Electrochemistry of proteins at the interface between two immiscible electrolyte solutions

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Summary. The electrochemistry of proteins at the interface between two immiscible electrolyte solutions (ITIES) is discussed, with detection capabilities based on protein-facilitated anion transfer, and which has enabled protein detection via adsorptive stripping voltammetry. Targeting an enzymatic biomarker has achieved detection at picomolar concentrations, whilst studies into structural aspects of proteins at the ITIES have revealed formation of oligomers (cytochrome c) and unfolded protein (lysozyme) together with alterations to the protein secondary structures.

Introduction

Electrochemistry at the interface between two immiscible electrolyte solutions (ITIES)[1] provides a powerful means to harness interfacial charge transfer processes for a range of possible applications[2]. The behaviour of ionised biomolecules at such interfaces has been of interest and the interactions of proteins, in particular, with this interface opens up possibilities for label-free detection. This Opinion focuses on recent results pertaining to the understanding of protein behaviour at the ITIES and to uses of that behaviour in label-free detection (sensing) strategies.

Methods for the detection of proteins are highly significant in solving societal problems in biological and biomedical sciences. The ability to measure protein concentrations and activities can be used as a means to understand important cellular processes and even to help in the rapid diagnosis of disease. In the latter case, certain proteins can serve as indicators of the disease and so detection of those proteins enables the detection of the disease, perhaps at an earlier stage than might be achieved if relying solely on patients' physical symptoms. Such proteins are referred to as biomarkers, and the opportunity to detect these in a label-free manner at extremely low concentrations in complex biological fluids such as blood, serum and saliva opens up opportunities to contribute to disease diagnostics and management[3].

Proteins at the ITIES

Whilst the electrochemical behaviour of proteins at the ITIES has a long history, it is only in the last decade that significant understanding has been developed. Vanysek et al.[4] studied

proteins at water-nitrobenzene interfaces and found that adsorption occurred but no ion transfer current associated with the proteins was observed. Studies of the polypeptide protamine[5] revealed a direct ion transfer process attributed to the polypeptide transferring into the organic phase. Studies of protein behaviour at the ITIES in the presence of surfactants [6-9] revealed that the formation of reverse micelles in the organic phase could be used as a strategy to transfer proteins across the ITIES and enable their label-free detection. In such studies, however, Shinshi et al.[10, 11] reported an interesting control experiment in the surfactant-induced electrochemical extraction of proteins at the ITIES. In the absence of surfactant, the coloured protein cytochrome c was seen to not transfer across the interface, despite the presence of a substantial current response in the voltammogram. This transfer was attributed to the transfer of background electrolyte anions from the organic phase to the aqueous phase to complex with the cationic cytochrome c under the conditions employed[10]. Subsequent studies with a range of proteins at the ITIES[12-15] and microITIES[16, 17] in the absence of surfactant revealed that proteins can be detected as long as the pH of the aqueous phase is lower than the isoelectric point of the protein. This allows the anion of the organic phase to complex with the cationic protein, hence providing a charge transfer process can transfer across the ITIES to complex with the protein, and this complex adsorbs at the interface. Indeed, electrochemical parameters indicate also that multi-layer films of adsorbed protein are formed at the interface. Systematic studies of pH with a range of proteins revealed that this indeed was the case (lysozyme,[15] insulin,[13] haemoglobin,[12] cytochrome c,[10, 11] myoglobin[17], serum albumin[18]) and most recently, ferritin[19] and thrombin[20] have been shown to react exactly as predicted. Such behaviour has been summarised in a range of reviews/chapters.[21, 22] Figure 1 summarises current thinking about the nature of protein electrochemistry at the ITIES. Interestingly, in the recent example of ferritin electrochemistry at the ITIES, Sakae et al.[19] found that the protein was electroactive at the ITIES when present in the aqueous phase at sufficiently low pH and the electrochemistry was also dependent on the organic phase electrolyte. Moreover, repetitive cyclic voltammetric sweeps resulted in deposition of an opaque film at the interface. Using the example of protamine, a highly cationic polypeptide that is an antidote to heparin in surgical procedures, Amemiya et al recently asked the question whether this undergoes adsorption or transfer at a thin film gelled ITIES.[23] Previous studies at a microITIES indicated that protamine transferred across the liquid-liquid interface when the organic phase contained dinonylnaphthalenesulfonate as ionophoric recognition agent[5]. Combining experiment and simulation[23] they found that protamine transferred from aqueous to organic phase, in the presence of organic phase dinonylnaphthalenesulfonate, but that more energy was needed to drive the transfer than the adsorption. With tetraphenylborate anions (or its derivatives) in the organic phase, Trojanek et al. found that the protamine-anion pair was adsorbed at the aqueous side of the interface[24].

The direct determination of proteins can be achieved to some extent with this approach, for example leading to detection of albumin[18] and a range of other proteins[25, 26]. However, improved detection limits are achieved by exploiting the fact that proteins adsorb at the interface as a protein-organic anion complex. The adsorption process can be

employed as a preconcentration step prior to voltammetric determination. This resulted in the adsorptive stripping voltammetric (AdSV) detection of lysozyme (30 nM),[27] haemoglobin (40 nM)[28] and insulin (10 nM)[29]. When combined with differential pulse voltammetry, AdSV enabled detection of 10 nM lysozyme[30]. These studies revealed a substantial build-up of protein material at the interface, which is usually gelled in the organic phase to promote mechanical stability and re-useability. Many monolayers of protein are deposited during the adsorption step[27]. Nevertheless such analytical studies have always indicated a selectivity problem, associated with the inability of electrochemistry at the ITIES to distinguish sufficiently between proteins.

Some approaches to selectivity might be achieved by exploiting slight differences in protein adsorption potentials, so as to enable selective preconcentration of one protein in the presence of others, as exemplified by the selective detection of insulin in the presence of bovine serum albumen[29]. Another approach is to exploit the acid-base chemistry of proteins by adjusting the pH of an aqueous phase mixture so that only the target protein is cationic and detectable. This was demonstrated for the selective detection of amylin (rat) in mixture with a number of proteins, and made use of the fact that amylin's pI was higher than the proteins present so that it could be detected (noting that in this case, detection was based on an amylin ion transfer process, not complexation with organic phase anion)[31]. It is anticipated that sufficient selectivity for practical applications will not be achieved by these approaches, but ways to harness biomolecular recognition reagents at the ITIES will be developed, such as the attachment of aptamers at the ITIES[20]. In this case, it was found that addition of a cationic surfactant to the organic phase promoted the adsorption of the aptamer to the ITIES as a complex with the surfactant.

Ultrasensitive detection via the ITIES

A key advancement in the detection of biomarker proteins via electrochemistry at the ITIES was achieved by exploiting catalytic (enzymatic) activity of the protein. In this case, prostate specific membrane antigen (PSMA) could be detected at picomolar (pM) concentrations by combining (Scheme 1) the analyte-catalysed reaction, in which a peptide is hydrolysed to release glutamate, with a second enzymatic reaction which produces protons and ammonium cations[32]. The facilitated ion transfer of protons at the ITIES, using the ionophore octadecyl isonicotinate (ETH1778), then enabled PSMA detection, by cyclic voltammetry or square wave voltammetry. Notable was the ability to detect PSMA, a biomarker for prostate cancer, at concentrations of 5.9 ng/mL (69 pM) by CV and 0.3 ng/mL (3.5 pM) by SWV, below the concentrations necessary for biomedical diagnostics (~3.5 nM, 294 ng/mL)[32]. This measurement approach opens up the prospect to detect other biomarkers at biomedically-relevant concentrations by relying on their catalytic activity. Of course, the behaviour of enzymes at the ITIES has been the subject of study for a number of years, as exemplified by glucose oxidase[33-37] and cholinesterases[38, 39].

Electrochemistry of enzymes at the ITIES

The behaviour of GOx at the ITIES has been subjected to a number of studies, due to its widespread use in glucose biosensing. For example, Pereira et al.[36] linked the GOx-

catalysed oxidation of glucose to the ITIES to achieve the detection of glucose, based on an ion transfer process. However, electron transfers have also been explored for GOx and other enzymes from a fundamental viewpoint rather than seeking to develop a glucose biosensor. Georganopoulou et al.[33] studied electron transfer between aqueous phase GOx and organic phase dimethylferricinium, while Mirceski et al.[37] found coupled ion-electron transfer reactions for GOx and decamethylferricinium in a three-phase system. GOx adsorption at the ITIES was reported[34, 37]. Similar observations were reported for fructose dehydrogenase[40], undergoing electron transfer reaction with organic phase dimethylferricinium, and with adsorption at the interface. Whether such reactivity can be exploited in an electroanalytical strategy remains to be seen, as stability of the enzyme is crucial to biosensor performance and the indicated adsorption will undoubtedly influence enzyme activity.

What's happening to the protein?

In order to better understand the protein detection mechanism at the ITIES and any impact of that on the protein structure, a range of studies by non-electrochemical techniques have been undertaken. Studies confirming the complexation of cationic protein with organic phase anions using a quartz crystal analysis method have been reported by Ellis et al.[41]. Electrospray ionisation mass spectrometry (ESI-MS) in a two-phase flow system indicated the formation of lysozyme-organic anion complexes, as expected from the electrochemical responses, only when the interface was positively polarised[42]. Subsequently, the mass spectral characterisation of lysozyme following its electroadsorption (i.e. first step of the AdSV process) at an interface between liquid aqueous and gelled organic phases revealed the partial unfolding of this protein at the interface, using the technique of electrostatic spray ionisation - MS[43]. Importantly, the MS data were consistent with the formation of protein multi-layer films at the interface, with the protein closest to the interface being most unfolded and protein furthest from the interface least unfolded. The classic biochemical method of gel electrophoresis has also been applied to the characterisation of protein electroadsorbed at the ITIES, with either both phases as liquids or the organic phase gelled by addition of poly(vinylchloride)[44]. Specifically, electroadsorbed Cytochrome c at the interface was found to contain dimers and trimers as well as higher order oligomers[44].

Protein-facilitated ion transfer may result in alterations in protein secondary structure, which may account, at least in part, for the formation of the dimers and oligomers detected. Vibrational spectroscopy, particularly Fourier transform infrared (FTIR) spectroscopy, is well suited to analysis of the average secondary structure of proteins that adsorb, accumulate or aggregate at the ITIES. Due to the inherent sensitivity of FTIR spectroscopy to the oscillating bond dipole associated with molecular vibrations, characteristic absorbance bands are observed for functional groups found in proteins. Here, the strong dipole of the carbonyl group of the amide bond produces an intense absorbance band (Amide I band) in the mid-infrared spectral range (ca. 1700 – 1600 cm^{-1}). Furthermore, the secondary structure of proteins is stabilised by a hydrogen-bonding network across the carbonyl group of the amide bonds, which in turn impacts on the frequency of the molecular vibration. As a result, changes in the protein secondary structure causes a shift in the position of the Amide I band

absorbance maxima[45, 46]. Application of this strategy to a series of proteins adsorbed at the gelled ITIES has revealed changes in the secondary structure, specifically consistent with the formation of aggregated anti-parallel β-sheets[47].

Finally, the power of computational modelling approaches to understanding the structures of biological systems can also be applied to characterising proteins at interfaces. In the case of non-polarised interfaces, i.e. at open circuit, molecular dynamics (MD) simulations have shown that protein interactions at organic/water interfaces can involve conformational changes, depending on the amino acid sequence and the secondary and tertiary structure of the protein, and of course the hydrophobicity of the organic phase [48, 49]. Recently we reported the first MD simulation study of a protein at a polarised liquid/liquid interface, in this case lysozyme at low pH at the water/1,2-dichloroethane interface. [50] Lysozyme was found to adsorb rapidly and change conformation into a multitude of unfolded states with similar free energies. Lysozyme was seen to interact with the organic phase through regions rich in non-polar amino acids (Figure 3). Exposure of hydrophobic and hydrophilic regions on the surface of an adsorbed protein will ultimately determine the mechanism of anion complexation and the subsequent pathways of ion transfer across the ITIES.

Conclusion and Outlook

The present state of protein electrochemistry at the ITIES indicates that protein complexation with organic phase anions and formation of adsorbed layers is a universal behaviour. A recent report on ferritin behaviour indicates that this is a general approach to protein detection. However the impact of the ion complexation, the low pH employed in most cases, and of interfacial adsorption in altering the protein structures has hardly been touched on. Some MS, IR and computational data to-date indicate that proteins unfold and undergo some change in secondary structure. Comprehensive studies of such systems are required, both in situ and ex situ, as well as linking to protein reactivity (e.g. enzymes) at the interface, as the foundations for new bioanalytical strategies exploiting adsorption or other behaviour.

Acknowledgements

We thank Dr. M. Arooj for provision of Figure 3.

Figures and Schemes

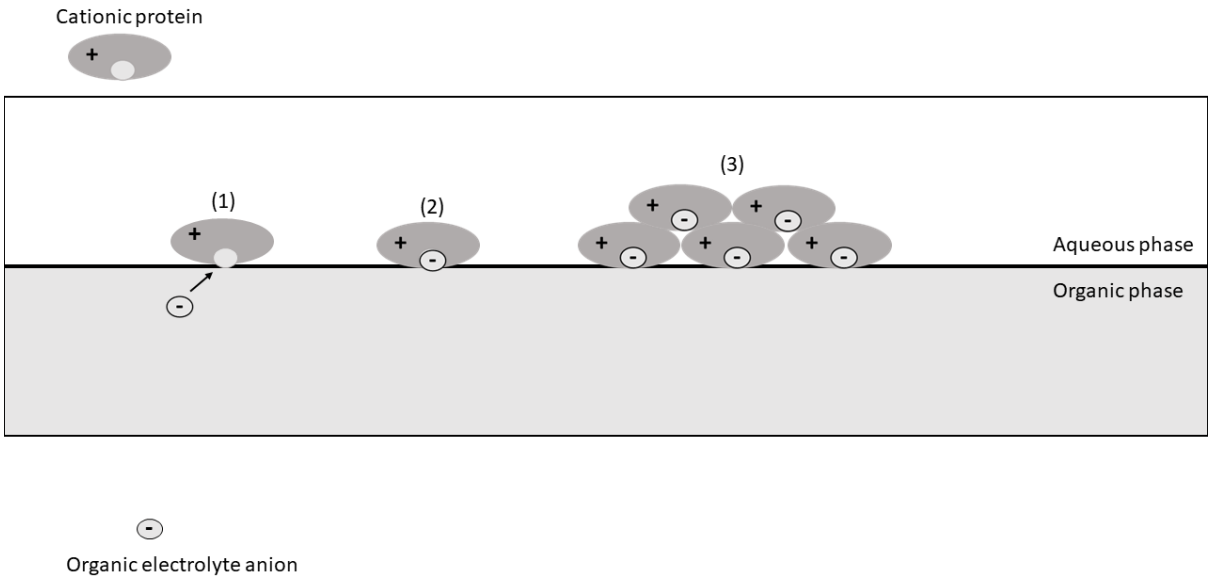


Figure 1. Cartoon summarising protein electrochemistry at the ITIES based on facilitated ion transfer of the organic phase anion by cationic protein. (1) complexation of organic anion by aqueous phase cationic protein; (2) adsorption at the interface; (3) formation of multilayer film.

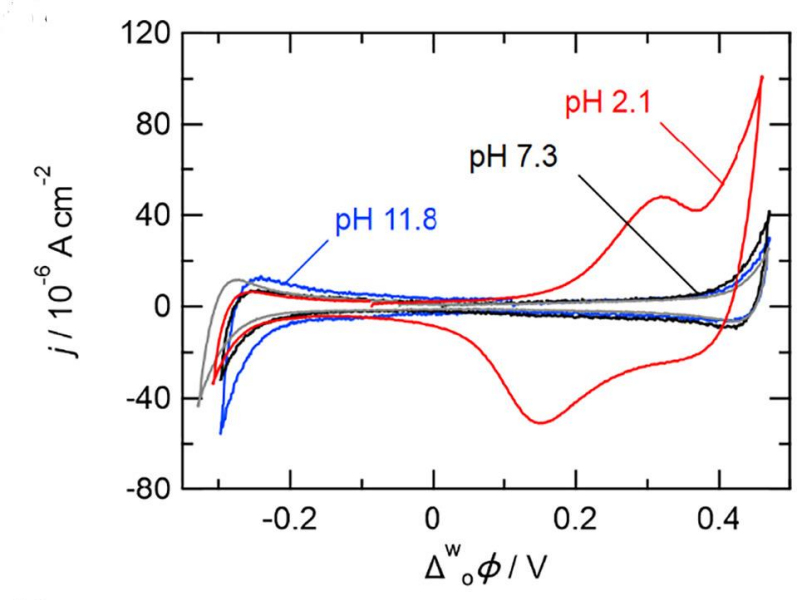
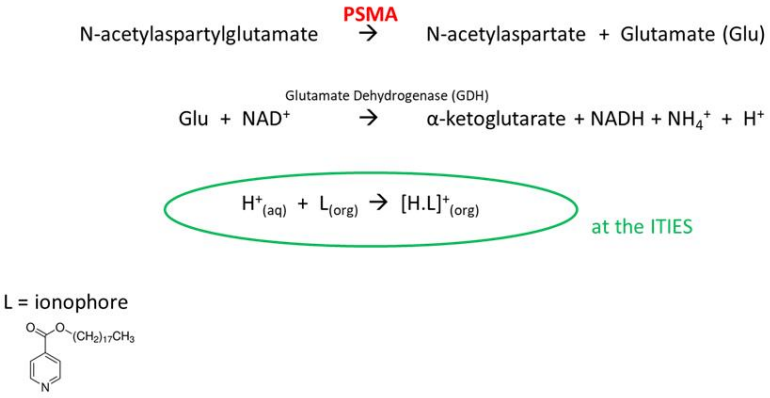


Figure 2. Cyclic voltammograms of ferritin at the ITIES with aqueous phase pH as indicated. Ferritin concentration: 1 µM. Voltammetric sweep rate: 100 mV s^{-1}. Reprinted from H. Sakae, Y. Toda, T. Yokoyama, Electrochemical behavior of ferritin at the polarized water|1,2-dichloroethane interface, Electrochemistry Communications, vol. 90, Pages 83-86, Copyright (2018), with permission from Elsevier.



Scheme 1. Summary of the reactions employed to detect the enzyme prostate specific membrane antigen (PSMA) by electrochemistry at the ITIES.

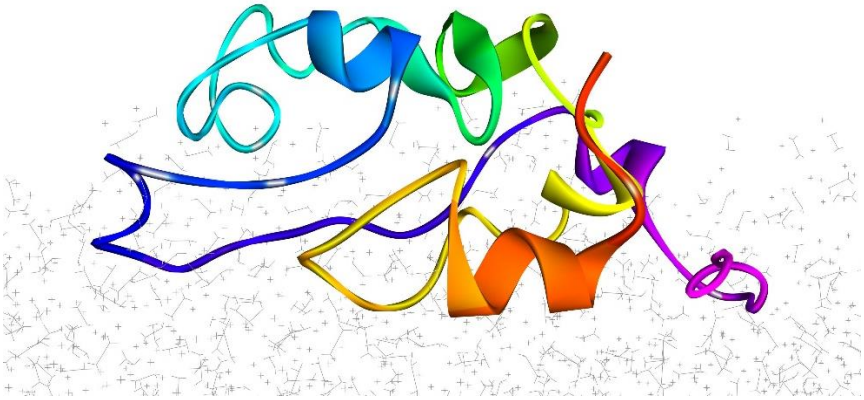


Figure 3. Ribbon representation of the partially unfolded structure of lysozyme adsorbed at the water/1,2-dichloroethane interface.

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