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ELECTROCHEMICAL SENSORS FOR DIRECT REAGENTLESS MEASUREMENT OF SUPEROXIDE PRODUCTION BY HUMAN NEUTROPHILS

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Electrochemical sensors based on immobilised cytochrome *c* or superoxide dismutase for the measurement of superoxide radical production by stimulated neutrophils are described. Cytochrome *c* was immobilised covalently at a surface-modified gold electrode and by passive adsorption to novel platinised activated carbon electrodes (PACE). The reoxidation of cytochrome *c* at the electrode surface upon reduction by superoxide was monitored using both xanthine/xanthine oxidase and stimulated neutrophils as sources of the free radical. In addition, bovine Cu/Zn superoxide dismutase was immobilised to PACE by passive adsorption and superoxide, generated by xanthine/xanthine oxidase, detected by oxidation of hydrogen peroxide produced by the enzymic dismutation of the superoxide radical. A biopsy needle probe electrode based on cytochrome *c* immobilised at PACE and suitable for continuous monitoring of free radical production was constructed and characterised.

KEY WORDS: Electrochemical sensors, superoxide, immobilised cytochrome *c*, superoxide dismutase, modified electrode, activated carbon, neutrophils, free radicals.

INTRODUCTION

Reactive oxygen-derived species, particularly the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), act as mediators which perpetuate inflammation in disease states.[1] These species are released during the respiratory burst of neutrophils[2] and are implicated in the pathogenesis of post-ischaemic tissue injury and rheumatoid arthritis.

In a previous paper, we described an electrochemical method of measuring O_2^- production by neutrophils based on the reversible electrochemistry of cytochrome *c* in solution at a surface-modified gold electrode.[3] This simple, rapid and sensitive technique was used to detect O_2^- production in neutrophil enriched plasma samples.

This paper describes the development of reagentless sensor systems which could be applied to continuous monitoring for direct measurement of free-radical activity *in vivo*. This could enable tissue damage to be attributed causally to radical production.

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At present O_2^- activity can only be inferred from measurement of presumed end-products *in vitro*.

A method of covalent binding of cytochrome *c* to a surface-modified gold electrode has been employed.[4] In addition, a novel platinised carbon electrode material (PACE) was used for passive adsorption of cytochrome *c*. This electrode material has previously been shown to bind enzymes and proteins efficiently.[5] The reaction of O_2^- with cytochrome *c* immobilised on gold and PACE was investigated electrochemically. Cytochrome *c* reduced by O_2^- was reoxidised at the electrode surfaces via direct electron transfer[4] by poising the electrodes at an oxidising potential sufficiently positive of the cytochrome *c* formal potential, resulting in the generation of an oxidation current which was proportional to the O_2^- concentration.

$$\text{cyt c (III)} + O_2^- \longrightarrow \text{cyt c (II)} + O_2$$

$$\text{cyt c (II)} \xrightarrow{\text{electrode}} \text{cyt c (III)} + e^-$$

A simple PACE-based sensor incorporating immobilised cytochrome *c* has been constructed within a 2.6 mm internal diameter biopsy needle. This system was used successfully to measure O_2^- production by stimulated human neutrophils and may be suitable for continuous *in vivo* monitoring.

In a further configuration we have investigated an enzyme electrode system based on the immobilisation of superoxide dismutase (SOD) on PACE. SOD catalyses the conversion of O_2^- to H_2O_2 at a rate close to diffusion control:[6]

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

O_2^- can then be estimated by oxidation of H_2O_2 by PACE at +320 mV:[7]

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

MATERIALS AND METHODS

Electrochemistry

A 1 ml volume electrochemical cell, machined from perspex, contained a 1.5 mm diameter gold working electrode set in a slight recess, a 1.5 mm diameter platinum auxiliary electrode, and a 1.5 mm diameter Ag/AgCl reference electrode. During current vs time measurements, the cell contents were stirred using a Rank Brothers electronic stirrer and magnetic follower. A low-noise, battery-operated bipotentiostat, constructed by the Electronics Workshop, University of Newcastle upon Tyne, was used to polarise the electrodes and the current responses were recorded on a Gould BS-271 chart recorder. Platinised activated carbon (PACE) electrodes were obtained from Cambridge Life Sciences plc.

A 2.6 mm internal diameter stainless steel biopsy needle (Shrimpton and Fletcher) was used to construct a prototype small probe electrode (Figure 1). This was manufactured by insulating the inside of the needle using a PTFE sleeve and placing a titanium rod contact within the needle such that a 0.5 mm recess, suitable for incorporation of 1.5 mm diameter PACE discs in a push-fit arrangement, was produced. A stainless steel contact rod was attached to the titanium rod for electrical connection. The outer barrel of the biopsy needle was used as a combined auxiliary and pseudo-reference electrode.[8]

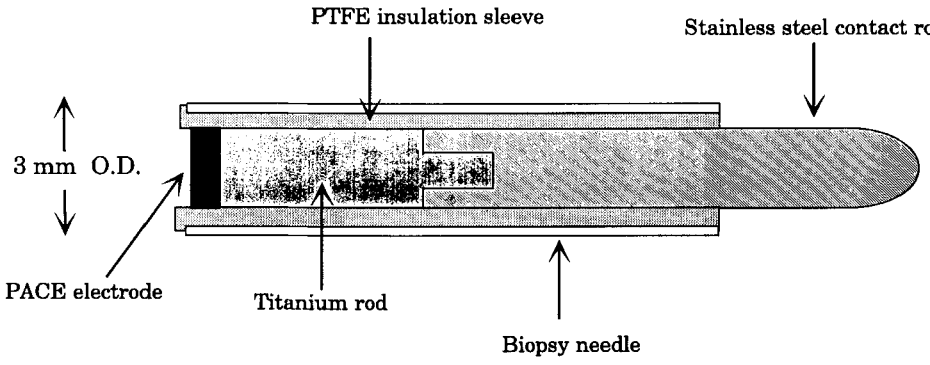

FIGURE 1 Schematic diagram of the biopsy needle electrode incorporating PACE-cyt *c*.

Immobilisation of Cytochrome c on Gold Electrodes

The surface of the gold working electrode was modified by immersion in a 10 mM solution of N-acetyl cysteine (Sigma) for 1 minute at room temperature followed by copious rinsing with distilled water. Cytochrome *c* (type VI, Sigma) was then immobilised at the electrode surface via peptide coupling using the following procedure: a 20% solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, Sigma) was prepared in distilled water and the surface-modified electrode immersed in this solution for 10–20 minutes at room temperature. Thereafter, the activated electrode was placed in a 2 mM solution of cytochrome *c* in phosphate buffer, pH 5.5, at 5°C for approximately 20 hours to complete the immobilisation. The electrode was then rinsed with distilled water and stored at 5°C in phosphate buffered saline, (PBS), pH 7.4. Physical studies of these electrodes have been reported elsewhere.[4]

The prepared electrode was polarised at +25 mV versus Ag/AgCl and the rate of change of current as a function of concentration of enzymically generated O_2^- was measured.

Immobilisation of Cytochrome c on PACE

A $1cm^2$ piece of PACE was immersed in a 10 mg/ml solution of cytochrome *c* in PBS and shaken gently on a Dynatech microtitre plate shaker for 5 hours. The cytochrome *c* solution was then replaced by fresh solution and shaking continued for a further 5 hours. After this time the PACE incorporating adsorbed cytochrome *c* (PACE-cyt *c*) was rinsed thoroughly and stored in PBS at 5°C.

To assemble the electrochemical cell for use with PACE-cyt *c*, a drop of 5% (w/v) hydroxyethyl cellulose gel (Fluka Chemicals Ltd) in PBS was placed on the clean gold working electrode to effect an efficient electrical connection. Thereafter, a 1.5 mm diameter disc of PACE-cyt *c*, cut with a hole punch, was placed in the recess formed by the gold contact to give an interference fit. A few drops of PBS was then applied to the electrode and a piece of polycarbonate membrane (PCM, Nuclepore, 0.05 μm pore size, Costar Ltd) placed over the electrode holder and held in place by an 'O' ring. The electrode was polarised at +25 mV vs Ag/AgCl and the rate of change of current as a function of enzymically generated O_2^- was measured.

Immobilisation of Superoxide Dismutase on PACE

Bovine Cu/Zn superoxide dismutase (SOD), a gift from Grünenthal GmbH, was immobilised on PACE using an identical method to that described for immobilisation of cytochrome *c*. A 1.5 mm diameter disc of PACE-SOD was placed in the electrochemical cell and a PCM applied as described previously. A polarizing potential of +320 mV versus Ag/AgCl was used for estimation of H_2O_2 produced by the enzymic dismutation of O_2^-:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{electrode}} O_2 + 2H^+ + 2e^-$$

There are two sources of H_2O_2 and hence of current in this system: that from the SOD reaction and that from the Xa/XOD reaction. It was therefore necessary to subtract from the combined current that due to the Xa/XOD reaction alone. This was measured and eliminated using a second electrode consisting of PACE with immobilised bovine serum albumin[9] (BSA, Fraction V, Sigma) in conjunction with the bipotentiostat poised at +320 mV versus Ag/AgCl.

Generation of O_2^- by Xanthine/Xanthine Oxidase

The rate of enzymic generation of O_2^- at room temperature was measured in a 20 mM sodium phosphate buffer solution, containing 150 mM sodium chloride (BDH) and 100 mM sodium perchlorate (Sigma). O_2^- was generated as an intermediate during the oxidation of xanthine (Xa) by xanthine oxidase (XOD, Genzyme):

$$Xa + O_2 + H_2O \rightarrow \text{Uric acid} + H_2O_2$$

A stock solution of 15 mM xanthine (Sigma) was prepared in 0.1 M potassium hydroxide (Sigma) and added to the electrochemical cell containing phosphate buffer to give a final concentration of 0.5 mM. When a stable baseline response had been obtained (ca 5 min), XOD was added to give final enzyme concentrations over the range 0.06–0.8 μM. The rate of current production during the re-oxidation of reduced cytochrome *c* was measured as a function of XOD concentration. In addition, spectrophotometric measurements of the rate of cytochrome *c* reduction with increasing XOD concentration were carried out at 550 nm.[3]

Measurement of O_2^- Produced by Human Neutrophils

Polymorphonuclear leukocytes were prepared from freshly collected venous blood using heparin (12.5 units/ml) as anticoagulant as described previously.[3] The cells isolated were 80% neutrophils and were greater than 95% viable, as judged by trypan blue exclusion. Cell numbers were estimated using a haemocytometer.

Differing numbers of resuspended neutrophils in Hank's Balanced Salt Solution (HBSS) were added to the electrochemical cell containing 5 mM glucose in HBBS such that the final volume was always 1 ml. After a steady background current had been obtained, the respiratory burst was elicited by addition of 2 μl of phorbol 12-myristate 13-acetate (PMA, Sigma) diluted in AnalaR dimethyl sulphoxide (BDH) to a concentration of 0.2 mg/ml. The rate of change of current using each PACE configuration was recorded.

RESULTS AND DISCUSSION

We have shown previously that cytochrome *c* electrochemistry in solution can be used to measure specifically O_2^- produced by human neutrophils and that this method correlates well with spectrophotometric measurements of O_2^- production.[3] The major advantage of electrochemical methods is that they can operate in undiluted biological samples without the need for extensive sample pretreatment to remove spectral interferences. However, one disadvantage with the previous method was the requirement for soluble cytochrome *c* to be added to the biological sample. In the context of producing stable, essentially reagentless sensors suitable for reusable O_2^- measurement with the capability for continuous monitoring of O_2^- production, we have investigated immobilisation of cytochrome *c* at different electrode surfaces and have assessed the relative performance characteristics of these systems. In addition, we have investigated the use of the highly specific enzyme SOD immobilised at PACE for the measurement of H_2O_2 produced by the dismutation of O_2^-.

Comparison of Electrode Configurations using Enzymically Generated O_2^-

The electrochemical characteristics of cytochrome *c* immobilised at an N-acetyl cysteine modified gold electrode have been described in detail elsewhere.[4] Figure 2a shows a calibration curve for O_2^- production by Xa/XOD using such a surface-modified 1.5 mm diameter gold electrode. A linear relationship between the initial rate of change of current ($nA\ min^{-1}$), due to reoxidation of O_2^- reduced cytochrome *c*, and the concentration of XOD (μM) was observed ($y = 36.3x + 2.6$, $r = 0.99$, $n = 6$). The sensitivity of this system, defined as the rate of change in current density per unit concentration of XOD, was calculated as 2.05 $\mu A\ cm^{-2}\ min^{-1}\ \mu M^{-1}$.

A similar calibration curve for cytochrome *c* immobilised by passive adsorption to PACE (Figure 2b) also produced a linear response over the range of XOD concentrations used ($y = 601x + 14.7$, $r = 0.99$, $n = 6$), however the sensitivity obtained was greatly increased: 34.0 $\mu A\ cm^{-2}\ min^{-1}\ \mu M^{-1}$. This increase in sensitivity is thought to arise from the much larger effective surface area of PACE compared with a planar gold electrode. PACE is an extremely porous material[5] capable of binding much larger amounts of cytochrome *c* than a gold electrode of the same diameter covered by a monolayer of immobilised N-acetyl cysteine[4] to which cytochrome *c* is subsequently bound covalently.

Due to the highly effective nature of enzyme sensors constructed using a combination of PACE and peroxide producing enzymes,[5] it was decided to investigate immobilisation of SOD at PACE for the direct enzymic determination of O_2^- produced by Xa/XOD. This was carried out using the dual electrode system described earlier to eliminate the electrochemical response due to H_2O_2 produced directly by the Xa/XOD reaction. A calibration curve for SOD immobilised on PACE also showed a linear relationship between the initial rate of change of current and the XOD concentration (Figure 2c, $y = 347x - 14.2$, $r = 0.99$, $n = 6$). The sensitivity of this measurement system was 19.6 $\mu A\ cm^{-2}\ min^{-1}\ \mu M^{-1}$.

Thus, of the 3 electrode configurations described, the PACE-cyt *c* electrode was the most sensitive for detection of enzymically generated O_2^- and was therefore considered to be the most suitable for use in combination with stimulated human neutrophils. Indeed, further characterisation of this system demonstrated good reproducibility of measurement. When tested repeatedly ($n = 6$) with 1.0 μM XOD,

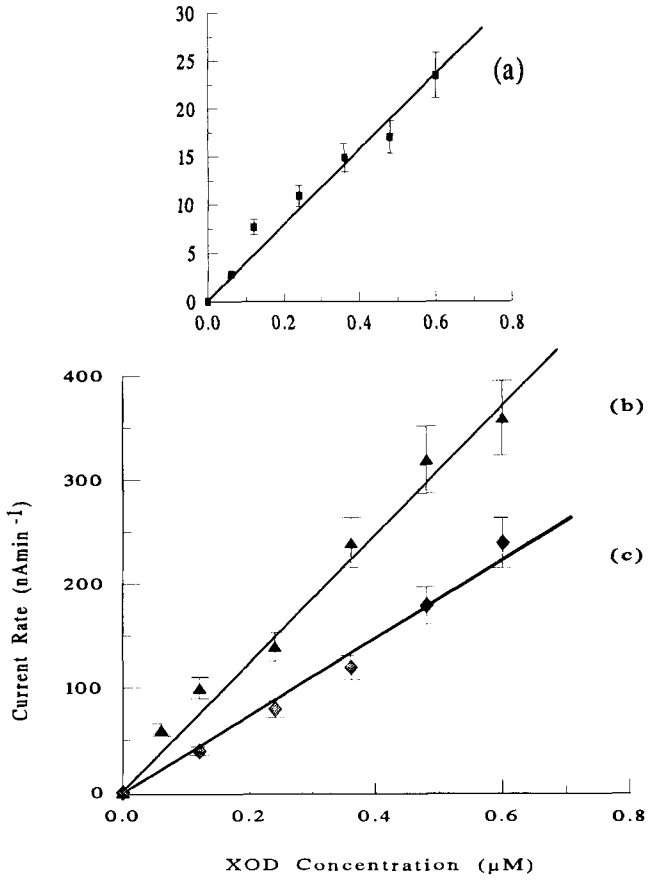

FIGURE 2 Electrochemical measurement of O_2^- production as a function of XOD concentration using the enzymic generating system. In all cases each point represents the mean (± SD) of triplicate measurements. (a) Cytochrome *c* immobilised covalently on an N-acetyl cysteine modified gold electrode. Measurement carried out at +25 mV vs Ag/AgCl. (b) Cytochrome *c* immobilised by passive adsorption on PACE. Measurement carried out at +25 mV vs Ag/AgCl. (c) Superoxide dismutase immobilised by passive adsorption on PACE. Measurement carried out by oxidation of enzymically produced H_2O_2 at +320 mV vs Ag/AgCl.

a coefficient of variation of 8.6% was obtained. In addition, the current response was totally inhibited upon the addition of SOD (1.6 mg/ml, specific activity 3300 U mg^{-1}) showing the specificity of the electrochemical technique for O_2^-. PACE-cyt *c* electrodes were stable for 3 months when stored at 5°C in PBS.

In order to assess the utility of a SOD enzyme electrode for potential *in vivo* monitoring, the PACE-SOD electrode was also tested with stimulated human neutrophils.

Electrochemical Measurement of O_2^- Produced by Stimulated Neutrophils

Figure 3 shows examples of typical response curves using PACE-cyt *c* towards O_2^- produced by increasing numbers of stimulated human neutrophils from different

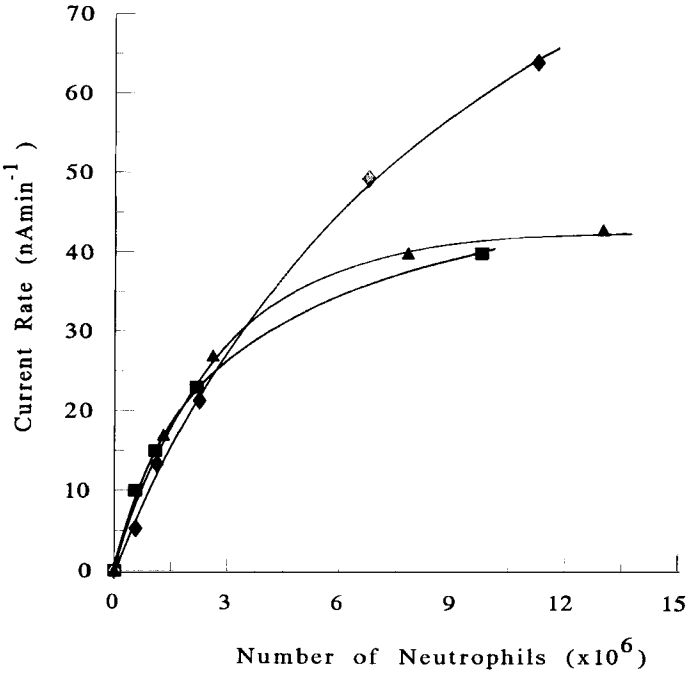

FIGURE 3 Electrochemical measurement, using PACE-cyt *c* in the perspex cell, of O_2^- generated by isolated, stimulated human neutrophils from 3 different donors. The cells were stimulated at room temperature by the addition of 0.4 μg ml^{-1} PMA.

donors. Although the rate of current production varied directly with the number of neutrophils, the response curve was not linear, showing saturation behaviour at higher neutrophil numbers. As can be seen in Figure 3, the magnitude of the response was not solely dependent on the number of neutrophils tested, since equal numbers of neutrophils obtained from different donors gave different responses. These observations can probably be explained as a feature of biological variation within the donor population in the degree and magnitude of neutrophil activation by PMA. The reproducibility of measurement (n = 4) for one particular donor using 11 $\times$ 10^6 neutrophils was of the order of 5% (mean current rate = 76 $\pm$ 2.3 nA min^{-1}).

The PACE-SOD electrode, although successful with the Xa/XOD enzymic O_2^- generating system, gave no current response to stimulated neutrophils. This anomalous result may be due to the very low flux of H_2O_2 at the electrode surface due to the extremely high dismutation rate constant of the enzyme (2×10^9 M^{-1} s^{-1}),[6] the efficient oxidising characteristics of the electrode surface destroying H_2O_2 before a detectable concentration (ca 1 μM) can accumulate.

Biopsy Needle O_2^- Electrode

A biopsy needle electrode was constructed in a manner suitable for simple interchangeable incorporation of 1.5 mm diameter discs of PACE-cyt *c* (Figure 1). This electrode configuration was considered a prototype useful for direct *in vivo* measurement of free radical activity *in situ* without the need to add extraneous cell

stimuli. All of the materials used in the manufacture of this system have been shown in other studies to be biocompatible. Preliminary *in vitro* studies with this electrode configuration have demonstrated that, with the simple instrumentation used, measurable currents from O_2^- production by isolated human neutrophils can be detected from at least 1.4×10^5 cells. It is intended to use this electrode further for determination of the presence of O_2^- in samples from sites of reperfusion injury, as a prelude to the development of an *in vivo* probe for O_2^- measurement.

CONCLUSION

The 3 electrochemical sensors described, based on immobilised redox proteins and enzymes, were specific for measurement of the superoxide anion, and the oxidation current $i \propto [XOD] \propto [O_2^-]$. The PACE-cyt *c* system demonstrated the greatest sensitivity and showed acceptable reproducibility.

The PACE-cyt *c* electrode was used successfully for the direct reusable, reagentless measurement of O_2^- produced by stimulated human neutrophils, thereby demonstrating a simple, rapid, sensing system. These qualities should make this sensor configuration ideal for adaptation towards continuous *in vivo* monitoring of O_2^- production in certain disease states. Initial studies towards an implantable sensor have shown promise and further work is in progress.

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References

1. J.M. McCord and R.S. Roy (1982) The pathophysiology of superoxide: Roles in inflammation and ischemia. *Canadian Journal of Physiology and Pharmacology*, **60**, 1346–1352.
2. J.A. Badwey, J.T. Curnette and M.L. Karnovsky (1979) The enzyme of granulocytes that produces superoxide and peroxide. *New England Journal of Medicine*, **300**, 1157–1160.
3. C.J. McNeil, K.A. Smith, P. Bellavite and J.V. Bannister (1989) Application of the electrochemistry of cytochrome *c* to the measurement of superoxide radical production. *Free Radical Research Communications*, **7**, 89–96.
4. J.M. Cooper, K.R. Greenough and C.J. McNeil (1992) Direct electron transfer reactions between immobilised cytochrome *c* and modified gold electrodes. *Journal of Electroanalytical Chemistry*, in press.
5. H.P. Bennetto, G.M. Delaney, J.R. Mason, C.F. Thurston, J.L. Stirling and D.R. DeKeyzer (1987) Immobilised enzyme electrodes. *International Patent*, WO87/07295.
6. J.M. McCord, J.D. Crapo and I. Fridovich (1977) Superoxide dismutase assays: A review of methodology. In Superoxide and Superoxide Dismutases (A.M. Michelson, J.M. McCord and I. Fridovich, eds.). Academic Press, London, New York, San Francisco, pp. 11–17.
7. J.M. Cooper, C.J. McNeil and J.A. Spoors (1991) Amperometric enzyme electrode for the determination of aspartate aminotransferase and alanine aminotransferase in serum. *Analytica Chimica Acta*, **245**, 57–62.
8. S.J. Churchouse, W.H. Mullen, F.H. Keedy, C.M. Battersby and P.M. Vadgama (1986) Studies on needle glucose electrodes. *Analytical Proceedings*, **23**, 146–148.
9. C.J. McNeil, J.A. Spoors, J.M. Cooper, K.G.M.M. Alberti and W.H. Mullen (1990) Amperometric biosensor for rapid measurement of 3-hydroxybutyrate in undiluted whole blood and plasma. *Analytica Chimica Acta*, **237**, 99–105.

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