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Electrochemical signature of hen egg white lysozyme at the glycerol-modified liquid-liquid interface

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ABSTRACT

Electrochemical characterization of hen egg white lysozyme (HEWL) at a glycerol-modified interface between two immiscible electrolyte solutions (ITIES) was conducted using a microporous silicon membrane-supported gelled-1,6-dichlorohexane|water-glycerol interface. The electrochemical response of HEWL under these conditions is of interest for the system's potential application to the formation of isorefractive emulsified-ITIES, which offer practical opportunities for spectrophotometric analysis of interfacial processes. Importantly, the voltammetric signature for HEWL seen under glycerol-rich conditions was similar but with some differences from that for glycerol-free conditions. Specifically, the potential at which facilitated transfer of the organic phase electrolyte anion tetrakis-(4-chlorophenyl) borate ($TPBCl^-$) occurred was shifted to lower potential with increasing glycerol concentration. However, features in the voltammetry associated with adsorption/desorption processes were observed to remain constant. The simple ion transfer response of tetraethylammonium cations (TEA^+) at the same glycerol-modified ITIES provides insight into the nature of changes that determine the atypical HEWL signature. Lower ion transfer current with respect to increasing glycerol concentration and a shift in transfer potential were the key findings here. The results indicate that the electrochemistry which determines the HEWL signature is similar in environments that are rich in glycerol or purely aqueous.

1. Introduction

Voltammetry at the interface between two immiscible electrolyte solutions (ITIES) provides a platform for the non-redox detection of analytes in a range of solution environments [1]. This system has had particular success applied to quantitative analysis of biomacromolecules [2], where the detection mechanism is suggested to involve facilitated ion transfer of the organic phase electrolyte anion by a cationic protein, and adsorption of this protein-anion complex on the interface [3]. There has been a significant amount of work directed towards understanding the behaviour of proteins at the ITIES, with a view to improving detection selectivity for medically and biologically important species. In a more general sense, a better understanding of the way proteins and other large biomolecules interact with polarised liquid-liquid interfaces is also important for drug delivery applications [4] as well as study into biological systems [5]. Voltammetric studies have traditionally been the preferred tool for investigating these systems [2], however in recent years novel mass spectrometry techniques have broken new ground in elucidating the structure of these analytes at the polarised interface [6,7]. To date, studies employing such techniques have delivered insight into the interfacially-mediated formation of protein-anion complexes [7] and protein tertiary structure, with respect to interfacial proximity and applied potential [6]. There are many well established techniques for characterising protein structure; spectrophotometric techniques such as Fourier transform infra-red (FTIR) [8] and circular dichroism (CD) spectroscopies [9] are of particular interest. It is, however, difficult to characterise proteins within an ITIES system as the geometry of the system, often at the micro- or nanoscale, prohibits the use of regular laboratory spectrophotometric systems. In addition, the characterisation method must not significantly affect the physical environment within which the protein analyte resides, and upon which the interfacial environment is dependent. The studies reported in this paper concern voltammetry under glycerol-rich conditions which represent those required to implement an *in situ* spectrophotometric analysis of the polarised ITIES.

The recently-established refractive index matched emulsion (RIME) [10,11] system consists of an organic phase dispersed in a glycerol-water aqueous phase, the proportions of which render the

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emulsion isorefractive. It follows that an emulsion stabilised by addition of a surface active protein (Pickering emulsion), such as hen egg white lysozyme (HEWL), may then be subjected to bulk phase spectrophotometric measurements, such as CD or FTIR, in order to characterise the protein structure as a function of various parameters, e.g. interfacial potential, solvent composition, pH, etc.

To implement the refractive index matched ITIES, it is necessary to modify the character of one or both phases. A surprisingly limited body of work has dealt with modification of the aqueous phase of the ITIES. Previously, frozen [12] and gelled aqueous phases [13–15] have been employed and in these cases, the aim has been to modify the mechanical properties of the aqueous phase. Indeed, gelling of the aqueous phase by addition of a polymer, such as agar, agarose, κ-carrageenan or chitosan, is commonly employed to modulate aqueous phase diffusion processes [15], but no work has reported using glycerol as an additive, despite the clear potential for probing the chemically-dependent nature of interfacial interactions and analyte conformation. Of course, the chemical composition of the phases defines not only the solvation energy, and consequently the potential at which processes occur (Gibbs energy of transfer), but also the analyte's structure. The latter may be especially important in the case of large bio-macromolecules, which often exhibit a structural dependence on their solvation environment that is more significant, in terms of molecular activity and function, than typically observed for much smaller molecules. HEWL exhibits some dependence on glycerol-water solvent system composition [16], which is important to note as it also exhibits complex and structurally dependent interactions with the ITIES [3,6,7,17]. By contrast, any solvation dependence exhibited by small analyte ions, such as tetraethyl ammonium cations (TEA^+), ought to result in comparatively insignificant changes to the electrochemical response, as the ion transfer (IT) mechanism that small tetraalkylammonium ions undergo is expected to be insensitive to solvent-induced conformational change. It is therefore interesting to compare the voltammetric responses of HEWL and TEA^+ in discussing glycerol-modification of the ITIES.

HEWL is a model bio-macromolecular polyionic analyte, chosen for its surface activity, tolerance to relatively low pH, and high denaturation temperature (ca. 74.8 °C [18]). Previous studies employing HEWL as an analyte at the ITIES proposed three mechanistic features: facilitated ion-transfer (FIT) of the organic phase electrolyte anion by HEWL; adsorption of this anion-HEWL complex; and, on scanning back to lower potentials, desorption of the anion-protein complex [3,17]. These proposed phenomena correspond to characteristic features in the cyclic voltammetry. The aim of the work reported here was to determine whether formation of a refractive index-matched ITIES enabled similar electrochemical behaviour of HEWL as obtained at an unmodified ITIES. By modifying a typical non-emulsified ITIES system incrementally with glycerol, it is possible map the observed electrochemical response to changes in the composition. The results presented form a foundation for future development of refractive index matched emulsified-ITIES systems, which use glycerol to render the interface isorefractive.

2. Experimental

Voltammetry was conducted at a microporous silicon supported micro-ITIES array [19] in which the membrane consisted of eight hexagonally arranged pores of radius 26.6 μm. The aqueous phase was modified with different concentrations (wt%) of glycerol and contained HCl (10 mM) with either tetraethylammonium (TEA^+) or HEWL as the target analyte. The organic phase was bis (triphenylphosphoranylidene)ammonium tetrakis-(4-chlorophenyl) borate (BTPPATPBCl) (10 mM) [20] in 1,6-dichlorohexane (1,6 DCH) (spectrophotometric grade 99.5%) gelled with polyvinylchloride (10% w/v). Electrochemistry conducted at the glassy carbon disk electrode (GCE) (3 mm diameter) was that of three redox couples prepared from hexaammineruthenium(III) chloride, potassium hexacyanoferrate(II) trihydrate and ferrocenecarboxylic acid (FCA) (Alfa Aesar), where all were dissolved in potassium chloride (0.1 M in water) at 0.001 M with the exception of FCA, which was diluted by a factor 10 times from a saturated aqueous solution (0.1 M KCl). All materials were purchased from Sigma Aldrich, Australia, and used as received, unless otherwise stated.

Electrochemical measurements were conducted using an AUTOLAB PGSTAT302N potentiostat (Metrohm, The Netherlands). The cell was operated in two-electrode mode where the reference electrodes in each phase were Ag/AgCl, connected via an organic reference solution in the case of the organic phase, and also served as the counter electrodes. The ITIES was modified by addition of up to 80 wt% glycerol, which is representative of the solvent conditions required to render the constituent phases isorefractive [11].

3. Results and discussion

The transfer potential at the ITIES represents the Gibbs free energy of transfer for a particular species and is dependent on the difference in solvation energy of that species in either phase [21]. Furthermore, the potential at which any response is observed is directly related the Gibbs free energy required to drive that process. With this understanding, it becomes instructive to examine how the position of a particular response on the potential scale changes with respect to modification of the ITIES system composition. Keeping in mind that the purpose of pursuing the refractive index-match ITIES system is to investigate the nature of protein at the electrified ITIES, it is important to understand how the interfacial interaction with HEWL responds to an increasingly glycerol rich environment.

3.1. HEWL at the glycerol modified interface

The aqueous phase is prepared containing HEWL (20 μM), which is considered fully protonated in the presence of HCl (10 mM) [3]. Fig. 1*(a)* shows the typical cyclic voltammetry response for HEWL at the unmodified micro-ITIES array. Several key features in the voltammetry have previously been associated with mechanistic steps [3,17,22]. The pre-peak (Fig. 1a, forward scan, 0.86 V) is associated with adsorption of a HEWL/$TPBCl^-$ complex to the interface. This complex, thought to form by complexation of the initially transferred $TPBCl^-$ ion(s) with protein near the interface, then adsorbs at the interface. The main peak (indicated in Fig. 1*c, forward scan, 1.05 V*) is proposed to be the result of organic phase electrolyte anion transfer facilitated by HEWL in a FIT mechanism. The main-peak and background-electrolyte transfer peak, appear severely convolved for the 0 and 20 wt% cases (Fig. 1*a and 1b*). Finally, the desorption-peak (Fig. 1a, reverse scan, 0.66 V) is associated with desorption of the HEWL/$TPBCl^-$ complex from the interface (Fig. 1, *reverse scan*). This desorption process may involve the decomplexation of the adsorbed species and the subsequent back-transfer of the organic phase anion, which provides the charge transfer step employed in detection of proteins by this approach [23–25], or simply the desorption of the complex to the bulk aqueous phase. The shape and position of the reverse peak remain constant with respect to glycerol concentration, while the peak area decreases gradually with increasing glycerol content. This behaviour suggests that the reverse peak observed for high percentage glycerol systems is still representative of the same desorption process observed for the glycerol-free systems presented here, and reported previously