was used to draw solution through the cell using arbitrary rates 1 to 99. The pump was controlled by a LabJack U3-HV ADC DAC with a Windows interface. Tubing entering and leaving the cell was made from ethylene tetrafluoroethylene (ETFE, VWR). The tubing for the peristaltic pump tubing was made from TygonMHLL (Bennet Scientific) consisting of a chemically resistant core with an internal diameter of 0.7 mm. The manufacturer's value for typical mass sensitivity in liquid is $1.8\ \text{ng cm}^{-2}$,¹ equivalent to $< 0.3\%$ of a monolayer of *MvBOx*. All experiments were done at 25°C.

The QCM-D and E-QCM-D setups were placed in an insulated box. The box contained a fan for convective temperature regulation and the QCM-D setup and solutions were preheated by placing on an aluminium plate controlled by a temperature-controlled water recirculator set to 25°C.

2.1.1 Sensor specifications

The sensors (Q-Sense) were 14 mm diameter, gold-coated, AT-cut quartz crystals with a titanium adhesion layer and a fundamental frequency of (4.95 ± 0.05) MHz. All the frequency and dissipation changes presented in this thesis are from the seventh harmonic of the fundamental frequency however the 1st, 3rd, 5th, 7th, 9th, 11th and 13th harmonics were all recorded during measurements. When changing solutions, the 7th harmonic is less affected by crystal mounting and small mechanical vibrations, is more surface sensitive, and still gives a good signal-to-noise ratio compared to the higher frequencies. A proportional-integral-derivative (PID) controller regulated the temperature of the QCM-D and E-QCM-D module and the measured temperature of the QCM cell was constant within 10 mK. The dependence of the fundamental frequency on temperature (i.e., $d(\Delta f/f)/dT$) of AT-crystals at 25 °C is $-3.3 \times 10^{-7} \text{ K}^{-1}$,² so temperature variations should not lead to frequency variations greater than 16 mHz in f_0 ($\sim 0.3 \text{ ng cm}^{-2}$). In practice, however, we observed larger variations in frequency and dissipation, usually in the range of ± 7 –14 Hz and ± 0.5 ppm for Δf_7 and Δd_7 , respectively. The sensor surface is remote from the point at which the E1 system measures the temperature, so the entire liquid handling system (cell, fluid reservoir, tubing and pump) was placed on top of an aluminium plate through which water held at 25 °C was circulated, then surrounded by an insulated box in which the air was circulated by an electric fan. The thermal coupling between the flow modules and QCP101 chamber platform was improved with thermal conducting paste (RS components, RS 217-3835). Other sources of variation arise from the mounting of the crystal in the cell and the effect of the repeated cleaning procedure on the gold surface.

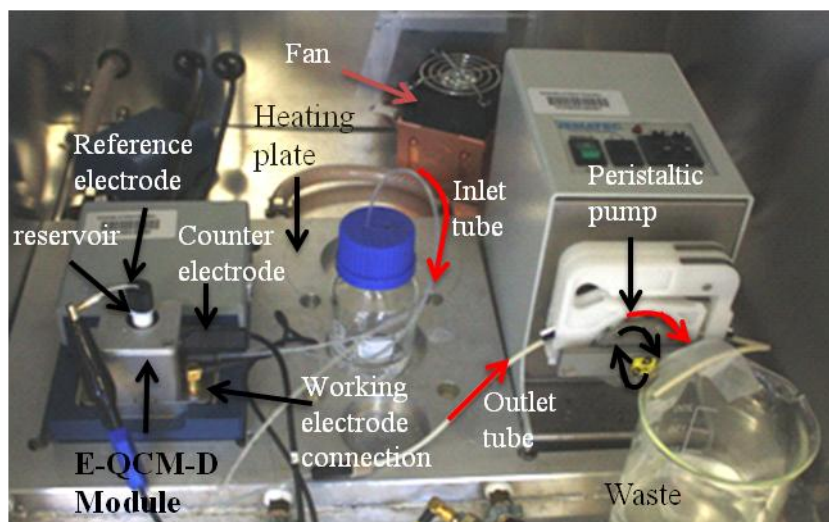


Figure 2.1. Photograph of the E-QCM-D setup. Red arrows indicate physical components of the system. Black arrows indicate the direction of fluid flow

2.1.2 Enzyme loading

Enzyme was introduced to the cell, typically using 25 μL volumes, by stopping the peristaltic pump, reversing the flow momentarily to produce a suspended drop at the end of the entry tube, placing the tube in the enzyme solution and restarting the pump in the forward direction. After enzyme was introduced, the procedure was repeated to flow buffer through the cell. Each exchange took less than 1 min. The dead time for liquids to flow from the inlet to the cell was 2 min.

2.1.3 Electrochemical setup

The electrochemical cell used a three-electrode configuration: the gold-coated QCM sensor (exposed geometric area of 0.80 cm^2) as the working electrode; a 0.2 mm thick, 2.25 cm^2 square platinum plate running parallel to the sensor surface as the counter electrode; and a $\text{Ag}|\text{AgCl}||3\text{M NaCl}$ reference electrode (BASi RE-6, $E_{\text{SHE}} = E_{\text{ref}} + 0.209\text{ V}$) at 25°C . The distance between the working electrode and counter electrode was $0.7\text{--}0.8\text{ mm}$; the distance between working and reference electrode was $0.9\text{--}1.0\text{ mm}$. The

potential was set and the current was measured by an Ivium CompactStat, using the accompanying IviumSoft control software (version 2.178). Buffer was always kept in a sealed glass bottle and oxygenated throughout the experiment with at least 30 min pre-oxygenation prior to the start of each experiment (see section 2.4.7).

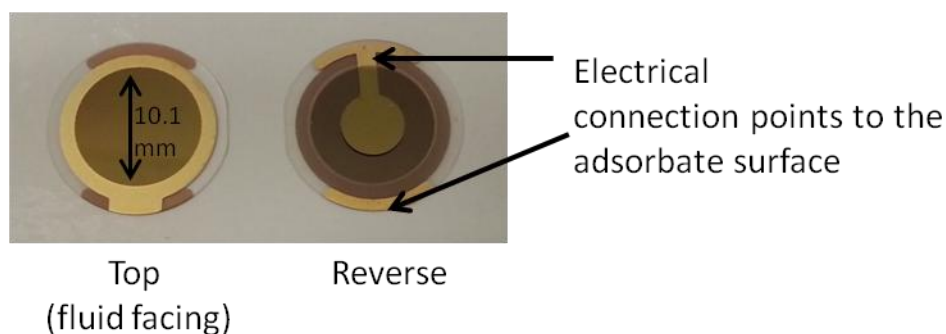


Figure 2.2. An E-QCM-D sensor along with dimensions and the position of the electrical contacts.

2.2 Fluid flow rate dependence

The flow rate of the liquid running over the crystal in the E-QCM-D setup is controlled by a peristaltic pump with a digital control with arbitrary speeds of 0-99. For the experiments used within this thesis, buffer was brought to the surface of the sensor at the arbitrary rate of 2 (0.1 ml min^{-1}). The speeds for the arbitrary units were measured by timing the period taken for flowing a fixed volume of buffer (2 ml) through the cell (Figure 2.3).

Figure 2.3A shows the calibration curve effect fitted with a second order polynomial. The maximum change in reductive current ($-4.8 \mu\text{A}$) occurs at a pump speed of 50 which equates to a flow rate of 2.86 ml min^{-1} . Increasing the pump speed produced moderate increases in current however flow rates greater than 0.5 ml min^{-1} mechanically disrupted the stability of the electrochemical cell (Figure 2.4). For this reason a pump speed of 2 (0.1 ml min^{-1}) was used throughout this thesis

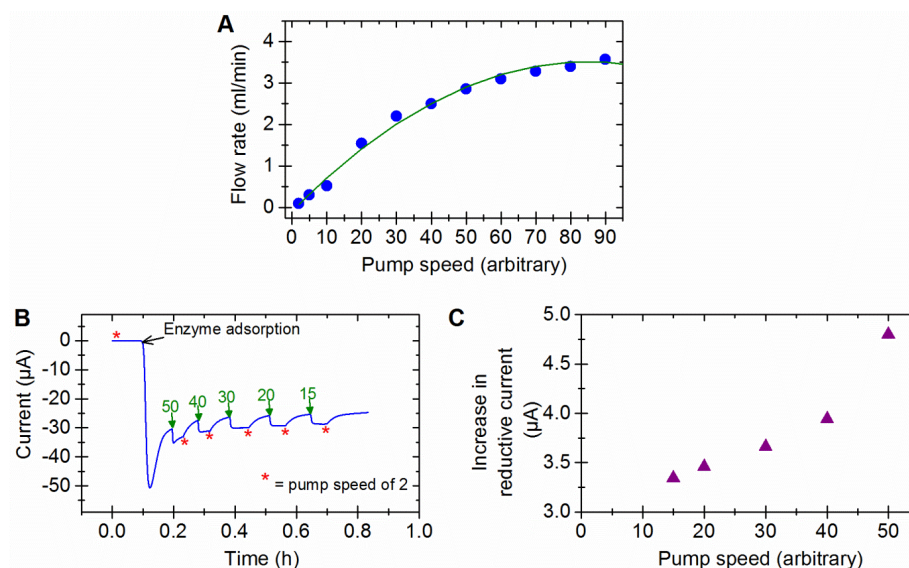


Figure 2.3. Calibration curve and fluid flow dependence. Panel A shows a calibration curve for pump speed and flow rate. Panel B shows the effect of increasing the flow rate on the reductive current generated after protein adsorption. Panel C shows the increase in reductive current after each change in pump speed.

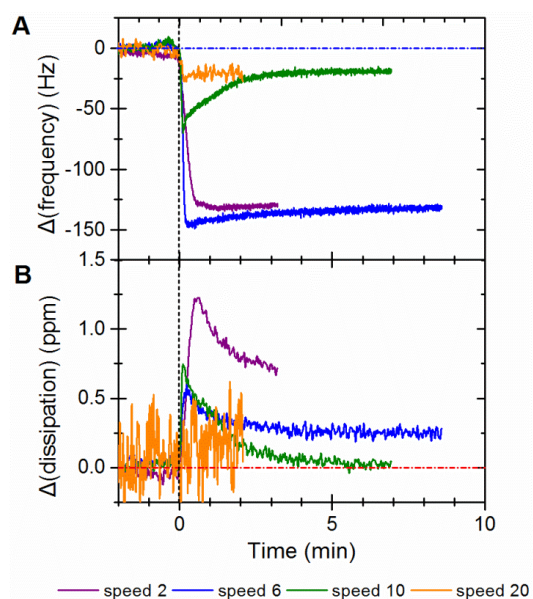


Figure 2.4. Frequency and dissipation responses for adsorption of 25 μL MvBOx (25 mg ml⁻¹) at different pump speeds. Speed 2 = 0.1 ml min⁻¹, speed 6 = 0.3 ml min⁻¹, speed 10 = 0.52 ml min⁻¹, speed 20 = 1.54 ml min⁻¹.

2.3 Material sources

Chemicals were purchased and used without further purification. All water was purified via reverse osmosis and ion exchange to a resistivity of 18.2 MΩ cm at 25 °C using a MilliQ water purifier.

Table 2.1 Sources of chemicals

Chemical	Purity and source
(1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide hydrochloride (EDC)	98+%, Acros Organics
3-mercaptopropionic acid (3MPA)	>99%, Fluka
3-mercapto-1-propanol (3MPOL)	95%, Sigma
3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP)	>98%, ProChem Inc
4-acetamidothiophenol (4AATP)	95%, Sigma
4-hydroxythiophenol (4HTP)	97%, Sigma
4-mercaptobenzoic acid (4MBA)	90%, Sigma
4-mercaptobutyric acid (4BUA)	Sigma
6-mercaphexanoic acid (6MHA)	90%, Sigma
8-mercaphooctanoic acid (8ODA)	95%, Sigma
11-mercaphoundecanoic acid (11UDA)	95%, Sigma
ammonium bicarbonate	≥99.5%, Sigma
benzenethiol	≥98%, Sigma
citric acid monohydrate	≥99%, Sigma
<i>D</i> -(+)-glucosamine hydrochloride	≥99%, Sigma
dimethyl sulfoxide, DMSO	≥99.9%, Fisher
di-Hydrogen orthophosphate dihydrate	99.0–101.0%, Fisher
di-Sodium hydrogen orthophosphate anhydrous	99.5+%, Fisher
dithiothreitol (DTT)	≥99.5%, Sigma
HAuCl ₄ •3H ₂ O	99.99%, Alfa Aesar
hydrochloric acid, HCl 37%	Fisher
hydrogen peroxide (30% w/v)	Scientific Laboratory Supplies
hydroxylamine hydrochloride	>97%, Fisher
<i>N</i> -hydroxysuccinimide	98+%, Acros Organics
<i>N</i> -hydroxysuccinimide ester of fluorescein-5-EX	Sigma
iodoacetamide	≥99%, Sigma
pepsin (from porcine gastric mucosa)	≥250 units/mg, Sigma
sodium borohydride, NaBH ₄	99%, Sigma
sulphuric acid (1.83 g ml ⁻¹ specific gravity)	>95%, Fisher
tri-Sodium citrate dihydrate	≥99%, Sigma
tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	>98%, Sigma
trypsin (from porcine pancreas)	>10,000 units/mg, Sigma

2.3.1 Buffer preparation

Solutions of the acid and base form of the buffer were made up to the required concentrations using deionised water rather than addition of strong acid or base. The final buffers were then made up to the desired pH by titration of the acid solution and the base solution at the desired temperature, which was typically 25°C. The pH was measured using an Accumet 950 pH meter (Fisher Scientific).

2.4 QCM sensor surface treatment

2.4.1 Cleaning

Residual contamination on new QCM sensors was removed by placing them under a ozone-generating UV lamp in air for 30 min. Sensors were reused, typically 4-5 times, by oxidizing the surface with piranha solution (3:1 v:v H₂SO₄: H₂O₂) for 20 min with constant heating at 60°C, followed by rinsing in water. The crystals were dried under a stream of house nitrogen from boil-off, then ozone treated as described. Unlike the cleaning of stationary gold electrodes, the QCM crystals were not scanned in 0.1 M NaOH or 0.1 M H₂SO₄ as this resulted in the thin layer of gold being etched from the surface of the quartz.

2.4.2 Gold stationary electrodes

2.4.2.1 Cleaning procedure

The gold stationary electrodes used for reductive desorption experiments were first mechanically cleaned by polishing with 1 µm α-alumina followed by 0.3 µm α-alumina on polishing pads. The electrodes were then rinsed and sonicated in ethanol for 10 min, to

remove residual alumina particles, and then treated with ozone for 20 min. This was followed by an electrochemical cleaning procedure by first cycling the potential between 0.2 V to -0.8 V vs. SHE in 0.1 M NaOH. The electrode was then transferred to 0.1 M H_2SO_4 and the potential was cycled between 0.2 V and -1.5 V vs. SHE. Typically 10 scans were carried out in NaOH and 15-20 scans were carried out in acid. The scan rate was 50 mV s^{-1} and scans were repeated until they resembled those shown in Figure 2.5.

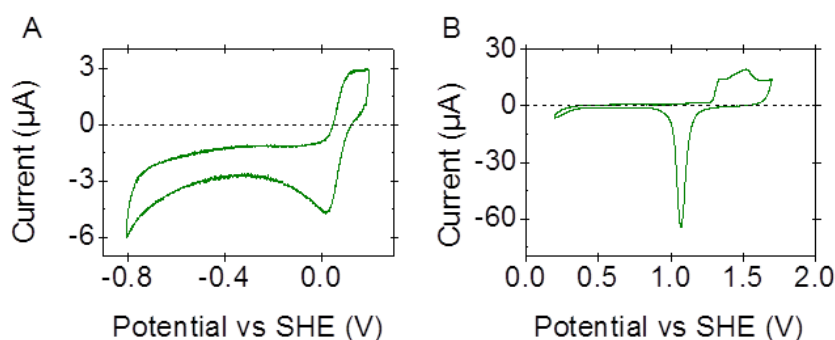


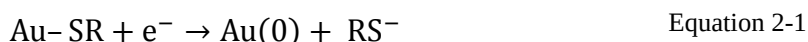
Figure 2.5. Electrochemical cleaning of gold. Panel A shows the basic cleaning in 0.1 M NaOH and Panel B shows the acid clean in 0.1 M H_2SO_4 .

2.4.3 Organothiol surface modification

Surface modification solutions consisted of 10 mM thiol with 1.3 mM TCEP (used as a reducing agent) in ethanol. Solutions were sonicated for 20 min until TCEP was fully dissolved. Freshly cleaned sensors were immersed for 15 h in these solutions under a nitrogen atmosphere. (Consistent with the reports of Z. Cao and co-workers, we have observed gold dissolution by QCM from oxygenated thiol solutions.)^{3,4} Surface coverage of the thiol layer was determined by reductive desorption in basic anaerobic aqueous solution (section 2.4.4).⁵ After surface modification, the crystals are rinsed with water and ethanol, dried in a stream of nitrogen gas, then mounted in the E-QCM-D cell.

2.4.4 Electrochemical desorption of thiol

An approach for testing for the presence of an alkanethiolate modification on gold and in turn, the surface coverage of the monolayer, is based on the coulometric measurement for the charge passed during the one-electron reduction, and subsequent desorption, of the gold thiolate bond (Au-SR) in alkaline solution.⁵ The process to be measured at the electrode is shown by Equation 2-1.⁶



A typical reductive desorption experiment (Figure 2.6) is carried out using deoxygenated 0.1 M NaOH solution. The strong basic conditions are to avoid interference from dihydrogen evolution.

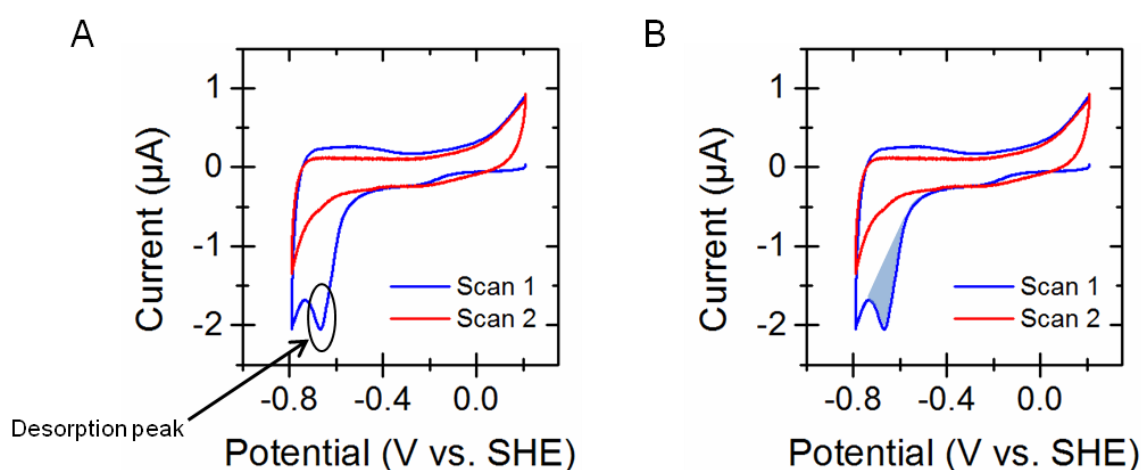


Figure 2.6. A typical reductive desorption. A stationary gold electrode with surface area 0.035 cm^2 was modified with 3-mercaptopropan-1-ol under a nitrogen gas atmosphere in 10 mM ethanolic thiol solution. Panel A shows the desorptive peak on the first scan as the potential is swept from $0.21 - 0.79 \text{ V vs. SHE}$ at 50 mV s^{-1} . The second scan lacks this desorptive peak indicating desorption of the monolayer. Using the known surface area of the electrode, the area of the desorptive peak, highlighted in panel B, will give the surface coverage of the thiol modification.

The reductive desorption peak in Figure 2.6 is seen at -0.65 V . The electrode potential that the reduction occurs at is greatly dependant on the strength of the inter-chain van der

Waals interactions after adsorption. Longer chain thiol molecules and those with aromatic residues have stronger inter-chain van der Waals interactions and therefore desorption peaks occur at more negative potentials. The area of the desorptive peak can be integrated to give the surface density (equation Equation 2-2). It is assumed that reductive desorption is a one-electron process and the number of thiol molecules desorbing is equivalent to the number of electrons transferred from the electrode.⁷

$$\Gamma = \frac{\int i dE}{n\nu FA} \quad \text{Equation 2-2}$$

where Γ is the surface density, i is the current, E is the applied potential, n is the number of electrons transferred, ν is the scan rate, F is the Faraday constant and A is the area of the electrode. On the second and subsequent scans, the lack of a desorptive peak indicates that the thiol has dissociated from the surface.

2.4.5 DTSSP coupling

Gold sensors were modified using 10 mM 3,3'-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) with 1.3 mM TCEP in water and left for 3 h adapted from a literature procedure.⁸ After longer time periods DTSSP precipitated out of solution leaving white deposits on the crystal surface. The crystal was loaded in the same way as described in section 2.4.3. Enzyme was also loaded using the same procedure however the peristaltic pump was turned off for 45 min to allow for the succinimidyl groups to react with the lysine residues of bilirubin oxidase. After the reaction had proceeded the pump was turned back on and fluid flow resumed (see chapter 4).

2.4.6 Glucosamine coupling

Gold sensors were first modified using 3-mercaptopropionic acid (3MPA) using the same procedure described in 2.4.3. The sensor was then removed from the ethanolic thiol solution, rinsed with ethanol and water and dried under a stream of nitrogen gas. The sensor was then immersed in a solution containing 10 mM EDC and 5 mM NHS in deionised water and left for 1 hour. The sensor was then removed, rinsed with water and dried under nitrogen. The final step was to immerse the sensor in a solution of 10 mM glucosamine in water for 1 hour. The sensor was again rinsed and ready for mounting into the E-QCM-D cell.

2.4.7 Variable oxygen saturation

The concentration of O₂ was set by rotameters using oxygen gas (in-house). The flow rate of oxygen was generally between 0.90-0.95 cm³ min⁻¹ at a gauge pressure set between 0–1 bar and the gas was flowed through the Schott bottles containing buffer solution. Gas-solution equilibrium was reached after 30 minutes based on a volume of 40 mL buffer solution.

2.5 Enzyme purification

Bilirubin oxidase from *Myrothecium verrucaria* (MvBOx) was obtained from Amano Enzyme. The enzyme was purified as for protein crystallization.⁹ Lyophilized MvBOx powder was dissolved in 10 mM sodium phosphate, pH 7.0 at 4°C, then centrifugally dialyzed to remove small-molecular-weight contaminants (Vivaspin, 30 kDa MWCO PES). This was then further purified by weak anion exchange (DEAE Sepharose) and by hydrophobic interaction (HiTrap Phenyl Sepharose) chromatography at 4°C. The protein was eluted using 10 mM sodium phosphate, pH 7.0 at 4°C, with a 0–0.4 M ammonium

sulphate gradient for the anion exchange, and 50 mM sodium phosphate, pH 6 at 4°C, with a 1.25–0 M ammonium sulphate reverse gradient for hydrophobic interaction. Typical yields of purified protein were $\approx 3\%$ by mass. Protein concentrations were determined using the Beer-Lambert law based on $A_{280\text{nm}}$ measured from enzyme aliquots using the molar extinction coefficient, $\epsilon_{280\text{nm}} = 9.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, calculated from the enzyme amino acid sequence by the online software ProtParam.¹⁰

Laccase from *Trametes versicolor* (TvL) was obtained as a crude powder from Sigma. The crude powder was dissolved in 10 mM sodium acetate, pH 5.5 at 5 °C, and then filtered through two filters (GFA, 0.45 μm , 0.22 μm). This was then centrifugally dialyzed to remove small-molecular-weight contaminants (Vivaspin, 30 kDa MWCO PES). This was then further purified by weak anion exchange (Q Sepharose, 10 mM sodium acetate, pH 5.5 at 5 °C, with a 0–0.1 M ammonium sulphate gradient) followed by hydrophobic interaction (HiTrap Phenyl Sepharose, 20 mM sodium phosphate, pH 4.7 at 5 °C, with a 1.65–0 M ammonium sulphate reverse gradient) at 5 °C.

2.5.1 Blank enzyme activity

Rotating graphite electrodes were used to test the enzyme activity after purification in a typical three electrode setup (see Introduction 1.5.3.5) with an Ag|AgCl|3M NaCl reference electrode and platinum wire counter electrode. A small amount of purified enzyme ($\sim 5 \mu\text{L}$) was pipetted onto the working electrode surface (surface area of 0.03 cm^2). The electrode was then immersed into a pre-oxygenated 0.1M sodium phosphate pH 6.0 buffer solution. Buffer was oxygenated throughout the experiment with at least 20 min pre-oxygenation prior to the start of each experiment. The electrode was rotated at 4000 rpm and the resulting oxygen reducing catalytic current was measured using an Ivium CompactStat, using the accompanying IviumSoft control software (version 2.178).

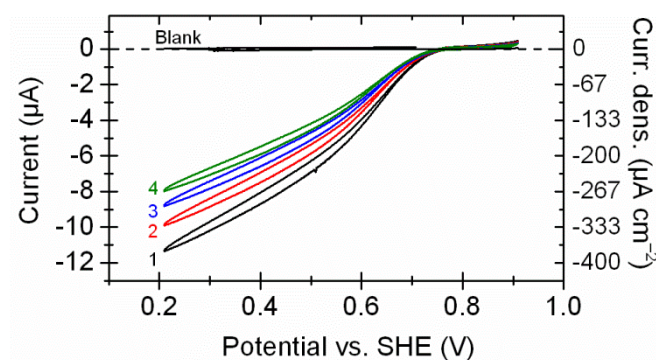


Figure 2.7 Typical electrocatalytic wave of 25 mg ml⁻¹ MvBOx (5 µL) on a pyrolytic graphite rotating disc electrode. Showing the blank graphite electrode followed by scans 1, 2, 3 & 4 of 4 scans cycled between 0.91-0.21 V vs. SHE after enzyme spotting. Conditions: O₂-saturated 0.1 M sodium phosphate buffer pH 6.0 at 25 °C, rotation rate 4000 rpm, scan rate 10mV s⁻¹.

2.6 Degree of labelling and reactive lysine determination

2.6.1 Fluorescein-5-EX (F5EX) labelling

MvBOx was labelled with an amine-reactive fluorophore based on a procedure from Molecular Probes (now Invitrogen).¹¹ Aliquots of 15.2 mM Fluorescein-5-EX succinimidyl ester (F5EX) in DMSO were mixed with aliquots of 0.24–0.25 mM MvBOx in 0.1 M sodium bicarbonate pH 8.3 to give dye:protein molar ratios of up to 72. The total volume of the reaction mixture was 110 to 200 µl. The mixture was incubated in the dark with stirring at room temperature for 1 h, then 0.1 ml of freshly prepared 1.5 M hydroxylamine pH 8.5 was added to conjugate to unreacted F5EX and stop further reaction with the protein. The protein was separated from unconjugated dye by dialysis through a 30 kDa MWCO filter (Vivaspin, GE Healthcare).

The degree of labelling (DoL) was determined spectrophotometrically ($\lambda_{\max}^{\text{F5EX}} = 494 \text{ nm}$) in a 1cm path length quartz cuvette and Equation 2-3:

$$\text{DoL} = \frac{A_{494 \text{ nm}}^{\text{mix}} \epsilon_{280 \text{ nm}}^{\text{MvBOx}}}{(A_{280 \text{ nm}}^{\text{mix}} - \text{CF } A_{494 \text{ nm}}^{\text{mix}}) \epsilon_{494 \text{ nm}}^{\text{F5EX}}} \quad \text{Equation 2-3}$$

where $A_{280\text{ nm}}^{\text{mix}}$ and $A_{494\text{ nm}}^{\text{mix}}$ are the recorded optical densities for the labelled protein at 280 nm and 494 nm, respectively, $\epsilon_{494\text{ nm}}^{\text{F5EX}} = 6.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{280\text{ nm}}^{\text{protein}} = 9.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and the correction factor (CF) = 0.30. The correction factor is the ratio of the absorbance of the free dye at 280 nm to its absorbance at λ_{max} (i.e., $\epsilon_{280\text{ nm}}^{\text{F5EX}}/\epsilon_{494\text{ nm}}^{\text{F5EX}}$).

2.6.2 Trypsin digest

Solutions of 1 mg ml^{-1} MvBOx, 100mM dithiothreitol (DTT, for reduction of disulphide bonds in the protein), 100 mM iodoacetamide (to cap free cysteine thiolates), and $20 \text{ }\mu\text{g ml}^{-1}$ trypsin were made in 50 mM ammonium bicarbonate. $2.1 \text{ }\mu\text{l}$ of the DTT solution was added to $50 \text{ }\mu\text{l}$ of the protein solution, the mixture was vortexed then incubated for 45 min at 37°C . After the solution returned to room temperature, $8.7 \text{ }\mu\text{l}$ of the iodoacetamide solution was added, the mixture vortexed again, then incubated in the dark for 45 min, at room temperature. A further $2.2 \text{ }\mu\text{l}$ of DTT solution was added, the mixture vortexed, then incubated at 37°C for 10 min. $20 \text{ }\mu\text{l}$ of trypsin solution was added to the mixture, which was then incubated for 12 h at 37°C . Final solution frozen at -21°C and stored for 7 days until analysed using mass spectrometry. Trypsin cleaves at Arg and Lys residues.

2.6.3 Pepsin digest

$50 \text{ }\mu\text{l}$ MvBOx (15 mg ml^{-1} , 0.1M sodium phosphate buffer pH 6.0) was acidified with $20 \text{ }\mu\text{l}$ 0.04 M HCl. To this solution, $20 \text{ }\mu\text{l}$ pepsin (0.3 mg ml^{-1}) in water was added (71:1) and the mixture vortexed briefly. The mixture was incubated for 12 h at 37°C , heated to 95°C for 15 min. then cooled to room temperature and frozen at -21°C . Pepsin exhibits preferential cleavage for hydrophobic, aromatic groups.

2.6.4 MALDI mass spectrometry

All MALDI mass spectrometry experiments were carried out on an Ultraflex II instrument (Bruker Daltonics) in positive reflectron mode. A solution of matrix (α -cyano-4-hydroxycinnamic acid) was applied to the protein sample and allowed to dry before analysis.

2.7 Protein structure

Protein crystal structures were downloaded from the protein data bank at <http://www.rcsb.org/pdb/home/home.do> and the molecular modelling program PyMOL was downloaded from <http://pymol.sourceforge.net/> and used to show protein crystal structures and the coordination of the copper sites in bilirubin oxidase (*MvBOx*).

2.8 Dynamic light scattering

Dynamic light scattering (DLS) measurements were carried out using DLS in a Malvern Zetasizer Nano System (He-Ne laser, 633 nm). A volume of 600 μL of *MvBOx* was centrifuged at a concentration of 0.1 mg ml^{-1} in 0.1 M sodium phosphate buffer at pH 6.0 and 0.1 M sodium acetate buffer at pH 4.0. 600 μL aliquot of enzyme was added to a quartz cuvette and measurements were taken over a period of 60 and 80 seconds respectively.

The intensity or count rate measures the amount of scattered light expressed as photons detected per second and is proportional to the size and concentration of the particles in solution.

2.9 References

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(11) Invitrogen Amine-Reactive Probe Labeling Protocol | Life Technologies.
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Chapter 3: Electrochemical Cell Design

3 Electrochemical cell design

This chapter outlines the initial problems encountered with the original commercial electrochemical cell provided by Q-sense (QEM 401), alongside designs and drawings detailing changes that were made. The two stages of redesign are documented along with the associated difficulties that arose at each stage, and the measures taken to rectify each problem. The design process involved discussions with university design workshops, sourcing of raw materials, computer-aided design of the cell, and thorough user testing of the new E-QCM-D module. The total time taken for the whole redesign process was approximately 8-9 months.

3.1 Commercial cell

The original commercial module had significant deficiencies and each problem encountered, rendered the equipment unusable for extended periods of time (3-4 weeks). The first major problem with the commercial module was in the positioning of the reference electrode, which entered from the side of the cell (horizontally) and was sealed with a small O-ring (Figure 3.1).

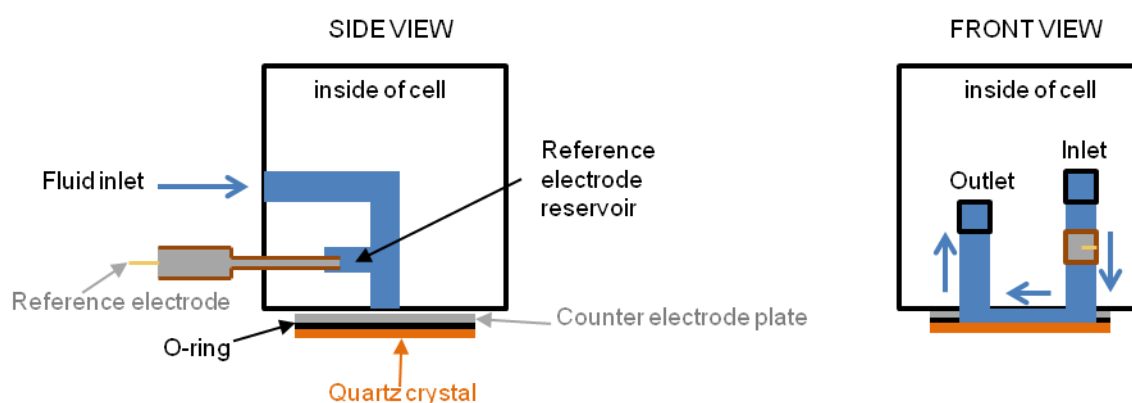


Figure 3.1. Schematic of the commercial Q-sense E-QCM-D cell design. The side view shows the entrance for the reference electrode and the reservoir in which the reference electrode sits. The front view shows the fluid flow path over the crystal (and through a predrilled hole in the counter electrode plate). The fluid is pulled through the cell via a peristaltic pump.

The Q-sense electrochemical module failed frequently due to the ease with which air could be trapped around the reference electrode entrance into the cell. Further to this, as a result of the sealing of the reference electrode with the O-ring, the cell was prone to pulling air bubbles into the flow chamber. The introduction of air bubbles into the cell breaks the connection between the reference and working electrodes. This loss of connection causes the potentiostat to apply an increased voltage to the working electrode to try and maintain the set potential between the working and reference electrodes. This increased voltage resulted in the stripping of both the gold layer and titanium adhesion layer (beneath the gold) (Figure 3.2). This was a significant problem as preparation time for each experiment would require an overnight modification, and in addition the cost of replacing damaged QCM crystals was high (£50).

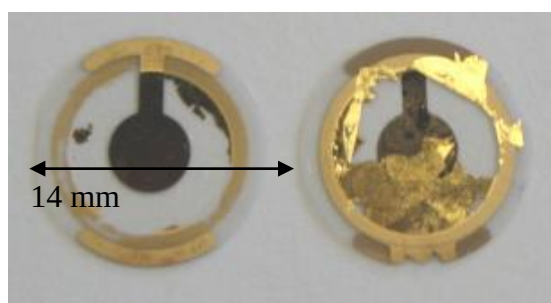


Figure 3.2. A QCM crystal with the gold coating stripped from the quartz crystal surface due to oxidation of the titanium adhesion layer.

The crystals were also very fragile due to crystals being very thin (0.3 mm). This was a concern because the lack of secure housing for the crystal, when placed in the module chamber (O-ring, Figure 3.1A), resulted in numerous crystal breakages occurring when the module was assembled and tightened ready for experimentation. Each time a crystal breakage occurred within the chamber, the entire module would need disassembling and cleaning, rendering the module unusable for short periods of time (4-7 days).

Another problem, during experimentation, was that the bubbles that were taken into the cell resulted in significant fluctuations in frequency and dissipation responses, which

were unrelated to mass changes on the crystal. This caused discrepancies in the frequency analysis and gave unreliable data (Figure 3.3). This rendered any experiment useless and time was also lost in the preparation and modification of the crystals (1-2 days).

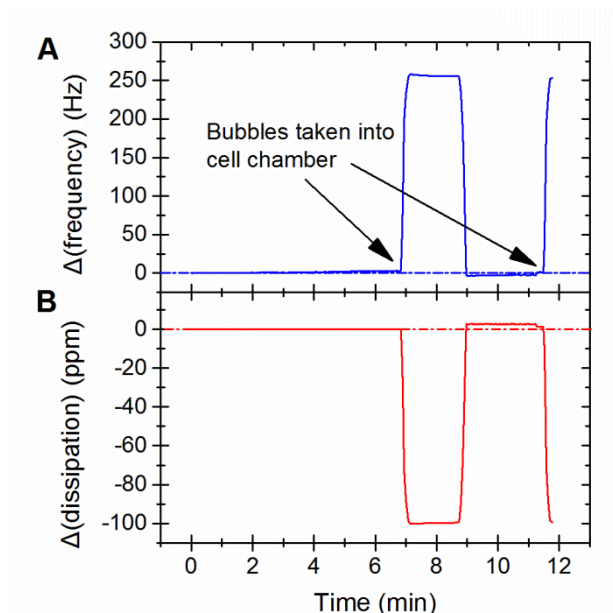


Figure 3.3. Frequency and dissipation response for the effect of bubble formation in the poorly sealed reference electrode chamber. 0.1 M Sodium phosphate buffer at pH 6.0, flowing at 0.1 ml min^{-1} . Seventh harmonic shown.

3.2 Improvements to the cell

3.2.1 First redesign

Figure 3.5 shows the breakdown of individual components and shows the key areas of the new cell design. Figure 3.5A and 3.5B show the inlet and outlet fluid channels with Figure 3.5B highlighting the entire fluid flow path chamber with the connected reference electrode reservoir.

The cell was redesigned to have the reference electrode vertically enter into a small reservoir. A reverse ferrule was introduced to keep a seal on the tip of the reference electrode (positioned around the bottom of the reference electrode, Figure 3.4) which ensured the reference electrode was sealed sufficiently to keep air bubbles out and to

maintain an ionic connection with the fluid flow path. This also helped keep the reference electrode well positioned above the small fluid inlet hole and help maintain a good seal.

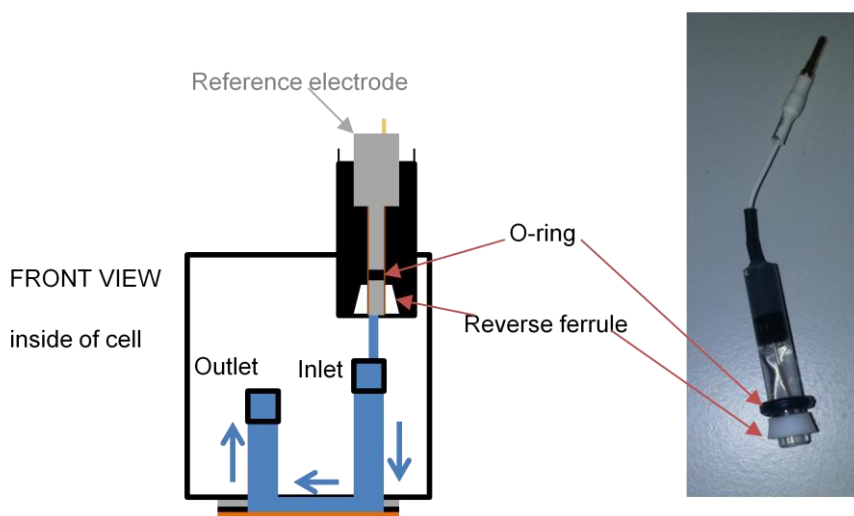


Figure 3.4. Schematic drawing of the new cell design incorporating the new vertically aligned reference electrode reservoir and reverse ferrule setup. The photograph shows how the reference electrode fits through the O-ring then the reverse ferrule.

The Pt sheet ensures a constant connection with the cell volume. Figure 3.5F shows the components making up the connection between counter electrode and the inner cell. Note the metal barrel in Figure 3.5F fits into the top opening in Figure 3.5E. The screw then connects to the gold pin which protrudes into the cell to make a connection with the Pt sheet. The Pt sheet is aligned by the inlet and outlet holes, above the main cell body. A gasket (black acetal, Delrin®) is placed above this, which fixes the platinum sheet in place. The O-ring (1.0 mm thickness, made from the high-chemically resistant polymer Chemraz®) is then positioned in the circular opening within the gasket to form a seal for when buffer flows into the cell chamber.

Figure 3.5G shows the individual components used to construct the cell before experimentation. Figure 3.5H is the constructed cell as described and Figure 3.5I is the fully constructed module with the outer casing and working, reference and counter electrode connections highlighted.

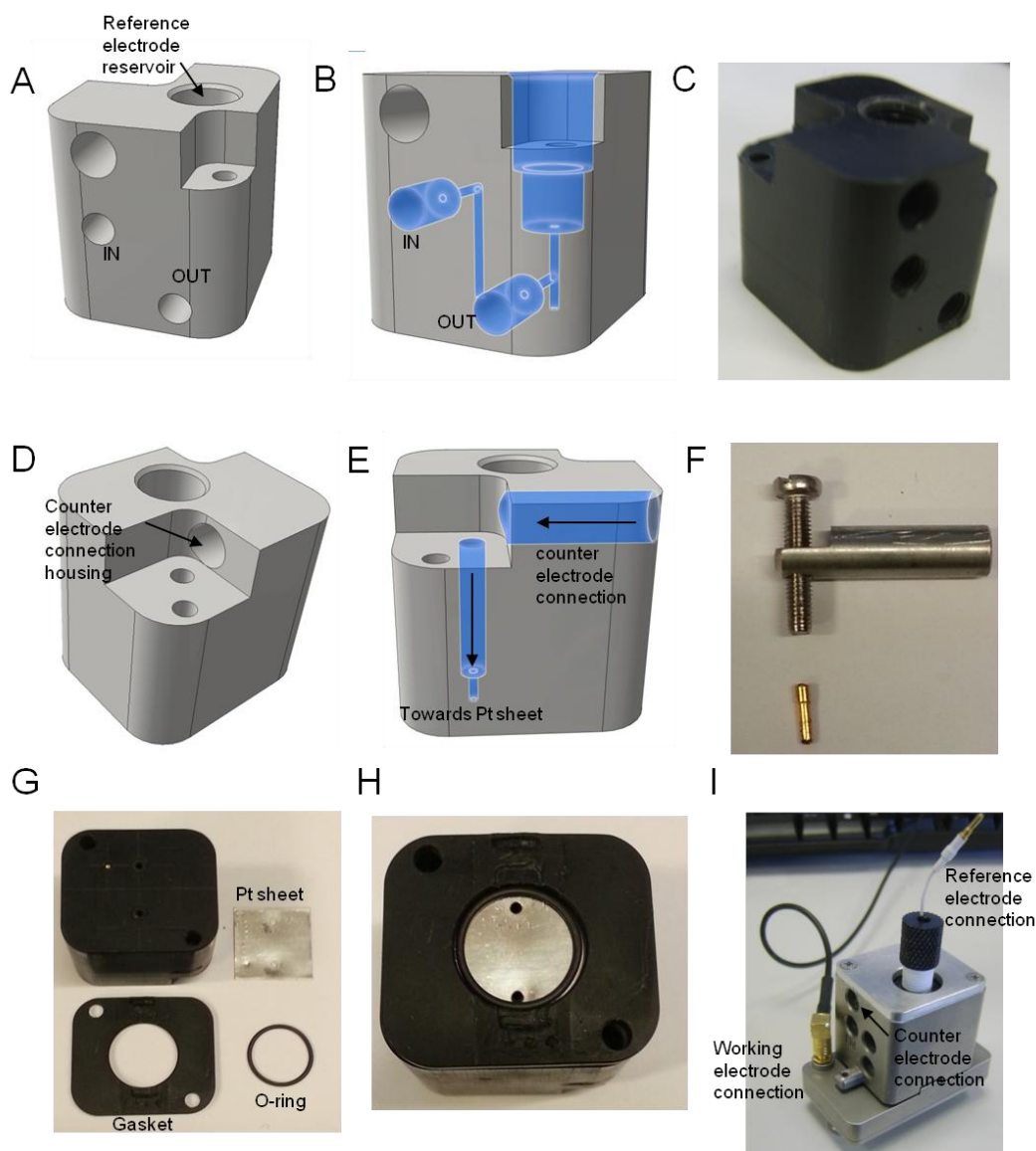


Figure 3.5. Panel A shows the side view of the redesigned cell detailing the fluid inlet and outlet holes along with the new reference electrode reservoir at the top of the module. Panel B shows the fluid flow path for the inlet and outlet and how the new reference reservoir maintains an ionic connection with the fluid. Panel C shows a photograph of the real module machined out of delrin. Panel D shows the opposite side view to panel A with an arrow indicating where the counter electrode connection is housed. Panel E shows the upper counter electrode housing where the metal contact sits and the lower housing which contains the screw and gold contact pin which makes contact with the platinum sheet. Panel F shows how the counter electrode connection is constructed. Panel G shows the individual components for assembling the cell including the platinum sheet which is positioned to align the two holes above the inlet and outlet holes. This allows fluid to pass over the platinum sheet. The gasket sits on top of the sheet and an O-ring is placed in the spacing (shown in Panel H). Panel I shows an assembled module with working electrode and reference electrode connections in place. The pin for the counter electrode from the potentiostat fits in the hole marked. The images in A, B, D and E were designed using AutoCAD 2013 and Inventor Fusion 2013.

3.2.1.1 Effects of temperature

The quartz crystals used throughout this thesis are ‘AT’ cut crystals. The relationship between temperature and frequency for these crystals is cubic, with an inflection point near room temperature. As a consequence the AT-cut quartz crystal is most effective when operating at or near room temperature. The crystal is well suited to applications requiring a high degree of frequency stability over a wide temperature range.¹

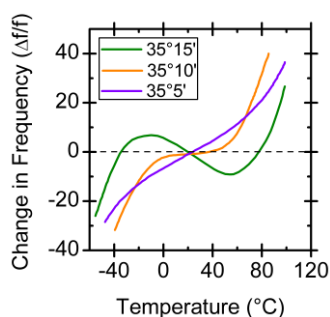


Figure 3.6. Cubic frequency versus temperature relationship highlights AT cut crystals will give only small degrees of variation in frequency at temperatures close to 25°C or room temperature. The angle of cut is given in the legend. This figure is adapted from Sauerbrey et al.¹

A more significant contributor to the temperature sensitivity of frequency measurements is the change in temperature arising from fluid flowing through the cell. The resonance frequency is dependent on the viscosity and density of the fluid and in turn this is dependent on the fluid temperature according to the Arrhenius equation:

$$\mu(T) = \mu_0 \exp\left(\frac{E}{RT}\right) \quad \text{Equation 3-1}$$

where $\mu(T)$ is viscosity at given temperature, μ_0 is the viscosity at a reference temperature and E is the viscosity temperature coefficient. This means that as temperature increases, the viscosity of a liquid will decrease and may affect any baseline responses during QCM–D measurements.

The standard non-electrochemical flow cell was originally made out of PTFE. A peristaltic pump (Ismatech) was used to draw solution through the cell. A temperature regulator was situated underneath the module and this helped maintain the temperature of the fluid resulting in minimal temperature variation. The new cell is made of black poly(oxymethylene) plastic (black acetal, Delrin®) which was chosen due to its good electrical insulating properties alongside being chemically inert and easily machined. However, black acetal is a poor thermal conductor, having a heat conductivity two orders of magnitude lower than that of titanium (Table 3.1).

Table 3.1 Thermal properties of black acetal (Delrin®) vs. titanium

	Delrin®	Titanium
Thermal Conductivity ($\text{W m}^{-1} \text{K}^{-1}$)	0.3-0.4	22
Heat Capacity ($\text{kJ kg}^{-1} \text{K}^{-1}$)	1.0-1.3	0.52
Density (kg m^{-3})	$1.3-1.4 \times 10^3$	4.5×10^3
Volumetric Heat Capacity ($\text{MJ m}^{-3} \text{K}^{-1}$)	1.6	2.3

Consequently temperature gradients can exist within this cell where the solution running through is not equilibrated to the same temperature throughout the flow chamber. As a result of this lack of control over the flowing solution temperature, the crystal frequency may vary causing period oscillations in the frequency and dissipation traces.

3.2.2 Second redesign

The problems with the first design became evident after approximately 18 months. These were caused by deformation of the Pt sheet over time and sealing problems within the cell and within the reference electrode reservoir, causing fluid to leak during experiments.

Liquid escaped from the reference electrode reservoir and shorted the connection between the reference electrode and the working electrode due to the reverse ferrule

system (Figure 3.4) not retaining liquid in the reservoir. As a result, crystal surfaces were rendered unusable. This has been addressed in the new design (Figure 3.9B). The flow path to and from the chamber had a weak seal between the counter electrode sheet and the point where the liquid enters/exits the crystal chamber (Figure 3.4B) causing distortions in the sheet due to inefficient O-ring positioning and sealing. This lack of sealing caused bubbles of air to form between the platinum sheet and the inlet/outlet holes. These pockets of air feed into and become trapped in the chamber, leading to displacements in the frequency and dissipation readings. The mis-positioning of the platinum sheet also caused liquid to leak onto the opposite side of the sheet and as a result, the frequency traces were severely affected as the excess liquid is sensed as an increase in mass (Figure 3.7). This also caused problems with the electronic connections as the leaking liquid caused corrosion of the gold contacts and over time these also needed replacing.

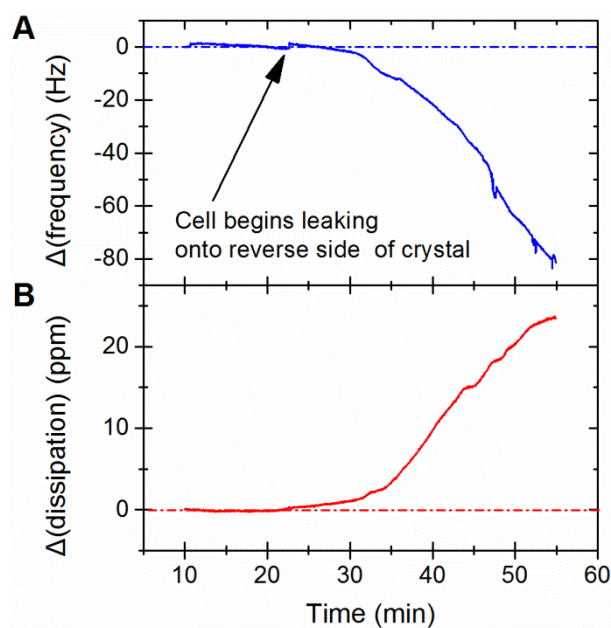


Figure 3.7. Frequency and dissipation traces showing the effect of fluid leakage onto the reverse side of the crystal during data acquisition. 0.1 M Sodium phosphate buffer at pH 6.0, flowing at 0.1 ml min^{-1} . Seventh harmonic used. The reason for the change in frequency is because the increasing amount of water on to the reverse of the crystal is sensed as an increase in mass.

To solve these problems, a thicker O-ring (1.8 mm) was introduced to ensure a better seal for the inner cell chamber shown in Figure 3.8 (right hand panel). This also helped resolve the issue of crystal breakages detailed in Section 3.1 and when the full module was assembled the crystal was able to rest more securely against the thicker O-ring.

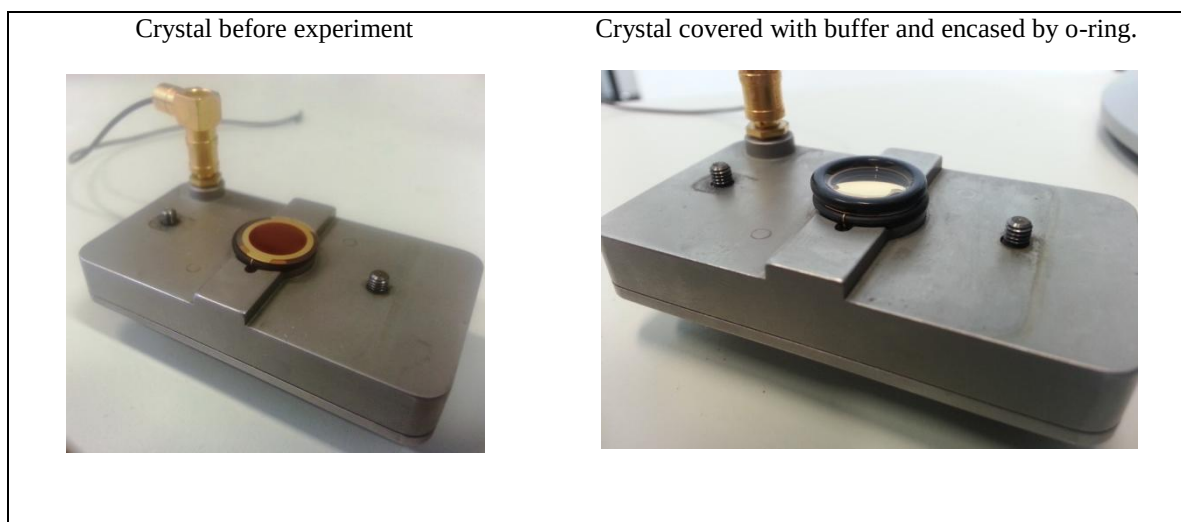


Figure 3.8. Photographs taken showing a QCM crystal mounted before an experiment (left) and after an experiment with the cell volume and O-ring (right). In this case the O-ring being used is the new thicker O-ring. The total cell volume is 40 μL , however as the cell had been taken apart, some residual volume was lost when taking the image.

The reference electrode reservoir was then redesigned to provide a more secure reference electrode housing. This was done by introducing a ferrule design into the machining of the cell which now positions and holds the reference electrode securely (Figure 3.9B). This has minimised the volume of liquid in the reservoir to ensure connection between the cell chamber and reference electrode. The gasket, which is the thin machined component used to hold the platinum sheet in place (Figure 3.9G) now has a thicker edge (0.5 mm) to ensure the platinum sheet is held more firmly against the inlet/outlet liquid openings.. This new cell design has proven successful in producing consistent and reliable E-QCM-D data and has been used throughout this thesis.

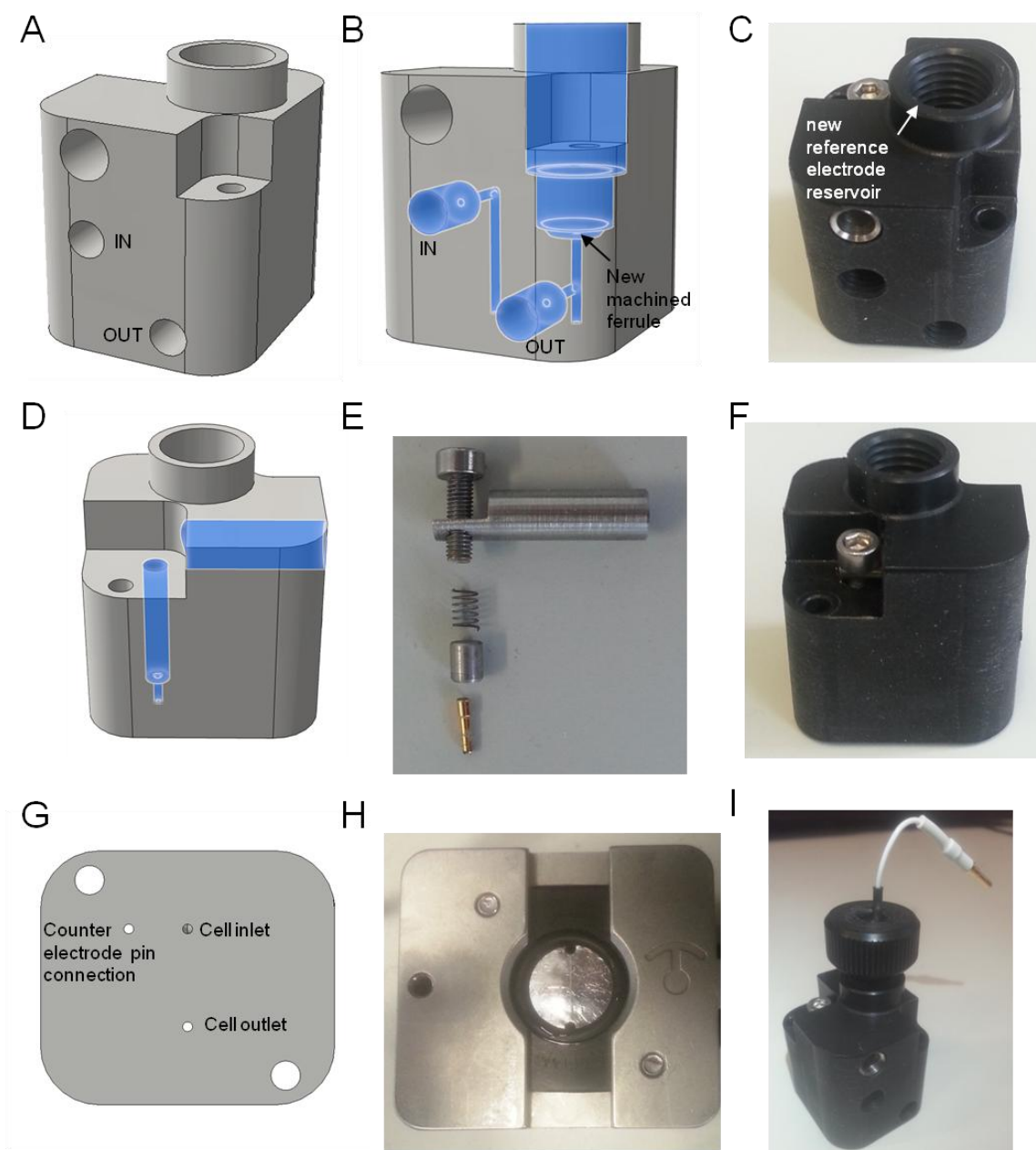


Figure 3.9. Panel A shows the side view of the redesigned cell detailing the fluid inlet and outlet holes along with the new reference electrode reservoir protruding from the top of the module. Panel B shows the fluid flow path for the inlet and outlet and the new ferrule machined into the bottom of the new reference electrode reservoir. Panel C shows a photograph of the real module machined out of delrin with the new reference electrode housing highlighted. Panel D shows the opposite side view to panel A highlighting where the counter electrode connection is housed. Panel E shows the individual components used to construct the counter electrode connection. Panel F shows how the counter electrode connection is constructed. Panel G shows the bottom of the module and where the fluid leaves and re-enters the module during operation. It also shows the exit point for the gold contact pin for the counter electrode connection. Panel H shows the assembled module with a thicker O-ring to keep a better seal of the liquid during measurements. Panel I shows an assembled module with the reference electrode in place. The images in A, B, D, E and G were designed using AutoCAD 2013 and Inventor Fusion 2013.

3.3 Conclusion

In this chapter the development of the Q-sense electrochemical module (QEM401) has been detailed. The major problems with this commercially available E-QCM-D module were the poor positioning of the reference electrode, which led to inefficient sealing, intake of air bubbles into the cell, and the stripping of the gold and titanium layers.

The final design has solved these problems by combining a more secure housing for the reference electrode to sit in whilst also ensuring a minimum volume of fluid remains in the chamber to prevent loss of connection. The advantage of the new reference electrode reservoir is that it is designed for commercially available reference electrodes (RE-6, BASi) and therefore sourcing and replacing reference electrodes is less problematic.

Further problems with leaking and positioning of the platinum counter electrode sheet have been solved using a thicker inner gasket to provide a better seal against fluid leaking. This secure housing, alongside the more secure positioning of the crystal during experimentation (thicker O-ring) has significantly cut down on the cost of replacing broken or damaged crystals, and has also reduced the associated ‘down-time’ previously required when disassembling and cleaning the cell due to broken crystals.

In addition to this, a new connection has been incorporated, between the potentiostat counter electrode connecting pin and the platinum sheet. The platinum sheet now sits permanently beneath the inner chamber and remains in constant connection with flowing liquid.

Future considerations would include the machining of the cell from titanium so as to obtain better temperature control during experiments. However, machining titanium is impractical and titanium also exhibits high electrical conductivity, and therefore an alternative would be to anodise the finished cell before experiments. The advantage of

using Delrin is that the newly designed module suffers less ‘down-time’ than the original commercial design. The low cost means that multiple modules can be manufactured, and if difficulties do arise, there is no time lost between replacing modules.

The CFB group is currently working with the University of Manchester Intellectual Property Team (UMIP) on patent and design rights for this module. Several research groups in the UK have also shown interest in the new module and the results obtained using the redesigned cell.

3.4 References

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Chapter 4:
Sources of activity loss in bilirubin
oxidase adsorbed to thiol-modified gold

4 Sources of activity loss in bilirubin oxidase

4.1 Overview

The E-QCM-D was used to show that the catalytic lifetime of *MvBOx* adsorbed on, or covalently attached to an organothiol-modified electrode, is shortened from days to hours when the applied potential is cycled to below a critical potential (ca. 0.6 V vs. SHE at pH 6.0). One of the barriers to the practical use of redox enzymes in fuel cells as well as biosensors and industrially useful enzymatic transformations, is the proteins' slow loss in activity with time. The adsorbed mass, stiffness of the protein adlayer, and electrocatalytic activity was measured, and it was concluded that during chronoamperometric control, structural rearrangements and water loss are the primary culprits in activity loss in these cathodes. The effect was most pronounced when simulating a changing power load by cycling the potential. Changes in the position of the catalytic wave (halfway point of the steepest part of the wave (E_{cat})) were seen most prominently when cyclic voltammetry was ongoing during successive enzyme adsorptions. Covalent attachment did not mitigate activity loss, and both, simple adsorption and covalent binding were compared to a second multicopper oxidase, *TvL*, suggesting that inactivation is dependent more on structural rearrangements than enzyme type. Enzyme-coated cathodes that receive their electrons directly from an electrode, rather than via redox mediators, will need additional stabilisation before they will be durable enough for miniature fuel cells from which a sustained power output is required.

4.2 Chronoamperometric Studies

To probe how enzyme activity changes with time under conditions approximating constant load in a fuel cell, this section describes the results from chronoamperometry (CA) experiments, where *MvBOx* was adsorbed onto 3MPA-modified and bare gold surfaces. This section then looks at experiments where the potential was stepped between high and low potentials, and probes the effect on the resulting catalytic activity. The figures presented in this chapter are the result of a series of replicate and reproducible experiments, where a minimum of three repeats were carried out for each experiment.

4.2.1 Enzyme adsorption on a 3MPA-modified sensor

In this measurement, *MvBOx* was adsorbed to a 3MPA-modified gold sensor in pH 6.0 phosphate buffer pre-saturated with O_2 , at 25°C. A potential of 0.21 V vs. SHE was applied 7.5 min after the enzyme was injected and held constant for ca. 8 h. This potential is low enough to drive enzymatically catalyzed O_2 reduction, but sufficiently high enough to avoid O_2 reduction on bare gold.

The trace in Figure 4.1A is typical: the change in adsorbed mass decreased by about an eighth over the first 2.5 h, then remained stable for the next 5 h at $0.68 (\pm 0.01) \text{ mg cm}^{-2}$. An initial steady-state current of $-110 \text{ }\mu\text{A}$ (Figure 4.1C) was observed and the current magnitude was up to $30 \text{ }\mu\text{A}$ larger for the first 1.0–1.5 min while dissolved O_2 near the sensor surface was depleted. The initial turnover frequency of the enzyme, k_{cat} , under chronoamperometric control (constant potential) is about 35 s^{-1} , about 7 times lower than values presented for *MvBOx* in the absence of mass-transport limitations,¹ but more than 10 times higher than previous work by Kamitaka et al. using an E-QCM setup.²

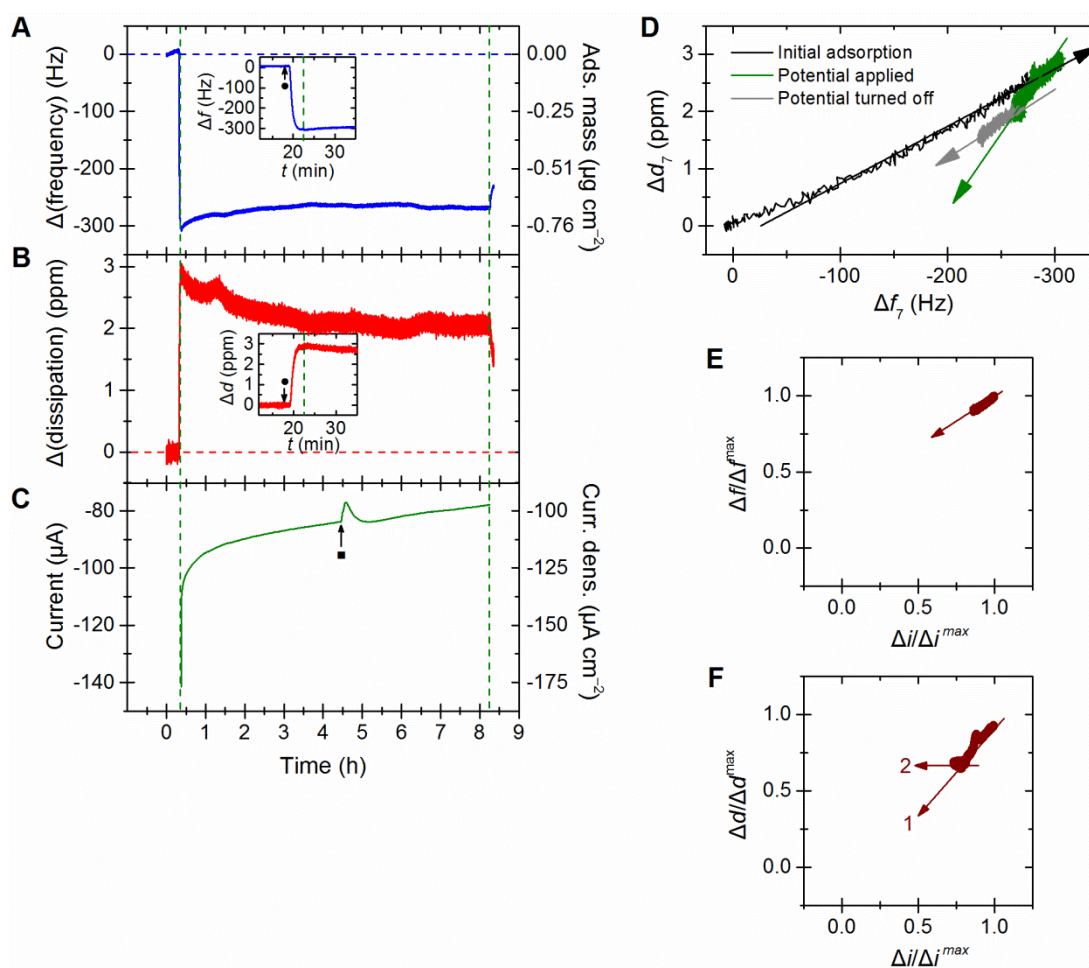


Figure 4.1. E-QCM-D response with time for MvBOx adsorbed onto a 3-mercaptopropionic acid layer. Panel A and B show the frequency and dissipation response respectively. Panel C shows the O₂ reduction current. Panel D shows the Δd vs. Δf plot highlighting the periods during adsorption, when a potential is held and after the potential is turned off. Panel E and F show the frequency-activity plot and dissipation-activity plot respectively. Panel F shows the dissipation-activity plot with arrows highlighting the decrease in current with Δd (1) followed by a steady response in Δd (2). Insets in panel A and B show the frequency and dissipation response during adsorption (25 μl enzyme at 24 mg ml^{-1}) Circle (●) indicates when MvBOx was injected. Green dashed line shows when a potential of 0.21 V vs. SHE was applied. Note that Δf^{max} relates to the maximum magnitude of change in the frequency. Conditions: 0.1M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min^{-1} , projected electrode area 0.80 cm^2 . Flow of O₂ lowered for 10 min at (■).

Over the course of the measurement, the current decayed by approximately 30% of its initial value. Although the initial adsorbed mass of enzyme correlates with the initial magnitude of the catalytic current, the lack of any changes in adsorbed mass i.e., mass loss over time, does not parallel the changes seen in catalytic activity and the change in

mass remains relatively stable while current continues to fall. Like the current magnitude, the dissipation also drops by about 30% over time (Figure 4.1B). This value takes into account the post ‘overshoot’ baseline which is the stable dissipation response i.e., the response after the dissipation initially spikes on adsorption, before returning to a stable value after approximately 10 minutes after adsorption.

The magnitude of $\partial d/\partial f$ is more than two times higher when a potential is applied (black vs. green trace, Figure 4.1D) suggestive of a change in organisation/structural arrangement of the protein. The first-order time constant for current loss of *MvBOx* on 3MPA was 2.9 h compared to 2.2 h on bare gold.

The dissipation-activity plot in Figure 4.1F shows a gradual decrease (arrow 1) followed by a stable response (arrow 2) suggesting that mechanical changes in the enzyme could be the source of activity loss, due to the correlation between adlayer stiffness (decreasing d over time) and the fall in activity.

4.2.2 Chronoamperometry on a bare gold sensor

In this measurement, *MvBOx* was adsorbed to a bare gold sensor surface and a potential of 0.21 V vs. SHE was applied for ca. 2.6 h. The reason for this measurement was to act as a comparison to the adsorption on the thiol–modified surface in Figure 4.1.

The E–QCM–D response of *MvBOx* was carried out on bare gold and the change in frequency in Figure 4.2 is of a lower magnitude compared to the 3MPA–modified gold surface (Figure 4.1) and the magnitude of catalytic current is also lower, within the normal range of variation that was observed. When a constant potential is applied to *MvBOx* on bare gold (ca. 28 min) there is a similar mass loss (~ 25 Hz, $0.06 \mu\text{g cm}^{-2}$) to that seen in Figure 4.1. The short, vertical portion of the dissipation-activity trace (Figure 4.2F, arrow 1) arose from a short increase in dissipation after the potential was turned on,

after which the trace returned to its more typical linear correlation with a slope near 1 (arrow 2). After approximately 2 h the dissipation response became stable (arrow 3).

The Δd vs. Δf gradient during adsorption on bare gold (black line, Figure 4.2D) was $0.007 \text{ ppm Hz}^{-1}$ compared to a gradient of 0.01 ppm Hz^{-1} in Figure 4.1D. The reduced dissipation change and overall reduced Δd vs. Δf gradient for bare gold suggests an increased rigidity when enzyme adsorbs to bare gold.

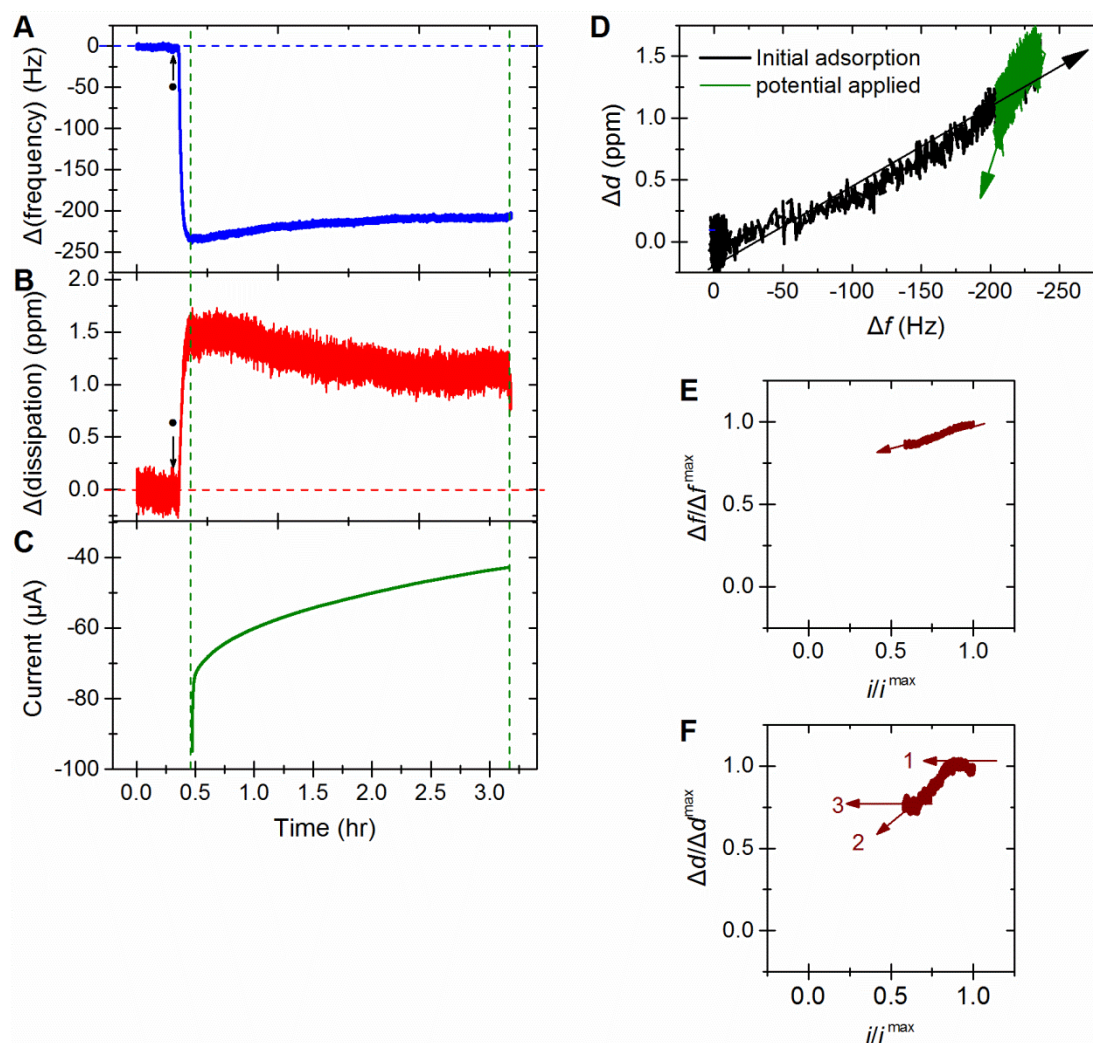


Figure 4.2. E-QCM-D response with time for MvBOx adsorbed on a bare gold crystal surface: (A) frequency change, (B) energy dissipation change, (C) O_2 reduction current. Circle (●) indicates when 25 μl MvBOx (24 mg ml^{-1}) was injected. Green dashed line shows when a potential of 0.21 V vs. SHE was applied. Panel D shows the Δd vs. Δf plot highlighting the regions during adsorption and when a potential is applied. Panel E and F show the frequency activity and dissipation-activity plots during chronoamperometry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^{\circ}C$, 0.1 $ml \text{ min}^{-1}$. Current density based on geometric area of 0.80 cm^2 , seventh harmonic measured.

This is further supported by comparing to the 3MPA-modified measurement: on bare gold, the gradual decrease in dissipation before a stable response occurs over 1.5-2 h compared to almost 3.5 h on the 3MPA-modified surface.

4.2.3 Switching between high and low potential

Catalysis occurs at reductive potentials, however to simulate the effect of varying the potential load on an enzymatic biofuel cell, the potential was switched between high and low potentials, effectively switching enzyme catalysis on and off. The higher potential of 0.86V vs. SHE was chosen because at this potential, oxygen catalysis is no longer thermodynamically favourable, and the enzyme cannot turnover. In this experiment, a reducing potential of 0.21 V was held for 900s, after being held at 0.86V for 60s, 300s, and 600s (i.e., 900s at 0.21V followed by 60s at 0.86V, followed by 900s at 0.21V).

Each 0.86 V step is highlighted by a drop in both mass and dissipation followed by a return in stability when reverted to 0.21V. The first application of 0.86V (60s) caused a 10% drop in the mass ($\sim 0.05 \mu\text{g cm}^{-2}$) but over time, the extent of disturbance in both traces was reduced and the mass loss in each case also drops. As the experiment proceeds, the changes in frequency and dissipation are negligible.

Figure 4.3 shows the current loss trace during the measurement. Over the course of the measurement the normalised activity loss was 0.61 ($(\Delta i / \Delta i^{\text{max}}) / t$). Interestingly, there was a discrepancy between the expected current loss (before switching to 0.86V) and the actual loss (after switching back to 0.21V).

In Figure 4.4 the expected current loss for each 0.21V step is plotted using a first order exponential (dashed traces). Each time the potential is switched back to 0.21 there is a reduction in the ‘actual’ versus ‘expected’ current response. When a potential of 0.86 V is applied for 60 s and then switched to 0.21V, there is a difference of $\sim 11\%$ between actual and expected activity (crimson versus orange trace, Figure 4.3D).

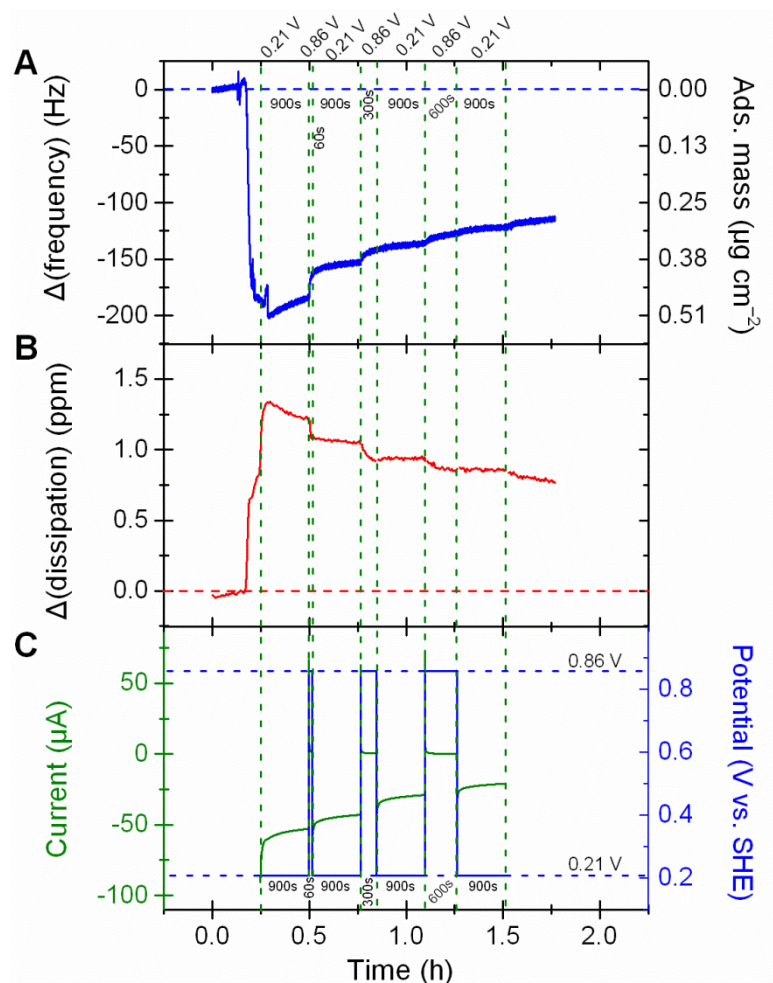


Figure 4.3. The effect of switching chronoamperometry between high and low potentials on the current response of *MvBOx* (24 mg ml^{-1}) adsorbed on a 3MPA layer. Panel A and B show the frequency and dissipation response to switching the applied potential between 0.86 and 0.21 V vs. SHE. The potential of 0.86 V was held for increasing time periods (60 s, 300 s, and 600 s, labelled in panel A and panel C) in between 900 s intervals of holding at 0.21 V. Panel C shows the changes in current response, while changing the applied potential over time. Panel D shows the current response with expected current loss trendlines (fitted to a 1st order exponential) given after each period of holding potential at 0.86 V. Conditions: O_2 -saturated 0.1 M sodium phosphate buffer pH 6.0 at 25°C flowing at 0.1 ml min^{-1} with continuous O_2 bubbling; seventh harmonic shown.

When 0.86 V is applied for 300 s, there is a difference of 33% between actual and expected activity (orange versus blue trace) when switched back to 0.21 V. Finally, the potential is held at 0.86 V for 600 s and when switched back to 0.21 V, there is a 23% difference between the actual and expected values. These observations suggest that holding the enzyme at high potential is likely causing inactivation.

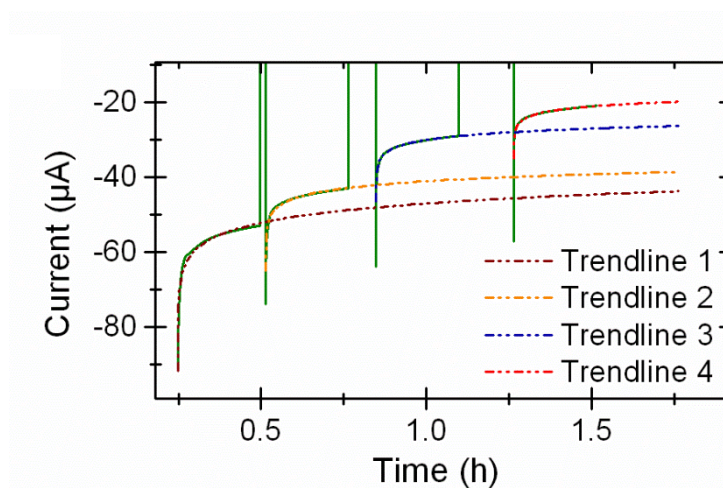


Figure 4.4 The effect of shifting the potential on catalytic current. The ‘expected’ current loss trendlines (dashed traces, fitted to a 1st order exponential) highlight the difference between the actual current loss responses after each period of holding the potential at 0.86V. Conditions: O₂-saturated 0.1 M sodium phosphate buffer pH 6.0 at 25 °C flowing at 0.1 ml min⁻¹ with continuous O₂ bubbling.

This inactivation is further highlighted by a drop in the adsorbed mass efficiency ($\Delta i/\Delta f$) upon each application of a reducing potential. This parameter describes the change in catalytic current generated for every unit change in frequency. In Figure 4.4, there is a decrease in the adsorbed mass efficiency each time the potential switches from a non-reductive to a reductive potential i.e., from 0.86 V to 0.21 V (Table 4.1).

Table 4.1 Table showing the change in adsorbed mass efficiency upon each application of a reducing potential.

Measurement	Efficiency ($\mu\text{A Hz}^{-1}$)
Potential held at 0.21V	0.28
Returning to 0.21V after 60s at 0.86V	0.27
Returning to 0.21V after 300s at 0.86V	0.21
Returning to 0.21V after 600s at 0.86V	0.17

Table 4.1 suggests that the longer the enzyme is held at high potential the greater the loss in efficiency, when a reducing potential is applied. The high potential appears to destabilise the enzyme film.

4.2.4 Comparison of chronoamperometry measurements

To compare the chronoamperometry measurements, the normalised current loss $((\Delta i/\Delta i^{max})/t)$ was considered in both the bare gold and 3MPA measurements. Table 4.2 shows that normalised current loss on the bare gold measurement was more than twice that in the 3MPA measurement, which suggests there is a greater retention of current on the 3MPA-modified surface. The first-order time constants for current loss also suggest that the stability of response on the 3MPA-modified surface was increased.

Table 4.2 Table showing the current loss, time constants and adsorbed mass efficiency of MvBOx adsorbed to 3MPA-modified gold when the potential is held constant (Figure 4.1) and when the potential is switched between high and low potentials (Figure 4.3). Current loss and efficiency loss normalised to time duration of experiment.

Measurement	Starting current (- μ A)	Final current (- μ A)	Normalised current loss $(\Delta i/\Delta i^{max})/t$	Time constant (τ , hr)	Normalised efficiency loss (μ A Hz ⁻¹ h ⁻¹)
3MPA CA (Figure 4.1)	110	78	0.08	2.9	0.01
Bare gold CA (Figure 4.2)	90	43	0.18	2.2	0.072
CA switching between potentials (Figure 4.3)	91	21	0.18	-	0.26

The normalised adsorbed mass efficiency loss in Table 4.2 shows that MvBOx adsorbed to bare gold experiences a greater loss in catalytic efficiency compared to being adsorbed on 3MPA. This loss in efficiency is three times larger when switching between high and low potentials, which suggests that applying a non-reducing potential greatly diminishes catalytic competency.

4.2.5 Discussion of Chronoamperometry measurements

The current magnitude at the beginning of the experiment on 3MPA (Figure 4.1C) was up to 30 μ A larger for the first 1.0–1.5 min while dissolved O₂ near the sensor surface was

depleted. However, this could also be a result of there being an initial peak in activity when the enzyme first adsorbs. In this measurement, both the dissipation and activity drops by 30% while the frequency remains stable over approximately 8 h.

Adsorption of proteins contributes towards this dissipated energy and softer films experience more inelastic collisions than more rigid films. The formation of softer films, therefore, produces higher Δd values and rigid/stiffer films produce lower Δd values (Introduction). This change can give insight into conformation changes during protein layer formation.³ Therefore, the change in dissipation over the 8 h measurement is indicative of a stiffening of the enzyme after adsorption, with the concurrent loss in activity suggestive of enzyme inactivation. Thus, the term ‘film loss’ is not strictly accurate here. The slow loss of activity in *MvBOx* must be due to some non-desorptive process like structural/mechanical rearrangements, conformation changes or cofactor loss.

The strong correlation between adlayer stiffness (inversely related to Δd) implicates structural changes as a possible source of activity loss. The effect of an applied potential on protein structure also appears in the plot of Δd_7 vs. Δf_7 (Figure 4.1D) which normalises dissipation to adsorbed mass to quantify viscoelastic losses.^{4,5} The change in magnitude of $\partial d/\partial f$ in Figure 4.1D is indicative of two distinct regimes under which the enzyme exists; 1) before and 2) during the application of a potential. This change in magnitude is more than two times higher when the potential is applied: the protein layer has stiffened under the applied potential.

When comparing the bare gold example, the dissipation decreases and stabilises, over a shorter time frame. The decrease in dissipation, therefore, suggests that the secondary structure of *MvBOx* could be changing, but over a shorter time frame, compared to 3MPA. One possible reason for this is that the secondary structure on bare gold is kept intact less readily than on 3MPA-modified gold. This idea is consistent with circular

dichroism spectra taken of cytochrome b562 protein molecules, which show that protein secondary structure is more effectively retained when adsorbed to thiol-modified gold surfaces than to bare gold surfaces.⁶

The reason for both the decreasing dissipation and decreasing current could be due to denaturation, and this idea is consistent with E-QCM work done by Kamitaka *et al.*, which follows the adsorption and electrocatalysis of *MvBOx* on bare Au sensors. In this study, they suggest that the lower catalytic current density on bare gold, compared to that on carbon electrodes, is a result of enzyme denaturation. Therefore, in combination with the lower catalytic activity seen in the bare gold measurement, and assuming a better retention of secondary structure on 3MPA, enzyme denaturation is possible.

In addition, proteins undergo conformational changes upon adsorption to a solid surface, and it is generally accepted this is because the conformation of a protein that corresponds to the free energy minimum in solution, typically, does not correspond to the free energy minimum of the same protein once it is in contact with a surface. The protein-surface contact area brings about an increase in free energy and therefore, proteins tend to maximize their footprint through conformational rearrangement.⁷⁻⁹ This structural rearrangement is further driven by an entropy gain, resulting from a loss of ordered secondary structure, and in addition to the release of counter ions or solvation molecules.¹⁰ To this end, the conformational changes that occur on surface adsorption, lead to observable effects such as a reduced amount of adsorbed protein (loss of water molecules) and an altered secondary structure.¹¹ This is particularly important because it suggests that the mass loss following adsorption, in both Figure 4.1 and 4.2, could be attributed to a loss in solvation/water molecules from the enzyme which accompany a slow change in conformation/mechanical arrangement of the enzyme. Therefore, loss of secondary structure, with some possible dehydration, is a possible explanation for the

dissipation, mass and current responses seen in Figure 4.1 and Figure 4.2. The idea of a loss in secondary structure over time is consistent with a recent study by Pankratov *et al.* where, over the period of three hours they observed more than a 50% loss in activity of MvBOx immobilized onto a bare gold electrode, under constant potential control at 400 mV. This 3 hour loss in activity is paralleled in the measurement in Figure 4.2. Furthermore, in combination with their AFM and ellipsometry data, showing an irreversible character for adsorption, they suggest that the time decay for activity is due to a gradual deactivation and conformational change in MvBOx.¹² For these reasons, MvBOx-coated cathodes for use in fuel cell systems will need additional treatments such as structural scaffolds, before they will be durable enough to be incorporated into miniature fuel cells, where a sustained power output is required.

In Section 4.2.4, Figure 4.3 shows that when the potential is switched between 0.86 and 0.21 V vs. SHE, respectively, there is a potential-induced instability. This is more apparent when the first potential of 0.21V is applied. This causes an immediate jump in the dissipation trace and suggests a disruptive effect on the enzyme structural arrangement. There was also an instantaneous increase of approximately 0.3 ppm when the potential was switched; however, this was most likely down to the applied potential disturbing the potential that drives the crystal.

The difference seen between the ‘actual’ and ‘expected’ current values suggests that the enzyme may be undergoing an inactivation process when held at high potential. In this situation the enzyme does not recover current, and therefore the measured activity diverges from the expected value when switching back to low potential.

Each time the enzyme is held at high potential, there is a decrease in the dissipation response, compared to the relatively stable response when held at low potential. This suggests that there could be some structural rearrangement/stiffening (decrease in d),

which in turn, could play a part in the inactivation that causes the discrepancy between ‘actual’ and ‘expected’ activity. This instability in both current and dissipation (structural arrangement) could pose a problem for incorporating such enzymes into real systems, where a variable load is common.

An interesting point to consider is the possibility of a field effect on the protein that causes inactivation. One way of addressing this is to test the effect of altering the size of the potential step during the application of a potential.

The inactivation of the enzyme adlayer at high potential poses the idea that there could be a reactive species being generated at the surface of the electrode which causes inactivation. If such a species exists, one possible method for determining whether the generation of the species is potential dependent is to hold the enzyme adlayer at different high, non-reducing potentials, and observe the rates of activity/efficiency loss. Another typical experiment to approach this problem would involve holding the enzyme at a low potential for a short period, before switching to high potential, and holding, for a longer period (i.e. 8+ hours), and then returning back to the low potential. The difference in current before and after this high potential step could be directly related to any long term change in dissipation, and may give further information on any inactivation processes.

Another question which arises, is whether there exists a potential related to either the isoelectric point of the protein, the surface, or both, and whether this causes a change in the mechanical properties of the enzyme that are associated with inactivation. This could help to understand whether potential dependency affects the utilisation of *MvBOx* in a fuel cell setting.

4.3 Cyclic voltammetry studies

To simulate the effect of a variable load on an enzymatic biofuel cell, cyclic voltammetry (potential cycling) was carried out on *MvBOx* adsorbed to bare and 3MPA-modified gold (Figure 4.5). This was done to see if a constantly varying (cycling) load would have a similar effect on adlayer activity to that seen in Figure 4.3.

4.3.1 Potential cycling on 3MPA-modified gold

In this measurement, *MvBOx* was adsorbed to a 3MPA-modified gold surface and potential cycling was carried out over the potential range 0.91–0.21V vs. SHE for 60 scans.

When the applied potential is kept constant, like in Figure 4.1, the mass remains relatively stable, however, cycling the potential causes an immediate drop in adsorbed mass (Figure 4.5A) where, typically, the mass drops by 20–25% over ca. 15 min. The catalytic current falls by roughly 30% over the same period (Figure 4.5C). Catalytic currents above ca. 110 μA produced voltammograms with a flattened shape indicative of mass transport polarisation, so the current at 0.31 V vs. SHE on the reductive sweep was used for activity loss calculations.

The activity dissipation showed a linear relationship, similar to the constant potential measurement (Figure 4.5A, inset), however it again had a non-zero intercept. After 10–20 potential cycles, the frequency trace stabilises, but the O_2 reduction activity continues to fall. Parallel measurements made on bare gold showed similar results, with potential cycling resulting in a 43% mass loss over ca. 15 min, indicative of a less stable layer (Figure 4.6). During potential cycling, both the frequency and dissipation traces showed periodic oscillations that were slightly out of phase (green dashed line, Figure 4.5D E & F) with the applied potential (Figure 4.5C). These oscillatory changes (Figure 4.5D) have

maximum peak-to-peak amplitude of ca. 9 Hz (0.02 ng cm^{-2}) which would equate to roughly 3% of an enzyme monolayer.

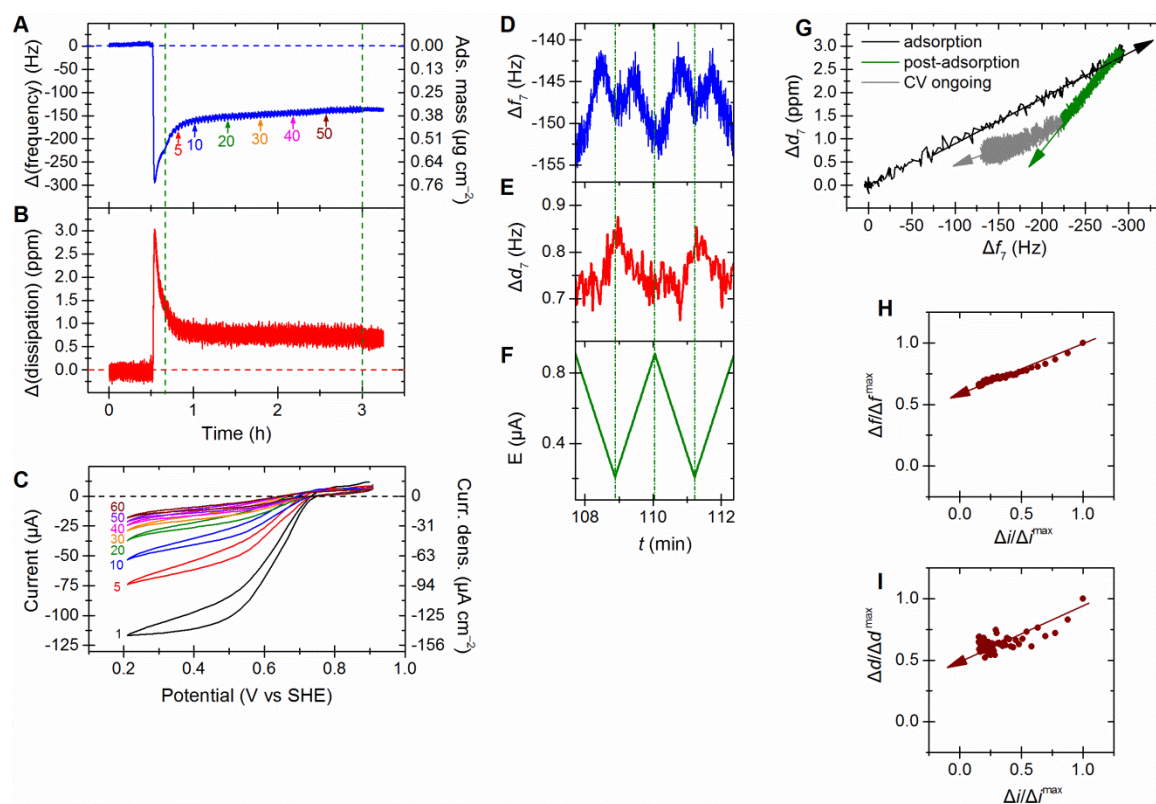


Figure 4.5. Effect of potential cycling on *MvBOx* adsorbed on a 3MPA modified QCM crystal. Frequency (A) and dissipation (B) response for the addition of *MvBOx*. Panel C shows scans 1,5, 10, 20, 30, 40, 50 and 60 of 60 CVs. cycled between 0.91-0.21 V vs. SHE, 10 mV s^{-1} . Green dashed lines show the period of CVs. with the arrows marking the start of the numbered cycles. Panels D & E show the frequency and dissipation responses with applied potential for scans 30 and 31 where effects of CV give rise to an oscillatory effect (sets of double peaks (slightly out of phase with applied potential shown in panel F, green dashed lines). Panel G shows the Δd vs. Δf relationship during adsorption before, during and after potential cycling. Panel H & I show the frequency-activity and dissipation-activity plots during potential cycling. Conditions: O_2 -saturated 0.1 M sodium phosphate buffer pH 6.0 at 25°C flowing at 0.1 ml min^{-1} with continuous O_2 bubbling; seventh harmonic shown.

The effect of the potential cycling on the adlayer is indicated by a distinct change in the Δd vs. Δf plot (Figure 4.5G) where there is a shift in gradient: the magnitude of $\partial d / \partial f$ during potential cycling (Figure 4.5G, grey trace) is a third lower than protein adsorption (black trace); the enzyme is now in a stiffened conformation (with some possible enzyme

desorption). The frequency response is stable however; there is a consistent fall in activity in the voltammograms (84% loss over 60 scans, Figure 4.5C).

4.3.2 Potential cycling on bare gold

As a comparison to the 3MPA-modified measurement, *MvBOx* was introduced to a bare gold surface and potential cycling was carried out over 80 scans.

Figure 4.6 shows the potential cycling carried out on *MvBOx* adsorbed to a bare gold QCM sensor. Compared to the typical mass drop of 20–25% in the first 15 min. in Figure 4.5, the parallel measurements made on bare gold showed a 33% mass loss over 15 min. indicating a less stable layer. On bare gold, the E_{cat} also shifted to a more negative potential (Figure 4.6C), suggesting that the 3MPA modification facilitates more efficient electron transfer, compared to non-modified bare gold. This could also be the reason why there is a lower current seen for *MvBOx* on bare gold during constant potential control.

The frequency trace in Figure 4.6 exhibits a stable response after around 3.5 h, compared to around 1.5-2 h in Figure 4.5.

The peak to peak amplitude of the oscillatory distortions showed a similar value of ca. 9 Hz (Figure 4.6) to that seen in the 3MPA example and suggests that this oscillatory change is enzyme dependent rather than surface specific. After 60 scans on bare gold the overall activity loss was around 81% which is comparable to the 3MPA example of 84%.

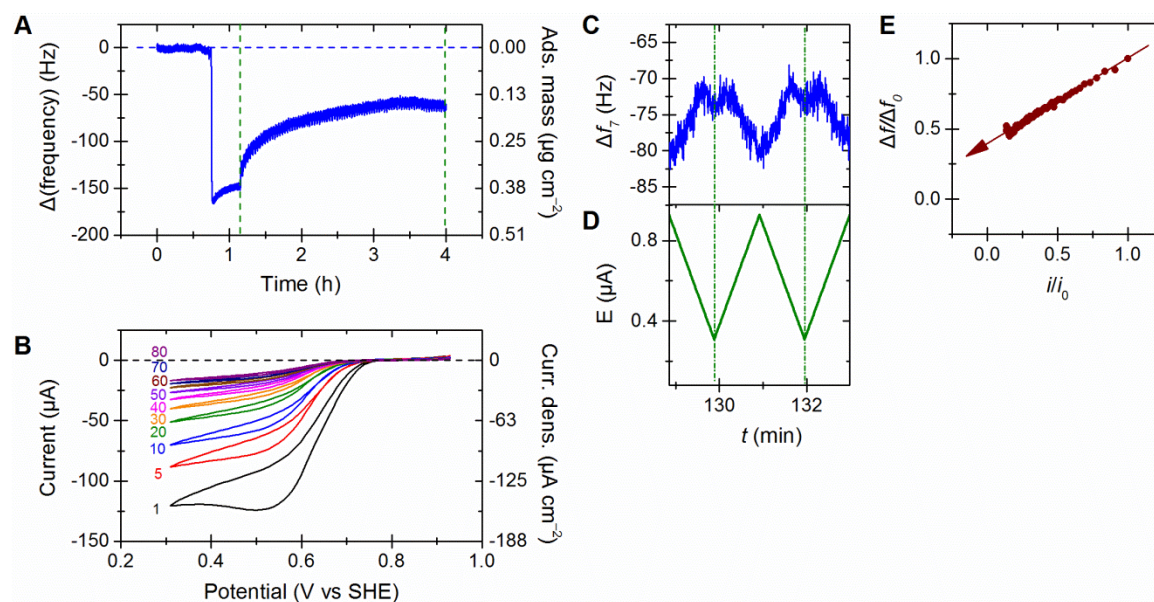


Figure 4.6 The effect of potential cycling on MvBOx adsorbed on a bare gold QCM crystal. Panel A shows the frequency response for the addition of MvBOx (24 mg ml⁻¹). Panel B shows scans 1, 5, 10, 20, 30, 40, 50, 60, 70 and 80 of 80 scans cycled between 0.93-0.31 V vs. SHE at 10mV s⁻¹. Green dashed lines show the period of CVs with the arrows marking the start of the numbered cycles. Panels C & D show the frequency responses with applied potential for scans 30 and 31 where the effects of CV give rise to an oscillatory effect (sets of double peaks (slightly out of phase with the applied potential shown in panel E, green dashed lines)). Panel E shows the frequency-activity during potential cycling. Conditions: O₂-saturated 0.1 M sodium phosphate buffer pH 6.0 at 25 °C flowing at 0.1 ml min⁻¹ with continuous O₂ bubbling; seventh harmonic shown.

Table 4.3 indicates that the starting currents, final currents and current loss for potential cycling of MvBOx on 3MPA-modified and bare gold surfaces were comparable. However there was a slightly greater loss in adsorbed mass efficiency on the bare gold surface over the course of potential cycling.

Table 4.3 Table showing the activity and efficiency of MvBOx adsorbed on 3MPA-modified and bare gold for the experiments carried out in Figure 4.1, Figure 4.5 Figure 4.2 and Figure 4.6. Currents taken at lower vertex potential.

Measurement	Current at scan 1 (– μA)	Current at final scan (– μA)	Normalised current loss ($\Delta i/\Delta i^{max}$)/t(h)	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Final efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($\mu\text{A Hz}^{-1}\text{h}^{-1}$)
3MPA CV (Figure 4.5)	117	18 (scan 60)	37%	0.52	0.15	0.16
Bare gold CV (Figure 4.6)	121	17 (scan 80)	32%	0.81	0.3	0.19

4.3.3 Discussion of cyclic voltammetry on bare and 3MPA-modified gold

When comparing the cyclic voltammetry experiments, the starting currents, and current loss are similar in both bare gold and 3MPA measurements (Table 4.3). The interesting difference here is that, the loss in adsorbed mass efficiency during potential cycling is slightly greater for the bare gold measurement. This observation indicates the importance of pre-modifying a surface in order to retain catalytic competency of the adlayer. This also relates back to the discussion in Section 4.2.5, where it was discussed that the breakdown of secondary structure, which is more readily encountered on bare gold, could be the reason for diminished activity and hence reduced efficiency.

A study by Salaj-Kosla *et al.* found that adsorbing enzyme onto nanoporous gold surfaces, capped with a polymer coating, increased current densities. Therefore a similar method may help address this observed instability of MvBOx immobilisation onto bare gold for direct electron transfer in fuel cell systems.¹³ These observations indicate the advantages of utilising a modified surface over a non-modified surface, for protein adsorption, with the primary focus on retaining catalytic competency.

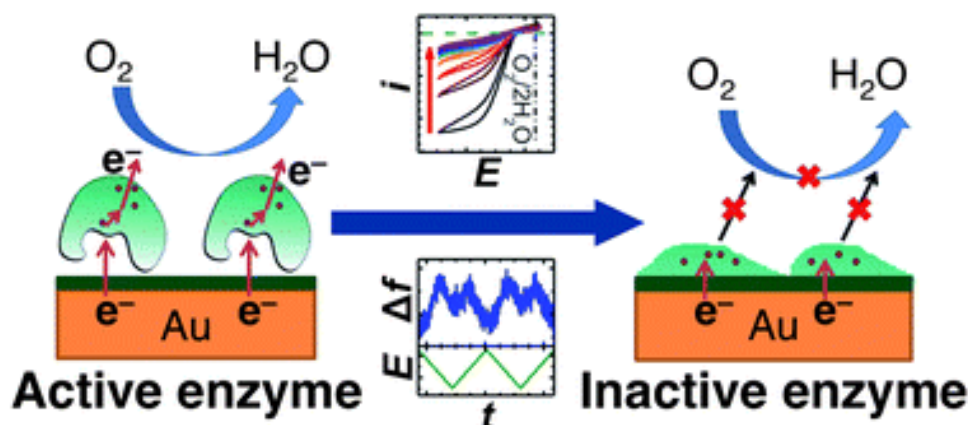
The QCM is used to measure hydrated mass, and approximately 45% of the protein's mass is entrained water (see Appendix II, Published manuscripts, EES paper, SI) which contributes to frequency changes of the sensor.^{4,5} Therefore, one possible reason for the potential cycling-induced mass loss, in both measurements, could be as a result of the enzyme layer extensively dehydrating (see discussion in Section 4.2.5).

The idea of dehydration is also consistent with a study by Goda and Miyahara, where they used a field effect transistor (FET) sensor, capable of determining changes in the charge density upon nonspecific protein adsorption, to suggest that an increase in surface charge density is a result of a dehydration and condensation step during protein adsorption.¹⁴ However, in this case as the frequency continues to increase (i.e., as the

mass decreases), some desorption is also a possibility and cannot be ruled out. In addition, the decrease in adsorbed mass efficiency with time, during potential cycling, further supports the idea that the adsorbed enzyme is undergoing some sort of inactivation. Further experiments could include modelling surface-bound protein behaviour to look at the elastic modulus and density change at the start and end of scans to corroborate the evidence shown in the study by Goda and Miyahara.¹⁴

Further to this, Rabe *et al.* suggest that surface-induced structural rearrangement of surface bound proteins is driven by an entropy gain, resulting from a loss of ordered secondary structure, in addition to the release of counter ions or solvation molecules.¹⁰ This in turn corroborates the idea that the enzyme adlayer could be experiencing dehydration. The processes of dehydration and conformational rearrangement may in turn, relate to the large current loss seen in the cyclic voltammograms in Figure 4.5C, indicative of an enzyme inactivation process.

The periodic oscillations, seen in both the bare gold and 3MPA measurements, suggest that when varying the potential, the electric field near the enzyme–surface interface may repeatedly distort the adsorbed protein, causing it to repeatedly expel mass. This mass could be in the form of water or ions, which in turn, hastens enzyme denaturation. This hypothesis is consistent with STM observations on single molecules of another MCO, laccase from *Streptomyces coelicolor*, which showed different heights for reduced and oxidised enzyme on a thiol-modified gold electrode.¹⁵ This idea of potential cycling-induced destabilisation and possible dehydration is shown in Scheme 4.1.



Scheme 4.1 Cartoon illustrating the effect of potential cycling on the adsorbed enzyme adlayer. The frequency changes and distortion caused by potential cycling destabilise the adlayer, and encourage a breakdown in secondary structure. This breakdown in structure results in a reduction in the oxygen reducing ability of the enzyme resulting in inactivation. Taken from EES publication¹⁶

These potential cycling oscillations could also be contributing to the destabilisation of the enzyme secondary structure. Further to this, in comparison to applying a constant potential, potential cycling highlights two possible factors that contribute towards enzyme inactivation: dehydration and conformational rearrangement/stiffening.

Interestingly, there could also be other possible reasons for inactivation. One method may be through the generation of reactive chemical species near the surface of the electrode which are inactivating the protein. This process may be potential dependent and there could be a potential window over which enzyme activity is more/less efficient. This is a similar observation/idea from the experiment when the potential was switched between high and low potentials (Figure 4.3). One possible method of testing a potential dependence is by holding the enzyme at a range of different potentials and looking at the rate of activity loss over a fixed period of time. Potential cycling could also be administered using different scan rates and over different potential ranges, to help distinguish between EC' routes of inactivation and more simple mechanisms.

An interesting avenue for research would be to further probe the hydration layer of the enzyme by carrying out electrochemical surface plasmon resonance studies. The E-SPR

technique combines voltammetric analysis with surface plasmon resonance in much the same way as the E-QCM-D with the surface of interest doubling as the working electrode. However, with SPR, it is the dry mass which is measured and so carrying out E-SPR in parallel with E-QCM-D would help corroborate any structural perturbations occurring in either dry or hydrated mass. This would also help in understanding whether the associated mass loss on potential cycling is indeed related to a dehydration step.

4.3.4 Combined effects of cyclic voltammetry and chronoamperometry

To contrast the effects of chronoamperometry and cyclic voltammetry in the same adlayer (to control for the variations that occur between adlayers), each technique was applied sequentially on the same layer of *MvBOx* (Figure 4.7). The idea here was that the potential cycling might precondition the adlayer before applying a constant potential. Looking at the rate of loss of adsorbed mass efficiency over the course of the constant potential application could act as a comparison to probe the mechanisms of inactivation.

For the measurement in Figure 4.7, potential cycling was first carried out for 5 scans before a constant potential was applied and left for ca. 7h. This potential was then turned off before a final set of 10 scans were carried out.

In Figure 4.7D, after *MvBOx* adsorption, the potential was cycled five times (scan time 124 s) causing a 25% mass loss (ca. $0.15 \mu\text{g cm}^{-2}$) and an activity loss of about a third (44 μA) (Figure 4.7D lower panel & 4.4F). The potential was then held constant at 0.21 V for ca. 7 h. During this time the frequency trace remained relatively stable between -146 and -132 Hz (Figure 4.7A), but the current dropped by more than half ($\sim 52\%$) (Figure 4.7B). Finally, the potential was cycled for 10 scans (Figure 4.7E), during which the activity dropped by about 29% (lower panel) and the mass dropped by about a tenth to roughly 60% of its value before the start of the second cyclic voltammetry step.

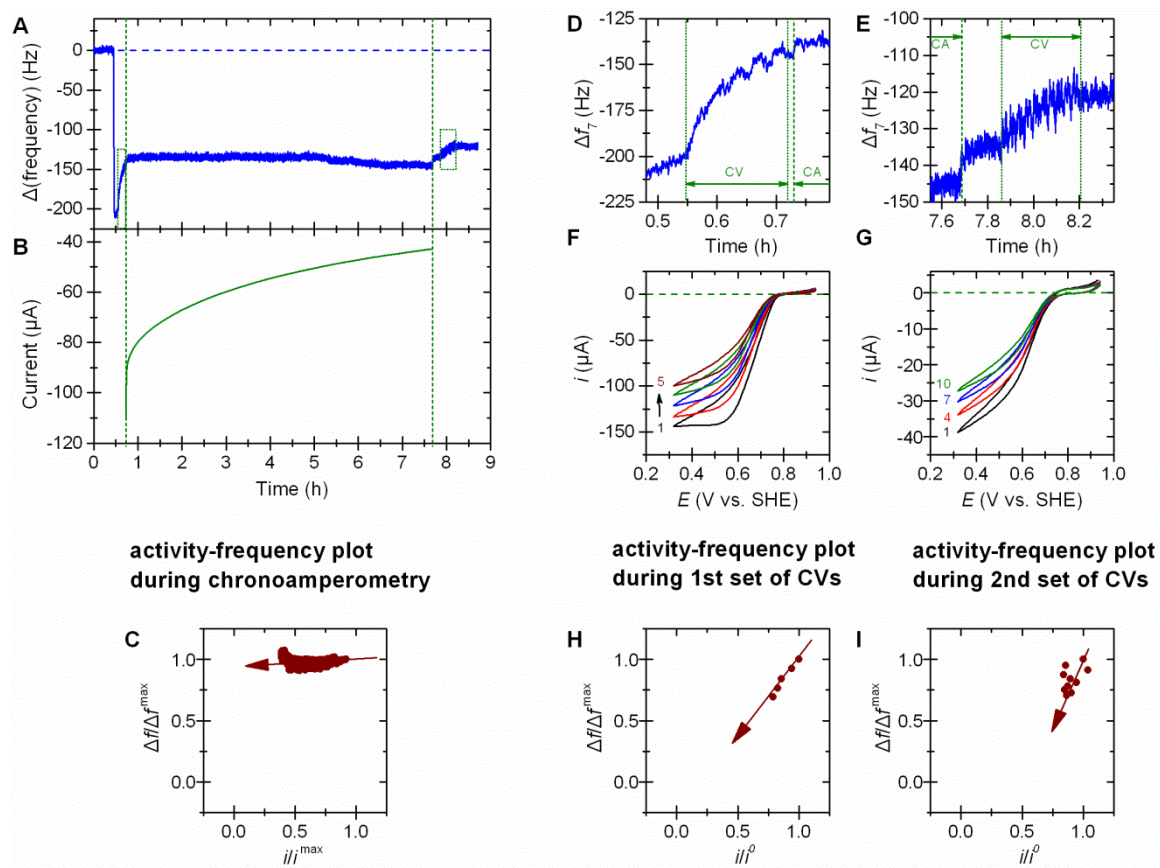


Figure 4.7. The effect of potential cycling before and after applying a constant potential. Panel A and B show the frequency change and chronoamperometric current response with time for *MvBOx* adsorbed on a 3MPA modified QCM sensor respectively. Circle (●) indicates when *MvBOx* (24 mg ml⁻¹) was injected. Green dashed lines indicate the application of a constant potential of 0.21V vs. SHE. Panel C shows the frequency-activity plot during chronoamperometry. Panel D shows the frequency response during the first 5 voltammograms (Panel F) carried out before chronoamperometry. Panel E shows the frequency response for 10 scans (Panel G) carried out after chronoamperometry. Panel H & I show the frequency-activity plots for the first 5 CVs and final 10 CVs, respectively. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. CV: 0.92-0.32 V vs. SHE, 10 mV s⁻¹.

These observations are further highlighted in the $\Delta f / \Delta f^{\max}$ vs. $\Delta i / \Delta i^{\max}$ plots. In Figure 4.7C, there is a strong correlation between a stable frequency and a fall in current. In contrast to this, when potential cycling is carried out, there is a reduction in mass partnered with a reduction in current (Figure 4.7F and Figure 4.7G). These plots serve to make the differences between holding a constant potential, and cycling the potential, more evident. Holding a constant potential indicates enzyme inactivation over time, with

little change in mass. Potential cycling indicates a greater destabilisation effect on adsorbed mass, along with a greater activity loss over a shorter time.

Figure 4.8 shows the effect of changing the potential control on the adsorbed mass efficiency. During the first five scans there is a drop in efficiency of $0.1 \mu\text{A Hz}^{-1}$. This sharp drop in efficiency is continued during chronoamperometry until, at approximately 1 h, the gradient of the plot shallows. During the course of chronoamperometry there is a decrease of $0.42 \mu\text{A Hz}^{-1}$. The final 10 scans cause a marked shift in the plot and over the course of the 10 scans there is a decrease in the efficiency of $0.05 \mu\text{A Hz}^{-1}$.

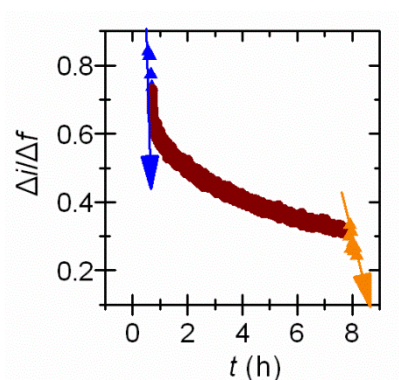


Figure 4.8 The effect of potential cycling and constant potential control on the adsorbed mass efficiency of MvBOx adsorbed onto 3MPA-modified gold. Triangle (▲) indicates the change in efficiency with time during the first 5 CVs. Circle (●) indicates the change in efficiency with time during chronoamperometry. Triangle (▲) indicates the change in efficiency with time during the final 10 CVs.

Table 4.4 shows the mass loss and activity loss during each phase of the experiment. The greatest activity loss was found when holding the potential constant; however, there was a greater activity loss over a shorter time frame, during potential cycling.

Table 4.4 Table showing the mass loss and activity loss for each stage of cyclic voltammetry (CV) and chronoamperometry (CA) carried out in the measurement in Figure 4.7. NB the duration of each potential control measurement differs and therefore are not readily comparable.

Measurement	Time (min)	Relative activity loss
1 st step - 5 CVs	10	33 %
2 nd step – CA	420	52 %
3 rd step - 10 CVs	20	29 %

4.3.5 Discussion of the combined effects of constant and cycling potentials

Figure 4.7 further highlights the difference between applying a constant potential and a cycling potential to adsorbed enzyme and the results have been summarised in Table 4.4. There is a greater mass loss during the potential cycling steps, compared to an almost zero mass loss during chronoamperometry. When the potential is held constant, the mass also remains relatively constant, but does not correlate with the fall in activity. Holding a constant potential in between two potential cycling steps, further indicates the possibility of an inactivation process resulting from a conformational rearrangement (stable frequency + activity loss) which is consistent with the hypothesis suggested in Section 4.3.3.

The changes in adsorbed mass efficiency in Figure 4.8 highlight the effects of constant and cycling potential on film stability, suggesting that the efficiency and catalytic competency of the enzyme adlayer is directly associated to the type of potential control being administered. This strengthens the idea that the potential cycling is causing destabilisation not only to the mass on the surface but to the catalytic competency of the enzyme layer. Interestingly, there is an efficiency loss during chronoamperometry, suggesting that although the enzyme consistently exhibits inactivation over time, potential cycling hastens this inactivation.

However, the idea that potential cycling directly causes the denaturation of the protein is difficult to prove. For example, if there are two parallel plates at a fixed distance with a potential difference about equal to the working electrode potential, this will give an electric field that will vary between about 0 V m^{-1} to 10^3 V m^{-1} . The force on a point charge is equal to the product of the field and the charge, and assuming there is a net charge of 1–10 (plus or minus), the generated force would be approximately 10^{-16} to 10^{-15} N. According to a study by Stirnemann *et al.* which gives details on the forces required to

mechanically denature a protein, this force may be smaller than required to denature the protein.¹⁷ However, there may still be smaller scale mechanical motions occurring at the surface of the electrode, and testing the enzyme at different scan rates and over different potential ranges may give some idea of the kinetics of the process that are occurring, to help distinguish between EC' routes and simpler mechanisms.

4.3.6 *Potential range dependence*

To further simulate the effect of a variable load on an enzymatic biofuel cell, the range over which potential cycling was varied to understand if there was a potential dependence on the activity loss. This would let us know if there is a thermodynamic component to the activity loss rather than just a mechanical effect from the electric field.

In these measurements *MvBOx* was adsorbed onto 3MPA-modified gold surfaces and potential cycling was administered over three different potential ranges: 0.91–0.31 V, 0.81–0.31 V and 0.91–0.61 V vs. SHE.

For the largest potential range, there is a frequency change of –308 Hz (addition of $0.78 \mu\text{g cm}^{-2}$) (Figure 4.9A) and although Figure 4.9E (0.81–0.31 V) and Figure 4.9I (0.91–0.61 V) both have different amounts of enzyme going down, the normalised frequency–activity plots show that the rate of cycling-induced mass loss is similar (upper panels in Figure 4.9D, H and L).

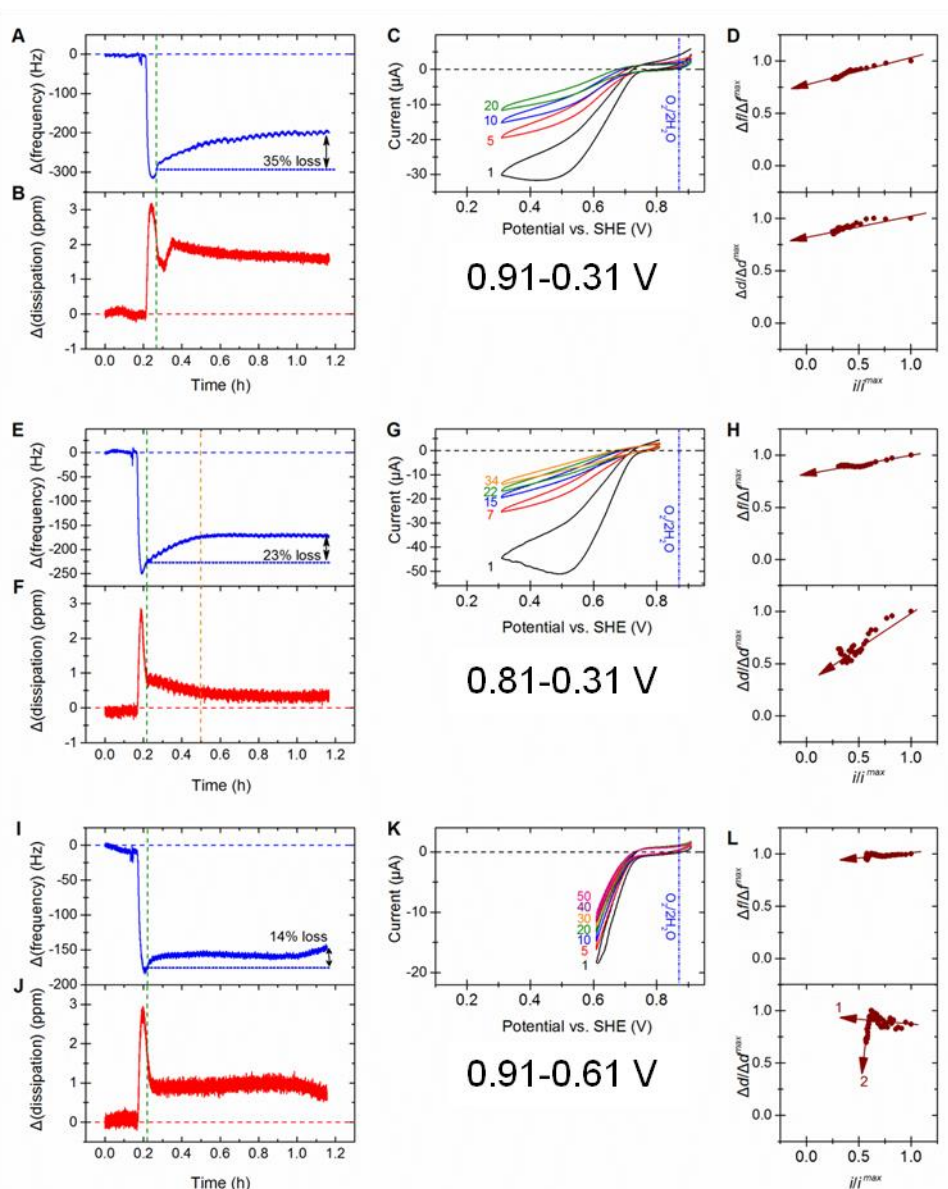


Figure 4.9. The effect of changing the potential ranges. Panels A, B and C show the frequency, dissipation and cyclic voltammetry when cycling potential between 0.91–0.31 V vs. SHE, respectively. Panel D shows the frequency-activity (upper) and dissipation-activity plots (lower) respectively. Panels E, F and G show the frequency, dissipation and cyclic voltammetry for potential cycling between 0.81–0.31 V vs. SHE, respectively. Panel H shows the frequency-activity (upper) and dissipation-activity (lower) plots respectively. Panels I, J and K show the frequency, dissipation and cyclic voltammetry for potential cycling between 0.91–0.61 V vs. SHE, respectively. Panel L shows the frequency-activity (upper) and dissipation-activity (lower) plots, respectively. Green dashed lines in frequency response plots indicate when CVs were run. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. Scan rate: 10 mV s⁻¹.

Over the widest range, there is a relative 35% loss in mass over 1 hr. This mass loss decreases to 23% when lowering the high potential vertex by 0.1V, and further still to

14% when cycling over the narrowest range (shifting the low potential vertex by +0.3V). The slopes for the normalised frequency-activity plots in Figure 4.9D, H and L (upper panels) highlight this increased stability, with the slope decreasing as the potential range is narrowed (Table 4.5).

Table 4.5 Table showing the slopes of the normalised frequency-activity plots in Figure 4.9, for each potential range.

Potential range	Slope of $\Delta f/\Delta i$
0.91–0.31V	0.32
0.81–0.31V	0.13
0.91–0.61V	0.035

Looking at the dissipation response for the range 0.81–0.31 V, the Δd vs. Δf plots show subtle changes in the mechanical properties of *MvBOx*. When potential cycling is started (green dashed lines in Figure 4.9F), the magnitude of the $\partial d/\partial f$ immediately decreases by a factor of about 10 from 0.073 to 0.0075 ppm Hz⁻¹ (Figure 4.10B). There is a gradual decrease in the dissipation response over ca. 0.3 h after potential cycling begins which could be the result of a slow change in the mechanical properties of the adlayer. When the dissipation becomes stable (after the orange dashed line, Figure 4.9F) there is a further change in the $\partial d/\partial f$ gradient, which decreases by a factor of 10 to 8 x 10⁻⁴ ppm Hz⁻¹. There is also a correlating pattern of mass loss during these changes. The activity loss during potential cycling for the 0.91–0.31 and 0.81–0.31 V was comparable: ~67% in Figure 4.9C and ~70% in Figure 4.9G.

Lowering the value of the high-potential vertex from 0.91 to 0.81 V, increased stability where the frequency became stable over 30 min (Figure 4.9E) compared to a lack of stability (continually increasing Δf) in Figure 4.9A. This increased stability was seen in the Δd vs. Δf plots: the Δd vs. Δf gradient during potential cycling for the 0.91–0.31 V range was 0.026 ppm Hz⁻¹ compared to a value of 0.0075 ppm Hz⁻¹ for the 0.81–0.31 V

range, where the lower gradient suggests less of an effect on the mechanical stability of the film (Figure 4.10A & B).

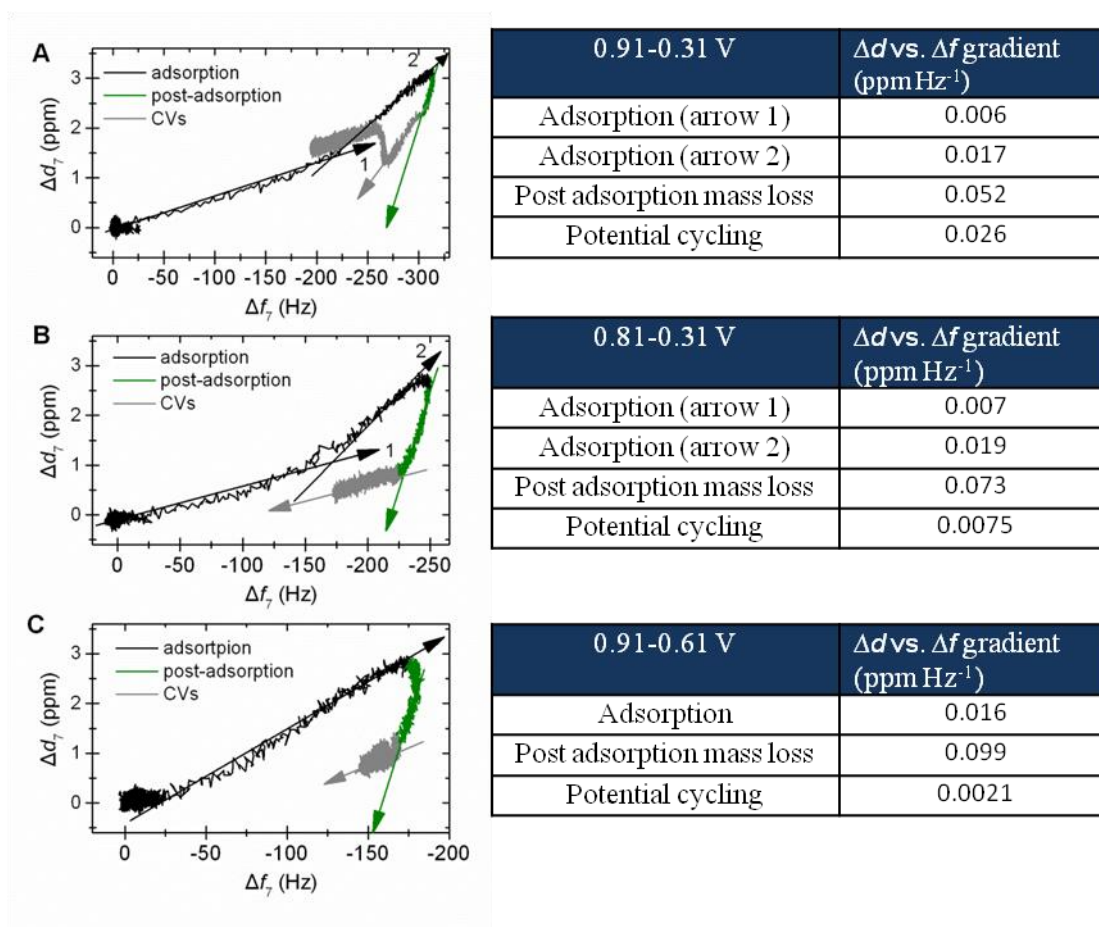


Figure 4.10 The changes in gradient during enzyme adsorption and potential cycling for the scan range 0.91–0.31 V (A), 0.81–0.31 V (B) and 0.91–0.61 V (C) vs. SHE, respectively.

When cycling the potential over the narrower potential range (0.91–0.61 V), the response in the dissipation changes (Figure 4.9J): after the initial ‘spike’ response, the dissipation immediately gives a stable response and this remains stable over the remainder of potential cycling.

This stability is also seen in the reduced Δd vs. Δf gradient (Figure 4.10C) for the period of potential cycling when both frequency and dissipation traces are stable (ca. 0.3 h onwards). The amount of mechanical change per unit mass is reduced when the potential is kept above 0.61V.

Both the frequency and dissipation showed an unexplained small increase towards the end of the measurement (arrow 2, panel L). Raising the lower-potential vertex changed the pattern of inactivation to be closer to that of the chronoamperometry case.

4.3.7 Scan rate dependence

The charge in the electric field distorts the protein and as a result, the rate at which the distortions affect the protein can be probed by changing the scan rate. In the following measurement, *MvBOx* was adsorbed to 3MPA-modified gold and the potential cycling was carried out using a scan rate of 1mV s^{-1} (10x slower than the typical scan rates used in this chapter) over ca. 14 h.

Figure 4.11 shows the effects of decreasing the scan rate 10 fold. The adsorbed mass decreased by $\sim 72\%$ over 2.5 hr and then remained stable ($49 \pm 1\text{ Hz}$) for a period of ~ 11.5 hr. During this stable frequency, the current continued to decrease. Directly comparing this with the result in Figure 4.1 the activity loss over 8 hr is 94% compared to just a 32% reduction over the same time period, in Figure 4.1C. In Figure 4.11C the first voltammogram has a flattened shape indicative of mass transport polarisation, with a final current loss of $30.8\text{ }\mu\text{A}$ (96%) after 14 h.

During potential cycling, the frequency and dissipation traces again showed periodic oscillatory changes that were slightly out of phase (green dashed line, Figure 4.5D & E) with the applied potential (Figure 4.11F). These oscillatory changes (Figure 4.5D) in comparison have a lower maximum peak-to-peak amplitude of approximately 5 Hz ($0.01\text{ }\mu\text{g cm}^{-2}$) and as a result, the set of double-peaks are less pronounced. Interestingly, these oscillations are more difficult to observe at the highest scan rate (see Appendix).

The adsorbed mass efficiency decreases by $0.1\text{ }\mu\text{A Hz}^{-1}$ to a value of $0.02\text{ }\mu\text{A Hz}^{-1}$ at the end of the 14 h measurement. The change in current generated per unit change of dissipation ($\Delta i/\Delta d$) also showed a similar decrease. A reduction in this parameter

indicates that there is less activity per energy dissipated, and because the dissipation is stable from 1.5 h onwards, this could suggest that the enzyme has transitioned to a state which results in a slow inactivation over time (Figure 4.12B).

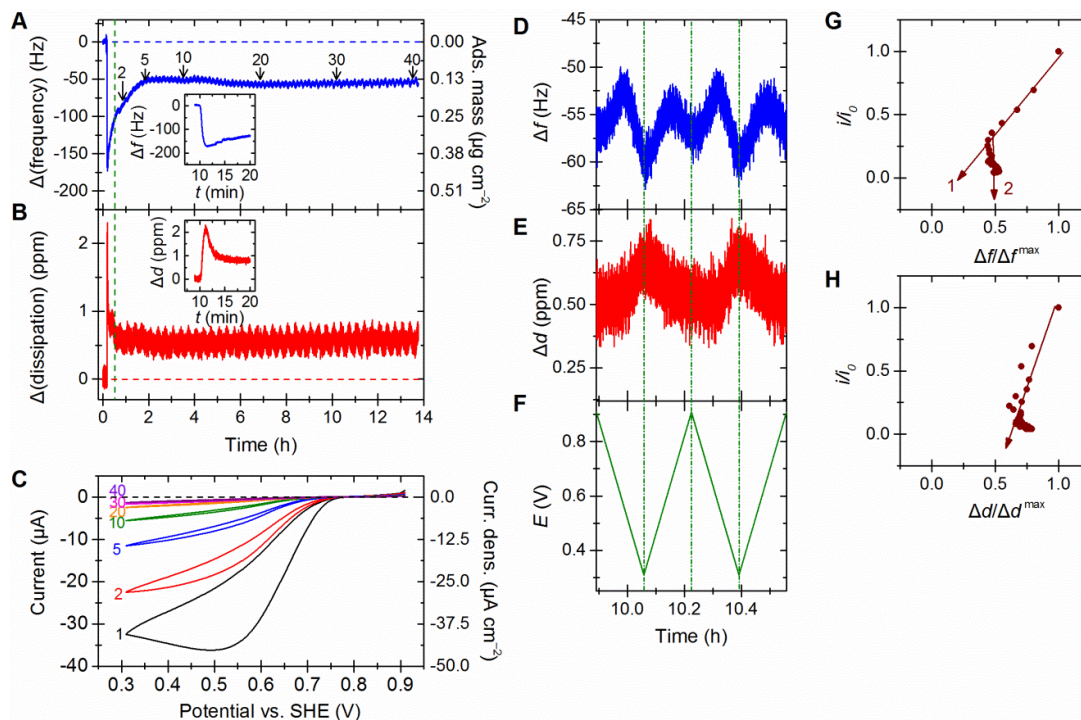


Figure 4.11. Decreasing the scan rate during potential cycling. Panel A and B show the frequency and dissipation responses respectively. The insets show the adsorption phase for both traces respectively. Panel C shows scans 1, 2, 5, 10, 20, 30 and 40 of 47 scans with each scan numbered in Panel A. Panels D & E show the frequency and dissipation responses for scans 30 and 31 where the effects of the CVs give rise to an oscillatory effect (sets of double peaks (slightly out of phase with the applied potential shown in panel F, green dashed lines). Panel G and H show the frequency-activity and dissipation-activity during potential cycling respectively. The arrows in Panel G indicate initial adsorption followed by mass loss (arrow 1) and a stable response during cycling (arrow 2). Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. CV: 0.91–0.31 V vs. SHE, 1 mV s⁻¹.

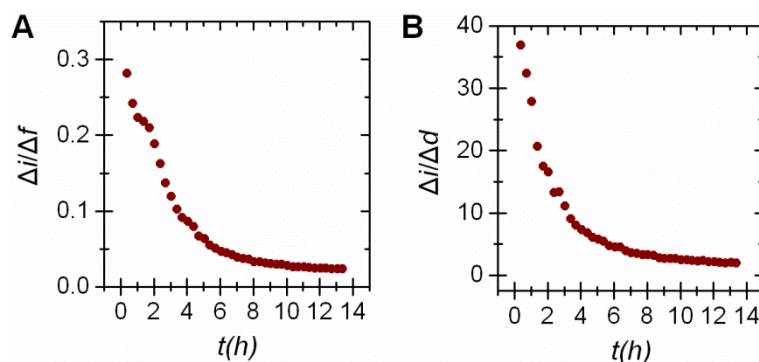


Figure 4.12 Plots showing the adsorbed mass efficiency ($\Delta i/\Delta f$) (A) and changes in $\Delta i/\Delta d$ (B) during potential cycling at 1 mV s^{-1} .

Carrying out potential cycling using slower scan rates leads to much larger losses in activity, however the mass losses are comparable to those from higher scan rates (Table 4.6, see Appendix).

Table 4.6. Effect of scan rate on adlayers of *MvBOx*. Current was measured at the lower vertex potential and fractional losses are based on the current values from the lower vertex potential on the first scan and the scan 20 minutes after. Total scan times for each measurement are the same. Potential range: 0.91–0.31 V vs. SHE.

Scan rate (V s^{-1})	No. Scans	Relative current loss	Relative mass loss
0.1	100	66%	30%
0.01	10	57%	24%
0.001	1	31%	27%

4.3.8 Discussion of potential range and scan rate dependence on mass loss

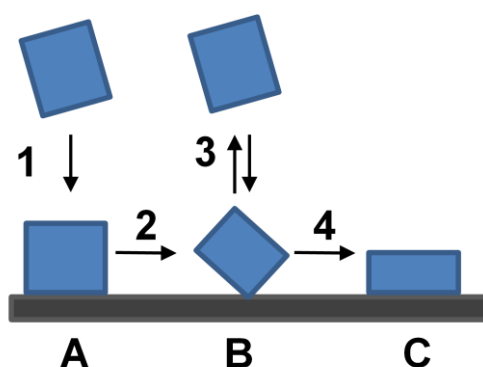
The presence of two regimes in the dissipation traces for the 0.81–0.31 potential range measurements (Figure 4.9E &F), suggests that the surface bound enzyme could be undergoing a multistep process: 1) an initial soft layer formation followed by an immediate stiffening of the layer (peak response in dissipation), followed by 2) a slow change in rigidity over time (gradual decrease in dissipation response following potential cycling, and lowered magnitude of $\partial d/\partial f$). Interestingly, the continuing increase in

frequency (mass loss) while this occurs could point to a loss of hydration/solvation molecules associated with a breakdown in the secondary structure of the enzyme.

This idea is consistent with the three states model for protein adsorption developed by Rabe *et al.* based on the data obtained from studying the adsorption of β -Lactoglobulin on a hydrophilic glass surface (Scheme 4.2).¹⁸ This model consists of three different adsorbed states; an initial irreversible state (A), a reversible intermediate adsorbed state (B), and a final irreversible state (C).¹⁸ Proteins adsorb on the surface via the initial irreversible state (State A, Scheme 4.2). The transition between the initial, and the intermediate state (transition 2, Scheme 4.2), is a result of lateral protein-protein interactions and takes place once a critical surface coverage has been exceeded. According to experimental observations, this transition proceeds rapidly suggesting an orientation change rather than a conformational rearrangement. The coverage-dependent first transition implies that large numbers of surface adsorbed proteins abruptly change their surface affinity, from irreversible to reversible (transition 2), which is the key element of the overshooting effect in this model. This step in the model is consistent with the initial peak that is seen in the dissipation; a high dissipation shows a soft layer formation and the proceeding rapid stiffening could actually be due to a change in surface affinity followed by a rapid change in orientation. After this transition, there may be some degree of desorption (transition 3).

The intermediate state in the model undergoes a further transition into the final relaxed state (transition 4, Scheme 4.2). This transition is much slower and therefore most likely involves some conformational rearrangements. This final step in the model is consistent with the idea of there being a more gradual decrease in the dissipation response after adsorption, indicative of a mechanical/conformational rearrangement (Figure 4.9F). In line with the hypothesis proposed in Section 4.2.5, the slower transition to an irreversible

state, here proposed by Rabe et al., again suggests a breakdown in secondary structure after adsorption and supports the possibility of protein denaturation. The slower transition in Figure 4.9F, appears to be initiated by potential cycling (green dashed line) suggesting potential cycling causes, or maybe accelerates, the enzyme denaturation process. The relaxed state proposed by the model could possibly be an inactive state and over time, as more enzyme transitions to this irreversible state, there is a loss of catalytic activity and efficiency. This slow inactivation over time is mirrored in the measurement taken at 1 mV s^{-1} , where a reduction in the $\Delta i/\Delta d$ parameter (Figure 4.12B) (indicating there is less activity per energy dissipated) and a relatively stable dissipation, suggests the enzyme could have irreversibly transitioned to a state which exhibits a slow inactivation over time.



Scheme 4.2 A schematic showing a model for protein adsorption and relaxation. The protein is first transported to the surface (1). The protein adsorbs via an irreversible state (A). There is a transition (2) from the irreversible initial state (A) and intermediate reversible state (B) and once in this state, there could be some enzyme desorption (3). There is a slow conformation change over time (4) which appears to be hastened when applying potential cycling compared to a constant potential.

The adsorbed adlayer may be rearranging its structure over a relatively long period of time (transition 4), in order to optimise surface interactions.¹⁹ However this tendency may be hindered, if the rate of arrival of protein to the surface is comparable with, or larger than, the rate of structural reorganisation. Therefore, at higher concentration the structural/orientation changes in the enzyme may be similar to those immediately

following adsorption. An interesting route would therefore be to look at the changes in dissipation, $\Delta d/\Delta f$ gradient and catalytic efficiency at lower concentrations and understand the extent to which catalytic competency and long term performance is affected.

Raising the potential of the low-potential vertex above ca. 0.6 V significantly improved stability and a stable Δf was achieved after 12 min (arrow 1 panel L), with an absence of the secondary, gradual change in conformation mentioned above. This indicates a potential-dependant destabilisation effect, as the rate of mass loss is reduced when potential is kept above 0.6 V. The stable dissipation i.e. absence of any gradual change in the dissipation after the initial ‘peaked’ response (Figure 4.9J), suggests that raising the potential may also help control against potential-induced conformational changes. The idea that there is a critical lower potential for enzyme catalysis, is consistent with electrochemical observations on another fuel cell enzyme, the [FeFe] hydrogenase HydA1 from *Chlamydomonas reinhardtii*, which showed inactivation at low (reductive) potentials when adsorbed onto modified rotating disc graphite electrodes.²⁰

When looking at the scan rate dependence measurement, over the period of 20 minutes, mass losses are comparable to higher scan rates as shown in Table 4.6, however as the slow structural rearrangement has a duration of ~2-2.5 hours, decreasing the scan rate to 1 mV s⁻¹ led to a similar mass loss but only about half the activity loss.

The idea here is that the charge in the electric field distorts the protein. That is, if the protein’s charged groups are distorted slowly (lower dE/dt), it has time to change shape in a way that is reversible and maintains catalytic competence. If the scan rate increases, then there will come a point where the protein deforms irreversibly. However, at the slower scan rate, there is a 92% loss in the adsorbed mass efficiency, over 14h, suggesting

that, although decreasing the scan rate may temporarily reduce activity loss, inactivation of the enzyme adlayer over time is still a concern.

One way to investigate this idea of protein distortion is to cap the surface Glu, Asp and Lys residues with uncharged but polar groups and look at the extent of change in the catalytic competence across the different scan rates. When incorporating this enzyme into a fuel cell, the ideal result would be to combine minimum loss in activity over time, with stability. At lower scan rates, this may be possible.

An interesting observation was that, once the frequency response became stable during potential cycling in Figure 4.11, the pattern of activity loss resembled the original chronoamperometry measurement in Figure 4.1, whereby the current continued to decrease with no apparent change in mass. This effect was also mirrored in Section 4.3.4 where potential cycling was carried out before a constant potential was held, resulting in a stable adlayer and negligible mass loss. These observations suggest that the potential cycling may be conditioning the enzyme adlayer, such that, the enzyme transitions to an irreversible, relaxed state which exhibits stable frequency and dissipation but experiences continual inactivation.

This section further highlights the destabilising effect of potential cycling over a longer period of time. Over the first two hours of the measurement in Figure 4.11, the dissipation shows a gradual decrease, after an initial ‘peaked’ response, which then stabilises. This could suggest a multi step process: the initial increase in dissipation in the ‘peaked’ response indicates the enzyme forming a soft layer which is immediately followed by an abrupt change in surface affinity and orientation change. This is then followed by a slow structural rearrangement (denaturation).^{10,18}

The findings from this section show that the catalytic lifetime of *MvBOx*, adsorbed onto organothiol-modified gold electrodes, is significantly reduced when the applied potential is cycled to below a critical potential (0.6 V vs. SHE at pH 6.0).

4.4 E-QCM-D control measurements

The following measurements consist of control experiments determining the effects of applying potential control in the absence of redox enzyme. The effect of varying the oxygen saturation during constant potential control was also looked at to understand whether oxygen reactive species were the reason for inactivation of surface bound enzyme. The catalytically inactive protein, Bovine serum albumin (BSA), was then monitored under constant and varying potential control, followed by a set of measurements that looked at the effect of combining *MvBOx* (active) and BSA (inactive) protein solutions during adsorption. The final measurement in this section was taken to compare the behaviour of *MvBOx* to a second multicopper oxidase, laccase from *Trametes versicolor* (*TvL*).

4.4.1 Absence of enzyme

In this set of control measurements, potential control was carried out on 3MPA-modified gold crystals in the absence of enzyme using potential cycling (Figure 4.13A, B & C), and holding a constant potential (Figure 4.13D, E & F).

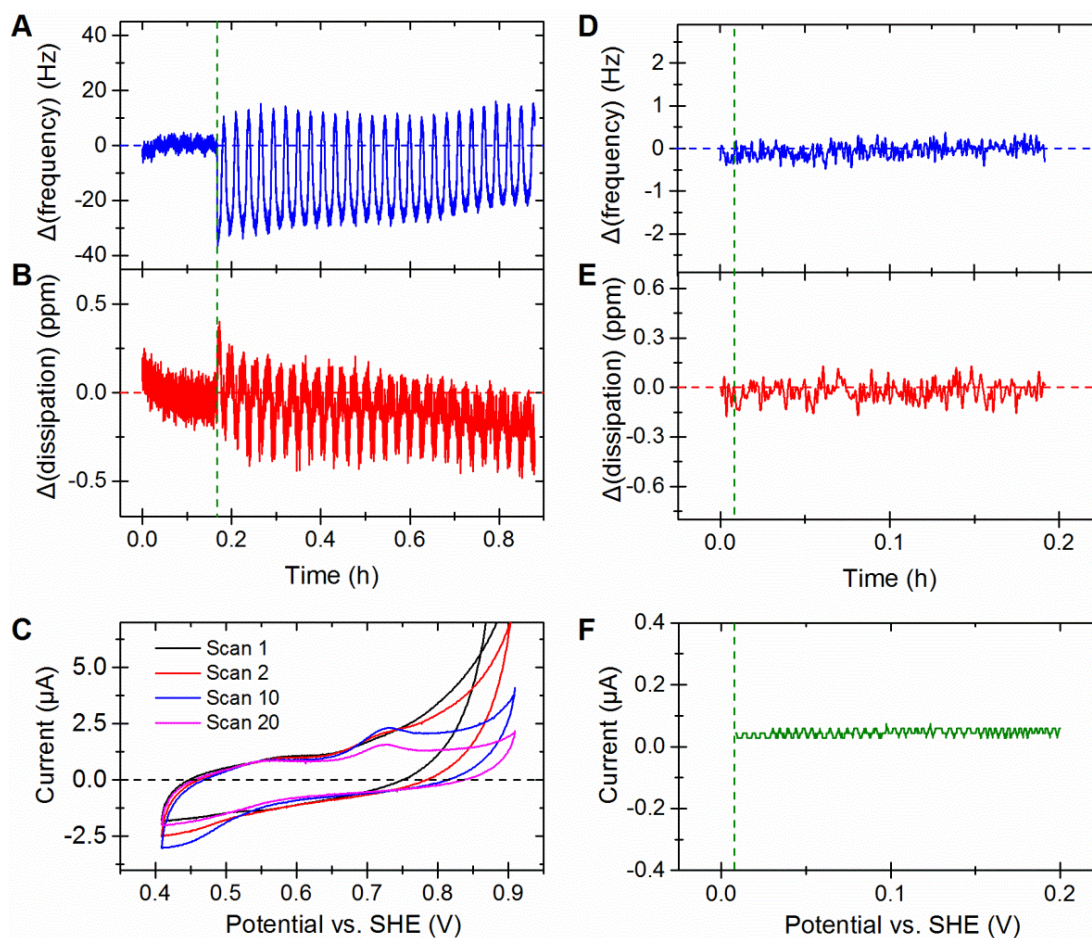


Figure 4.13. Frequency, dissipation, cyclic voltammetric and chronoamperometric responses with time for a 3MPA-modified gold-coated QCM sensor in the absence of enzyme. Panel A and B show the frequency and dissipation response during cyclic voltammetry. Panel C shows scan 1, 2, 10 and 20 of 25 scans carried out between 0.91–0.41 V vs. SHE at 10 mV s⁻¹. Panel D and E show the frequency and dissipation response to the application of a constant potential of 0.21 V vs. SHE. Green dashed line indicates the start of chronoamperometry. Panel F shows the near-zero chronoamperometric response. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown.

The cycling induced oscillations in the Δf and Δd traces in Figure 4.13A and 4.13B are similar to those in Figure 4.5, but with an amplitude about three times higher (28 Hz, 0.07 $\mu\text{g cm}^{-2}$) than when *MvBOx* is adsorbed 3MPA (Figure 4.5). This suggests that the observed mass and dissipation changes are possibly related to bulk fluid changes near the surface of the crystal, rather than changes in the protein shape.

The traces in Figure 4.13C show the onset of O₂ reduction around 0.5 V vs. SHE. This could be the reason for the enzyme losing catalytic competency below 0.6 V, however it

is uncertain what is being generated on the surface around this potential, at pH 6, that could mechanically breakdown the protein. Holding a constant potential gave a near-zero current response and no change was observed in either the frequency or dissipation traces (Figure 4.13D, E & F).

4.4.2 *Effects of variations in oxygen saturation*

To test for the possibility of hydrogen peroxide evolution being the source of inactivation (from the two-electron reduction of O₂, see Introduction), the effect of swapping between oxygen and argon flow during constant potential experiments was carried out.

Enzyme was introduced to the crystal and a constant potential of 0.21 V vs. SHE was applied after adsorption. After a stable frequency and dissipation had been reached (ca. 30 min), the solution was switched to argon saturated buffer solution. After approximately 55 min, the buffer solution was switched back to oxygenated buffer.

Figure 4.14 shows a trace with a peak in the dissipation, followed by a steady frequency response and an exponential decrease in catalytic current. Changing the buffer to argon saturation buffer causes the current magnitude to decrease with a first-order time constant of about 780 s; switching back to oxygen causes the current magnitude to increase with a first-order time constant of about 450 s.

As indicated by the dark green dotted line in panel C (a first-order exponential extrapolation of the current measured before the argon flush), the current magnitude after reoxygenation is slightly higher than would be expected in the absence of an argon purge. This suggests that hydrogen peroxide is unlikely to be causing enzyme inactivation and the slight overshoot in current may be due to some reactivation of the enzyme.

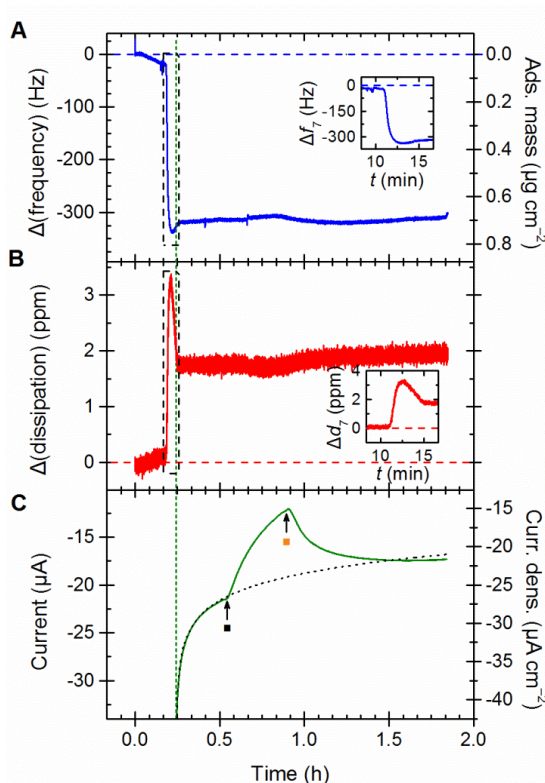


Figure 4.14. The effect of changing from oxygen saturated solution to argon saturated solution. Panel A and B show the frequency and dissipation responses and Panel C shows the current response with time for MvBOx adsorbed onto a 3MPA-coated QCM. The green dashed line indicates a potential of 0.21 V vs. SHE applied. Inset in A and B show the enzyme adsorption. Square (■) indicates the switch to argon gas saturated buffer and (■) back to oxygen saturated solution. Conditions: 0.1 M sodium phosphate pH 6.0, 25 °C, 0.1 ml min⁻¹, seventh harmonic shown.

4.4.3 Catalytically inactive protein

In these measurements, Bovine serum albumin (BSA), a globular protein similar in size to MvBOx,^{21,22} was adsorbed on a 3MPA-coated QCM sensor (Figure 4.15) and potential cycling was carried out. The reason for these measurements was to understand whether the changes in frequency and dissipation were comparable when potential control was applied to a non-catalytic protein with similar size and dimensions as MvBOx. Once BSA was adsorbed, potential cycling was administered for 30 scans.

In the first hour of potential cycling there was a 17% loss in mass compared to a mass loss of 34% when potential cycling was carried out on MvBOx (Figure 4.5). There is also no oxygen reduction present in the cyclic voltammograms (Figure 4.15D) which rules out

the idea that it could be an O_2 species being generated that causes the film death in *MvBOx* adlayers during potential cycling.

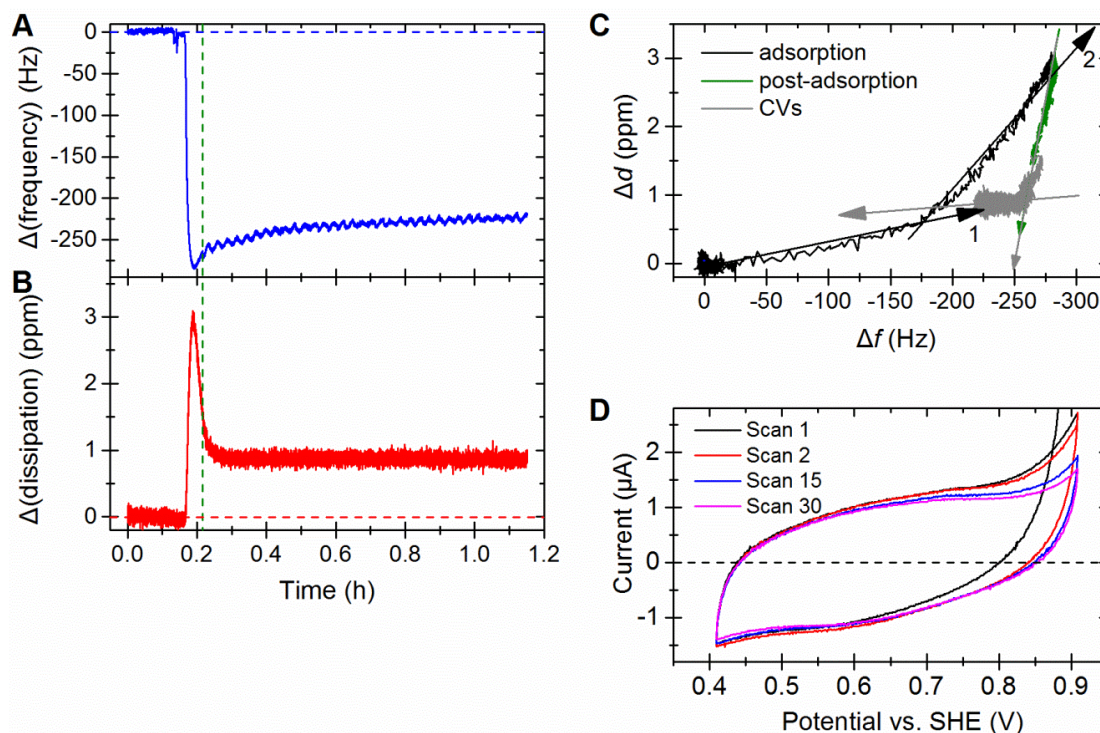


Figure 4.15. The effect of potential cycling on BSA adsorbed on a 3MPA-modified gold-coated QCM sensor. Panel A and B show the frequency and dissipation responses for the adsorption of BSA (24 mg ml^{-1}). The green dashed line indicates the start of cyclic voltammetry. Panel C shows the Δd vs. Δf plot during the measurement. Panel D shows scans 1, 2, 15 and 30 of 30 scans carried out between 0.93–0.41 V vs. SHE, 10 mV s^{-1} . Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , seventh harmonic shown.

When the potential cycling was started (grey line), there was no significant change in gradient from the post-adsorption gradient (green line). This suggests that the non-catalytic protein is more stable against potential cycling than *MvBOx*.

An interesting observation during BSA adsorption was that adsorption occurred in two clear phases (Figure 4.15C, black arrows 1 and 2). During the first phase, the $\Delta d/\Delta f$ gradient is $0.003 \text{ ppm Hz}^{-1}$ (arrow 1) and the magnitude of this gradient increases by around a factor of 10 during the second adsorption phase (0.02 ppm Hz^{-1} , arrow 2). This suggests a two step process, slightly different to that seen in Section 4.3.8: an initial rigid

adsorption, indicated by the shallow gradient and lower dissipative change per frequency change (arrow 1), followed by the build up of a more loosely bound or ‘floppy’ layer, indicated by the higher dissipation per unit mass (steeper $\Delta d/\Delta f$ gradient, arrow 2).

4.4.4 BSA concentration measurements

A concentration study of BSA adsorption on 3MPA-modified gold was carried out where BSA was adsorbed to 3MPA-modified surface at increasing concentrations. This was carried out as a control for the *MvBOx* concentration measurements (see Appendix III, Published manuscripts, EES publication – SI) and to compare the behaviour between catalytic and non-catalytic protein on frequency and dissipation responses.

Figure 4.16 shows the concentration dependence of BSA adsorption onto 3MPA. As the concentration increases, there is a maximum saturation of BSA of around $0.68\text{--}0.7\ \mu\text{g cm}^{-2}$ and the value of the ‘post-wash off’ response (Δf^{∞}) is similar across all concentrations ($-215 \pm 10\ \text{Hz}$). The highest concentration, however, results in a dissipation peak which is broader and takes longer to stabilise. For both *MvBOx* and BSA adsorption, it was difficult to calculate k_{on} and k_{off} values due to there not being enough time to approach equilibrium to accurately assess these details (this would probably need a static cell). There is also a need for much lower concentrations as k_{on} is most likely high enough that it cannot be resolved at the high concentrations that are used within these measurements, with a 1second time resolution.

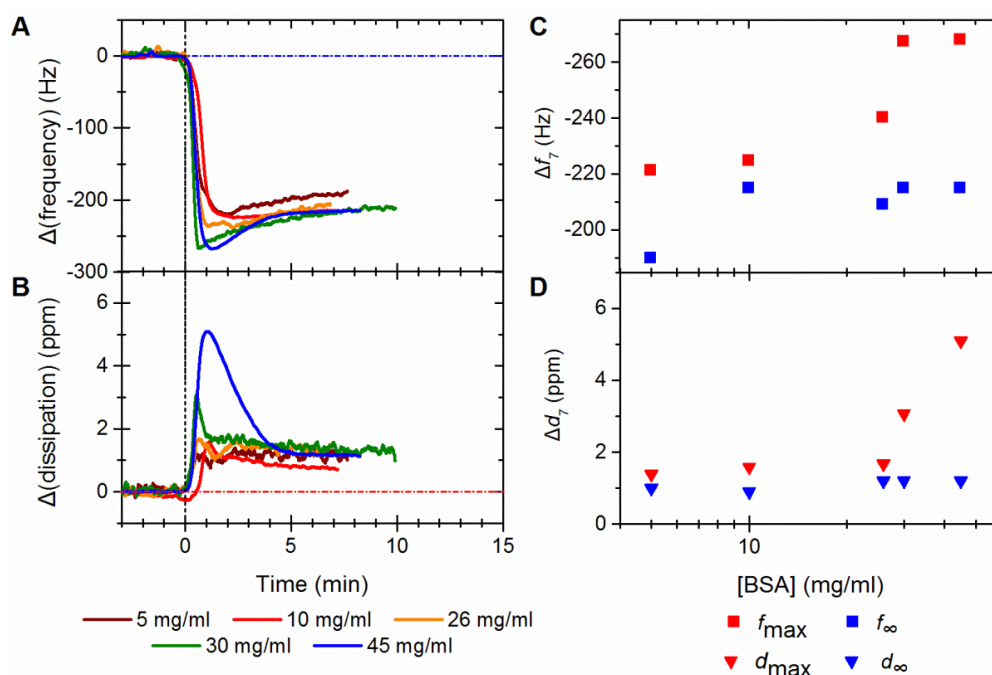


Figure 4.16. QCM-D responses for BSA concentration study adsorbed onto 3MPA modified quartz crystals. Panel A and B show frequency and dissipation responses for different BSA concentrations, respectively. Panels C and D show the maximum changes in frequency and dissipation (f_{max} , d_{max}) and the final stable response after adsorption (f_{∞} , d_{∞}) at each concentration. Note the x-axis is logarithmic ($\log_{10}$). Conditions: 0.1 M sodium phosphate pH 6.0, 25 °C, 0.1 ml min⁻¹, Flow rate of O₂ at 90 cm³ min⁻¹, seventh harmonic.

These measurements suggest that, although initial mass loading of BSA may be different, there is little effect of concentration on the final mass that remains adsorbed. This is in contrast to that seen in concentration studies of MvBOx on 3MPA-modified gold surfaces where the final mass that remains adsorbed increases with increasing concentration. When looking at the changes in dissipation; greater concentrations lead to larger ‘peaked’ responses however the final stable dissipation values (Δd^{∞}) are similar across all concentrations (1.25 ± 0.1 ppm) which is similar to that seen in concentration studies of MvBOx.

4.4.5 Combined and sequential adsorption of BSA with bilirubin oxidase

In this section, the adsorption of MvBOx was combined with the catalytically inactive BSA to simulate the possibility of having a non-catalytically active layer containing

regions or ‘holes’ into which catalytically active protein can absorb. This is based on an idea that BSA exhibits a lower degree of ordered structure on hydrophilic surfaces such as 3MPA, resulting in an increasing footprint and relaxation of the molecule after adsorption.²³ Another reason for pursuing this idea was that BSA remains stable against potential cycling and thus incorporating *MvBOx* into a layer of BSA could protect against any possible potential cycling-induced inactivation. It would also be interesting to see if there is a higher catalytic efficiency per mass of *MvBOx* due to the stabilisation advantage of having a monolayer but with less competition for O₂.

In the measurement, BSA was introduced to the surface followed by *MvBOx* (sequential adsorption). In the second set of measurements, co-adsorptions were carried out at different concentrations ratios of BSA and *MvBOx*, where *MvBOx* concentration was kept constant. This helped in understanding whether the balance between activity and stability could be controlled using different ratios of catalytic and non-catalytic protein.

4.4.5.1 Sequential adsorption

In this measurement BSA (24 mg ml⁻¹) was introduced to a 3MPA-modified surface before a solution of *MvBOx* (24 mg ml⁻¹) was introduced. Potential cycling was carried out on the resulting layer to look for any signs of catalysis from the adsorbed *MvBOx*.

The frequency and dissipation responses in Figure 4.17A and Figure 4.17B for the adsorption of BSA show a large soft layer formation ($\Delta f = -291$ Hz, $0.74 \mu\text{g cm}^{-2}$) with an overshoot of ~ 18 Hz ($0.05 \mu\text{g cm}^{-2}$) before a stable response ($\Delta f^{\infty} = -273$ Hz). There is a 38% loss of mass during the buffer wash, and a 94% reduction in dissipation. The magnitude of $\partial d/\partial f$ is more than three times higher during the ‘buffer wash’ (0.017 ppm Hz⁻¹) compared to BSA adsorption (0.058 ppm Hz⁻¹).

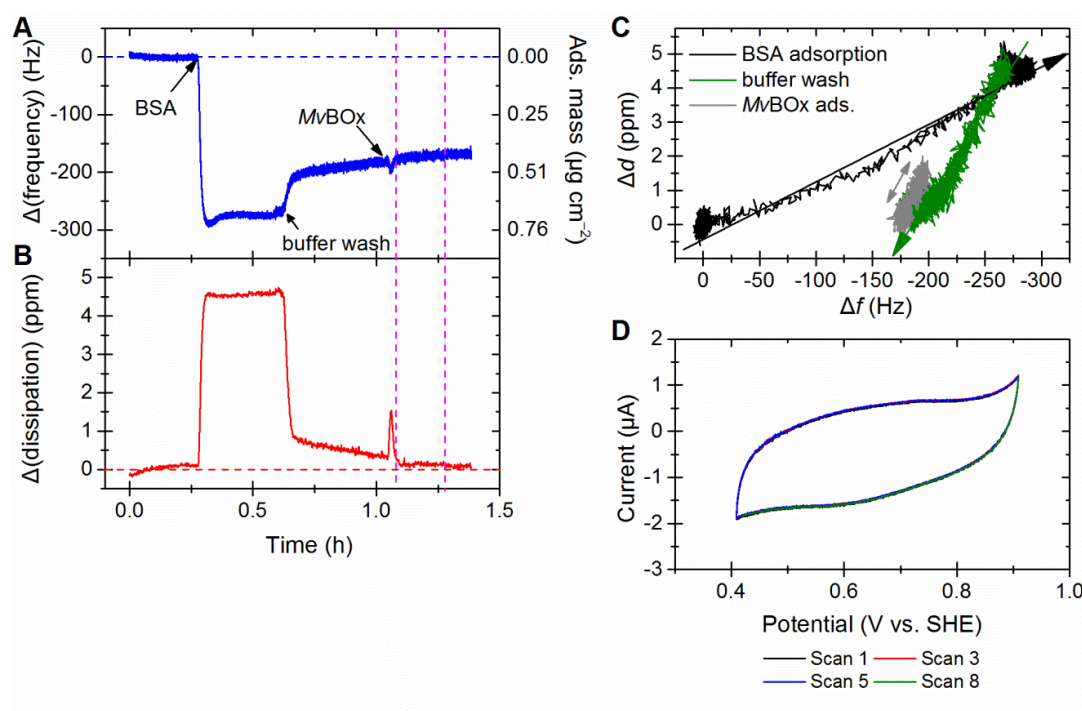


Figure 4.17. The sequential adsorption of MvBOx onto a layer of adsorbed BSA on 3MPA-modified gold-coated quartz crystals. Panel A and B shows the frequency response and dissipation response for protein adsorption. Panel C shows the Δd vs. Δf plot during both adsorptions. Panel D shows the cyclic voltammogram after MvBOx adsorption on to the BSA layer, carried out between 0.93–0.31 V vs. SHE. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 °C, and 0.1 ml min⁻¹. Flow of O_2 set to 90 cm³ min⁻¹, seventh harmonic measured. For the injection of BSA, the inlet tubing was immersed in a solution of BSA and left. Once a steady post-adsorption response (Δf^∞ & Δd^∞) was seen the inlet tubing was then switched back into buffer (buffer wash). MvBOx (24 mg ml⁻¹) and BSA (24 mg ml⁻¹).

When MvBOx is introduced, there is a temporary increase in both mass (0.04 $\mu\text{g cm}^{-2}$) and dissipation (1.8 ppm), however both the change in frequency and dissipation are non-permanent and the responses immediately shift back to the pre-adsorption response. This suggests a bulk-solution effect. The cyclic voltammogram (Figure 4.17D) shows the complete absence of a catalytic wave, indicating the complete lack of oxygen reduction catalysis. It was evident from the concentration studies that there was a saturated adlayer of BSA adsorbed to the surface and therefore it was expected that MvBOx catalysis would not be seen.

4.4.5.2 Combined adsorptions

In the coadsorption measurements, three different BSA concentrations were used: 5, 10 and 25 mg ml⁻¹. In each case, a constant potential was held during adsorption and was then switched off, before potential cycling was applied to the layer.

Figure 4.18 shows the co-adsorption of BSA (5 mg ml⁻¹) with MvBOx (24 mg ml⁻¹) onto a 3MPA-modified gold-coated quartz crystal.

After adsorption, there is an overshoot resulting in a small mass loss ($\Delta f = +17.5$ Hz, ~ -0.04 $\mu\text{g cm}^{-2}$), however the dissipation trace shows a stable response ($\Delta d = 2.1 \pm 0.01$ ppm). When the potential is switched off, there is a 57% decrease in the dissipation trace alongside a larger mass loss of (-0.24 $\mu\text{g cm}^{-2}$).

When potential cycling is started, the activity of the MvBOx/BSA layer increases. There is a small shift in E_{cat} (-0.03 V) over the course of 30 min (15 scans) indicating a change in the orientation of the enzyme.

Figure 4.18E (upper panel) gives an approximation of the magnitude of $\partial i / \partial f$ from the point when the enzyme first begins to show catalytic activity. The adsorbed mass efficiency ($\Delta i / \Delta f_7$) for the combined layer is $0.07 \mu\text{A Hz}^{-1}$. Normalising this to enzyme concentration (multiplying by $((24 + 5)/24)$) gives an efficiency of $0.087 \mu\text{A Hz}^{-1}$.

In Figure 4.18E (lower panel) the dissipation-activity plot suggests that maximum activity correlates with a maximum dissipation, however activity begins to drop while dissipation remains constant, indicated by the short horizontal section of Figure 4.18E (lower panel).

During potential cycling, there is more than a 50% loss in activity over the course of 30 min and during this period both the dissipation and frequency converge to a steady response. The magnitude of $\partial d_7 / \partial f_7$ during CVs (grey trace, Figure 4.18D) was roughly

1.5 times lower than the mass loss that followed chronoamperometry being turned off (green trace): the combined protein layer has undergone significant stiffening.

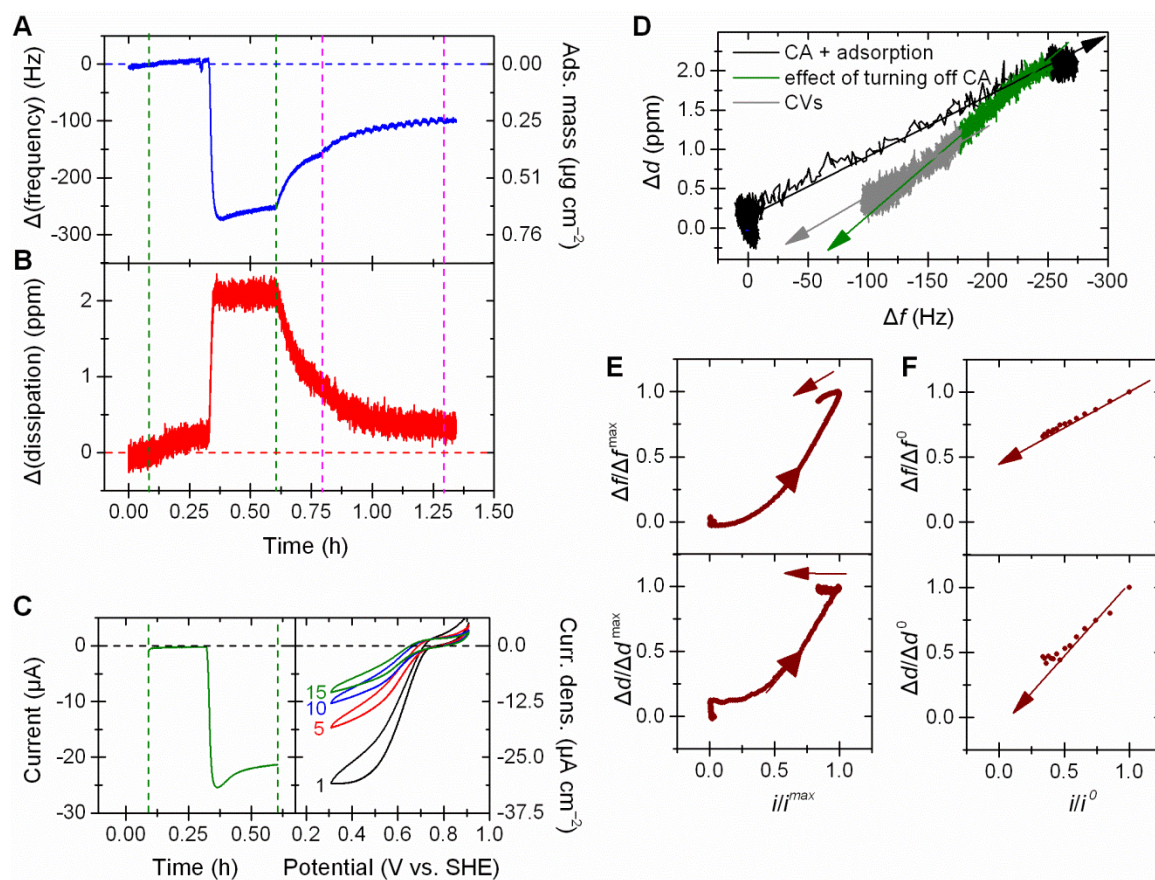


Figure 4.18. The effect of combined adsorption of BSA (5 mg ml⁻¹) and MvBOx (24 mg ml⁻¹) onto a 3MPA modified gold-coated crystal. Panel A and B show the frequency and dissipation responses and Panel C shows the chronoamperometric current (left) and CVs (right). Green dashed lines indicate when chronoamperometry was carried out and magenta dashed lines indicate CVs. Panel E shows the frequency-activity (upper) and dissipation-activity (lower) plots during chronoamperometry. Panel F shows frequency-activity (upper) and dissipation-activity (lower panel) plots during potential cycling between 0.91-0.31 V vs. SHE. Arrows show the direction in which measurement proceeds. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, and 0.1 ml min⁻¹. Flow of O₂ at 90 cm³ min⁻¹, seventh harmonic measured.

The adsorption and dissipation changes, when the potential was held constant, showed an overall increase when increasing the concentration of BSA, highlighted in Table 4.7. The rate of activity loss during chronoamperometry is also higher at higher BSA concentrations (17% activity decay at 5 mg ml⁻¹ BSA vs. 47% activity decay at 10 mg ml⁻¹ over 0.25 h). At the highest concentration of BSA there is a more ‘peaked’ response

in the dissipation trace (see Appendix) similar to the measurement of BSA on a 3MPA-modified surface in Figure 4.15. The measurement using the lowest concentration of BSA also showed the highest adsorbed mass efficiency (when the potential was turned off).

Table 4.7 Table showing the measured parameters during chronoamperometry when increasing the concentration of BSA. [#]Values for $i/\Delta f$ have been normalised for *MvBOx* content. Full figures are included in Appendix

[<i>MvBOx</i>] (mg ml ⁻¹)	[BSA] (mg ml ⁻¹)	$\Delta f^{\max}$ (Hz)	Δf^{∞} (Hz)	$\Delta d^{\max}$ (ppm)	Δd^{∞} (ppm)	$\Delta i^{\max}$ (μ A)	Δi^{∞} (μ A)	$i/\Delta f$ (μ A/Hz) [#]
24	5	-266	-246	2.12	2.12	-25.4	-21.3	0.087
24	10	-277	-273	2.25	2.25	-16.5	-9.2	0.071
24	25	-277	-210	4.7	0.25	-3	n/a	n/a

Table 4.8 compares the currents taken from cyclic voltammograms during the coadsorption of *MvBOx* and BSA. The highest current is seen when using the lowest concentration of BSA. When the BSA concentration is doubled, the current taken from the first scan is 72% lower. At the highest concentration of BSA, there is a distinct lack of activity.

Table 4.8. Table comparing the currents (activity) taken from the cyclic voltammograms during coadsorption of *MvBOx* and BSA. Currents taken at lower potential vertex.

[<i>MvBOx</i>] (mg ml ⁻¹)	[BSA] (mg ml ⁻¹)	Scan 1 current ($-\mu$ A)	Scan 5 current ($-\mu$ A)	Scan 10 current ($-\mu$ A)	Scan 15 current ($-\mu$ A)	Current loss (%)
24	5	25	15	10	8.5	66%
24	10	7	2.7	2.3	2.1	70%
24	25	2.7	2	2	-	

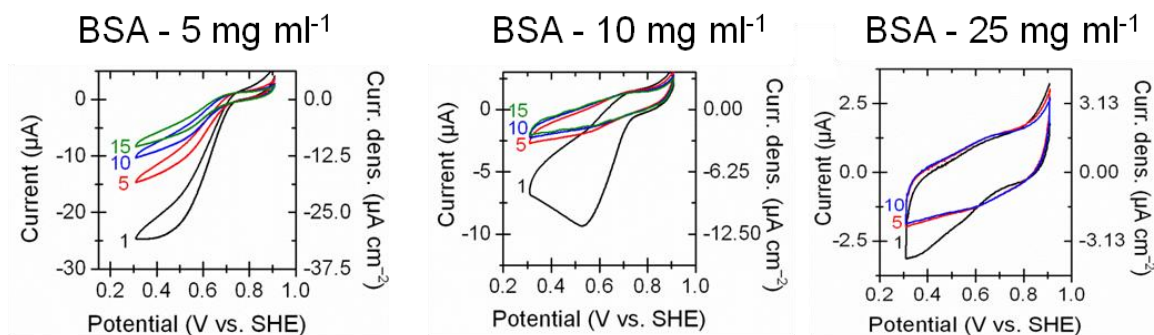


Figure 4.19 Comparison of cyclic voltammograms for the varying concentrations of BSA used in the coadsorption with *MvBOx*. Full figures are included in Appendix

Figure 4.19 compares the voltammograms taken from cyclic voltammograms during the coadsorption of *MvBOx* and BSA. At 5 mg ml⁻¹ BSA, the waveshape resembles the sigmoidal shape characteristic of electrocatalysis and exhibits the highest activity. However, at 10 mg ml⁻¹, the response indicated that the enzyme activity was reduced as evidenced by the lower current observed in the cyclic voltammograms. At the highest BSA concentration, there is evidence of a catalytic wave, however, activity remains consistently low throughout the following voltammograms. This suggests that, at higher concentrations, BSA may be outcompeting *MvBOx* for surface adsorption. Interestingly, this is also highlighted by the normalised $i/\Delta f$ (Table 4.7), which indicates that coadsorption with the lowest BSA concentration has a higher efficiency per adsorbed mass of *MvBOx*.

4.4.6 Discussion of sequential and combined adsorptions using BSA

During sequential adsorption of BSA and *MvBOx*, the magnitude of $\partial d/\partial f$ is more than three times higher during the ‘buffer wash’ (0.017 ppm Hz⁻¹) compared to adsorption (BSA adsorption (0.058 ppm Hz⁻¹). This suggests that during the buffer wash, the BSA may be rigidifying or undergoing some mechanical rearrangement on the sensor surface. This rearrangement could be a conformational shift (lower d) and this idea is consistent with a study by Roach *et al.* where grazing angle infrared spectroscopy (GA-FTIR) shows

BSA exhibiting a lower degree of ordered structure on hydrophilic surfaces such as 3MPA.²³ Their findings suggest that after the buffer wash removes excess BSA from the surface, the remaining protein undergoes relaxation and increases in footprint, hence the reason for the lower dissipation.

A study by Norde and Giacomelli, showed that, when comparing the secondary structure of BSA, before adsorption and after homomolecular displacement from hydrophilic silica particles, that structural perturbations induced by adsorption to the silica, were reversible i.e. BSA completely regains its native structure.⁸ Therefore an interesting route to go down would be to monitor dissipation and the $\Delta d/\Delta f$ plots for responses relating to structural changes of BSA. The adsorption of *MvBOx* into a BSA layer may be dependent on this reversible process.

BSA also adapts its conformation to variations in environmental conditions and more specifically, as a consequence of pH changes, BSA undergoes reversible conformational isomerisation, of which there have been several pH dependent forms of BSA reported.^{24,25} Therefore, another potential avenue of research would be to monitor dissipation traces at different pH, to determine the effect of any such conformational changes when BSA is adsorbed in combination with *MvBOx*.

In Figure 4.18, after adsorption, there is an overshoot resulting in a small mass loss ($\Delta f = +17.5$ Hz, $\sim -0.04 \mu\text{g cm}^{-2}$). However the dissipation trace shows a stable response ($\Delta d = 2.1 \pm 0.01$ ppm) suggesting that the conformation of the adsorbed species is relatively stable. The lower dissipation also suggests that the species on the surface is more rigidly adsorbed (lower Δd) compared to the higher dissipation when adsorbing just BSA.

There is an interesting observation when the potential is switched off, there is a 57% decrease in the dissipation trace alongside a larger mass loss of ($-0.24 \mu\text{g cm}^{-2}$). This suggests that holding the potential at 0.21V vs. SHE, may hold the conformation of the

adsorbed species, before a rigidification occurs once the potential is switched off. However, this hypothesis is difficult to prove as it is unknown the exact ratio of each species of protein that is adsorbed.

In Figure 4.18E (lower panel) the dissipation-activity plot suggests that maximum activity correlates with a maximum dissipation, however activity begins to drop while dissipation and mass both remain relatively constant (arrow '1', Figure 4.18E). This lack of change in both responses is difficult to distinguish, as the enzyme-BSA adsorption ratio is unknown. However, one possible explanation is that the enzyme may have changed orientation, which agrees with $-0.03\text{ V } E_{\text{cat}}$ shift in the voltammograms. Interestingly, the first scan in the voltammograms shows a higher current compared to when the constant potential is switched off. This could suggest that cycling the potential immediately orients the enzyme for more efficient electron transfer whilst in the presence of BSA.

At the highest concentration of BSA in the combined adsorption measurements, there is a more 'peaked' response in the dissipation trace (see Appendix) similar to the measurement of just BSA on a 3MPA-modified surface in Figure 4.15. This suggests that, because the adsorption pattern is similar to that in Figure 4.15, BSA adsorption is dominant at the higher concentrations, and outcompetes *MvBOx* adsorption. This is further supported by the reduced oxygen catalysis in the cyclic voltammograms. Another explanation for this could be that, as the BSA concentration increases, there is a higher degree of inactivation in *MvBOx*, which is indicated by the lower efficiency per adsorbed mass of *MvBOx* (Table 4.7). One possible measurement would be to introduce a solution of both proteins onto a surface, where a constant potential is being held, and look at the increase in activity of the combined adlayer throughout adsorption. This could be done over a variety of concentrations and then normalised to the content of *MvBOx* to

understand how different concentration ratios of BSA affect the catalytic activity and efficiency of *MvBOx*. Another experimental study would be to label the different proteins in solution (i.e., radioactively or fluorescently) to determine their contributions to the total adsorbed mass.

4.4.7 Measurements using a second multicopper oxidase

A second commercially available multicopper oxidase, laccase from *Trametes versicolor* (*TvL*) was adsorbed to a gold-coated quartz crystal modified with 3-mercaptopropyl-1-propanol, with the adsorption and subsequent cyclic voltammetry performed as for Figure 4.5. Figure 4.20 shows 50 potential cycles and E-QCM-D responses that parallel those observed for *MvBOx*.

During potential cycling, both the frequency and dissipation traces again showed periodic oscillatory changes that were slightly out of phase with the applied potential (Figure 4.5C). However these oscillatory changes had maximum peak-to-peak amplitude of ca. 27 Hz (0.07 ng cm^{-2}), roughly three times larger than in Figure 4.5D, and the presence of the ‘double peak-single trough’ was replaced with a ‘single peak-single trough’ pattern of oscillation.

There is an 85% activity loss in Figure 4.20C which is comparable to the 82% loss seen in Figure 4.5D, however, there is hysteresis present in the voltammograms, which fully disappears at scan 24 (48 min). The dissipation during this period decreases by 26% before giving a stable response of $\Delta d = 0.95 \pm 0.02 \text{ ppm}$.

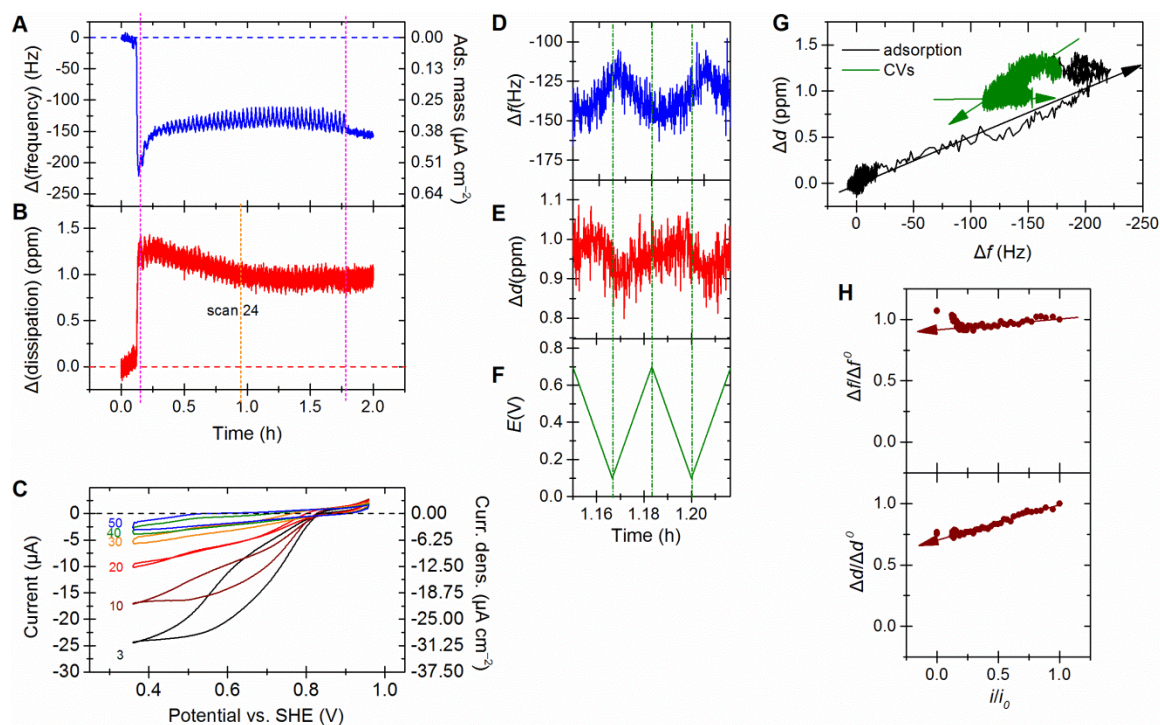


Figure 4.20. The effect of potential cycling on TvL adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropyl (3MPOL). Panel A and B show the frequency and dissipation response. The magenta dashed line indicates when cyclic voltammetry was started and ended. Panel C shows scans 3, 10, 20, 30, 40 and 50 of 50 scans carried out. The first scan, scan 3, is shown because the CVs were started during the adsorption phase and therefore scan 1 did not indicate the highest maximum current. Panel D & E show the frequency and dissipation responses for scans 30 and 31 with the potential cycling-induced oscillatory effect (single peaks slightly out of phase with the applied potential shown in panel F, green dashed lines). Panel G shows the Δd vs. Δf plot highlighting both the adsorption and potential cycling phases of the measurement. Panel H shows the frequency-activity (upper) and frequency-activity (lower) plots with arrows used as visual guides. Conditions: 25 °C, 0.1 M sodium citrate pH 4.0 (pre-saturated with O_2), 0.1 ml min⁻¹, seventh harmonic. Potential range: 0.97–0.36 V vs. SHE, 10 mV s⁻¹.

4.4.8 Discussion of potential cycling on second multicopper oxidase

The change in the oscillatory peaks during potential cycling, when using TvL, suggests that the cycling-induced distortion of the enzyme may be dependent on the structural composition of the adsorbed enzyme. There is an 85% activity loss in the potential cycling measurement on TvL (Figure 4.20C) which is comparable to the 82% loss seen with MvBOx in Figure 4.5D. However, there is hysteresis present in the voltammograms suggesting that TvL is switching between two different states, which then fully disappears

at scan 24 (48 min). This is consistent with typical voltammograms for the electrocatalysis of TvL that show a similar activation process, present in the first potential cycle of TvL (indicated by hysteresis), that disappears in subsequent scans.²⁶

The structure of the high potential MCOs are similar and therefore the pattern of activity loss is likely to be similar also. The implications of these comparisons between TvL and MvBOx suggest that inactivation may be independent of protein type, and more related to structural rearrangements of the adsorbed enzyme.

4.5 Successive adsorptions of bilirubin oxidase

In this section, equal amounts and concentrations of MvBOx were introduced onto 3MPA-modified surfaces in two successive steps. Potential cycling was carried out to assess the stability and lifetime of building up enzyme layers within an enzymatic fuel cell. The focus of this section was to ascertain whether loss in activity over time could be corrected by using fresh enzyme.

In the first measurement, MvBOx is adsorbed to a 3MPA-modified gold surface at pH 6 phosphate buffer, and potential cycling is carried out over approximately 2.3 h (60 scans), over the range 0.91–0.31 V vs. SHE. Potential cycling is stopped and a second addition of MvBOx is added to the surface and potential cycling is carried out a second time for approximately 2 h (50 scans).

Figure 4.21 shows the result of two successive adsorptions of MvBOx onto a 3MPA modified crystal. When the potential is cycled, it causes an immediate drop in adsorbed mass (Figure 4.21A) by 20–25% over ca. 15 min.

In this second adsorption, the mass increase is a third lower (increase of 105 Hz, 0.27 $\mu\text{g cm}^{-2}$) than the first adsorption, highlighting the possibility that the limited secondary

adsorption may be dependent on the amount of desorption of enzyme, and thus space created, from the first adsorption.

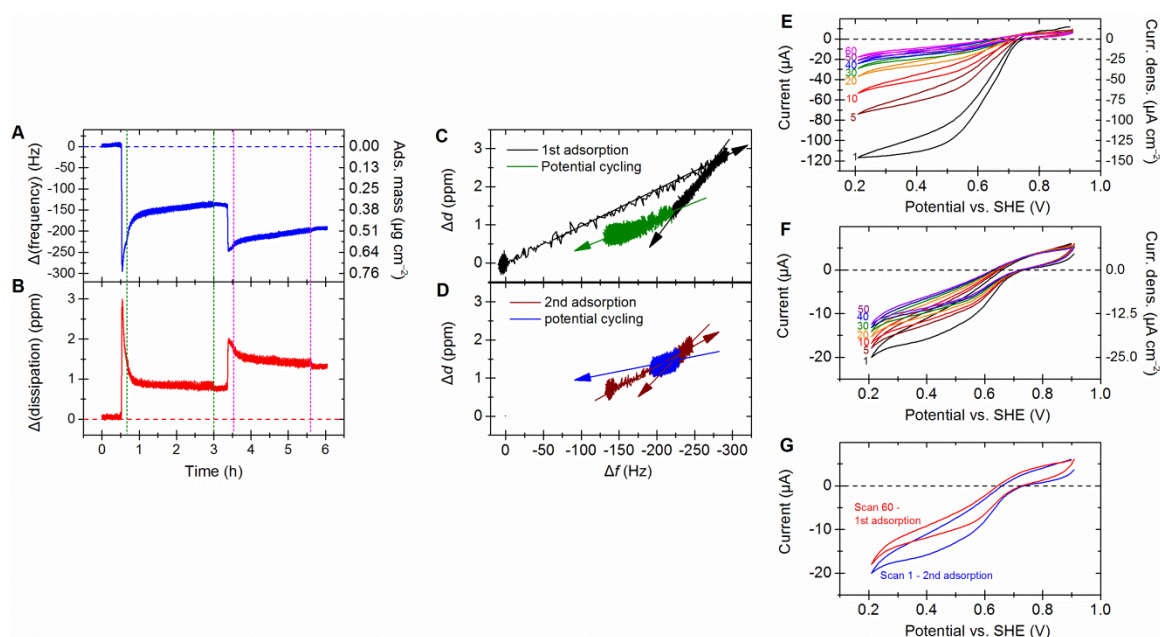


Figure 4.21. The effect of multiple enzyme adsorptions on catalytic activity. Panel A and B show the frequency and dissipation responses to two successive adsorptions of *MvBOx* on a 3MPA-modified gold-coated QCM sensor, respectively. The green dashed lines (ca. 0.6 h to 3 h) indicate 60 CVs carried out after the first adsorption. The magenta dashed lines (ca. 3.5 h to 5.5 h) indicate the CVs carried out after the second adsorption. Panel C shows the Δd vs. Δf plot up to the end of the first set of CVs. Panel D shows the Δd vs. Δf plot from just after the second adsorption to the end of the second set of CVs. The arrows indicate the direction of the change in gradient. Panel E shows scans 1, 5, 10, 20, 30, 40, 50 and 60 of 60 scans carried out after the first adsorption. Panel F shows scan 1, 5, 10, 20, 30, 40 and 50 of 50 scans carried out after the second adsorption. Panel G shows the increase in current between the 60th scan in Panel E and the first scan in Panel F. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. Cyclic voltammetry: 0.91-0.31 V vs. SHE, 10 mV s⁻¹.

The Δd vs. Δf relationship is shown in Figure 4.21C. The black trace shows the adsorption and subsequent mass loss following the first addition. Upon starting the potential cycling (green line), there is a distinct change in the Δd vs. Δf regime with a shallower gradient synonymous with a change in enzyme conformation. Values for $\partial d/\partial f$ are taken using linear regression analysis.

On addition of the second load of enzyme, the Δd vs. Δf gradient for adsorption (wine coloured line, 0.0096 ppm Hz⁻¹) shows a similar gradient to adsorption during the first

addition (black line, $0.0097 \text{ ppm Hz}^{-1}$), indicating that adsorption follows a similar process. The catalytic current degrades simultaneously during the first adsorption, falling by about a third (Figure 4.21E). During the second set of CVs the magnitude of $\partial d/\partial f$ is 1.5 times higher than the first set of CVs (blue trace ($0.0059 \text{ ppm Hz}^{-1}$) versus green traces ($0.0088 \text{ ppm Hz}^{-1}$)) suggesting that the potential cycling is causing a higher degree of dissipative changes per unit mass on this secondary layer, compared to the primary adsorption.

When the second load of enzyme is introduced, there is a small increase in activity highlighted by Figure 4.21G ($\sim 3 \mu\text{A}$). The voltammograms in Figure 4.21E and Figure 4.21F also show a downward turn, or ‘tail’ at the low potential vertex. Over time, this becomes more pronounced.

4.5.1 Potential cycling ongoing through adsorption

In this set of measurements, *MvBOx* was twice adsorbed onto a 3MPA–modified gold surface with ongoing potential cycling throughout both adsorptions of enzyme. The measurement was carried out using 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2) at 25°C delivered to the QCM cell at a rate of 0.1 ml min^{-1} . Potential cycling was carried out over the range 0.91-0.31V vs. SHE with a scan rate of 10 mV s^{-1} . The second load of enzyme was injected at the beginning of scan 40.

The reason for carrying out this measurement was to understand if the potential cycling effect had a direct impact on the stability of the incoming/adsorbing enzyme and whether the ongoing potential cycling would affect the adsorption and subsequent activity of the enzyme.

In Figure 4.22, potential cycling was started before enzyme adsorption and the adsorption showed a maximum frequency change of -263 Hz ($0.67 \mu\text{g cm}^{-2}$) compared to the reduced amount of adsorption in the previous measurement (-290 Hz in Figure

4.21A). The maximum current from catalysis was not seen until scan 6 (Figure 4.22C) and over the course of the first 40 min. (scan 6 to scan 25) min there was a shift of -0.153 V in the position of E_{cat} (Table 4.9).

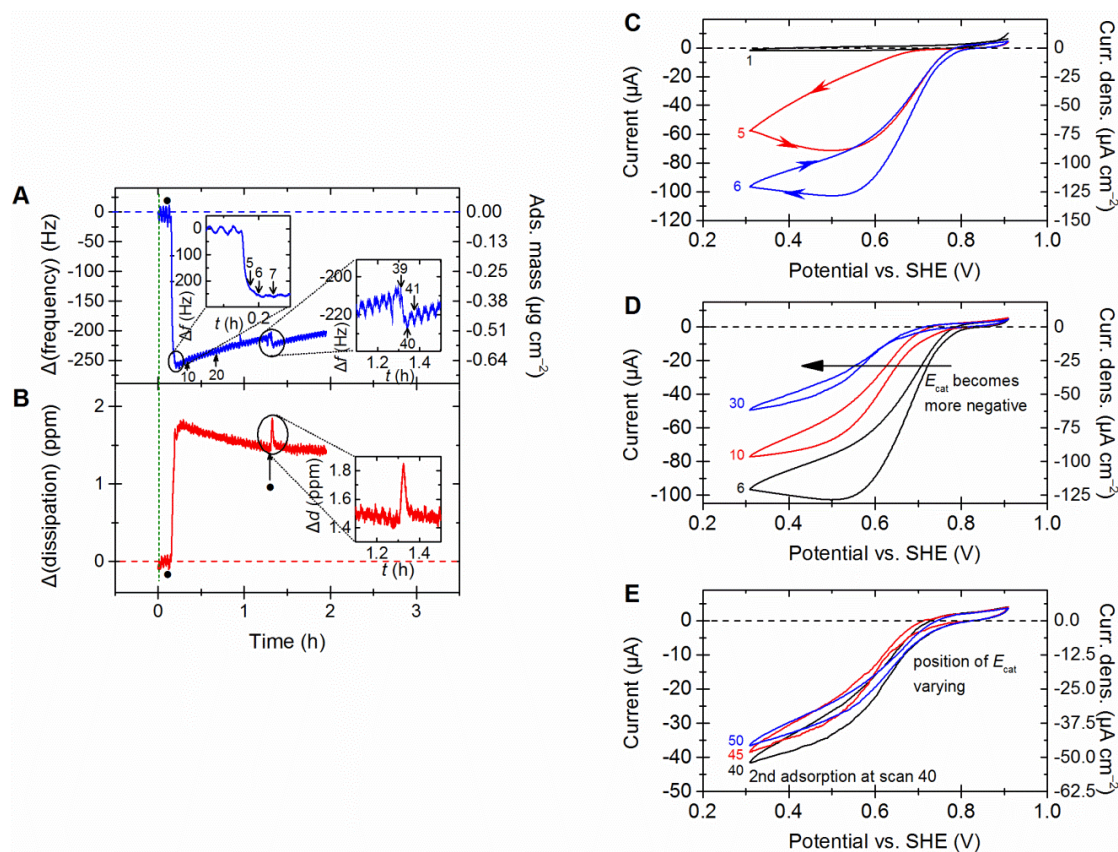


Figure 4.22. The effect of ongoing potential cycling during multiple adsorption of MvBOx onto a 3MPA modified QCM. Panel A and B show frequency and dissipation response for two adsorptions of MvBOx. Circle (●) indicates MvBOx injection (24 mg ml^{-1}). The second load of enzyme was injected at the start of scan 40 (Panel A, right inset). Panel B inset indicates the dissipation response to this second load. The green dashed line indicates the start of cyclic voltammetry. Panel C shows CVs before, during and after the first adsorption of enzyme, labelled corresponding to the inset in Panel A. The maximum current response was seen in scan 6. Panel D shows the large change in E_{cat} during voltammetry. Panel E indicates the current decay over in the 10 scans following the second adsorption. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , seventh harmonic shown. Cyclic voltammetry: $0.91\text{--}0.31 \text{ V}$ vs. SHE, 10 mV s^{-1} .

Table 4.9 Table showing the position of E_{cat} versus scan number during the first adsorption

Scan number	E_{cat} (V vs. SHE)
6	0.749
10	0.649
15	0.567
20	0.601
25	0.596
30	0.617

Alongside this shift in the E_{cat} , there was also a 51% decrease in activity from scan 6 to scan 30 (50 min, Figure 4.22D). There was reduction in this rate of activity loss after the second adsorption (Figure 4.22E) with only an 8% activity loss over the next 10 scans following adsorption. The position of E_{cat} is still variable throughout the 10 scans following the second adsorption.

A more detailed analysis of the changes in Δd vs. Δf gradient is shown in Figure 4.23, where there are three distinct regimes during the measurement (Figure 4.23C). On the second addition of enzyme, small increases in the adsorbed mass (~ 2 Hz, 4.8 ng cm^{-2}) were observed, however this was not of the same magnitude as in Figure 4.21 (~ 15 Hz, $0.036 \text{ } \mu\text{g cm}^{-2}$). On the second adsorption step, in Figure 4.23C, there is a temporary increase in the magnitude of $\partial d/\partial f$ (0.15 ppm Hz^{-1} , 5a) before returning to a value three times lower in magnitude than before the second adsorption: the gradient at arrow 5c is $0.0025 \text{ ppm Hz}^{-1}$ compared to the gradient of arrow 4, which is $0.0073 \text{ ppm Hz}^{-1}$).

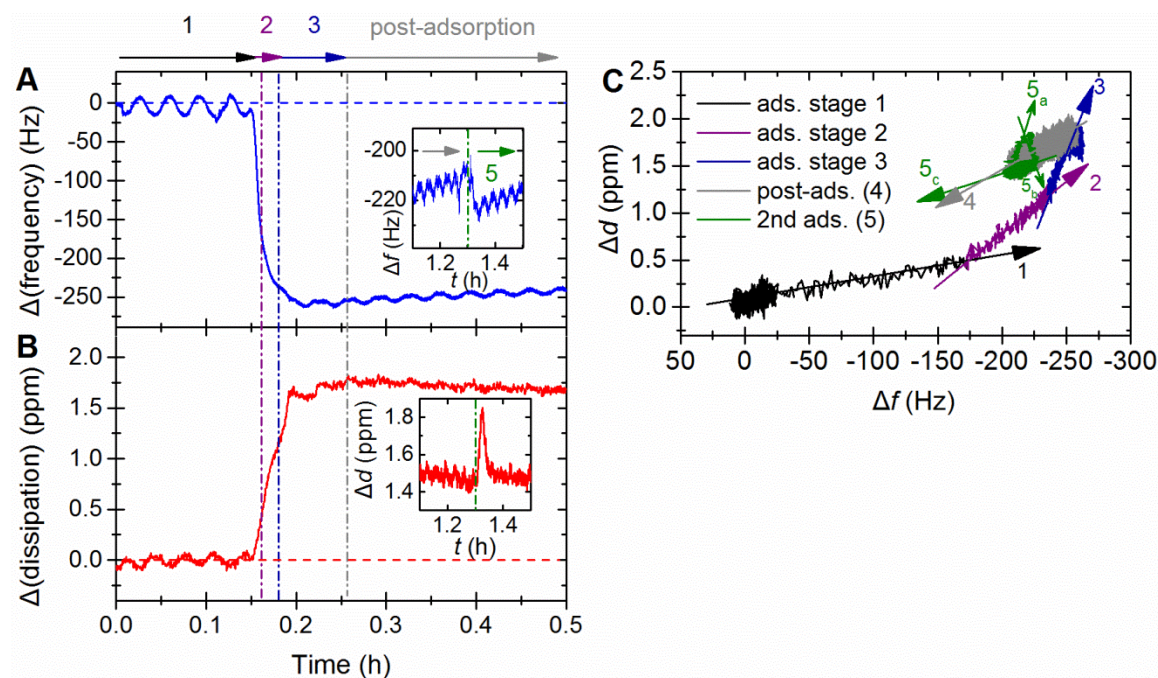


Figure 4.23. The effect of multiple adsorptions during ongoing potential cycling on the Δd vs. Δf gradient. Panel A and B show the frequency and dissipation traces. Panel C shows the Δd vs. Δf plot. The arrows along the top of panel A indicate when each adsorption phase starts in line with the numbered phases in Panel C. Arrows 1, 2 and 3 indicate the three step adsorption process during the first adsorption. The grey arrow indicates the mass loss over time post-adsorption. The green arrows represent the second addition of enzyme and highlight the immediate increase in dissipation (5a), followed by the immediate stiffening/denaturation (5b) before resuming a stable response (gradient of stage 4 = gradient of stage 5c).

4.5.2 Discussion of successive adsorption measurements and continuous potential cycling measurements

During the multiple adsorption measurement, the second adsorption causes a permanent increase in the dissipation response and therefore suggests the build up of an increasingly soft layer of enzyme. One possible reason for the increase, in both frequency and dissipation during the second adsorption, is that this enzyme is replacing the enzyme that could be desorbing during the first round of potential cycling.

In both the first and second adsorption, there exists a two step process in the dissipation where the second step is a slow mechanical change in the adlayer after adsorption. This is consistent with the Rabe *et al.* three states model for protein adsorption¹⁸ (discussed in Section 4.3.8). The transition from an initial irreversible state

to an intermediate state involves a change in surface affinity of the protein during adsorption, which can be attributed to the sharp peak increase in dissipation and temporary soft layer formation, followed by a rapid change in orientation, which can be attributed to the rapid decrease in dissipation after the peak response, giving a more rigid layer.

On addition of the second load of enzyme, the Δd vs. Δf gradient (wine coloured line, 0.01 ppm Hz^{-1}) shows a similar gradient to the initial adsorption (black line, $0.009 \text{ ppm Hz}^{-1}$) indicating that adsorption follows a similar process as the first adsorption. The catalytic current degrades simultaneously during the first adsorption, falling by about a third (Figure 4.21E). During the second set of CVs the magnitude of $\partial d/\partial f$ is 1.5 times higher than the first set of CVs (blue trace ($0.006 \text{ ppm Hz}^{-1}$) versus green traces ($0.009 \text{ ppm Hz}^{-1}$)) suggesting that potential cycling on the secondary layer causes a greater dissipation response per unit . In turn, this could mean that the secondary layer stiffens more readily and in general is more sensitive to potential cycling.

On addition of the second load of enzyme, there is a small increase in activity highlighted by Figure 4.21G. This is significant because it confirms that introducing additional enzyme to the surface increases the catalytic activity. However, the voltammograms in Figure 4.21E and F also show a downward turn, or ‘tail’ at the low potential vertex. Over time, this ‘tail’ becomes more pronounced in the voltammograms, and could be a signature for a change in conformation change, which in turn leads to poor orientation of the enzyme. It could also be an indication that the enzyme has become distorted in a way that the copper coordination has changed thus dropping the potential, resulting in the tail shape.

An interesting set of experiments to overcome enzyme inactivation and incorporate a greater load of enzyme onto an electrode, and also to possibly protect enzyme from

potential cycling induced destabilisation, could be to incorporate layers of *MvBOx* in a layer-by-layer assembly (LbL). A popular method of constructing these layer-by-layer assemblies is by creating layers of polyelectrolyte ions interspaced with oppositely charged enzyme molecules.^{27,28} This immobilization strategy has clear applications in enzyme electrocatalysis over a range of different electrode surfaces. In a study by Tsujimura *et al.*, Bilirubin oxidase (BOD) was immobilized as a multiple layer in a cationic polymer (poly-l-lysine) matrix on a carbon electrode surface. Under convective conditions where O₂ depletion was negligible near the electrode surface, a steady-state voltammogram was obtained with a limiting current density of about 1 mA cm⁻². In the study, they say that construction of multiple enzyme layers is useful for increasing the current density in DET-type bioelectrocatalysis: the maximum current density in this study is as large as 845 μA cm⁻², which is about three times larger than that at a monolayer enzyme-modified electrode.²⁹ This kind of experimental setup would be useful in optimising the thickness and concentration of the immobilized enzyme layer, and understanding how this would affect the long term performance of using such enzymes in enzymatic fuel cells.

To further investigate the idea of changes in copper coordination, circular dichroism (CD) could be used to study the secondary structure of the protein. Circular dichroism measures the difference in the absorption of left-handed circularly polarised light and right-handed circularly polarised light. Proteins have different structural elements that have characteristic CD spectra, due to each element having different degrees of absorption of left and right polarised light. Therefore, as the CD spectra of proteins are dependent on the proteins structural elements i.e. its conformation, CD can be used to observe how the secondary structure changes with environmental conditions such as temperature and pH, which in turn might have an effect on copper coordination. In a

study by Gotoh et al., the copper coordination of bilirubin oxidase was probed using CD and it was found that the CD spectra differed between the oxidised and reduced forms of enzyme. It was inferred that, when the copper coordination state changed, subtle changes in the amino acid residues around the copper sites caused changes to the ligand-metal bond distances at the copper site, which in turn induced changes in secondary structure.³⁰ Therefore taking a CD spectrum of the enzyme, before and after potential cycling, could help understand if there are potential-induced changes to the copper coordination.

In the continuous potential cycling experiments, potential cycling was started before enzyme adsorption and carried on throughout. Over the course of the first 0.75 h min there was a shift of -0.15 V in the position of E_{cat} , meaning a greater thermodynamic driving force is needed for O_2 reduction. This also suggests that the continuous potential cycling contributes to the mis-orientation/poor positioning of the enzyme on the electrode surface, thereby resulting in poor electron transfer. Interestingly, the change in E_{cat} could also be as a result of a change in the copper coordination in the enzyme. This potential cycling-induced instability to the adsorbing enzyme is in contrast to the observations in an early study by Kleijn and Norde, where they suggest that the adsorption of protein are not affected by imposing an external potential to the sorbent surface.¹⁹

A clearer view of the mechanics of the surface, or at least the distribution of charge on the enzyme surface could provide an understanding as to the effects of potential cycling on the mechanical properties of the enzymes. There may be excessive steric hinderance between enzyme particles on a crowded surface for an orientation change, however, the potential cycling could be stretching out the enzyme which in turns affects/distorts the T1 primary electron entry point. This phenomenon could be probed using electron paramagnetic resonance (EPR) to look at changes of the EPR active T1 and T2 copper centres within the enzyme, which are involved in electron transfer processes.

On the second addition of enzyme, small increases in the adsorbed mass were observed, however this was not of the same magnitude as the increase in mass on the second adsorption. The continuous potential cycling appears to be limiting the amount of mass adsorbed to the electrode surface. However, in the measurement taken in Figure 4.21, the first set of scans were left running for approximately 2 h and therefore it is possible that the enzyme underwent some relaxation process which made it feasible for further adsorption of protein.

In addition to this, and in contrast to that seen in Figure 4.21, there is no increase in the catalytic activity after adsorption (Figure 4.22E). One possible explanation for this is that the small amount of enzyme adsorbing to the surface, during the second adsorption, immediately inactivates. Another view is that the current measurements are not sensitive enough to detect whether the addition of <1% of a monolayer of enzyme made a difference in the already decaying catalytic current.

On the second addition of enzyme in Figure 4.23C, the Δd vs. Δf gradient showed a reversible change in the Δd vs. Δf gradient (arrow 5a, 5b & 5c) indicating that, unlike the measurement in Figure 4.21, the addition of further enzyme had no permanent effect on the binding regime. The dissipation response increased very slightly after the initial peak response (Figure 4.23B, inset) and enzyme still adsorbed. This suggests that the enzyme could be adsorbing in an inactivated state with an almost immediate rigidification (drop in Δd) of the enzyme. This hypothesis would require further experimental work to be carried out and one such avenue could be to use E-SPR and monitor the adsorption of the second amount of enzyme. In a study by Nakamoto et al., E-SPR was used to synchronise redox and optical signals in relation to the redox changes of an osmium–hydrogen peroxidase film on an array of nanometre-sized pores in Au.³¹ In a similar fashion, E-SPR could be used to investigate any potential cycling-induced conformation changes.

Caruso *et al.* showed the advantages of the use of QCM alongside SPR and AFM to identify wet and dry ferritin coverage, thickness and density on Au substrates. E-SPR has also been applied to investigate redox-induced conformation changes of immobilised proteins.³² In a similar fashion, the use of the QCM with SPR could help reveal information on the thickness, density and any conformational change of the second layer of enzyme, and therefore would give a better understanding of the ongoing processes behind the reduced responses in the dissipation, and also the reason behind not seeing an increase in the catalysis after the second adsorption.

These observations suggest that administering an ongoing variable load has two effects: mechanical changes occur in the enzyme, resulting in a greater thermodynamic push for catalysis (negative change in E_{cat}) and secondly, the addition of further enzyme does not contribute to an increase in the catalytic activity of the layer.

Another alternative route to go down would be to incorporate a matrix or scaffold, which promotes electron transport and improves long term performance. A study by Salaj-Kosla *et al* showed improved electrocatalytic activity by electrodepositing a polymer layer or ‘cap’ on adsorbed bilirubin oxidase with reported current densities of up to 0.8 mA cm^{-2} .¹³ Improving the stability and long term performance of the adsorbed layer could possibly be more beneficial than to correct for losses by the introduction of more enzyme.

4.6 Covalent attachment of bilirubin oxidase

To control for the possibility that diminishing activity is due to enzyme desorption and factor out the effect of mass losses from the surface, *MvBOx* was covalently linked to a thiol-modified gold crystal via an amide linkage. The chemical linker was 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) and formed a thiol layer on the gold

surface, with an active hydroxysuccinimide group for amide bond formation with one of the lysine side chains on the protein's exterior, similar to EDC–NHS-type coupling reactions.

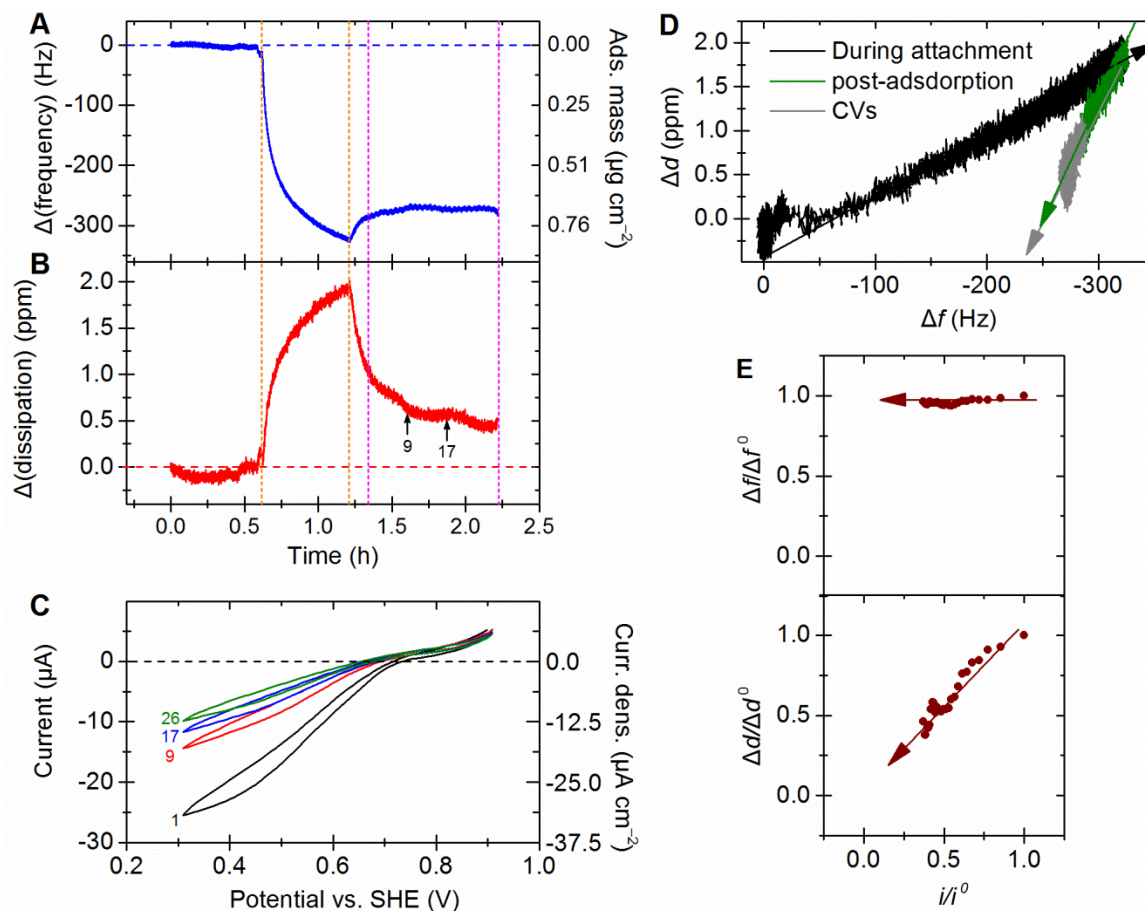


Figure 4.24. The effect of potential cycling on MvBOx covalently attached via a DTSSP-modified gold-coated quartz crystal. Panel A and B show the frequency and dissipation responses. After addition of MvBOx (24 mg ml^{-1}), buffer flow was stopped for approx. 40 min (orange dashed lines) to allow adsorption and reaction with surface layer (reaction adapted from the literature).^{33,34} Flow was resumed for ca. 10 min before voltammetry was started (magenta dashed lines). Panel C shows scans 1, 9, 17 and 26 of 26 scans carried out: 0.91–0.31 V vs. SHE, 10 mV s^{-1} . The arrows in panel B indicate start of selected cycles and correspond to numbering in Panel C. Panel D shows the Δd vs. Δf plot highlighting the attachment, post-adsorption and potential cycling phases. Panel E shows the frequency-activity (upper) and dissipation-activity (lower) plots during potential cycling (arrows used as a visual guide). Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , seventh harmonic shown.

After MvBOx was introduced to the DTSSP-modified surface, the buffer flow was stopped for approx. 40 min, to allow for the reaction with surface layer (reaction adapted

from the literature).^{33,34} The buffer flow was resumed for ca. 10 min before 26 scans were carried out between 0.91- 0.31 V vs. SHE at a scan rate of 10 mV s⁻¹.

Unlike the simple adsorption examples, the mass dropped by only 6% in the first seven cycles then stabilised between 0.68 and 0.70 $\mu\text{g cm}^{-2}$. Despite this, the catalytic activity still dropped off rapidly (Figure 4.24C), diminishing by 80% in less than 1 h. The position of E_{cat} decreases by ~ 0.1 V, meaning that the covalently attached enzyme needs a greater driving force (~ 10 kJ mol⁻¹) to drive electrocatalysis, assuming a series of 1 electron processes rather than a 4 electron process, compared to the voltammograms in Section 4.3.1 (potential cycling of *MvBOx* on 3MPA, Figure 4.1). The increased charge-transfer resistance is also evident in the shape of the voltammograms in Figure 4.24C: a more linear, ohmic response dominates the catalytic waveshape (compare to Figure 4.5B).

The magnitude of $\partial d/\partial f$ during the adsorption (black trace) was 0.007 ppm Hz⁻¹ which is lower than the gradient during adsorption in the chronoamperometry case (0.01 ppm Hz⁻¹). The magnitude of the $\partial d/\partial f$ gradient (Figure 4.24D) showed only a slight increase from before potential cycling (0.02 ppm Hz⁻¹) to after potential cycling (0.03 ppm Hz⁻¹). The effect of potential cycling on mass loss is significantly reduced, shown by the near-zero gradient in Figure 4.24E (upper panel). There is only an overall 6% mass loss during potential cycling compared to $\sim 20\%$ mass loss in Figure 4.5 (Table 4.10). The slope for the normalised frequency-activity plot in Figure 4.24E shows this effect more clearly with a slope of 0.069 indicating very little mass loss in relation to current loss. Compared to a slope of 1.06 in the normalised dissipation-activity plot, there is a direct correlation between a change in the mechanical properties of the adlayer and the fall in activity seen in the voltammograms.

When comparing the potential cycling measurements between the covalently attached *MvBOx* and *MvBOx* on 3MPA-modified gold (Figure 4.5), there is a clear increase in the

current at the lower potential vertex of the first voltammogram in the 3MPA-modified gold example. The rate of current loss (over 26 scans) is also higher in the 3MPA example. However, there is a higher mass loss compared to the covalently attached MvBOx (Table 4.10).

Another interesting point shown in Table 4.10, is that the normalised adsorbed mass efficiency loss $((\Delta\text{efficiency}/\Delta\text{efficiency}^{\text{max}})/t)$ is approximately a factor of 2.5 lower when MvBOx is attached covalently compared to simple adsorption to a 3MPA-modified surface.

Table 4.10 Table comparing the starting current, current loss and mass loss during potential cycling of MvBOx on 3MPA-modified and MvBOx covalently attached to DTSSP-modified gold surfaces.

Potential cycling measurements	Starting current (μA)	Current loss over 26 CVs	Mass loss during CV	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Final efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($\mu\text{A Hz}^{-1} \text{h}^{-1}$)
MvBOx						
Simple adsorption (3MPA)	-117	72 %	20 %	0.52	0.22	0.24
Covalent attachment (DTSSP)	-26	63 %	6 %	0.091	0.035	0.62

4.6.1 Reactive Dye Labelling and Mass Spectrometry

Fluorescein 5-EX (F5EX) labelling was carried out to determine which lysine residue was involved in covalent attachment to the activated DTSSP surface. F5EX labelling was carried out, as Fluorescein 5-EX is an amine reactive probe and the succinimidyl ester terminal group allows for attachment to the amine group of the Lys residue. In this way, the Lys residues involved in DTSSP attachment can be identified.

At a dye-to-protein molar ratio of ca. 25, the degree of labelling (DoL) saturated at about one dye molecule per protein molecule ($\text{DoL} = 1.09 \pm 0.10$, Figure 4.18A inset). Most of the lysines accessible to react with F5EX, were ruled out: of the 11 lysines in the sequence of MvBOx (Figure 4.26), three did not appear differently in the labelled and unlabeled samples: Lys220, Lys251 and Lys314. Lys118 and Lys429 have $<2.5 \text{ \AA}^2$ of

their atoms on the protein surface (using the FindSurfaceResidues.py algorithm on PyMOLwiki).³⁵ Although Lys118 is accessible to react with dye molecules, it is too buried to couple to surface groups.

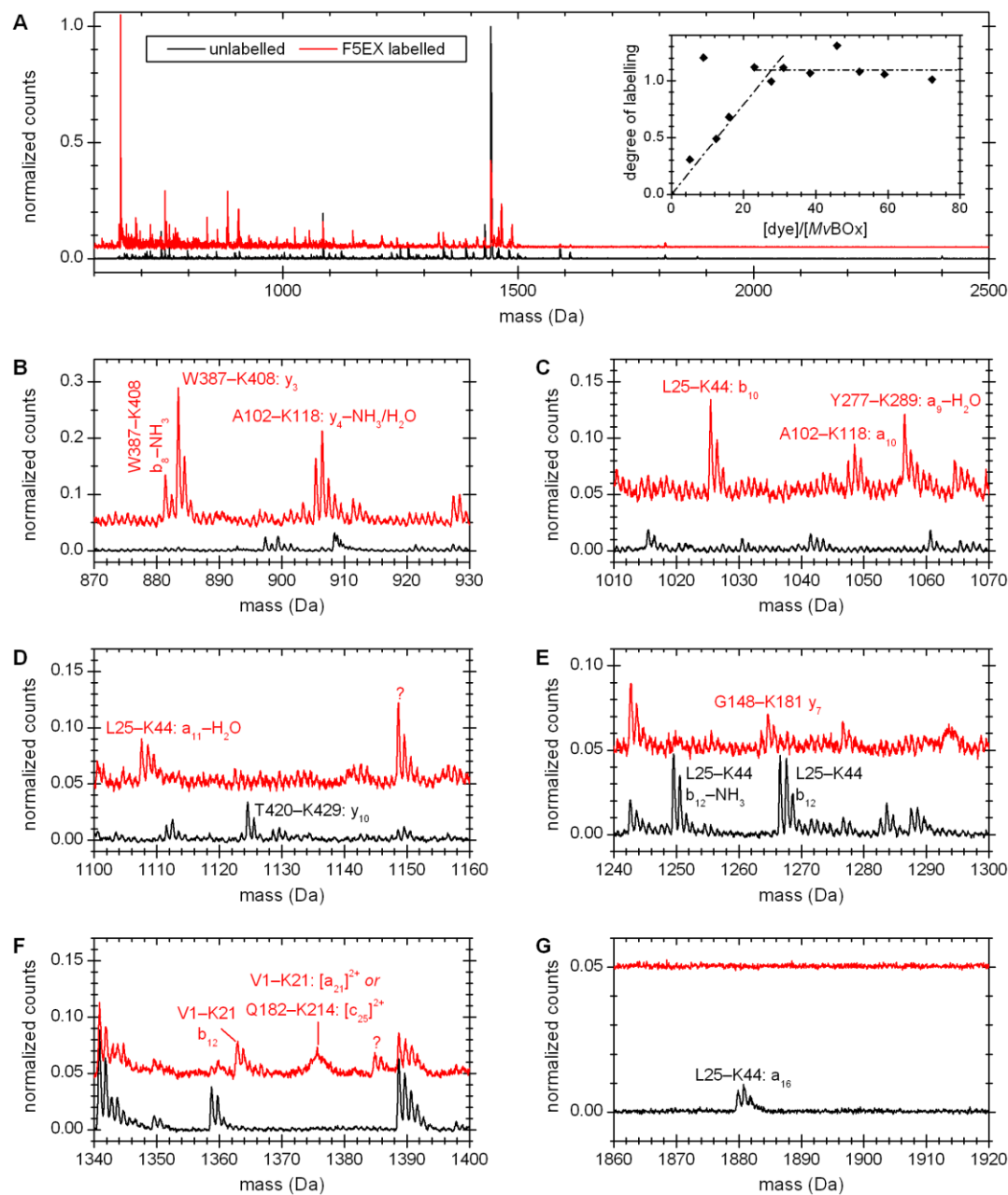


Figure 4.25. Determination of the location of reactive lysine side chains in MvBOx by reaction with fluorescein 5-EX (F5EX). Panel A compares the full MALDI mass spectra of enzyme that has been labelled with F5EX (red) and that which has not (black). The inset shows the degree of labelling as a function of enzyme concentration. The lines are to guide the eye. Panels B–F show specific regions where the two mass spectra differ. Peak labels give the peptide sequence and ion type.^{36–38} The full scales for the unlabeled and labelled spectra are 2.9×10^4 and 1.9×10^4 counts, respectively. Figure taken from Singh *et al.* with full permission from the Royal Society of Chemistry.¹⁶

The main new contributions to the mass spectrum of the labelled protein, compared to the unlabelled protein, were the peaks shown in Figure 4.18B, which correspond to Lys408 and Lys118. Small peaks appeared in fragments containing labelled Lys181 (Figure 4.18E) and Lys210 or Lys214 (Figure 4.18F). Small peaks disappeared from the spectrum of the unlabeled protein corresponding to Lys429 (Figure 4.18D) and Lys44 (Figure 4.18E&G). Trypsin cleaves at Arg and Lys residues, so the F5EX labels will always be at the C-terminus of any peptide fragment.

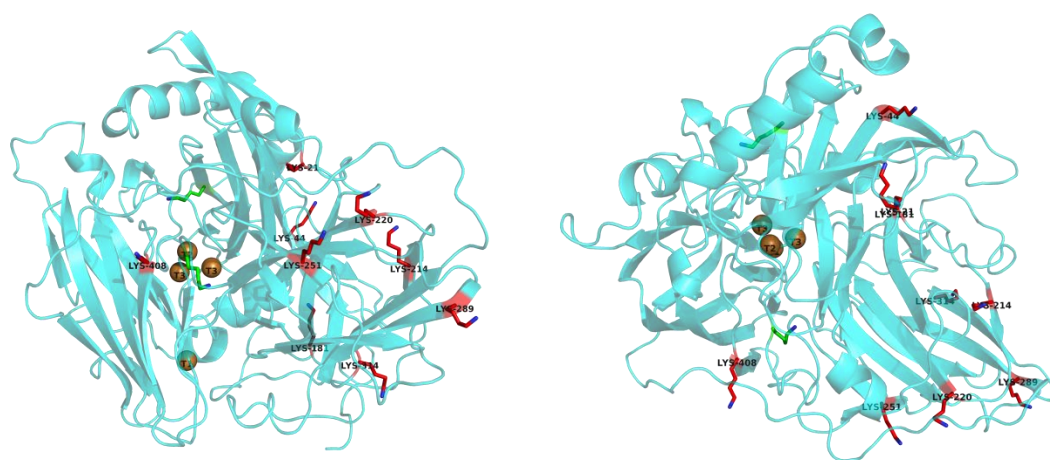


Figure 4.26. PyMOL visualization of the location of the lysine residues in *MvBOx* (PDB code 2xll).²¹ The polypeptide backbone is shown as the cyan cartoon. The coppers are shown as spheres labelled with their spectroscopic designation. The nine lysine side chains that have at least $2.5 \text{ \AA}^2$ on the surface are in red; the two that do not are in green. The image on the right is rotated 90° along a horizontal axis. Figure taken from Singh *et al.* with full permission from the Royal Society of Chemistry.¹⁶

4.6.2 Covalent attachment of laccase

To compare the effect of covalent attachment on enzymes other than *MvBOx* (Figure 4.24) *TvL* was attached to a DTSSP-modified gold crystal (Figure 4.27). The covalent attachment occurs through a single reactive lysine residue, Lys174,³⁹ which is $\sim 32 \text{ \AA}$ away from the T1 centre, too far for efficient electron transfer from the electrode.⁴⁰ The loss of activity is further highlighted in the near-absence of a catalytic wave upon potential cycling (panel C).

When potential cycling was carried out, there was initial increase in the dissipation trace after which it gradually decreased before showing a stable response (0.35 ± 0.05 ppm) (Figure 4.27B).

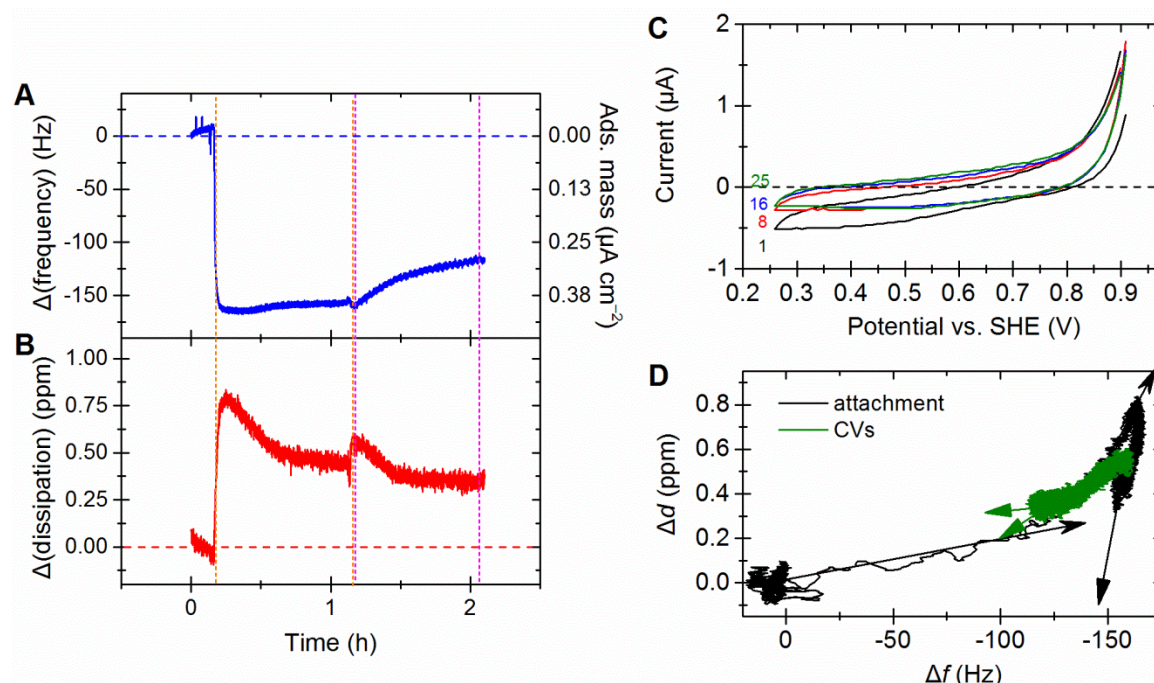


Figure 4.27. The effect of potential cycling on TvL covalently bound via a DTSSP-modified QCM sensor. Panel A and B show the frequency and dissipation traces. The orange dashed lines represent the time left for enzyme to react with surface layer (reaction adapted from the literature) during which time the peristaltic pump was turned off. The magenta dashed line shows when potential cycling began. Panel C shows CV scans 1, 8, 16 and 25 of 25 scans. Panel D shows the Δd vs. Δf plot. The black arrows indicate the adsorption and post adsorption regimes and the green arrows indicate the changes in regime during potential cycling. Conditions: O_2 -saturated 0.1 M sodium citrate buffer pH 4.0 at 25 °C flowing at 0.1 ml min^{-1} with continuous O_2 bubbling, seventh harmonic shown. Cyclic voltammetry: 0.91-0.36 vs. SHE, 10 mV s^{-1} .

4.6.3 Discussion of covalent measurements

Covalent attachment did not stabilise the catalytic activity. It also decreased the efficiency of electron transfer from the electrode to the enzyme. When *MvBOx* is covalently attached using the DTSSP linker, the comparatively small mass loss during potential cycling, partnered with the 80% loss in activity suggests that although the enzyme may be fixed more permanently on the electrode surface, inactivation is still

prevalent. The position of E_{cat} , a characteristic potential defined by the maximum of the change in current with potential on a swept-potential voltammogram, shifts by -0.1 V, meaning that the covalently attached enzyme needs a greater driving force (~ 10 kJ mol $^{-1}$) to drive electrocatalysis compared to the simple adsorption measurements seen earlier in this section (Section 4.2.1). The increased charge-transfer resistance is also evident in the shape of the voltammograms in Figure 4.24C: a more linear, ohmic response dominates the catalytic waveshape (compare to Figure 4.5B) further suggesting poor electron transfer.

Table 4.10 shows that although simple adsorption of *MvBOx* to the 3MPA results in higher currents, the rate of current loss is higher and there is a greater mass loss during potential cycling compared to the DTSSP-modified surface. In the covalent example, both the mass loss and current loss are smaller. This highlights that the enzyme retains a higher percentage of catalytic current and is attached more securely. However, an important observation is that, although the DTSSP attachment does not mitigate current loss completely, there is a smaller percentage drop over 26 scans compared to the 3MPA example. The percentage loss in adsorbed mass efficiency is similar in both cases, suggesting that irrespective of attachment strategy, the enzyme may inactivate at the same rate. This hypothesis would however require further experimentation and the need for testing a wider variety of surfaces and attachment strategies e.g., the use of electrostatic interactions or LbL assembly,⁴¹ metal-ion chelation using histidine-tagged proteins,⁴² and affinity binding using active site directed surface modifications.⁴³

The magnitude of $\partial d/\partial f$ during the adsorption (black trace) is lower than the gradient during adsorption of *MvBOx* on 3MPA under chronoamperometric control (0.01 ppm Hz $^{-1}$, Figure 4.1D, black trace) suggesting that the adsorption process results in a more rigid formation on the surface with a lower change in dissipation per mass unit.

The magnitude of the $\partial d/\partial f$ gradient (Figure 4.24D) showed only a slight increase from before potential cycling (0.02 ppm Hz⁻¹) to after potential cycling (0.03 ppm Hz⁻¹), suggesting that the covalently attached enzyme does not undergo the same level of potential cycling-induced destabilisation. The negative linear dissipation-activity in Figure 4.24E suggests that there could be a direct relationship between the fall in current and change in conformation or more specifically that structural changes are responsible for activity loss, even if they are not accompanied by a large mass loss.

When looking at the mass spectrometry measurements, the strongest evidence points to the most reactive exposed lysine side chain, and hence the one most likely to be involved in the covalent attachment, being Lys408, with some reactivity in Lys 21 and Lys181. All three of these groups are more than 1.5 nm from the enzyme's active sites, too far for tunnelling rates compatible with biological processes.⁴⁰ Lys408 is 2.59 nm from the type 1 copper (the primary electron acceptor)⁴⁴ and 2.15 nm from the type 2 copper; Lys21 is 3.23 nm from the type 1 and 2.03 from a type 3 copper; and Lys181 is 1.94 nm from the type 1 copper. These distances were measured from the terminal nitrogen atom. The most prominent peak, corresponding to the F5EX-labelled Lys408 fragment in Figure 4.25B, suggests that Lys408 is the most likely Lys residue to be targeted. Although the covalent attachment of DTSSP to the reactive Lys on MvBOx is not directly proven, the mass spectrometry data provides indirect evidence to suggest that Lys408 is the most likely residue involved in covalent attachment. In addition, the poor electron transfer that is seen in Figure 4.24C, compared to the voltammograms of MvBOx on 3MPA (Figure 4.5), is in agreement with the enzyme attaching via the Lys408 residue, as the enzyme is positioned further from the type 1 copper site (primary electron entry point) which therefore results in poor enzyme orientation and a lowered catalytic response. The enzyme exhibits a poor rate of electron transfer as a result of being

positioned further from the type 1 copper site, however, there is still some degree of enzyme inactivation (causing the activity loss), and a possible breakdown in structure, or denaturation (drop in dissipation while the mass remains stable, refer back to Section 0).

In contrast to the observations for *MvBOx* in Figure 4.24, the frequency magnitude increased when potential cycling was carried out on covalently attached *TvL*. There was a 27% mass loss when the potential was cycled however the true effect of this loss on catalytic activity is difficult to quantify as the catalytic current was low (Figure 4.27C).

In a study by Hamdan *et al.*, covalent attachment by amide linkage stabilised a [NiFe] hydrogenase for over 2 h in response to potential steps up to 250 mV.⁴⁵ [FeFe] hydrogenases covalently bound to electrode surfaces showed activity decay time constants ranging from hours to days depending on immobilisation method.⁴⁶ The observed behaviour above is dependent on a combination of protein structure and electrode composition and these issues are important when incorporating enzyme into fuel cell environments.

4.7 Conclusion

The catalytic activity of *MvBOx* on thiol-modified gold significantly decreases over time, and this inactivation can be attributed to a breakdown in secondary structure on the electrode surface, as opposed to enzyme desorption. Further to this, the enzyme likely undergoes a two stage process involving an initial rapid change in conformation, followed by a secondary slower change. When the potential is cycled to simulate variable loads on a fuel cell cathode, periodic oscillations at the electrode surface cause distortions in the enzyme which further exacerbates the inactivation of the enzyme. There is also a partnered mass loss which can be attributed to a combination of a breakdown in secondary structure and possible loss of solvation/hydration. Raising the potential of the low-potential vertex above ca. 0.6 V significantly improved stability and a stable mass

response, with an absence of the secondary, gradual change in conformation mentioned above.

Addition of further enzyme to an already electroactive enzyme layer can help replenish any loss in activity, however ongoing potential cycling renders this addition obsolete whilst also causing a significant shift in the position of E_{cat} , and ultimately leading to poorer electron transfer.

Attempts to attach the enzyme more permanently via covalent attachment did not improve catalytic stability and also resulted in lower power output due to poor orientation of surface bound enzyme. Comparison between a second multicopper oxidase suggested that enzyme inactivation is independent of enzyme type. These observations suggest that without additional treatments such as structural scaffolds, which act to help retain protein secondary structure and/or slow conformational rearrangement, or administering overpotential limits, MCO-coated cathodes may be unsuitable for long-term power generation in enzymatically catalysed fuel cells where a varying load is present.

4.8 References

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Chapter 5: Electrocatalytic activity of bilirubin oxidase and its dependence on surface chemistry and pH

5 Electrocatalytic activity of bilirubin oxidase and its dependence on surface chemistry and pH

5.1 Overview

The E–QCM–D was used to show that the catalytic activity of *MvBOx* is highly dependent on the pH and chemical functionality of the surface to which it binds. Gold QCM crystals were modified with alkane and phenyl thiols, and terminated with different functional end groups, whilst monitoring chronoamperometric and cyclic voltammetric currents during oxygen reduction. The activity, mass loading and dissipation responses of *MvBOx* adsorbed onto carboxyl functionalised surfaces was studied whilst varying the pH and chain length of the thiol modifier. Adsorption of the enzyme was also monitored on hydrophobic surfaces exhibiting rapid inactivation, and changing the surface functionality to hydroxyl and acetamido groups misorientated the enzyme, resulting in a change in the position of the catalytic wave (halfway point of the steepest part of the wave (E_{cat}). Affinity binding of *MvBOx* to a glucosamine functionalised surface (chitosan analogue) was also studied showing an increased stability and catalytic current and an E_{cat} similar to that seen on carboxyl terminated surfaces. This chapter highlights how the nature of the surface directly affects the position of E_{cat} and how changing the pH not only alters the E_{cat} but can also alter the mass loading mechanics and the Δd vs. Δf relationship during enzyme–surface interactions.

The data in this chapter was obtained by the use of bilirubin oxidase from *Myrothecium verrucaria* (*MvBOx*). Surface functionality experiments were carried out using gold–coated quartz crystals (resonance frequency 4.95 ± 0.05 MHz) modified with alkane–terminated and phenyl–terminated thiols (10 mM), in pH 6.0, 0.1 M sodium phosphate buffer. For the measurements looking at pH variation, pH 4.0, 0.1 M sodium citrate buffer and pH 5.0–7.0, 0.1 M sodium phosphate buffers were used. Chronoamperometry measurements were held at

0.21 V vs. SHE and cyclic voltammetry was carried out with a scan rate of 10 mV s⁻¹. Potentials are given vs. SHE unless otherwise stated.

The results will be presented by working through a typical measurement and comparing and discussing trends across the range of measurements in that section. The figures presented in this chapter are the result of a series of replicate and reproducible experiments, where a minimum of three repeats were carried out for each experiment. All complete figures are included in the Appendix.

5.2 pH measurements

This section describes the results from E-QCM-D experiments where *MvBOx* was adsorbed to 3MPA-modified gold-coated QCM crystals over the pH range 4 to 7. These measurements were carried out to probe how enzyme activity changes with pH under conditions approximating both constant and varying loads within a fuel cell.

In a typical measurement from this section, *MvBOx* was introduced to a 3MPA-modified gold surface at pH 6.0 while a constant potential of 0.21 V vs. SHE was applied on the electrode surface. After ca. 1 h, this constant potential was turned off and left for around 10 min, before potential cycling was then applied over the range 0.91–.21 V vs. SHE.

In Figure 5.1, *MvBOx* was adsorbed onto a 3MPA-modified surface at pH 6.0 and showed a maximum frequency change ($\Delta f^{\max}$) of -254 Hz (0.65 $\mu\text{g cm}^{-2}$) and maximum dissipation change ($\Delta d^{\max}$) of 2.17 ppm (Figure 5.1A & B). This was partnered with a maximum current ($\Delta i^{\max}$) of -34.6 μA with a current loss time constant of 0.26 h and gave a final current (Δi^{∞}) of -15.5 μA . The current trace showed a ‘peaked’ response and this was consistent in all observations. The mass adsorbed, dissipation and activity here are lower than recorded in chapter 4 and this was attributed to the fact that a constant potential was held throughout the adsorption process, which affected the mass adsorption and catalytic activity of the enzyme

adlayer, similar to the effect seen during continual potential cycling (Chapter 4, Section 4.5.1).

While the potential was held constant, the dissipation dropped by 0.33 ppm over the first 20 min, before giving a stable response. The frequency increased by *ca.* -14 Hz ($0.04 \mu\text{g cm}^{-2}$) over the same time and the current dropped by 55%. The magnitude of $\partial i / \partial f$ (adsorbed mass efficiency) gave a value of $1.36 \mu\text{A Hz}^{-1}$ which is similar to when potential cycling was carried out on *MvBOx* in chapter 4.

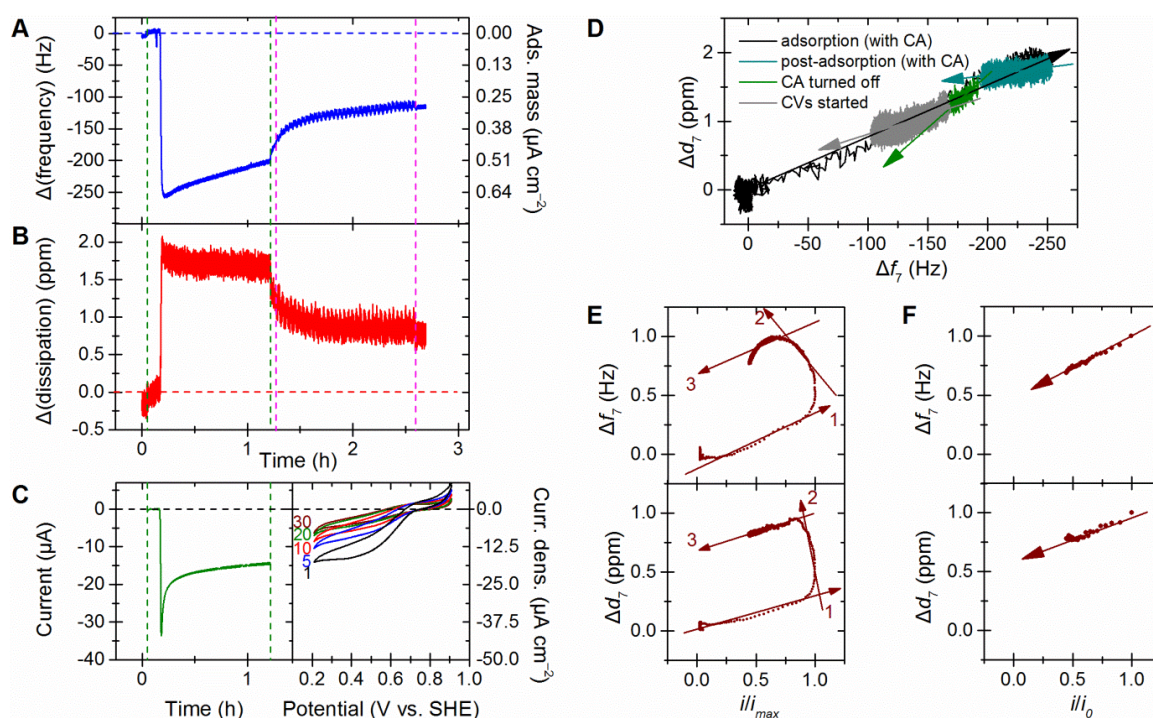


Figure 5.1 E-QCM-D response with time for *MvBOx* adsorbed on a 3MPA layer at pH 6.0 sodium phosphate buffer (pre-saturated with O_2). Panel A and B show the frequency and dissipation response, respectively. Panel C (left) shows the O_2 chronoamperometric reductive current. Panel C (right) shows potential cycling scans 1, 5, 10, 20 and 30 of 30 scans. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled from 0.91–0.21 V vs. SHE. Panel D shows the Δd vs. Δf plot with each regime highlighted and labelled. Panel E shows the frequency-activity (upper) and dissipation-activity (lower) plots during chronoamperometry. Panel F shows the frequency-activity plots (upper) and dissipation-activity plots (lower) during cyclic voltammetry. Conditions: 25 °C, 0.1 ml min^{-1} . Flow of O_2 at $90 \text{ cm}^3 \text{ min}^{-1}$, seventh harmonic shown.

The $\Delta d / \Delta f$ gradient during the adsorption phase (black trace, Figure 5.1D) has a value of $0.0076 \text{ ppm Hz}^{-1}$ and this decreases by over a factor of 3 to $0.002 \text{ ppm Hz}^{-1}$ for the post-

adsorption response (cyan trace), which relates to the period following $\Delta f^{\max}$ and $\Delta d^{\max}$ in panel A and B. When the potential was switched off, the magnitude of $\partial d/\partial f$ increased 6 fold to $0.012 \text{ ppm Hz}^{-1}$ (green trace), which again was similar across all measurements. When potential cycling began (grey trace), the shift in gradient again changed to $0.005 \text{ ppm Hz}^{-1}$ and the efficiency of adsorbed mass dropped to $0.16 \text{ } \mu\text{A Hz}^{-1}$, indicative of a slowly inactivating *MvBOx* layer. This was also seen as a reduction in activity in Figure 5.1C (right panel) with a 55% decrease in activity over the course of 30 CVs (1 h).

When holding the potential constant, the change in frequency as a function of activity is shown in Figure 5.1E (upper panel). A key observation was that the total mass ($\Delta f^{\max}$) was adsorbed was seen after the maximum current was observed (arrow 1). Figure 5.7E shows that the peak current, $\Delta i^{\max}$, was seen at approximately half of the maximum mass adsorption and half maximum dissipation change, suggesting that the enzyme begins to inactivate while the adsorption process is still ongoing. When the adsorbed layer reaches a maximum dissipation, there was already an approximate 10% loss in current ($\Delta d^{\max} \cong 0.90 \Delta i^{\max}$, arrow 1 and 2 Figure 5.7E lower panel) with an even greater loss in current at maximum adsorption ($\Delta f^{\max} \cong 0.62 \Delta i^{\max}$, arrow 1 and 2 Figure 5.7E upper panel). Figure 5.8F shows the frequency-activity (upper panel) and dissipation-activity (lower panel) plots during potential cycling. There is a decrease in both traces correlating to an overall 55% activity loss over 1 h.

Table 5.1. Table showing the measured parameters of *MvBOx* adsorbed to 3MPA-modified surfaces across pH 4-7 during constant potential control.

pH	$ f ^{\max}$ (Hz)	$ f ^{\infty}$ (Hz)	$d^{\max}$ (ppm)	d^{∞} (ppm)	$ i ^{\max}$ (μA)	$ i ^{\infty}$ (μA)	E_{cat} (V vs. SHE)
4	503	431	5.6	5.4	110	107	0.754
5	278	172	2.6	2.5	59	50	0.703
6	247	196	2.1	1.8	35	17	0.647
7	212	126	1.5	1.2	18	6	0.624

As the pH increased from 4 to 7, the values for the maximum frequency change ($\Delta f^{\max}$), maximum dissipation change ($\Delta d^{\max}$) and maximum activity ($\Delta i^{\max}$) at 0.21V vs. SHE all showed a decrease (Table 5.1).

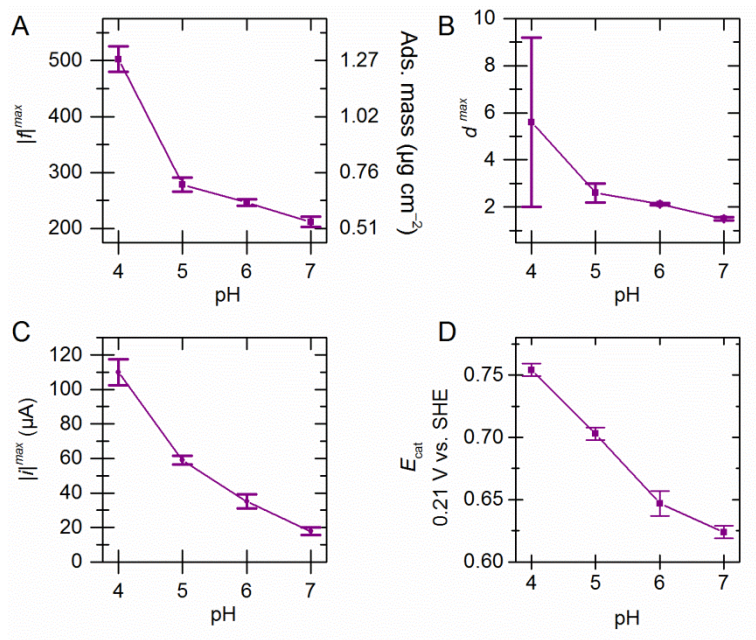


Figure 5.2 Plots of (A) maximum frequency change, (B) maximum dissipation change, (C) maximum activity and (D) change in the position of E_{cat} during MvBOx adsorption onto 3MPA-modified surfaces at varying pH.

Figure 5.3 shows a significant change in the typical response seen across the pH measurements. At pH 4 the enzyme adsorption was highest: -503 Hz ($1.27 \mu\text{g cm}^{-2}$) compared to the relative magnitudes of adsorption at pH 5 to 7 (Table 5.1). Unlike adsorption at higher pH, at pH 4 the increased mass adsorption was relatively stable throughout the experiment. This was in contrast to the typical mass loss seen, which occurred when turning the potential off. Although there was no significant mass loss during potential cycling, the enzyme activity decreased by over 80% during cycling (Figure 5.3C, right panel).

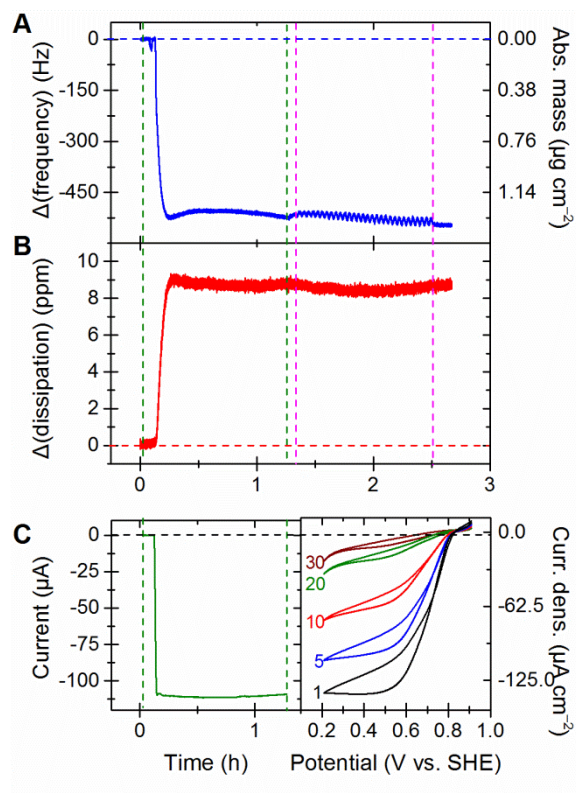


Figure 5.3. E-QCM-D response with time for MvBOx adsorbed on a 3MPA layer at pH 4.0. Panel A shows frequency and Panel B shows the dissipation response. Panel C (left) shows O_2 chronoamperometric reductive current during chronoamperometry. Panel C (right) shows CVs of scans 1, 5, 10, 20 and 30 of 30 scans. Green dashed lines show the application of 0.21 V vs. SHE. Magenta dashed lines show when potential cycling was carried out from 0.91-0.21 V vs SHE at 10 mV s⁻¹. Conditions: 0.1 M sodium citrate pH 4.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹. Flow of O_2 controlled at 90 cm³ min⁻¹, seventh harmonic shown.

Figure 5.4 shows the changes in Δd vs. Δf relationship at pH 4. Figure 5.4A shows the initial adsorption gradient of $\Delta d/\Delta f$ was 0.0009 ppm Hz⁻¹ (arrow 1). The gradient then increased to 0.02 ppm Hz⁻¹. The final part of adsorption saw a drop in the gradient to 0.004 ppm Hz⁻¹ (arrow 3). The frequency-activity plot in Figure 5.4B (upper panel) shows that the first signs of catalysis occurred while the mass adsorbed was negligible. The highest current was also seen while the dissipation response was negligible (Figure 5.4B upper panel).

The Δd vs. Δf plots at pH 5 to 7 showed only a two step change during adsorption, compared to the three step change in gradient in Figure 5.4A compared (summarised in Table 5.2) with pH 6 showing the least obvious change from a linear adsorption gradient.

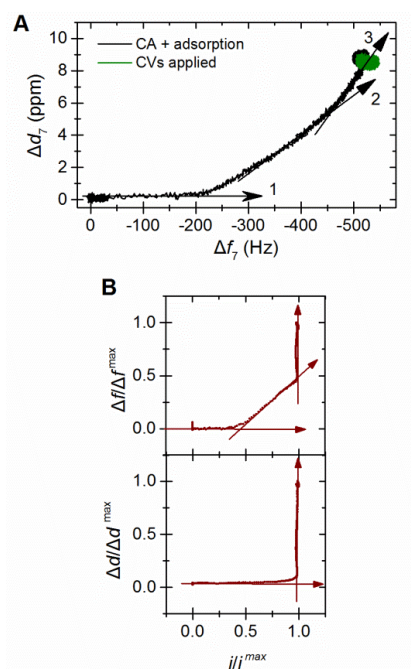


Figure 5.4 Panel A shows the Δd vs. Δf plot with the three changes in gradient indicated by arrows 1, 2 and 3. Panel B shows the frequency-activity (upper) and dissipation-activity (lower) plots during protein adsorption when holding the potential constant at 0.21 V vs. SHE.

Table 5.2 Changes in $\Delta d/\Delta f$ gradient during enzyme adsorption

pH	$\partial d/\partial f$ - step 1 (ppm Hz ⁻¹)	$\partial d/\partial f$ - step 2 (ppm Hz ⁻¹)	$\partial d/\partial f$ - step 3 (ppm Hz ⁻¹)
4	0.0009	0.02	0.04
5	0.005	0.01	n/a
6	0.006	0.008	n/a
7	0.003	0.01	n/a

Table 5.3 Table showing the time taken to reach 95% maximum frequency during *MvBOx* adsorption onto 3MPA–modified gold, with increasing pH.

pH	Time taken for 0.95 $\Delta f^{\max}$ (s)
4	756
5	662
6	698
7	415

Table 5.3 shows the rate of adsorption of *MvBOx* with increasing pH, using the time taken to achieve 95% of the maximum frequency, and suggesting that adsorption is faster as the pH increases.

5.2.1 Comparison of electrochemical measurements

To compare the electrochemical measurements across pH, the maximum current, and adsorbed mass efficiency loss during chronoamperometry, at each pH was considered. The current decay constant for the current loss during chronoamperometry was also considered. Table 5.1 highlights the electrochemical data for *MvBOx* adsorbed to 3MPA–modified gold and shows a pattern of decreasing catalytic activity with increasing pH. This is evident from the i^∞ values (current value immediately before potential is turned off) and suggests that *MvBOx* retains greater catalytic ability when adsorption is carried out at pH 4.

Table 5.4 shows the adsorbed mass efficiency ($\Delta i/\Delta f$) of the enzyme adlayer, immediately before constant potential control is turned off. Although there is a higher catalytic activity at the lowest pH (as shown in Table 5.1), the efficiency at pH 4 and pH 5 is the same before greatly reducing at pH 6 and further still at pH 7. However, the current decay time constant (τ) increased with increasing pH suggesting an increased stability of catalytic current at the expense of lower starting activity at higher pH.

Table 5.4 Table showing the current decay constants and adsorbed mass efficiency during constant potential control at 0.21 V vs. SHE from pH 4-7. The time constants at the beginning of the current decay deviate strongly from a single exponential in every case and as a result the decay has both fast and slow components. The time constants reported here are for the slow component.

pH	τ (h)	Efficiency ($\mu\text{A Hz}^{-1}$)
4	n/a	0.22
5	0.08	0.22
6	0.26	0.080
7	0.46	0.048

Figure 5.5 compares the voltammograms of 30 scans carried out on *MvBOx* on 3MPA over each pH, once a constant potential had been turned off. At pH 4.0, the waveshape resembles the sigmoidal shape characteristic of electrocatalysis and exhibits the highest activity. As the pH increases there is a reduction in the current at the lower vertex potential in

the first scan. Each set of voltammograms show a downward turn, or ‘tail’ at the low potential vertex (pH 4.0 scan 10 onwards, pH 5.0 scan 5 onwards, pH 6.0 scan 5 onwards) which becomes more pronounced over time, and at pH 7.0 the presence of this downward tail is present throughout the 30 scans. This could be a signature for a change in conformational arrangement and could also suggest that the enzyme has become distorted in a way that the copper coordination has changed thus dropping the potential, resulting in the tail shape. Interestingly, this change in arrangement appears to be both time-dependent and pH dependent.

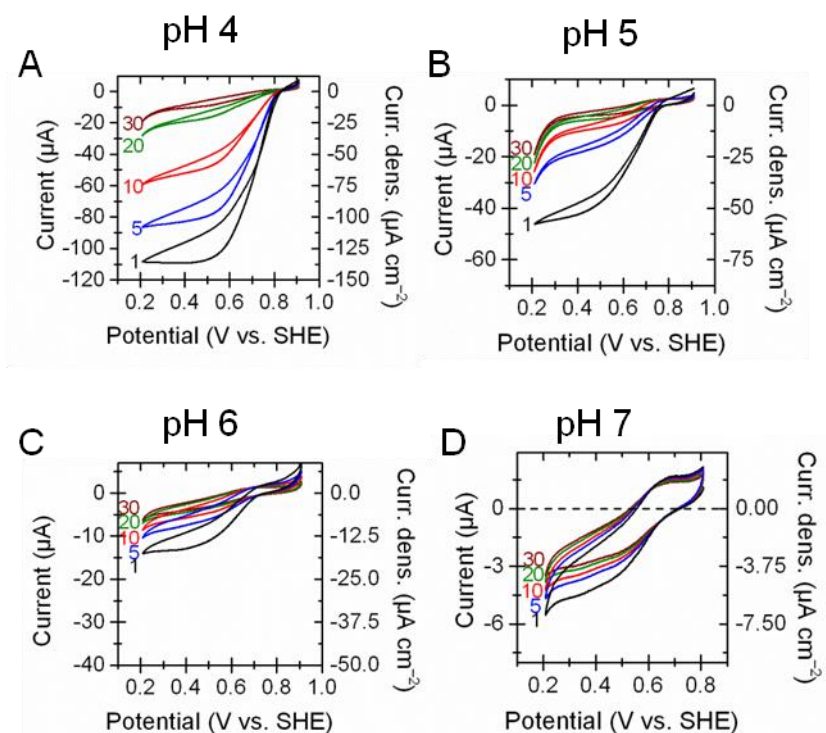


Figure 5.5 Potential cycling voltammograms for MvBOx on 3MPA-modified gold at 0.1M sodium citrate buffer pH 4.0 (A), 0.1 M sodium phosphate buffer pH 5.0 (B), 0.1 M sodium phosphate buffer pH 6.0 (C) and 0.1 M sodium phosphate buffer pH 7 (D). Condition: 0.91-0.21 V vs SHE at 10 mV s⁻¹, buffer pre-saturated with O₂, 25 °C.

Table 5.5 further indicates how there is a greater retention of catalytic activity at higher pH, with both the lowest activity loss and lowest efficiency loss observed at pH 7. These observations suggest that at high pH, under potential cycling, an adlayer with low initial

activity has a higher stability in both efficiency and activity. Interestingly however, looking at Table 5.1, the reason for the lack of activity at higher pH is down to a change in the position of E_{cat} and therefore an adlayer exhibits stable activity at the cost of a greater thermodynamic push needed to initiate catalysis, when the pH is increased.

Table 5.5. Activity loss and adsorbed mass efficiency loss during potential cycling over 30 scans

pH	Activity loss	Efficiency loss ($\mu\text{A Hz}^{-1}$)
4	82 %	0.17
5	58 %	0.07
6	55 %	0.03
7	36 %	0.009

5.2.2 Reductive desorption of thiol modifications

Reductive desorption of thiol modifications was used as proof of modification, and was also used to show that the thiol was still present on the gold surface after a typical E-QCM-D measurement (Figure 5.6).

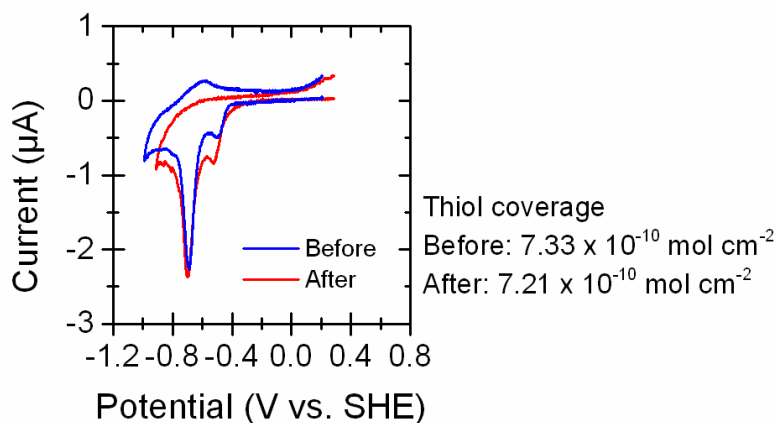


Figure 5.6. Reductive desorption of 3-mercaptopropionic acid on gold. Blue trace indicates the reductive desorption peak of 3MPA (-0.701 V vs. SHE) immediately after overnight modification. Red trace indicates the reductive desorption peak (-0.710 V vs. SHE) after the end of an adsorption experiment, in this case, the experiment carried out for Figure 5.1. The thiol surface coverage before and after enzyme adsorption is given.

The surface coverage of thiol was in the same magnitude before and after protein adsorption. However, the potential for the reductive desorption of the thiol, after protein adsorption, was shifted to a more negative potential (Figure 5.6) than the reductive desorption

immediately after modification. One possible reason for this is that, because of residual enzyme/protein on the surface, a greater thermodynamic push is needed to initiate the one electron reduction of thiol from the surface.

5.2.3 Discussion of pH effects

The atypical results seen at pH 4 suggest that electrostatic interactions affect protein adsorption. As a result of the pH being lower than both the pKa of 3MPA which has been reported at 5.2¹ and the isoelectric point (pI) of MvBOx (4.2)², there is a net positive charge on the enzyme suggesting a favourable interaction with the net negatively charged carboxylic terminated surface. Adsorption rates are high when protein and substrate carry opposite charges which are a direct result of the electrostatic attractions accelerating the migration of the protein towards the surface. However, the total mass load on adsorption is generally maximized at (or near) the isoelectric point,³⁻⁵ and this is consistent with the observations in the above results, that show the amount of adsorbed enzyme decreased with increasing pH. This also suggests that electrostatic protein-protein repulsions need to be minimized near the pI, therefore allowing a higher packing density on the surface.⁶ DLS measurements suggest the aggregation of MvBOx at pH 4 and that there could be a cluster-like adsorption process which occurs. However due to the greater loss in efficiency during potential cycling, at pH 4.0 this cluster-like adsorption may need further stabilisation. This is important in the design of fuel cell environments as there should be an optimum adsorption-activity ratio where the environmental conditions encourage a favourable interaction with the surface of choice, whilst also allowing the enzyme to operate with high efficiency and catalytic activity.

Charge screening methods could also be used to understand the effect of electrostatic interactions on enzyme adsorption. Jensen *et al.* highlight changes in the adsorption of enzyme and resulting enzyme activity on flat and nanostructured gold electrodes.

Conformational changes were found to be dependent on surface topology.⁷ In a similar fashion introducing structural scaffolds which help to fix the enzyme conformation would be an attractive route to follow.

The decrease in the dissipation response, with increasing pH, suggests that the structure of the enzyme experiences a higher degree of stiffening. The suggestion is that as the repulsion effect between enzyme and surface (due to the increased ionisation of the carboxyl terminal groups) increases, the enzyme layer exhibits a lower dissipative change and hence a lower conformational flexibility: the protein adsorbs more rigidly. This increased rigidification purports to a breakdown in secondary structure (see Chapter 4, Section 4.2.5). As pH increases a protein denatures faster, and this denaturation means that the amount of hydrophobic interactions in the protein interior will decrease as the protein unfolds, resulting in a substantial increase in the conformational entropy during adsorption.⁸ Therefore a more qualitative look at the rate of adsorption (time taken to reach $0.95\Delta f^{\max}$, Table 5.3) indicates that adsorption is generally faster at higher pH and this fits with the above assumption that, when the secondary structure of the protein breaks down more readily, there is a greater driving force for adsorption and therefore adsorption proceeds faster. This is an important factor when setting conditions for fuel cell enzyme catalysis as pH will affect conformational arrangement as well as affecting catalytic competency.

An alternative approach to looking at the effect of pH would be to study the secondary structure of the protein using circular dichroism (CD). In this technique, different structural elements, such as amide bonds in the peptide backbone, have characteristic CD spectra due to each element causing changes in the degree of absorption of left-handed and right-handed plane polarised light. As the CD spectra of proteins are so dependent on their structural elements i.e. their conformation, CD can be used to monitor conformational changes that occur during changes in binding interactions on surfaces.^{9,10} This would be useful when

looking at changes in the structural/molecular environment of the protein at different pH, and then relating these back to dissipation, mass, and catalytic current measurements that are taken using the E-QCM-D.

The overall amount of enzyme adsorption decreased with increasing pH, and this could be due to the repulsive effect between the net negative charge of *MvBOx* (at $\text{pH} > \text{pI}$) and the increasing net negative charge of the surface 3MPA groups (at $\text{pH} > \text{pKa}$). Proteins adsorbing at positively or negatively charged interfaces generally tend to expose oppositely charged patches to the surface which drives adsorption. Therefore, although the net charges should in theory repel each other, in some cases, proteins carrying net charges can adsorb to surfaces with similar charge,¹¹ such is the case in the above observations..

An important observation, throughout these measurements was that there was also an approximate 15-20 s time lag between the first sign of catalytic current and the first signs of both mass adsorption and dissipation changes. Due to this lag, the process of enzyme inactivation will most likely be occurring on a similar time scale to enzyme adsorption, and therefore, activity loss begins before a maximum frequency is reached.

The decrease in activity was expected as supported by a study by dos Santos *et al.* where they report a decrease in catalytic activity with increasing pH,¹². However in combination with the lower activity there is also a shift in the position of E_{cat} in the catalytic voltammograms and therefore the higher pH may also be causing a change in the orientation of the enzyme or at least changing the electron transfer process. Interestingly, the adsorbed mass efficiency (during chronoamperometry) remains stable from pH 4 to pH 5 before falling by more than 50% when changing from pH 5 to pH 6 and suggests catalytic efficiency is highest at low pH. Conversely, during potential cycling, there is a greater loss in efficiency at lower pH which suggests that efficiency of the adsorbed adlayer is sensitive to the type of potential control being applied. Increasing the pH also resulted in both an increase in the

current decay time constants and a decrease in the activity loss during potential cycling. This suggests the need for fine-tuning the pH conditions to balance the need for a high starting current/higher catalytic efficiency and greater stability in catalytic current over time.

5.3 Chain length dependence on catalytic activity

To further investigate the effect of distance on the electrocatalytic activity of adsorbed enzyme, this section describes the results from E-QCM-D experiments where *MvBOx* was adsorbed to gold-coated QCM crystals modified with carboxylic acid terminated thiol linkers with varying chain length (C_n -COOH where $n = 2, 3, 5, 7$ and 10) at pH 6. The thiol linkers used to modify the gold surfaces were 4-mercaptobutyric acid (4MBA), 6-mercaptohexanoic acid (6MHA), 8-mercaptooctanoic acid (8MOA) and 11-mercaptoundecanoic acid (11MUA), with a comparison to *MvBOx* on 3MPA from the previous section (Figure 5.7).

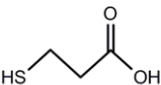
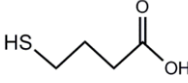
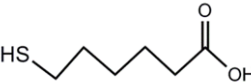
Abbreviation	Structure	Length
3MPA		5.5 Å
4MBA		8.6 Å
6MHA		11.6 Å
8MOA		13.6 Å
11MUA		19.1 Å

Figure 5.7. Diagram showing the structures of carboxyl terminated thiol linkers with varying chain lengths. Lengths given from sulphur to furthest hydrogen atom, using Chem3DPro 12.0.

5.3.1 Chain length measurements

In the first measurement, the adsorption of *MvBOx* to 4-mercaptobutyric acid (4MBA) modified gold is shown in Figure 5.8 and is used as the typical measurement. In this measurement, *MvBOx* was introduced to a 4MBA-modified gold surface while a constant

potential of 0.21 V vs. SHE was applied on the electrode surface. After ca. 1.25 h this constant potential was turned off and left for around 10 min, before potential cycling was then applied over the range 0.81–0.21 V vs. SHE for 30 scans.

Figure 5.8 shows a maximum mass adsorption of -315 Hz ($0.8 \mu\text{g cm}^{-2}$) and maximum dissipation change (Δd^{max}) of 4.23. The maximum current response gave a value of $-46.06 \mu\text{A}$ (i^{max}) which was higher than the adsorption of *MvBOx* onto 3MPA example in chapter 4. When the potential was turned off there was a similar mass loss process to that seen in the previous section, although this was not mirrored in the dissipation trace (Figure 5.8B).

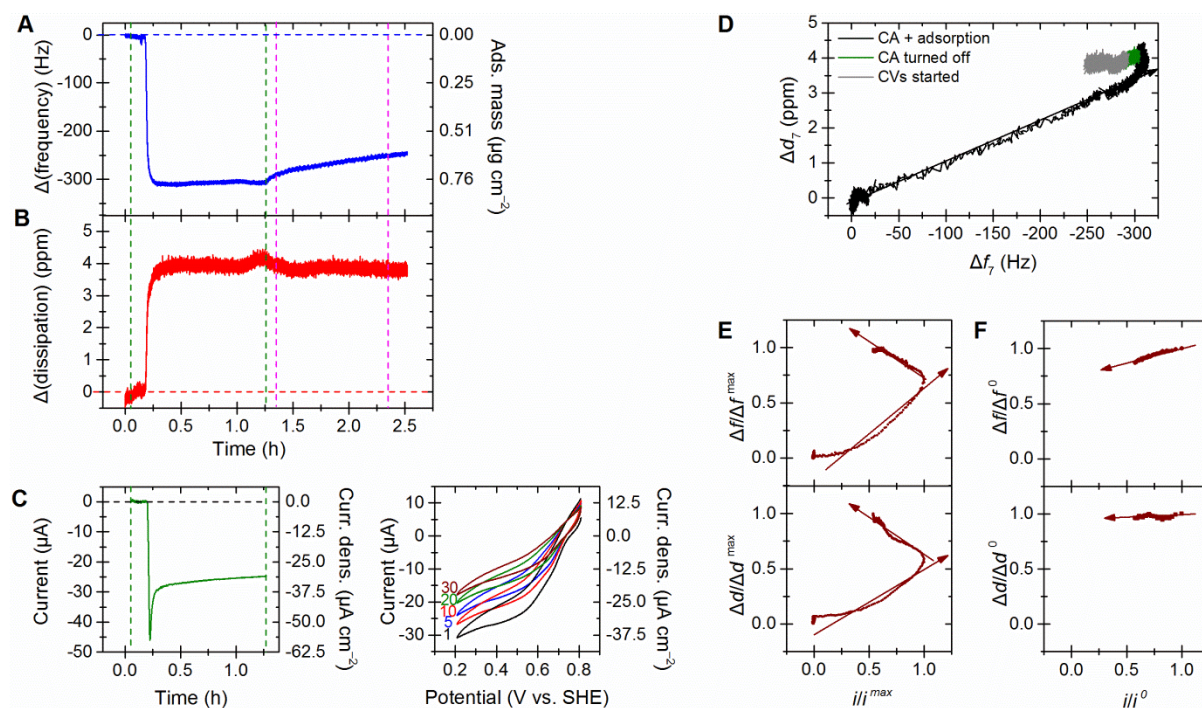


Figure 5.8 E-QCM-D response with time for *MvBOx* adsorbed on a 4MBA layer. Panel A and B shows the frequency and dissipation response, respectively. Panel C (left) shows the O_2 chronoamperometric reductive current at 0.21 V vs. SHE. Panel C (right) shows the CV scans 1, 5, 10, 20 and 30 of 30 scans. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled from 0.81–0.21 V vs. SHE. Panel D shows the Δd vs. Δf plot with each regime highlighted. Panel E shows the frequency-activity (upper) and dissipation-activity (lower) plots for chronoamperometry. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^\circ\text{C}$, 0.1 ml min^{-1} . Flow of O_2 at 90 $\text{cm}^3 \text{min}^{-1}$, seventh harmonic shown.

In Figure 5.8, during the application of potential, there was a decrease in the adsorbed mass efficiency between $i^{\max}$ ($\Delta i/\Delta f = 0.21 \mu\text{A Hz}^{-1}$) and i^{∞} ($\Delta i/\Delta f = 0.08 \mu\text{A Hz}^{-1}$) suggesting a fall in catalytic competency (Figure 5.8C).

The $\Delta d/\Delta f$ plot showed a more linear adsorption profile (black trace, Figure 5.8D) with a gradient of $0.013 \text{ ppm Hz}^{-1}$ suggesting a less rigid adsorption compared to the adsorption profile of 3MPA ($0.006 \text{ ppm Hz}^{-1}$).

Figure 5.8E indicates a similar phenomenon described in Section 5.2 where catalytic current is seen before enzyme adsorption. This lag decreased as the carbon chain increased to give a more linear correlation between frequency change and current (see Appendix).

As the chain length was increased the general trend was that mass adsorption ($\Delta f^{\max}$) decreased, the rigidity of the enzyme layer increased (decreasing $\Delta d^{\max}$) and the maximum current, $i^{\max}$, also decreased (Table 5.6).

Table 5.6 Table showing the frequency, dissipation and electrochemical parameters measured by the E-QCM-D for MvBOx adsorption onto thiol-modified gold surfaces with different carbon chain lengths. Data given to 3sf.

Carbon chain (n)	$ f ^{\max}$ (Hz)	$ f ^{\infty}$ (Hz)	$d^{\max}$ (ppm)	$ d ^{\infty}$ (ppm)	$ i ^{\max}$ (μA)	$ i ^{\infty}$ (μA)
3	247	196	2.13	1.84	35.0	16.5
4	315	308	4.65	4.34	46.0	24.7
6	155	145	1.75	1.69	22.8	16.5
8	249	249	5.97	5.19	21.6	9.43
11	82.2	55.3	1.13	0.88	3.11	1.10

In the previous section, when chronoamperometry was turned off, there was an associated mass loss and fall in dissipation. Figure 5.7 shows that as the carbon chain length increases, this magnitude of mass loss associated with turning the constant potential off, decreases. The shortest thiol chain shows a frequency increase of 82 Hz after chronoamperometry was turned off and this decreased to 59 Hz on 4MBA. The amount of mass loss as a direct result

of turning the potential off was negligible for the longer chain thiols (Table 5.7). This is seen more clearly in the frequency traces in Figure 5.9.

Table 5.7 The relationship between turning the constant potential off and the resulting mass loss of *MvBOx* on 3MPA and 4MBA surfaces. The effect of potential cycling did not affect the rate of mass loss.

Carbon chain (<i>n</i>)	Frequency increased after CA turned off (Hz)	Max mass loss after CA turned off ($\mu\text{g cm}^{-2}$)
3	82.2	0.21
4	59.4	0.15

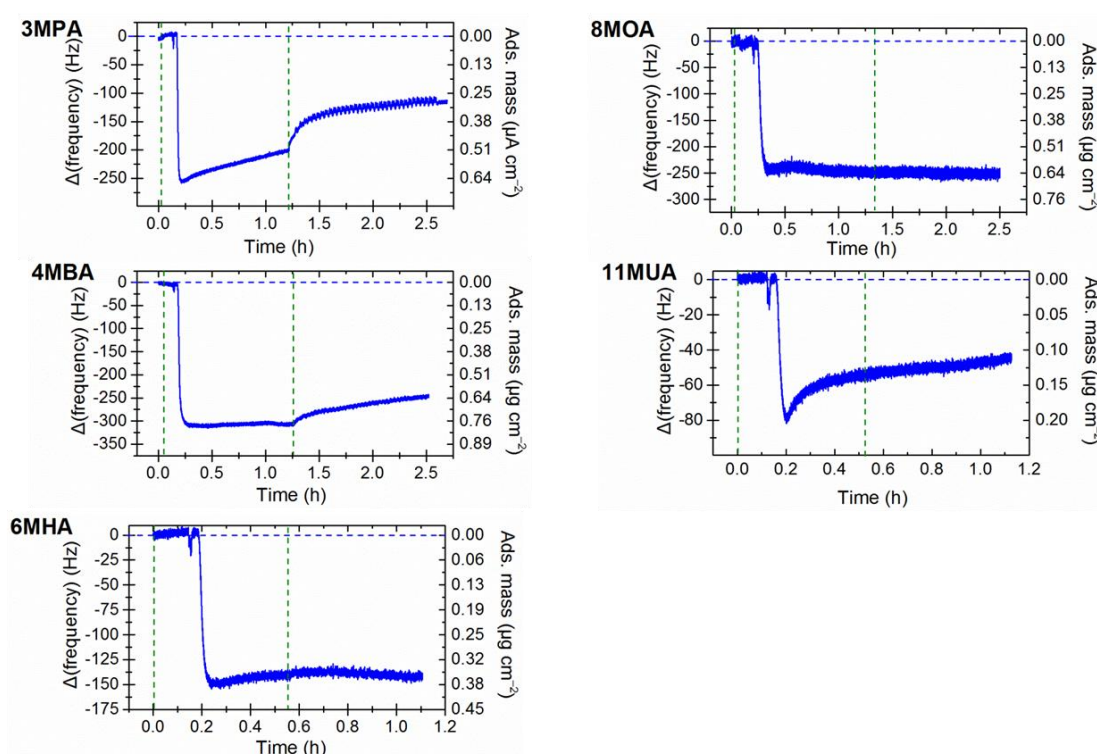


Figure 5.9 Frequency responses from the adsorption of *MvBOx* onto gold-coated quartz crystals modified with 3-mercaptopropionic acid (3MPA), 4-mercaptopbutyric acid (4MBA), 6-mercaptophexanoic acid (6MHA), 8-mercaptopoctanoic acid (8ODA) and 11-mercaptopundecanoic acid (11MUA). Green dashed lines indicate the time when a potential of 0.21 V vs. SHE was applied and turned off

The gradients of $\Delta d/\Delta f$ show an increase in magnitude as chain length increases indicating that the adsorbing layer is softer as the distance from the surface increases.

Table 5.8 Gradients of $\Delta d/\Delta f$ across all carbon chain lengths during the adsorption process. This refers to the black trace in Panel D in all figures for varying chain length in appendix)

Carbon chain (<i>n</i>)	$\Delta d/\Delta f$ (ppm Hz ⁻¹)
3	0.006
4	0.010
6	0.011
8	0.016
11	0.013

5.3.2 Comparison of electrochemical measurements

5.3.2.1 During chronoamperometry

The shorter chains showed the highest catalytic activity (Table 5.6), however as these values are based on a peak current, the time constants for activity decay and the loss in adsorbed mass efficiency gives a better comparison. During chronoamperometry, the 4MBA surface showed the highest current decay time constant, however this was at the expense of having a greater loss in efficiency of adsorbed mass. The 3MPA surface showed a current decay time constant 4 times lower than 4MBA and with a greater loss in efficiency (Table 5.9 & Figure 5.10).

Table 5.9. The effect of carbon chain length on current decay time constants, activity loss, and adsorbed mass efficiency, during chronoamperometry. The time constants at the beginning of the current decay deviate strongly from a single exponential in every case and as a result the decay has both fast and slow components. The time constants reported are for the slow time decay.

Carbon chain (<i>n</i>)	τ (h)	Normalised efficiency loss ($\mu\text{A Hz}^{-1} \text{h}^{-1}$)	Highest peak current, $ i ^{\text{max}}$ (μA)
3	0.261	0.19	35.0
4	0.662	0.13	46.0
6	0.125	0.17	22.8
8	0.332	0.09	21.6
11	0.137	0.07	3.11

In comparison to the shorter chain lengths, 6MHA showed the lowest loss in efficiency but also exhibited the lowest current decay time constant. The longer chain thiols showed had greater retention of efficiency however this stability was offset by lower starting currents.

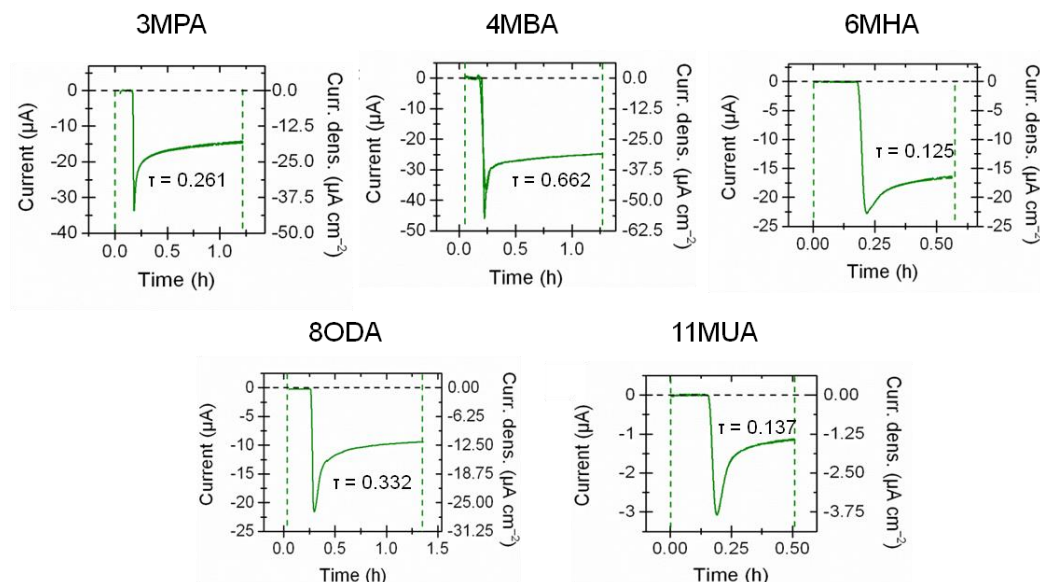


Figure 5.10 Comparison of the current generated while holding a constant potential at 0.21 V vs. SHE on thiol-modified surfaces. Current decay time constants are indicated on each trace.

5.3.2.2 During potential cycling

During potential cycling the 4MBA surface showed the highest E_{cat} , however this surface also displayed the highest activity loss which was a great deal higher than the stable current seen on the 6MHA surface. As the carbon chain length increased there was a greater stability in the activity compared to the shortened chain thiols however, this was again at the cost of smaller currents, and there was also a negative shift in the potential of E_{cat} meaning a greater thermodynamic push was needed for catalysis (Table 5.10), indicative of the increasing electron transfer distances with increasing thiol chain length.

The slope for the change in efficiency with time ($(\Delta i/\Delta t)/i$) is highest for the shorter chain thiols, however there is a greater retention in efficiency i.e. a smaller slope for carbon chain length ≥ 6 . The higher efficiency on the 8ODA and 11MUA surfaces is at the cost of lower

starting activity and a lower E_{cat} . The 6MHA surface has the highest starting efficiency (taken at the lower potential vertex for the first scan) and the combination of starting activity, position of E_{cat} , and efficiency appears to be most finely balanced using the 6MHA surface.

Table 5.10 Normalised activity loss and potential of E_{cat} with varying carbon chain length of thiol modifier.

Carbon chain (n)	Normalised activity loss ($\mu\text{A h}^{-1}$)	E_{cat} (V vs. SHE)	Starting efficiency	Slope of $\Delta\text{efficiency}$
3	6.9	0.647	0.08613	-0.02409
4	13	0.684	0.10777	-0.03316
6	1.4	0.639	0.12056	-0.01938
8	2	0.559	0.04865	-0.00669
11	0.50	0.359	0.00429	-0.000183

5.3.3 Discussion of chain length effects

On increasing the length of the carbon chain in the thiol modifier, the layer between the electrode surface and enzyme becomes thicker, resulting in a decrease in adsorbed mass. However, a benefit of increasing the chain length is that the adsorbed layer shows a lower sensitivity to the applications of an applied potential, or in this case when an applied potential is turned off, as shown in Figure 5.9.

Another important observation was that there was an increasing $\Delta d/\Delta f$ gradient as chain length increased. In alignment with the decreasing Δd^{max} with increasing chain length, this suggests that the adsorbed adlayer undergoes stiffening at an increasing rate when the distance from the surface increases. This is an interesting route for further experimental work, to test for conformational changes (different magnitudes of $\partial d/\partial f$) that different functional groups may induce, and relating this back to electrocatalytic competency.

The electrocatalytic activity of *MvBOx* decreases as the chain length increases. Tominaga *et al.* carried out a similar study on the dependence on carbon chain length of thiol-modified

surfaces on MvBOx adsorption and reported a pattern similar to the measurements above. Although in contrast, they reported a value of $\sim 20 \text{ nA cm}^{-2}$ current density on an 11MUA surface compared to a higher value of $1.88 \mu\text{A cm}^{-2}$ (using a surface area of 0.8 cm^{-2}).

The mechanical rigidity is greatest on the 11MUA layer with the smallest overall dissipation change during the measurement ($\Delta d = 1.1$). However the enzyme, although adsorbing more rigidly, must maintain a degree of structural integrity for electron transfer to occur and therefore may undergo structural reorganisation or orientation change. This idea of a structural reorganisation is consistent with the findings by Tominaga *et al.* where they suggest that a more ordered molecular conformation of the enzyme is needed to permit electron transfer over longer distances.¹³ These changes in mechanics of the enzyme adlayer plays into the efficiency of the enzyme, where the increased distance between enzyme and surface, presumably requires a greater mechanical change to facilitate electron transfer. This change is reflected in the lower starting currents, negative shift in the E_{cat} , and reduced starting efficiency during potential cycling, all indicative of a changing orientation and in addition, an inactivating adlayer. However, it appears difficult to distinguish between this rearrangement and a breakdown of secondary structure using just frequency and dissipation measurements.

In the previous section, it was seen that turning off the potential caused a disturbance to the frequency and resulted in the net loss of mass from the surface. As the carbon chain length was increased, this effect was less evident, suggesting that the increased thickness of the modified layer protects the enzyme from the field effect of the electrode, near the surface of the crystal.

Smaller chain thiols tend to give higher currents and increasing time constants which is attractive for use in fuel cell applications, however, due to the enzyme being positioned closer to the electrode surface these layers are more unstable towards potential cycling and exhibit

higher activity loss. Catalytic efficiency was highest on the 6MHA surface and showed the greatest stability in efficiency. Tailoring a surface for enzyme adsorption involves balancing the need for placing the enzyme closer to the surface for improved electron transfer efficiency but far enough away to be affected by the electric field, and also provide catalytic efficiency which is stable over time.

5.4 Surface chemistry dependence on catalytic activity

In this section, the effect of changing the surface chemistry groups is discussed. *MvBOx* was adsorbed to gold-coated QCM crystals modified with thiol molecules and terminated with hydrophobic functional groups: benzenethiol and 4-mercaptoacetanilide (4MPAA), and hydrophilic functional groups: 4-mercaptobenzoic acid (4MBZ) and 4-mercaptophenol (4MPOH) (Figure 5.11). The reason for carrying out these measurements was to see the effect of changing surface chemistry on frequency, dissipation and activity responses and understand how hydrophobic/hydrophilic surfaces affect enzyme catalytic competency.

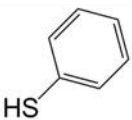
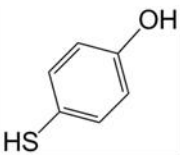
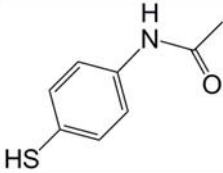
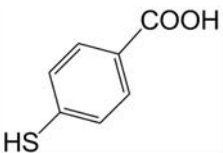
Abbreviation	Structure	Length
Benzenethiol		5.7Å
4MPOH		7.0Å
4MPAA		9.9Å
4MBZ		8.2Å

Figure 5.11 Structures of thiol modifiers with varying functional group chemistry with abbreviation and length. (Lengths are given from sulphur to furthest hydrogen atom, using Chem3DPro 12.0)

5.4.1 Surface chemistry measurements

The adsorption of *MvBOx* to benzenethiol is shown in Figure 5.12 and is used as the measurement against which surface chemistry measurements are compared. In this measurement, *MvBOx* was introduced to a benzenethiol-modified gold surface while a constant potential of 0.21 V vs. SHE was applied on the electrode surface. This potential was then switched off before potential cycling was applied over the range 0.91–0.31 V vs. SHE for 15 scans.

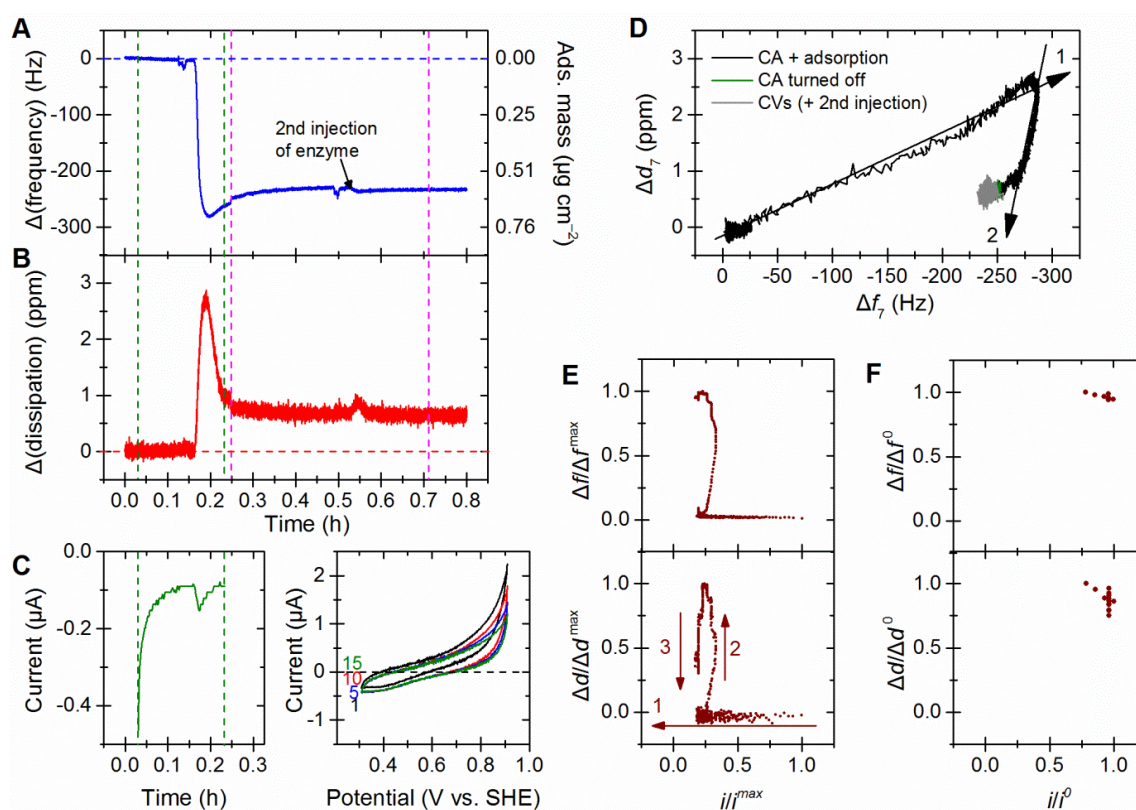


Figure 5.12. E-QCM-D response with time for *MvBOx* adsorbed non-covalently on a benzenethiol layer at pH 6.0 phosphate buffer. Panel A and B show the frequency and dissipation responses, respectively. Panel C (left) shows the O_2 chronoamperometric reduction current when a potential of 0.21 V vs. SHE. Panel C (right) shows scans 1, 5, 10 and 15 of 15 scans. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show then the potential was cycled from 0.91–0.31 V vs. SHE. 2nd injection of enzyme is highlighted in A (arrow) showing a mass increase of $\sim 112 \text{ ng cm}^{-2}$. Panel D shows the Δd vs. Δf relationship. Panel E shows the frequency-activity (upper panel) and dissipation-activity (lower panel) plots for chronoamperometry. Panel F shows the frequency-activity (upper panel) and dissipation-activity (lower panel) plots for cyclic voltammetry. Conditions: 25 °C, 0.1 ml min^{-1} , Flow of O_2 at $90 \text{ cm}^3 \text{ min}^{-1}$, seventh harmonic shown.

In Figure 5.11, the measurement of *MvBOx* adsorbed to a benzenethiol-modified surface gave a maximum frequency change ($\Delta f^{\max}$) of -282 Hz ($0.72\text{ }\mu\text{g cm}^{-2}$) and a maximum ‘peak’ dissipation ($\Delta d^{\max}$) of 2.78 suggesting both a dense and softer layer adsorbing to the surface. However the maximum current change ($\Delta i^{\max}$) during adsorption was $-0.05\text{ }\mu\text{A}$ (Figure 5.11C, left panel) highlighting a reduced catalytic activity with a current decay of 0.015 h. Although Figure 5.12C (right panel) shows the slight presence of a catalytic wave, the general lack of catalysis renders panels E and F essentially meaningless. The $\Delta d/\Delta f$ gradient during adsorption in Figure 5.12D is 0.007 ppm Hz^{-1} , which is similar to the gradient seen in the example of *MvBOx* on 3MPA in the pH measurements. This suggests that, although the mechanical change per unit mass during adsorption is similar, i.e., the adsorption mechanism is similar, the benzene functionality is in some way responsible for the reduced activity. The post adsorption gradient (arrow 2, Figure 5.12D) is 0.08 ppm Hz^{-1} which is a factor of 10 higher than the post-adsorption gradient in the 3MPA example. A second addition of enzyme was introduced at 0.54 h (Figure 5.12A) and although this showed a permanent mass increase ($\Delta f = -8\text{ Hz}$, $0.02\text{ }\mu\text{g cm}^{-2}$), the dissipation ‘peaked’ before returning back to its pre-addition response.

The highest amount of adsorbed enzyme was seen for 4MPAA with the lowest adsorption on the hydrophilic 4MBZ surface. Both hydrophobic surfaces show high increases in dissipation however the post-adsorption fall in dissipation ($d^{\max}$ to d^{∞}) is higher for benzenethiol than for 4MPAA suggesting a greater stiffening process on the hydrophobic surfaces. The 4MPAA surface also shows catalytic current although still comparatively low ($\Delta i^{\max} = -8\text{ }\mu\text{A}$ at 0.21V vs. SHE) and with a time constant of 0.33 h (Table 5.11).

Table 5.11 Table showing the frequency, dissipation and electrochemical parameters measured during *MvBOx* adsorption onto different surface modifications

Surface	$ f ^{\max}$ (Hz)	$ f ^{\infty}$ (Hz)	$d^{\max}$ (ppm)	d^{∞} (ppm)	$ i ^{\max}$ (μA)	$ i ^{\infty}$ (μA)
benzenethiol	282	235	2.78	0.53	0.05	n/a
4MPAA	299	264	3.04	2.79	8	5.2
4MPOH	232	173	1.76	1.31	28.3	7.1
4MBZ	142	127	1.58	1.46	21.2	15

5.4.2 Comparison of electrochemical measurements

5.4.2.1 During chronoamperometry

4MPOH has the highest current decay time constant partnered with the highest peak activity (Figure 5.13 & Table 5.12) suggesting a favourable interaction with *MvBOx*.

Table 5.12 Table showing the current decay time constants, normalised efficiency loss and highest peak current for benzenethiol, 4-mercaptoacetanilide (4MPAA), 4-mercaptophenol (4MPOH) and 4-mercaptobenzoic acid (4MBZ). There was 100% loss in activity on the benzenethiol layer and therefore efficiency loss was also 100%.

Surface	τ (h)	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($(\Delta\text{eff} / \Delta\text{eff}^{\max}) \text{h}^{-1}$)	Highest peak current, $ i ^{\max}$ (μA)
Benzenethiol	0.015	-	-	0.05
4MPAA	0.33	0.03	0.33	8
4MPOH	0.53	0.23	0.83	28.3
4MBZ	0.17	0.22	0.91	21.2

However the 4MPOH and the 4MBZ surfaces also showed similarly high efficiency loss (Table 5.12) suggesting that, although the hydroxyl and carboxyl units contribute to a high *MvBOx* peak activity, the enzyme itself loses efficiency more readily on these surfaces. Interestingly, carrying out chronoamperometry at a lower potential than 0.21 V would reveal more information on the current decay time constants and would give a true account of the current decay while a constant potential is held.

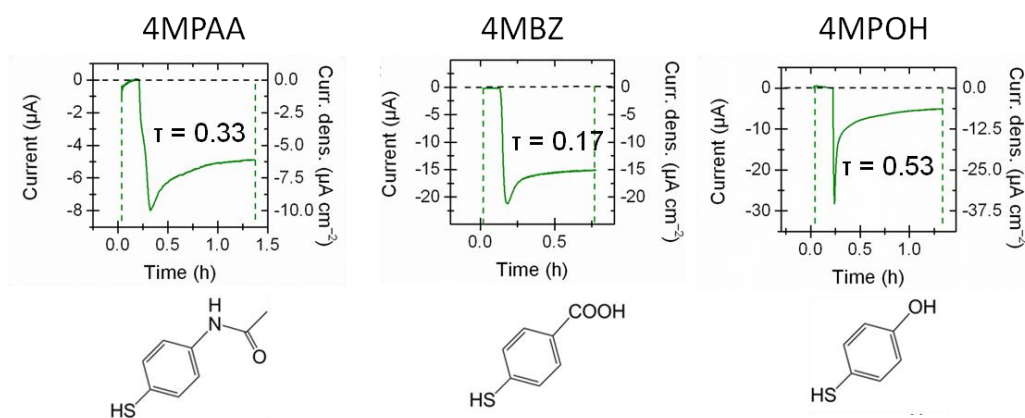


Figure 5.13 Comparison of the current traces for *MvBox* adsorption to 4MPAA (A), 4MBZ (B) and 4MPOH (C) modified gold surfaces. Current decay time constants are highlighted

5.4.2.2 During potential cycling

The waveshape for catalysis also changed across all surfaces. On the benzenethiol surface there is reduced catalytic activity, with only a small presence of a catalytic wave. In the voltammograms for 4MPOH and 4MPAA, a more linear, ohmic response is observed indicating a change in the electron transfer through the enzyme, compared to the more typical sigmoidal response in the 4MBZ voltammogram (Figure 5.14).

The lack of catalysis in the benzenethiol voltammograms suggests there is some enzyme inactivation occurring. The voltammograms for the 4MPAA and 4MPOH surfaces indicated a greater thermodynamic push needed for catalysis, due to the more negative E_{cat} (Table 5.13).

However, an interesting observation was seen during potential cycling. The general pattern was that of a decrease (i.e., negative shift) in the position of E_{cat} , going from the hydrophilic 4MBZ layer, down to the more hydrophobic 4MPAA layer, with negligible catalysis on the benzenethiol layer (Table 5.13). This was an important observation as it further strengthens the idea that the acetamido group of 4MPAA results in poor electron transfer such that a higher driving force for catalysis is needed. The oscillatory changes due to potential cycling were also more pronounced with peak to peak amplitude of ~ 30 Hz suggesting significant distortion of the enzyme (see 4MPAA in Appendix).

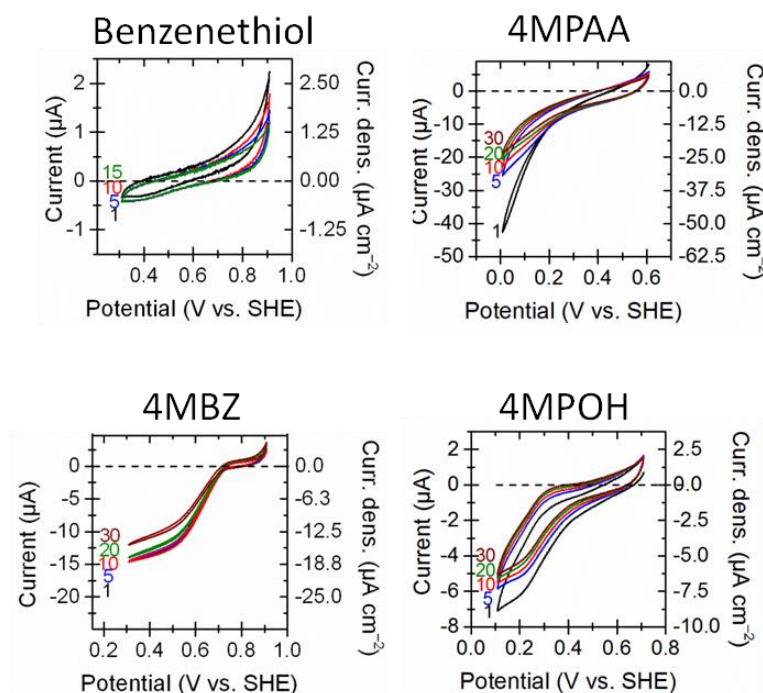


Figure 5.14 Comparison of the cyclic voltammograms generated when *MvBOx* was adsorbed on thiol-modified surfaces. Benzenethiol potential range: 0.91–0.31 V vs. SHE, 4MPAA potential range: 0.61–0.01 V vs. SHE, 4MBZ potential range: 0.91–0.31 V vs. SHE, 4MPOH potential range: 0.71–0.11 V vs. SHE.

Table 5.13. The effect of the different surface functionality on the position of E_{cat} .

Surface	E_{cat} (V vs. SHE)
benzenethiol	n/a
4MPAA	0.21
4MPOH	0.31
4MPZ	0.67

Table 5.14 Table showing the starting efficiency, normalised efficiency loss and relative current loss for *MvBOx* on each surface modifier

Surface	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($(\Delta\text{eff} / \Delta\text{eff}^{\text{max}}) \text{h}^{-1}$)	Relative activity loss
Benzenethiol	-	-	-
4MPAA	0.163	0.53	56%
4MPOH	0.0103	0.44	29%
4MBZ	0.108	0.10	20%

During potential cycling the starting adsorbed mass efficiency is highest for the 4PAA surface however this surface also exhibits the highest efficiency loss over time and has the highest relative activity loss (Table 5.14). The 4MBZ surface has a starting efficiency a third lower than 4MPAA, however it retains efficiency more readily, suggesting the carboxyl layer gives the *MvBOx* adlayer more stability in terms of catalytic competency.

5.4.3 Discussion of surface chemistry effects

In the most qualitative picture of adsorption, if there is a domain on the enzyme surface that is hydrophobic and the adsorbate surface is also hydrophobic, once adsorption begins conformation changes will cause it to unravel and expose its hydrophobic core to the surface. This results in a reduction of ordered structure due to adsorption induced conformation changes.¹⁴ Therefore it can be supposed that the benzenethiol surface causes rapid inactivation of the adsorbed enzyme, and that there could also be a breakdown in secondary structure or denaturation. After the second addition of enzyme, the lack of change in the voltammograms partnered with the permanent increase in the mass adsorbed, further suggests that any enzyme that adsorbs on the surface immediately inactivates. The adsorption profile suggests a similar degree of mechanical change per unit mass, as in the 3MPA example in the earlier section on pH measurements, and therefore it is possible that the inactivation is a result of the hydrophobic surface.

Hydrophobic surfaces adsorb proteins more strongly than hydrophilic surfaces and thus adsorb a greater load of protein, where the protein unfolds and spreads its hydrophobic core over the surface owing to the thermodynamic driving force to reduce the net hydrophobic surface area of the system exposed to the solvent.^{15,16} It is therefore likely that a protein may undergo conformational rearrangement in a way that the hydrophobic residues on the surface of the protein come into closer contact with the hydrophobic surface.^{17,18} It is then safe to

assume that there could be an element of denaturation or a breakdown of secondary structure when the enzyme spreads on the surface, and in turn this is a likely cause for the diminished activity seen on the benzenethiol surface. This idea is supported in a study by Haynes and Norde, where they looked at lysozyme and α -lactalbumin adsorbed on polystyrene and hematite surfaces using micro-DSC (differential scanning calorimetry). This technique can observe thermally induced structural transitions in proteins, and they indicate that surface adsorbed proteins are less stable than native protein dissolved in solution. They further suggest that this instability, and the amount of secondary structure lost upon adsorption, increases on hydrophobic surfaces.¹⁹

The 4MPOH surface shows high starting activity and a higher efficiency of adsorbed mass when chronoamperometry is started, which is in line with a study by Cracknell *et al.*, where they indicated the inner binding pocket of *MvBOx* to be predominantly hydrophilic in nature and therefore likely to preferentially interact with hydrophilic residues.²⁰ However the catalytic waveshape is significantly altered and, like 4MPAA, the catalysis is seen over a lower potential range meaning that the hydroxyl group is contributing towards a misorientation of the enzyme. The significant loss in efficiency while a potential is held constant, compared to 4MBZ, suggests that the carboxylic acid group promotes better electron transfer which is consistent with the findings by Tominaga *et al.*¹³ where they indicate carboxyl terminated thiols better promote electron transfer with adsorbed *MvBOx*.

These results are critical in assessing the applicability of similar enzymes for use in fuel cell systems. The observations suggest the need for a surface which avoids the rapid inactivation, and likely denaturation, on hydrophobic residues, with the need for a suitably hydrophilic surface, which does not alter the orientation of the enzyme but instead allows *MvBOx* to maintain a stable current response and efficiency over time.

5.5 Affinity binding

This section describes the results from E-QCM-D experiments where *MvBOx* was adsorbed to a layer of glucosamine-modified gold-coated QCM crystals. This method uses EDC-NHS chemistry to activate the carboxyl function group on 3MPA for attack by the amine group on glucosamine. The glucosamine unit acts as an analogue of the linear polysaccharide chitosan which is oxidised by bilirubin oxidase and has been commonly used on the surface of structured electrodes for the immobilization of *MvBOx*.^{21,22} The idea was to look at the effect of affinity binding on the frequency, dissipation and electrochemical properties of the adsorbed adlayer.

In the measurement shown in Figure 5.15, *MvBOx* was introduced to a 3MPA-modified gold surface while a constant potential of 0.21 V vs. SHE was applied on the electrode surface. After ca. 1 h this constant potential was turned off and left for around 5 min, before potential cycling was then applied over the range 0.81–.21 V vs. SHE.

The maximum adsorption ($\Delta f^{\max}$) was -252 Hz ($0.64 \mu\text{g cm}^{-2}$) and a maximum dissipation ($\Delta d^{\max}$) of 2.42. Both responses were stable during chronoamperometry giving values of -240 Hz ($0.61 \mu\text{g cm}^{-2}$) (Δf^{oc}) and 2 ppm (Δd^{oc}).

The $\Delta d/\Delta f$ gradient for adsorption was $0.009 \text{ ppm Hz}^{-1}$ (black arrow, Figure 5.15D) suggesting a lower degree of conformational rearrangement during adsorption compared to the carboxyl terminated surfaces in section 5.3. When chronoamperometry was turned off the gradient of $\Delta d/\Delta f$ was of a similar magnitude to the adsorption.

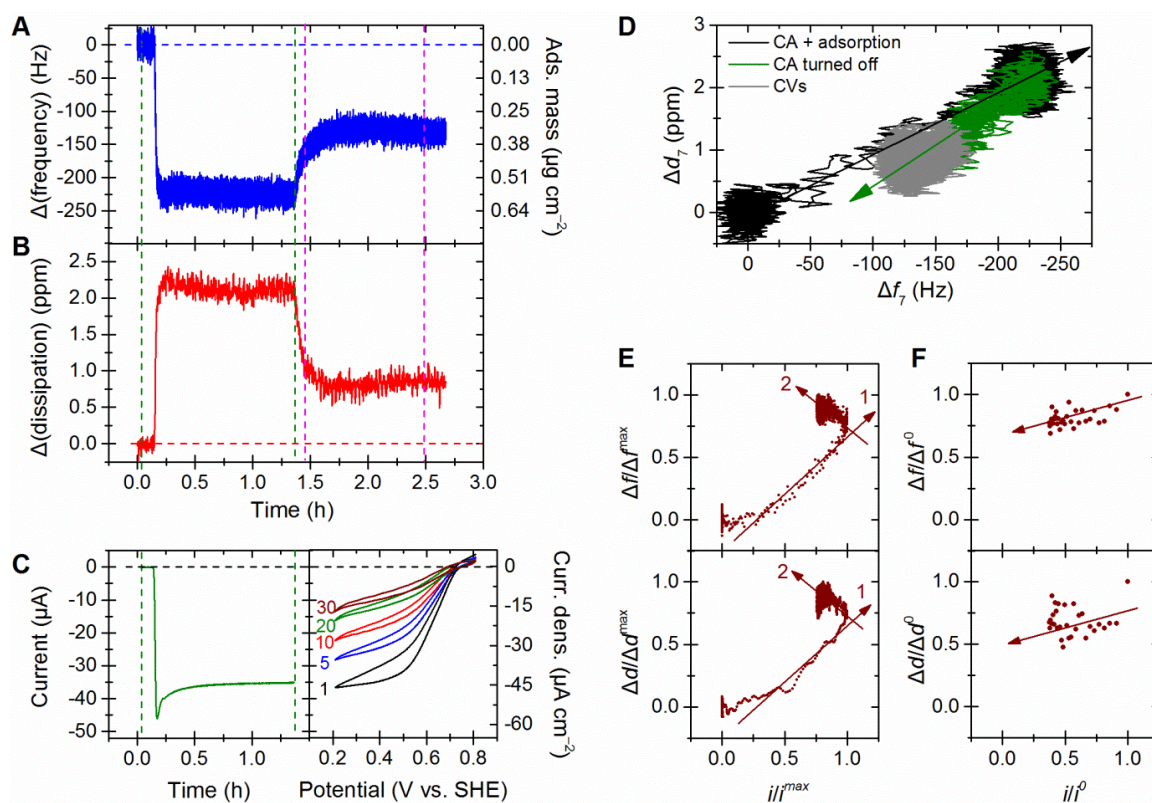


Figure 5.15 E-QCM-D response with time for MvBOx adsorbed non-covalently on a glucosamine modified layer at pH 6.0 sodium phosphate buffer (pre-saturated with O₂): (Panel A) frequency change, (Panel B) energy dissipation change, (Panel C, LHS) O₂ chronoamperometric reduction current and (Panel C, RHS) cyclic voltammetry showing scans 1, 5, 10, 20 and 30 of 30 scans. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show then the potential was cycled from 0.81–0.21 V vs. SHE. Panel D shows the Δd vs. Δf relationship. Panel E shows the frequency-activity and dissipation-activity plots for chronoamperometry. Panel F shows the frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0, 25 °C, 0.1 ml min⁻¹, Flowrate of O₂ at 90 cm³ min⁻¹, seventh harmonic shown.

5.5.1 Comparison of electrochemical measurements

5.5.1.1 During chronoamperometry

During chronoamperometry, the maximum current change (Δi^{max}) was 46 μA : a factor of over 1.5 higher than the highest peak activity in the surface chemistry measurement (see Table 5.12, Section 5.4.2). The current stabilised at -35 μA , and exhibited a current decay time constant of 0.20 h. interestingly, the starting efficiency (taken at i^{max}) is also higher for glucosamine than all surface chemistry measurements and there is a greater stability/retention

in efficiency (Table 5.15). The glucosamine surface appears to promote high activity and high efficiency which is more stable over time than the surfaces used in Section 5.4

Table 5.15 Table showing the current decay constant, normalised efficiency loss, peak activity for *MvBOx* adsorbed to a glucosamine-modified gold electrode while a constant potential of 0.21 V vs. SHE applied, compared to surface chemistry measurements.

Surface	τ (h)	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($(\Delta\text{eff} / \Delta\text{eff}^{\text{max}}) \text{h}^{-1}$)	Highest peak current, $ i ^{\text{max}}$ (μA)
Glucosamine	0.20	0.22	0.27	46
4MPAA	0.33	0.03	0.33	8
4MPOH	0.53	0.23	0.83	28.3
4MBZ	0.17	0.22	0.91	21.2

The frequency-activity and dissipation-activity plots (Figure 5.15E upper and lower panels) both showed more linear relationships

5.5.1.2 During potential cycling

The first interesting observation was that, as the frequency dropped after the constant potential was turned off, the efficiency of the adlayer increased from 0.16 $\mu\text{A Hz}^{-1}$ (ca. 1.4 h) to 0.22 $\mu\text{A Hz}^{-1}$ (at the lower potential vertex during the first voltammogram). There was a further drop of 50% in the efficiency over the 30 scans carried out, with a relative activity loss of 61% (Table 5.16). During this period, however both the frequency (after an initial mass loss) and dissipation traces remained stable. The position of E_{cat} (0.68 V vs. SHE) was similar to the 4MBZ modifier suggesting that the glucosamine acts in a similar fashion to the carboxyl surface modifiers, and promotes better electron transfer, that is, there is less of a thermodynamic push needed for catalysis.

Table 5.16 Table showing the starting efficiency, normalised efficiency loss, relative activity loss and position of E_{cat} for MvBOx adsorbed to a glucosamine-modified gold electrode during potential cycling.

Surface	Starting efficiency ($\mu\text{A Hz}^{-1}$)	Normalised efficiency loss ($(\Delta\text{eff} / \Delta\text{eff}^{\text{max}}) \text{h}^{-1}$)	Normalised activity loss ($(\Delta i / \Delta i^{\text{max}}) \text{h}^{-1}$)	E_{cat} (V vs. SHE)
Glucosamine	0.22	0.52	0.61	0.68
4MPAA	0.163	0.53	0.56	0.21
4MPOH	0.0103	0.44	0.29	0.31
4MBZ	0.108	0.10	0.20	0.67

Another interesting observation showed that during the potential cycling the oscillatory changes were present but with a much smaller peak-to-peak amplitude of just 0.35 Hz.

5.5.2 Discussion of affinity adsorption effects

The glucosamine layer allows MvBOx to adopt an orientation that promotes facile electron transfer. This is shown by the stability in both the frequency and dissipation traces which suggests that the enzyme is adsorbing in a favourable manner, with a stable dissipation response and no detected conformational change. This is further evidenced by the E_{cat} potential of 0.68 V vs. SHE which is similar to the E_{cat} potentials seen on the shorter-chain carboxyl-terminated surfaces. However, potential cycling again seems to inactivate the adlayer more readily with a greater comparative.

An interesting point here is that there is a small window between the potential being turned off and starting the potential cycling (~ 5 min) where the mass loss is equal to ~ 68 Hz. This mass loss does not contribute to a fall in activity of the enzyme, and the efficiency increases from the point when the constant potential is turned off to the current at the lower potential vertex in the first voltammogram. This suggests that, although there is a stiffening process occurring, the enzyme adlayer retains its activity. One reason for the mass loss could be the loss of a hydration layer or salvation molecules associated with the enzyme adlayer. This idea is consistent with a study by Pasche *et al.* where they suggest that during protein

adsorption to polyethylene glycol (PEG) brushes, the hydrophilic chains in the brushes act as a scaffold for structural water during protein adsorption. In a similar manner, the glucosamine layer may hold water molecules, and when the potential is turned off the loss of mass might be due to a loss of structured water on the surface.²³ A further experiment would be to observe any conformational changes when switching between constant potential and potential cycling, using E-SPR. As E-SPR measures the dry mass of protein during adsorption, this experiment could reveal the degree of hydration surrounding a protein molecule, when comparing E-QCM-D measurements. In addition, the predominantly hydrophilic character of the glucosamine modification may reduce the formation of strong hydrophobic forces and therefore the rate of breakdown of the secondary structure is also reduced. This hypothesis would however need further work using contact angle measurements to understand the difference between other hydrophilic groups such as the hydroxyl terminated surfaces in the previous section which do not display the same degree of stability as glucosamine.

5.6 Conclusions

When changing the pH, there is a need to carefully balance the high starting current/higher catalytic efficiency with greater stability over time. There is an increased rigidification during adsorption, in the pH measurements, which suggests a change in secondary structure. There is evidence to suggest that, as pH increases, enzyme adsorption is faster and this is down to the enzyme secondary structure breaking down more readily at higher pH. This is an important factor when setting conditions for fuel cell enzyme catalysis as pH will affect conformational arrangement as well as affecting catalytic competency.

Increasing chain length also contributes to a decrease in catalytic activity of *MvBOx*. The mechanical rigidity decreases as carbon chain length increases and it can be said that there is a structural reorganisation that occurs to permit electron transfer over longer distances. This

also results in reduced starting efficiency, lower starting currents and a negative shift in the E_{cat} , on the largest carbon chain modifiers. However, although there is a change in the mechanical properties of the protein, protein that adsorbs through affinity interactions appears to exhibit a greater activity, greater efficiency retention over time partnered with reduced mass loss from the surface.

In the surface chemistry measurements, hydrophobic residues appear to induce a breakdown in the secondary structure of the protein and ultimately cause enzyme inactivation. Tailoring the surface towards hydrophilicity increases the activity but in some cases can lead to a change in orientation and waveshape during catalysis.

These observations suggest that a careful fine tuning is needed to create a surface which: 1) allows *MvBOx* to adsorb with little change in conformation (i.e. to decrease the rate of secondary structure breakdown), 2) positions the enzyme in an orientation which promotes efficient electron transfer and is stable against the effects of potential cycling, and 3) promotes the generation of suitably high currents with long term stability. The requirements suggested are critical in assessing the applicability of enzymes for fuel cell catalysis.

The E-QCM-D provides information on surface adsorption, mechanical changes and catalytic efficiency and activity. This allows for a more comprehensive study on a variety of factors which can affect catalytic ability of redox enzyme that are incorporated into fuel cell systems.

5.7 References

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Chapter 6: Summary

6 Summary

6.1 Introduction

This chapter provides a summary of the findings from Chapter 4 and 5 alongside avenues for future research and new frontiers for enzyme adsorption using the electrochemical quartz crystal microbalance with dissipation (E-QCM-D).

6.2 Enzyme conformation and inactivation

In this thesis, it has been shown how an E-QCM-D can be used to optimise the immobilisation of electron-transfer catalysts, by providing mass-specific catalytic activity, diagnostic tools for failure analysis and a way to monitor how actual operating conditions affect long-term performance.

The effects of administering potential control of *MvBOx* immobilized onto thiol-modified gold have been investigated in Chapter 4. It has been shown that on application of a constant potential, dissipative analysis, together with activity measurements, suggests a change in mechanical properties of the enzyme adlayer over time. This can be attributed to a breakdown in secondary structure of the enzyme which, in turn, results in loss of activity and a decrease in catalytic competency.

When a constant potential is held and then switched between high and low potentials (i.e., turning catalysis on and off), there is a change in the stability of adsorbed enzyme and there is an inherent loss of activity resulting when holding at high potential. Varying the scan rate over which potential cycling is carried out, showed how the efficiency of the enzyme, regardless of the rate at which the cycling induced-distortions occur, was greatly diminished over time. The idea here is that the charge in the electric field distorts the protein. That is, if the protein's charged groups are pulled slowly (lower dE/dt), it has time to change shape in a way that is reversible and maintains catalytic competence.

However, at the slower scan rate, there is still significant loss in the adsorbed mass efficiency over time, suggesting that, although decreasing the scan rate may temporarily reduce activity loss, inactivation of the enzyme adlayer over time is still a concern. Potential range measurements indicated that when the potential range is kept above a lower vertex potential of 0.6 V vs. SHE, the enzyme adlayer shows greater stability in both mass and dissipation responses.

Applying a continuous cycling potential further highlighted the detrimental effect of potential cycling, where the ongoing cycling causes inactivation as well as a possible orientation change, implied by the negative shift in the potential of the steepest section of the catalytic wave (E_{cat}).

During protein adsorption, structural rearrangement of the protein is driven by an entropy gain, pertaining to a loss of ordered secondary structure, in addition to the release of counter ions or solvation molecules. Therefore, as the QCM is used to measure hydrated mass, there is suggestion that potential cycling-induced mass losses could be a result of adlayer dehydration. For this reason, additional treatments such as structural scaffolds will be needed before these enzymes will be durable enough to be incorporated into miniature fuel cells.

Attaching the enzyme more permanently via covalent bonding did not improve catalytic stability and also resulted in lower activity because of longer distances for interfacial electron transfer. Comparison to a second multicopper oxidase suggested that enzyme inactivation is independent of enzyme type. The structures of the high-potential MCOs used for fuel cell cathodes are similar,¹ so the patterns of activity loss are likely to be the similar for all three-domain MCOs. For example, laccase from *Trametes versicolor* (TvL) showed similar patterns of mass and activity loss, though the magnitudes differed (Chapter 4). The observed behaviour depends on protein structure and electrode

composition, however, for example, covalent attachment by amide linkage stabilised a [NiFe] hydrogenase over 2 h in response to potential steps up to 250 mV,² and covalently bound [FeFe] hydrogenases showed activity decay time constants ranging from hours to days depending on immobilisation method.³

Olejník *et al.* also showed the difference in electroactivity between laccase immobilised on positively charged versus negatively charged surfaces. Although similar masses of enzyme were immobilised, the resulting activity was far greater on the positively charged surface, which indicates a direct relationship between enzyme orientation, surface topology and catalytic current.⁴

These observations suggest that without additional treatments, such as structural scaffolds which act to address changes in the mechanical properties of an enzyme, namely loss of secondary structure, or overpotential limits, MCO-coated cathodes may be unsuitable for long-term power generation in enzymatically catalysed fuel cells.

6.3 Surface chemistry, pH and length dependence

Chapter 5 examined the effects of changing pH, surface chemistry and varying the chain length of thiol-modifiers for surface adsorption of *MvBOx*. The pH measurements suggest the need for fine tuning to balance between high starting current/higher catalytic efficiency (at low pH) with greater stability in catalytic current over time (at higher pH).

The measurements on carbon chain length indicate the need for an optimum distance between enzyme and electrode where the enzyme is stable against the effect of potential control (turning potential on or off) but also retains sufficient conformational integrity to promote facile electron transfer.

The surface chemistry measurements highlight how the E-QCM-D explicitly relates the high degree of denaturation on hydrophobic surfaces to the complete loss in activity

of the enzyme. However, changing the functional group present on the hydrophobic residue increases activity and suggests the orientation of enzyme plays a critical part in activity loss. Tailoring surfaces for hydrophilicity suggests that –COOH groups on the surface promote a better orientation of *MvBOx*.

These observations suggest that a careful fine tuning is needed to create a surface which: 1) allows *MvBOx* to adsorb with little change in conformation (i.e. to control for the likely breakdown in secondary structure upon adsorption), 2) positions the enzyme in an orientation which promotes efficient electron transfer and is stable against the effects of potential cycling, and 3) promotes the generation of suitably high currents with long term stability. These requirements are critical for assessing the use of enzymes for fuel cell catalysis. They also suggest the importance of carrying out efficiency measurements, alongside dissipation and frequency measurements, to paint a more comprehensive picture on the applicability and effectiveness of utilising such enzymes in real fuel cell applications.

6.4 Future work

Throughout this thesis there has been a strong suggestion of conformation changes occurring in the enzyme, which contribute to the inactivation and loss of catalytic competency in the enzyme. An alternative approach is to study the secondary structure of the protein using circular dichroism (CD). Circular dichroism measures the difference in the absorption of left-handed circularly polarised light and right-handed circularly polarised light, and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups). Proteins have different structural elements which have characteristic CD spectra due to each element causing changes in the degree of absorption of left- and right-handed plane polarised light. The secondary structure of a protein is also

sensitive to its environment i.e., temperature and pH, and therefore, as the CD spectra of proteins are dependent on their structural elements i.e. their conformation, circular dichroism can be used to observe how secondary structure changes with environmental conditions or on interaction with other molecules. CD can also be used to monitor conformational changes that result from changes in binding interactions on surfaces.^{5,6}

In a study by Kondo *et al.*, circular dichroism was used to determine how structural changes, in a range of proteins including BSA and horse cytochrome *c*, can be identified by shifts in the CD spectra, that relate to the structural flexibility of the protein, when adsorbed to silica particles.⁷ In tandem with the E-QCM-D, circular dichroism would allow the development of a more expansive picture on the mechanics of protein conformation changes in surface bound protein. Environmental effects such as temperature, pH and electrostatic interactions can be probed using both techniques to understand the extent to which these conditions have an effect on protein conformation/structure, and then relate this back to the catalytic competency of the adsorbed enzyme. Alongside the E-QCM-D, circular dichroism could also be used to monitor the potential induced effects on conformation during a constant potential application, and also before, during and after potential cycling. This would prove vital to assess the applicability of having such enzymes used in fuel cell systems. This technique could also be used to compare the conformational/orientation changes occurring in a range of multicopper oxidases to assess the applicability of each class of enzyme, to its use as a fuel cell catalyst. This potential dependence study using a range of techniques could then be extended further afield to look at the effect of applied potentials on amperometric biosensors that incorporate enzymes like glucose oxidase, and microbial fuel cells that rely on the electron transfer capability of bacteria, such as *Shewanella* and *Geobacter*, to act as cathode materials.

Another method for studying the structural changes occurring within an enzyme is by using attenuated internal reflectance infrared spectroscopy (ATR-IR). This is a type of spectroscopy where infrared light is passed through a prism at an angle exceeding the critical angle for internal reflection, and in such a way that it reflects at least once off the internal surface in contact with the sample. This results in an evanescent wave that extends into the sample and therefore ATR-IR can be used to produce IR spectra for proteins in solution. The sensitivity of IR bands to changes in the molecular environment makes it a suitable technique for monitoring the adsorption of proteins to surfaces, in particular any structural changes such as loss of the alpha helix and/or changes in beta sheet structure.⁸ Due to the long IR wavelength used in this technique, light scattering problems are also virtually nonexistent (compared to CD), and therefore highly aggregated materials or large protein fragments can be investigated.

In a study by Iwaki et al., redox-induced protonation state changes of the Glu residue in bilirubin oxidase were studied by ATR-FTIR spectroscopy. By monitoring IR bands of the carboxylic acid C=O stretch in the wild-type and Glu463-Gln mutant enzymes, the Glu463 residue was found to be unprotonated in the oxidised form, and protonated in the reduced form. This provided direct evidence for proton uptake by the Glu residue, suggesting the importance of this residue in proton donation (and associated change in molecular environment) to the activated oxygen species in the catalytic cycle.⁹

FTIR techniques have previously been developed to automatically subtract the IR spectrum of water to obtain a spectrum of just the protein^{10,11} and this provides an attractive method for factoring out the effects of water on the protein adlayer. In combination with the dissipative analysis from the E-QCM-D, this would help paint a more robust picture for the conformational/orientation changes that occur on/after protein adsorption to surfaces.

It has also been suggested in this thesis that potential cycling could be stretching out the adsorbed enzyme which in turns affects/distorts the T1 primary electron entry point. This phenomenon could be probed using electron paramagnetic resonance (EPR) to look at changes in the EPR active T1 and T2 copper centres within the enzyme, which are involved in electron transfer processes.

Using EPR, Kjaergaard et al. showed how the resting oxidized form of bilirubin oxidase from *Magnaporthe oryzae* had similar features to the resting form in *Trametes versicolor* laccase, which were characterised by ‘normal’ T2 Cu EPR features: an adsorption shoulder at 330 nm and a short T2 Cu–Cu distance. They then used these features to distinguish between resting oxidised and alternative resting oxidised forms, where the alternative resting oxidised form lacked adsorption intensity at 330 nm, and also had a longer T3 Cu–Cu distance. These differences were then correlated to changes in activity and redox state of the two forms.¹² In a similar fashion, EPR could be used to look at the copper sites in the enzyme before, during, and after potential cycling to understand whether any changes in copper coordination are responsible for enzyme inactivation. Taking EPR spectra periodically over longer durations to look at the change in copper coordination and correlate this to any breakdown in secondary structure that may occur.

Using electrochemical surface plasmon resonance (E–SPR) with the E–QCM–D for this type of measurement would also help understand changes in conformation and possible stiffening/denaturation processes. The E–SPR technique combines voltammetric analysis with surface plasmon resonance in much the same way as the E–QCM–D, with the surface of interest doubling as the working electrode. The last few years has seen an increase in the development of this technique with increased focus on the monitoring of redox processes. In a study by Nakamoto *et al.*, E–SPR was used to synchronise redox and

optical signals in relation to the redox changes of an osmium–hydrogen peroxidase film on an array of nanometre-sized pores in gold.¹³ E-SPR has also been applied to investigate redox-induced conformation changes of immobilised proteins. Zhai et al. showed that the conformation changes identified in cytochrome c adsorbed onto SAMs were heavily dependent on the double-layer capacitance of the electrode surface.¹⁴

The use of E-SPR in tandem with the E-QCM-D can help to develop knowledge on surface-specific phenomena by combining the high sensitivity to surface changes of surface plasmon resonance and the intrinsic viscoelastic properties measured by the QCM. Combining the hydrated mass detection technique of the QCM and the dry mass detection in SPR, together with catalytic efficiency measurements, would also allow for a more quantitative description of surface dependant enzyme processes and the extent to which adlayer hydration plays in redox enzyme performance.

6.5 Future applications

One particularly lucrative frontier is “industrial bioelectrocatalysis”: electrode-driven, enzyme-catalysed organic synthesis. Cytochrome P450 enzymes are an especially promising candidate for this application because they have been shown to be active in layer-by-layer films¹⁵ and membrane mimics.¹⁶ P450 enzymes catalyse oxygen-insertion reactions with high stereoselectivity and regioselectivity¹⁷ and have been engineered to oxidise non-natural substrates.¹⁸ The methods we have outlined can be applied to both P450 monolayers as well as P450 enzymes in more complex surface assemblies. Electrochemically driven cycling of NAD(P)⁺/NAD(P)H is another widely useful target well suited to optimisation by E-QCM-D. Instead of driving an enzyme directly that requires NAD(P)H, an enzyme such as lactate dehydrogenase¹⁹ or a hydrogenase subunit²⁰ can be immobilised instead to drive coenzyme recycling of the NAD(P)H-

dependent enzyme in solution. The E-QCM-D can be readily applied to develop surface chemistry and structures to support these coenzyme-recycling proteins.

Finally, an exciting emerging area is the use of electrochemically driven enzymes to convert CO₂ into organic materials such as formate for use as feedstock chemicals.^{21,22} The small volume of the E-QCM-D cell allows the local liquid above an enzyme film to be changed rapidly to explore the effects of substrate and inhibitor concentration, pH and contaminants.

Many properties of *MvBOx* have been determined using the E-QCM-D technique. Amongst these properties, the effect of enzyme adsorption to an electrode surface on the enzyme structure and function has been detailed. By modifying gold sensor surfaces with thiol chemistry and monitoring simple adsorption, covalent attachment and to an extent, affinity binding, the use of *MvBOx* as a fuel cell cathode was assessed. The electron transfer between electrode and enzyme was directly related to both changes in conformation and changes in positioning (i.e. orientation). Further optimisation of electrode surfaces is needed, to correct for these changes so as to resolve issues of long-term stability and activity which are a result of possible enzyme denaturation and inactivation.

E-QCM-D technology has reached a point where a sophisticated instrument is partnered with rigorous data analysis methods to calculate mass loading, thickness, density and viscoelasticity in soft films. The ease of sensor modification and real-time monitoring of both enzyme immobilisation and subsequent electrochemically driven reactions make the E-QCM-D a unique surface-sensitive testbed for applied bioelectrochemistry.

6.6 References

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Appendices

Appendix I Chapter 4 measurements

I.1 Scan rate measurements

Potential cycling at 10 mV s⁻¹

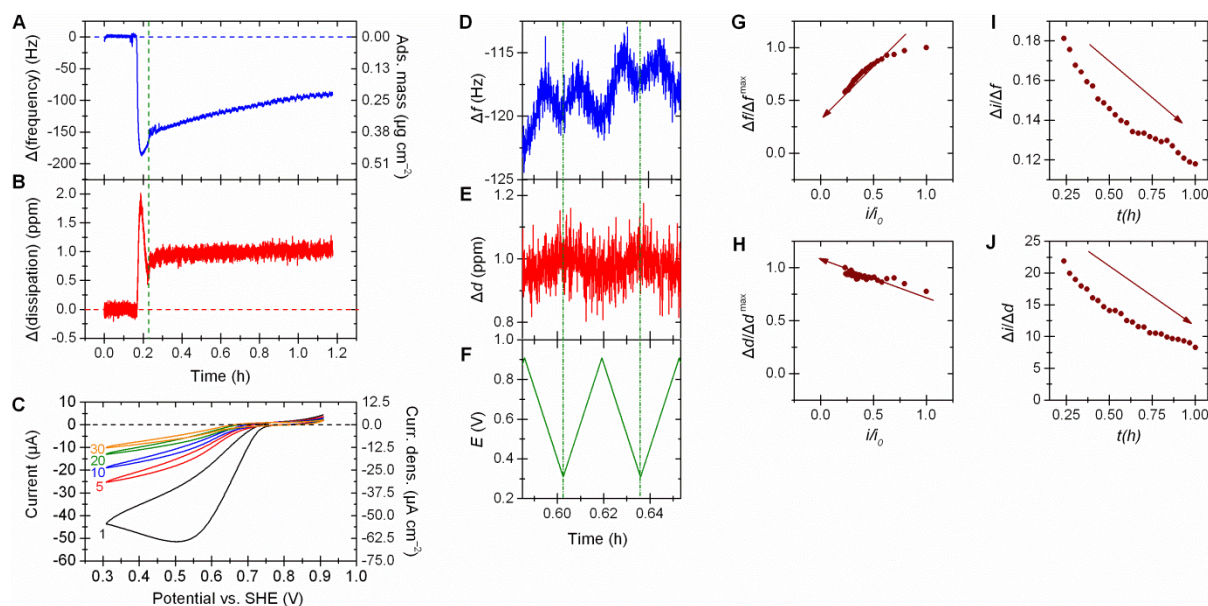


Figure I.1 Potential cycling at scan rate of 10 mV s⁻¹. Panel A and B show the frequency and dissipation responses respectively. The insets show the adsorption phase for both traces respectively. Panel C shows scans 1, 5, 10, 20 and 30 of 30 scans with each scan numbered in Panel A. Panels D & E show the frequency and dissipation responses for scans 24 and 25 where the effects of the CVs give rise to an oscillatory effect (sets of double peaks in the frequency slightly out of phase with the applied potential shown in panel F, green dashed lines). Panel G and H show the frequency-activity and dissipation-activity during potential cycling respectively. The arrows in the panels are to show direction of loss. Panel I and J show the efficiency-time and Δi/Δd-time plots during potential cycling. Arrows show the direction of efficiency loss. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. CV: 0.91–0.31 V vs. SHE. Experiment carried out with the help of Trevor McArdle (CFB group).

Potential cycling at 100 mV s^{-1}

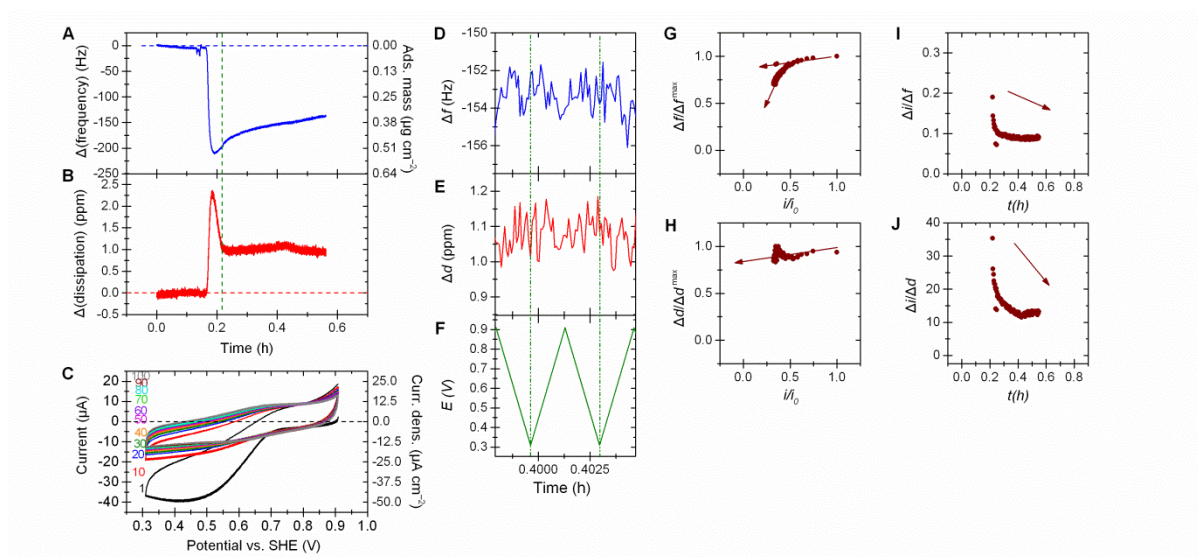


Figure I.2 Potential cycling at scan rate of 100 mV s^{-1} . Panel A and B show the frequency and dissipation responses respectively. The insets show the adsorption phase for both traces respectively. Panel C shows scans 1, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 of 100 scans (numbered in Panel A). Panels D & E show the frequency and dissipation responses for scans 55 and 56 where the effects of the CVs give rise to an oscillatory effect. Panel G and H show the frequency-activity and dissipation-activity during potential cycling respectively. The arrows in the panels are to show direction of loss. Panel I and J show the efficiency-time and $\Delta i/\Delta d$ -time plots during potential cycling. Arrows show the direction of efficiency loss. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , seventh harmonic shown. CV: 0.91–0.31 V vs. SHE. Experiment carried out with the help of Trevor McArdle (CFB group).

I.2 Combined adsorptions of bilirubin oxidase and BSA

I.2.1 Coadsorption of BSA (10 mg ml^{-1}) and MvBOx

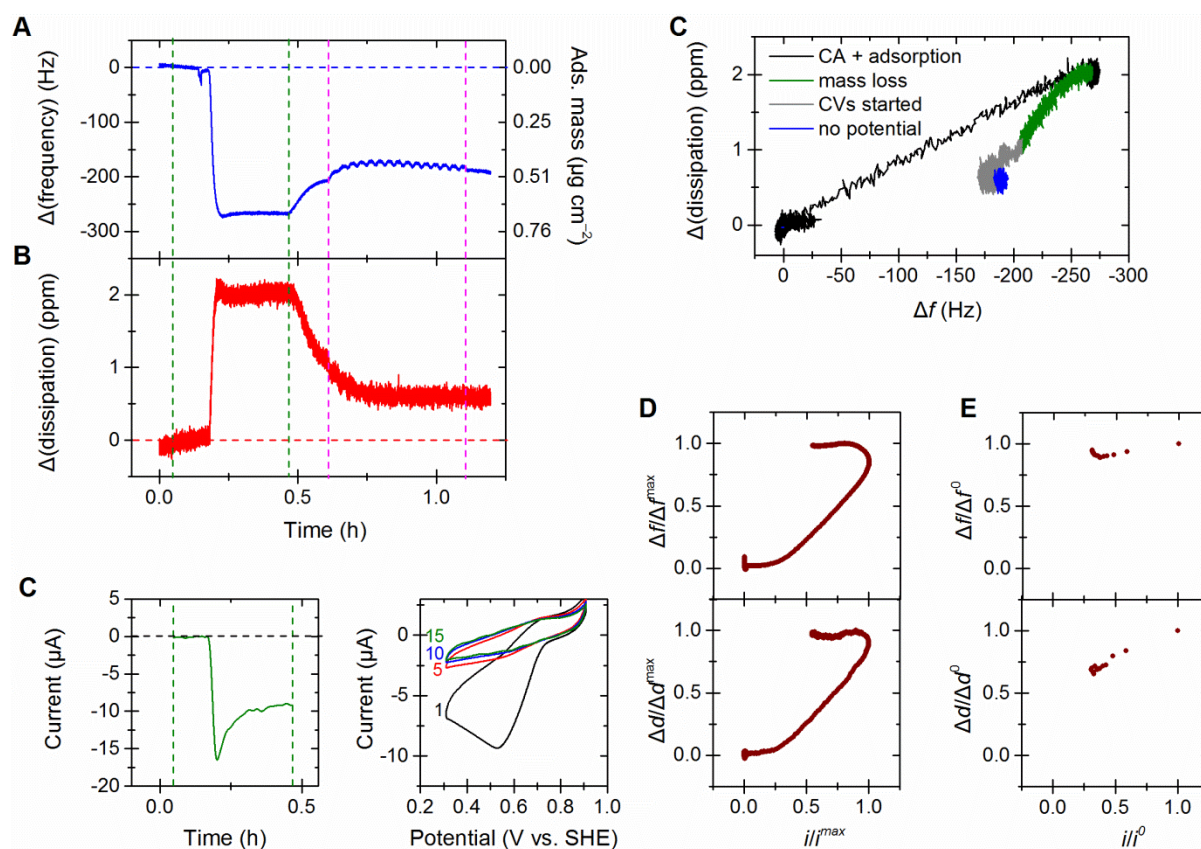


Figure I.3. E-QCM-D response for the coadsorption of BSA (10 mg ml^{-1}) and MvBOx (24 mg ml^{-1}) onto a 3MPA modified gold-coated quartz crystal. Panel A and B show the frequency and dissipation responses respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when potential cycling was carried out. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scan 1, 5, 10 and 15 of 15 scans carried out from 0.91-0.31 V vs. SHE. Panel D shows frequency-activity plots (upper panel) and dissipation-activity plots (lower panel) during chronoamperometry. Panel E shows frequency-activity plots (upper panel) and dissipation-activity (lower panel) during potential cycling. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^{\circ}C$, and 0.1 ml min^{-1} . Flowrate of O_2 at $90 \text{ cm}^3 \text{ min}^{-1}$, seventh harmonic measured.

I.2.2 Coadsorption of BSA (24 mg ml⁻¹) and MvBOx

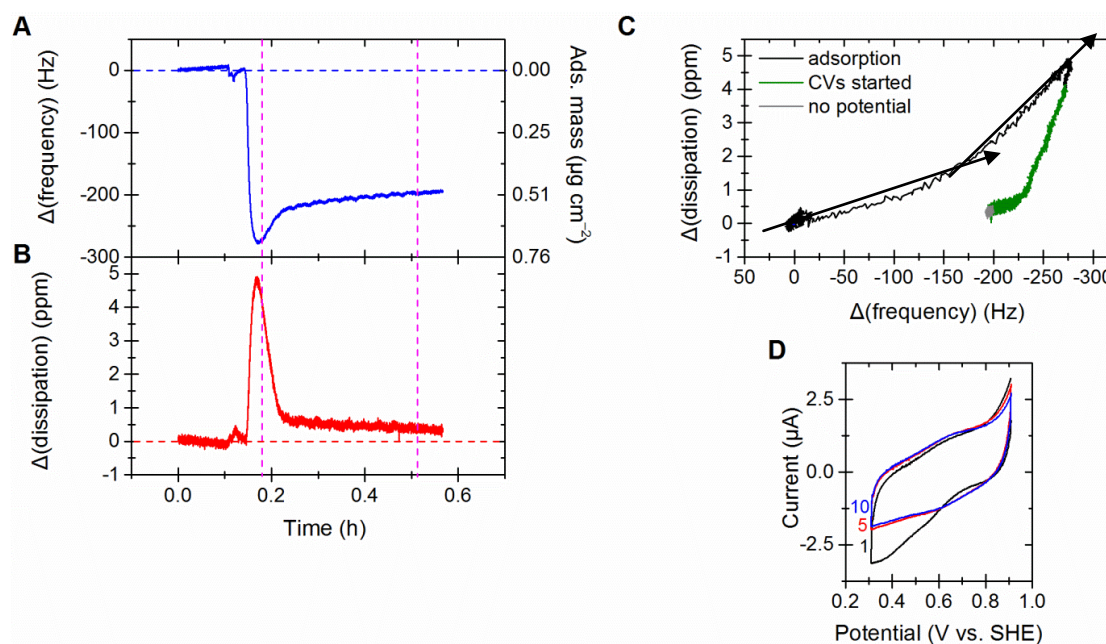


Figure I.4. E-QCM-D response for the coadsorption of BSA (24 mg ml⁻¹) and MvBOx (24 mg ml⁻¹) onto a 3MPA modified gold-coated quartz crystal. Panel A shows the frequency change and panel B shows the dissipation where introduction of protein to the system is indicated by labelled arrows. Panel C shows the Δd vs. Δf plot during both adsorptions. Panel D shows Δf vs. current (upper panel) and Δd vs. current (lower panel) while holding a constant potential of 0.21V vs. SHE during chronoamperometry. Panel E shows Δf vs. current (upper panel) and Δd vs. current (lower panel) during potential cycling between 0.91-0.31 V vs. SHE. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, and 0.1 ml min⁻¹. Flowrate of O₂ at 90 cm³ min⁻¹, seventh harmonic measured.

Appendix II Chapter 5 measurements

II.1 pH measurements

II.1.1 Bilirubin oxidase on 3MPA at pH 4

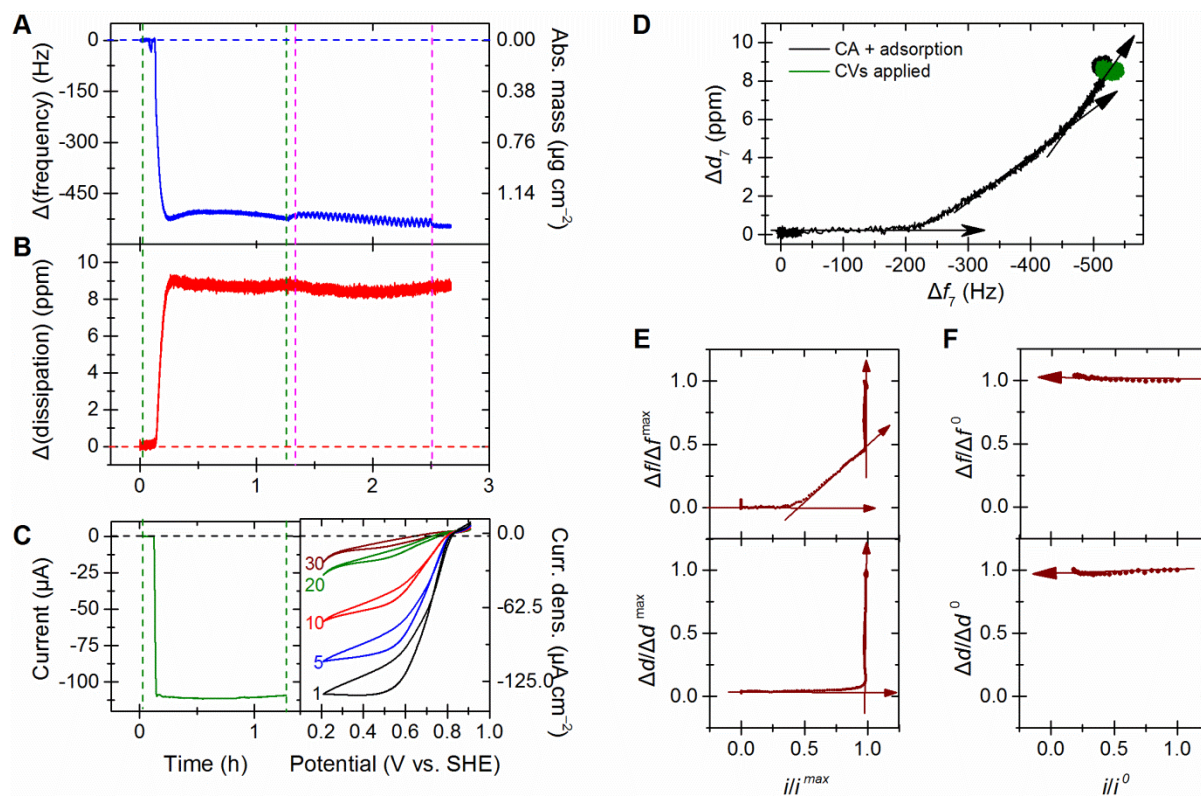


Figure II.1E-QCM-D response with time for MvBOx adsorbed on a 3MPA layer at pH 4.0. Panel A and B show the frequency and dissipation response, respectively. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out 0.91–0.21 V vs. SHE. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled. Panel D shows the Δd vs. Δf plot. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium citrate pH 4.0 (pre-saturated with O_2), 25 °C, 0.1 ml min^{-1} . Flow of O_2 at $90 \text{ cm}^3 \text{ min}^{-1}$, seventh harmonic shown.

II.1.2 Dynamic light scattering results

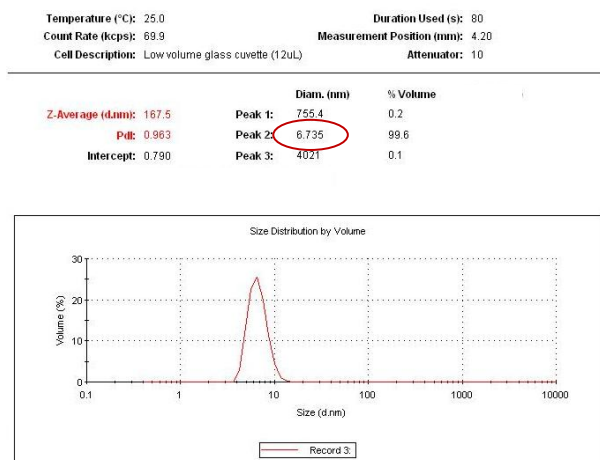


Figure II.2. Dynamic light scattering result of *MvBOx* in pH 6.0, 0.1M sodium phosphate buffer solution. The diameter of the enzyme particles is circled in red.

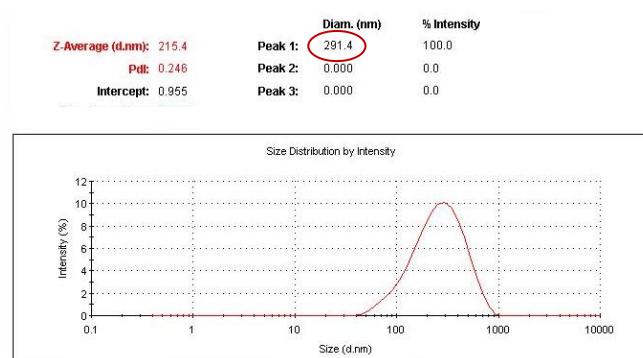


Figure II.3. Dynamic light scattering result of *MvBOx* in pH 4.0, 0.1M sodium citrate buffer solution. The diameter of the enzyme particles is circled in red

II.1.3 Bilirubin oxidase on 3MPA at pH 5

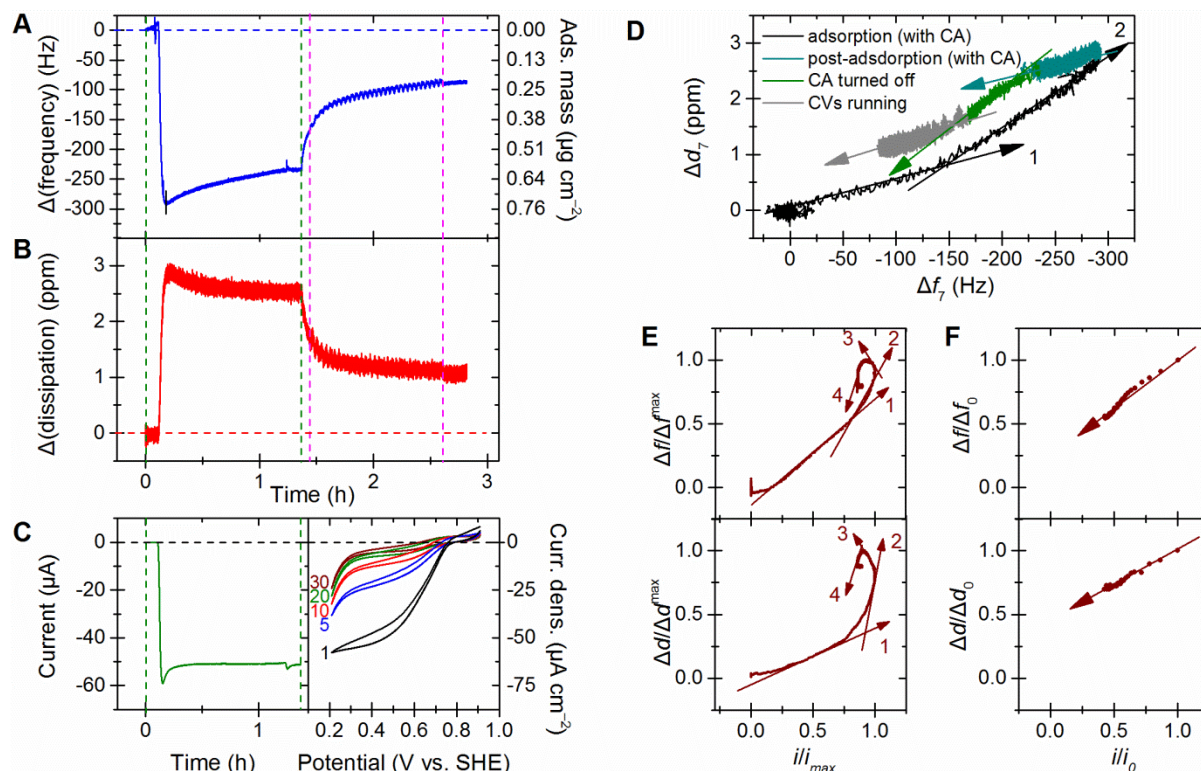


Figure II.4 E-QCM-D response with time for MvBOx adsorbed on a 3MPA layer at pH 5.0 sodium phosphate buffer. Panel A and B show the frequency and dissipation responses, respectively. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out 0.91–0.21 V vs. SHE. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 5.0 (pre-saturated with O_2), 25 $^{\circ}\text{C}$, 0.1 ml min^{-1} . Flow of O_2 at 90 $\text{cm}^3 \text{min}^{-1}$, seventh harmonic shown.

II.1.4 Bilirubin oxidase on 3MPA at pH 7

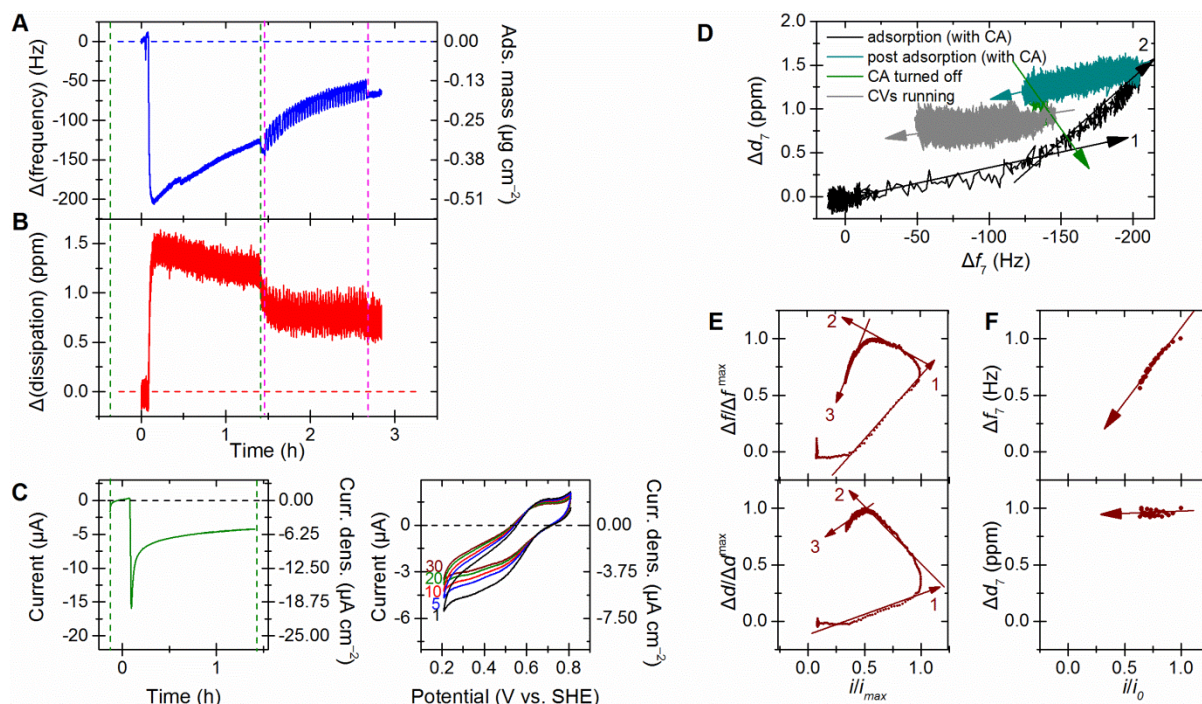


Figure II.5 E-QCM-D response with time for MvBOx adsorbed on a 3MPA layer at pH 7.0 sodium phosphate buffer. Panel A and B show the frequency and dissipation responses, respectively. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out 0.81–0.21 V vs. SHE. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 7.0 (pre-saturated with O_2), 25 $^{\circ}C$, 0.1 ml min $^{-1}$. Flow of O_2 at 90 cm 3 min $^{-1}$, seventh harmonic shown.

II.2 Chain length experiments

II.2.1 Bilirubin oxidase on 6MHA

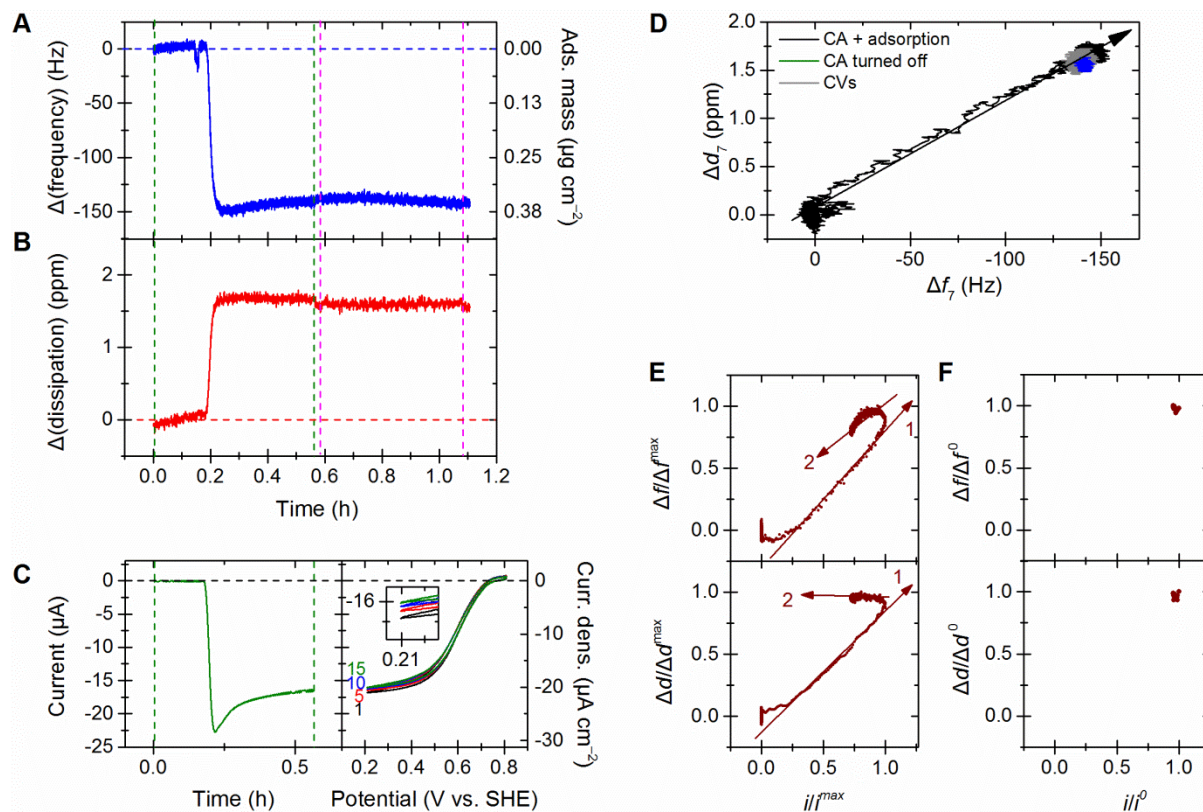


Figure II.6. E-QCM-D response with time for *MvBOx* adsorbed non-covalently on a 6MHA layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.81–0.21 V vs. SHE. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10 and 15 of 15 scans carried out. Inset shows the stability in current at lower vertex potential. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 7.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹. Flow of O_2 at 90 cm³ min⁻¹, seventh harmonic shown.

II.2.2 Bilirubin oxidase on 8MOA

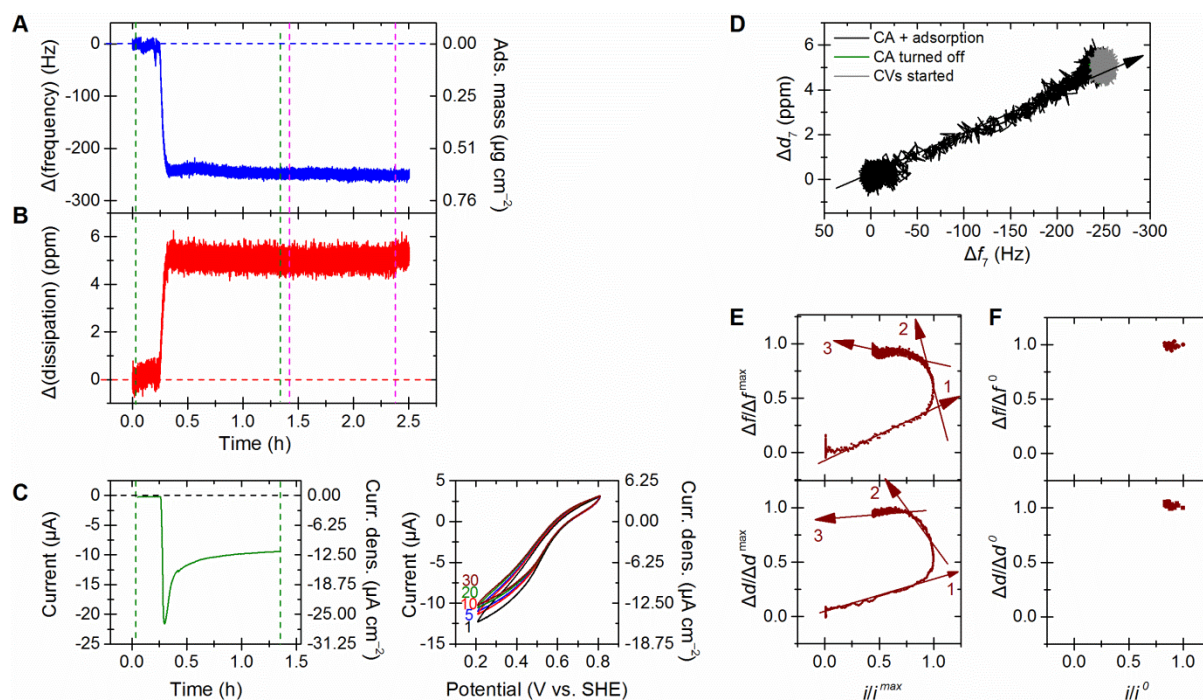


Figure II.7. E-QCM-D response with time for MvBox adsorbed non-covalently on an 8MOA layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.81–0.21 V vs. SHE. Panel C (left) shows the O_2 chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 °C, 0.1 ml min⁻¹. Flow of O_2 at 90 cm³ min⁻¹, seventh harmonic shown.

II.2.3 Bilirubin oxidase on 11MUA

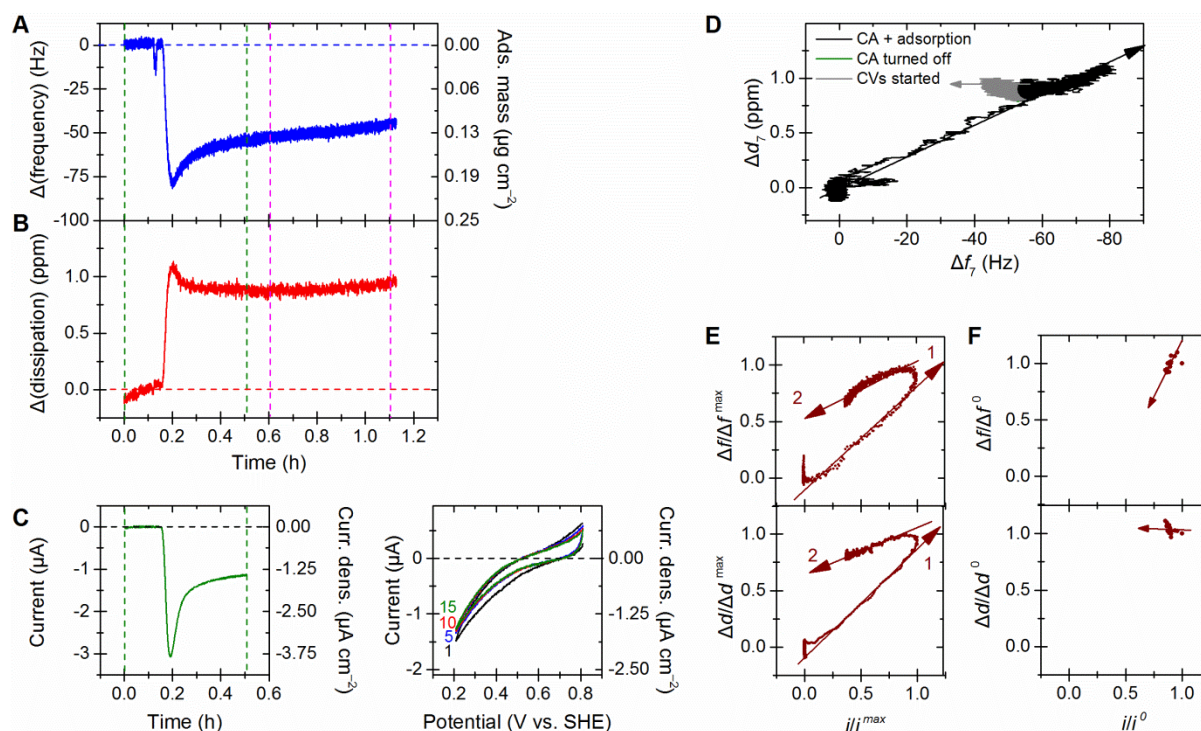


Figure II.8 E-QCM-D response with time for MvBOx adsorbed non-covalently on a 11MUA layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.81–0.21 V vs. SHE. Panel C (left) shows the O₂ chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10 and 15 of 15 scans carried out. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹. Flow of O₂ at 90 cm³ min⁻¹, seventh harmonic shown.

II.3 Surface chemistry experiments

II.3.1 Bilirubin oxidase on 4-mercaptophenol

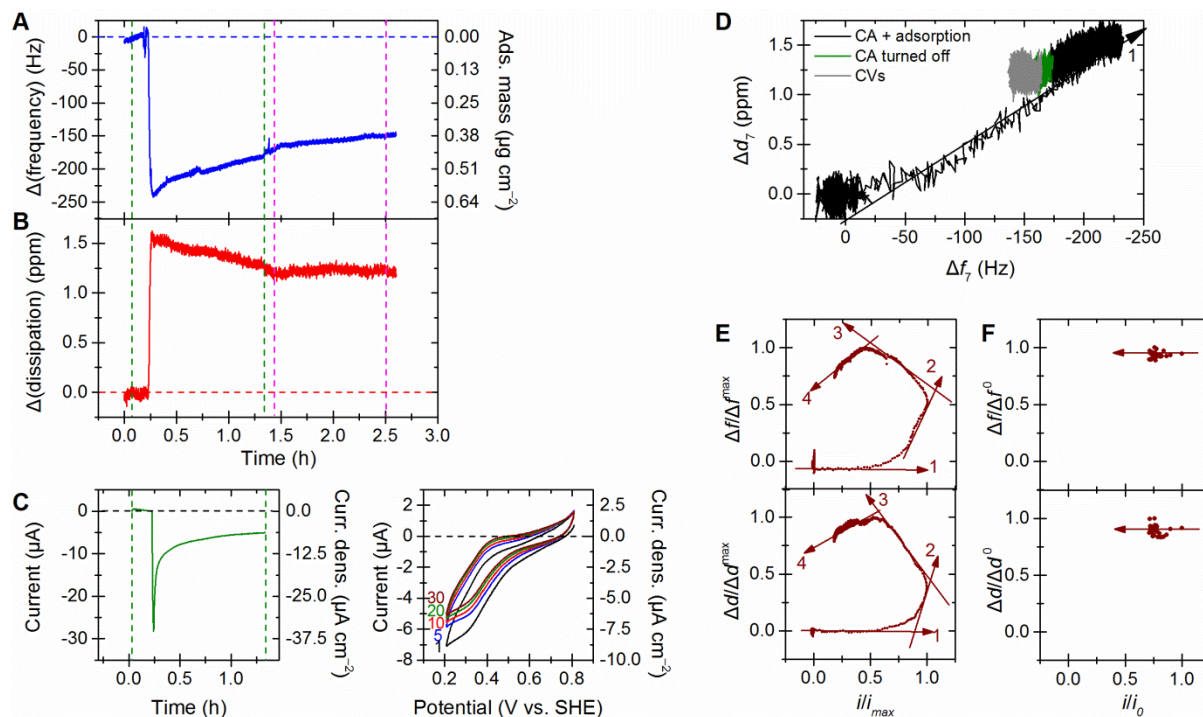


Figure II.9 E-QCM-D response with time for MvBOx adsorbed non-covalently on a 4MPOH layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.81–0.21 V vs. SHE. Panel C (left) shows the O₂ chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹. Flow of O₂ at 90 cm³ min⁻¹, seventh harmonic shown.

II.3.2 Bilirubin oxidase on 4-mercaptoacetanilide

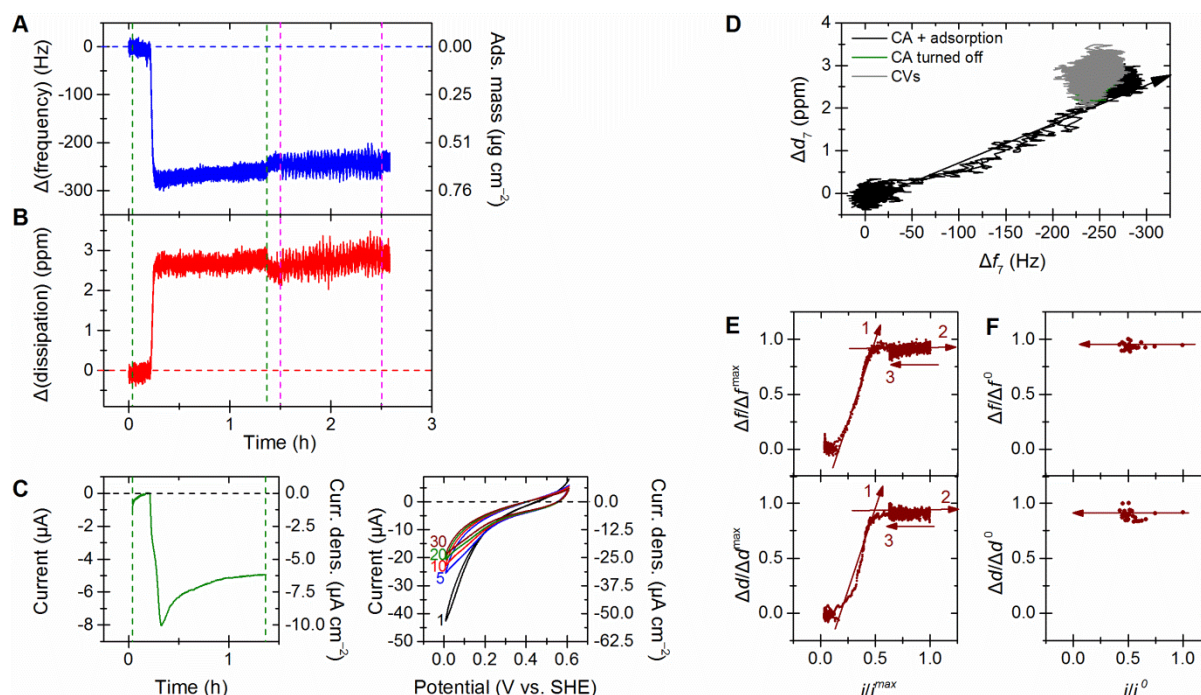


Figure II.10 E-QCM-D response with time for MvBOx adsorbed non-covalently on a 4MPAA layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.61–0.01 V vs. SHE. Panel C (left) shows the O₂ chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹. Flow of O₂ at 90 cm³ min⁻¹, seventh harmonic shown

II.3.3 Bilirubin oxidase on 4-mercaptobenzoic acid

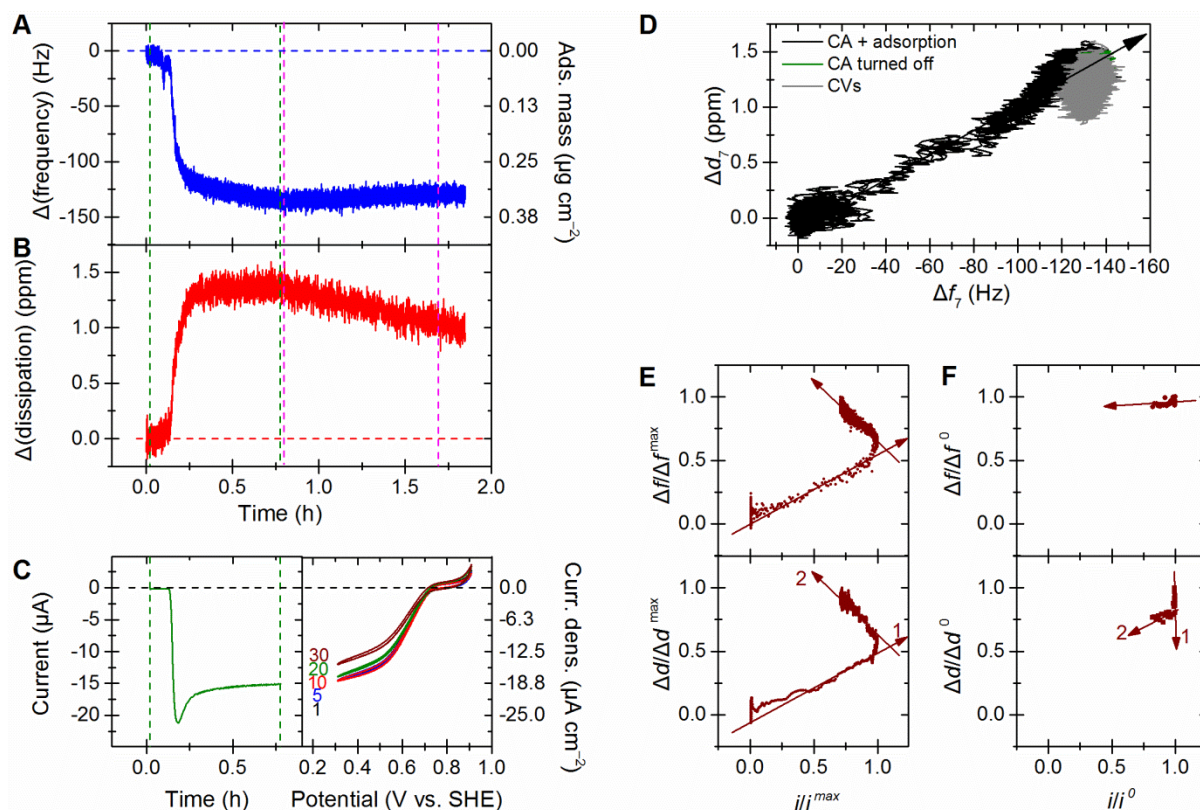
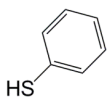
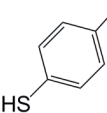
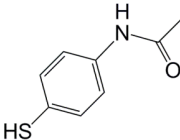
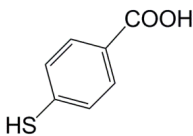
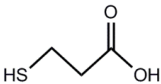
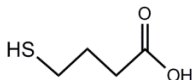
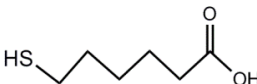


Figure II.11 E-QCM-D response with time for MvBOx adsorbed non-covalently on a 4MPBZ layer at pH 6.0. Panel A and B show the frequency and dissipation responses, respectively. Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Magenta dashed lines show when the potential was cycled at 0.91–0.31 V vs. SHE. Panel C (left) shows the O₂ chronoamperometric reductive current and Panel C (right) shows scans 1, 5, 10, 20 and 30 of 30 scans carried out. Panel D shows the Δd vs. Δf plot with each regime labelled. Panel E shows the frequency-activity (upper) and dissipation-activity plots (lower) for chronoamperometry. The arrows are to guide the eye and the numbers indicate the order in which the trace changes. Panel F shows frequency-activity and dissipation-activity plots for cyclic voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹. Flow of O₂ at 90 cm³ min⁻¹, seventh harmonic shown

II.4 Reductive desorption data for all surface modifiers

Table II.1 Summary of thiol modifiers of gold along with length of molecule (length from sulphur to furthest hydrogen atom, using Chem3DPro 12.0) and surface coverage (using reductive desorption voltammograms on a stationary gold electrode, with surface area 0.035 cm^2).

Thiol	Length (Å)	Surface coverage $\Gamma(\text{mol cm}^{-2})$	Desorption peak potential (V vs. SHE)
Benzenethiol 	5.7	3.09×10^{-10}	-0.741
4-hydroxythiophenol 	7.0	2.83×10^{-10}	-0.831
4-acetamidothiophenol 	9.9	2.34×10^{-10}	-0.826
4-mercaptobenzoic acid 	8.2	2.92×10^{-10}	-0.691
3-mercaptopropionic acid 	5.5	7.33×10^{-10}	-0.701
4-mercaptobutyric acid 	8.6	7.17×10^{-10}	-0.776
6-mercaptohexanoic acid 	11.6	6.51×10^{-10}	-0.836
8-mercaptooctanoic acid 	13.6	5.86×10^{-10}	-0.906
11-mercaptoundecanoic acid 	19.1	4.58×10^{-10}	-0.931

Appendix III Published manuscripts

The publication in *Energy and Environmental Science* has been included with full permission from the Royal Society of Chemistry.

The publication in *ChemCatChem* has been included with full permission from John Wiley & Sons Publishing.

Sources of activity loss in the fuel cell enzyme bilirubin oxidase†

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Electrochemical quartz crystal microbalance measurements with energy dissipation monitoring (E-QCM-D) show that the catalytic lifetime of the O₂-reducing multicopper oxidase bilirubin oxidase from *Myrothecium verrucaria*, adsorbed on or covalently attached to a organothiol-modified gold electrode, is shortened from days to hours when the applied potential is cycled to below a critical potential (0.6 V vs. SHE at pH 6.0)

Immobilised oxidoreductases show a loss in electrocatalytic activity with time, colloquially termed ‘film loss’, which limits their applicability to technological applications such as heterogeneous biocatalysis, biosensor development and fuel cells.^{1–4} We used the multicopper oxidase (MCO) bilirubin oxidase from *Myrothecium verrucaria* (MvBOx) as a model protein to dissect the mechanisms of film loss, using an electrochemical quartz crystal microbalance with dissipation measurement (E-QCM-D). This technique allows us to simultaneously measure the hydrated mass of the enzyme adsorbed on the sensor surface, its mechanical coupling to the sensor and its catalytic competence.

MCOs have been advanced as catalysts for the four-electron reduction of O₂ to water, a key reaction in low-temperature fuel cells.⁵ Bilirubin oxidases carry out this reaction at near-neutral pH values, at a lower overpotential than Pt,^{6,7} and can produce currents limited only by transport of O₂ into solution.⁶

Many methods exist for preparing oxidoreductases for heterogeneous electrocatalysis, including uses in enzyme-catalysed fuel cells.² A common and relatively straightforward method is *via* direct adsorption on graphite electrodes.⁸ The

Broader context

The multicopper oxidases are a family of copper-containing enzymes that includes laccases and bilirubin oxidases. They have been studied as efficient replacements for platinum and its compounds to catalyse the four-electron reduction of O₂ to H₂O in low-temperature, enzymatically catalysed fuel cells. Enzymes' specificity, selectivity against inhibition, and ability to function at near-neutral pH values mean they can generate power in living plants or animals. One of the barriers to the practical use of redox enzymes in fuel cells—as well as biosensors and industrially useful enzymatic transformations—is the proteins' slow loss in activity with time. We simultaneously measured electrocatalytic activity, adsorbed mass of enzyme, and the stiffness of this protein adlayer and concluded that structural rearrangements and water loss are the primary culprits in activity loss in these cathodes. The effect was most pronounced when we simulated a changing power load by cycling the potential. Covalent attachment did not mitigate activity loss. Enzyme-coated cathodes that receive their electrons directly from an electrode, rather than *via* redox mediators, will need additional stabilisation before they will be durable enough for miniature fuel cells from which a sustained power output is required.

roughness and heterogeneous surface chemistry of abraded graphite supports many enzymes for analytical protein film electrochemistry,⁹ but complicates any analysis of activity loss mechanisms. Immobilising enzymes in redox-active polymers,⁶ within porous electrodes¹⁰ or on high surface-area dispersions^{11,12} creates a reservoir of surplus enzyme that can mask underlying activity loss. In this work, the electrode is the surface of the QCM sensor, a flat, polished gold surface modified with the short-chain thiol 3-mercaptopropionic acid (3MPA) to produce a chemically homogeneous surface for stable adsorption of MvBOx and efficient direct electron transfer to it.^{13,14}

An E-QCM has been used previously to follow the adsorption and electrocatalysis of MvBOx on bare gold sensors,¹⁵ but their work was carried out in static conditions with dilute enzyme solutions, did not include dissipation analysis, and presented adlayer masses equivalent to ~10 layers of enzyme. Moreover, we have found the simultaneous energy dissipation analysis, which they could not do, is crucial to understanding the

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† Electronic supplementary information (ESI) available: Materials and methods; calculations; images of bare sensor surface; controls with bare gold, azide-inhibited MvBOx, BSA and TvL; variations in scan parameters and oxygenation; plots of Δd_f vs. Δf_r . See DOI: 10.1039/c3ee00043e



mechanisms of activity degradation, particularly when the applied potential is not constant. Also, Olejnik *et al.* used an E-QCM to study the activity and lifetime of covalently bound laccase, a related MCO, but they measured activity only under constant applied potential.¹⁶

Fig. 1 shows a typical adsorption of *MvBOx* on a 3MPA-modified gold-coated sensor. Buffer saturated with O_2 was passed continually through the measurement cell except at *ca.* 15 min when the enzyme was injected. For thin, rigid and evenly distributed layers, the mass adsorbed on a QCM sensor is proportional to the change in the resonant frequency of the crystal's shear vibration modes;¹⁷ in this work the proportionality constant equals $-17.9 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ for the fundamental frequency and -2.55 for the seventh harmonic, which was used for its sensitivity to changes close to the surface and insensitivity to variations in sensor mounting (see ESI†). The 300 Hz drop in the resonant frequency of the seventh harmonic (Fig. 1A) is consistent with the formation of a hydrated enzyme monolayer of protein on the sensor surface (see ESI†). At the

same time, the energy dissipation of the crystal increased to a maximum of about 3 ppm (Fig. 1B).

Dissipation is the sum of all energy losses in the system per oscillation cycle.¹⁸ Adsorption of proteins contributes towards this dissipated energy and this change can give insight into conformation changes during protein monolayer formation.¹⁹ Softer films experience more inelastic collisions than more rigid films. The formation of softer films, therefore, produces higher Δd values.

The surface of the QCM sensor also serves as a working electrode to quantify the catalytic activity of the protein film and any resistance to electron transfer from the sensor to the proteins' metal centres. The current is proportional to the areal density of the enzyme on the surface and its turnover frequency (see ESI†), thus providing a sensitive method to monitor catalytic activity at the same time as changes in mass and stiffness of the adlayer.

To probe how enzyme activity changes with time under conditions approximating constant load in a fuel cell, a potential of 0 V vs. Ag|AgCl|3 M NaCl was applied 7.5 min after the enzyme was injected and held constant for *ca.* 8 h. The applied potential is low enough to drive enzymatically catalyzed O_2 reduction, but too high for O_2 to be reduced on bare gold. An initial steady-state current of $-110 \mu\text{A}$ (Fig. 1C) was observed in comparison to near-zero reduction current in the presence of buffer saturated with argon (see ESI†). The current magnitude was up to $30 \mu\text{A}$ larger for the first 1.0–1.5 min while dissolved O_2 near the sensor surface was depleted. The initial turnover frequency of the enzyme (k_{cat}) under constant potential conditions is about 35 s^{-1} (see ESI†), about $7\times$ lower than values presented for *MvBOx* in the absence of mass-transport limitations,⁷ but more than $10\times$ higher than previous work using an EQCM setup.¹⁵ Over the course of the measurement, the current decayed to about 30% of its initial value.

Although the initial adsorbed mass of enzyme correlates with the initial magnitude of the catalytic current, subsequent changes in adsorbed mass with time do not parallel changes in catalytic activity. The trace in Fig. 1A is typical: the mass dropped by about an eighth over the first 2.5 h, then remained stable for the next 5 h at $(0.68 \pm 0.01) \mu\text{g cm}^{-2}$. In contrast, changes in energy dissipation with time show an almost linear correlation with catalytic activity (Fig. 1C inset). Like the current magnitude, the dissipation also drops by about 30% over time.‡ The changes of *MvBOx* on bare gold sensors are almost identical (see ESI†).

Thus, the term 'film loss' is not strictly accurate here: the slow loss of activity in *MvBOx* must be due to some non-desorptive process like structural changes or copper loss. The strong correlation between adlayer stiffness (inversely related to Δd) and activity implicates structural changes as the primary source of activity loss. The effect of applied potential on protein structure also appears in the plot of Δd_7 vs. Δf_7 (see ESI†), which normalises dissipation to adsorbed mass to quantify viscoelastic losses.^{19,20} The magnitude of $\partial d_7/\partial f_7$ is more than two times higher when a potential is applied: the protein layer stiffens substantially under applied potential.

To simulate the effect of a variable load on an enzymatic biofuel cell, complementary E-QCM-D measurements were

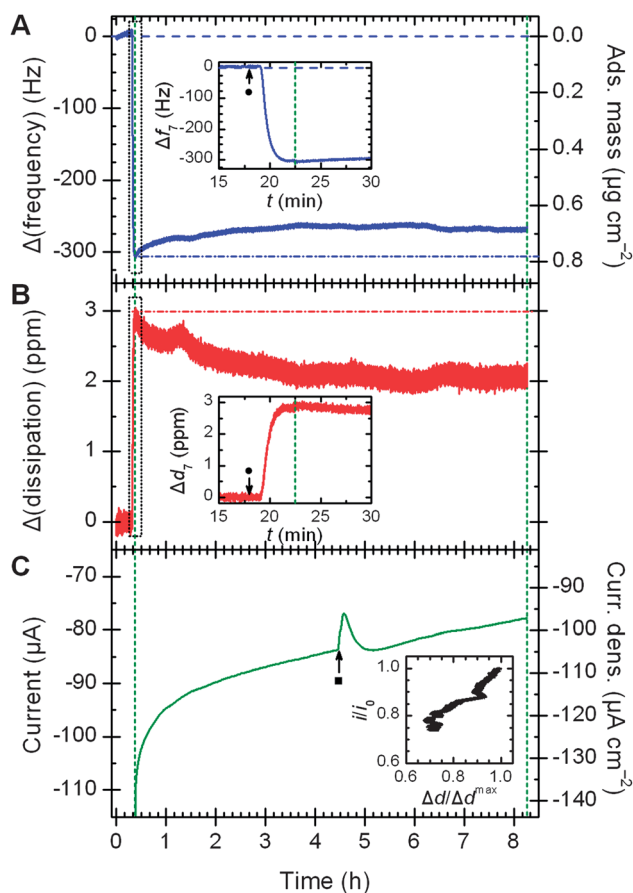


Fig. 1 E-QCM-D response (7th harmonic) with time for bilirubin oxidase adsorbed non-covalently on a 3-mercaptopropionic acid layer: (A) frequency change, (B) energy dissipation change, and (C) O_2 reduction current. Insets in (A) and (B) highlight sensor response after adding $25 \mu\text{l}$ 24 mg ml^{-1} enzyme (●). Green dashed lines show when a potential of 0.21 V vs. SHE was applied. Inset in (C) shows the correlation between dissipation value and electrocatalytic activity. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , projected electrode area 0.80 cm^2 . Flow rate of O_2 lowered for 10 min at ■.



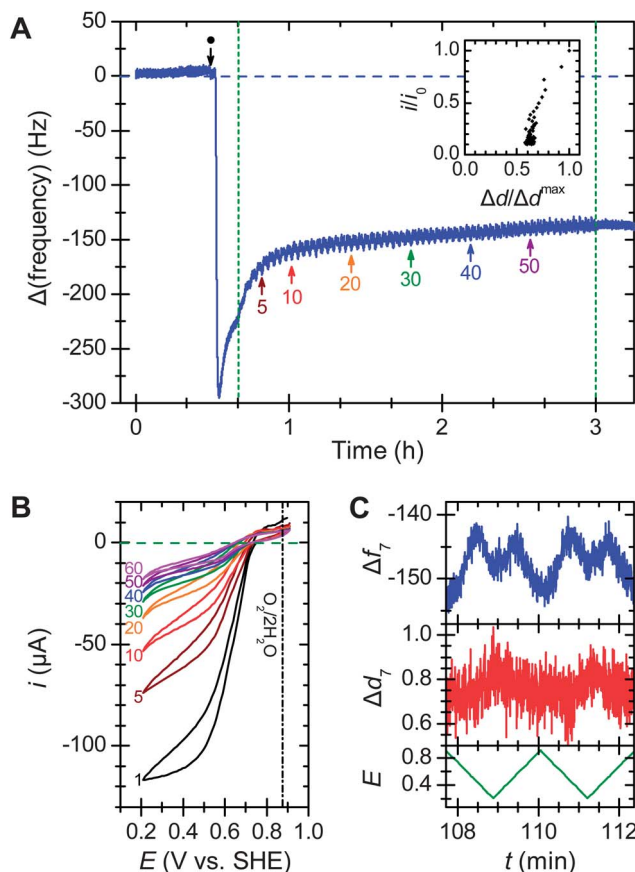


Fig. 2 Effect of potential cycling on bilirubin oxidase adsorbed on a 3-mercaptopropionic acid layer. (A) Frequency response from the addition of 24 mg ml⁻¹ MvBOx (●) followed by 60 potential cycles between 0.91 and 0.11 V vs. SHE at 10 mV s⁻¹ (green dashed lines); arrows mark the start of numbered cycles. Inset shows the relationship between enzyme activity (current at the lowest potential for that scan) and energy dissipation for each scan, normalized to values from scan 1. (B) Selected scans from the cyclic voltammetry. (C) Frequency (Hz) and dissipation (ppm) responses with applied potential (V vs. SHE) for scans 30 and 31. Conditions: O₂-saturated 0.1 M sodium phosphate buffer pH 6.0 at 25 °C flowing at 0.1 ml min⁻¹ with continuous O₂ bubbling; 7th harmonic.

recorded with the potential cycling (Fig. 2). In contrast to the relatively stable mass when the applied potential is held constant, cycling the potential causes an immediate drop in adsorbed mass (Fig. 2A): the mass typically drops by 20–25% over *ca.* 15 min. The catalytic current degrades simultaneously, falling by about a third in the same period (Fig. 2B).§ As in the constant potential cases, the correlation between activity and dissipation (Fig. 2A inset) was nearly linear, albeit with a non-zero intercept.

If the scan range is kept above 0.6 V vs. SHE, the pattern of mass and activity loss is like that for chronoamperometry. Decreasing the scan rate to 1 mV s⁻¹ led to the same mass loss but only about half the activity loss. Changing the step size had no significant effect on either current loss or mass loss (see ESI†).

After about 10–20 potential cycles, the frequency trace stabilises, but the O₂ reduction activity approaches zero. A QCM measures hydrated mass and about 45% of the protein's mass is entrained water (see ESI†) that contributes to frequency

changes of the sensor,^{19–21} suggesting that the enzyme adlayer extensively dehydrates during potential cycling. However, in this case some desorption cannot be ruled out.

When the potential was cycled, both the frequency and dissipation traces showed periodic changes that were slightly out of phase with the applied potential (Fig. 2C). The frequency traces showed multiple peaks and a maximum peak-to-peak amplitude of *ca.* 9 Hz (0.02 μg cm⁻²), about 3% of an enzyme monolayer. The varying potential or electric field near the surface may repeatedly distort the adsorbed protein, causing it to repeatedly expel mass in the form of water or ions, and hastening its denaturation. This hypothesis is consistent with STM observations on single molecules of another MCO, laccase from *Streptomyces coelicolor*, that showed different heights for reduced and oxidised enzyme on a thiol-modified gold electrode.¹⁴

To control for the possibility that diminishing activity is due to enzyme desorption, MvBOx was covalently linked to a thiol-modified gold crystal *via* an amide linkage between an activated carboxylate on the surface and one of the lysine side chains on the protein's exterior (Fig. 3), identical to EDC-NHS-type coupling reactions. After uncoupled enzyme was washed away, the potential was cycled. Unlike the adsorbed samples, the mass dropped by only 6% in the first 7 cycles then stabilised between 0.68 and 0.70 μg cm⁻². Despite this, the catalytic activity still

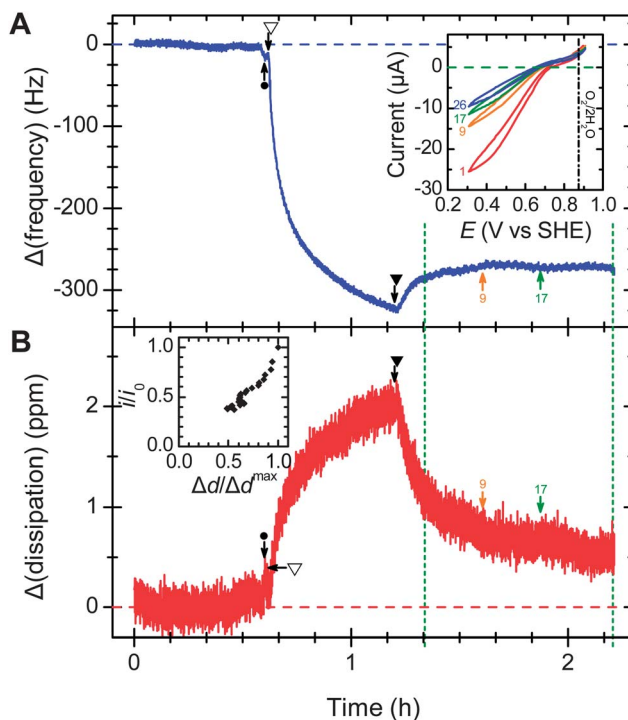


Fig. 3 Effect of potential cycling on (A) frequency response and (B) dissipation response for bilirubin oxidase covalently bound *via* an amide linkage to a thiol-modified QCM sensor. After addition of 25 μL of 24 mg ml⁻¹ MvBOx (●), buffer flow stopped (▽) to allow adsorption and reaction with surface layer (reaction adapted from the literature)²² then resumed (▼) for *ca.* 10 min before voltammetry. Potential cycled 26 times between 0.91 and 0.31 V vs. SHE (green dashed lines). Arrows indicate start of selected cycles and correspond to numbering in top inset. Lower inset relates activity and dissipation as in Fig. 2. Other conditions as in Fig. 2.

dropped off rapidly (Fig. 3A inset), diminishing by 80% in less than 1 h. The activity *versus* dissipation trace (Fig. 3B inset) displayed the same characteristic correlation, again suggesting that structural changes are responsible for activity loss, even if they are not accompanied by a large mass loss.

Covalent attachment did not stabilise the catalytic activity. It also decreased the efficiency of electron transfer from the electrode to the enzyme. The position of E_{cat} , a characteristic potential defined by the maximum of the change in current with potential on a swept-potential voltammogram, shifts by -0.1 V, meaning that the covalently attached enzyme needs a greater driving force (~ 10 kJ mol $^{-1}$) to drive electrocatalysis compared to the adsorbed sample. The increased charge-transfer resistance is also evident in the shape of the voltammograms in the inset of Fig. 3A: a more linear, ohmic response dominates the sigmoid shape characteristic of electrochemical-chemical (EC) reactions (compare to Fig. 2B).

Reactive dye labelling measurements identified three reactive lysine side chains on MbOx through which the enzyme could couple to the surface (see ESI†). All of these groups position both of the enzyme's electron acceptors more than 2 nm from the electrode surface, too far for interfacial electron transfer rate compatible with biochemical processes.²³

Controls using catalytically inactive bovine serum albumin (BSA) on 3MPA-layers showed stable frequency and dissipation traces when the potential was cycled.

The structures of the high-potential MCOs used for fuel cell cathodes are similar,²⁴ so the patterns of activity loss are likely to be the similar for all three-domain MCOs. For example, laccase from *Trametes versicolor* (TvL) showed similar patterns of mass and activity loss, though the magnitudes differed (ESI†). The observed behaviour depends on protein structure and electrode composition, however. For example, covalent attachment by amide linkage stabilised a [NiFe] hydrogenase over 2 h in response to potential steps up to 250 mV,²⁵ and covalently bound [FeFe] hydrogenases showed activity decay time constants ranging from hours to days depending on immobilisation method.¹ in activity that was attributed to fast desorption and slower denaturation processes.

Also, an enzyme can be stabilised by its immediate environment: surface modifications and buffered polyelectrolytes let some fuel cell enzymes retain some activity for months.^{3,26} The current goal, therefore, is to relate extended catalytic stability to enzyme structure and the enzyme's interface with the electrode and other surroundings.

Conclusions

We applied E-QCM-D as a real-time probe of the potential-dependent stability of enzymes used as catalysts in fuel cell cathodes. The catalytic activity of MbOx drops rapidly when the applied potential is cycled to simulate variable loads on a fuel cell cathode. Although this activity loss was accompanied by some loss of mass from the adlayer, covalent attachment did not improve catalytic stability and also resulted in lower power output because of longer distances for interfacial electron transfer. Catalytic activity decreased even when the adlayer's

mass remained stable, such as when the applied potential was constant. Decreasing catalytic activity always correlated with increasing layer stiffness, implicating structural rearrangements over desorption as the primary mechanism of activity loss. These observations suggest that without additional treatment or overpotential limits, MCO-coated cathodes may be unsuitable for long-term power generation in enzymatically catalysed fuel cells.

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Notes and references

† The dissipation trace for enzyme loadings greater than about 0.6 mg (10 nmol) initially spikes but returns to a stable baseline in about 10 min, indicative of loosely bound protein (ESI†). The value of Δd^{max} is taken from the post-spike baseline.

§ Reduction current magnitudes above 110 μ A produced CV scans with a flattened shape indicative of mass-transport polarisation, so the current at 0.4 V vs. SHE on the reductive sweep was used for activity loss calculations.

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Electronic Supplementary Information for

Sources of activity loss in the fuel cell enzyme bilirubin oxidase

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Section S1 Material sources

Bilirubin oxidase from *Myrothecium verrucaria* (MvBOx) was obtained from Amano Enzyme. The enzyme was purified as for protein crystallization.¹ Lyophilized MvBOx powder was dissolved in 10 mM sodium phosphate, pH 7.0 at 4 °C, then centrifugally dialyzed to remove small-molecular-weight contaminants (Vivaspin, 30 kDa MWCO PES). This was then further purified by weak anion exchange (DEAE Sepharose) and by hydrophobic interaction (HiTrap Phenyl Sepharose) chromatography at 4 °C. The protein was eluted using 10 mM sodium phosphate, pH 7.0 at 4 °C, with a 0–0.4 M ammonium sulfate gradient for the anion exchange, and 50 mM sodium phosphate, pH 6 at 4 °C, with a 1.25–0 M ammonium sulfate reverse gradient for hydrophobic interaction. Typical yields of purified protein were $\approx 3\%$ by mass. Protein concentrations were determined using the Beer-Lambert law based on $A_{280\text{nm}}$ measured from enzyme aliquots and using $\epsilon_{280\text{nm}} = 9.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, calculated from the enzyme amino acid sequence by the online software ProtParam – ExPASy Proteomics Server (<http://www.expasy.ch/tools/protparam.html>).

Laccase from *Trametes versicolor* (TvL) was obtained as a crude powder from Sigma. The crude powder was dissolved in 10 mM sodium acetate, pH 5.5 at 5 °C, then filtered through two filters (GFA, 0.45 μm , 0.22 μm). This was then centrifugally dialyzed to remove small-molecular-weight contaminants (Vivaspin, 30 kDa MWCO PES). This was then further purified by weak anion exchange (Q Sepharose, 10 mM sodium acetate, pH 5.5 at 5 °C, with a 0–0.1 M ammonium sulfate gradient) followed by hydrophobic interaction (HiTrap Phenyl Sepharose, 20 mM sodium phosphate, pH 4.7 at 5 °C, with a 1.65–0 M ammonium sulfate reverse gradient) at 5 °C.

Dimethyl sulfoxide (DMSO, $\geq 99.9\%$, Fisher), hydroxylamine hydrochloride ($>97\%$, Fisher), hydrochloric acid (37%, Fisher), sodium dihydrogen orthophosphate dihydrate (99.0–101.0%, Fisher), disodium hydrogen orthophosphate anhydrous (99.5+%, Fisher), trisodium citrate dihydrate ($\geq 99\%$, Sigma), citric acid monohydrate ($\geq 99\%$, Sigma), ammonium bicarbonate ($\geq 99.5\%$, Sigma), iodoacetamide ($\geq 99\%$, Sigma), dithiothreitol (DTT, $\geq 99.5\%$, Sigma), trypsin (from porcine pancreas, $>10,000$ units/mg, Sigma), pepsin (from porcine gastric mucosa, ≥ 250 units/mg, Sigma), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, $>98\%$, Sigma), sulphuric acid (1.83 g mL^{-1} specific gravity, $>95\%$, Fisher), hydrogen peroxide (30% w/v, Scientific Laboratory Supplies), 3-mercaptopropionic acid (3MPA, $>99\%$, Fluka), 3-mercapto-1-propanol (3MPOL, 95%, Sigma), 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP, $>98\%$, ProChem Inc.). The *N*-hydroxysuccinimide ester of fluorescein-5-EX (F5EX, CAS no. 1196157-68-2) was purchased from Sigma Aldrich, stored in the dark at -20 °C. All chemicals were used as received without further purification. All

water was purified via reverse osmosis and ion exchange to a resistivity of 18.2 MΩ cm at 25 °C using a MilliQ water purifier.

Section S2 Equipment

QCM-D data were acquired using a Q-Sense E1 system with accompanying Q-Soft data acquisition software. QCM-D measurements without concurrent electrochemical analysis used a Q-Sense Flow module 401 (total volume ~140 μl, volume above sensor surface ~40 μl, non-sensor surfaces Ti or Viton). E-QCM-D measurements used a modified Q-Sense QEM401 module (volume above sensor surface ~100 μl). The PTFE insert on the electrochemistry module was replaced by a custom-made insert made of poly(oxomethylene) (Delrin) for better wettability, to accommodate a standard 5.7 mm OD reference electrode, and to provide a more robust seal when liquid was pulled through the cell. The manufacturer's value for typical mass sensitivity in liquid is 1.8 ng cm⁻²,² equivalent to <0.3% of a monolayer of *MvBOx*.

The sensors (Q-Sense) were 14mm diameter, gold-coated, AT-cut quartz crystals with a Ti adhesion layer and a fundamental frequency of (4.95 ± 0.05) MHz. All the frequency and dissipation changes presented are from the seventh harmonic of the fundamental frequency (35 MHz, $C = 125 \text{ ng cm}^{-2} \text{ Hz}^{-1}$). This harmonic is less affected by crystal mounting and small mechanical vibrations when changing solutions, is more surface sensitive, and still gives a good signal-to-noise ratio compared to the higher frequencies. The measured temperature of the QCM cell was constant within 10 mK. The dependence of the fundamental frequency on temperature (i.e., $d(\Delta f/f)/dT$) of AT-crystals at 25 °C is $-3.3 \times 10^{-7} \text{ K}^{-1}$,³ so temperature variations should not lead to frequency variations greater than 16 mHz. In practice, however, we observed larger variations in frequency and dissipation, usually in the range of ±7–14 Hz and ±0.5 ppm for Δf_7 and Δd_7 , respectively. The sensor surface is remote from the point at which the E1 system measures the temperature, so the entire liquid handling system (cell, reservoir, tubing and pump) was placed on top of an aluminium plate through which water held at 25 °C was circulated, then surrounded by an insulated box in which the air was circulated by an electric fan. The thermal coupling between the flow modules and QCP101 chamber platform was improved with thermal conducting paste (RS components, RS 217-3835). Other sources of variation arise from the mounting of the crystal in the cell and the effect of the repeated cleaning procedure on the gold surface.

Enzyme was introduced to the cell by stopping the pump, reversing the flow for a few seconds to produce a suspended drop at the end of the entry tube, placing the tube in the enzyme solution and restarting the pump in the forward direction. After enzyme was introduced, the procedure was repeated to flow buffer through the cell. Each exchange took less than 1 min. The dead time for liquids to flow from the inlet to the cell was 2 min.

The electrochemical cell used a three-electrode configuration: the gold-coated QCM sensor (exposed geometric area of 0.80 cm^2) as the working electrode; a 0.2mm thick, 2.25 cm^2 square platinum plate running parallel to the sensor surface as the counter electrode; and a Ag|AgCl|3M NaCl reference electrode (BASi RE-6, $E_{\text{SHE}} = E_{\text{ref}} + 0.209\text{ V}$). The distance between the working electrode and counter electrode was 0.7–0.8 mm; the distance between working and reference electrode was 0.9–1.0 mm. The potential was set and the current was measured by an Ivium CompactStat, using the accompanying IviumSoft control software.

Buffer was kept in a sealed glass bottle and oxygenated for 20 min before each experiment. Increasing the pump speed produced moderate increases in current. For example, an O_2 -reduction current of $-69\text{ }\mu\text{A}$ at 0.1 ml min^{-1} increased by 9% when the flow rate was increased to 0.3 ml min^{-1} . No significant current increase was seen upon increasing the flow rate to 0.5 ml min^{-1} and flow rates greater than 0.5 ml min^{-1} mechanically disrupted the stability of the electrochemical cell. (Conditions for example: $25\text{ }\mu\text{l}$ of 12 mg ml^{-1} $MvBOx$, $25\text{ }^\circ\text{C}$, 0.1 M sodium phosphate pH 6.0, 0 V vs. reference.)

Section S3 QCM sensor surface treatment and protein adsorption

Section S3.1 Cleaning

Residual contamination on new QCM sensors was removed by placing them under a ozone-generating UV lamp in air for 30 min. Sensors were reused by oxidizing the surface with piranha solution (3:1 v:v conc. H_2SO_4 :30 wt% H_2O_2) for 20 min with constant heating at 60°C , followed by rinsing in water. The crystals were dried under a stream of house nitrogen from boil-off, then ozone treated as described.

Section S3.2 Organothiol surface modification

Surface modification solutions consisted of either 10 mM DTSSP, 10 mM 3MPA or 10 mM 3MPOL mixed with 1.3 mM TCEP in ethanol. Solutions were sonicated for 20 min. before freshly cleaned sensors were immersed for 15 h under a nitrogen atmosphere (for 3MPA) or 3 h in air (for DTSSP). (Consistent with the reports of Z. Cao and co-workers, we have observed gold dissolution by QCM from oxygenated thiol solutions.)^{4,5} Surface coverage of the thiol layer was determined by reductive desorption in basic anaerobic aqueous solution,⁶ giving surface coverages between 9.6×10^{-11} and $6.7 \times 10^{-10}\text{ mol cm}^{-2}$, depending on the size and length of the thiol layer. After surface modification, the crystals are rinsed with water and ethanol, dried in a stream of nitrogen gas, then mounted in the E-QCM cell.

Section S3.3 Introduction of protein to the cell

The fluid flow through the cell is controlled via peristaltic pump located and operated outside the cell, at a rate of 0.1 ml min^{-1} . The pump was stopped and the inlet tubing switched to a microcentrifuge tube containing $25\text{ }\mu\text{L}$ of protein at the desired concentration. After addition, the pump was temporarily switched

to flow in an anticlockwise to eliminate air bubbles from the flow path. At this point the pump was turned back to clockwise and the fluid flow was resumed.

Section S3.4 DTSSP coupling

The addition of protein was done in the same way however upon the QCM-D response in the frequency and dissipation traces, the peristaltic pump was turned off for 45 min to allow for the succinimidyl groups to react with the lysine residues of *MvBOx*. After the reaction had proceeded the pump was turned back on and fluid flow resumed.

Section S4 Relating electrocatalytic current to enzyme activity

Electrocatalytic activity is related to the areal density of the enzyme on the surface and its turnover frequency according to Eqn. S1:

$$i = -n F A \Gamma k_{\text{cat}} \quad (\text{S1})$$

where i is the catalytic current (negative for reductive processes by convention), n is the number of electrons involved in the reaction (4 for the reduction of O_2 to $2\text{H}_2\text{O}$), F is Faraday's constant, A is the electroactive surface area of the electrode accounting for surface roughness ($0.80 \text{ cm}^2 \times 1.6$, see Section S7), Γ is the coverage of the enzyme (4.2 pmol cm^{-2} for a saturated monolayer, see Section S8), and k_{cat} is the enzyme's intrinsic rate constant/turnover frequency.

Section S5 Degree of labelling and reactive lysine determination

Section S5.1 Methods

Section S5.1.1 Fluorescein 5-EX (F5EX) labelling

MvBOx was labelled with an amine-reactive fluorophore based on a procedure from Molecular Probes (now Invitrogen).⁷ Aliquots of 15.2 mM F5EX in DMSO were mixed with aliquots of 0.24–0.25 mM *MvBOx* in 0.1 M sodium bicarbonate pH 8.3 to give dye:protein molar ratios of up to 72. The total volume of the reaction mixture was 110 to 200 μl . The mixture was incubated in the dark with stirring at room temperature for 1 h, then 0.1 ml of freshly prepared 1.5 M hydroxylamine pH 8.5 was added to conjugate to unreacted F5EX and stop further reaction with the protein. The protein was separated from unconjugated dye by dialysis through a 30 kDa MWCO filter (Vivaspin, GE Healthcare).

The degree of labelling (DoL) was determined spectrophotometrically ($\lambda_{\text{max}}^{\text{F5EX}} = 494 \text{ nm}$) in a 1 cm path length quartz cuvette and Eqn. S2:

$$\text{DoL} = \frac{A_{494 \text{ nm}}^{\text{mix}} \epsilon_{280 \text{ nm}}^{\text{MvBOx}}}{(A_{280 \text{ nm}}^{\text{mix}} - \text{CF } A_{494 \text{ nm}}^{\text{mix}}) \epsilon_{494 \text{ nm}}^{\text{F5EX}}} \quad (\text{S2})$$

where $A_{280\text{ nm}}^{\text{mix}}$ and $A_{494\text{ nm}}^{\text{mix}}$ are the recorded optical densities for the labelled protein at 280 nm and 494 nm, respectively, $\epsilon_{494\text{ nm}}^{\text{F5EX}} = 6.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{280\text{ nm}}^{\text{protein}} = 9.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and the correction factor (CF) = 0.30. The correction factor is the ratio of the absorbance of the free dye at 280 nm to its absorbance at λ_{max} (i.e., $\epsilon_{280\text{ nm}}^{\text{F5EX}}/\epsilon_{494\text{ nm}}^{\text{F5EX}}$). The values for the dye were recorded at pH 8.0.

Section S5.1.2 Trypsin digest

Solutions of 1 mg ml⁻¹ MvBOx, 100mM dithiothreitol (DTT, for reduction of disulphide bonds in the protein), 100mM iodoacetamide (to cap free cysteine thiolates), and 20 µg ml⁻¹ trypsin were made in 50mM ammonium bicarbonate. 2.1 µl of the DTT solution was added to 50 µl of the protein solution, the mixture was vortexed then incubated for 45 min at 37 °C. After the solution returned to room temperature, 8.7 µl of the iodoacetamide solution was added, the mixture vortexed again, then incubated in the dark for 45 min. at room temperature. A further 2.2 µl of DTT solution was added, the mixture vortexed, then incubated at 37 °C for 10 min. 20 µl of trypsin solution was added to the mixture, which was then incubated for 12 h at 37 °C. Final solution frozen at -21 °C and stored for 7 d until mass spec analysis.

Section S5.1.3 Pepsin digest

50 µl MvBOx (15 mg ml⁻¹, 0.1M sodium phosphate buffer pH 6.0) was acidified with 20 µl 0.04M HCl. To this solution, 20 µl 0.3 mg ml⁻¹ pepsin in water was added (71:1) and the mixture vortexed briefly. The mixture was incubated for 12 h at 37 °C, heated to 95 °C for 15 min. then cooled to room temperature and frozen at -21°C.

Section S5.1.4 MALDI mass spectrometry

All MALDI mass spectrometry experiments were carried out on an Ultraflex II instrument (Bruker Daltonics) in positive reflectron mode. A solution of matrix (α -cyano-4-hydroxycinnamic acid) matrix was applied to the protein sample and allowed to dry before analysis.

The spectra taken of the protein after the trypsin digest are plotted in Figure S1. Peaks were labelled according to the conventions of Roepstorff and Fohlman⁸ and Johnson and co-workers,^{9, 10} guided by the tutorial article from Wysocki and co-workers.¹¹ These assignments are summarized in Table S1 and Table S2. No differences were observed in the peak positions after the pepsin digest.

Section S5.2 Results and Analysis

At a dye-to-protein molar ratio of ca. 25, the degree of labelling saturated at about one dye molecule per protein molecule (DoL = 1.09 ± 0.10 , Figure S1 inset). The main new contributions to the mass spectrum of the labelled protein were the peaks shown in Figure S1B, which correspond to Lys408 and Lys118. Small peaks appeared in fragments containing labelled Lys181 (Figure S1E) and Lys21 or Lys214 (Figure S1F). Small peaks disappeared from the spectrum of the unlabelled protein corresponding to Lys429 (Figure S1D)

and Lys44 (Figure S1E&G). Trypsin cleaves at Arg and Lys residues, so the F5EX labels will always be at the C-terminus of any peptide fragment. So, with the exception of the doubly charged a21 fragment in Figure S1F, a, b and c ions are not labelled. There was no definitive pair of labelled peaks corresponding to the appearance of one shifted by 475.07 Da and the disappearance of its parent.

Most of the lysines can be ruled out: of the 11 lysines in the sequence of *MvBOx* (Figure S2), three do not appear differently in the labelled and unlabelled samples: Lys220, Lys251 and Lys314. Lys118 and Lys429 have $<2.5 \text{ \AA}^2$ of their atoms on the protein surface (using the FindSurfaceResidues.py algorithm on PyMOLwiki).¹² Although Lys118 is accessible to react with dye molecules, it is too buried to couple to surface groups.

The strongest evidence points to the most reactive exposed lysine side chain being Lys408, with some reactivity in Lys 21 and Lys181. All three groups are more than 1.5 nm from the enzyme's active sites, too far for tunnelling rates compatible with biological processes:¹³ Lys408 is 2.59 nm from the type 1 copper (considered the primary electron acceptor)¹⁴ and 2.15 nm from the type 2 copper; Lys21 is 3.23 nm from the type 1 and 2.03 from a type 3 copper; and Lys181 is 1.94 nm from the type 1 copper. These distances were measured from the terminal nitrogen atom.

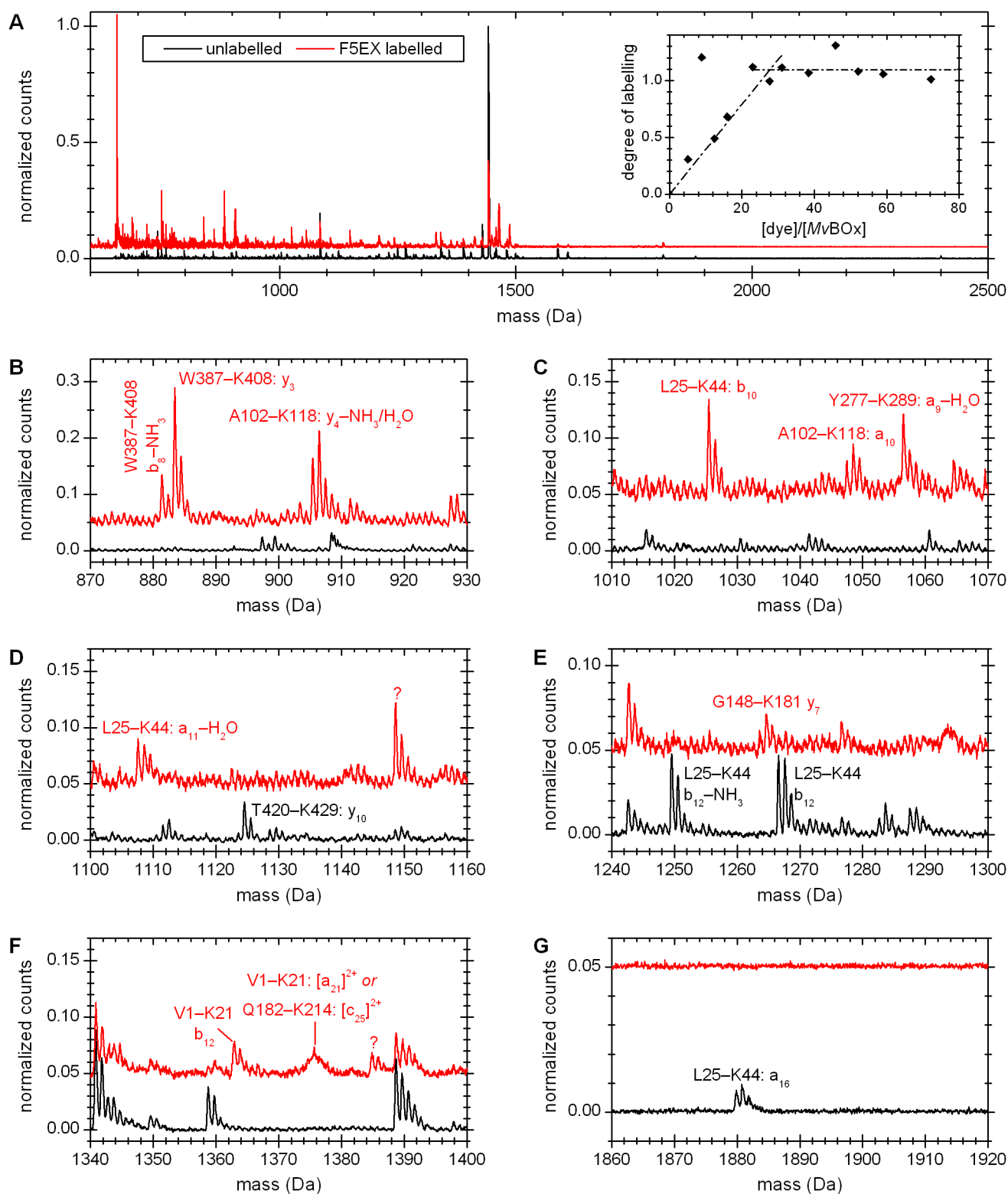


Figure S1. Determination of the location of reactive lysine side chains in MvBOx by reaction with fluorescein 5-EX (F5EX). Panel A compares the full MALDI mass spectra of enzyme that has been labelled with F5EX (red) and that which has not (black). The inset shows the degree of labelling as a function of enzyme concentration. The lines are to guide the eye. Panels B–F show specific regions where the two mass spectra differ. Peak labels give the peptide sequence and ion type.⁸⁻¹⁰ The full scale for the unlabelled and labelled spectra are 2.9×10^4 and 1.9×10^4 counts, respectively.

Table S1. Additional peaks in MALDI mass spectrum of fluorescein 5-EX-labelled *MvBOx* compared to unlabelled *MvBOx* after trypsin digest

Mass (Da)	Fragment	Assignment
881.4	W387–K408	$b_8\text{--NH}_3$
884.4	W387–K408	y_3
905.4	A102–K118	$y_4\text{--H}_2\text{O}/\text{NH}_3$
1025.5	L25–K44	b_{10}
1048.5	A102–K118	a_{10}
1056.5	Y277–K289	$a_9\text{--H}_2\text{O}$
1107.6	L25–K44	$a_{11}\text{--H}_2\text{O}$
1148.5	unknown	
1264.6	G148–K181	y_7
1362.8	V1–K21	b_{12}
1374.	V1–K21	a_{21}^{2+}
	Q182–K214	c_{25}^{2+}
1384.9	unknown	

Table S2. Missing peaks in MALDI mass spectrum of fluorescein 5-EX-labelled *MvBOx* compared to unlabelled *MvBOx* after trypsin digest

Mass (Da)	Fragment	Assignment
1124.5	T420–K429	y_{10}
1249.5	L25–K44	$b_{12}\text{--NH}_3$
	Q182–K214	c_{25}^{2+}
1266.6	L25–K44	b_{12}
1879.9	L25–K44	a_{16}

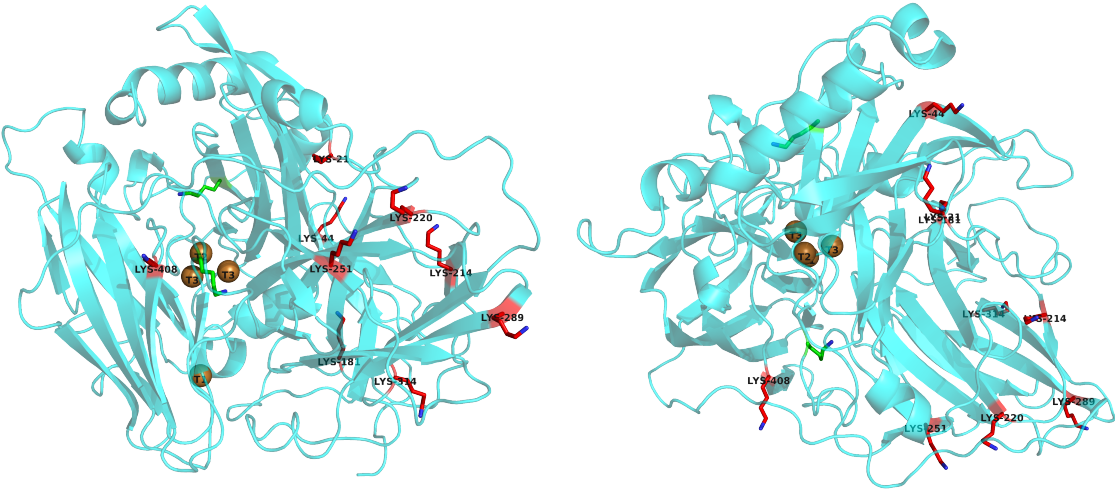


Figure S2. PyMOL visualization of the location of the lysine residues in *MvBOx* (PDB code 2xll).¹ The polypeptide backbone is shown as the cyan cartoon. The coppers are shown as spheres labelled with their spectroscopic designation. The nine lysine side chains that have at least 2.5 Å² on the surface are in red; the two that do not are in green. The image on the right is rotated 90° along a horizontal axis.

Section S6 Effect of protein concentration on protein adsorption isotherm

Oxygenated buffer was flowed over gold-coated crystals modified with 3-mercaptopropionic acid (Figure S3) and those with no surface modification (Figure S4) until a steady baseline was observed in the frequency and dissipation traces. For each concentration, 25 μL of enzyme was flowed through the cell. Both sets show a maximum frequency change for the seventh harmonic of 275–325 Hz ($0.70\text{--}0.83\text{ }\mu\text{g cm}^{-2}$, cf. Section S8). The dissipation traces are markedly different: the adlayer on bare gold initially has a much higher viscoelasticity (i.e., higher dissipation) then stiffens within 1–2 min. There is also a much sharper decrease in mass after adsorption on bare gold. These are both characteristics of substantial dehydration and denaturation of the protein.

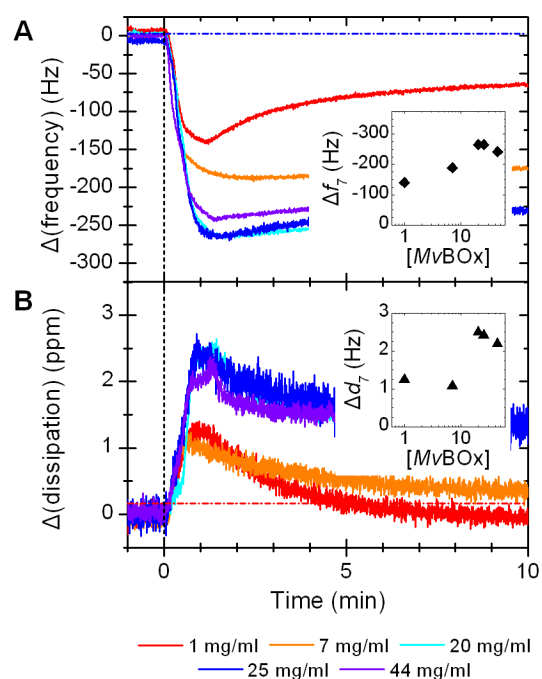


Figure S3. The effect of enzyme concentration on adsorption onto 3-mercaptopropionic acid–modified gold-coated quartz crystals. Panel **A** shows the frequency change at different concentrations of enzyme. Panel **B** shows dissipation change at different concentrations. The insets show the relationship between concentration and highest magnitude of frequency and dissipation change. Conditions: 7th harmonic, 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^\circ\text{C}$, 0.1 ml min^{-1} .

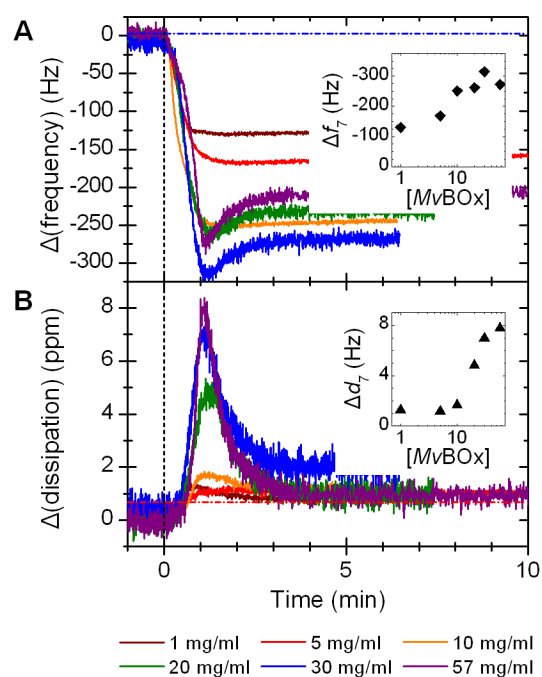


Figure S4. The effect of enzyme concentration on adsorption onto bare gold-coated quartz crystals (i.e., without organothiol modification). Panel **A** shows the frequency change at different concentrations of enzyme. The inset in panel **A** shows the relationship between concentration and peak frequency change. Panel **B** shows the dissipation change at different concentrations. The insets show the relationship between concentration and highest magnitude of frequency and dissipation change. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹

Section S7 Electroactive area (surface roughness) determination

The accessible surface area of the gold-coated QCM sensors was determined by the electrochemical formation and stripping of a gold oxide monolayer in solution according to an IUPAC method.¹⁵ The QCM crystal was suspended in 0.1 M H₂SO₄, typically immersing 90 mm² of the gold surface. The potential was cycled at 50 mV s⁻¹ from 0.45 V to 1.6 V (vs. Ag|AgCl|3 M NaCl) until a stable voltammogram was recorded (30 cycles). The potential was then cycled once over the same range at 10 mV s⁻¹ and the charge transferred was calculated from the stripping peak at about 1.08 V vs. ref, corrected with a linear baseline (Figure S5). Backscattered electron images of the sensor surface (Figure S6) appeared to show a polycrystalline surface with a profusion of 5 μm-wide cracks. We used the IUPAC-recommended value of 390 ± 10 μC cm⁻² to relate the area of the stripping peak to the surface area of the gold accessible to oxygen.¹⁵ The ratio of the electrochemically accessible surface area to the geometric surface area (roughness factor) was 1.5–1.6. These roughness factors are consistent to those reported by Kamitaka et al. for their Seiko sensors (1.5–1.7).¹⁶

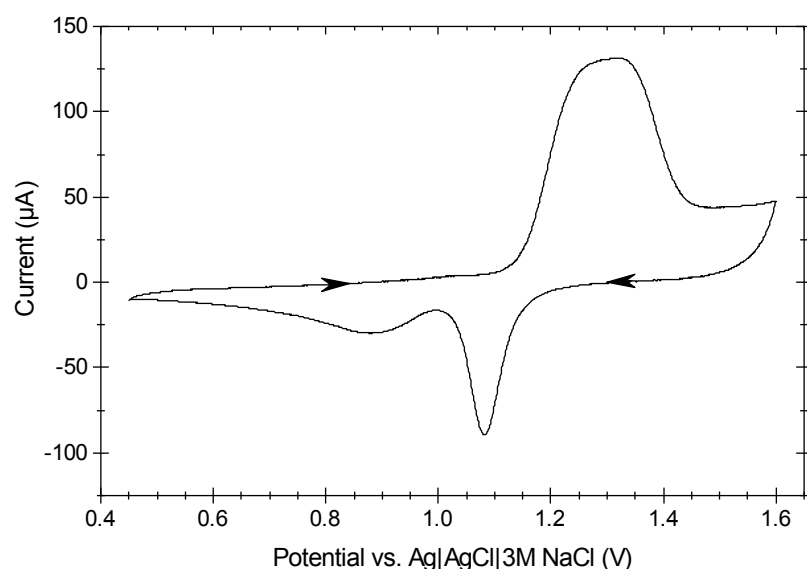


Figure S5. A representative example of the final cyclic voltammogram associated with gold oxide formation and stripping on a Q-Sense QCM sensor.

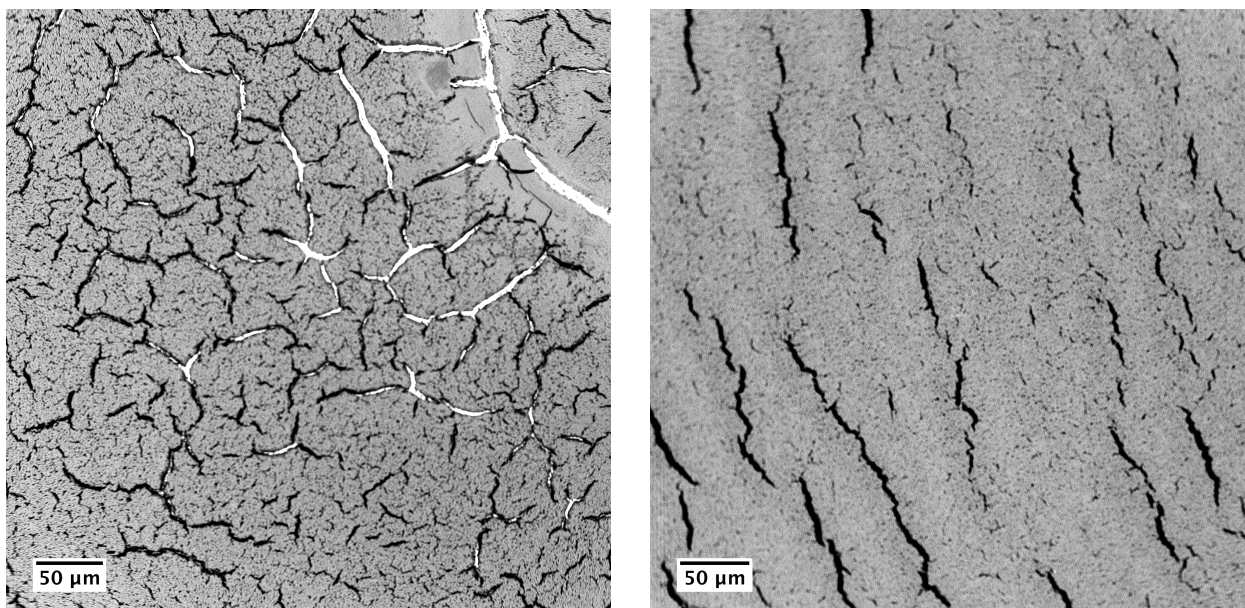


Figure S6. Representative scanning electron micrographs taken of the surface of a Q-Sense gold-coated QCM sensor. The image on the left was taken near the crystal centre and the right image was taken toward the edge of the crystal. The crystals were new and were prepared by leaving under an ozone-generating lamp for 20 min. The above images are from a backscattered electron SEM (Phenom Pro G2 desktop SEM).

Section S8 Estimation of the mass required for surface saturation

A first estimate of the frequency change expected from a saturated monolayer of *MvBOx* can be obtained by simply dividing the QCM sensor's active area by the macromolecule's footprint (ca. 40 nm², based on PDB 2xll),¹ then applying the Sauerbrey equation:³

$$\Delta m = -\frac{\sqrt{\rho_q \mu_q}}{2 f_1^2 N} \Delta f_N \quad (\text{S3})$$

$$\Delta m = -\frac{C}{N} \Delta f_N \quad (\text{S4})$$

where Δm is the mass adsorbed, ρ_q and μ_q are the density and shear modulus of AT-cut quartz (2.648 g cm⁻³ and 2.947×10^{11} g cm⁻¹ s⁻², respectively), f_1 is the fundamental frequency of the sensor (4.95 ± 0.05 MHz), N is the harmonic of the sensor, and Δf_N is the frequency change of that harmonic. For the Q-Sense E1, the proportionality constant, C , is 17.87 ng cm⁻² Hz⁻¹.

Table S3. Contributions to the hydrated mass of *MvBOx*

Component	Mass (Da)
peptide chain	59955
copper ions four per macromolecule	254
ordered glycans four <i>N</i> -linked <i>N</i> -acetylglucosamines per macromolecule	812
water $V_{\text{cell}} = 631.75 \text{ nm}^3$, 4 macromolecules/cell assumes 53.3% solvent, $\rho_{\text{water}} = 10^{-21} \text{ g nm}^{-3}$ (of which there are 316 ordered water molecules per macromolecule = 5922 Da)	50520
<i>total</i>	111541

Monolayer coverage of close-packed *MvBOx* macromolecules on a perfectly smooth sensor surface is 4.2 pmol cm^{-2} . Corrections must be applied for the roughness of the sensor surface (Section S7) and the mass of the water that is mechanically coupled to the protein. We assumed that solvent content of the three-dimensional X-ray structure could approximate the mass of water in a closely packed monolayer of the protein to calculate a hydrated mass of 111.5 kDa (Table S3). This gives a maximum adsorbed mass on a smooth electrode of $0.47 \text{ } \mu\text{g cm}^{-2}$ ($\Delta f_1 = -26.0 \text{ Hz}$, $\Delta f_7 = -182 \text{ Hz}$), and $0.78\text{--}0.80 \text{ } \mu\text{g cm}^{-2}$ on our electrodes ($\Delta f_1 = 44 \text{ Hz}$, $\Delta f_7 = -306\text{--}308 \text{ Hz}$, 7.2 pmol cm^{-2}). The latter values are consistent with the observations presented in the main text.

Section S9 Enzyme adsorption on a bare gold sensor

The measurements shown in Figure S7 parallel those shown in Figure 1 in the main text. The change in frequency occurring here is to a lesser extent than with the 3MPA-modified gold surface and the magnitude of catalytic current is also lower, within the normal range of variation we observed. When a chronoamperometric potential is applied ($\sim 28 \text{ min}$) there is a large apparent mass loss ($\sim 25 \text{ Hz}$, $0.06 \text{ } \mu\text{g cm}^{-2}$) which may be due to removal of loosely bound enzyme or mechanical effects (cf. Figure 2 in the main text). The short, horizontal portion of the current vs. dissipation trace (Figure S7 inset) arose from a short increase in dissipation after the potential was turned on, after which the trace returned to its more typical linear correlation with a slope near 1. The first-order time constant for current loss was 2.2 h.

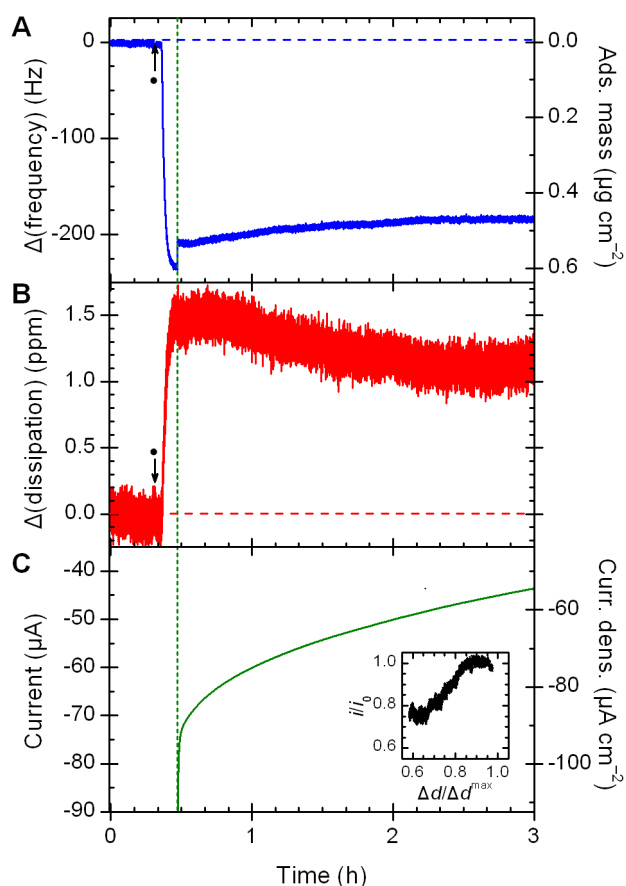


Figure S7. E-QCM-D response (7th harmonic) with time for bilirubin oxidase adsorbed on a bare gold crystal surface: **A** frequency change, **B** energy dissipation change, **C** O_2 reduction current. Circle (●) indicates when 25 μl 24 mg ml^{-1} $MvBOx$ was injected. Green dashed line shows when a potential of 0.21 V vs. SHE was applied. Inset in C shows the correlation between dissipation and electrocatalytic current. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^\circ\text{C}$, 0.1 ml min^{-1} . Current density based on geometric area of 0.80 cm^2 .

Section S10 Combined effects of cyclic voltammetry and chronoamperometry

We compared the effects of constant and variable potentials on the same layer of $MvBOx$ (Figure S8). After $MvBOx$ adsorption, the potential was cycled five times (Figure S8B, scan time 124 s) causing a 25% mass loss (ca. $0.15 \mu\text{g cm}^{-2}$) and an activity loss of about a third ($44 \mu\text{A}$). The shape of the first reductive scan shows flattening that is characteristic of mass-transport limitations, which would mask the true activity of the protein layer, so the actual activity loss is probably higher. At 0.45 V, the activity loss is about 60%. The potential was then held constant at 0 V for ca. 7 h. During this time the frequency trace remained relatively stable between -146 and -132 Hz, but the current dropped by 52%. Turning the potential on and off caused momentary fluctuations in the frequency trace, similar to that seen in Figure S7. Finally, the potential was cycled 10 more times (Figure S8C) during which the activity dropped by about a third and the mass dropped by about a tenth to about 60% of its value before the start of the voltammetry.

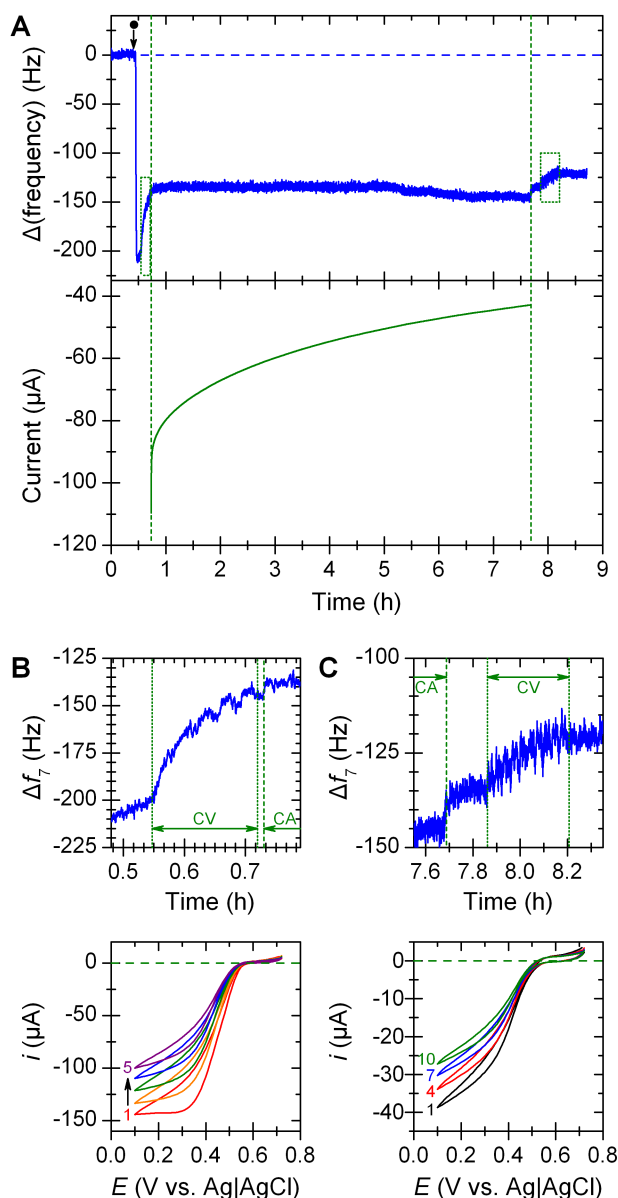


Figure S8. Frequency and current responses with time for *MvBOx* adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropionic acid. The top part of panel **A** shows the frequency change. Circle (●) indicates when 25 μl 24 $mg\ ml^{-1}$ *MvBOx* was injected. The periods when the potential was cycled (panels **B** and **C**) are indicated by dotted green boxes. The period when a constant reducing potential was applied is indicated by dashed green vertical lines. The bottom part of panel **A** shows current response to constant potential. Panel **B** shows frequency and current responses to five potential cycles before a constant potential was applied. Panel **C** shows this data for 10 potential cycles after a constant potential was applied. Data for frequency traces averaged over 21 points (6 s). Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^{\circ}C$, 0.1 $ml\ min^{-1}$, seventh harmonic shown. Cyclic voltammetry (CV): 0.72 $\rightarrow$ 0.10 $\rightarrow$ 0.72 V vs. Ag|AgCl|3M NaCl, 10 $mV\ s^{-1}$. Chronoamperometry (CA): constant applied potential of 0 V vs. Ag|AgCl|3M NaCl.

Section S11 QCM measurements in the absence of electrocatalysis

Cycling the potential on thiol-modified gold crystals without enzyme (Figure S9) produces oscillations in the Δf & Δd traces as for Figure 1, but with an amplitude about three times higher than when *MvBOx* or BSA is adsorbed. The traces in panel C show the onset of O₂ reduction around 0.5 V vs. SHE. A bump on the oxidative scan appears around 0.72 V vs. SHE after the first scan.

Bovine serum albumin (BSA), a globular protein with similar dimensions to *MvBOx*, was adsorbed on a 3MPA-coated QCM sensor (Figure S10). Unlike the rapid mass loss observed for an *MvBOx* adlayer when the potential was cycled (Figure 2), the frequency response for the BSA adlayer increased slightly to a value about 80% of its initial magnitude. The frequency response of an azide-inhibited *MvBOx* adlayer (Figure S11) was closer to that of the uninhibited enzyme, with a large, rapid drop in adsorbed mass. As expected, no electrocatalytic activity was observed for either BSA or azide-inhibited *MvBOx*.

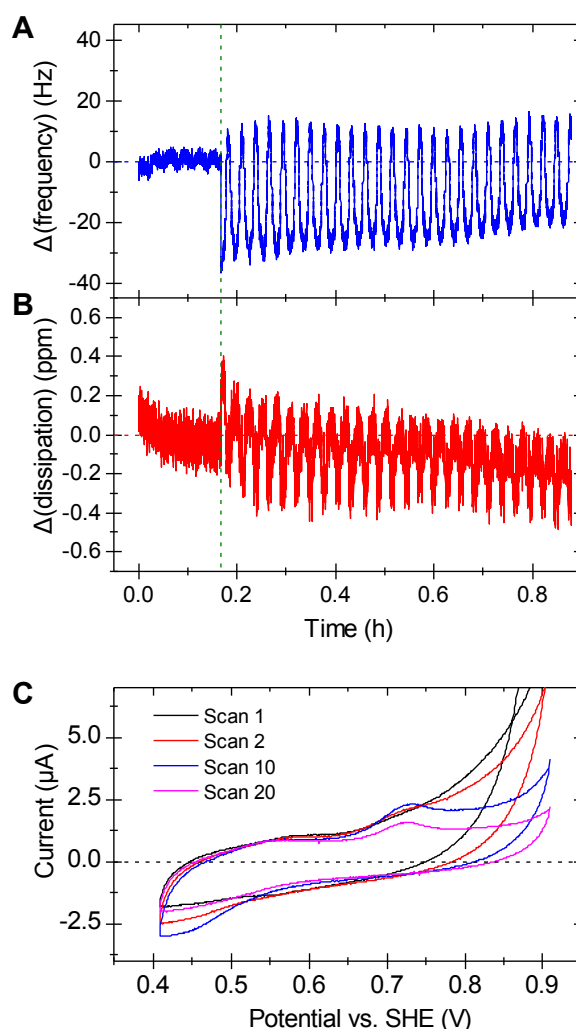


Figure S9. Frequency, dissipation and cyclic voltammetric responses with time for a gold-coated QCM sensor modified with 3-mercaptopropionic acid. Panel A shows the frequency change, panel B shows the dissipation response and panel C shows selected CV traces. The vertical green dashed line indicates the start of the voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. Cyclic voltammetry: 0.7 → 0.2 → 0.7 V vs. Ag|AgCl|3M NaCl, 10 mV s⁻¹.

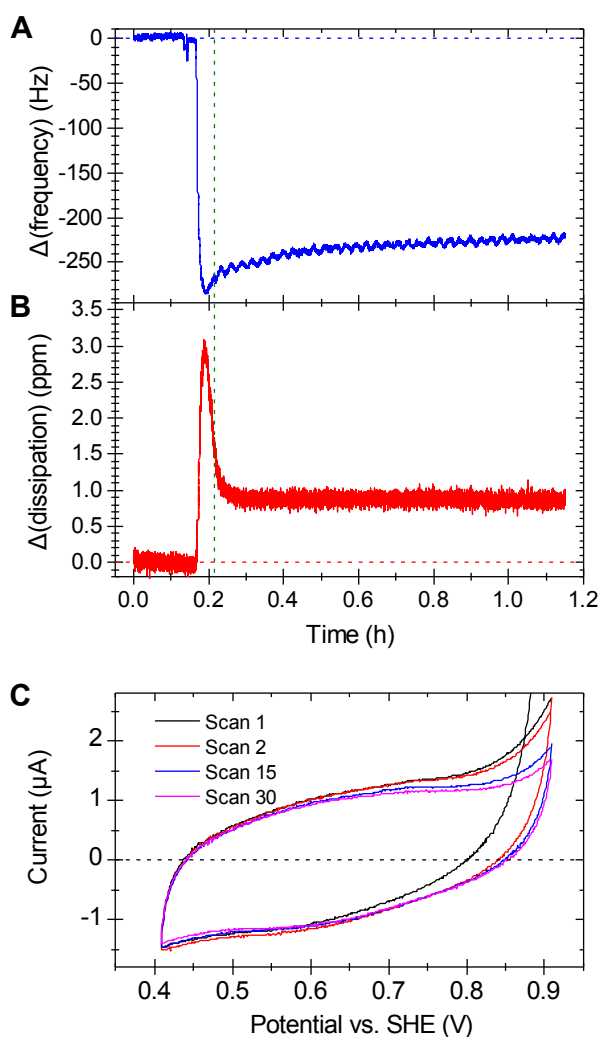


Figure S10. Frequency, dissipation and cyclic voltammetric responses with time for bovine serum albumin ($25 \mu\text{l } 24 \text{ mg ml}^{-1}$) adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropionic acid. Panel **A** shows the frequency change, Panel **B** shows the dissipation response, and Panel **C** shows selected CV scans. The vertical green dashed line indicates the start of the voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25°C , 0.1 ml min^{-1} , seventh harmonic shown. Cyclic voltammetry: $0.70 \rightarrow 0.20 \rightarrow 0.70 \text{ V vs. Ag|AgCl|3M NaCl}$, 10 mV s^{-1} .

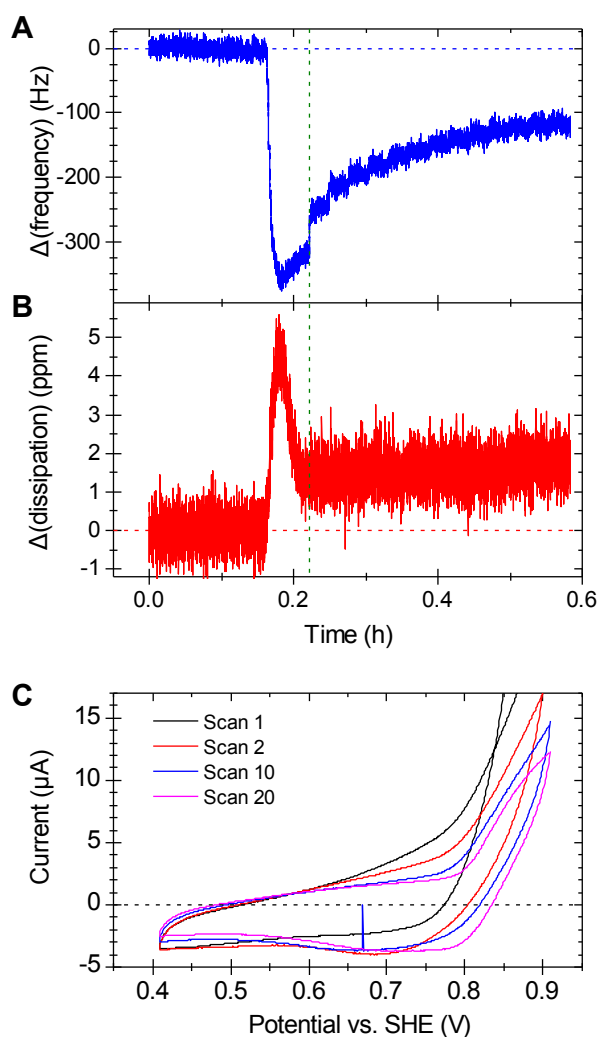


Figure S11. Frequency and current responses with time for *MvBOx* inhibited with 0.1 mM azide ($25 \mu\text{l}$ 24 mg ml^{-1}) adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropionic acid. Panel **A** shows the frequency change, Panel **B** shows the dissipation response, and Panel **C** shows selected CV scans. The vertical green dashed line indicates the start of the voltammetry. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2) with 0.1 mM sodium azide, 25°C , 0.1 ml min^{-1} , seventh harmonic shown. Cyclic voltammetry: $0.70 \rightarrow 0.20 \rightarrow 0.70 \text{ V vs. Ag|AgCl|3M NaCl}$, 10 mV s^{-1} .

Section S12 Effect of changing CV scan parameters

Changing to potential range over which the cyclic voltammetry significantly affected both mass and activity loss (Figure S12). Lower the value of the high-potential vertex made no difference to stability, while raising the potential of the low-potential vertex above ca. 0.6 V vs. SHE significantly improved stability and changed the pattern of inactivation to be closer to that of the chronoamperometry case.

Mass and activity are also sensitive to scan rate. Lower scan rates (e.g., Figure S13) lead to much larger losses in activity even though the mass losses are comparable to those from faster scan rates (Table S4).

There was no appreciable difference in the QCM traces when the size of the potential steps for the CVs was varied between 0.1 mV and 10 mV.

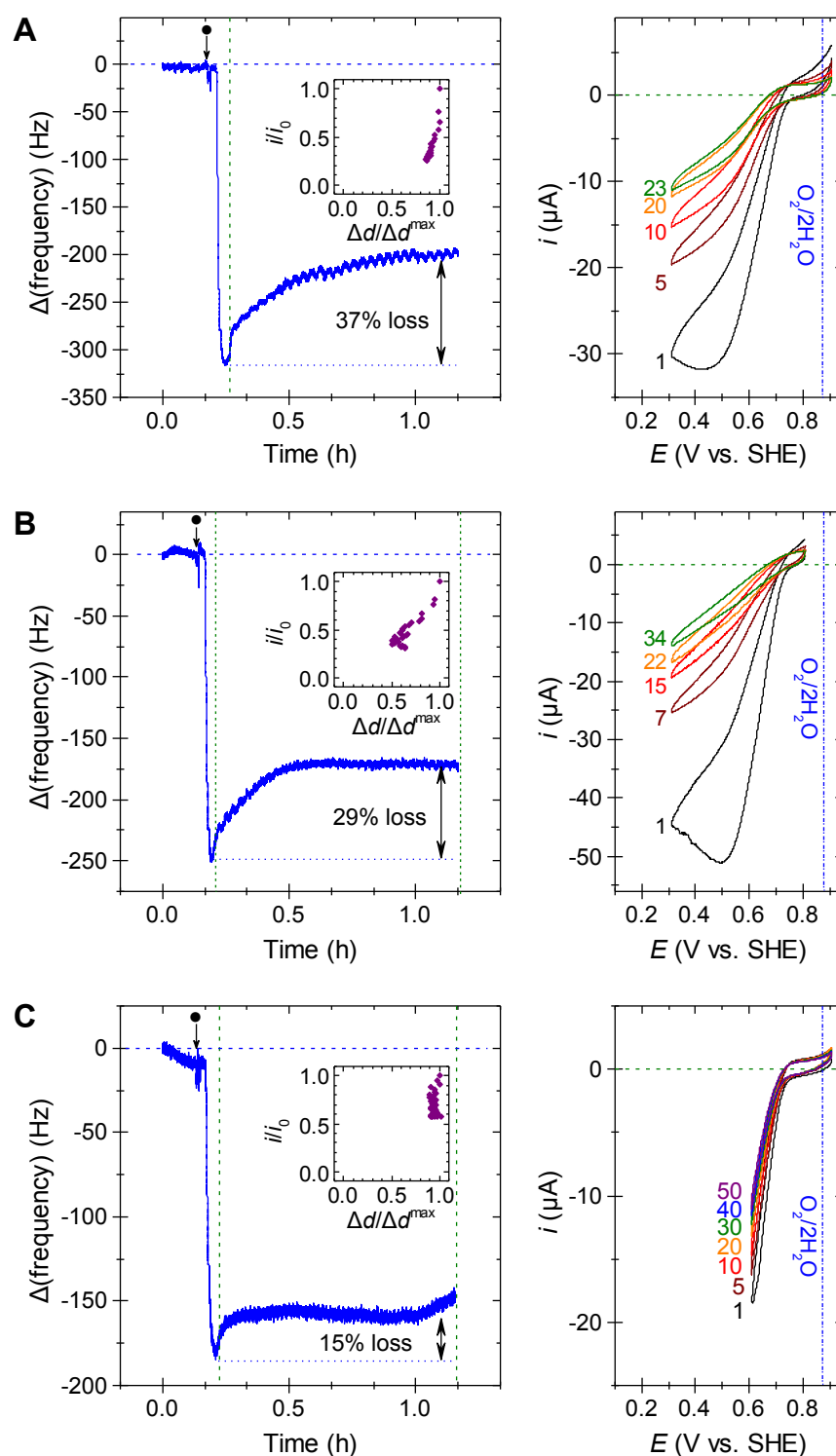


Figure S12. Frequency and electrochemical response to *MvBOx* adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropionic acid. Panel **A**: 0.70 to 0.10 V vs. Ag|AgCl|3M NaCl (full range); Panel **B**: 0.60 to 0.10 V vs. reference (keeping below potential for four-electron O_2 reduction); Panel **C**: 0.70 to 0.40 V vs. reference (keeping above potential where some reduction occurs in the absence of protein, cf. **Figure S9**). Green dashed lines indicate the period over which cyclic voltammetry scans were run. Circle (●) indicates when 25 μl 24 mg ml^{-1} *MvBOx* was injected. The insets show the relationship between dissipation and electrocatalytic current. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^\circ\text{C}$, 0.1 ml min^{-1} , seventh harmonic shown. Scan rate: 10 mV s^{-1} .

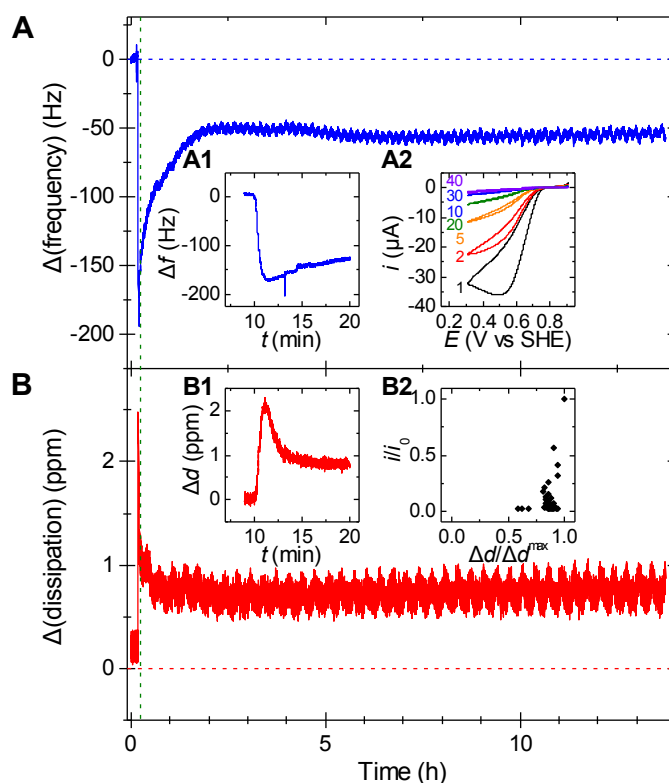


Figure S13. Frequency, dissipation and current responses to $MnBOx$ adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropionic acid when the applied potential is scanned 10 $\times$ more slowly than the case shown in Figure 2. Panel **A** shows the frequency change. The insets **A1** and **A2** show, respectively, a zoom of frequency during adsorption and cyclic voltammetry scans 1, 5, 10, 20, 30 and 40 of 46 scans. Panel **B** shows the dissipation response. Inset **B1** shows a zoom of the dissipation trace during adsorption. Inset **B2** shows the relation between current and dissipation during the experiment. The green dashed line indicates when cyclic voltammetry was started. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O_2), 25 $^{\circ}C$, 0.1 ml min $^{-1}$, seventh harmonic shown. Cyclic voltammetry: 0.70 $\rightarrow$ 0.10 $\rightarrow$ 0.70 V vs. Ag|AgCl|3M NaCl, 1 mV s $^{-1}$.

Table S4. Effect of scan rate on adlayers of $MnBOx$. Fractional losses are based on readings taken immediately cyclic voltammetry began and 20 minutes after.

scan rate (mV s $^{-1}$)	no. scans	current loss	mass loss
0.1	100	66%	30%
0.01	10	57%	24%
0.001	1	31%	27%

Section S13 Effects of variations in oxygen saturation

The effect of swapping between oxygen and argon flow during constant potential experiments is illustrated in Figure S14. The trace shows a typical pattern of a peak in the dissipation from the formation of a loosely bound layer that washes away quickly, followed by a steady frequency response and an exponential decrease in catalytic current. Changing the buffer purge gas to argon causes the current magnitude to decrease with a first-order time constant of about 780 s; switching back to oxygen causes the current magnitude to increase with a first-order time constant of about 450 s. As indicated by the dotted line in panel C (a first-order exponential extrapolation of the current measured before the argon flush), the current magnitude after reoxygenation is slightly higher than would be expected in the absence of an argon purge.

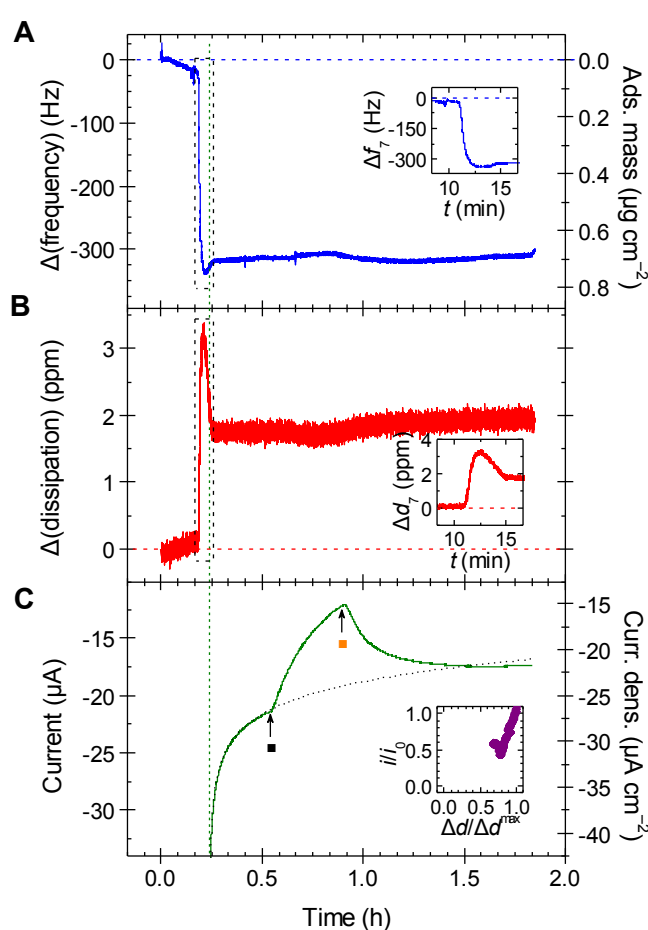


Figure S14. Frequency, dissipation and current response with time for *MvBOx* adsorbed onto a gold-coated QCM sensor modified with 3-mercaptopropionic acid. The green dashed line shows when a potential of 0 V vs. Ag|AgCl|3M NaCl was applied. Inset in **A** and **B** shows zoomed trace of enzyme adsorption. Square (■) indicates the switch to argon gas saturated buffer solution and (■) indicates the switch back to oxygen-saturated solution. Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown. Current density based on projected electrode area of 0.80 cm².

A ‘mixed mode’ measurement, in which both constant and cycled potentials were used, is shown in Figure S16. After protein adsorption, the applied potential was held constant, then argon-saturated buffer was flowed through the cell. While the oxygen was being displaced, the measurements swapped to cyclic voltammetry. Toward the end of the potential cycling, the oxygen-saturated buffer was reintroduced; some activity remained. After 25 cycles, the potential was again held constant. Both the current and frequency response at this stage were very stable.

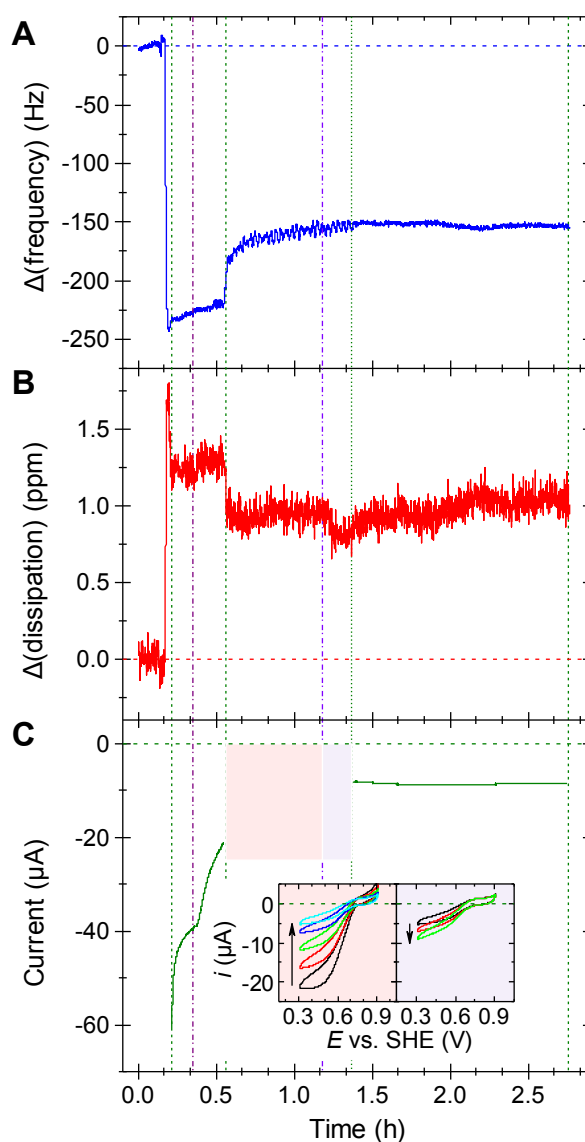


Figure S15. The effect of oxygen on the (A) frequency, (B) dissipation and (C) current response with time for *Mv*BOx adsorbed onto a gold-coated QCM sensor modified with 3-mercaptopropionic acid. The vertical green dashed lines represent the start and end of chronoamperometry measurements (0.1 V vs. Ag|AgCl|3M NaCl). The vertical green dotted lines represent the start and end of cyclic voltammetry (0.70 → 0.10 → 0.70 V vs. reference). The vertical purple dash-dot lines indicate when the solution flowing through the cell was changed to argon saturated buffer. The insets in Panel C show the decay in electrocatalytic current as O₂-saturated buffer is displaced by argon-saturated solution (left inset; scans 1, 3, 6 and 19 shown) and the partial recovery of current after the argon-saturated buffer is replaced by O₂-saturated solution (right inset, scans 19, 21 and 24 shown). Conditions: 0.1 M sodium phosphate pH 6.0 (pre-saturated with O₂), 25 °C, 0.1 ml min⁻¹, seventh harmonic shown.

Section S14 Measurements on a second multicopper oxidase

We also used a second commercially available multicopper oxidase, laccase from *Trametes versicolor* (*TvL*). The protein was adsorbed to a gold-coated quartz crystal modified with 3-mercaptopropyl, with the adsorption and subsequent cyclic voltammetry performed as for Figure 2 from the main text. Figure S16 shows 50 potential cycles and E-QCM-D responses that parallel those observed for *MvBOx*.

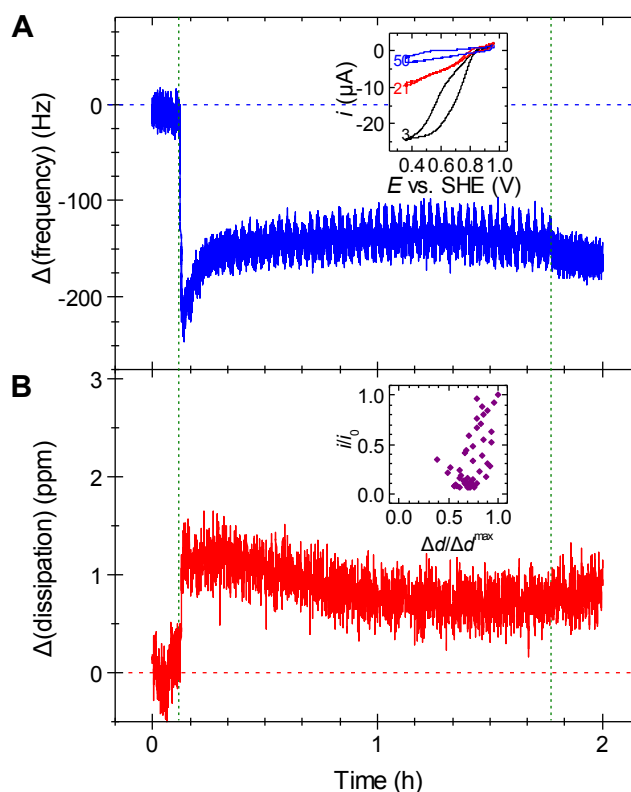


Figure S16. Frequency (Panel A) and dissipation (Panel B) response to *TvL* adsorbed on a gold-coated QCM sensor modified with 3-mercaptopropyl. The green dashed line indicates when cyclic voltammetry was started and ended. Inset: Cyclic voltammetry of *TvL* on 3-mercaptopropyl. Inset in A shows scans 3, 21 and 50; inset in B shows the relationship between current and dissipation at each low-potential vertex. Conditions: 25 °C, 0.1 M sodium citrate pH 4.0 (pre-saturated with O_2), 0.1 ml min⁻¹, seventh harmonic. Potential range: 0.75 → 0.15 → 0.75 V vs. Ag|AgCl|3M NaCl, 10 mV s⁻¹.

To compare the effect of covalent attachment to enzymes other than *MvBOx* (Figure 3, main text) we attached *TvL* to a DTSSP-modified gold crystal. The covalent attachment occurs through a single reactive lysine residue, Lys174,¹⁷ which is ~32 Å away from the T1 centre, too far for efficient electron transfer from the electrode.¹³ The loss of activity is further highlighted in the near absence of a catalytic wave upon potential cycling (panel A inset). In contrast to the observations for *MvBOx* in Figure 3, the frequency magnitude increased when the potential was cycled, though the current vs. dissipation trace (panel B inset) was similar.

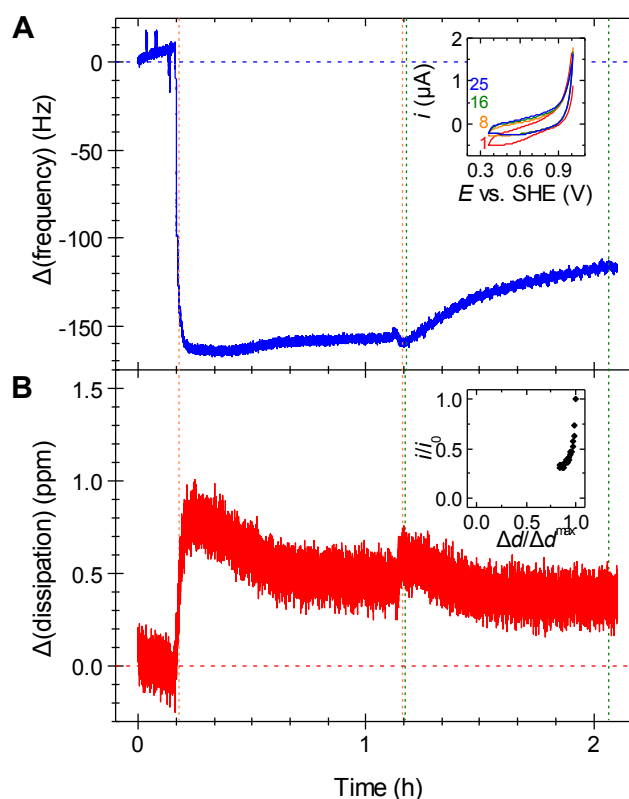


Figure S17. Frequency and dissipation response with time for TvL covalently bound via an amide linkage to a DTSSP-modified QCM sensor. The orange dashed lines represent the time left for enzyme to react with surface layer (reaction adapted from the ref. 18) during which time the peristaltic pump was turned off. The green dashed line shows when potential cycling began with the inset in **A** showing the cyclic voltammograms from scans 1, 8, 16 and 25 of 25 scans. **B** shows the dissipation during enzyme adsorption and the subsequent change on potential cycling. Conditions: O₂-saturated 0.1 M sodium phosphate buffer pH 6.0 at 25 °C flowing at 0.1 ml min⁻¹ with continuous O₂ bubbling, seventh harmonic shown. Cyclic voltammetry (CV): 0.80 → 0.15 → 0.80 V vs. Ag|AgCl|3M NaCl, 10 mV s⁻¹.

Section S15 Additional Δd_7 vs. Δf_7 traces

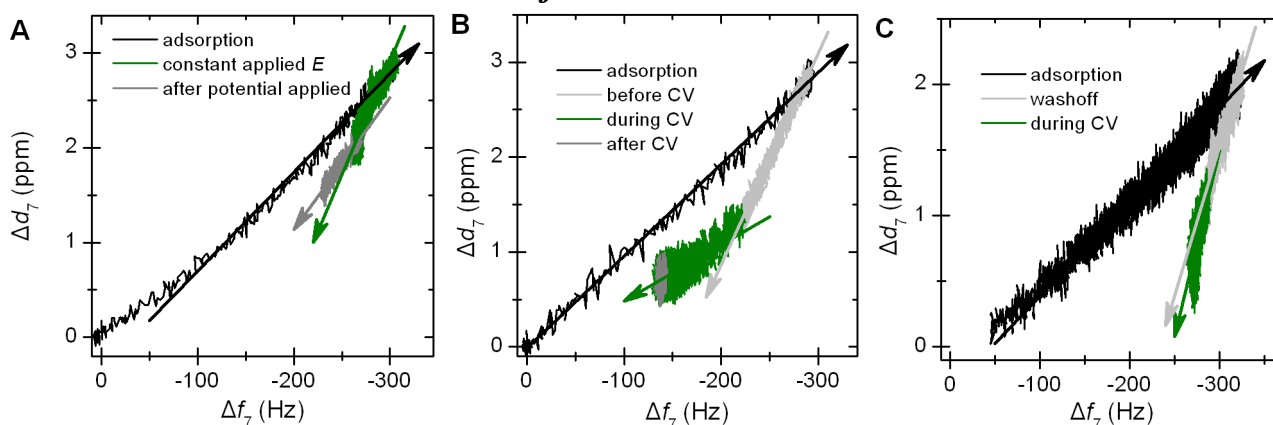


Figure S18. Plots showing correlation between energy dissipation and frequency change for the data plotted in **A** Figure 1, **B** Figure 2 and **C** Figure 3 in the main text.

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Electrochemical Quartz Crystal Microbalance with Dissipation Monitoring: A Technique to Optimize Enzyme Use in Bioelectrocatalysis

Kulveer Singh^[a, b] and Christopher F. Blanford^{*[b]}

This Concept article outlines how an electrochemical quartz crystal microbalance with dissipation monitoring (E-QCM-D) is used as a tool to follow adsorption and inactivation processes of immobilised oxidoreductases and to determine their specific activity simultaneously. Plots that relate activity to the adsorbed mass and dissipation have distinct features that can be

used to diagnose the mechanisms of long-term activity loss. Along with model plots, this article highlights some of the key advances made by using the technique and how it has been applied to biocatalytic processes in which electrons are transferred directly between an electrode and an immobilised enzyme.

Introduction

The quartz crystal microbalance (QCM) is a technique used commonly to measure the adsorption of small amounts of material [ng cm^{-2}] onto the surface of a quartz crystal, which can be held in either gas or solution. The application of a sinusoidally alternating potential to the crystal face generates a mechanical oscillation in the quartz wafer. The addition of material to the surface of the crystal decreases its resonant frequency. If the added mass is thin, rigid and evenly distributed, then the frequency change for the N th harmonic of the resonator, Δf_N , is proportional to the added mass according to the Sauerbrey equation [Eq. (1)]:^[1]

$$\Delta m = -\frac{C}{N} \Delta f_N \quad (1)$$

in which Δm is the areal mass change and C is the Sauerbrey constant [approximately $18 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ for an AT-cut quartz crystal with a fundamental frequency of 5 MHz (AT: the first temperature-compensated cut for a quartz crystal)].

Quartz sensors are cut from larger quartz crystals in a specific orientation. The examples in this article use an AT-cut crystal, which minimises the temperature dependence of the resonant frequency near 25°C and has pure shear modes when it oscillates.^[1] Thin layers of metal are sputtered or evaporated on to

both sides of the crystal and are used as electrical connection points to drive the crystal oscillation.

The applied potential gives rise to vibrations in two directions: the first runs parallel to the applied field (normal to the crystal surface), and the second propagates as a transverse shear wave through the crystal in the thickness direction (Figure 1 A). Standing shear waves are generated if the thickness of the crystal (t_q) is twice the acoustical wavelength [Eq. (2)]:

$$t_q = \frac{v_q}{2f_1} = \frac{\lambda_q}{2} \quad (2)$$

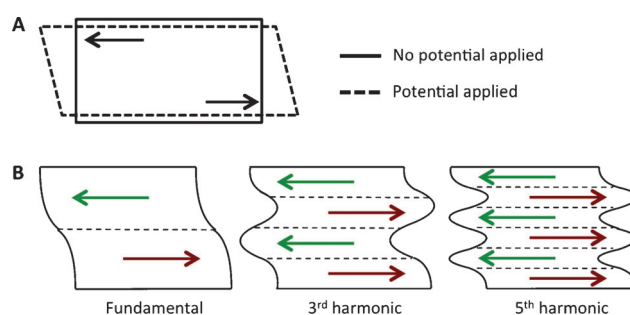


Figure 1. A) Changes in crystal shape for a quartz crystal operating in thickness shear mode. B) How these modes appear at odd harmonics of the fundamental resonance frequency. The dashed lines represent planes with zero displacement.

in which v_q is the velocity of the acoustic wave generated in the quartz crystal, λ_q is the wavelength of the acoustic wave in quartz and f_1 is the fundamental frequency.

Quartz crystals may be operated at the fundamental or higher odd harmonics. The fundamental frequency of quartz crystals is related to the thickness of the crystal [Eq. (2)], and so for an AT-cut crystal that resonates at a fundamental fre-

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quency of 5 MHz, the crystal thickness is approximately 0.33 mm, whereas a 15 MHz crystal is a third as thick.^[1]

Quartz crystals that operate in thickness shear mode also resonate at odd multiples (harmonics) of the fundamental frequency (Figure 1B). At lower harmonics, acoustic energy trapping is inefficient and leads to a higher sensitivity to bulk changes and external mechanical vibrations. Energy trapping refers to the idea that the energy contained in the mechanical oscillations of the crystal are confined to area of the crystal used as the electrode. The energy trapping is less confined at lower harmonics and these are, therefore, more sensitive to mounting and small vibrations during setup.^[2] It is desirable to operate the crystals at higher frequencies for increased mass sensitivity because of the linear relationship between harmonic number and frequency change [Eq. (1)]. However, thinner crystals, which have a higher fundamental frequency, are more fragile so a compromise is needed in which the crystals that operate at lower fundamental frequencies are driven at higher harmonics. The use of high-frequency harmonics for analysis rather than a thinner crystal with a high fundamental frequency gives the same mass sensitivity without the accompanying fragility. However, the higher harmonics experience overlap between anharmonic sidebands (those nodes that are perpendicular to the plane of the frequency resonator), which cause interference between actual signals and spurious responses and ultimately result in a low signal-to-noise ratio.^[3]

In solutions, the crystal oscillations create a shear wave in the solution above it. For a Newtonian fluid, this wave has a characteristic decay length, δ [Eq. (3)].^[4]

$$\delta = \sqrt{\frac{\eta_{\text{fluid}}}{\pi N f_1 \rho_{\text{fluid}}}} \quad (3)$$

in which η_{fluid} and ρ_{fluid} are the shear viscosity and density of the fluid above the crystal, respectively. So for a 5 MHz crystal in pure water around room temperature ($\eta_{\text{fluid}} = 0.9 \text{ MPa s}$, $\rho_{\text{fluid}} = 1 \times 10^3 \text{ kg m}^{-3}$), the penetration depth of the fundamental frequency is 0.24 μm . This decreases to 0.09 μm for the seventh harmonic. The result of this is that higher harmonics are more surface sensitive than lower ones. This effect has been used to study the penetration of lipid membranes by short peptide chains.^[5]

When material adsorbs on the crystal surface, the increase in the mass of the crystal is measured as a decrease in frequency (Figure 2). If evenly distributed, added mass can be seen as a direct increase in the thickness of the crystal itself.

The Sauerbrey equation is only accurate for rigid films, which are unlikely to form from large proteins.^[6] Also, proteins are often hydrated heavily, and hence the QCM is a detection technique for the dynamic (hydrated) mass of biological molecules. As the size and the structural flexibility of the biological molecules increases, a second parameter is introduced to account for the resistance of a soft adlayer to vibration and its ability to store mechanical energy, that is, its viscoelasticity.

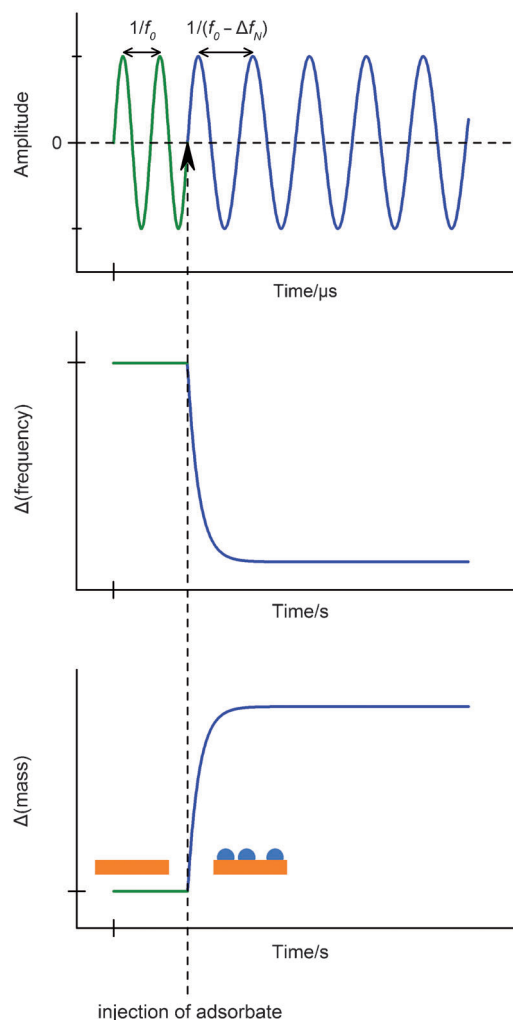


Figure 2. The change in frequency and mass on injecting an adsorbate species onto a quartz crystal sensor.

Viscoelastic energy losses

“Dissipation” quantifies the vibrational energy an oscillator loses per cycle and is a measure of the viscoelastic dampening from an adsorbate on a QCM sensor. It is used to assess the rigidity and arrangement of an adlayer immediately after adsorption and to monitor how that layer changes over time, for example, through structural rearrangements or denaturation.^[7]

The idea is similar to that of a tuning fork. If the fork is struck, it will resonate at a given frequency until all vibrational energy is lost. If at some point while it resonates, the fork were to be submerged into liquid, the vibrational energy would be dampened and the resonance would decay more rapidly. In the same way, a larger (softer) layer deposited on the crystal dampens the oscillation of the resonator, more energy is dissipated and the resonance decays more quickly than for a more rigidly adsorbed layer (Figure 3C and D).

If we consider adsorbed protein, the layer is a composite of the adsorbed species together with trapped water molecules.^[6] To this end, the dissipation can be used to look at how water associated with the protein can affect the stability of different

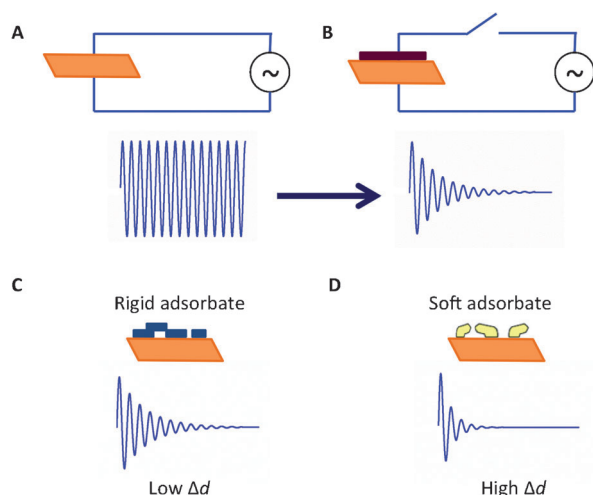


Figure 3. Schematic illustration of one method to quantify dissipation and how dissipation varies between rigid and soft adlayers. A) An alternating potential is applied across the crystal to drive its oscillation at its resonant frequency. B) If the circuit is opened, the voltage amplitude will decay exponentially with time with a decay time constant inversely proportional to dissipation [Eq. (6)]. C) The oscillations of rigid adlayers are well coupled to the sensor to generate a relatively slow decay (high τ) and lower dissipation changes. D) Soft layers experience more inelastic collisions and higher decay rates (low τ) and, therefore, higher dissipation changes.

surface-bound species and what this means for structure–function relationships.

Dissipation is a dimensionless number defined by Equation (4):

$$D = \frac{\text{Energy dissipated per cycle}}{\text{Energy stored} \times 2\pi} \quad (4)$$

One method to measure the dissipation is to monitor the decay in the amplitude of the voltage oscillation of a crystal after the drive potential has been turned off (Figure 3A and B). The rate of decay depends on the environment of the crystal, especially the adsorbate. The amplitude of the oscillation (A) is fitted to an exponentially damped sinusoid from the point at which the resonator drive circuit is switched off ($t=0$) [Eq. (5)]:

$$A(t) = A_0 \exp(-t/\tau) \sin(2\pi f_{\text{app}} t), \quad t \geq 0 \quad (5)$$

in which τ is the decay time constant, which is related to dissipation by Equation (6):

$$D = \frac{1}{\pi f \tau} \quad (6)$$

For thin films in a liquid, both the frequency and dissipation responses are dominated by the bulk liquid acoustic response,^[4b] so are plotted as a change relative to baseline conditions such as a clean crystal in aqueous buffer. Thin viscoelastic adlayers in solution will always lead to a smaller frequency response than a rigid layer would give, so the use of the Sauerbrey equation [Eq. (1)] will give an underestimate of the mass of a protein film.^[4b]

Plots of frequency change (Δf) versus time can be used to identify the adsorption mechanism (e.g., Langmuir/random sequential adsorption, multilayer adsorption). Plots of dissipation change (Δd) with time can show differences in adsorbate packing, structural evolution and denaturation.^[8] Two films of comparable mass (and thus comparable Δf) are shown in Figure 4A; the less rigidly bound proteins that correspond to the blue trace produce a much higher change in dissipation. The frequency change for the formation of a stabilised monolayer of a hydrated 60 kDa globular protein is approximately $(35\text{--}40)N \text{ Hz}$,^[9] in which N is the harmonic number. (Some software displays $\Delta f/N$ instead of Δf to make it easier to compare the responses of multiple harmonics simultaneously.) The dissipa-

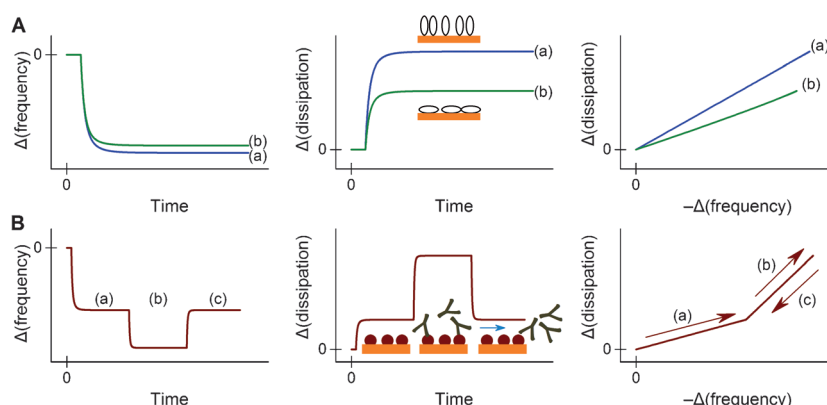


Figure 4. A) How QCM-D can be sensitive to protein orientation: The vertically aligned molecules (a) are more flexible than those lying on the surface (b), so have a higher dissipation for a similar adsorbed mass. The dissipation versus frequency plot shows the difference in gradient between the two orientations. The orientation of the adsorbed species is, therefore, a necessary factor to consider if biomolecular interactions are investigated with the QCM. B) Reversible binding of antibody to antigen: (a) Relative frequency and dissipation changes on antigen adsorption; (b) Antibody adsorption, which results in a large increase in dissipation because of the elongated and more “wobbly” structure of the antibodies; (c) Antibody binding is fully reversible because the dissipation returns to its original value. This appears in the dissipation versus frequency plot as a reversible change in the gradient. An additional coating to prevent non-specific binding is not shown.

tion response from the same protein film is approximately 1–5 ppm and shows a non-linear, non-monotonic dependence on N .^[4b] The resolution of Δf and Δd for an adlayer in a liquid is $\approx 0.1 \text{ Hz}$ and $\approx 0.1 \text{ ppm}$, respectively,^[10] so the technique is very sensitive to changes in adsorbed mass and moderately sensitive to changes in viscoelastic changes.

Plots of Δd versus Δf for an adsorption typically show regions with different gradients. The gradient normalises the

energy dissipation of the adlayer to its mass, so changes in slope indicate changes in adsorbate–surface interactions during the adsorption and desorption process. An idealised example in which an antigen layer adsorbs on the sensor followed by the reversible binding of a comparatively less rigid antibody layer is shown in Figure 4B. On a Δd versus Δf plot, the second adsorption would appear as a steeper line.

Typical data from the adsorption of a ≈ 60 kDa globular protein are presented in Figure 5. The frequency and dissipation

the higher harmonics of a 5 MHz crystal, which complicates modelling. Also, this means that some caution must be used if changes in slope on a Δd versus Δf plot are characterised qualitatively because steeper slopes will not always correspond to less rigid adlayer formation. In the case of the formation of a purely viscous (Newtonian) adlayer, Δd will not change with adsorbed mass, and an areal mass density (e.g., ng cm^{-2}) is calculated because the thickness and density cannot be determined independently.^[4b]

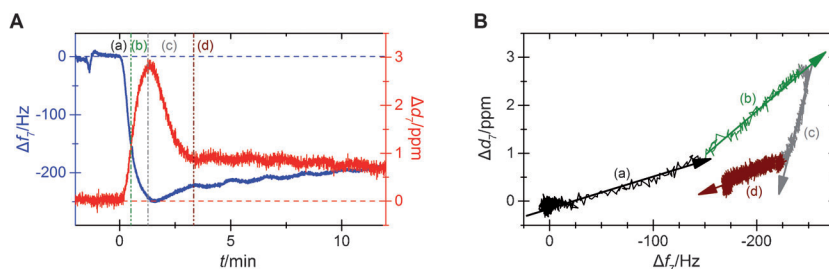


Figure 5. A) Typical frequency and dissipation responses for the adsorption of *MvBOx* on a layer of 3-mercaptopropionic acid on an Au-coated quartz sensor. B) Four distinct regimes in the Δd versus Δf plot labelled (a)–(d). If we apply the model from Rabe and co-workers,^[12] the dominant processes in these regimes are (a) protein adsorption before a critical surface coverage is reached, (b) adsorption of a reversibly bound form of the protein, (c) desorption of reversibly bound protein, and (d) slow desorption of “irreversibly” bound protein. Conditions: $f_1 = 4.95$ MHz, $N = 7$, 0.1 M sodium phosphate pH 6.0, 25 °C, 0.1 mL min⁻¹ flow, 25 μL of 24 mg mL⁻¹ *MvBOx*.

traces in panel A appear to show three regimes: an initial adsorption over ≈ 1 min, a rapid phase of stiffening and mass loss over 2–3 min, and a more gradual mass loss. The Δd versus Δf plot in panel B shows that there are actually four distinct adsorption and desorption regimes. The non-monotonic adsorption curve is frequently observed in protein adsorption studies and is known as “overshoot”.^[11] Although the QCM traces could be explained by the formation of flexible, reversibly bound secondary layers atop a stiffer primary adlayer, the adsorbed mass is consistent with the formation of just a single layer. A model from Rabe and co-workers provides a more consistent explanation by including a reversibly bound form of the protein that only arises above a critical surface concentration.^[12] The slopes of regions 2 and 3 in panel B are steeper, so this reversible form must also be bound less rigidly than the initial layer.

The mechanics of a viscoelastic protein adlayer can be parameterised by using four variables: thickness, density, shear viscosity and shear modulus. In theory, these parameters can be determined by recording Δf and Δd for more than one harmonic, which allows all four variables to be monitored with time. This is difficult in practice, however, because of non-monotonic behaviour in Δf and Δd with changes in shear modulus and viscosity: the dissipation and frequency responses to changes in viscosity and shear modulus reverse for some combinations of these two variables.^[4b] A typical monolayer formed from a ≈ 60 kDa globular protein such as bilirubin oxidase from *Myrothecium verrucaria* (*MvBOx*) or bovine serum albumin has a viscosity of ≈ 4 MPa s and a shear modulus of ≈ 1 MPa; these values are near the response turning points for

Motional resistance

The measurement of motional resistance, the energy lost within the vibrating area of a resonating crystal, is an alternative method to quantify energy dissipation from an adlayer. Oscillating mechanical systems can be represented as an equivalent Butterworth–van Dyke electrical circuit, which consists of a “static” arm that represents the electrical characteristics of a resonator and a “motional” arm that represents its mechanical properties.

The static arm consists of a capacitance, C_o , which arises from the electronic capacitance of the sensor. The motional arm is modelled by a motional resistance, R_m , which relates to the energy losses of the sensor; a motional capacitance, C_m , which is attributed to the stored energy during oscillation and is related to the nature of adsorbate; and a motional inductance, L_m , which models the kinetic energy of the resonator system and represents the inertial component of displaced mass during oscillation (Figure 6A).

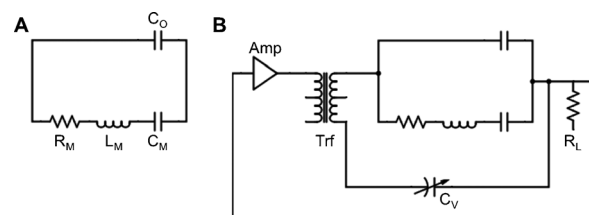


Figure 6. A) Butterworth–van Dyke model for a quartz crystal resonator that consists of static and motional arms. B) Introduction of a voltage amplifier (Amp) and a transformer (Trf) that inverts the applied voltage driven into a variable capacitor, C_v to cancel out the static capacitance, C_o .^[13]

Provided there is a sufficient amplification gain, the voltage returned on a load resistor (R_L) positioned outside the series circuit (Figure 6B) will sustain the oscillation of the crystal. This load resistance is a combination of the motional resistance of quartz and the resistance caused by energy losses on mass loading.

In an oscillating system, the static capacitance generates a lead current that is driven into the load resistor with

a known resistance and this must be cancelled. To this end, an amplifier (Amp) is introduced into the circuit that drives a transformer (Trf) with two secondary windings, one of which inverts the amplified voltage. This then generates a current through a variable capacitor, C_v and cancels out C_o ($C_v = C_o$). In a series resonance, the reactance of L_m and C_m then cancels and leaves just R_m and R_L , which needs a gain, A_v to maintain oscillation [Eq. (7)]:

$$A_v = \frac{R_m + R_L}{R_L} \quad (7)$$

In this way, if the gain is known, the motional resistance of the adsorbed species can be measured as shown in Equation (8):

$$R_m = R_L (A_v - 1) \quad (8)$$

Geelhood et al. have shown that as long as the static quartz capacitance is properly cancelled, typical dissipation measurements are in good agreement with measurements of motional resistance and are related to each other by Equation (9):^[14]

$$D = \frac{R_m}{\omega L_m} \quad (9)$$

in which ω is the radial frequency.

Electrochemical quartz crystal microbalance

QCM experiments initially focused on the use of its mass-sensing capability in the gas-phase monitoring of oxidation rates at metal surfaces, metal plating and gas-phase adsorption/desorption processes.^[15] The idea to use a piezoelectric sensor in the gas phase was first suggested by King in 1964.^[16] Sauerbrey then developed a gravimetric method to analyse coupled mass in the gas phase.^[1] In later work by Tsionsky and Gileadi, gas-phase adsorption on the QCM was studied under constant pressure while the concentration of an adsorbate in a carrier gas was varied.^[17] In the 1980s, Kanazawa and Gordon showed that a QCM could be used to study adsorption processes in liquids.^[18] Nomura and Hattori then showed that measurements can be taken with the crystal immersed in a liquid to detect micromolar concentrations of cyanide in solution.^[19] Caruso et al. showed the advantages of the use of QCM alongside surface plasmon resonance (SPR) and AFM to identify wet and dry ferritin coverage, thickness and density on Au substrates for potential use in immunosensors.^[20]

The first combination of QCM with electrochemistry was described in a report by Jones and Mieux.^[21] They used Δf measurements to establish concentration–mass relationships for Cd layers on QCM electrode surfaces that were electrodeposited in an external electrochemical cell.^[21] They later used this method to analyse a mixture of metals and establish sensitivity factors for each metal at each associated deposition voltage.^[22] A true electrochemical quartz crystal microbalance (E-QCM) combines the QCM with an electrochemistry module in situ

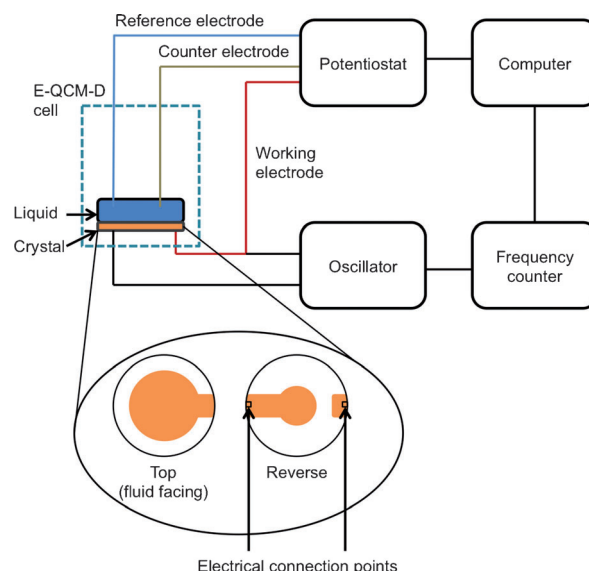


Figure 7. A typical E-QCM setup that employs a three-electrode electrochemical cell in which the mass sensor surface doubles as the working electrode.

(Figure 7) to allow mass changes at electrode surfaces to be directly correlated to redox processes.^[23] The first reported application of a E-QCM was from Nomura and Iijima for the determination of Ag^+ concentrations by electrodeposition.^[24] More recently, E-QCM has been applied to a variety of industrial applications that range from probing structural changes in DNA for clinical diagnostics,^[25] investigating inhibition of Cu corrosion^[26] and following thermo-electric film deposition.^[27]

For the electrochemical analysis of protein adlayers, Au electrode surfaces are frequently modified by organothiols, which form self-assembled monolayers (SAMs) to present a tailored, uniform chemical functionality to the adsorbed protein. Schneider and Buttry used E-QCM to quantify the surface coverage of alkylthiol monolayers by monitoring both mass loss and the charge transferred during reductive desorption,^[28] and a further study by Kawaguchi et al. reported a simultaneous cation adsorption during the reductive process.^[29] Cordas et al. used an E-QCM to monitor the formation of SAMs terminated with iron porphyrin electrocatalysts, which were used for potential-driven dioxygen reduction in the cell.^[30]

Protein adsorption

Redox-active proteins play an important role in heterogeneous bioelectrocatalysis,^[31] biosensors^[32] and electrodes in enzyme-catalysed biofuel cells.^[9,33] A combination of the ability of the E-QCM to quantify adsorption and electron-transfer processes with dissipation measurements (i.e., E-QCM-D) provides a way to link them with changes in the structure of a thin enzyme film.^[31e,33a,b,34]

Jensen et al. highlighted differences between the mass adsorption of an enzyme and the resulting enzyme activity on flat and nanostructured Au electrodes.^[33a] Changes in structural integrity associated with enzyme adsorption were studied over time and found to be dependent on surface topology, which

has implications in biosensor development and surface structure design.

Olejnik et al. have shown the difference in electroactivity between laccase immobilised on positively charged versus negatively charged surfaces. Although similar masses of enzyme were immobilised, the resulting activity was far greater on the positively charged surface, which indicates a direct relationship between enzyme orientation, surface topology and catalytic current.^[33b]

An E-QCM has been used to follow the adsorption and electrocatalysis of MvBOx on bare Au sensors.^[35] They suggested that the lower catalytic current density on Au, compared to that on carbon electrodes, was a result of enzyme denaturation. Further to these findings we published a study on the combination of chronoamperometric control, potential cycling and dissipation measurements of MvBOx on modified Au electrodes that detailed the non-desorptive elements that contribute to activity loss.^[9]

Electrochemical measurements and QCM-D are frequently applied to the formation and analysis of layer-by-layer (LbL) assemblies of redox-active proteins and poly-electrolytes. Hodak and co-workers used an E-QCM-D to monitor thiol adsorption followed by the LbL assembly of ferrocene-modified poly(allylamine) and glucose oxidase to Au. They reported successful catalytic glucose oxidation with activity that increased with increasing layer build up.^[36] Others have used E-QCM-D to study assemblies that incorporate cytochrome c,^[31d] and QCM-D with ex situ electrochemistry to study assemblies of glucose oxidase and sugar-binding lectins.^[32d,e] Other structured, protein-containing electrodes studied by using an E-QCM-D include those made of porous Au,^[32a] Au nanoparticles,^[33c] glancing angle deposition (GLAD),^[33a] supported lipid membranes^[31c] and graphene-family nanomaterials.^[32c]

Application of the E-QCM-D to redox enzyme catalysis

The E-QCM-D has a significant advantage over other surface techniques as it allows the simultaneous monitoring of the electroactive current generated by redox enzyme catalysis with concurrent mass and viscoelastic monitoring. The real benefit of this technique comes from the ability to normalise biocatalytic activity to adsorbed mass.

To a first approximation, the change in frequency is proportional to the change in mass according to the Sauerbrey equation [Eq. (1)], and the adsorbed mass is proportional to the molar surface coverage of enzyme, Γ_{enz} [Eq. (10)]:

$$\Delta m = -\frac{C}{N} \Delta f_N = M_H \Gamma_{\text{enz}} \quad (10)$$

in which M_H is the hydrated molar mass of the enzyme, that is, the mass of the macromolecule plus any mechanically coupled fluid associated with it.^[9, 33a, 37]

The magnitude of the catalytic current, i_{cat} , generated by the enzyme is also proportional to surface coverage and the turnover frequency [Eq. (11)]:

$$i_{\text{cat}} = -nFA\Gamma_{\text{enz}}k_{\text{cat}} \quad (11)$$

in which n is the stoichiometric number of electrons transferred for a reduction or oxidation, F is the Faraday constant, A is the electrode area and k_{cat} is the apparent catalytic rate constant of the enzyme. The value of k_{cat} will depend on the applied electrochemical potential, substrate and product diffusion, and enzyme conformation. The negative sign in Equation (11) is indicative of a reductive process; a positive sign would be used for an electrochemical oxidation.

We can combine Equations (10) and (11) to show how the changes in current and mass can be used to estimate the relative turnover frequency of the enzyme during catalysis [Eq. (12)]:

$$\frac{i_{\text{cat}}}{\Delta f} = \frac{CnFA}{NM_H}k_{\text{cat}} \quad (12)$$

To a first approximation, all of the terms on the right-hand side of Equation (12) are constant for a given oxidoreductase. (The hydrated mass of a protein will often decrease in the first stages of adsorption, which lowers the value of M_H .) Thus, the change in slope with time for plots of i_{cat} versus Δf shows how the electrocatalytic competence of an enzyme film evolves with time with no interference from protein desorption.

For oxidoreductase adlayers, plots that combine frequency change, dissipation change, time and catalytic current data can be used to distinguish various post-adsorption processes (Figure 8).

An idealised adsorption is shown in Figure 8A: the oxidoreductases form a single, stable layer in an orientation for efficient electron transfer. The blue lines show the expected frequency change and red lines show the expected dissipation changes. Both the dissipation change and catalytic current response (green lines) are directly proportional to the adsorbed mass. Thus, the associated plots of current versus Δf and Δd (purple lines) are linear and monotonic. If the protein is not in an orientation that permits efficient electron transfer (Figure 8B), the plots appear superficially identical to those in Figure 8A, but the current generated per unit mass adsorbed is lower.

If loss in catalytic activity is solely because of desorptive processes (Figure 8C), dissipation and current will still be proportional to adsorbed mass during the desorption process. So the trace of current versus Δf and Δd after the point of maximum activity (dashed brown lines) will exactly overlap the trace from the initial adsorption (purple lines).

The denaturation of the protein (Figure 8D) can produce a similar trend in activity loss to enzyme desorption. However, the brown and purple traces do not overlap. Denaturation often leads to layer stiffening (a decrease in Δd) and some loss of mechanically associated water (an increase in Δf). The drop in activity per unit of adsorbed mass means that the average turnover frequency of the enzyme has decreased [Eq. (12)].

Finally, Figure 8E and F show two model E-QCM-D “overshoot” responses like those seen in Figure 5. Multilayer forma-

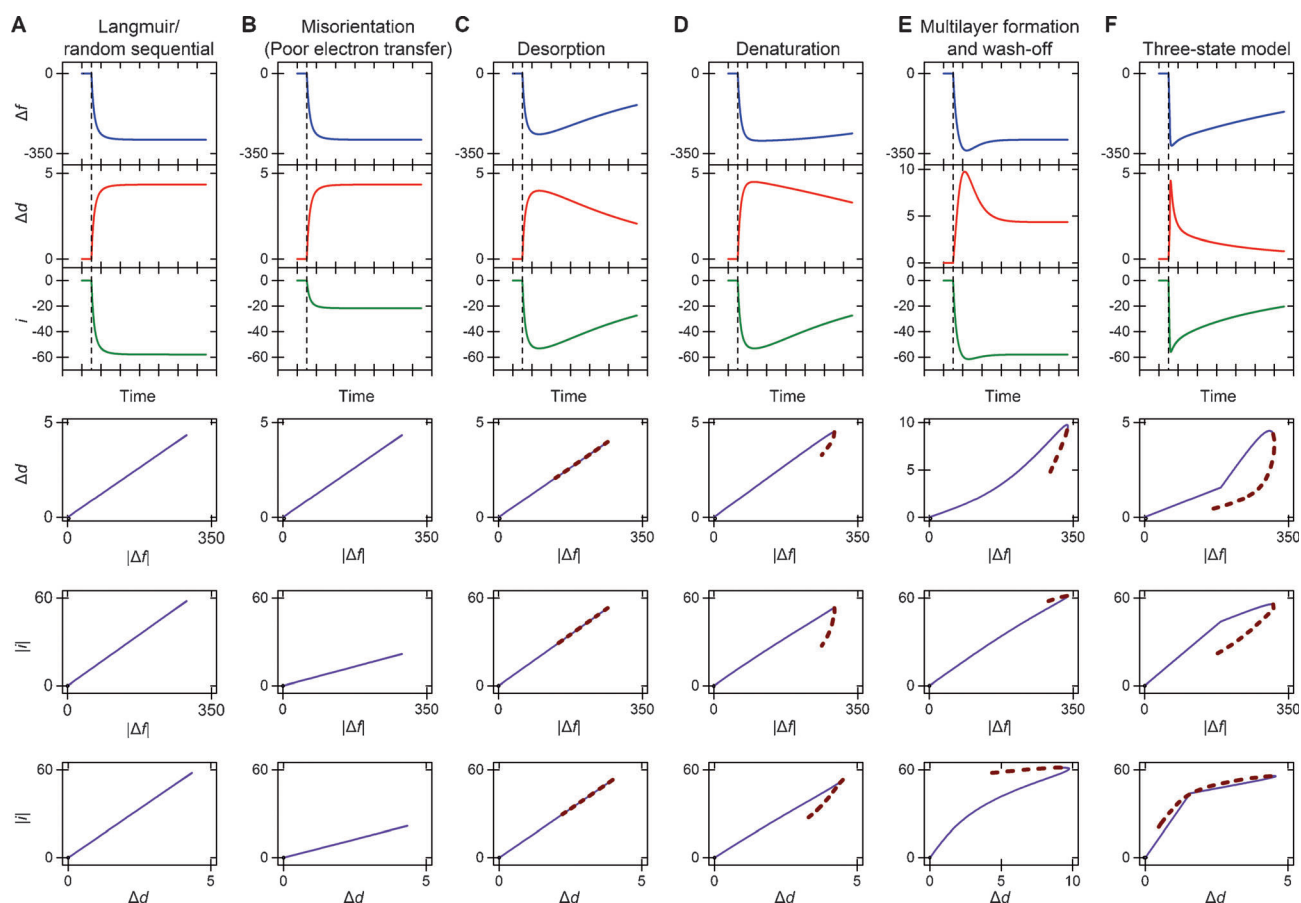


Figure 8. Model E-QCM-D responses for the adsorption of an oxidoreductase and common post-adsorption phenomena. Each vertical panel shows, from the top down, the frequency response (—), the dissipation response (—) and the catalytic current (—) followed by dissipation versus frequency, activity versus frequency, and activity versus dissipation plots. A) Idealised adsorption of a monolayer with no subsequent desorption or denaturation. B) A similar case, but in which the enzyme is orientated in a way that does not promote efficient electron transfer, which leads to a smaller current response or a given mass of adsorbed enzyme. C) Simulation of enzyme desorption from the electrode surface. No structural changes are included so the current response is proportional to the adsorbed mass and the adsorption (—) and desorption (---) lines overlap in the other plots. D) Effects of denaturation accompanied by some dehydration. E) Effect of multilayer formation with a pronounced peak in the dissipation as the surface forms multilayers of enzyme. This layer contributes little to the overall activity then desorbs rapidly. F) Adsorption based on a three-state model from Rabe and co-workers.^[12] It shows a superficially similar response for frequency, dissipation and activity, but there are clear differences in the plots that compare activity to the frequency and dissipation responses.

tion is modelled in Figure 8E: the first adlayer is relatively stiff, tightly associated with the surface and orientated for efficient electron transfer with the electrode. Subsequent layers are less rigid, more weakly attached and have less efficient interfacial electron transfer. This secondary layer dissipates a disproportionate amount of energy for its mass and contributes little to the catalytic current. Thus the dissipation curve shows a pronounced peak, whereas the frequency and current traces show relatively small dips. The current versus dissipation plot shows two adsorption regimes, then deviates in the opposite direction from the identical plot in Figure 8D. The three-state model proposed by Rabe and co-workers is simulated in Figure 8F.^[12] The dissipation versus frequency plot shows four distinct regions similar to those shown in Figure 5B, with a clear inflection point on the purple adsorption trace at a critical surface concentration. The current versus frequency desorption trace (dashed) deviates in the opposite direction from that in the multilayer desorption model, whereas the current versus dissipation desorption trace follows a multi-stage path that de-

pends on the mass and viscoelastic parameters associated with the three enzyme states.

E-QCM-D versus electrochemical surface plasmon resonance

SPR is the process by which polarised light excites the collective oscillation of free electrons in a metal film. The result is an intensity loss in the reflected light beam as a result of light absorption by surface plasmons. The angle of the incident light is varied to minimise the reflected intensity. Surface adsorption events and molecular rearrangements change the refractive index of a composite film and shift the resonant angle. In contrast to QCM measurements, which measure the mass of the protein and any mechanically coupled water, the amount of protein on a surface is inferred from changes in the refractive index.

Electrochemical SPR (E-SPR) combines voltammetric analysis with SPR in much the same way as the E-QCM-D. The surface of interest doubles as the working electrode. Reference and

counter electrodes are fed into the cell, so that electrochemical processes and SPR signals can be monitored simultaneously.

The last few years has seen an increase in the development of this technique with increased focus on the monitoring of redox processes. In a study by Nakamoto et al., E-SPR was used to synchronise redox and optical signals in relation to the redox changes of an osmium–hydrogen peroxidase film on an array of nanometre-sized pores in Au.^[38] E-SPR has also been applied to investigate redox-induced conformation changes of immobilised proteins. Zhai et al. showed that the conformation changes identified in cytochrome c adsorbed onto SAMs were heavily dependent on the double-layer capacitance of the electrode surface. Modification of the Au surface with alkanethiol, however, reduced excess surface charge and enhanced the SPR signal by a factor of 2.7.^[39] In E-SPR, a change in the applied electrode potential can shift the resonance angle by changing the molecular coverage, index of refraction and average thickness of the layer. This can be a problem: potential changes of less than 1 V induce shifts in the SPR angle that are hundreds of times greater than shifts associated with protein conformation changes.^[40] In comparison, the potential that drives the oscillation of the quartz crystal can sometimes be affected by the applied potential during electrochemical monitoring.^[41] We observe a frequency shift of ≈ 1 ppm ($\approx 0.1 \mu\text{g cm}^{-2}$); again this can be reduced if the crystal is pre-modified with thiol coatings.

In E-SPR, the detection limit can be defined as the minimum detectable angular shift and illustrated by the angular position of the sharp decrease in reflected light intensity or SPR “dip”. This in turn is dependent on instrumental parameters such as prism material, wavelength of light and refraction unit. GE Healthcare provide an SPR instrument that can detect the adsorption of 100 Da molecules ($\approx 0.01 \text{ ng cm}^{-2}$) and changes in films up to ≈ 150 nm thick.^[42] For comparison, the Q-Sense E-QCM-D module (QEM401) has a mass detection limit of around 0.5 ng cm^{-2} (equivalent to $< 0.1\%$ of a monolayer of globular 60 kDa protein) and can measure films up to a few micrometres thick, which depends on the viscoelasticity of the film.^[4b, 43]

Conclusions and future implications

Catalysts often represent a significant fraction of the cost of a process or device. In this article, we showed some ways by which an electrochemical quartz crystal microbalance with dissipation monitoring (E-QCM-D) can be used to optimise the immobilisation of electron-transfer catalysts by providing mass-specific catalytic activity, diagnostic tools for failure analysis and a way to monitor how actual operating conditions affect long-term performance.^[9]

One particularly lucrative frontier is “industrial bioelectrocatalysis”: electrode-driven, enzyme-catalysed organic synthesis. Cytochrome P450 enzymes are an especially promising candidate for this application because they have been shown to be active in layer-by-layer films^[31g] and membrane mimics.^[44] P450 enzymes catalyse oxygen-insertion reactions with high stereoselectivity and regioselectivity^[45] and have been engineered to oxidise non-natural substrates.^[46] The methods we have out-

lined can be applied to both P450 monolayers as well as P450 enzymes in more complex surface assemblies.

Electrochemically driven cycling of $\text{NAD(P)}^+/\text{NAD(P)H}$ is another widely useful target well suited to optimisation by E-QCM-D. Instead of driving an enzyme directly that requires NAD(P)H , an enzyme such as lactate dehydrogenase^[47] or a hydrogenase subunit^[48] can be immobilised instead to drive coenzyme recycling of the NAD(P)H -dependent enzyme in solution. The E-QCM-D can be readily applied to develop surface chemistry and structures to support these coenzyme-recycling proteins.

Finally, an exciting emerging area is the use of electrochemically driven enzymes to convert CO_2 into organic materials such as formate for use as feedstock chemicals.^[49] The small volume of the E-QCM-D cell allows the local liquid above an enzyme film to be changed rapidly to explore the effects of substrate and inhibitor concentration, pH and contaminants.

E-QCM-D technology has reached a point at which sophisticated instruments are partnered with rigorous data analysis methods to calculate thickness, density, viscosity and shear modulus in protein films. The use of electrochemical surface plasmon resonance spectroscopy in tandem with the E-QCM-D can help to develop knowledge on surface-specific phenomena by combining the high sensitivity to surface changes of surface plasmon resonance and the intrinsic viscoelastic properties measured by the QCM.

The ease of sensor modification and real-time monitoring of both enzyme immobilisation and subsequent electrochemically driven reactions make the E-QCM-D a unique surface-sensitive testbed for applied bioelectrochemistry.

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