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Authors	Serleti, Alessandro;Salaj-Kośła, Urszula;Magner, Edmond
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ARTICLE TYPE

The spatial and sequential immobilisation of cytochrome c at adjacent electrodes

Alessandro Serletti, Urszula Salaj-Kosla, Edmond Magner*

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Two adjacent electrode surfaces were modified in a sequential manner with self-assembled thiol layers from the same solution using conditions (aqueous buffer at neutral pH) suitable for applications with proteins. A faradaic response was obtained from the redox protein, cytochrome c, independently immobilised at each surface.

Biofuel cells rely on the use of biocatalysts, predominantly as either isolated enzymes,¹ or in the form of mitochondrial² or microbial³ fuel cells to generate power from a fuel source. Both types of biofuel cell have been extensively investigated for potential applications ranging from clinical uses to the remediation of waste materials.¹⁻⁴ One of the challenges in the development of more efficient biofuel cells is to perform multi-step oxidation of a fuel such as glucose to enhance the power output of the cell.⁵ Enzymatic biofuel cells in particular face a significant limitation in this regard due to the specificity of enzymes, limiting their use to single substrates. Enzyme cascades can alleviate this limitation by enabling the sequential and more complete oxidation of the fuel.^{6, 7} Such sequential reactions mimic cascade reactions in cells where the product of one enzyme serves as the substrate for an adjacent enzyme with the rate of reaction controlled by the concentrations of substrates and co-factors and the activity of the enzymes involved. A prototype biofuel cell using this approach was described for the conversion of methanol to carbon dioxide by employing three NAD⁺ dependent enzymes: alcohol (ADH), aldehyde (AldDH) and formate (FDH) dehydrogenases resulting in a cell with an overall power output of 0.68 mW cm⁻².⁶ A similar type of cell with the addition of a quaternary ammonium bromide modified Nafion membrane provided a higher power output of 1.55 mW cm⁻².⁸ Recently a biofuel cell prepared using a hydrogel containing three immobilised NAD⁺ dependent enzymes: ADH, AldDH, and FDH had a current density of 26 mA cm⁻² when utilising ethanol as a fuel.⁷ On using formaldehyde and formate as intermediate fuels (two and one enzyme systems, respectively) lower current densities of 16 and 6 mA cm⁻² were observed. While substantial increases in output (>4 fold for the aADH, aAldDH, FDH system⁷) can be enabled through the use of multiple enzymes, methods of precisely locating the enzymes at the desired location and sequence have not been described. Such an approach has the potential to enable more efficient oxidation of the fuel.

A wide range of methods of immobilizing enzymes has been

described.^{9, 10} One of the most widely used methods of immobilising enzymes on electrodes entails the use of self-assembled-monolayers (SAMs).¹¹⁻¹³ Modified electrodes are prepared by chemisorption of the head groups of the chains of alkyl thiols onto the metal surface, usually by passive adsorption of thiols dissolved in solutions of ethanol over relatively long time periods (>12 hr).¹⁴ These surface modifiers are widely used in bio-electrochemical applications that entail interfacing biological components with the surface of the electrode. Electron transfer reactions of the redox protein cytochrome c (*cyt c*) have been extensively examined as a model for electron transfer reactions in proteins.¹⁵ *Cyt c* possesses a number of lysine residues close to the haem edge of the protein¹⁵ that facilitate interactions with redox enzymes such as cytochrome c oxidase. These residues can also be utilised to promote electron transfer at SAM modified electrodes and enable rapid rates of electron transfer when the haem prosthetic group is correctly oriented at a short distance from the surface.^{11-13, 16}

Electrochemical methods can be used to spatially define the location of electrode modification with features defined by the area of the electrode. Application of an appropriate potential can also be used for the rapid formation and desorption of SAM layers, with electrode modification occurring on time scales of minutes as opposed to the longer time scales (hours) used for solution deposition.¹⁷⁻²¹ Typically, SAM formation is performed in nonaqueous solutions, due to the limited solubility of thiols in aqueous buffer. An example of the electrochemically promoted adsorption and desorption of an alkanethiol is that of dodecanethiol in a solution containing 0.5 M KOH in aqueous ethanol.²² A linear correlation between the length of the alkanethiol and the desorption potential was observed. Monolayers of both single and mixed thiols (of mercaptopropionic acid and octadecanethiol) have been prepared by application of a potential of 0.5 V for 5 min, using conditions suitable for applications with proteins (pH 7, 5 mM phosphate, 0.1 M LiClO₄).¹⁷ The desorption of a dodecanethiol SAM on gold has also been accomplished at neutral pH.²³ Taken together, these reports indicate that both the adsorption and desorption processes can be performed in a benign environment for proteins and biological materials. In this report we describe a method to adsorb and desorb mixed alkanethiol layers on adjacent electrodes at neutral pH where the modified electrodes are subsequently used for the sequential immobilisation of the model

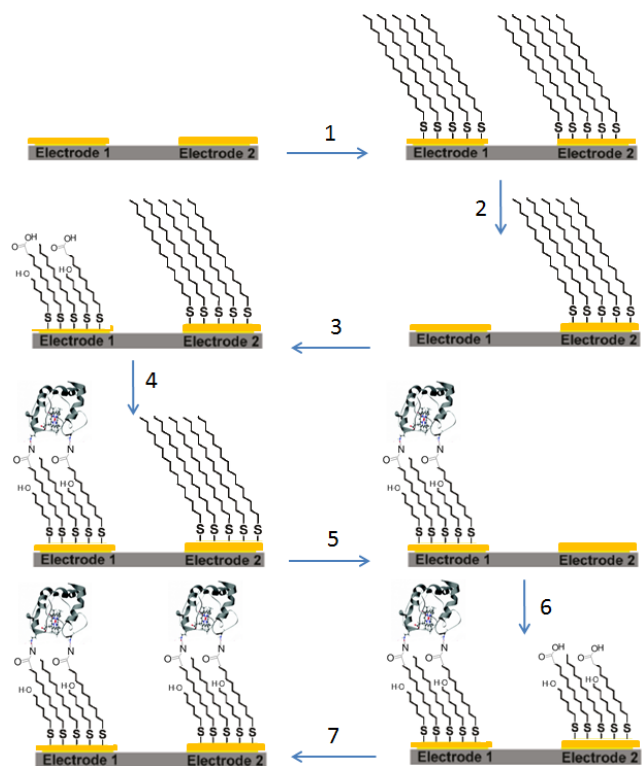


Fig. 1. Schematic representation of the steps utilized for the immobilisation of *cyt c* on two adjacent gold electrodes; 1. electrochemical deposition of blocking SAMs on A and B, 2. electrochemical desorption of blocking SAM from A, 3. electrochemical deposition of functionalised SAM on A, 4. immobilisation of *cyt c* on functionalised SAM on A, 5. Electrochemical-desorption of blocking SAM from B, 6. Electrochemical deposition of functionalised SAM on B, 7. Immobilisation of *cyt c* on functionalised SAM on B.

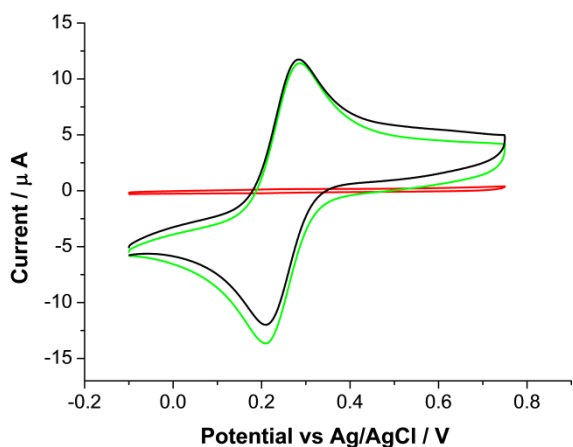


Fig. 2. Cyclic voltammograms of $K_3Fe(CN)_6$ (2 mM) in 150 mM KCl at a gold electrode (—), after electrochemical deposition of 1-hexadecanethiol (—), and electrochemical desorption of 1-hexadecanethiol (—).

redox protein, *cyt c*. Such an approach can be utilised to pattern surfaces for the immobilisation of proteins in a specific manner.

The approach used is outlined in schematic form in Fig. 1. A layer of 1-hexadecanethiol (in 50% aqueous ethanol (50% v/v) was deposited electrochemically on two gold disc electrodes (A

and B) to create two separate passivated surfaces (step 1). Electrochemical adsorption of SAMs can be achieved using a range of conditions, e.g. the adsorption of octadecanethiol was accomplished by using an applied potential of 0.5 V for 5 min.¹⁷ The adsorption of 1-hexadecanethiol was examined in detail over a range of potentials, scan rates and number of scans. The optimal conditions entailed scanning between 0.4 and 0.6 V (10 mVs⁻¹, 100 scans). Complete coverage of the electrode surfaces by 1-hexadecanethiol was indicated by the absence of a faradaic response for $K_4Fe(CN)_6$ (Fig. 2). Desorption of 1-dodecanethiol has been achieved by using an applied potential of -1.8 V (5 min, 0.1 M phosphate, pH 7.5).²³ The optimal conditions for the removal of the 1-hexadecanethiol layer (step 2) entailed scanning the potential between -1.5 and 2.0 V (50 scans at a scan rate of 50 mVs⁻¹). Electrode A was cleaned using this method. The removal of the SAM layer was accompanied by the evolution of H_2 which assists in preventing the re-deposition of thiols, as described previously.²³ This cleaning step resulted in a clean gold surface as evidenced by the faradaic response of $K_3Fe(CN)_6$ (Fig. 2) which was equivalent to that obtained prior to the adsorption of the first hexadecanethiol layer.

The electrochemically cleaned gold electrode A was then modified (step 3) with a mixed thiol layer of 6-mercapto-1-hexanol and 11-mercapto-undecanoic acid. Such a layer has been used to bind *cyt c* in an orientation that enables reversible electron transfer at the modified electrode surface.¹² It is necessary that the time required for this step should be as short as possible in order to prevent thiol exchange¹⁹ between the mixed thiols in solution and the thiol layer at the insulated electrode, B. A range of potentials, scan rates and number of scans were examined. Deposition of the mixed thiol layers occurred on application of a fixed potential for a period of 5 minutes, however full coverage of the electrode surface was not obtained as is evident from the observed faradaic responses to $K_3Fe(CN)_6$ (Fig. S1). On screening a range of conditions, efficient deposition of the mixed thiol layer was achieved by applying a single potential cycle between 0 and 1.5 V at a scan rate of 50 mVs⁻¹. No faradaic response was obtained for a solution of $K_3Fe(CN)_6$, indicating that a complete monolayer had been formed (Fig. S2).

Covalent attachment of *cyt c* was achieved using -cyclohexyl-N'-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfo-nate (CMC) activated thiols to anchor the protein (step 4).²⁴ The faradaic response was strongly dependent on the method used to prepare the mixed thiol monolayer. For example, deposition of the monolayer by scanning the potential over a series of potentials yielded no faradaic response and/or significant increases in the peak potentials (Fig. S3). On screening a range of conditions, deposition of the mixed thiol layer using a single potential scan (0 – 1.5 V, scan rate of 50 mVs⁻¹) yielded a quasi-reversible response, with a value of 20 mV (Ag/AgCl) for E^0 , in good agreement with published data.^{12, 13, 25}

Removal of the insulating dodecanethiol layer on electrode B was performed using the same approach as for electrode A (step 5). This step was successfully achieved as demonstrated by the faradaic response observed with $K_3Fe(CN)_6$, while the faradaic response of cytochrome c at electrode A was unaffected. Electrochemically assisted deposition of a mixed SAM layer was accomplished on electrode B (step 6), followed by

immobilisation of *cyt c* on to CMC activated thiols (step 7). Again it is important to minimize the time required for this process in order to prevent thiol exchange, as described earlier, for electrode A. A quasi-reversible faradaic response was obtained for cytochrome *c* immobilised at electrode B. During the procedures used to clean, modify and immobilise *cyt c* on electrode B (steps 5-7), the response of electrode A was unaffected.

To demonstrate the feasibility of the approach described here, *cyt c* was immobilised on two adjacent (6 mm apart), electrically insulated electrodes (Fig. 3). The surface coverage of *cyt c* was 30 pmol cm⁻², close to full monolayer coverage (Fig. 4). The higher degree of surface coverage observed in comparison to previous reports (*ca.* 0.5 monolayers)^{11, 12} may arise from the smooth surface (roughness factor of 1.0) of the sputtered gold layer. Variations in the surface coverage of *cyt c* immobilised on electrodes A and B of 20% were observed and can be ascribed to variations in the preparation of the electrodes.



Fig. 3. Image of sputtered gold electrodes.

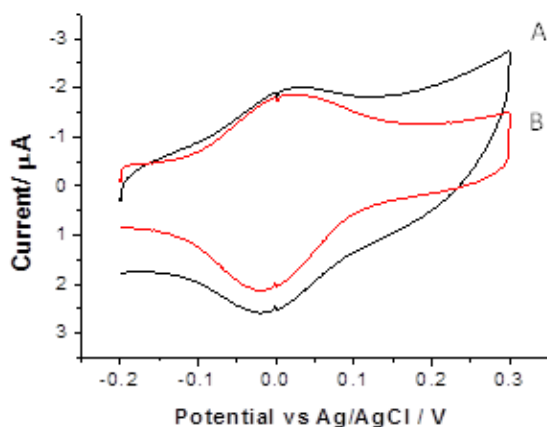


Fig. 4. Cyclic voltammograms of *cyt c* covalently immobilised on a HS(CH₂)₁₀COOH/HS(CH₂)₆OH modified gold electrode in 4.4 mM K₂HPO₄-KH₂PO₄ buffer, pH 7 at a scan rate of 100 mV s⁻¹, (A) electrode A, (B) electrode B at the end of the procedure.

In summary, the ability to independently address and pattern the surfaces of two adjacent electrodes with a protein has been demonstrated in a manner that enables each electrode to be modified sequentially under conditions of neutral pH without affecting the neighbouring surface. Such an approach has the potential to enable the specific patterning and immobilisation of enzymes in cascade reactions. The system described here is a proof of concept device, with relatively large electrode areas and inter-electrode spacing. While such an approach has potential applications in flow through devices, emulation of biological

cascades will require systems that are separated by less than 1 µm. Work is currently in progress to develop such miniaturised systems. Amongst other applications, the use of this approach has the potential to enhance the power output of biofuel cells, an approach that is currently under investigation.

Notes and references

Department of Chemical and Environmental Sciences & Materials and Surface Science Institute, University of Limerick, Limerick, Ireland. Fax: 353 61 213529; Tel: 353 61 202629; E-mail: edmond.magner@ul.ie.

† Electronic Supplementary Information (ESI) available: materials, methods of electrode modification, supplementary figures. See DOI: 10.1039/b000000x/.

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