Protein modulation of electrochemical signals: application to immunobiosensing†

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Herein we present a label-free immunobiosensor based on the modulation of amperometric signals of surface bound redox species when immersed in a protein environment which is applicable to either the detection of antibodies or the detection of small molecules such as drugs or pesticides.

This communication stems from work by us,[1–3] and others,[4–6] into direct electron transfer to glucose oxidase (GOx). In that work we observed that the immersion of the redox active centre of GOx, flavin adenine dinucleotide (FAD)—which was attached to an electrode surface *via* a self-assembled monolayer (SAM)—into a protein environment causes an attenuation of the electrochemistry of the redox species. A related observation was made by Yan *et al.*[7] who found that, within a mixed SAM with one component containing a viologen species and the other a biotin moiety, binding of anti-biotin IgG antibodies to the interface attenuated the viologen electrochemistry. Furthermore, both Tender and co-workers[8] and Kraatz and co-workers[9,10] have exploited this phenomena as the basis of a biosensor for monitoring proteins. However, in all these examples, the interaction of the redox active species with the proteins was ill-defined as the immersion of the redox species in the protein could be due to either nonspecific adsorption of protein to the interface or a specific interaction. Hence the origins of the attenuation remained obscured.

In our previous work with FAD and GOx we proposed that the attenuation in electrochemistry was due to either: (1) a change in electronic coupling to the redox active species upon protein immersion. Such a change would be possible because the flexibility of the adenine dinucleotide component of the molecule meant that, upon binding with the protein, there was the possibility of a change in conformation of the isoalloxazine ring (where the electrochemistry occurs) relative to the self-assembled monolayer to which it is attached. (2) the inability of ions to access the redox species and balance the change in charge of the redox species upon the change in oxidation state. However, with FAD the situation is complicated by the redox species undergoing a two-electron transfer process where the counter ion was a proton. or (3) both of the above.[2] Therefore, to ensure both a specific interaction between protein and that there was no significant change in conformation of the redox species we designed the electrode interface in Fig. 1. With this interface the electrochemistry is a simple one-electron process with an anionic counter-ion and the conformational space sampled by the ferrocene is largely unperturbed by immersion in the protein. These features combined rule out the possibility of a change in electronic coupling due to a change in conformation and any ambiguity regarding the electrochemical process was removed. The ferrocene is attached to the end of a oligo(phenylethynylene) molecular wire (MW) and biotin as a model epitope for selective binding by an IgG antibody was attached to the ferrocene. An antibody rather than streptavidin was employed because the lower affinity of an IgG antibody for biotin relative to streptavidin meant the protein specifically bound to the interface could be displaced. Streptavidin binding to biotin is has such a high affinity it is virtually irreversible. The MW serves the dual purposes of being rigid, thereby allowing access to the biotin by the binding antibodies without hindrance from the surface, and being an efficient conduit for electron transfer, which is necessary as the ferrocene is located approximately 20 Å above the electrode surface. Furthermore, the oligo(ethylene glycol) (OEG) species

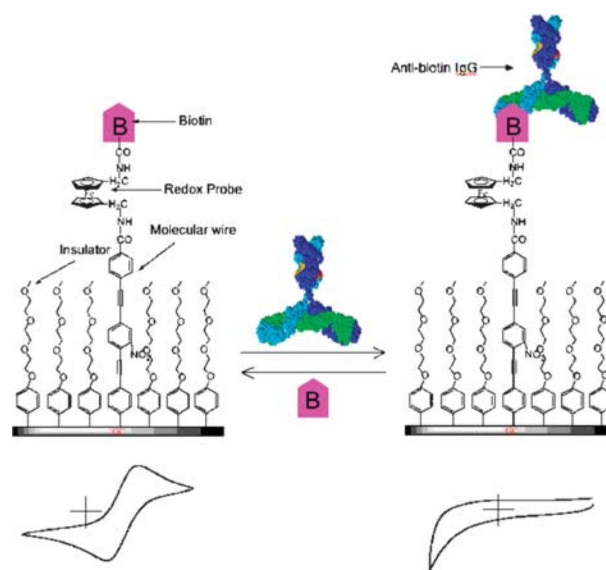

Fig. 1 Schematic representation of antibody targeting to the biotin modified GC surfaces resulting in attenuation of electrochemistry. Note antibody not depicted to scale.

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which also has two functions: blocking access to the electrode surface by other species in solution, and ensuring that anti-biotin IgG antibodies will only specifically bind to the biotins attached to the ends of the MW since OEG molecules have been shown to resist non-specific adsorption of proteins to an interface.[11–14]

To prepare the electrode construct in Fig. 1 glassy carbon electrodes modified *via* reductive adsorption of aryl diazonium salts were employed due to the greater stability this system offers over the gold–alkanethiol system.[14] A 20 : 1 mixture of an OEG–MW was adsorbed onto the electrode. The molecular wire was terminated by carboxylic acid moieties that, upon activation using carbodiimide chemistry, allowed the attachment of ferrocene dimethylamine. To the free amine of the ferrocene was attached sulfo-NHS-biotin to give the final electrode interface. The binding of anti-biotin IgG antibodies to the interface then served to immerse the ferrocene in a protein environment. We have previously characterized this interface in some detail for protein electrochemistry and have demonstrated the dual capability of the oligo(ethylene glycol) to resist protein adsorption as well as ensuring specific interaction of proteins with the MW.[14]

As implied at the bottom of Fig. 1, the electrochemistry of the ferrocene moieties is expected to be strongly present prior to incubation of the electrode in anti-biotin IgG and suppressed once the electrode is exposed to the antibody. As is evident from the cyclic voltammograms in Fig. 2(a) this is what is observed (see Fig. S4, ESI,† for equivalent square wave voltammograms). After attachment of the ferrocene to the MW there is a strong ferrocene electrochemistry with a formal potential of 349 mV (*vs.* Ag/AgCl) with the area of the peak corresponding to 1.28×10^{-11} mol cm^{-2} of ferrocene on the electrode surface. The coverage of aryl diazonium salt derived species was determined to be approximately 4.1×10^{-10} mol cm^{-2} from the charge passed during the reductive adsorption. Considering the theoretical maximum coverage for aryl diazonium salts is 12×10^{-10} mol cm^{-2}, this indicates that either a monolayer or a sub-monolayer forms on the electrode, as distinct from a multilayer as observed for many aryl diazonium salts.[11] After attachment of the biotin to the interface there was no significant suppression of the peak current (peak height relative to background). However after incubation in the anti-biotin IgG antibody solution the current decreased significantly. A series of controls were performed to verify that the change in current was, indeed, due to the immersion of the ferrocene into the protein, following the specific interaction of the anti-biotin IgG with the biotin (see Fig. S5–7, ESI†). Replacement of ferrocene dimethylamine with ethylene diamine led to no electrochemistry. When the interface was assembled with the ferrocene dimethylamine but in the absence of biotin, only a minor suppression of the electrochemistry was observed; consistent with the OEG molecules resisting nonspecific adsorption of protein. Finally, incubation of the complete interface with bovine serum albumin as a different protein or with an anti-pig IgG as an antibody that is not specific for biotin, also showed only a very minor decrease in electrochemistry (at a protein concentration of 0.5 mg mL^{-1} only 0.1% of the current reduction was due to non-specific adsorption of protein in each case). Thus the only significant decrease in current is observed when the complementary antibody is complexed with the interface. As there can be no change in conformation of the ferrocene, unlike FAD, upon protein binding we believe these results are evidence for the restriction of counter ions to access the ferrocene once immersed in a protein environment, thereby hindering the electron transfer, as the cause of the attenuation of the electrochemistry.

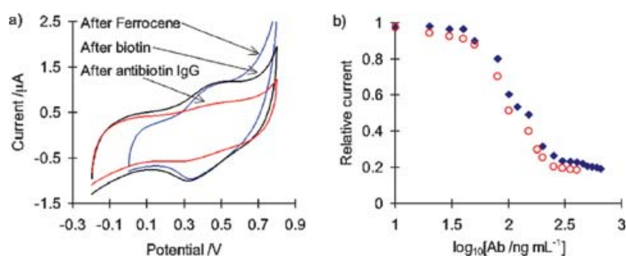

Fig. 2 (a) Cyclic voltammograms of mixed monolayer of MW and PEG modified GC surfaces after the step-wise attachment of ferrocenedimethylamine, biotin and 0.5 μM anti-biotin in 0.05 M phosphate buffer (0.05 M KCl, pH 7.0) at a scan rate of 100 mV s^{-1}. The ferrocene was first covalently attached to the mixed monolayer modified GC surfaces through the formation of an amide bond followed by the attachment of NHS-biotin. Anti-biotin was finally complexed using its affinity for the surface bound biotin. (b) Calibration plot of relative current against the logarithm of the anti-biotin concentration for two different electrodes to demonstrate the reproducibility of the system.

The ability of the antibody to specifically bind to the electrode interface and to attenuate the long-range charge transfer to the ferrocene moiety provides an opportunity to develop a biosensor for detecting the presence of antibodies in a sample. The magnitude of the current suppression is influenced by the antibody concentration as shown in Fig. 2(b) for two separate electrodes. The electrode responds to anti-biotin between ~30 to 500 ng mL^{-1} with a lowest detected concentration of 30 ng mL^{-1}. The immunosensor response and hence its construction was also reproducible; nine immunosensors were prepared by following identical steps and their response toward anti-biotin IgG led to a relative standard deviation of only 6%.

Fig. 2(b) illustrates the ability of the electrode to detect the presence of antibodies in a solution in what is essentially a label free method that requires the user to do no more than expose the sensing interface to the sample of interest. Therefore, protein modulation of electrochemical signals for detecting proteins is a competing technologies to other label-free detection systems such as evanescent wave devices,[15] acoustic wave devices.[16] microcantilevers[17] and nanowire field effect transistors.[18] There are, however, very few technologies that have the potential to produce portable handheld devices for the detection of small molecules without requiring the user to do anything other than expose the sensor to the sample; what we refer to as 'user-intervention free'. The reversibility of immunobinding reactions provides the potential for producing an immunobiosensor that can do just that *via* a displacement assay. To demonstrate this, an electrode interface was prepared with the anti-biotin IgG attached to the interface such that the electrochemistry of the ferrocene species was

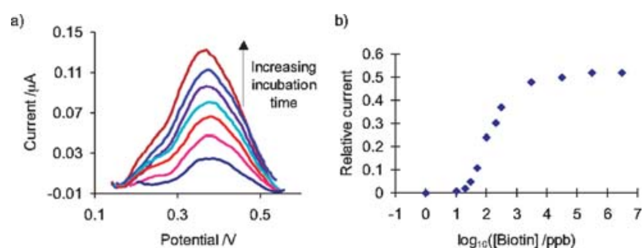

Fig. 3 (a) Relative current of anti-biotin modified GC surface after exposure of 0.3 mg mL^{-1} free biotin for different times determined using square wave voltammetry (SWV). (b) A calibration curves showing the variation is relative current with the logarithm of the concentration of biotin in solution. Relative current is the current before the exposure of free biotin divided by the current after exposure of free biotin.

suppressed (see Fig. S9, ESI†). Exposing this sensing interface to a sample which contained free biotin established a competition for the surface bound antibodies. A consequence of such a competition is that a proportion of the antibodies will dissociate from the surface-bound biotin moieties and complex with the biotin in solution. An increase in the electrochemical signal is then expected. Such an increase in current is illustrated in Fig. 3(a) where over a period of 20 min the peak current increased by 100 nA. Further, the extent of the current increase was dependent on the concentration of biotin in the solution. The calibration plot demonstrates the classical sigmoidal variation in peak current with the logarithm of the concentration of the biotin over a concentration range from 30 ng mL^{-1} to 0.3 mg mL^{-1} with a lowest detected concentration of 30 ng mL^{-1}. It is important to note that due to the high affinity of the anti-biotin IgG for the surface bound biotin, not all the anti-biotin leaves the electrode surface and the peak current is observed to rise to only 50% of the peak current recorded prior to complexing the antibody onto the electrode surface. A control was performed where the electrode with complexed antibody was exposed to a phosphate buffer solution that does not contain biotin over the same time period; in this case, there was only a modest increase in current density, possibly due to displacement of nonspecifically adsorbed anti-biotin from the surface (Fig. S10, ESI†). These results illustrate that it is the competition that is responsible for the removal of the anti-biotin; thus showing that the system can operate as an immunobiosensor.

The importance of the immunobiosensor presented here is that it is user-intervention free and compatible with portable instrumentation. The criterion for user-intervention free is important as this is essentially the criterion that differentiates an analytical method which requires trained personnel from one that the general public, untrained in measurement science, can perform. There are very few immunobiosensing concepts developed for small molecules whose operation simply requires the biosensor to be exposed to the sample.[19] Surface plasmon resonance has been shown to be able to monitor small molecules binding to proteins using benchtop instruments.[20,21] Similarly, the nanowire FETs of Lieber and coworkers have also been able to monitor the competition of the drug inhibitor Gleevac™ with ATP by binding to protein tyrosine kinase Alb; but this technique requires a difference in charge between ATP and Gleevac™ for a difference in conductivity through the nanowire to be achieved.[22] The concept we present here is, in principle, generic to any antibody–small molecule pair.

To summarize, we have demonstrated the modulation of the electrochemistry of a ferrocene moiety, connected to a glassy carbon electrode *via* a molecular wire, upon immersion in a protein environment. The interface of the modified electrode is designed such that only specific interactions of proteins with the ferrocene occur, which provides evidence that the modulation of the electrochemistry may be being controlled by the accessibility of counter ions to balance the change in charge of the ferrocene species upon oxidation/reduction. This phenomenon has been demonstrated to allow the detection of antibodies in solution *via* the attenuation of electrochemistry as antibodies bind to the interface. Conversely, with antibodies bound to the electrode surface, the presence of a small molecule analyte in solution causes a competition for the antibodies, some of which are displaced from the electrode surface, with a concomitant increase in electrochemical signal.

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