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Design of Maleimide-Functionalised Electrodes for Covalent Attachment of Proteins through Free Surface Cysteine Groups

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Abstract: Mixed two-component monolayers on glassy carbon are prepared by electrochemical oxidation of *N*-(2-aminoethyl)acetamide and mono-*N*-Boc-hexamethylenediamine in mixed solution. Subsequent *N*-deprotection, amide coupling and solid-phase synthetic steps lead to electrode-surface functionalisation with maleimide, with controlled partial coverage of this cysteine-binding group at appropriate dilution for covalent immobilisation of a model redox-active protein, cytochrome *c*, with high coverage ($\approx$7.5 pmol cm^{-2}).

Modification of electrode surfaces is fundamental to the development of new, more efficient electrochemical devices for sensing, catalysis and energy conversion. The desired redox catalyst or recognition molecules must be stable on the surface, preferably through covalent immobilisation, and their redox state easily modulated by the potential applied to the underlying bulk electrode. The particular case of modification of electrodes with large biomolecules, especially redox enzymes, poses special challenges. To address the redox centre of the enzyme directly, its orientation on the surface has to be controlled in order to minimise the electron transfer distance and thus avoid the necessity of using diffusing redox mediators. This requires surfaces that can selectively bind a specific functional group (often artificially introduced by protein engineering) of the enzyme. In addition, steric considerations must be taken into account. The binding group on the surface should be able to access the target functional group of the protein and be laterally distributed to accommodate the bulky macromolecule. Moreover, the polarity/charge of the modified electrode surface around the binding group should be compatible with the protein surface around the target functional group of the protein to prevent the repulsion or denaturation of the enzyme.

A number of reliable procedures exist to create close-packed and mixed monolayers on electrode surfaces.[1–3] Monolayers can be readily assembled on gold from thiols or disulfides in solution,[4–6] however the layers thus formed are dynamic due to the mobility of thiols on gold and thiol exchange can occur in mixed monolayers, with the fraction of longer chains on the surface increasing upon prolonged incubation in the deposition solution, making the surface composition different to that of the solution.[6,7] Mixed thiols on the surface also segregate into homogeneous islands over time, and monolayer stability is limited, particularly for short thiols and mixtures.[8] These problems can be avoided with covalently modified carbon substrates, but there has been significantly less research into mixed monolayers on carbon. Gooding et al. have investigated the formation of mixed layers from reaction of glassy carbon (GC) surfaces with mixtures of differently substituted aryl diazonium salts, generated in situ. X-ray photoelectron spectroscopy (XPS) indicates formation of the mixed layer, although the ratio on the surface differs from that of the solution, with the more easily reduced diazonium salt giving greater surface coverage than expected from its concentration in solution.[9] Mixed 4-carboxyphenyl/phenyl layers have been formed from reduction of a mixture of the corresponding (pre-prepared) benzoic acid/benzene diazonium salts on GC electrodes and ferrocene methylamine subsequently coupled to the carboxylic acid functionalised surface.[10] Comparison with analogous ferrocene-terminated thiol layers on gold reveals the functionalised GC electrodes to have lower electron transfer rates, suggesting formation of multilayers, which is commonly observed for diazonium derived organic layers.[11,12] Other mixed layers have also been prepared for protein electrochemistry; mixed polyethylene glycol (PEG) and oligo(phenylethynylene) (molecular wire) layers have been prepared by reduction of a mixture of PEG- and oligo(phenylethynylene)-based diazonium salts onto GC electrodes. PEG was used as a group to stop the non-specific adsorption of proteins onto the electrode and ferrocene methylamine or horseradish peroxidise was coupled to the molecular wire, allowing direct electron transfer.[13] This work did not focus on precise control of the partial coverage; mixtures containing 5% (molar content) of the molecular wire were used and the surface layers, interrogated by voltammetry, shown to contain 3.7% covalently coupled ferrocene. Assess-

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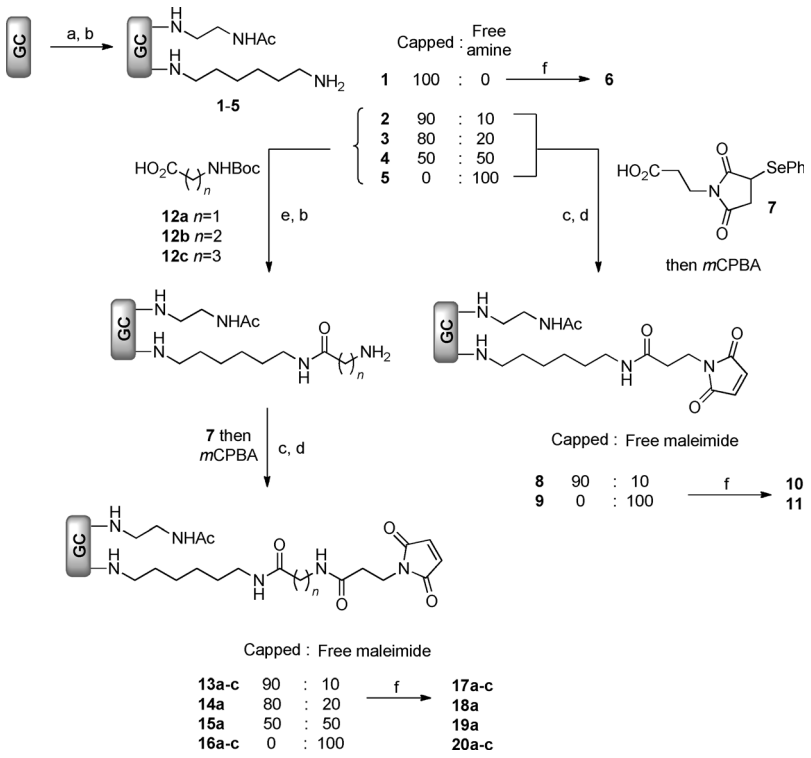
ment of the extent of control over surface coverage relied on the assumption that the relative surface coverages directly reflected the relative concentrations of the two diazonium salts in solution. Most recently, the same group has investigated the use of supramolecular interactions between two diazonium salts to form binary films with a 1:1 composition.[14]

In our approach, we functionalise GC by the oxidation of mono-Boc-protected diamines which, following deprotection, can be further modified using solid-phase synthesis to achieve the desired molecular architectures.[15–20] The method of carbon modification using amine oxidation was introduced in 1990 by Barbier et al.,[21] but to our knowledge, preparation of mixed monolayers from amine mixtures has not been attempted. In this Communication, we report functionalisation of GC electrodes with two-component monolayers derived from a mixed amine solution in which one component is a mono-Boc-protected diamine. Subsequent Boc-removal results in a mixed monolayer containing fractional coverage of a free amine, facilitating further stepwise coupling of spacer and terminal maleimide functionality. In this way, we obtain electrodes with controlled fractional coverage of a maleimide group for cysteine binding. An important feature of this strategy is that it gives a modified electrode which can spontaneously react with the engineered protein in buffer solution, allowing the most efficient use of small quantities of purified enzyme. The influence of maleimide coverage and the total length of the linker (controlled by the introduction of a range of spacers) upon protein binding is assessed using cytochrome *c* as a model redox protein. Cytochrome *c* from bovine heart was chosen due to an accessible free cysteine at its surface (Cys 17) in proximity to the haem redox cofactor.[22]



Scheme 1. Sequential electrochemical and solid-phase preparation of functionalised electrodes **6**, **10**, **11** and **17**–**20**. Subscripts **a**, **b** and **c** refer to $n=1$, 2 and 3, respectively. Prior to modification, 3 mm diameter (0.071 cm^2) glassy carbon (GC) rod electrodes were individually polished with silicon carbide paper (grade P1200, 3 M) followed by alumina lapping film (5 μm, 3 M) and alumina slurries (1.0 and 0.3 μm) on polishing cloths. Reagents and conditions: a) *N*-(2-*a*minoethyl)acetamide:mono-*N*-Boc-hexamethylenediamine (mono-*N*-Boc-HDA) in mixed solution (1:0, 9:1, 8:2, 1:1 or 0:1 ratio in MeCN) with a total amine concentration of 10 mM, nBu_4N^+ BF_4^-(150 mM), 2.1 V vs. Ag/AgCl, 180 s; b) 4.0 M HCl in 1,4-dioxane, 1 h; c) **7**, HBTU, iPr_2NEt, DMF, 16 h; d) *m*CPBA, MeCN, 0 °C, 1 h then RT, 1 h; e) **12a**, **b** or **c**, HBTU, iPr_2NEt, DMF, 16 h; f) cytochrome *c* (20 mM in phosphate buffer pH 7), 4 °C, 16 h. The ratio "Capped:Free" refers to the ratio of the concentrations of the two amines used in the initial electrochemical immobilisation step (step a).

We first prepared a series of electrodes, each with a full maleimide-terminated monolayer, but of varying length of tether of the terminal maleimide group to the surface (Scheme 1). In each case, the GC surface was functionalised by oxidation of mono-*N*-Boc-1,6-hexanediamine (HDA), followed by *N*-deprotection to give **5**. Direct HBTU-mediated coupling of **5** with **7**, or sequential coupling of a further *N*-Boc protected spacer **12** followed by *N*-deprotection before coupling with **7**, gave electrodes **9** and **16a**–**c**, respectively, following cleavage of the phenylselenyl group. Direct amide coupling of surfaces bearing free amine functionality with 3-maleimidopropionic acid (MPA)[23] would obviate the need for subsequent maleimide deprotection (i.e., for selenyl cleavage), however coupling of MPA with **5** was capricious in our hands and the use of **7**, readily prepared by phenylselenation of MPA (Supporting Information), proved convenient since oxidative elimination of the selenyl protecting group was facile under very mild conditions.[24] With the free maleimide moiety thus exposed, electrodes **9** and **16a**–**c** were finally treated with cytochrome *c* and its presence and quantity on the surface of the final redox protein-functionalised electrodes **11** and **20a**–**c** was investigated by cyclic voltammetry (Supporting Information).

Figure 1a shows typical results for the voltammetry of the cytochrome *c* modified electrodes. The baseline currents were subtracted using the algorithm described in the Supporting Information. This fits the background to the voltammogram using anchor points lying away from the position of cytochrome *c* peaks and using a B-spline function. The effectiveness of this procedure was checked using an unmodified glassy carbon electrode treated in the same way. Figure 1b shows the background-subtracted voltammetry. In all cases, the cytochrome *c* peaks were centred at ≈0.01 V versus SCE in

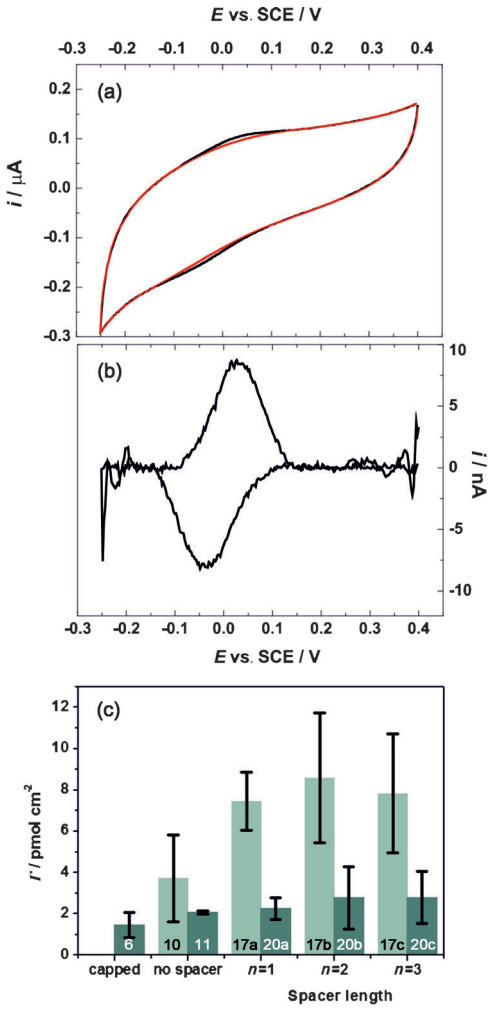


Figure 1. a) Raw (black) cyclic voltammogram of electrode **17a** overlaid with baseline from a polished GC control electrode (red). b) Background-corrected cyclic voltammogram of electrode **17a** derived from baseline subtraction from the raw data (Supporting Information). Voltammograms were recorded at pH 7, in 20 mM phosphate buffer with 100 mM $NaClO_4$ at 20 mV s^{-1}. c) Dark bars: Cytochrome *c* coverage on electrodes modified with a full monolayer of maleimide-terminated linker of variable length **11**, **20a**–**c**. Cytochrome *c* coverage on a (maleimide-free) 'capped' electrode (**6**) is given for comparison. Light bars: Cytochrome *c* coverage on electrodes modified with a 10% mixture of maleimide-terminated linker of variable spacer length (**10**, **17a**–**c**). Standard deviation (3 replicates of each electrode) is indicated.

agreement with published studies.[25] The surface coverage of cytochrome *c* was estimated from the area under the curve.

Surface coverages of cytochrome *c* for full coverage with maleimide linkers of different length (**11**, **20a**, **20b** and **20c**) are shown in Figure 1c (dark bars). The coverage on a control electrode **6**, obtained by treatment of a fully *N*-acetyl 'capped' surface (bearing no maleimide group) with cytochrome *c* is also included. Similar values of coverage, 2–3 pmol cm^{-2}, are obtained, irrespective of the spacer used. Moreover, the amount of cytochrome *c* on the surface in functionalised electrodes **11** and **20a**–**c** is comparable to that found on control electrode **6**. This suggests that non-specific binding is the predominant mode of protein retention on the electrode, perhaps because the maleimide terminated linkers form a compact layer of uniform thickness and binding between maleimide and the Cys17 residue of cytochrome *c* is hindered since the protein bulk cannot be sterically accommodated. Small increases in coverage observed for electrodes **20b** and **20c** with the longer linkers could result from greater disorder in the layer, with a higher number of individual maleimide groups sterically accessible. Figure 1c also shows analogous results obtained with lower maleimide coverages (light bars), and we will return to these data below.

For cytochrome *c* orientated towards the electrode by attachment through Cys 17, the theoretical surface coverage, assuming the crystallographic dimensions and close packing of the molecules on the surface, is about 11 pmol cm^{-2}. This is significantly larger than the values in Figure 1c for cytochrome *c* coverage of 2–3 pmol cm^{-2} on electrodes **11** and **20a**–**c** and suggests that close packing of the maleimide linkers might block their accessibility towards Cys 17 since accessibility is compromised by a local concave protein surface topography. To test this idea, we investigated the effect of diluting the coverage of the maleimide linker on the electrode surface by making mixed monolayers. The HDA linker and length of the co-immobilised acetyl capped linker were selected based on the cytochrome *c* crystal structure and location of Cys 17 (Supporting Information).

Mixed two component monolayers on the GC surface were prepared by electrochemical oxidation of a mixed solution of *N*-(2-aminoethyl)acetamide and mono-*N*-Boc-HDA, by stepping the potential to 2.1 V. From preliminary voltammetric investigation, it is known that at this potential both amines are oxidised at a diffusion controlled rate, and therefore stoichiometric amounts of the corresponding amine radicals are formed. If we assume similar reactivities for the two radicals formed, we expect the ratio of the surface coverages of the two amines to be similar to the ratio of their solution concentrations. Following *N*-deprotection, the resulting electrodes **2**, **3**, **4** and **5** are therefore expected to bear surface monolayers containing, respectively, 10, 20, 50 and 100% free amine functionality available for the subsequent solid-phase steps, reflecting the percentage component of mono-*N*-Boc-HDA in the first oxidation step. Electrodes **2**–**5** were each coupled with spacer **12a** before *N*-deprotection of the spacer unit, coupling with **7**, phenylselenyl cleavage and binding of each member of the resulting electrode series **13a**–**16a** with cytochrome *c* to give **17a**–**20a** (Scheme 1). Cyclic voltammetry of **17a**–**20a** clearly indicates that the surface concentration of maleimide in **13a**–**16a** leads to a pronounced effect on the amount of immobilised cytochrome *c* (Figure 2, Supporting Information).[26] Dilution of the binding group on the surface results in increased coverage of the protein, reaching (7.5 ± 1.4) pmol cm^{-2} for surfaces modified from a mixture of amines containing 10% HDA. In an attempt to maximise the cytochrome *c* coverage, a 1% HDA coverage was also investigated, however the reproducibility of the voltammetric signal of the corresponding final (cytochrome *c* bound) electrodes was poor, and is not included in the analysis.

Electrode **2**, modified with a mixed monolayer containing 10% free amine, was thus chosen as a starting point to investi-

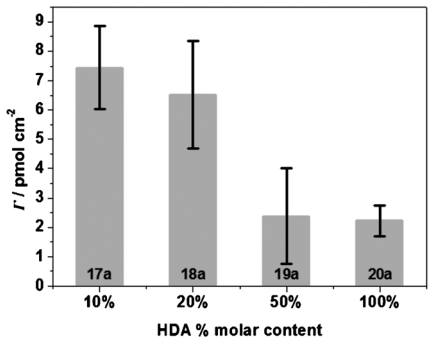


Figure 2. Cytochrome *c* coverage on electrodes of fixed tether length **17a**–**20a**, derived from electrodes **2**–**5**, respectively. 'HDA% molar content' refers to the composition of mixed amine solutions used to prepare **2** (10% HDA), **3** (20% HDA), **4** (50% HDA) and **5** (100% HDA) in the first surface-functionalisation step. Standard deviation (3 replicates of each electrode) is indicated.

gate the effect of the total length of a maleimide-terminated linker on binding of cytochrome *c*. Four different variants were prepared; the shortest linker was obtained by coupling **7** directly with **2** to yield **8**, and for longer linkers the coupling of **7** was preceded by the introduction of a spacer (one of **12a**–**c**) to eventually give maleimide-terminated electrodes **13a**–**c**. Upon reaction of **8** and **13a**–**c** with the protein, surfaces **10** and **17a**–**c** (also denoted as 'no spacer' and $n=1$–3, respectively) were obtained, with cytochrome *c* bound via four different tethers of distinct length. Surface coverage of cytochrome *c* upon each electrode was determined by cyclic voltammetry (Figure 1c, light bars, Supporting Information). The increase in the length of the linker is initially followed by a similar trend in the coverage of cytochrome *c*. The coverage doubles from electrode **10** to **17a**, that is, after introduction of the shortest spacer, followed by a moderate increase with further elongation of the linker (**17b**). The use of the longest linker, however, results in a drop in the cytochrome *c* coverage (**17c**) to a level comparable with that of **17a**. Whilst a longer, more flexible linker can improve the access and therefore the binding of a maleimide group to the cysteine residue of cytochrome *c*, the trend appears to have a limitation. The longest linkers are prone to the formation of more disordered layers with higher likelihood of entanglement of aliphatic chains. This in turn can cause a decreased efficiency of the subsequent binding steps, in our case coupling of **7** to the terminal amine as well as binding of cytochrome *c* to the terminal maleimide. Similar behaviour was previously reported for electrodes modified with tethered metal complexes.[19]

In conclusion, we have described a generic, modular approach to the modification of carbon electrodes with maleimide groups. The maleimide group is chosen because it undergoes spontaneous reaction with free cysteine groups at room temperature in aqueous buffer, making it an excellent choice for the immobilisation of proteins genetically engineered to contain free cysteine at suitable surface positions. By using electrochemical oxidation of mixtures of mono-*N*-Boc-diamine and *N*-(2-aminoethyl)acetamide, followed by *N*-deprotection and subsequent solid-phase synthesis steps, we have been able to control dilution of the maleimide group upon the GC electrode surface and independently vary the length of the linker to this binding group. The efficacy of this approach has been demonstrated using bovine heart cytochrome *c* as a redox-active model protein. Our results show that for full surface coverage of maleimide the final surface coverage of the protein is close to that for non-specific adsorption and we postulate that this is because the high surface coverage of maleimide leads to significant steric hindrance towards the reaction of the cysteine on the cytochrome *c* surface. Consistent with this hypothesis, when the maleimide groups on the electrode surface are diluted by co-immobilisation of mono-Boc-protected diamine with *N*-(2-*a*minoethyl)acetamide, we find significantly higher coverages for cyctochrome *c*, as judged by the electrochemistry of the immobilised protein. Further support for this interpretation is provided by study of the effect of the length of the electrode surface attachment linker for maleimide, upon the electroactive coverage. We find that for the shortest linker the coverage is again lower, and steric restrictions upon coupling between maleimide and cysteine are consistent with the crystal structure of the protein (Supporting Information).

Our results emphasise the importance of careful design of the electrode surface architecture for efficient attachment of cysteine-modified proteins, and demonstrate that this can be achieved by the flexible synthetic approach described herein.

Experimental Section

Detailed experimental procedures for solid-phase preparation of electrodes **6**, **10**, **11** and **17**–**20**, cyclic voltammograms, and methods of peak extraction for electrodes **6**, **10**, **11** and **17**–**20**, are provided in the Supporting Information.

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Keywords: cyclic voltammetry · electrochemistry · proteins · solid-phase synthesis

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[26] Coupling of ferrocenyl hexanethiol to a GC surface functionalised with a full maleimide-terminated monolayer was attempted in order to establish a model with which to then identify any impact of the mixed monolayer upon the efficiency of thiol binding. However, voltammetric investigation (electrode cycling in CH_3CN with $n Bu_4N^+BF_4^-$ from 0 to 1.1 V vs. Ag/AgCl for an initial 10 cycles at 100 mV s^{-1}) indicated that non-specific absorption of the redox-active species compromises accurate measurement of coupling efficiency since a large and broad-shouldered current at 0.74 V vs. Ag/AgCl, not seen in the reverse scan, appears which we attribute to loosely bound multiple ferrocene species that desorb from the surface quickly.

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