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Stripping voltammetric detection of insulin at liquid–liquid microinterfaces in the presence of bovine albumin

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Abstract

Electrochemistry at the interface between two immiscible electrolyte solutions (ITIES) provides a platform for label-free detection of biomolecules. In this study, adsorptive stripping voltammetry (AdSV) was implemented at an array of microscale ITIES for the detection of the antidiabetic hormone insulin. By exploiting the potential-controlled adsorption of insulin at the ITIES, insulin was detected at 10 nM via subsequent voltammetric desorption. This is the lowest detected concentration reported to-date for a protein by electrochemistry at the ITIES. Surface coverage calculations indicate that between 0.1 and 1 monolayer of insulin forms at the interface over the 10 – 1000 nM concentration range of the hormone. In a step toward assessment of selectivity, the optimum adsorption potentials for insulin and albumin were determined to be 0.900 V and 0.975 V, respectively. When present in an aqueous mixture with albumin, insulin was detected by tuning the adsorption potential to 0.9 V, albeit with reduced sensitivity. This provides the first example of selective detection of one protein in the presence of another by exploiting optimal adsorption
potentials. The results presented here provide a route to the improvement of detection limits and achievement of selectivity for protein detection by electrochemistry at the ITIES.

**Keywords:** insulin, albumin, adsorptive stripping voltammetry, ITIES, liquid – liquid interface.

1. **Introduction**

The ability to detect proteins and peptides using simple methods with miniaturised devices offers many benefits including the possibility for point-of-care measurements that may revolutionise diagnostic medicine and a wide range of industrial and environmental applications.\(^1\)-\(^3\) Advances in areas such as biomicroelectromechanical system (BioMEMS) and lab-on-a-chip technologies open up opportunities for the detection of important biological molecules such as disease indicators. Consequently, new strategies are constantly emerging that have the potential to contribute to a new generation of portable analytical and bioanalytical devices. Electrochemical methods have been greatly successful in the detection of biomolecules such as glucose in whole blood.\(^4\),\(^5\) Electrochemical measurement at interfaces between two immiscible electrolyte solutions (ITIES) is one such emerging area that may contribute to this revolution.\(^6\)-\(^8\) Electrochemistry at the ITIES provides scope for the detection of analyte species based on ion transfer reactions, rather than oxidation or reduction processes, and offers a label-free strategy for detection of proteins, amongst other target analytes.\(^9\)

Recently, electrochemistry at the ITIES has been examined as the basis for detection of biological macromolecules by various groups.\(^10\),\(^11\) Amemiya et al. detected heparin via ion–transfer voltammetry at the ITIES facilitated by ionophores, and recent studies have illustrated significant advances in terms of sensitivity and selectivity,\(^10\) e.g. the detection of heparin (0.13 unit mL\(^{-1}\)) at water - 1,2-dichloroethane interfaces in sheep blood plasma.\(^12\) The effect of serum proteins has also been studied in the detection of the β-blocker propranolol at a μITIES array. In this case, 0.05 μM propranolol was detected in the presence of bovine serum albumin by employing differential pulse stripping
voltammetry.\textsuperscript{13} Electrochemistry of the antidiabetic hormone insulin has been also investigated at the ITIES, but only at supraphysiological concentrations. The limits of detection for insulin at a millimetre-scale aqueous – 1,2 dichloroethane interface were 2.5 \( \mu \text{M} \) and 1 \( \mu \text{M} \) using cyclic voltammetry and square wave stripping voltammetry, respectively.\textsuperscript{14} Alternating current voltammetry enabled detection of 0.1 \( \mu \text{M} \) insulin via its adsorption at the ITIES.\textsuperscript{15} Cyclic voltammetry at a microITIES array enabled detection of 1 \( \mu \text{M} \) insulin.\textsuperscript{16} In these cases, the detection of insulin in the presence of other proteins was not assessed.

Diabetes mellitus is a metabolic disease associated with either a deficiency in insulin production (type 1 diabetes) or an ineffective use of insulin by the cells (type 2 diabetes).\textsuperscript{17} Thus, the determination of insulin is clinically important and an assortment of techniques have been employed for its detection, such as enzyme-linked immunosorbent assays,\textsuperscript{18} electrochemical impedance spectroscopy,\textsuperscript{19} mass spectrometry,\textsuperscript{20} microcantilever biosensors,\textsuperscript{21} capillary electrophoresis,\textsuperscript{22} and direct oxidative electrochemistry.\textsuperscript{23} The large variety of methods investigated for insulin detection is indicative of the on-going need for alternative methods for the detection of insulin which might be amenable to point of care testing.

This paper reports on the electrochemical behaviour and detection of insulin at an array of microscale ITIES. In this study, intentional adsorption of insulin at the electrified interface prior to the voltammetric measurement is employed for the first time. This has enabled the detection of nanomolar concentrations of insulin. Furthermore, the challenge of insulin detection in the presence of another protein, albumin, which is abundant in serum, has been explored. The results indicate that, although the voltammetric desorption peaks cannot be separated, the use of tuned adsorption potentials enables the detection of insulin in the presence of albumin.

2. Experimental details
2.1 Reagents.

All the reagents were purchased from Sigma-Aldrich Australia Ltd. and used as received, unless indicated otherwise. Mono-component zinc insulin (porcine) was purchased from CSL Laboratories, Australia. The gelified organic phase was prepared using bis(triphenylphosphoranylidene) tetrakis(4-chlorophenyl)borate (BTPPA$^+\text{TPBCl}^-$, 10 mM) in 1,6-dichlorohexane (1,6-DCH) and low molecular weight poly(vinyl chloride) (PVC).$^{24}$ The organic phase electrolyte salt BTPPA$^+\text{TPBCl}^-$ was prepared by metathesis of bis(triphenylphosphoranylidene)ammonium chloride (BTPPA$^+\text{Cl}^-$) and potassium tetrakis(4-chlorophenyl) borate ($K^+\text{TPBCl}^-$).$^{25}$ Aqueous stock solutions of albumin (from bovine serum, 98\%) and insulin (porcine) were prepared in 10 mM HCl (pH 2) on a daily basis and stored at +4 °C. All the aqueous solutions were prepared in purified water (resistivity: 18 MΩ cm) from a USF Purelab Plus UV.

2.2 Apparatus.

All experiments were performed using an Autolab PGSTAT302N electrochemical analyser (Metrohm Autolab, Utrecht, The Netherlands), controlled by the NOVA software supplied with the instrument. The silicon micropore arrays used to define the µITIES array were fabricated by photolithographic patterning and wet and dry silicon etching processes.$^{26}$ The fabrication procedure provided hydrophobic micropore walls, allowing the organic phase to fill the pores. The micropore array consisted of thirty micropores in a hexagonal close-packed arrangement, each with a diameter of 22.4 μm and a pore centre-to-centre distance of 200 μm. The microporous silicon membranes were sealed onto the lower orifice of a glass cylinder using silicone rubber (Acetic acid curing Selleys glass silicone). The gelled organic phase solution was introduced into the silicon micropore arrays via the glass cylinder, and the organic reference solution was placed on top of the gelled organic phase. The silicon membrane was then inserted into the aqueous phase (10 mM HCl, without or with albumin and/or insulin). Voltammetric experiments were then performed. The electrochemical cell arrangement used for the experiments was a two-electrode cell,$^{27}$ with one Ag|AgCl electrode in the organic phase
and one in the aqueous phase. The organic phase reference electrode was immersed in an aqueous reference solution of 10 mM BTPPA$^+\text{Cl}^-$ in 10 mM LiCl. All potentials are reported with respect to the experimentally-used reference electrodes. All voltammetric experiments were run at a sweep rate of 5 mV s$^{-1}$.

3. Results and discussion

3.1 Nanomolar detection of insulin.

Adsorptive stripping voltammetry (AdSV) at the µITIES array was used to detect insulin at nanomolar concentrations. AdSV functions by applying a selected potential difference at the liquid - liquid interface for a predetermined time, followed by implementation of voltammetry, in this case linear sweep voltammetry. When the biomolecule of interest adsorbs at the ITIES under the influence of the applied potential, imposition of a defined adsorption time results in preconcentration of the target analyte at the interface. This adsorption event is followed by voltammetry during which the adsorbed protein is subsequently desorbed from the interface, resulting in voltammetric peak. Figure 1 shows the AdSV of insulin over a concentration range from 10 nM to 1000 nM. This is below the previously reported detection limit for insulin at the ITIES.$^{14-16}$ Furthermore, the detection of 10 nM insulin provides the lowest detectable concentration of protein (or polypeptide) achieved to-date by electrochemistry at the liquid - liquid interface. This was achieved by applying an adsorption potential of 0.9 V for 450 s, in unstirred solution, followed by desorption of insulin from the interface, resulting in the voltammetric response. The adsorption potential was optimised, as will be discussed in section 3.2.
Figure 1. AdSV of insulin at the µITIES array. Insulin concentrations were 10, 25, 50, 75, 100, 250, 500, 750 and 1000 nM in aqueous 10 mM HCl. An adsorption time of 450 s was used at an adsorption potential of 0.9 V, in unstirred solution. The inset shows the plot of stripping voltammetric peak current versus the concentration, where the peak current was determined at the peak maximum for each concentration.

Surface coverage calculations were used to estimate the interfacial concentration of insulin in terms of monolayers. The experimentally determined charge was taken from the desorption peak and substituted into equation (1), providing a surface coverage value. This was done for each concentration of insulin studied over the 10 - 1000 nM range. For this analysis, the total charge under the peak was integrated, irrespective of the shift in the peak potential (Figure 1). Since insulin is the only protein present in solution for these experiments, all charge is attributed to it’s desorption from the interface.

\[ Q = z_i F A \Gamma \]  

(1)

In equation (1), \( Q \) is the charge under the corresponding desorption peak (C), \( z_i \) is the molecular charge on insulin (+6 at pH 2), \( F \) is Faraday’s constant (96485 C mol\(^{-1}\)), \( A \) is the total geometric area of the microinterfaces (1.18·10\(^{-4}\) cm\(^2\)) in the array, and \( \Gamma \) is the surface coverage (mol cm\(^{-2}\)).
experimentally determined surface coverage values were in the range 3 – 26 pmol cm\(^{-2}\). The theoretical surface coverage corresponding to a monolayer of insulin was calculated according to equation 2,\(^{28}\)

\[
\Gamma_{\text{mon}} = \frac{0.87}{\pi r_h^2 N_A} \quad (2)
\]

where \(\Gamma_{\text{mon}}\) is the surface coverage of a monolayer, \(r_h\) is the hydrodynamic radius of monomeric insulin (1.35 nm),\(^{29}\) \(N_A\) is Avogadro’s number and 0.87 is a factor to include the maximum occupied area when hexagonal close packing is assumed.\(^{28}\) The assumptions made by using this model are discussed by Herzog \emph{et al.}\(^{28}\) but nevertheless this should at least provide an insight into the adsorption behaviour of insulin at the low bulk solution concentrations depicted in Figure 1. Additionally, the hydrodynamic radius used assumes that monomeric insulin is present, which is a reasonable assumption at nanomolar concentrations. Were this surface coverage analysis conducted at micromolar insulin, then a hydrodynamic radius of up to ca. 2.7 nm would yield a lower relative surface coverage than obtained for purely monomeric insulin. The theoretical monolayer calculation results in a surface coverage of 25.2 pmol cm\(^{-2}\). This means that the experimentally determined surface coverages correspond to 0.12 – 1.03 of a monolayer, over the bulk insulin concentration range of 10 – 1000 nM. The peak currents (as determined at the peak maximum for each individual concentration) associated with this bulk concentration range were between 0.11 nA and 0.84 nA, respectively. The resulting calibration curve exhibits a non-linear relationship (Fig. 1 inset). This may be attributed to (i) the complex mechanism of detection involving protein adsorption, facilitated ion transfer (organic phase electrolyte anion) and desorption,\(^{14,16}\) (ii) changes in peak shape and size over the concentration range used, resulting in difficulty in peak comparisons from lower to higher concentrations, or (iii) insulin dimer, tetramer or hexamer formation at the interface. Despite this non-linearity, the present results show an order of magnitude improvement over previous work for detection of insulin at the ITIES.\(^{14-16}\)

3.2 \textit{Adsorptive stripping voltammetry of insulin and albumin}
As shown above for insulin, and previously for lysozyme\textsuperscript{29} and haemoglobin,\textsuperscript{30} the potential-controlled adsorption of the protein/polypeptide can be employed for efficient preconcentration and lead to improvements in detection limits. However, the issue of selectivity is still open to question. With an AdSV approach, two opportunities for selectivity are presented: the adsorption process and the detection process. Here, we present an analysis of the prospects for selectivity for one protein in the presence of another by examining insulin and albumin adsorption and detection. After an extensive characterization of insulin, the influence of bovine serum albumin on the electro-activity of insulin was investigated.

The optimum adsorption potentials of insulin and albumin were investigated using AdSV, where the adsorption time was held constant at 60 s, with a fixed concentration of protein. The adsorption potential was varied from 0.6 V to 1.0 V, after which linear sweep voltammetry from the adsorption potential to 0.4 V was implemented. Figure 2(A and B) shows the resulting voltammograms for 1 µM insulin and 1 µM albumin, respectively. A plot of the resulting peak currents against the adsorption potential is shown in Figure 2(D). Insulin (○) has its first observable voltammetric response following adsorption at 0.775 V, and its response peaks at an adsorption potential of 0.9 V, beyond which the response diminishes. Albumin (●) has its first observable voltammetric response following adsorption at 0.825 V, and its response peaks at an adsorption potential of 0.975 V, beyond which the current response also diminishes. Since the optimal adsorption potentials for the two proteins are different, it was postulated that selective adsorption of insulin in the presence of albumin could be achieved by careful selection of the adsorption potential. AdSV of a mixture of 1 µM insulin and 1 µM albumin (Figure 2(C)) was performed to characterise the adsorption behaviour of the mixture relative to the behaviour of the individual protein solutions. The resulting plot of peak current versus adsorption potential (Figure 2(D) – symbol ▼) showed a voltammetric response at 0.825 V that peaked with a maximum current response at 0.975 V. Unfortunately, the adsorption of the two proteins could not be resolved from one another in such an experiment. The adsorption behaviour of the mixture was expected to result in two adsorption maxima, corresponding to the adsorption of insulin at ~0.9 V and
albumin at ~0.975 V. From Figure 2(D) it can be seen that the mixture of insulin and albumin showed no independent behaviour, and it would appear that the presence of albumin affects the adsorption of insulin, possibly through protein–protein interactions or inhibition of the adsorption process.

Figure 2. Adsorptive stripping voltammetry of insulin, albumin and their mixtures at the µITIES array. AdSV of (A) 1 µM insulin, (B) 1 µM BSA and (C) a mixture of 1 µM insulin + 1 µM BSA in aqueous phase of 10 mM HCl, 60 s pre-concentration time at different applied potentials, from 0.6 V up to 1.0 V. Plot (D) shows the peak currents resulting from the varying applied adsorption potentials for (○) insulin (●) BSA and (▼) the mixture. Peak currents were estimated from a baseline extrapolated from the current at potentials lower than the peak maximum.

3.3. Detection of insulin in the presence of albumin

Although the selective adsorption approach did not provide clear-cut evidence that one protein could be selectively adsorbed in the presence of another, the detection of insulin in the presence and absence
of BSA was investigated using the AdSV approach. Albumin was used as a model interferent since it is an abundant protein present in serum that could potentially interfere in any detection method. Here, insulin detection was investigated with preconcentration at 0.9 V (the optimum adsorption potential for insulin, see section 3.2) for 60 s in the presence of 1 µM BSA. At this adsorption potential, albumin is not expected to produce a significant voltammetric response (Figure 2(D)). In Figure 3(A) the AdSV of 1 – 6 µM insulin in the presence of 1 µM BSA is shown, demonstrating an insulin dependent response. This demonstrates that by optimally tuning the adsorption potential, some degree of selectivity can be achieved, although with a cost to sensitivity, as shown in Figure 3(B). Although there is no concentration-dependent response linearity in the concentration range studied for both cases (absence and presence of albumin), insulin peaks were resolved in the presence of BSA in the aqueous solution. Similar results are achieved when the area under the desorption peak was evaluated against protein concentration, showing an insulin-dependent response with slightly reduced sensitivity (data not shown). The exact mechanism of disruption of linearity remains unclear at present, but this process could involve protein – protein interactions and/or inhibition of insulin adsorption by interfacially-adsorbed albumin. It is also possible that insulin self-association, which is concentration dependent at micromolar concentrations, might also contribute to the disruption of linearity. These putative phenomena result in a decrease in the sensitivity of the detection system. Nevertheless, this demonstrates that even though bovine serum albumin interferes with the electroactivity of insulin, detection of insulin is still achievable in the presence of another protein.

The challenge ahead is to improve the selectivity and sensitivity of this detection strategy. It is possible to make the measurement more selective by implementing a sample pre-treatment or separation step. For example, this could be a capillary electrophoretic separation combined with detection by electrochemistry at the ITIES. Alternatively, passage through a size-exclusion or other suitable filter membrane might be more practical to rapidly and conveniently separate the protein of interest.
from other proteins. Optimisation of these separation strategies will be important to ensure rapidity and accuracy.

**Figure 3.** Adsorptive stripping voltammetry of insulin in the presence of albumin. AdSV of insulin (1, 2, 3, 4, 5 and 6 µM) in the presence of 1 µM BSA (A) for an adsorption time of 60 s. (A) The dotted line is the response to the background electrolyte only and the dashed line is the response to 1 µM of albumin after 60 s preconcentration time at +0.9 V. Error bars are calculated from the standard deviations of peak currents from three separate calibration curves (for both Insulin and the mixture of insulin and albumin) performed on a single microinterface array. The array was washed between runs and background CV and LSV was performed to insure no carryover between experiments. (B) Plots of the current height of the insulin desorption peak in the absence (●) and the presence of albumin (○).
4. Conclusions

The detection of nanomolar insulin has been achieved by use of AdSV at the ITIES. This is the lowest observed concentration of protein detectable using electrochemistry at the ITIES reported to-date. Surface coverages evaluated from the electrochemical responses were between 0.1 and 1 of a monolayer, in contrast to the multilayer coverages obtained for lysozyme and haemoglobin.\textsuperscript{30,31} Investigation of the potential-dependent adsorption of both insulin and albumin revealed a 75 mV difference in optimal adsorption potential. By selecting an adsorption potential of 0.9 V, where no electrochemical response from albumin was expected, AdSV was able to successfully detect insulin in the presence of albumin. This work provides the basis for the use of electrochemistry at the ITIES as a bioanalytical tool and demonstrates for the first time that potential-dependent selective adsorption may offer a route to selective detection based on this strategy. However, improvements in sensitivity and selectivity remain as the immediate challenges.

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References


Insulin was detected voltammetrically following adsorptive accumulation at the microinterface array and in the presence of albumin.