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Structural changes in adsorbed cytochrome c upon applied potential characterised by neutron reflectometry

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Abstract

The structural behaviour of an electron-transfer protein—cytochrome c—at the 316L stainless steel electrode/aqueous interface was investigated over a range of applied potentials using neutron reflectometry (NR) supported by solution depletion isotherms, X-ray reflectometry and quartz crystal microbalance measurements. A custom-made electrochemical cell allowed *in situ* observation of the adsorbed protein across a range of applied potentials; models fitted to the NR data showed a compact inner protein layer at the metal/electrolyte interface and a further thicker but highly diffuse layer that could be removed by rinsing. The overall amount adsorbed was found to be strongly dependent on the applied potential and buffer pH. Subtle but significant changes in the structure of the adsorbed protein layer were seen as the potential was swept between ± 0.4 V, reflecting changing attractive/repulsive interactions between the protein's charged side groups and the surface. At greater applied potentials, irreversible changes in the stainless steel film structure were also observed and attributed to deuterium absorption into the metal.

Introduction

Cytochrome c is a mitochondrial membrane protein that plays a key role in electron-transfer processes within the cell. Such natural redox species have attracted increasing interest over recent years following the development of renewable energy technology devices including microbial fuel cells (MFCs) and biophotovoltaics (BPVs) that make use of microbes able to harvest energy from organic matter or from sunlight via photosynthetic mechanisms. Biofilms of bacterial cells are generally grown onto the anode in order to maximise electron transfer efficiency; the structure of key redox species at this interface is hence of key interest, particularly as their electron transfer to the surface is the rate-limiting step in many examples¹.

The redox-active haem centre of cytochrome c, found on the front face of the protein, is bordered by lysine residues that are positively-charged at pH 7 and hence direct the haem centre towards negatively-charged metal surfaces at which electron-transfer may occur². Several studies have shown that it is possible to tether cytochrome c to gold surfaces using negatively-terminated (e.g. carboxyl groups) self-assembled monolayers (SAMs), through which the protein redox centre is able to transfer electrons via quantum tunnelling preceded by the protein rearrangement to a 'redox active' state³. As the haem redox centre occupies a mere 0.6 % of the exposed protein surface, correct orientation is

critical in allowing electron transfer to occur between cytochrome c and its redox partners in its natural mitochondrial environment; changes to the charged surface groups even far away from the redox centre can have a significant adverse effect on the electron transfer efficiency because of their contributions to the overall dipole moment⁴.

Stainless steels and other metals have shown promise when used as anodes in MFCs⁵; 316L stainless steel has been successfully studied before using NR^{6,7} and was chosen as a model anode material that should remain relatively inert when exposed to electrolyte solution. The inert nature of the stainless steel arises from the outer surface oxide layer, which, though comprising both iron and chromium oxides, is highly enriched in chromium compared to the bulk metal composition and hence more resistant to corrosion^{8–14}. A larger, c-type cytochrome, OmcA, has been found to adsorb strongly to hematite (Fe₂O₃) surfaces and retain redox activity for a given time, after which the conformation or orientation of the adsorbed protein was assumed to change as peaks in the cyclic voltammogram disappeared^{15,16}.

In this work, neutron reflectometry (NR) was used to investigate changes in the structure of the cytochrome c protein at the 316L stainless steel/electrolyte interface as the applied potential was varied. NR is a powerful surface-study tool that gives well-resolved structural information concerning the thickness, roughness and composition of surface layers^{17,18}. Whereas X-rays are scattered from electron density, and hence are best-suited for analysis of metals and heavier elements, neutrons are scattered essentially independently of position across the periodic table and therefore are ideal probes of organic and biological species. The scattering length densities (SLDs) of various species used in this work are shown in Table 1. QCM-D (quartz crystal microbalance with dissipation) measurements were used to complement these data and demonstrate the importance of surface charge in protein adsorption.

	SLD / $\times 10^{-6} \text{ \AA}^{-2}$		SLD / $\times 10^{-6} \text{ \AA}^{-2}$
Si	2.07	Fe ₂ O ₃	7.18
SiO ₂	3.48	Cr ₂ O ₃	5.11
Fe	8.02	H ₂ O	-0.56
Cr	3.03	D ₂ O	6.34
Ni	9.41	Cytochrome c / 100 % H ₂ O	0.55
Mn	-3.05	Cytochrome c / 100 % D ₂ O	3.10

Table 1. SLD values. The SLD value for the cytochrome c protein is dependent on solvent contrast due to its high number of exchangeable protons (values calculated assuming 90 % exchange).

Experimental

Materials

316L stainless steel films were deposited onto silicon substrates (10 x 5 x 1 cm) to thicknesses of around 200 Å at the Nanoscience Centre, University of Cambridge, using electron beam evaporation. 316 L stainless steel powder (7 µm) was obtained from Sandvik Osprey; the powder surface area was found to be 0.3 m² g⁻¹, determined via N₂ adsorption and fitting to the BET equation¹⁹. Cytochrome c from bovine heart and all other materials were obtained from Sigma Aldrich; Indium tin oxide (ITO) powder (50 nm) was also characterised by N₂ adsorption and found to have a surface area of 7.3 m² g⁻¹. All experiments were conducted in phosphate buffered saline (PBS, 0.01 M, pH 7.4).

Solution depletion isotherms

Isotherms were collected by measuring the concentration change of a solution of known initial concentration of cytochrome c in PBS after tumbling with a high surface-area 316 L stainless steel or ITO powder (24 h) and centrifuging to remove the solid. The protein concentration was determined using UV/Vis spectroscopy by comparing the intensities at 410 nm to a calibration plot. The change in concentration is then attributed to surface adsorption.

XRR

XRR (X-ray reflectivity) profiles were collected at the Cavendish Laboratory, Cambridge using a Bruker D8 X-ray diffractometer with Cu target and a Goebel mirror. An accelerating voltage of 50 kV was used and a primary beam size of 0.1 mm. 0.35 mm Soller slits were inserted before the detector, which was operated in 1D mode. Following the NR measurements, further XRR measurements were conducted at the Materials Characterisation Laboratory at the ISIS Neutron facility using a Rigaku Smartlab X-ray diffractometer with rotating Cu anode, a 1D detector and scan speed of 0.125 deg. min⁻¹. All XRR data were fitted using GenX 2.0.0 software²⁰.

Figure 1. Simplified schematic of the custom-made 3-electrode NR cell. The protein molecules in solution/adsorbed at the film surface are depicted as green circles. The working electrode is the deposited thin film.

NR

Neutron reflectometry data were collected using the CRISP instrument at the ISIS neutron facility, Rutherford Appleton Laboratory; full details may be found elsewhere²¹. The instrument was used in nonpolarised mode and data were collected at scattering angles of 0.27°, 0.6° and 1.2. Silicon substrates coated with the 316L stainless steel films were cleaned by UV/ozone (30 min) prior to measurements. A custom-made 3-electrode cell was used, in which the working electrode was the deposited metal film on the silicon substrate and Pt wire and mesh were included in the trough beneath as counter and pseudo-reference electrodes (Figure 1). The films were initially characterised in PBS in two different contrasts (H₂O and D₂O) at 0.0 V before protein solutions were introduced to the cell and potentials applied, as detailed in the text below. A Biologic SP200 potentiostat was used

for the electrochemical measurements. Data were analysed using the RasCAL software with parameters modelled to minimise χ^2 . When modelling the protein layers, the SLD was kept fixed and a solvation % fitted to determine the extent of buffer in the surface layer. The quoted surface coverages were then determined using this value.

QCM-D

The 316 stainless steel sensors were cleaned prior to use in 1 % Hellmanex II (30 min), rinsed with ultrapure water (18 M Ω cm) and dried with N₂; they were then sonicated in 99 % ethanol (10 min), and rinsed and dried again before cleaning by UV/ozone (10 min). Experiments were conducted on a Q-sense E4 QCM at the Nanoscience Centre, University of Cambridge. The QCM instrument was cleaned with 2 % Hellmanex and rinsed thoroughly with ultrapure water before loading the sensors. Background solution (PBS) was flowed through and the sensors left for 24 h to equilibrate. Fresh electrolyte was then flowed through until the frequencies had stabilised. The sample solutions were then introduced to each sensor (flow rate 0.15 mL min⁻¹) and the frequency changes monitored. After the systems had reached equilibrium, background electrolyte was reintroduced to each one followed by ultra-pure water (UPW) until no further changes were observed. Data were analysed using the QSense Dfind 1.0 software. For Sauerbrey model fits, the f7 overtone was used; for viscoelastic model fits, a Voigt model was used.

Results

Isotherms

Figure 2. Solution depletion isotherms for cytochrome c on 316 L stainless steel powder (blue) and ITO powder (red). Experimental data are shown as points and Langmuir model fits as solid lines.

Solution depletion isotherms for the cytochrome c on the 316 L stainless steel powder and ITO powder are shown in Figure 2. Isotherms on both materials could be described by a simple Langmuir model fit, shown as solid lines, implying monolayer adsorption. The plateau value of $1.7 \times 10^{-7} \text{ mol m}^{-2}$ recorded for the ITO substrate is in almost exact agreement with that seen by Collinson *et al.* using a chronoabsorptometry technique for a similar ionic strength²². Albery *et al.* derive an adsorption value of $1.2 \times 10^{-6} \text{ mol m}^{-2}$ for ferricytochrome c adsorbed on a modified gold electrode with an adsorbed layer of 4,4'-bipyridyl²³. This considerably greater coverage value compared to those observed for the substrates in this work may relate to the bipyridyl coating, which permits a much higher rate of electron transfer from the redox protein to the gold electrode, and hence a greater binding constant. Interestingly, whilst cytochrome c clearly adsorbs to a lesser extent on the ITO (compared with the plateau value of $2.4 \times 10^{-7} \text{ mol m}^{-2}$ for the stainless steel), it is believed to retain more of its native structure on this surface compared to other metals, which may be beneficial to the electron transfer process²⁴.

NR

Figure 3. a) XRR data (points) and model fit (solid line) for the 316L stainless steel film for SS1 following deposition. b) SLD profile for the model fit.

The first stainless steel substrate ('SS1') was initially characterised by XRR in air and by NR under buffer solution to extract parameters pertaining to the metal layers; two layers were sufficient to fit the XRR data (Figure 3), comprising a 172 Å steel layer and 29 Å oxide, in good agreement with the estimated deposition thicknesses. The NR data required three layers to adequately describe the steel and its oxide, with a lower SLD layer closer to the silicon (Table 2). There are several possible reasons for this, arising from the different elemental sensitivities of X-ray vs neutron scattering. For example, it may reflect some inclusion of hydrogen during the deposition process, since the NR technique would be considerably more sensitive to this than the XRR. However, the overall thickness was in good agreement with the XRR.

Figure 4. a) NR reflectivity profiles (points) and model fits (solid lines) for SS1 with 2 mg ml⁻¹ cytochrome c at 0.00 V showing models using 1 layer (blue) and 2 layers (purple) protein. (Data offset vertically for clarity.) b) SLD profiles of the model fits (1 layer fit shown as a dashed line).

	Thickness / Å (± 2 Å)	SLD / x 10 ⁻⁶ Å ⁻²	Roughness / Å	Surface coverage / %	Amount adsorbed / mol m ⁻²
SiO ₂	20	3.48	2	-	-
Steel 1	50	6.13 (±0.09)	12	-	-
Steel 2	87	6.75 (±0.09)	4	-	-
Steel 3	79	6.26 (±0.09)	5	-	-
Voltage = 0.00 V					
CC1	22	3.10	7	32.1	1.14 x 10 ⁻⁷
CC2	68	3.10	15	5.0	
Voltage = -0.05 V					
CC1	22	3.10	8	31.7	1.13 x 10 ⁻⁷
CC2	64	3.10	11	5.0	
Voltage = -0.10 V					
CC1	21	3.10	8	32.4	1.25 x 10 ⁻⁷
CC2	70	3.10	14	6.2	
Voltage = -0.40 V					
CC1	27	3.10	7	28.0	1.17 x 10 ⁻⁷
CC2	78	3.10	15	4.0	
Voltage = +0.05 V					
CC1	23	3.10	9	32.0	1.22 x 10 ⁻⁷
CC2	74	3.10	14	5.0	
Voltage = +0.10 V					
CC1	22	3.10	9	32.0	1.17 x 10 ⁻⁷

CC2	73	3.10	14	5.0	
<i>Voltage = +0.40 V</i>					
CC1	24	3.10	7	28.0	1.00×10^{-7}
CC2	56	3.10	15	4.0	

Table 2. Fitted NR parameters for SS1 under buffer solution and with 2 mg ml⁻¹ cytochrome c at different applied voltages, given in the order measured ('SLD' denotes 'scattering length density'; 'CC' denotes 'cytochrome c'). SLD values for SiO₂ and the protein were kept fixed at their calculated values; the SLD values for the metal layer were modelled in the fit as their exact composition was unknown.

Subsequently, a 2 mg ml⁻¹ solution of cytochrome c in PBS/D₂O was introduced to the stainless steel surface and characterised at 0.00 V (Figure 4). A moderately good fit can be made to the data using one layer of cytochrome c, but the fit fails at higher Q_z values. Dividing into a more compact layer close to the surface and a second, highly diffuse outer layer improves the fit considerably. The fitted structural parameters are given in Table 2. The adsorbed protein was best described by a layer next to the stainless steel surface of 22 Å ($\pm$ 2 Å) thickness, 32.1 % surface coverage, and a further diffuse layer of 68 Å ($\pm$ 2 Å) thickness, 5.0 % coverage. By assuming a completely flat surface and a protein density of 1.37 mg ml⁻¹, the amount adsorbed at 0.00 V can be estimated as 1.14×10^{-7} mol m⁻². This is a considerably lower value than that seen in the solution depletion isotherm (Figure 2), where the plateau value was around 2.5×10^{-7} mol m⁻². This difference may be due to an overestimation in the depletion isotherm method, in which the powder is separated from the supernatant by centrifugation, which may cause some level of protein precipitation with the solid, or to differences in the surface groups of the powder compared to the thin film. The differences in the morphologies of the powder and thin film may also have some effect, as protein adsorption is thought to depend on surface roughness, even when accounting for differences in surface area, as in this case²⁵.

The diameter and length of cytochrome c from bovine heart have been reported as 25 Å and 36 Å respectively^{2,26}. The fitted thickness value here of 22 Å ($\pm$ 2 Å) for the innermost layer suggests a compact monolayer, despite the low surface coverage. The marginally lower-than-expected thickness may indicate some protein denaturing on the surface. The diffuse layer is probably caused by the occasional protein molecule loosely adhering to the surface, or to adsorbed protein already on the surface.

Figure 5a) NR data (points) and model fits (solid lines) for SS1 with 2 mg ml⁻¹ cytochrome c at 0.00, +0.40 & -0.40 V as labelled (data offset vertically for clarity). b) SLD profiles for model fits.

Figure 6. SLD profiles of sample 1 at 0.0 V and under applied voltages, as labelled. Protein layers region only shown.

When a potential was applied to the sample, subtle but significant changes in the protein layer are seen across the ± 0.40 V range (fitted parameters given in Table 2; SLD profiles shown in Figure 6). The small decrease in the amount in the second adsorbed protein layer at positively-applied potentials results from the increased repulsion between the positively-charged protein and metal surface. As this diffuse layer is most likely less firmly-adhered, it is more easily removed compared to the inner layer. Interestingly, at both extremes of the applied potential, -0.40 and +0.40 V, the change in the protein layer nearest the surface is almost identical—a small increase in the thickness by 3–5 $\text{\AA}$ accompanied by a decrease in surface coverage, as seen in Figure 5b. Whilst the overall charge of the cytochrome c protein is positive, the presence of numerous positively- and negatively-charged surface groups will lead to some level of repulsion with the surface for any given charge. Indeed, as the protein has a very strong dipole and hence is known to generally adsorb in relatively uniform fashion to any charged surface²⁷, it may be that by adsorbing the protein at 0.00 V, it has adopted an irreversibly-bound optimal conformation for that potential. When the potential is then swept between ± 0.4 V, it

is unable to reorient to a preferred position for each new surface charge and so can only pull away from the charge to minimise the repulsion.

This suggests that any structural changes in the adsorbed protein are caused by, or at least dominated by electrostatic effects and are essentially unrelated to the electron-transfer process between the redox protein and the metal surface. Conductivity and responsiveness to applied electric fields for proteins is generally known to be high due to their water content in solution²⁶. However, the electron-transfer between cytochrome c and the stainless steel surfaces used in this study is expected to be comparatively weak, as the ability of the protein's haem centre to interact with the electrode surface is strongly dependent on its orientation when adsorbed. Enhanced redox coupling has been seen for surfaces where the orientation is controlled to direct the haem centre towards the surface, for example bipyridyl-coated gold²³. These results suggest that choosing the optimal potential to apply to the surface prior to protein adsorption is critical in ensuring the protein adsorbs initially in the optimal configuration, particularly on metals, on which it is expected to bind irreversibly²⁴.

To further investigate the effects of the initial applied potential, a second stainless steel sample ('SS2') was prepared and characterised in the same way; for this sample, the metal could be described using just one layer of 218 Å (± 2 Å) and a 17 Å (± 2 Å) oxide, suggesting a smoother deposition process than for the previous sample.

Figure 7a) NR data (points) and model fits (solid lines) for SS2 with 2 mg ml⁻¹ cytochrome c at -0.50, -0.65, -1.10 and returned finally to 0.00 V as labelled (data offset vertically for clarity). b) SLD profiles for model fits.

	Thickness / Å (± 2 Å)	SLD / $\times 10^{-6}$ Å ⁻²	Roughness / Å	Surface coverage / %	Amount adsorbed / mol m ⁻²
Voltage = -0.50 V					
SiO ₂	17	3.48	2	-	
Steel 1	218	5.67 (± 0.09)	4	-	
Steel 2	17	6.75 (± 0.09)	11	-	

CC1	54	3.10	9	28.0	1.68 x 10 ⁻⁷
Voltage = -0.65 V					
SiO ₂	17	3.48	2	-	
Steel 1	212	5.68 (±0.09)	8	-	
Steel 2	41	6.48 (±0.09)	8	-	
CC1	33	3.10	12	32.9	2.41 x 10 ⁻⁷
CC2	57	3.10	12	18.8	
Voltage = -1.10 V					
SiO ₂	17	3.48	2	-	
Steel 1	118	5.68 (±0.09)	12	-	
Steel 2	89	5.74 (±0.09)	4	-	
Steel 3	45	6.48 (±0.09)	5		
CC1	60	3.10	12	24.0	2.71 x 10 ⁻⁷
CC2	68	3.10	15	14.8	
Voltage = 0.00 V at end of experiment					
SiO ₂	17	3.484	2	-	
Steel 1	78	5.68 (±0.09)	10	-	
Steel 2	136	5.82 (±0.09)	12	-	
Steel 3	35	6.53 (±0.09)	5		
CC1	60	3.10	12	18.5	2.89 x 10 ⁻⁷
CC2	100	3.10	26	15.0	

Table 3. Fitted NR parameters for SS2, given in the order measured.

Figure 8. Adsorbed amounts calculated from the NR model fits for SS1 (orange) and SS2 (blue) at various applied potentials.

2 mg ml⁻¹ cytochrome c in PBS/D₂O was then added at an applied potential of -0.50 V and the sample subjected to applied voltages between -1.00 and 0.00 V. The fitted NR data and SLD profiles are shown in Figure 7 and the fitted parameters listed in Table 3. At -0.50 V, the adsorbed protein could be adequately modelled with one layer, with a thickness of 54 Å (± 2 Å), suggesting the incorporation of

both the inner and diffusely-bound protein layers. The adsorbed amount was significantly higher than for any of the SS1 profiles; as surmised, this may imply the protein was able to adsorb initially in a preferential orientation that maximised the interactions of its positively-charged surface groups with the negatively-charged metal surface, leading to an increased protein surface coverage. (Small differences in the deposited film morphologies may also contribute to the differences in adsorption, although this is not expected to be the dominant effect.) The calculated amounts adsorbed for both samples are shown in Figure 8. The range of values (ca $1\text{--}2.9 \times 10^{-7} \text{ mol m}^{-2}$) encompasses those observed for the depletion isotherm (Figure 2). This may indicate that differences in the values calculated for 0.0 V reflect variation in surface charges for the two substrate types; it is not unlikely that an accumulated surface charge may have built up on the fine powder used for the depletion isotherm, leading to increased protein adsorption.

At -0.65 V, changes were seen in the reflectivity that could no longer be modelled by minor changes in the protein layer and by -1.10 V, the changes in fringe spacing were so great that it was clear the underlying metal layers must be altering; these changes remained when the potential was returned to 0.0 V (Figure S1). At -0.65 V, the changes could be modelled by a significant increase in thickness of the outermost steel layer, but as the applied potential became more negative, it was necessary to divide the steel into three layers, with the middle layer increasing slightly in SLD (Table 3). We avoid using the term 'oxide' for the outer layer in this instance, simply because it seems counterintuitive to talk of an oxide film growing in thickness upon decreasing potential. Whilst it could be possible that the oxide type (magnetite, hematite etc.) is changing depending on the applied potential, as reported from electrochemical deposition measurements²⁸, it seems more likely in this instance that the changes result from interactions with the background electrolyte; when steel undergoes cathodic polarisation, it is thought to absorb hydrogen from the aqueous environment^{29,30}, a phenomenon also seen previously for titanium measured by NR³¹; as the electrode was immersed in D₂O-based PBS buffer in this instance, it seems credible that in fact the higher-SLD ²D is being absorbed instead, leading to the observed gradual increase in SLD and growth of the higher-SLD outer layer. (The scattering lengths for ²D, Cr and O are 6.67 , 3.64 and $5.80 \times 10^{-15} \text{ m}$ respectively.) A model fitted to XRR data of the sample following the experiment (Figure 9) showed a decrease in X-ray SLD for the outer part of the steel film, which would agree with this hypothesis.

Figure 9. a) XRR data (points) and model fit (solid line) for the 316L stainless steel film for SS2 following the NR experiment. b) SLD profile for the model fit.

QCM-D

Figure 10. QCM-D changes in frequency (Δf , f_7 only shown in each case for ease of comparison) for cytochrome c on 316 stainless steel. (Protein solutions introduced at 85 s; * denotes replacement of the protein solution flow with PBS; at the points marked with ◇, the buffer was changed to ultra-pure water.)

The changes in frequencies, Δf , for the 316 stainless steel surfaces upon addition of solutions of cytochrome c at different concentrations and pHs are shown in Figure 10. When the protein solutions were introduced (85 s), an immediate and significant frequency shift was observed in each case, implying protein adsorption at the surface. When a Sauerbrey model is used to fit the data, a figure of approximately $4.0 \times 10^{-7} \text{ mol m}^{-2}$ adsorbed protein is calculated for the 2 mg ml⁻¹ sample at pH 7.4 at equilibrium, and $4.6 \times 10^{-7} \text{ mol m}^{-2}$ for the 2 mg ml⁻¹ sample at pH 10. These figures are significantly higher than the values predicted by either the depletion isotherms or neutron reflectometry (Figure 2Figure 8) and may reflect both an underestimation of surface area (as the QCM-D sensor surfaces

will have some roughness that is not accounted for in the calculations) and also the water associated with the adsorbed protein, which is detected by the QCM-D but not by the other techniques^{6,7,32,33}.

It is interesting that, although much higher concentrations were used than for the depletion isotherms (1 mg ml^{-1} is equivalent to approximately $8 \times 10^{-5} \text{ mol dm}^{-3}$), there is still an increase in the frequency change, and hence amount of protein adsorbed, between the 1 and 2 mg ml^{-1} protein samples. This may indicate that the Langmuir model shown in Figure 2 is not a true fit to the data at these higher concentrations. However, at pH 10, the equilibrium frequency shift is comparable for both concentrations, suggesting surface saturation had been reached.

The isoelectric point (IEP) of 316L stainless steel has been reported as ranging between pH 2.8 and 4.3, depending on the method of analysis used^{34,35}, whereas that of cytochrome c³⁶ is pH 10. At pH 7.4, therefore, there should be a strong electrostatic attraction between the positively-charged protein and negatively-charged stainless steel, whereas at pH 10, a weaker attraction might be expected. However, as is clear from Figure 10, the opposite trend is observed, and a greater amount of protein is adsorbed at the higher pH. This reflects the well-known phenomenon whereby proteins are seen to exhibit maximum adsorption around their IEP values, since there is less repulsion between adsorbed protein molecules, allowing them to form more closely-packed surface layers^{35,37}. There may also be a shift from predominantly electrostatic interactions between the protein and metal surface at pH 7.4 to hydrophobic interactions dominating at the higher pH, which could allow different orientations of the adsorbed protein on the surface; a similar trend was previously observed for cytochrome c adsorption on mesoporous silica surfaces³⁸.

Upon rinsing with PBS, around 10-20 % of the protein layer was removed in each case; this probably reflects removal of the more weakly-bound surface protein layer indicated by the NR results, whilst the more compact inner protein layer remains. More protein was removed when the rinsing medium was changed to ultra-pure water; as the screening effect of the buffer was diminished, the repulsion between the adsorbed protein molecules increased such that some level of desorption was preferable³⁹.

Conclusions

Neutron reflectometry measurements conducted in a custom-made 3-electrode sample holder have revealed subtle changes in structure for an adsorbed layer of cytochrome c protein at the 316L stainless steel electrode/electrolyte interface. The thickness of the protein layer adsorbed at 0.00 V was slightly less than the diameter of a single protein molecule in solution, implying the protein may have denatured to some extent upon adsorption; this was supported by the QCM-D measurements, which showed a significant extent of water association with the adsorbed protein. The thickness of this inner protein layer was found to increase when the applied potential was swept to $\pm 0.40 \text{ V}$, with an accompanying decrease in overall surface coverage, which was presumed to arise from repulsion

between the charged surface and side groups of the protein. The amount adsorbed was also found to depend on the initial applied potential; when the protein was introduced to the surface at -0.50 V, a greater amount was adsorbed at a higher thickness, possibly indicating the protein structure had remained more intact. QCM-D results showed that a greater amount of protein was also adsorbed close to its IEP of pH 10, as the protein molecules were less likely to repel each other and hence could pack more closely together on the surface. Clearly the structure and amount of the adsorbed protein is highly dependent on the balance of charges across both the protein and electrode surface.

At -0.65 V and increasingly negative potentials, changes within the 316L stainless steel film itself were observed, which could be modelled by assuming an ingress of the electrolyte into the metal; this was manifested as an increase in SLD for the NR data, due to the absorption of D₂O and a decrease in SLD for the XRR data.

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Associated Content: Raw data for the SS2, showing more clearly the permanent changes to the film after the applied potential.

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