Secondary Structural Changes in Proteins as a Result of Electroadsorption at Aqueous-Organogel Interfaces

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Abstract. The electroadsorption of proteins at aqueous-organic interfaces offers the possibility to examine protein structural rearrangements upon interaction with lipophilic phases, without modifying the bulk protein or relying on a solid support. The aqueous-organic interface has already provided a simple means of electrochemical protein detection, often involving adsorption and ion complexation; however, little is yet known about the protein structure at these electrified interfaces. This work focuses on the interaction between proteins and an electrified aqueous-organic interface via controlled protein electroadsorption. Four proteins known to be electroactive at such interfaces were studied: lysozyme, myoglobin, cytochrome c, and hemoglobin. Following controlled protein electroadsorption onto the interface, ex situ structural characterization of the proteins by FTIR spectroscopy was undertaken, focusing on secondary structural traits within the amide I band. The structural variations observed included unfolding to form aggregated anti-parallel β-sheets, where the rearrangement was specifically dependent on the interaction with the organic phase. This was supported by MALDI ToF MS measurement, which showed the formation of protein-anion complexes for three of these proteins, and molecular dynamic simulations, which modelled the structure of lysozyme at an aqueous-organic interface. Based on these findings, the modulation of protein secondary structure by interfacial electrochemistry opens up unique prospects to selectively modify proteins.

Introduction

Protein secondary structure and the resultant inter- and intra-molecular interactions determine the tertiary and quaternary structural arrangement of the protein which, in turn, govern protein function. Due to the underlying importance of protein secondary structures, such as α-helices and β-sheets, to higher order structure and function, protein secondary structures have been studied extensively and characterised with a variety of methods including X-ray crystallography, NMR, Fourier Transform infra-red (FTIR) spectroscopy, as well as circular dichroism (CD) and Raman spectroscopy.[1-7] Recently, β-sheet secondary structures have become an important focus in the biophysical sciences as it has been established that β-sheet secondary structures in protein aggregates are involved in numerous disease pathologies.[8, 9] In particular, many neurodegenerative conditions have been linked to protein aggregation by the formation of extended β-sheet secondary structures. Such pathologies include Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Prion disease and neurodegeneration following stroke. However, protein aggregation is not exclusively a pathology-related question, with tight control of protein aggregation required to regulate gelling processes in the food industry, for example.[10]

It is widely accepted that the majority of proteins, if not all, can unfold from a native conformation and adopt an aggregated β-sheet secondary structure if subjected to appropriate conditions.[11] Of pertinent interest is the ability of proteins to selectively fold into discrete, different β-sheet secondary structures, such as parallel or anti-parallel β-sheets. The adoption of a parallel or anti-parallel β-sheet secondary structure has been demonstrated to influence the cytotoxicity of fibrils or oligomers formed.[12] Discerning such interactions is necessary not only for an increased understanding of disease pathologies, but also to enable selective manipulation of protein structure to impart desirable properties in food products. As such, there is great interest, and a demonstrable need, for novel approaches to elucidate the chemical

pathways and interactions which may drive a protein to fold into a parallel- or anti-parallel β-sheet secondary structure.

There are a number of classical methods used to denature protein and induce aggregation. For example, it is well established that lysozyme will denature and aggregate when heated.[13] However, such a method is only suitable as a bulk, single phase manipulation of a sample, offering limited experimental control. Heating also shows little differentiation between proteins, as most proteins denature and aggregate under these conditions. Numerous other methods exist to manipulate protein secondary structure, including acidification or ion complexation as well as both chemical and physical disruption of bonding networks.[14] Similar to heating, these methods can generally only be applied to a single phase bulk sample.

Protein secondary structures have been shown to vary upon interaction with hydrophobic solvents or interfaces.[15] This property has offered scope to methodologically investigate protein secondary structure as a function of hydrophobicity and surface tension in a number of emulsion or micellar systems, or at the interface between an aqueous and organic phase.[7, 16-20] Extending from research at an aqueous-organic interface, the polarisable interface between two immiscible electrolyte solutions (ITIES), which is formed between aqueous and organic phases containing dissolved electrolyte species, offers a defect free, highly flexible substrate,[21] presenting an exciting, but largely unexplored opportunity to investigate the manipulation of protein secondary structure. Traditionally, the ITIES has been used to explore charge transfer via ion transfer (including facilitated ion transfer) and electron transfer reactions.[22] This enables a wide range of interfacial processes to be investigated including interfacial assembly and catalytic function.[23-25] To this end, protein electrochemistry at the aqueous-organic interface has been studied by many, including reports on the proteins of interest in this work: hemoglobin (Hb),[26, 27] myoglobin (Myo),[28] cytochrome c (Cyt c)[29, 30] and lysozyme (Lys).[31] While such studies have led to fine control and understanding of the electrochemical process, there is little information on the influence of the electrochemical interface on the secondary structures of the proteins.[21] From previous work, it was

proposed that electrochemically-induced protein adsorption at the ITIES occurs following the application of a positive potential bias to the aqueous-organic interface. This process is accompanied by facilitated ion transfer of the organic anion from the organic phase to form a complex with the protein.[32] As such, the ITIES offers the ability to exquisitely control and vary the organic anion-protein binding interactions at the interface, as distinct from manipulating protein in a bulk phase. Mechanically-improved ITIES systems were developed a number of decades ago by using gelled aqueous or organic phases to partially solidify one of the components, often using agar for aqueous phases or polyvinyl chloride for organic phases, resulting in a polarisable liquid-gel interface. Such phases produce similar electrochemical responses to those seen in liquid/liquid systems whilst expanding the range of experimental methods that can be applied at these soft interfaces.[33-35] Such aqueous-organogel interfaces have been used widely in studying the electrochemistry of proteins.[21, 28, 30, 36-38]

In this work, we identify the influence of electrochemical adsorption at the ITIES on the protein secondary structure by FTIR spectroscopy, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF/ToF MS), and molecular dynamics (MD) simulation. We present direct evidence that the ITIES-mediated protein assembly induces protein-specific changes in secondary structure, which are not observed in single phase interactions between the protein and organic anion. Furthermore, the propensity to adopt an anti-parallel β-sheet confirmation was found to differ between proteins. Such a finding highlights the potential of the ITIES-induced protein assembly to enable manipulation of protein secondary structure and enhance insight into the chemical and physical factors that influence such structures.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich Australia and used without further purification. HCl (6 M) was used to control solution pH and as an aqueous electrolyte. Samples in neutral pH were prepared using phosphate buffered saline (PBS, pH 7.4). Fresh solutions of lysozyme (hen egg white, >90%), cytochrome c (bovine heart, >95%), myoglobin (equine heart, >90%) and haemoglobin (bovine blood) were prepared each day and stored in the dark prior to use. The organic electrolyte used in all reactions, bis(triphenylphosphoranylidene) tetrakis (4-chlorophenyl)borate (BTPPATPBCl), was synthesised through a metathesis reaction between bis(triphenylphosphoranylidene) chloride (BTPPACl) and potassium tetrakis (4-chlorophenyl)borate (KTPBCl), and recrystallised in 1:1 acetone:ethanol.[39] Ultrapure water (resistivity of 18.2 MΩ cm, from a Milli-Q system) was used in all aqueous solutions. Fresh organic phase gel solutions were also prepared each day by addition of 10 wt% polyvinyl chloride (PVC) to the organic electrolyte (10 mM BTPPATPBCl in 1,2-dichloroethane (DCE)), with stirring, at 105 °C. The organic solutions were pipetted into the cells (glass Pasteur pipette tip, diameter 5 mm) whilst warm and allowed to cure for 1 hour prior to electrochemical measurements.

Electrochemistry

Electroadsorption of protein at the ITIES was carried out at the aqueous/organogel interface with the cell composition provided in Table 1 (cell schematic in the SI, Figure S1).

Ag/AgCl	1 mM BTPPACl 10 mM LiCl Aq	10 mM BTPPATPBCl 10 wt% PVC DCE	100 µM Protein 10 mM HCl Aq	Ag/AgCl

Table 1. Cell composition for protein electroadsorption at the liquid/organogel interface. The proteins used were haemoglobin, myoglobin, cytochrome c or lysozyme.

In this cell, homemade Ag/AgCl electrodes were used as counter electrodes in a 2-electrode cell. The interfacial area is ~0.20 cm^2. A blank cyclic voltammogram (i.e. only background electrolytes in each phase) was recorded on each fresh cell to ensure that there was no drift in the potential scale. All potentials reported here are relative to the Ag/AgCl electrodes employed. No ohmic compensation was applied during the electrochemical measurements. The protein was then added to the aqueous phase and a fixed potential was applied to the interface for 30 minutes. CV measurements in the presence of the protein were conducted in fresh cells and provided results in agreement with previous CVs of these proteins.[26-31] All electrochemical measurements were performed on an Autolab PGSTAT302N (Metrohm, The Netherlands).

Single phase experiments

A series of control experiments in single aqueous phase media were undertaken. These enabled us to examine the interaction of the proteins with a buffer, under heating, and in the presence of the organic anion, all within aqueous media. The PBS solutions contained 100 μM of the relevant protein along with 10 mM PBS (ca. pH 7.4). For the heat-denatured samples, the protein (100 μM) was dissolved in aqueous 10 mM HCl and heated to 62 °C for 30 minutes. For the organic anion complexation experiments, an aqueous solution was prepared containing 100 μM of the protein and 1 mM KTPBCl in 10 mM HCl; the samples were shaken vigorously and then allowed to stand for 1 hr. All aqueous samples were dropcast onto the ATR-FTIR crystal for measurement.

FTIR spectroscopy

Attenuated total reflection (ATR)-FTIR measurements were performed on a Nicolet iS50 FT-IR spectrometer (ThermoFisher Scientific, Massachusetts, USA) using a diamond ATR crystal. 128 accumulations were collected for each sample with a resolution of 4 cm^{-1}. ATR-FTIR measurements were performed on aqueous samples and on organogel surfaces. The aqueous samples were dropcast onto

the ATR crystal and allowed to partially dry, forming a thin film on the crystal surface before measurements. The organogel samples were removed from the electrochemical cells and allowed to partially dry before placing on the ATR crystal for measurement. For potential dependant assembly, 9 or ≥3 measurements were performed at 0.90 V and 0.30 V, respectively. Other samples were all subjected to three separate measurements. Initial data normalisation was performed using OPUS spectroscopy software (Bruker) and calculations of the second derivative spectra were conducted in Origin Pro (OriginLab, Northampton, MA).

MALDI-ToF Mass Spectrometry

Proteins were subjected to analysis following electroadsorption at the aqueous-organogel interface. Protein was removed from the gel surface by sonication (5 min) in 40 µL acetonitrile. 160 µL of water was added for a second sonication (5 min). The following day, 2 µL of sample was mixed with 2 µL 2% v/v trifluoroacetic acid. 2,5-dihydroxyacetophenone in ethanol (375 µL, 50 mM) was added to aqueous diammonium citrate (125 µL, 80 mM) and 2 µL of this preparation was mixed with the sample solution. 0.5 µL of this crystallising mixture was then spotted onto a MTP-384 ground steel target (Bruker Daltonics). MS acquisitions were externally calibrated using protein calibration standards 1 & 2 (Bruker Daltonics) prepared with the same matrices.

Spectra were acquired on an ultrafleXtreme MALDI-ToF/ToF instrument operating in linear positive ion mode in the m/z range 2000-20000 (DHAP). Spectra were acquired with laser repetition of 500 Hz and 500 shots per acquisition without partial sampling. 2000 (calibrant) and 4000 (sample) shots were collected manually. Calibrant spectra were smoothed prior to cubic enhanced external calibration. The spectra were analysed using R.[40]

Molecular dynamics simulation of lysozyme

The conformational behaviour of a single molecule of lysozyme upon electroadsorption at an aqueous/organic interface and anion complexation was characterised using MD simulations. Lysozyme was selected as the only protein to be investigated given that the other proteins (cytochrome C, myoglobin and haemoglobin) all showed evidence of significant aggregation at the interface upon electroadsorption. All MD simulations were performed using the same protocols reported in our earlier study of the adsorption of lysozyme at a polarised water/DCE interface.[41] All simulations were conducted using GROMACS 4.6.7 using the OPLS-AA force field for the protein, DCE and ions, and the TIP4P/2005 water model. Polarization was induced by applying an external electric field of strength 3.5×10^7 V m^{-1} directed from the aqueous to the organic phase. An adsorbed conformation of lysozyme at low pH was taken from this previous study as the starting structure for this work. To simulate facilitated ion transfer, a tetraphenylborate (TPB^-) anion was added to the organic (DCE) phase, along with a tetramethylammonium (TMA^+) counter ion to neutralize the system. Initially, a conventional MD simulation was performed until the anion made contact with the protein at the interface (which occurred after 27 ns of simulation time). At this point, replica exchange molecular dynamics (REMD) simulations were performed using 96 replicas at temperatures ranging from 298.15 to 408 K for 150 ns each, totalling 14.4 μs of simulation time. Configurations were saved every 10 ps for further analysis of secondary structure content in the reference replica at 298.15 K, which was done using the DSSP module implemented in GROMACS. DSSP assigns secondary structure using hydrogen bonding interaction and simple geometric criteria. Secondary structure was assigned for each residue based on the 3D coordinates of lysozyme at each snapshot of the simulation trajectory.

Results and Discussion

FTIR spectroscopy of electroadsorbed protein

As is now well established, protein adsorption at the ITIES can be controlled by manipulation of the applied potential.[42] The protein must be cationic to undergo the adsorption, therefore it is necessary to perform the experiments at a low pH (pH 2) to ensure that the protein is fully protonated.[31] Typically, cyclic voltammetry (CV) at the ITIES has been used to characterise the electrochemical adsorption of the proteins studied here.[21, 26, 43-45] Characteristic CVs recorded in this work for each protein in an aqueous/organogel configuration (Figure 1) are in agreement with previous reports.

Facile measurement of the FTIR response of the interfacial protein assemblies is made possible by using an organogel as the organic phase (Table 1). Following the electroadsorption procedure, the organogel phase may be removed from the electrochemical cell for analysis without the need for a substrate to collect the adsorbate or to re-suspend the protein. To examine the influence of the organic phase and the organic anion on the secondary structure of the protein, spectroscopic measurements were carried out on proteins following interfacial adsorption controlled by a fixed applied potential for 30 minutes. Applied potentials were chosen to replicate the open circuit potential, where no potential-driven protein adsorption is observed (0.30 V), and to induce interfacial adsorption for each protein (0.90 V), based on CVs at the liquid/organogel interface (Figure 1). Based on these CVs, 0.90 V accounts for any small variations between the proteins and for ohmic drop by providing a sufficient overpotential to that required for adsorption (ca. 0.70 V).

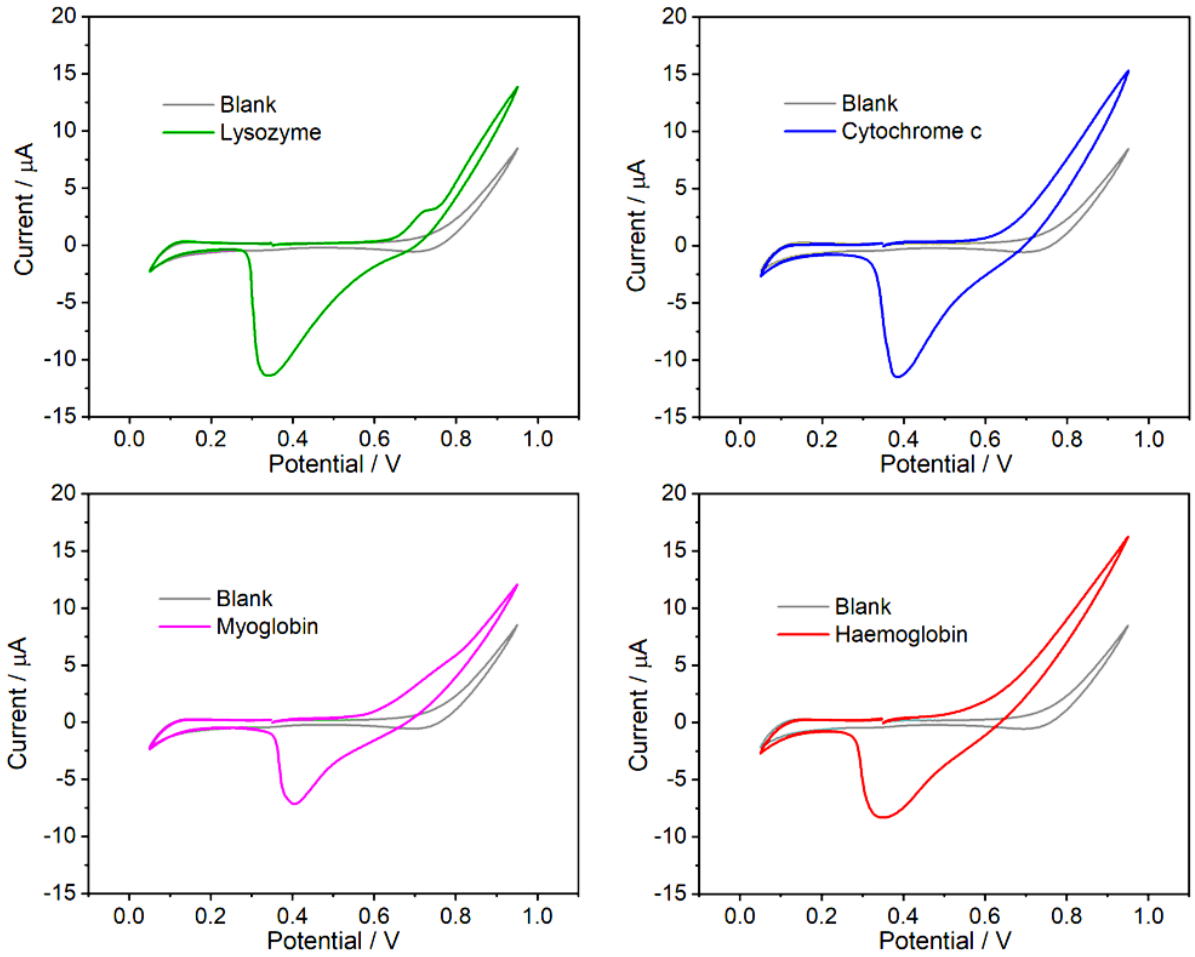

Figure 1. Cyclic voltammetry of lysozyme, cytochrome c, myoglobin and haemoglobin at a liquid/organogel interface. The CVs were conducted at a scan rate of 25 mV s^{-1}. The cell contained 100 µM of protein in each case (the full cell composition is included in Table 1).

The proteins examined provide clear features in the FTIR absorption spectra which are common for all proteins, described as the amide A, amide I and amide II features (Figure 2a). Following adsorption at 0.90 V, there was an intense feature present within the amide I region indicating a significant build-up of protein at the aqueous-organogel interface (Figure 2b, 2c). Due to the color of some of the proteins, it was also possible to see the interfacial deposit by eye following adsorption. This indicates deposition of a significant quantity of protein, probably many monolayers, as determined in previous studies.[31, 36-38] As reported in the literature, the spectra are sensitive to the protein environment.[46, 47] Each of the adsorbed

proteins provides a spectral response due to the presence of α-helices between 1651 and 1655 cm^{-1}. A feature around 1650 cm^{-1} can also be an indicator of an unordered protein structure.[3] In the native proteins (Figure S2), there is a feature at 1630 cm^{-1} present in the spectra of Hb, Myo and Cyt c. This feature indicates the presence of β-sheets within the protein. However, following interfacial electroadsorption, there is a shift in the position of the β-sheet peak for these proteins (Hb, Myo and Cyt c), to ~1625 cm^{-1}, and a second peak, at 1694-1696 cm^{-1}, has appeared. Absorption peaks at these positions have been reported to indicate the formation of intermolecular anti-parallel β-sheets due to transition dipole coupling.[3, 48, 49] These features point towards the formation of a significant quantity of rigid, irreversibly aggregated protein, suggesting oligomer formation.

In the spectra of the adsorbed proteins there is some variability in the intensity of the peaks observed in the second derivative (Figure S3 and S4). In the case of Hb, all samples show an intense peak at 1624-1625 cm^{-1} (anti-parallel β-sheet), whilst for Myo and Cyt c, some measurements show peaks at this position whilst others have no clear intensity in this region. This can be attributed to factors such as the number of possible protein orientations at the interface, the complex multilayer structures which form,[30] and the propensity of the protein to unfold on interaction with the organic phase. Whilst the intensity of this peak varies, there is no significant shift towards higher wavelengths, showing that the feature is a discrete indicator of aggregated anti-parallel β-sheets, with no intermediaries between these and native

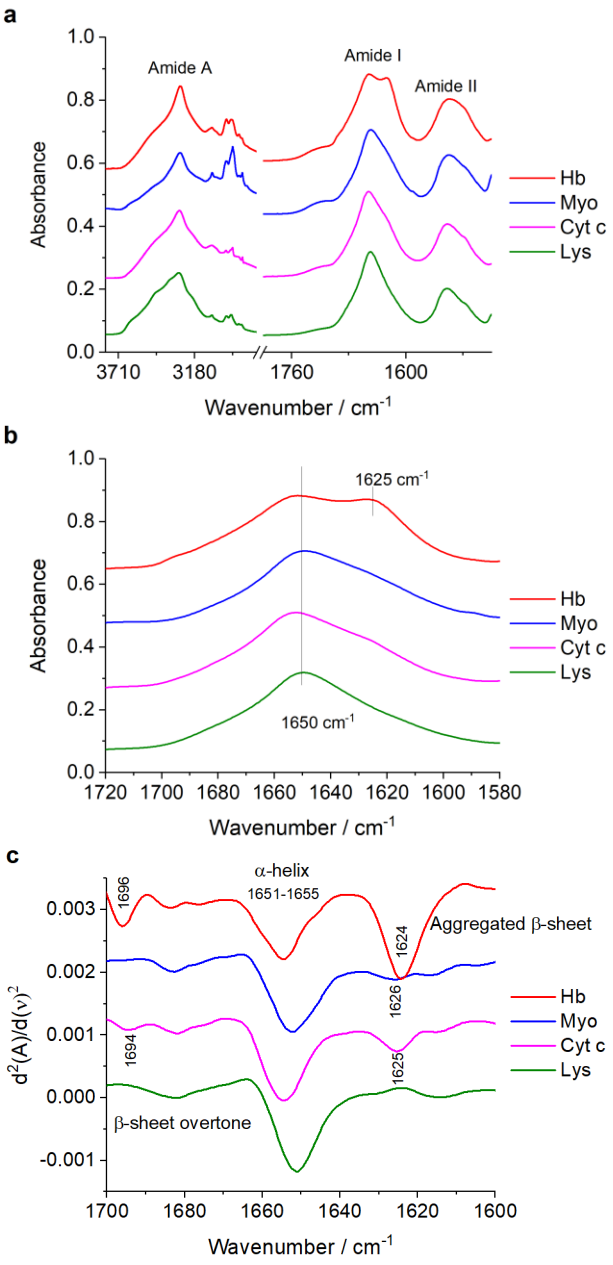

Figure 2. FTIR spectra for each of the proteins following electroadsorption at the aqueous/organogel interface, driven by a fixed applied potential of 0.90 V for 30 minutes. (a) shows the wavebands for the amide A, amide I and amide II features, (b) the amide I feature, and (c) the second derivative of the amide I peak, indicating the protein secondary structure. The protein abbreviations Hb, Myo, Cyt c and Lys correspond to hemoglobin, myoglobin, cytochrome c and lysozyme, respectively.

β-sheets observed. Work compiled by Zandomeneghi and co-workers[50] has identified the responses of amyloid fibrils between 1611 and 1630 cm^{-1}, quite distinct from native β-sheets which are observed between 1630 and 1643 cm^{-1}. In the control samples (Figure S2 and S6-8) there is little change in intensity or peak position between measurements, and in these control samples, the only beta-sheet feature observed was at 1630 cm^{-1} or higher with no feature present between 1694 and 1696 cm^{-1}. This indicates that no aggregated β-sheets were observed in the absence of adsorption at the liquid/organogel interface.

To examine the direct impact of potential on protein adsorption, measurements were also undertaken following application of 0.30 V for 30 min. This potential is below the onset of potential-driven protein adsorption and organic anion complexation. Therefore, any protein adsorption within this region is spontaneous rather than driven electrochemically. As a result, there is only a very small quantity of adsorbed protein expected. This makes analysis of the second derivative feature difficult. However, in samples where sufficient protein had adsorbed to allow examination of the second derivative of the amide I feature, the response was similar to that under potential-driven adsorption (Figure S4). Of significance is the response to Hb (Figure 3) whereby the spectrum indicates significant aggregated β-sheet formation on interaction with the organic surface alone, rather than through complexation with the organic anion, as phase transfer of the organic anion does not occur at this applied potential (0.3 V). This response also indicates that the applied potential is only acting to drive protein assembly at the interface and does not appear to have a direct effect on the protein structure. It can be expected that the polarisation of the interface will also result in the accumulation of supporting electrolyte at the interface. However, the similarity of the response from haemoglobin at OCP and following electroadsorption at 0.90 V suggests that the electrolyte, like the applied potential, does not alter the structural rearrangements that occur.

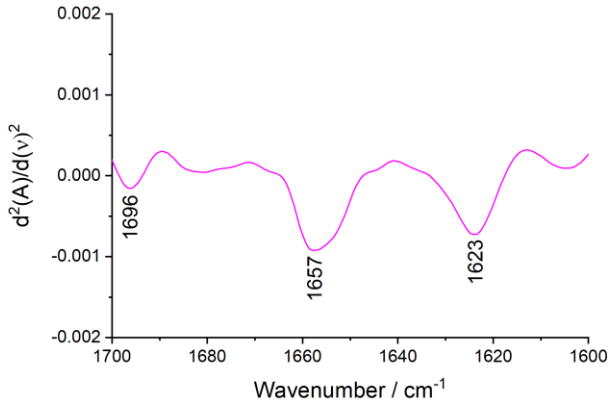

Figure 3. Second derivative of the amide I feature within the FTIR spectrum of haemoglobin. This measurement follows spontaneous protein adsorption at the aqueous/organogel interface under an applied potential of 0.30 V for 30 minutes.

Variation in structural rearrangement between proteins

The spectra collected following application of 0.90 V (Figure 2c) allow for the direct comparison of the four proteins studied. As proposed by Norde et al., the structural flexibility of proteins may be defined as hard or soft, whereby hard proteins have a more rigid, stable structure and soft proteins have a less stable, more flexible structure.[51-53] As such, it can be anticipated that harder proteins (Lys, Cyt c), would undergo less structural rearrangement due to stronger intermolecular interactions. In comparison, the softer proteins (Myo, Hb) should readily unfold to enhance the interaction with the organic phase. Evaluation of the spectra of the four proteins indicates that adsorbed Lys shows no major unfolding of the protein, because the only significant spectral feature is the peak at 1652 cm^{-1}, due to α-helices (Figure 2, Figure S3). This is to be expected given the presence within its structure of four disulphide bridges that stabilize its conformation. Our simulations (vide infra) reveal that the helical content is largely retained during adsorption, as most conformational changes occur in the turns, bends and some alpha-helical regions. There is no feature at 1624 cm^{-1} which relates to the formation of anti-parallel β-sheets. Most of the Lys structure is helical in nature, however the simulations show that the small amount of β-

sheet content is largely retained even after partial unfolding at the interface. In contrast, when Cyt c and Myo are adsorbed at the interface they show greater proclivity towards anti-parallel β-sheet formation, although the feature at 1624 cm^{-1} is not clearly resolved in all measurements. Finally, in the case of Hb, the largest protein analysed, anti-parallel β-sheet formation was observed in all six of the samples, with significant peaks at 1624 cm^{-1} and 1698 cm^{-1} (Figures 2 and S3). In some cases, this feature was more intense than that of the α-helix, suggesting significant alteration of the protein structure on interfacial adsorption. Overall, the observations point towards a link between the softness of the protein and the extent of structural rearrangement on interaction with the organogel surface.

Biphasic electrospray mass spectrometry data reported previously also suggested that Lys did not undergo significant aggregation upon interfacial adsorption.[32] Lys has, however, been shown to undergo partial unfolding at a liquid/organogel interface by electrostatic spray ionization mass spectrometry[38] and significant anti-parallel β-sheet formation through thermal treatment.[54] This variation between techniques can be due to the multilayer structures formed at the interface, where the protein at the surface may undergo unfolding but this may not propagate through the layers, as our own previous computer modelling has suggested that Lys does indeed unfold partially at such interfaces.[41]

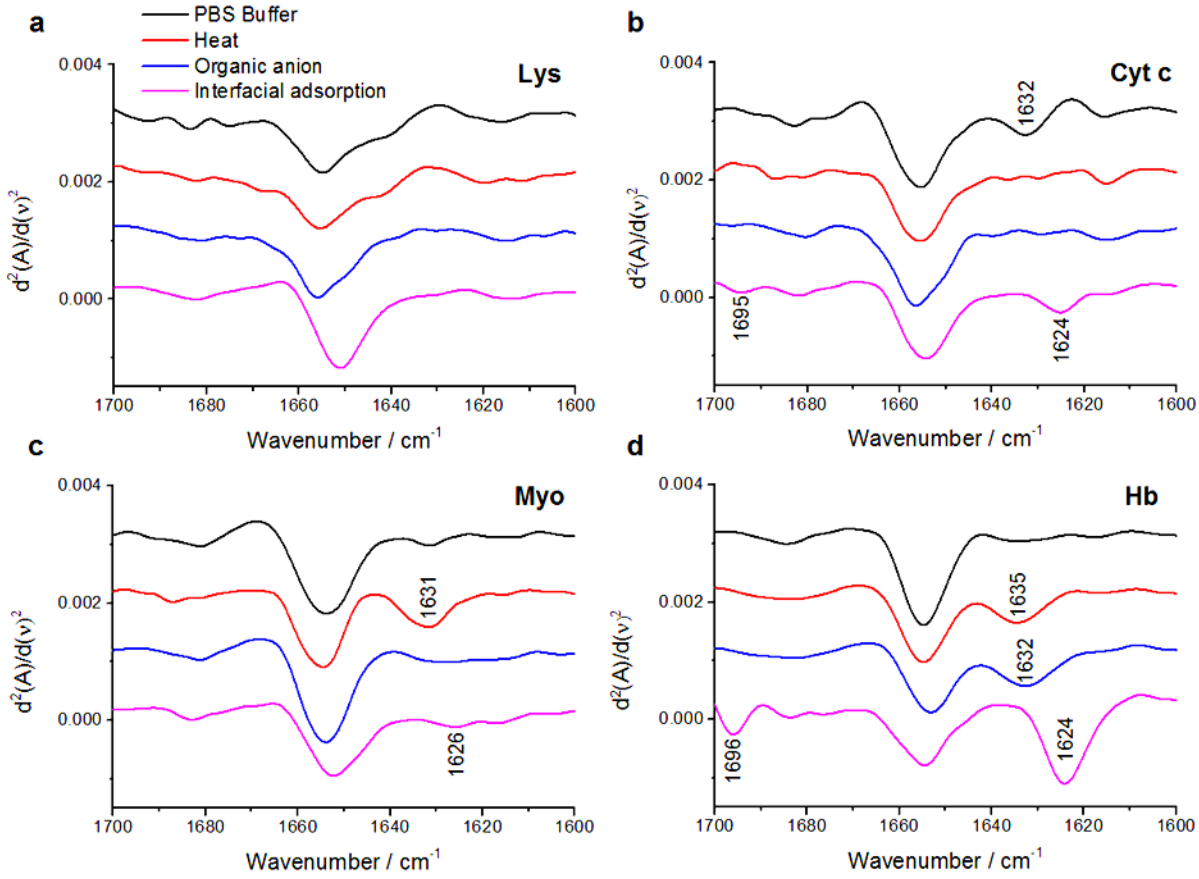

Figure 4. FTIR spectra showing the second derivative within the amide I band region for representative spectra of (a) Lys, (b) Cyt c, (c) Myo and (d) Hb, in buffer solution, after heating, after mixing with $TPBCl^-$, and following potential-dependent interfacial adsorption. The protein abbreviations Hb, Myo, Cyt c and Lys correspond to hemoglobin, myoglobin, cytochrome c and lysozyme, respectively.

Significance of the protein adsorption on the structural rearrangement

To establish the influence of the interfacial adsorption on the observed structural changes, the process was compared directly with spectra for proteins complexed with organic anions without interfacial or electrochemical stimuli and thermally-denatured proteins (Figure 4 and S6 and 7). Complexation of the

organic phase electrolyte anion is a prominent feature within CVs of proteins, occurring in combination with adsorption of protein at the interface, so that it is important to isolate the relative contributions of the anion and the organic phase on the interfacial structure of the protein.

The spectra presented in Figure 4 show that the important observation is that, whilst heating and ion complexation induce structural change within the soft proteins (Myo and Hb) including the formation of β-sheet structure (as evident by responses in the region 1631-1635 cm^{-1}), it is only through adsorption at the ITIES that anti-parallel β-sheet formation occurs (1624-1626 cm^{-1}).

MALDI-ToF MS following protein electroadsorption

The FTIR spectroscopy results were supported by MALDI-ToF MS of the proteins following adsorption on the organogel surface at 0.90 V (Figure 5 and Table S1). As for the FTIR spectroscopy, ex situ characterisation of the electroadsorbed protein was undertaken. Figure 5a shows the spectra following control experiments in which a potential was applied in the absence of protein, or the protein was present at open circuit conditions: the spectra indicate that no protein was detected under these conditions. Figure 5b shows the protein spectra obtained after protein electroadsorption at 0.90 V. Based on the assumption that the anion complexation does not impact ionization efficiency, and that adduction – rather than complexation – is not occurring in the gas phase,[32, 55, 56] the ratio between the peak intensity attributed to the protein-TPBCl complex $[protein+2H+TPBCl]^+$ and the signal of the protein $[protein+1H]^+$ showed the following trend Myo < Cyt c < Lys with ratios of 0.02, 0.08 and 0.2, respectively. This trend is in concurrence with the extent of unfolding of the four proteins (Hb > Myo > Cyt c > Lys) discussed above. Hb showed a significantly reduced signal compared to the other complexes (Figure S9). This may be due to either the low abundance or the low ionisation efficiency of Hb-anion conjugates. In addition, the absence of protein aggregates, which are postulated to form at the interface,[32] could be the result of complexes that are either too large to efficiently ionise or that do not survive the

MALDI process. The former observation is in line with previous work on electrochemically-induced oligomerisation of Cyt c.[30] It was also possible to detect all four proteins when co-electroadsorbed from their mixture at 0.90 V (Figure 5c), together with the corresponding anion complexes [protein+2H+TPBCl]$^+$ and protein [protein+1H]$^+$ (Figure S11 and S12) for the individually-electroadsorbed proteins.

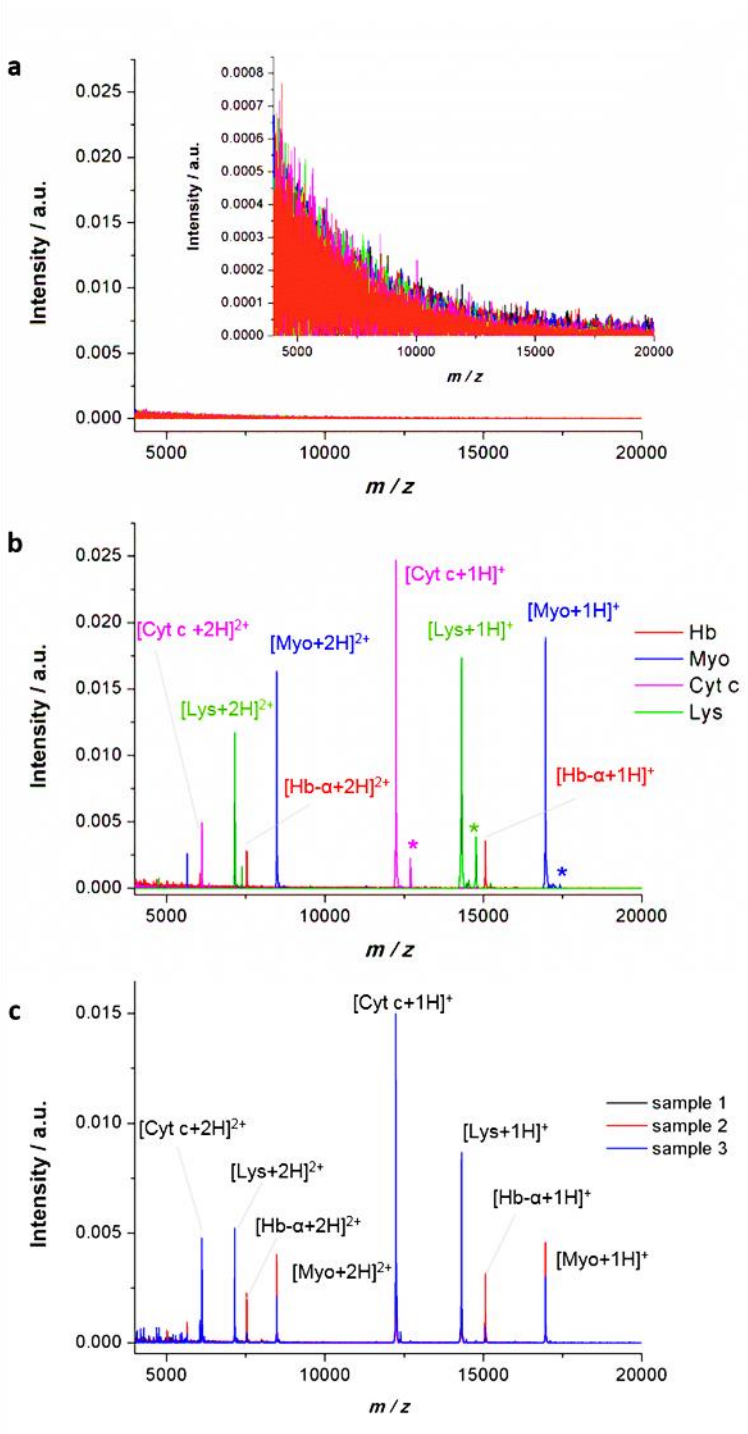

Figure 5. (a) MALDI-ToF/ToF spectra for control experiments corresponding to no protein in the aqueous phase at an applied potential of 0.30 V and 0.90 V and 100 μM of protein in the aqueous phase at open circuit potential for 30 min. (b) MALDI-ToF/ToF spectra for the individual proteins after electroadsorption at 0.90 V for 30 min. m/z values for all of the peaks are summarised in Table S1. (c)

MALDI-ToF/ToF spectra for triplicates of a mixture containing the four individual proteins after electroadsorption at 0.90 V for 30 min. **m/z* peaks correspond to protein complexes $[protein+2H+TPBCl]^+$ for Cyt c (*), Lys(*) and Myo (*). The protein abbreviations Hb, Myo, Cyt c and Lys correspond to hemoglobin, myoglobin, cytochrome c and lysozyme, respectively.

MD simulation of lysozyme during electroadsorption and anion complexation

Analysis of secondary structure content in Lys revealed the occurrence of conformational changes upon adsorption and anion complexation at the polarized aqueous/organic interface. Figure 6 shows that it is primarily bend, turn and α-helical structures in Lys that are affected. These changes in the structure of Lys mainly occurred in regions of the protein that mediated its adsorption at the interface. Multiple distinctive orientations of the adsorbed protein showed that amino acid residues from both the N- and C-termini along with central regions of the protein mediated interactions at the interface and were associated with local loss of secondary structure. These observations are consistent with our previously reported simulations of Lys at a polarised DCE/water interface without any anion present.[41] Overall these observations are consistent with the above experimental data, such that Lys only partially unfolds upon electroadsorption and anion complexation, with significant retention of helical structures and no significant changes in β-sheet content. The latter is further explanatory evidence for the lack of aggregation observed for Lys.

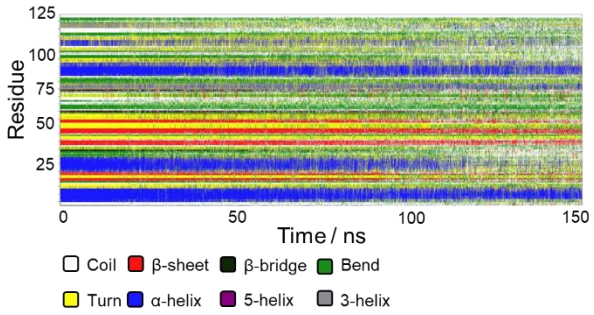

Figure 6. Time evolution of the secondary structure of lysozyme during the MD simulation. Each type of secondary structure is indicated by color.

Conclusions

Controlled electroadsorption at an aqueous/organogel interface was performed on the proteins haemoglobin, myoglobin, cytochrome c and lysozyme. The properties of the electroadsorbed proteins were then examined by FTIR spectroscopy and MALDI-ToF-MS. Analysis of the second derivative of the amide I feature within the FTIR spectra enabled the determination of protein structural alterations following interaction with the organic phase. The results show that on interaction with an organic phase, cytochrome c, myoglobin and haemoglobin undergo clear unfolding to form anti-parallel β-sheet aggregates, whilst the structure of lysozyme remained by comparison relatively unaltered showing no signs of aggregation mediated by β-sheets. The results of these experiments show that this structural rearrangement is specifically dependant on the interaction with the organic phase rather than the influence of the applied potential or complexation with the organic electrolyte anion. In this situation, the role of electrochemistry is simply to drive more protein material onto the interface, which makes detection of the protein easier. The MALDI-ToF-MS data indicate that intact protein and protein-organic anion complexes may be detected, confirming aspects of the proposed mechanism of protein electroadsorption.[21, 31, 32] Such aqueous/organogel interfaces therefore offer a unique platform to examine

the interaction between proteins and organic phases in biologically relevant systems, utilising electrochemistry to modulate the protein interfacial interaction in tandem with spectroscopic analysis.

ASSOCIATED CONTENT

Supporting Information. The supporting information contains a description of the electrochemical cell, further FTIR data and additional MALDI-ToF MS measurements. These files are available free of charge.

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