Direct Electron Transfer Reactions of Glucose Oxidase Immobilised at a Self-assembled Monolayer

Li Jiang,[a] Calum J. McNeil[b] and Jonathan M. Cooper*[a]

[a] *Department of Electronics and Electrical Engineering, University of Glasgow, UK G12 8LT*
[b] *Department of Clinical Biochemistry, The Medical School, University of Newcastle upon Tyne, UK NE2 4HH*

The direct electrochemistry of glucose oxidase, immobilised at a self-assembled monolayer of 3,3′-dithiobis-sulfocinnimidylpropionate (DTSSP) is reported, and electron transfer kinetics of the biocomposite assembly are discussed.

Over the last two decades there has been a continued interest in studying the direct electrochemistry of biological molecules at electrode surfaces.[1–7] Seminal work in this field[1,2] has centred on observing electron transfer (ET) reactions of soluble model proteins at modified electrodes, and has resulted in the observation of quasi-reversible electrochemistry of glucose oxidase (GOD, EC 1.1.3.4.) at a variety of suitably modified carbon[3–6] and gold surfaces.[7] More recently, there has been a shift of emphasis towards investigating reversible interfacial reactions involving small proteins, particularly cytochrome *c*, attached at the electrode either using ion-pairing[8] or *via* carbodiimide immobilisation procedures.[9,10] This arrangement of the biomolecule at the surface offers an advantage in that both solution resistance and desorptive losses of the protein can be negated. Moreover, such models have proved exciting for those involved in biosensor research, as they suggest that it may be possible to 'wire' proteins to electrodes without the need for ET mediators.[11]

We report, for the first time, on the reversible direct electrochemistry of the large glycoprotein enzyme GOD, attached at a self assembled monolayer. The enzyme–monolayer construction is immobilised at the electrode using a single step procedure involving a homo-bifunctional *N*-hydroxysuccimide ester, *e.g.* 3,3′-dithiobis-sulfosuccinimidylpropionate (DTSSP, Fig. 1), which is thiol-cleavable and assembles onto a gold surface. Information on the surface coverage of the enzyme, the rate constant (k_{ET}) for direct electron transfer between the enzyme-bound flavin adenine dinucleotide (FAD) prosthetic group and the modifed electrode (calculated using Laviron's simplest model[12]) and kinetic data for the enzyme catalysed reaction involving the oxidation of glucose is also presented.

The enzyme was immobilised by mixing the protein (10 mg ml^{-1}) with an excess of DTSSP (10 mmol dm^{-3} solution in 100 mmol dm^{-3} sodium phosphate buffer, pH 6.3) for 30 min. The non-water soluble analogue of DTSSP, dithiobis(succinimidylpropionate) DSP, may also be used as a 10 mmol dm^{-3} solution in Me_2SO–H_2O, 1:5 (*v/v*), and incubated at room temperature. In both situations, a gold electrode (area = 0.031 cm^2, polished with 0.05 μm alumina, treated with 25% H_2O_2 in H_2SO_4 and sonicated in water for 15 min) was incubated in the enzyme–cross-linker solution overnight at 4 °C. The enzyme is bound to DTSSP or DSP *via* a peptide bond between lysine groups on the surface of GOD and the DTSSP ester group.[13] Reductive cleavage of the disulfide site on the gold resulted in the formation of a monolayer of enzyme at the electrode, in such a configuration that it readily undergoes direct ET between the bound FAD centre and the electrode, Figs. 2 and 3. Alternatively, the cleaned gold was incubated in a buffered solution of 10 mmol dm^{-3} DTSSP for 4 h, and after washing, a solution of enzyme was added to the electrode. Surface coverage was estimated by integration of either the cathodic or the anodic curves of the *CV* (with the background apoGOD contribution subtracted) under anaerobic conditions at a scan rate of 5 mV s^{-1} (mean = 1.3×10^{-11} mol cm^{-2}), and indicated that both methods of immobilisation resulted in equivalent amounts of protein bound to the surface. Estimating the contact area of an enzyme molecule[14] as 6000 $Å^2$ and using a roughness factor of the modified surface of 2 indicates that the enzyme exists as a monolayer.[9]

The direct electrochemistry of the enzyme was investigated using CV. Fig. 2 demonstrates that the Au–DTSSP–GOD assembly readily undergoes reversible charge transfer, $E_{1/2}$ *ca.* −282 mV *vs.* Ag/AgCl, ΔEp = 102 mV at 5 mV s^{-1}. The redox peak currents for immobilised GOD were found to be directly dependent upon the potential scan rate. Fig. 3 demonstrates that the ET reaction is a surface bound process, which is confirmed by the persistence of the signal after repeated thorough washing. Using data from Fig. 3, the electron transfer rate constant k_{ET} was calculated as 0.026 s^{-1} (after Laviron[12]). Following overnight incubation of the GOD–electrode in guanidine·4HCl (to remove the FAD active centre from the enzyme[15]), the characteristic electrochemistry disappeared. The ET phenomena can therefore be attributed to the prosthetic group within the GOD shell, and not to free FAD (which may have dissociated away from the enzyme due to conformational changes during

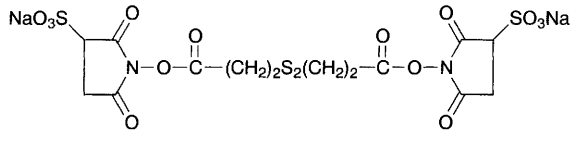

Fig. 1 3,3′-Dithiobis-sulfosuccinimidylpropionate, DTSSP

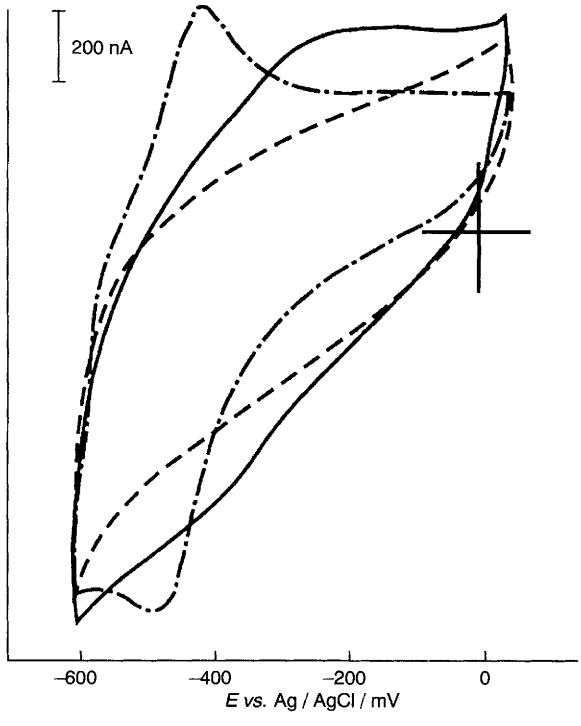

Fig. 2 Typical CVs of Au–DTSSP–apoGOD (----); Au–DTSSP–FAD (—·—·—); Au–DTSSP–GOD (—) in phosphate buffer (0.1 mol dm^{-3}, pH 6.3), potential scan rate 15 mV s^{-1}

immobilisation). Indeed, immobilisation of FAD at a DTSSP modified gold surface, also shown in Fig. 2, gives well-defined, reversible electrochemistry with $E_{1/2}$ = −395 mV, demonstrating that the redox potential of FAD bound within GOD shifts anodically by 113 mV with respect to the unbound species.

Enhancement of the oxidation current of the Au–DTSSP–GOD assembly, on addition of glucose, was observed under anaerobic conditions using CV. The working electrode was set at −600 mV, and a series of aliquots of degassed glucose solution (230 mmol dm^{-3} in buffer), were added to the electrochemical cell, maintained under a blanket of nitrogen. Results were recorded as the second scan, Fig. 4, demonstrating that the reduced form of GOD is catalytically regenerated by glucose in the absence of oxygen, and that the enzyme is directly re-oxidised on the electrode surface.

Finally, in order to demonstrate that the enzyme was immobilised on the self-assembled DTSSP monolayer in an active conformation, chronoamperometric measurement of the oxidation of enzymically produced H_2O_2 (at 700 mV *vs.* Ag/AgCl) in response to addition of glucose and its isomers was carried out under aerobic conditions. Using the current densities obtained, with estimates for k_{cat}, measured spectrophotometrically to be 512 s^{-1}, α, the ratio of hydrogen peroxide

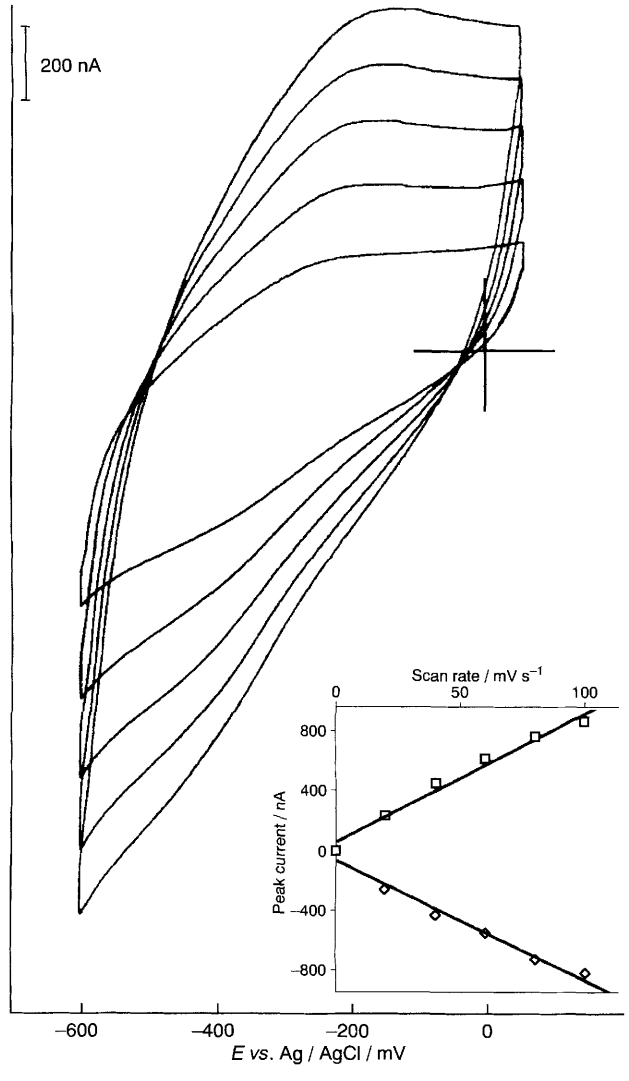

Fig. 3 CVs for Au–DTSSP–GOD assembly recorded in phosphate buffer (0.1 mol dm^{-3}, pH 6.3). The scan rate was increased from 20 to 100 mV s^{-1} in increments of 20 mV s^{-1}. Peak currents were measured by subtracting non-Faradaic currents recorded at an Au–DTSSP–apoGOD assembly (*e.g.* i_{pa} = 220 nA at −210 mV *vs.* Ag/Cl, scan rate 100 mV s^{-1}). Insert: linear plots of i_{pc} and i_{pa} *vs.* scan rate indicate that the electron transfer reaction is a surface bound process.

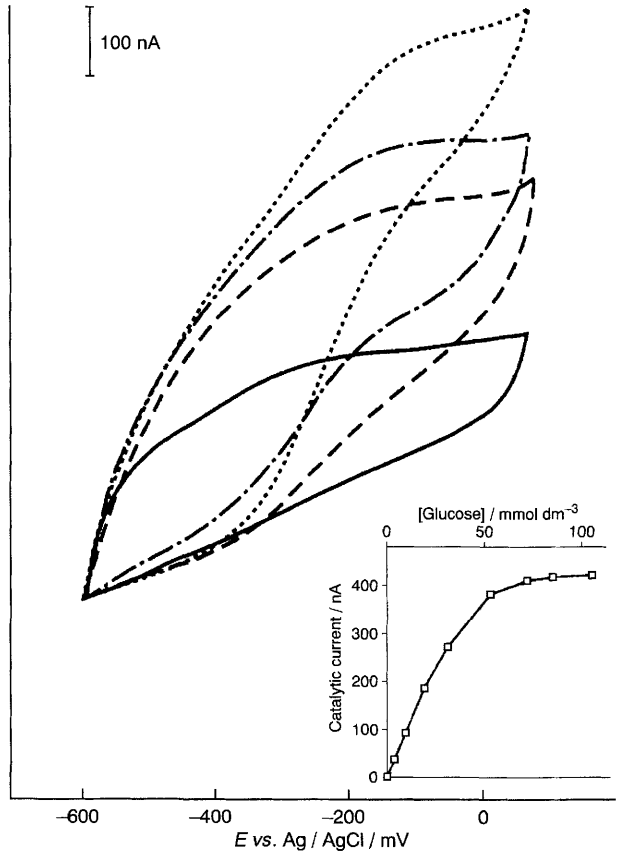

Fig. 4 CVs for Au–DTSSP–GOD in phosphate buffer (0.1 mol dm^{-3}, pH 6.3) (*a*) at 5 mV s^{-1} (——), showing catalytic enhancement of the FAD oxidation peak on addition of (*b*) 3.2 mmol dm^{-3} (----), (*c*) 32 mmol dm^{-3} (—·—) and (*d*) 72 mmol dm^{-3} (····) glucose solution in buffer. Insert: plot of i_{cat} *vs.* concentration of glucose/mmol dm^{-3}.

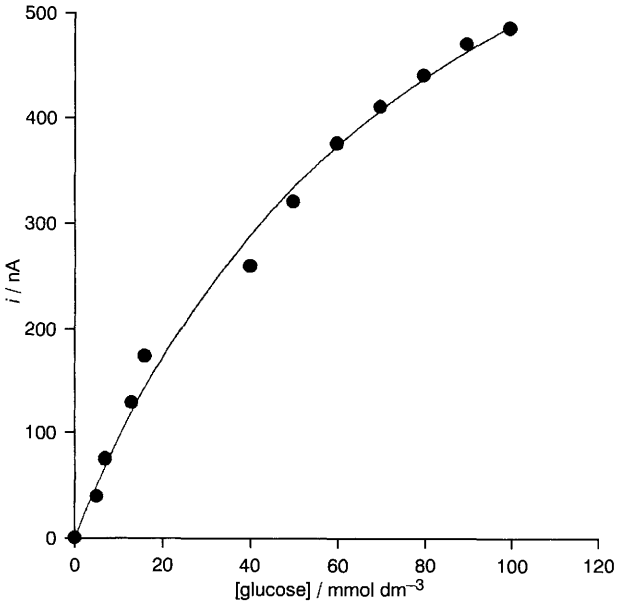

Fig. 5 The response of the Au–DTSSP–GOD biomolecular assembly to additions of glucose (0–100 mmol dm^{-3}) in phosphate buffer 0.1 mol dm^{-3}, pH 6.3). The data were fitted to curve using a non-linear least squares program.[16]

detected to that lost into bulk solution[16] to be 0.5, and the hydrodynamic radius as *ca.* 4.5 nm,[17] it was shown that there was sufficient active enzyme present to cover the electrode as a monolayer. The data (——) fitted to a previously developed algorithm, describing the kinetics of monolayers of enzymes immobilised within very thin (10 nm) electrochemically formed polymer matrices[16] using the Marquardt–Levenburg algorithm, Fig. 4. Enzyme kinetic parameters were determined as in eqns. (1) and (2), which are in close agreement with those previously determined for monolayers of GOD.[16]

$$\frac{\alpha K_s k_{cat} e_\Sigma l}{K_M} = 1.74 \times 10^{-6} \text{ cm s}^{-1} \quad (1)$$

$$\frac{\alpha K_{cat} e_\Sigma l}{1 + k_{cat}(kK_A a_\infty)} = 1.48 \times 10^{-10} \text{ mol cm}^{-2} \text{ s}^{-1} \quad (2)$$

By studying the catalytic activity with isomers of glucose at the immobilised enzyme–electrode assembly, it was shown that GOD was in its native conformation, and that it had not undergone structural rearrangement during immobilisation. Previously, conformational changes in immobilised GOD have been shown to reduce the substrate specificity of the enzyme.[7] However, the enzyme assembly described here retains its original selectivity, with no response shown to a range of isomers including D-(+)-mannose and 2-deoxy-D-glucose.

In summary, the immobilisation of GOD onto a DTSSP modified gold surface provides a highly selective and thermodynamically favourable ET pathway, which facilitates direct electrochemistry of GOD. The enzyme–monolayer construction provides an example of an enzyme in direct electrochemical communication with the electrode, a claim that is corroborated by the catalytic enhancement of the oxidation curve in the presence of its substrate, glucose.

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