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Mediation of glycosylated and partially-deglycosylated glucose oxidase of *Aspergillus niger* by a ferrocene-derivatised detergent

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A ferrocene-derivatised detergent, (11-ferrocenylundecyl) trimethylammonium bromide (FTMAB), when oxidised to the corresponding ferricinium ion, was found by electrochemical studies to be an effective electron acceptor for reduced glucose oxidase of *Aspergillus niger* (EC 1.1.3.4) and thus acts as a electron-transfer mediator between glucose oxidase and a working electrode held at a potential sufficiently positive to reoxidise reduced FTMAB. An increase in mediating activity was produced when FTMAB was present in concentrations above its critical micelle concentration. An 'enzyme electrode' was formed by adsorption of glucose oxidase and FTMAB surfactant on a graphite rod. The electrode functioned as an amperometric biosensor for glucose in phosphate-buffered saline solution. A mixed micelle of glucose oxidase and FTMAB, probably adsorbed on the electrode surface, appears to be advantageous for the amperometric determination of glucose. Additionally, glucose oxidase was treated with α-mannosidase. When this partially-deglycosylated glucose oxidase was incorporated in an enzyme electrode, a 100-fold increase in the second-order rate constant (k) for electron transfer between the enzyme and FTMAB was observed, together with increased current densities, with respect to the equivalent values for FTMAB and commercial glucose oxidase. The use of deglycosylated enzymes in biosensors is suggested.

Introduction

Glucose oxidase is a glycoprotein obtained from microbial and fungal sources that catalyses the oxidation of glucose to gluconic acid, with the concurrent formation of hydrogen peroxide from molecular oxygen [1]. The enzyme obtained from *Aspergillus niger* has a total molecular weight of approximately 160000 and is composed of two homologous protein subunits [2], each of which contains a tightly but non-covalently bound FAD group to participate in the necessary redox reactions [3,4].

The enzyme has frequently been employed as an element of a glucose biosensor where the biochemical event of glucose oxidation is coupled to a means of signal transduction, be it electrical, thermal or optical [5]. Due to the great clinical interest in a rapid and accurate determination of glucose in blood or other body fluids, glucose oxidase is widely employed in such biosensors and much reported in the literature in this respect. The majority of reports concern non-mediated or amperometrically-mediated enzyme electrodes. In the former configuration, glucose reacts with the oxidised FAD of glucose oxidase to form gluconolactone and reduced FAD; molecular oxygen then oxidises the reduced FAD with the formation of H_2O_2. Glucose oxidase may be combined with an O_2 or H_2O_2 sensitive electrode to follow the rate of consumption or production respectively, of these chemicals. However, fluctuations of the ambient concentration of oxygen in solution, from one sample to another for example, gives rise to fluctuating and thus unreliable responses.

One attempt to overcome this problem is to employ a xenogenous redox-active species to accept electrons from the reduced flavin group of glucose oxidase in place of O_2 and subsequently pass the electrons to an electrode held at fixed potential in order to regenerate the mediator. The currents thus produced are proportional to the concentration of glucose in solution as the glucose/glucose oxidase reaction is overall rate-limiting. Amongst the mediators documented in the literature are representatives of almost every class of chemical compound possessing fast, reversible electrochem-

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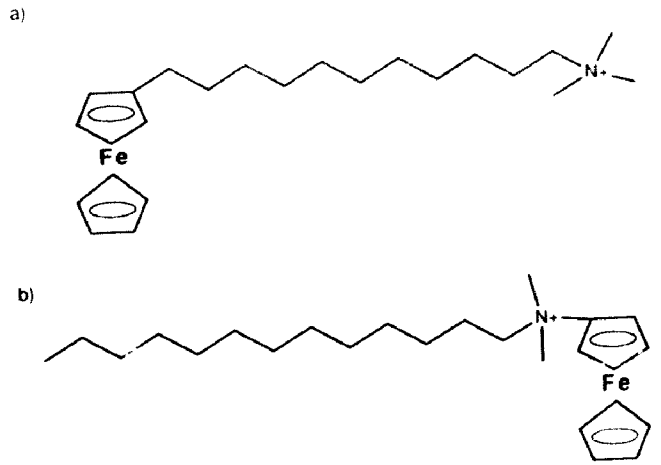

Fig. 1. Chemical structure of ferrocene detergents: (A) FTMAB as used in this study; (B) Compound used by Bourdillon and Madja [21].

istry: monomeric ferrocenes [6], glucose oxidase covalently modified with ferrocene [7,8], ferrocene derivatised polymers [9,10], conducting 'charge transfer salts' [11] 'redox gels' [12], octacyanotungstates [13], hexacyanoruthenate [14], nickel cyclams [15], and organic compounds such as quinones and benzoquinones [16].

Due to the extensive work of Hill and coworkers (for example see Ref. 17) the ferrocene family of compounds has been established as reliable mediators for glucose oxidase and other flavoproteins. Additionally, the behaviour of ferrocenes solubilised in detergent micelles has been well studied and reviewed [18–20]. The ferrocenes are generally only sparingly soluble in aqueous solution; their ferricinium ions are soluble but also unstable [19]. The use of detergents such as dodecyltrimethylammomium bromide (DDTAB) or sodium dodecyl sulphate (SDS) to solubilise ferrocenes indicates that such systems are capable of improved mediation between proteins (for example cytochrome *c*) and an electrode, relative to the ferrocene species alone, due to an enhanced effective solubility in aqueous solution [19]. However, we believe that only in a few cases [21,22] has a surfactant derivative of ferrocene combining detergent and redox moieties in the same molecule (see Fig. 1B) been employed as a mediator of glucose oxidase. Bourdillon and Madja [21] employed a rather complex electrode upon which a ferrocene surfactant and glucose oxidase were deposited. Löffler et al. [22] prepared ferrocenes with a long alkyl chain ring-substituent that were inherently insoluble in water but could be solubilised in micelles of non-redox-active 'tenside' detergent. However, they did note quasi-reversible electron-transfer at gold electrodes and high (10^5 M^{-1} s^{-1}) second-order rate constants for electron transfer from glucose oxidase.

In this paper we present results from a simple amperometric system employing a surfactant derivative of ferrocene (different to that used by Bourdillon and Madja [21]). Our findings indicate the importance of cohesive detergent/protein interactions, and comicellization of mediator and glucose oxidase, for rapid protein/mediator electron-transfer. The applicability of a partially-deglycosylated glucose oxidase in a biosensing system is also presented. We present results to show that in the case of a detergent-like mediator such as FTMAB, mediation is more effective with partially-deglycosylated glucose oxidase than with the commercial enzyme, suggesting that the use of deglycosylated glucose oxidase in biosensors may also be advantageous.

Materials and Methods

Electrochemical methods

A BAS-100 electrochemical analyser (Bioanalytical Systems (U.S.A.)) was employed to perform cyclic voltammetry, chronoamperometry and preparative controlled-potential thin layer electrolysis in electrochemical cells (Metrohm) of vols of 5–20 ml. A three-electrode cell was comprised of a standard calomel reference electrode (with respect to which all potentials are quoted), an auxiliary electrode of a platinum rod and a working electrode of glassy carbon (Metrohm) (surface area 7 mm^2) or an 'enzyme electrode' (see below). Cyclic voltammetry was performed with scan speeds of 5–100 mV s^{-1} and chronoamperometry with a 1 s time resolution. For preparative controlled-potential thin layer electrolysis of FTMAB, a 10 ml three compartment cell (Metrohm) with a platinum working electrode was employed with an applied potential of +350 mV. The electrolysis was followed by ultraviolet-visible spectrophotometry and was continued until there was no further change in the spectrum. All experiments, unless otherwise stated, were performed using phosphate-buffered saline solution (100 mM NaCl/10 mM Na_2HPO_4/0.1 mM EDTA/0.01% sodium azide/0.01 mM phenylmethylsulphonyl fluoride (PMSF), adjusted to pH 7.4 with conc. HCl). The concentration of azide used in this buffer did not effect the observed activity of glucose oxidase, relative to an identical buffer prepared without azide.

Determination of kinetic constant for mediation

The second-order rate constant, k, was derived following the experimental method described by Cass et al. [6]. Briefly, the magnitude of the catalytic current at a glassy carbon electrode was measured by cyclic voltammetry in a three-electrode cell in samples with increasing amounts of glucose oxidase in solution, FTMAB and glucose being in excess. The change in catalytic current with additions of glucose oxidase was compared to the diffusion currents obtained in the

absence of enzyme and thereby related to the rate constant by the scheme of Nicholson and Shain [23].

Preparation of enzyme electrode

Slabs of graphite (ZXF5-Q, 0.2 μm mean pore size, 15.4% porosity by volume, Poco Graphite Co., Decatur, Texas, U.S.A.) were cut to dimensions of $5 \times 6 \times 10$ mm. These blocks were drilled at one 5×6 mm face to enable the connection of a plastic coated copper wire which was anchored by silver amalgam to ensure electrical contact. The junction was fully insulated with inert silicon resin. The electrode was incubated in a solution of glucose oxidase or partially-deglycosylated glucose oxidase (10 mg/ml) and FTMAB (typically $5 \cdot 10^{-4}$ M) in phosphate buffered saline at 4°C. One or several electrodes were immersed in the incubation solution. For convenience, an incubation time of 16 h was used (i.e. overnight); however, an electrode incubated for 2 h gave equivalent results to those incubated for longer than this period. Electrodes were thoroughly rinsed with distilled water following withdrawal from the incubation solution. Unless otherwise stated, the electrodes were then used immediately for experiments.

Measurement of glucose oxidase activity, apparent activity and amount of glucose oxidase adsorbed on graphite electrodes

The specific activity of glucose oxidase in a solution, or the apparent activity of the enzyme adsorbed on electrodes, was monitored by an spectrophotometric assay based on peroxidase to measure production of H_2O_2 [24] where 1 unit, U, is defined as the amount of enzyme that will oxidise 1 μmol of glucose per min at pH 7.0 and 25°C. Determination of the apparent activity of glucose oxidase adsorbed on electrodes was performed by vortexing of an electrode with the peroxidase assay solution previously blanked at A_{500}, followed by measurement of the increase in A_{500} that had occurred during the period of vortexing. This was then interpreted as a rate of increase per s. The amount of protein adsorbed was assayed by shaking of an glucose oxidase/FTMAB electrode in 15 ml of phosphate-buffered saline solution for 24 h at room temperature. The amount of desorbed protein in the buffer was assayed with a Biorad kit [25]. It was assumed that all of the protein had desorbed from the surface as the electrode showed no apparent glucose oxidase activity after this procedure.

Partial deglycosylation of glucose oxidase

Glucose oxidase of *Aspergillus niger* was partially deglycosylated by a simple method similar to that of Kalisz et al. [26]. A solution of glucose oxidase (10 mg/ml) and α-mannosidase (0.5 mg/ml) in phosphate solution (30 mM NaH_2PO_4, pH 5) was incubated at 37°C for 36 h. The solution was then filtered using an Amicon ultrafiltration cell (model 8050) with a YM-100 membrane (molecular-weight cut-off of approximately 100000) to retain only glucose oxidase. The phosphate-only buffer was exchanged for the phosphate-buffered saline (pH 7.4) buffer specified above, with the final concentration of protein being 10 mg/ml.

Miscellaneous

FTMAB was obtained from Dojindo Chemicals, Japan. Glucose oxidase was supplied by Fluka, Switzerland, with an activity of 215 U mg^{-1}. All other reagents were purchased from Fluka, Sigma or Aldrich. Unless otherwise stated, experiments were performed at 20°C and most solutions were not degassed and thus contained dissolved oxygen. However, where necessary, solutions were saturated with nitrogen prior to use and the gaseous volume of the electrochemical cell was continuously purged with nitrogen using a system of rubber septa and syringe needles. Solutions were stirred magnetically. Surface tension measurements were performed with a Gouy-Chapman balance (Kruss, Hamburg) equipped with a Pt ring, at 20°C with a trough-cover to prevent rapid evaporation from the bath. Denaturing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [27] with an acrylamide concentration of 10%. Molecular weight standard proteins were from Boehringer. Ultraviolet-visible spectrophotometry was performed with a Hewlett-Packard HP 8450A diode-array spectrophotometer.

Results

Electrochemistry of FTMAB in solution

As has been previously demonstrated in a non-biosensor-oriented application [28–32] (11-ferrocenylundecyl)trimethylammonium bromide (FTMAB, Fig. 1A) and related compounds possess rapid, electrochemically reversible, heterogenous electron transfer with a range of electrode materials. It has been proposed that the compound has surfactant properties, with a critical micelle concentration of less than $1 \cdot 10^{-4}$ M [30,31] when the Fe atom of the ferrocenyl moiety is in the (II) oxidation state. Using a glassy-carbon electrode, with a solution of $5 \cdot 10^{-4}$ M FTMAB in phosphate-buffered saline (pH 7.4), and either glucose oxidase or glucose present, cyclic voltammetry (Fig. 2A(i)) shows a sharp peak in current corresponding to oxidation of the Fe (II) form to the Fe (III) form of FTMAB, with the peak current occurring at a potential of +95 mV, (scan speed of 25 mV s^{-1}). The oxidation peak resembles that of an electrode-surface adsorbed species in its shape and sharpness; however, the peak current, I_p, was linearly proportional to the root of the cyclic

voltammetry scan speed as one would expect from a diffusion-limited reaction; from the Randles-Sevcik equation [33] a diffusion coefficient of 1.69×10^{-7} cm^2 s^{-1} was determined. In the Fe (III) state, it has been proposed by Saji and co-workers (30, 31) that electrostatic repulsions cause the micelle to disperse. In concordance with their results, the broad reduction peak at +35 mV in the cyclic voltammogram suggests that the diffusion coefficient of the monomeric Fe (III) form is greater than that of the Fe (II) in micelles, i.e. the Fe (III) form may be ejected from a micelle composed of Fe (II) species. The standard redox potential of FTMAB in phosphate-buffered saline (pH 7.4) solution was determined to be +65 mV as measured at a glassy-carbon electrode.

In the presence of glucose (> 1 mM) and glucose oxidase (> $1 \cdot 10^{-6}$ M), the cyclic voltammogram of FTMAB displays a wave-form commonly referred to as a 'catalytic wave' (Fig. 2A(ii) and (iii)). The exact shape of the catalytic wave was time-dependent and corresponded to the macroscopic state of the solution/suspension; 1 min after addition of glucose and glucose oxidase, the cyclic voltammogram shows no reduction peak and the magnitude of the anodic current increases (Fig. 2A(ii)). Macroscopically, this corresponded to turbidity of the FTMAB/glucose oxidase/glucose suspension. After 5 min, the solution had clarified; this was accompanied by a more pronounced catalytic wave (Fig. 2A(iii)) as observed by cyclic voltammetry. We suggest that these time-dependent changes correspond to the reorganisation of FTMAB micelles around glucose oxidase; the protein is initially precipitated and then solubilised to form a mixed micelle, where mediation is most efficient. The catalytic wave has been rationalised [23] as evidence for fast electron transfer from reduced glucose oxidase to oxidised mediator (FTMAB ferricinium ion), coupled with fast, diffusion-controlled electrochemically reversible reoxidation of the reduced mediator at the electrode surface. The second-order rate constant for electron transfer from reduced glucose oxidase to FTMAB ferricinium ion was found to be $5 \cdot 10^3$ M^{-1} s^{-1}, thus in the range of values previously determined for the ferrocene family with glucose oxidase [6]. Additionally,

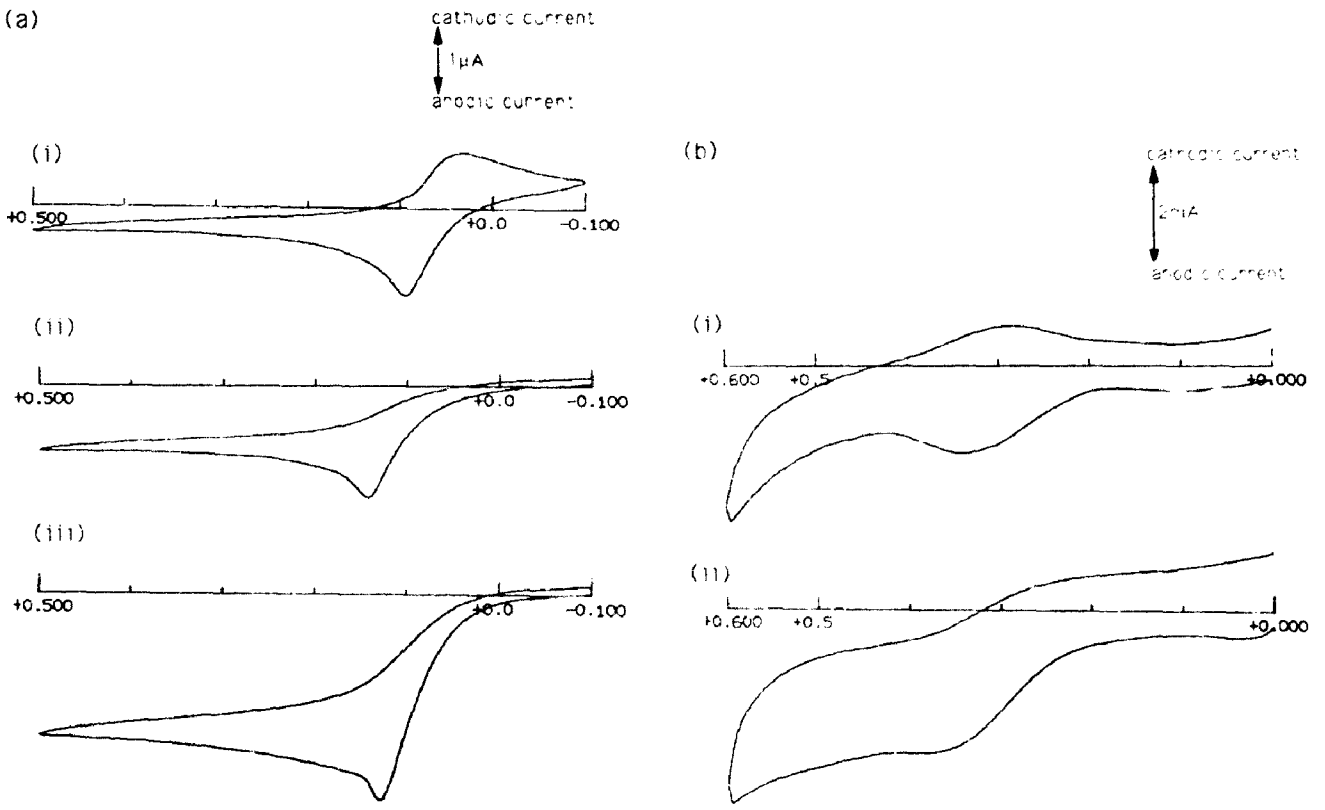

Fig. 2. Cyclic voltammograms (sweep rate of 25 mV s^{-1}) recorded at 20°C of FTMAB in solution and adsorbed on a graphite surface. (A) (i): $5 \cdot 10^{-4}$ M FTMAB in phosphate-buffered saline (pH 7.4) solution at a glassy carbon working electrode, no glucose oxidase or glucose present; (ii): as (i) but in the presence of glucose (64 mM) and glucose oxidase ($1 \cdot 10^{-6}$ M), cyclic voltammetry performed 1 min after glucose oxidase/glucose addition; (iii): as (ii) but 10 min after glucose oxidase/glucose addition. (B) FTMAB coadsorbed with glucose oxidase on surface of graphite as described in text. Cyclic voltammetry was performed in phosphate-buffered saline (pH 7.4) buffer both in the absence of glucose (i) and in the presence of glucose (ii) when a 'catalytic wave' was observed.

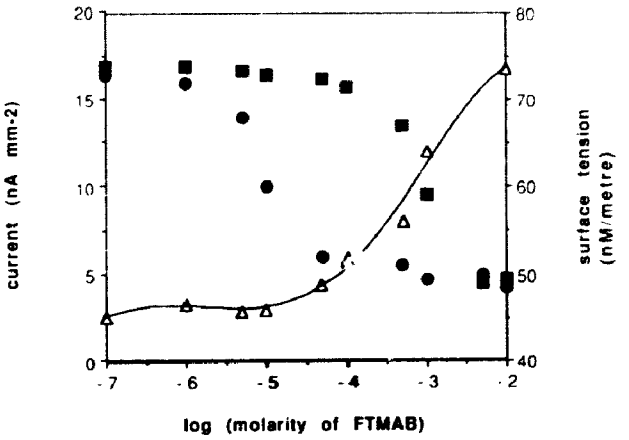

Fig. 3. The relationship of catalytic current to the critical micelle concentration of FTMAB. The catalytic current (△) was recorded at a glassy carbon electrode in phosphate-buffered saline solution (pH 7.4) of glucose oxidase ($1 \cdot 10^{-6}$ M), glucose (100 mM) and FTMAB (from $1 \cdot 10^{-7}$ to 10^{-2} M) at 20°C. The critical micelle concentrations of FTMAB (●) and FTMAB ferricinium ion (■) in phosphate-buffered saline (pH 7.4) buffer were determined by the measurement of surface tension. Temperature: 20°C.

the magnitude of the catalytic current varies with the concentration of glucose in solution (data not shown) as expected.

It was seen that the surface-active properties of FTMAB did not perturb the apparent activity of the glucose oxidase enzyme. No change of activity in a standard assay (see Materials and Methods) was seen following a 1 week incubation of the protein ($1 \cdot 10^{-6}$ M) in an $5 \cdot 10^{-4}$ M FTMAB solution in phosphate-buffered saline (pH 7.4) at room temperature. Equally, a detergent analogous in structure to FTMAB but lacking the ferrocene moiety, dodecyltrimethylammonium bromide (DDTAB), did not affect the observed activity of glucose oxidase under these conditions.

The effect of FTMAB concentration on its mediation of glucose oxidase

To quantify the effect of the concentration of FTMAB on the nature of the catalytic current produced in the presence of glucose, the magnitude of this current was measured in phosphate-buffered saline (pH 7.4) solutions containing a constant amount of glucose oxidase ($1 \cdot 10^{-6}$ M), glucose (200 mM) and varying concentrations of FTMAB (from $1 \cdot 10^{-7}$ to $1 \cdot 10^{-2}$ M). The magnitude of the catalytic current (i_c) at +400 mV was measured from cyclic voltammograms and the results are expressed graphically in Fig. 3. It can be seen that the relationship is not linear as one would expect from a simple effect of amount of FTMAB added. It was observed that FTMAB is indeed active as a mediator below a concentration of 10^{-5} M but that there is a rise in activity with FTMAB concentrations above that value. To complement the electrochemical data, the critical micelle concentrations of both the Fe (II) and (III) forms of FTMAB were determined by measurement of the variation of surface tension with FTMAB concentration (Fig. 3). For the Fe (II) form of FTMAB, a critical micelle concentration of $5 \cdot 10^{-4}$ M was obtained. This concentration corresponds closely to that above which mediating activity of FTMAB increases.

The Fe (III) form was formed by preparative controlled-potential thin layer electrolysis of FTMAB (II) ($1 \cdot 10^{-2}$ M solution in phosphate-buffered saline (pH 7.4)). Ultraviolet-visible absorption peaks at 626 and 546 nm were recorded. This solution, which showed foaming upon stirring or gas bubbling, was then diluted to give a range of concentrations between $1 \cdot 10^{-7}$ and $1 \cdot 10^{-2}$ M. The value for the critical micelle concentration of the Fe (III) form of FTMAB was significantly higher than that for the Fe (II) form, in the order of $1 \cdot 10^{-3}$ M. Therefore, some surface-activity was preserved but it is likely that micelles of the Fe (III) form are not stable at high concentrations due to charge repulsion. Additionally, the solvent will have a greater effect on the critical micelle concentration of the oxidised FTMAB compared to that of the reduced FTMAB and other values may well be determined in buffers of different compositions. The presence of protein ($1 \cdot 10^{-5}$ and $1 \cdot 10^{-6}$ M glucose oxidase, as in the electrochemical experiments) did not significantly change the critical micelle concentration as determined for either oxidation state of FTMAB.

Electrochemistry of co-adsorbed FTMAB and glucose oxidase

An 'enzyme electrode' may be formed by incubation of a piece of graphite in a solution of glucose oxidase and FTMAB as in Materials and Methods. FTMAB exhibits fast, reversible electron transfer when adsorbed (either in the presence or absence of glucose oxidase) on the surface of graphite of low porosity (Fig. 2B(i)) although the features of the cyclic voltammetry wave are somewhat masked by the capacitive current due to charging of the electrode surface itself. The redox potential of the adsorbed FTMAB, as judged from cyclic voltammetry, is approximately +320 mV, representing an increase of 255 mV when compared to that observed for the species in solution at a glassy carbon electrode (see previous section). This indicates that the reduced form of the FTMAB redox couple is preferentially stabilised when the FTMAB is adsorbed on a graphite surface. The positions of the oxidation and reduction peaks are separated by only 40 mV (at < 50 mV s^{-1} scan speed), suggesting that the redox waves correspond to adsorbed species with little mass transfer limitations to heterogenous electron transfer. This was supported by the observation that the peak oxidation current and the peak reduction current were

proportional to the cyclic voltammetry scan speed as one would expect for an adsorbed species [33]. A surface coverage of $2.9 \pm 1) \cdot 10^{-10}$ mol cm^{-2} was calculated (a continuous monolayer of FTMAB would correspond to approximately $1 \cdot 10^{-10}$ mol cm^{-2}). From the protein assay of glucose oxidase desorbed from the electrode surface as in Materials and Methods, a glucose oxidase coverage of $1.5 \cdot 10^{-11}$ mol cm^{-2} was determined. The apparent stoichiometry of adsorption will be discussed below. When the enzyme electrode is employed as the working electrode in a three-electrode system, a 'catalytic wave' is observed in the presence of glucose (and proportional to the glucose concentration), although due to the background capacitive current the wave is manifested as a net shift of the i/V relationship in the direction of oxidation (Fig. 2B(ii)) when compared to the equivalent cyclic voltammetry wave in the absence of glucose (Fig. 2B(i)).

Glucose sensing by glucose oxidase / FTMAB electrodes

Chronoamperometry was used to quantify the magnitude of the catalytic current, registered at a given potential, produced at an enzyme electrode immersed in phosphate-buffered saline (pH 7.4), following addition of aliquots of glucose (using a 1 M stock solution) to give final concentrations in the cellule of, typically, 1, 2, 4, 8, 16, and 32 mM. The working enzyme electrode was held at a potential corresponding to the oxidation peak observed for the redox couple of adsorbed FTMAB to ensure rapid electron transfer; thus with enzyme electrodes all measurements were performed at +350 mV. The trace of a typical chronoamperometric experiment is shown in Fig. 4. Upon addition of aliquots of 1 molar glucose solution in phosphate-buffered saline (pH 7.4), an immediate increase in current was observed with a maximum value being reached after 30 s. The rate of response was in fact limited by the mixing time of solution, as an increased rate of mechanical stirring gave a more rapid response which, however, suffered from more 'noise' when measuring the background current. The response was stable over a number of minutes, except at the higher concentrations of glucose (32 mM and above) where a 'tailing off' was seen after the peak current was reached. The response of identical electrodes prepared in the same incubation solution of FTMAB and glucose oxidase did not differ by more than 5% in their characteristics of response, although this discrepancy was nearer 10% when electrodes from different incubation batches were compared. The response of similarly prepared electrodes in both the presence and absence of saturating nitrogen is displayed (Fig. 5). The presence of saturating air reduced the current densities by some 30% (data not shown), representing the competition of ambient O_2 with FTMAB ferricinium ion for reduced glucose oxidase. However, the shape of the plot of response against glucose concentration, for

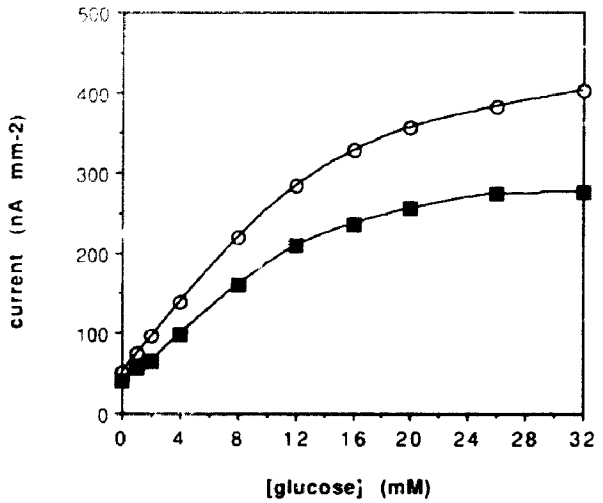

Fig. 5. Graph to show magnitude of chronoamperometric response of glucose oxidase/FTMAB enzyme-electrodes to addition of glucose. Enzyme-electrodes were assayed in phosphate-buffered saline (pH 7.4) buffer in the presence ( ) and absence (■) of saturating nitrogen. Electrodes were held at a constant potential of +350 mV and the steady-state currents were recorded. Temperature: 20°C.

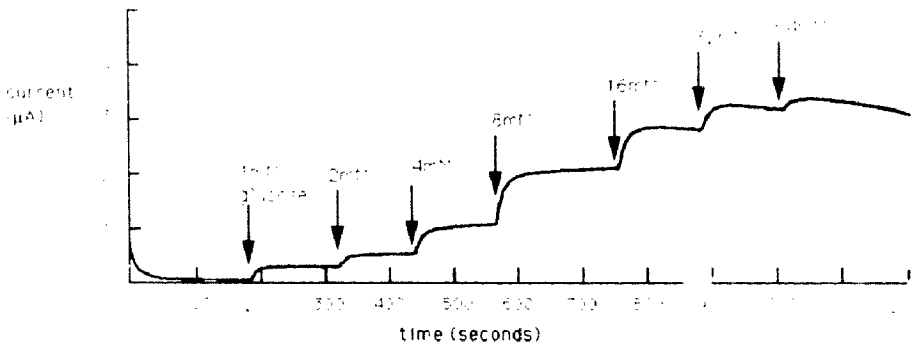

Fig. 4. Chronoamperometric experiment to show response in time of glucose oxidase/FTMAB enzyme-electrode to addition of aliquots of glucose, assayed in phosphate-buffered saline (pH 7.4) buffer. The electrode was prepared by incubation in a glucose oxidase/FTMAB solution as described in Materials and Methods. A constant potential of +350 mV was applied to the electrode. Temperature, 20°C.

example the limit of linearity of response, was similar in both cases. In addition, electrodes incubated with glucose oxidase and FTMAB did not show a significant response to glucose when the FTMAB concentration in the incubation solution was below its critical micelle concentration.

It was also found that electrodes, once incubated in a glucose oxidase/FTMAB solution, could be rinsed, left to dry for hours or days and then rehydrated prior to sensing of glucose. Performance was not significantly diminished. Limited ageing studies on the scale of weeks, using dry electrodes exposed to ambient conditions of temperature, light and air, showed a moderate loss of performance upon rehydration relative to a similar electrode that had not been dried (15% decrease of maximum current with 32 mM glucose, per week stored). Once immersed, activity is lost in a matter of hours through desorption of enzyme and mediator, as with an electrode that is not dried after removal from the glucose oxidase/FTMAB solution.

Preparation of partially deglycosylated glucose oxidase

Glucose oxidase was found to exhibit different electrochemical and physical properties after treatment with the enzyme α-mannosidase. After only 1 h of treatment as described in Materials and Methods, 98% of glucose oxidase originally in solution was macroscopically precipitated and formed feathery aggregates. For incubation times up to 12 h, the apparent glucose oxidase activity of this precipitate (when stirred and fully suspended in the solution) was unchanged relative to the commercial enzyme. After 36 h incubation apparent glucose oxidase activity was still 80% of the pre-incubation activity of the glucose oxidase solution. As monitored by the ratio of flavin absorption at 440 nm to aromatic amino-acid absorption at 280 nm (A_{440}/A_{280} for commercial enzyme = 0.15), the supernatant was enriched in FAD (A_{440}/A_{280} = 0.43) suggesting some loss of FAD (3% of the total present) from its binding site in glucose oxidase during the deglycosylation/precipitation process. However, the α-mannosidase-treated glucose oxidase has an A_{440}/A_{280} ratio of 0.2, a value for which we cannot readily account as it suggests enrichment of FAD or loss of protein.The latter possibility is ruled out by analysis of total protein retrieved after ultrafiltration of the treated sample.

As monitored by denaturing sodium dodecyl sulphate polyacrylamide electrophoresis (Fig. 6), commercial glucose oxidase from *Aspergillus niger* gives rise to one diffuse band (typical for glycoproteins) with an apparent molecular weight of 65–67000 for the single, dissociated subunit as judged against molecular weight standards. After 36 h incubation, a single, slightly sharper band with an apparent molecular weight of 62–65000 was observed (the α-mannosidase itself

Fig. 6. Denaturing SDS-polyacrylamide electrophoresis gel to show the effect of α-mannosidase treatment of *Aspergillus niger* glucose oxidase. Acrylamide concentration, 10%. Key (from left to right): lane 1, molecular weight standards; lane 2, bovine serum albumin; lane 3, ϕ phage; lane 4, commercial glucose oxidase (100 μg loaded); lane 5, glucose oxidase and α-mannosidase after 36 h incubation (see Materials and Methods) (100 μg loaded); lane 6, molecular weight standards; lane 7, commercial glucose oxidase (10 μg loaded). The molecular weight standards were (from top of gel): myosin, 200000 molecular weight, β-galactosidase, 116300; phosphorylase *b*, 97400; ovotransferrin, 76–78000; glutamate dehydrogenase, 55500; ovalbumin, 42700.

shows two bands on the gel, corresponding to molecular weights of approximately 25000 and 40000). This indicates that the protein species is still heterogenous but that the average molecular weight of the glucose oxidase molecules has decreased by approximately 5000. It is suggested that this net decrease in molecular weight is due to partial deglycosylation (5000 molecular weight corresponds to 30% of the total carbohydrate initially present) of the glucose oxidase by α-mannosidase. The precipitates of protein were not dispersed by FTMAB or DDTAB above their respective critical micelle concentrations. SDS, however, in a 5% solution, did disrupt the precipitate: all the protein in a sample entered an SDS-polyacrylamide electrophoresis gel. Commercial glucose oxidase incubated under the same incubation conditions but in the absence of α-mannosidase showed no change in apparent activity, no precipitation/aggregation and no change in the apparent molecular weight of the subunits.

The partially deglycosylated glucose oxidase, obtained by incubation for 36 h with α-mannosidase (following removal of the α-mannosidase by ultrafiltration as in Materials and Methods), was incorporated in enzyme electrodes exactly as with the commercial glucose oxidase.

Glucose sensing by partially-deglycosylated glucose oxidase / FTMAB electrodes

The responses of enzyme electrodes prepared with partially-deglycosylated glucose oxidase in place of commercial glucose oxidase are shown in Fig. 7. As controls, commercial glucose oxidase and FTMAB were incubated with a graphite electrode with the same conditions (pH, ionic strength of buffer, temperature), or combinations thereof, as used in the partial deglycosylation reaction. All electrochemical experiments were performed in phosphate-buffered saline (pH 7.4), that is, only the incubation conditions for the glucose oxidase / FTMAB electrodes were different. It was seen that partially-deglycosylated glucose oxidase / FTMAB electrodes gave current densities greater than commercial glucose oxidase / FTMAB electrodes. The electrochemical responses of glucose oxidase / FTMAB and partially-deglycosylated glucose oxidase / FTMAB electrodes to addition of glucose are compared in Table I. Incubation of electrodes with FTMAB and commercial glucose oxidase in solutions of lower pH and/or low ionic strength compared to phosphate-buffered saline (pH 7.4) did indeed give rise to increases in current densities but these were not as marked as those produced by the use of deglycosylated glucose oxidase (see Fig. 7). In cases where graphite rods were incubated in solutions of the the commercial enzyme with varying buffer compositions, the amount of protein adsorbed was always in the range of $1 \cdot 10^{-11}$ mol cm^{-2}, similar to that for the commercial enzyme in phosphate-buffered saline (pH 7.4). However, a detailed study of the observed activity of the adsorbed commercial enzyme was not carried out. The results serve as controls

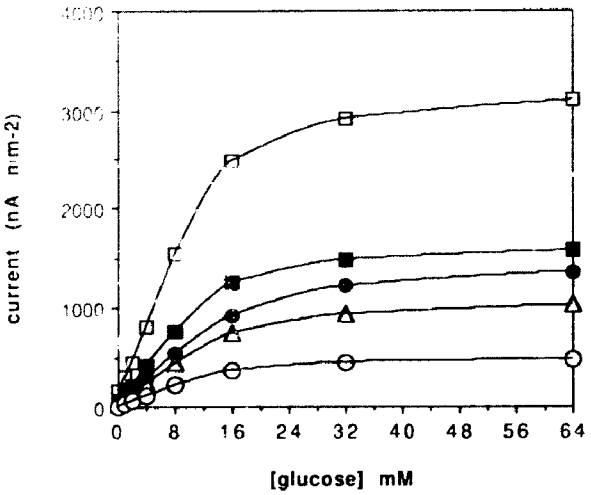

Fig. 7. Graph to show magnitude of amperometric responses of partially deglycosylated and glycosylated glucose oxidase/FTMAB enzyme-electrodes to addition of glucose. Graphite rods were incubated in a phosphate-buffered saline-solution (pH 7.4) of partially deglycosylated glucose oxidase (10 mg/ml) and FTMAB ($5 \cdot 10^{-4}$ M) (△) or in commercial glucose oxidase (10 mg/ml) and FTMAB ($5 \cdot 10^{-4}$ M) solutions of varying buffer composition: 30 mM phosphate (pH 5.0), (■); phosphate-buffered saline, (pH 5.0), (●); 30 mM phosphate (pH 7.4), (△); phosphate-buffered saline (pH 7.4), (○). All measurements were performed identically in phosphate-buffered saline (pH 7.4) buffer, with enzyme-electrodes having been rinsed in that buffer after incubation. The electrodes were held at a potential of +550 mV.

TABLE I

Comparison of physicochemical and electrochemical properties of commercial and partially deglycosylated glucose oxidase from Aspergillus niger

Glucose oxidase was treated with α-mannosidase to obtain a partially deglycosylated enzyme. Molecular weights were obtained by electrophoresis and apparent activity measured by a peroxidase-couple assay. Graphite electrodes were incubated in a phosphate-buffered saline (pH 7.4) solution of enzyme and FTMAB. Cyclic voltammetry and chronoamperometric techniques were used to characterise the mediation of the enzymes by a ferrocene-derivatised detergent, FTMAB. Full experimental details are presented in the Materials and Methods section of the text. Data represent mean ± S.E.

	Commercial glucose oxidase	Partially deglycosylated glucose oxidase
Molecular weight	$1.3 (\pm 0.3) \cdot 10^{5}$	$1.2 (\pm 0.3) \cdot 10^{5}$
Activity in solution (U mg)	50 (± 2)	38 (± 2)
Apparent activity in adsorbed state (U mg)	4 (± 0.5)	17 (± 2)
Amount adsorbed (mol cm^{-2})	$1.55 (\pm 0.1) \cdot 10^{-11}$	$1.05 (\pm 0.1) \cdot 10^{-11}$
Rate constant (k) with FTMAB ($M^{-1} s^{-1}$)	$5 (\pm 0.5) \cdot 10^{3}$	$2 (\pm 0.5) \cdot 10^{5}$
Current density of enzyme-electrode at 10 mM glucose (nA mm^{-2})	260 (± 10)	1750 (± 5)
K_m of enzyme-electrode (mM)[a]	9 (± 1)	8 (± 1)

[a] K_m was defined as that concentration of glucose which gave rise to a current density half that maximally observed with saturating glucose.

to demonstrate that the effect of deglycosylation is the major effect observed.

Discussion

The study of electrochemistry in micelles has received some attention [20,34] and in particular the effects of detergent species on the electrochemistry and mediating action of small, monomeric ferrocenes [18,35] and tetrathiovalene [36] have been thoroughly investigated. Due to coulombic and noncoulombic effects, the electrochemical properties of the surfactant solubilised molecule are frequently modified [37]. By appropriate choice of the detergent 'host', the electrochemical properties of the redox-active compound may be modified and often lead to enhanced rates of electron transfer [19]. The FTMAB molecule possesses redox and detergent properties. The ferrocene group could be envisaged as being buried in the middle of an insulating micellar structure; however, the heterogenous electron transfer of the FTMAB ferrocene with a glassy carbon or graphite remains electrochemically reversible, demonstrating that the dynamics of monomer association/dissociation with the micelle, or the dynamics of the assembled micelle, are sufficiently rapid not to limit heterogenous electron transfer.

It has been previously suggested [30,31] that the FTMAB ferricinium ion does not form a stable micelle. Our findings indicate that following complete electrochemical oxidation of FTMAB to the FTMAB ferricinium ion, the chemical retains some surfactant activity, with a critical micelle concentration of $1 \cdot 10^{-3}$ (against $5 \cdot 10^{-4}$ for the reduced form). Cyclic voltammetry shows that an adsorbed, mixed micelle of FTMAB (II) and glucose oxidase may exist. Even though it is the FTMAB (III) that interacts with the active site of glucose oxidase, we suggest that in solution, a micelle of FTMAB (II) provides a high local concentration of mediator. An increase in the mediating activity of FTMAB in solution is observed above a concentration of approx. $1 \cdot 10^{-5}$ M; this corresponds closely to the critical micelle concentration of the FTMAB (II) suggesting the importance of the micelle. Upon oxidation of FTMAB (II), the (III) form may be ejected from the micelle; this would promote interaction with a nearby molecule of glucose oxidase.

The second-order rate constant (k, $5 \cdot 10^3$ M^{-1} s^{-1}) for FTMAB ferricinium ion reaction with glucose oxidase is of the same order as those constants found for other, non-surfactant ferrocenes [6]. This shows again that the presence of the ferrocene group itself within the micelle (as the redox group is at the opposite 'end' of the molecule from the polar head-group) does not prevent interaction of a given monomer with the active site of glucose oxidase. The rapid association and dissociation of monomers of FTMAB with micelles may allow the transport of the reduced mediator away from the active site of the enzyme.

In the equivalent configuration for a small, monomeric ferrocene such as dimethylferrocene, the mediator is often mixed in a paste of paraffin oil with glucose oxidase, and a graphite powder to ensure electrical conduction of the resulting electrode. The paraffin oil is necessary due to the low solubility of ferrocene in water [18] and serves as a reservoir of mediator due to the high partition coefficient of ferrocene in the oil. In the monomeric ferrocene system, upon oxidation of the ferrocene at the electrode, the ferricinium ion thus formed is soluble and will diffuse to the active site of glucose oxidase where it may accept electrons from the reduced flavin group of glucose oxidase. However, when reduced to the ferrocene, the solubility is poor. In the case of oxidised FTMAB, the solubility of the reduced form of mediator is significant. To further understand the possible advantages conferred by the presence of a micelle, one would wish to investigate the kinetics of electron transfer from glucose oxidase to the oxidised FTMAB with bulk FTMAB concentration below the critical micelle concentration, for comparison. The sensitivity of the cyclic voltammetry-based method does not extend to this low concentration and stop-flow measurements would have to be used.

This model for association and mediation is not necessarily confirmed by the perceived stoichiometry of twenty adsorbed FTMAB to one adsorbed glucose oxidase (mol/mol, $2.9 \cdot 10^{-10}$ and $1.5 \cdot 10^{-11}$ mol cm^{-2}, respectively) which would not instinctively correspond to a complete micelle of FTMAB around glucose oxidase (approximately 100:1, see also Ref. 21). However, two very different assay principles are used to derive these values and it is likely that the electrochemical method for FTMAB, the protein assay and the activity assay for glucose oxidase are likely to probe greatly different populations of the respective molecules: FTMAB at or far from the electrode surface, adsorbed denatured and non-denatured glucose oxidase, etc and the associated errors may be significant. The manner of adsorption of FTMAB/glucose oxidase to surfaces warrants a separate investigation.

The electrochemical response to glucose of enzyme electrodes prepared with partially-deglycosylated glucose oxidase and the determination of the rate constant (k) for electron transfer from partially-deglycosylated glucose oxidase provides further information as to the mechanism of glucose oxidase/FTMAB mediation. Additionally, a number of interesting observations on the role of the polysaccharide chains of glucose oxidase in mediation can be made. It has been demonstrated here that in phosphate-buffered saline (pH 7.4) solutions the apparent activity of partially-deglycosylated glucose oxidase with respect to glucose and O_2 is the same as for commercial glucose oxidase. This

correlates with previous findings. Nakamura and coworkers [38,39] treated glucose oxidase with periodate to oxidase carbohydrate; the 10.5% total carbohydrate by weight was reduced to 5.5%. The activity of glucose oxidase and its gross structure was apparently unchanged by this treatment; the authors suggested that the polysaccharide moiety of glucose oxidase served only to increase the thermal stability to SDS detergent inactivation. In other cases in the literature [40], mention is made of a 'periodate-oxidised enzyme' as having been incorporated into enzyme electrodes but this species appears not to have been rigorously characterised; carboxyl groups were created but the extent of polysaccharide chain loss was not determined. No previous experimenters have remarked the precipitation of partially deglycosylated glucose oxidase or effects on the electrochemistry of the enzyme. It appears that only a minor loss (30%) of polysaccharide is sufficient to modify the electrochemical and physical properties of glucose oxidase.

Following partial deglycosylation of glucose oxidase, the more hydrophobic surface, with less steric interference by the polysaccharide chains, would allow easier access of detergents such as SDS and FTMAB. The increased current densities obtained with partially deglycosylated glucose oxidase/FTMAB electrodes, together with a striking, two orders of magnitude increase in k for FTMAB and partially-deglycosylated glucose oxidase, indicate that the surface conformation of glucose oxidase, as approximated by the amount and nature of the polysaccharide chains attached there, has a major influence on the interaction of FTMAB with the active site of the enzyme. Incubation of commercial glucose oxidase in buffers of pH 5 and low ionic strength (30 mM phosphate), the buffer used for the deglycosylation reaction with α-mannosidase, also increased the current densities produced by the resulting enzyme electrode, indicating variation in the apparent activity of the adsorbed protein. However, none of these circumstances had such a marked effect as partial deglycosylation of the enzyme.

Removal of the polysaccharide, or a portion thereof, would tend to increase the hydrophobic character of the glucose oxidase molecule. The observed precipitation suggests that contact between revealed, hydrophobic regions of different protein molecules leads to aggregation. The apparent glucose oxidase activity is higher in partially-deglycosylated glucose oxidase than in the commercial enzyme but only in the adsorbed state (Table I). Both the commercial enzyme and partially-deglycosylated glucose oxidase are less active when adsorbed but the partially-deglycosylated glucose oxidase aggregates retain more apparent activity following the process of adsorption. It is generally accepted that when proteins adsorb to a solid surface, they change their secondary, tertiary and quaternary structures, which may in turn lead to a change in biological activity [41]. The partially-deglycosylated glucose oxidase aggregates may be more resistant to these further structural changes induced by adsorption.

Our conclusions complement those made by Bourdillon and Madja [21]. These authors, using a surfactant molecule with the ferrocene moiety at the hydrophilic, positively charged headgroup, concluded that approximately 100 surfactant molecules aggregate around the entire glycoprotein shell of the glucose oxidase molecule, a number greater than one would expect from purely electrostatic considerations. They speculated that 'electrostatic and hydrophobic forces could thus be involved as well as intercalation' of the surfactant molecule into the protein structure. From our results, we see that the apparent activity of glucose oxidase or partially-deglycosylated glucose oxidase is unchanged by prolonged incubation in a solution (20 mM) of FTMAB (and equally DDTAB), demonstrating that the protein is not functionally perturbed by the presence and possible intercalation of such detergents. However, the deactivation of glucose oxidase by hexadecyltrimethylammonium bromide (HTAB) has been reported [42]; we suggest that the C_{16} acyl chain of HTAB denatures the enzyme readily compared to the C_{12} chain of DDTAB. We note that FTMAB and DDTAB do not disperse aggregates of partially-deglycosylated glucose oxidase whereas SDS does.

The formation of a surface adsorbed mixed micelle of glucose oxidase/FTMAB is indicated. Partial deglycosylation of glucose oxidase leads to more efficient mediation by FTMAB; we also suggest that the use of deglycosylated enzymes in biosensors may improve performance.

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References

1 Barker, S.A. and Shirley, J.A. (1980) In: Economic Microbiology (Burdon, R.H. and van Knippenberg, P.H., eds.) Vol. 5, pp. 455–465, Academic Press, New York.
2 O'Malley, J.J. and Weaver, J. (1972) Biochemistry 11, 3527–3532.
3 Weibel, M.K. and Bright, H.J. (1971) J. Biol. Chem. 246(9), 2734–2744.
4 Fisher, L., Spencer, R. and Walsh, C. (1976) Biochemistry 15(5), 1054–1064.
5 Janata, J. (1990) Anal. Chem. 62, 33R–44R.
6 Cass, A.E.G., Davis, G., Francis, G.D. and Hill, H.A.O. (1984) Anal. Chem. 56, 667–671.

7 Degani, Y. and Heller, A. (1987) J. Am. Chem. Soc. 91, 1285–1289.
8 Degani, Y. and Heller, A. (1988) J. Am. Chem. Soc. 110, 2615–2620.
9 Foulds, N.C. and Lowe, C.R. (1988) Anal. Chem. 60, 2473–2478.
10 Hale, P.D., Inagaki, T., Karan, H.I., Okamoto, Y. and Skotheim, T.A., (1989) J. Am. Chem. Soc. 111, 3482–3484.
11 Albery, W.J., Bartlett, D.N. and Craston, D.H. (1988) J. Electroanal. Chem. Interfacial Electrochem. 194, 223–235.
12 Gregg, B.A. and Heller, A. (1990) Anal. Chem. 62, 258–263.
13 Taniguchi, I., Miyamoto, S., Tomimura, S. and Hawkridge, F. (1988) J. Electroanal. Chem. 240, 333–339.
14 Crumbliss, A.L., Hill, H.A.O. and Page, D.J. (1986) J. Electroanal. Chem. 206, 327–331.
15 Taniguchi, I., Matsushita, K., Okamoto, M., Collin, J.-P. and Sauvage, J.-P. (1990) J. Electroanal. Chem. 280, 221–226.
16 Kulys, J.J. and Cénas, N.K. (1983) Biochim. Biophys. Acta 744, 57–63.
17 Frew, J.E. and Hill, H.A.O. (1987) Phil. Trans. R. Soc. Lond. B 316, 95–106.
18 Georges, J. and Desmettre, S. (1984) Electrochimica Acta 29, 521–525.
19 Yeh, P. and Kuwana, T. (1976) J. Electroanal. Chem. 123, 1334–1339.
20 McIntire, G.L. (1986) Am. Lab. 173–180.
21 Bourdillon, C. and Madja, M. (1990) J. Am. Chem. Soc. 112, 1795–1799.
22 Loffler, U., Wiemhofer, H.-D. and Gopel, W. (1990) Biosensors '90 Proceedings, pp. 161–162 Elsevier Amsterdam.
23 Nicholson, R.S. and Shain, I. (1964) Anal. Chem. 76, 706–723.
24 Bergmeyer, H.U. (1983) Methods of Enzymatic Analysis, 3rd ed., Vol II, pp. 267–273, Verlag Chemie, Weinheim.
25 Salecouddin, M., Ahmed, H. and Hosain, A. (1986) Appl. Biochem. 8, 761–767.
26 Kalisz, H.M., Hecht, H.-L., Schmiberg, D. and Schomburg, R.D. (1990) J. Mol. Biol. 213, 207–209.
27 Laemmli, U.K. (1970) Nature 227, 680–685.
28 Sato, T., Hoshino, K. and Aoyagui, S. (1985) J. Am. Chem. Soc. 107, 6865–6868.
29 Sato, T., Hoshino, K. and Aoyagui, S. (1985) J. Chem. Soc. Commun. 865–866.
30 Hoshino, K. and Sato, T. (1987) Chem. Lett. Chem. Soc. Jpn. 1439–1442.
31 Hoshino, K. and Sato, T. (1987) J. Am. Chem. Soc. 109, 5885–5883.
32 Hoshino, K. and Sato, T. (1988) Chem. Lett. Chem. Soc. Jpn. 693–696.
33 Bard, A.J. and Faulkner, L.R. (1980) Electrochemical Methods, Chap. 6, John Wiley and Sons, New York.
34 McIntire, G.L. (1990) Anal. Chem. 61, 25A–27A.
35 Ohsawa, Y. and Aoyagui, S. (1982) J. Electroanal. Chem. 136, 253–268.
36 Eddowes, M.J. and Gratzel, M. (1983) J. Electroanal. Chem. Interfacial Electrochem. 163, 31–44.
37 Kamau, G.N., Leipert, T., Shukla, S.S. and Rusling, J.F. (1987) J. Electroanal. Chem. 233, 173–187.
38 Nakamura, S. and Hayashi, S. (1974) FEBS Lett. 44(2), 327–330.
39 Nakamura, S., Hayashi, S. and Koga, K. (1976) Biochim. Biophys. Acta 445, 294–308.
40 Brooks, S.L., Ashby, R.L., Turner, A.P.F., Calder, M.R. and Clarke, D.J. (1987) Biosensors 3, 45–56.
41 Andrade, J.D. and Hlady, V. (1986) Adv. Polymer Sci. 79, 1–63.
42 Tsuge, H., Suzuki, M., Kito, N., Nakanishi, Y., Ohashi, K. and Aoki, K. (1984) Agric. Biol. Chem. 48, 19–28.