A novel *in vivo* nitric oxide sensor

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Graphite–epoxy electrodes modified with *N,N'-o*-phenylenebis(salicylidineiminato)iron(III) are shown to be capable of detecting nitric oxide *in vivo*.

Nitric oxide is a molecule with diverse biological roles but is difficult to detect *in vivo*. Several amperometric nitric oxide sensors have been developed which involve the oxidation of NO on carbon,[1,2] platinum[3] and gold.[4] However a large oxidising potential is required for the oxidation of NO which hinders selectivity *in vivo*, since any substance that is electroactive cathodic to this overpotential can cause interference. Selectivity can be improved by using a gas permeable or anionic membrane,[1–4] by chemical modification or both.[5] Electrocatalysis can reduce the overpotential and increase the selectivity towards NO. A NO electrode consisting of carbon fibre electrode modified with a polymeric nickel porphyrin compound has been reported by Malinski and Taha,[5] however, a robust easily prepared and reproducible probe suitable for tissue and single cell measurements has yet to be developed.

Graphite–epoxy electrodes were modified with *N,N'-o*-phenylenebis(salicylidineiminato)iron(III) ($[Fe^{III}(salphen)]^+$) and Nafion™; salphen was prepared by condensing 2 equiv. of salicylaldehyde with 1 equiv. of *o*-phenylenediamine in ethanol. The $[Fe^{II}(salphen)]$ complex was prepared by stoichiometric addition of an ethanolic solution of $FeCl_2{\cdot}4H_2O$ to salphen which had been dissolved in refluxing ethanol–acetone (20 : 3). The presence of atmospheric oxygen oxidises the $[Fe^{II}(salphen)]$ to $[Fe^{III}(salphen)]^+$.[6] Complexes of salphen are known to form stable nitroso and nitrosyl compounds and have already shown electrocatalytic behaviour towards small molecules such as CO_2.[7] Furthermore, a similar Schiff-base complex has shown electrocatalytic properties to oxygen.[8]

$[Fe^{III}(salphen)]^+$ (5% *m/m*) and graphite powder (ALFA, Ultra "F" Purity) were mixed by slightly warming the modified graphite mixture in the presence of a minimum amount of chloroform to obtain a homogeneous composition of paste. The ratios of graphite : epoxy resin (Ciba-Geigy) used were 1 : 1, 1 : 2 and 3 : 2 (*m/m*). The ratio 3 : 2 (*m/m*) has been reported to be the optimum ratio for minimising double-layer capacitance without introducing excessive resistance.[9] The resulting paste was packed into nylon injection canullae (0.6 mm and 1.0 mm o.d.). Electrical contacts were made with silver wire (125 μm). The electrode tips were cut and polished by hand using decreasing grain sizes (1.0–0.1 μm) of diamond paste. The electrodes were sonicated in reverse osmosis water after each polishing step to remove embedded abrasive.

Some electrodes were dip-coated with two coats of 0.5% Nafion™ diluted with propan-2-ol and water (1 : 1).[10] The first coat was left to dry for 30 min before the second coat was applied. The electrodes are inverted, left to air-dry overnight and then cured at 70 °C for 1 h. Thermal curing of solvent-cast Nafion™ is known to decrease the solubility and increase the crystallinity of the polymer, decrease its permeability to anions and neutral species and increase the biocompatibility.[10,11]

Potentiodynamic measurements were carried out on a three-electrode system. The counter and reference electrodes were platinum flag and home-made Ag/AgCl electrodes[12] respectively, with all potentials referred to this reference electrode. A custom built potentiostat was employed.[13] Digital control and data acquisition were achieved using an Opus PC386SX, Workbench software and WorkMate interface board.

The modified electrodes were activated by potential cycling in 0.5 mol dm^{-3} NaOH at 5 V s^{-1} between 0.6 and 2.0 V for 30 min[14] followed by 30 min each in 0.1 mol dm^{-3} H_2SO_4 and 0.1 mol dm^{-3} phosphate buffered saline (PBS) pH 7.4 at 1 V s^{-1} between −0.5 and +1.5 V.[15,16]

All experiments and calibration data used authentic aqueous solutions of NO prepared using a modification of methods published by Feelisch.[17] Nitrogen was purified before use by scrubbing with NH_4VO_3 (4% *m/v*) in 1 mol dm^{-3} H_2SO_4 and zinc amalgam to remove traces of oxygen. The empty gas collection bottle was flushed with nitrogen for 10 min. NO solutions were made up in reverse-osmosis water as suspended matter in water dramatically reduces the lifetime of NO in solution. The reverse-osmosis water was deoxygenated by bubbling with N_2 for at least 30 min. Commercially available NO (Aldrich) was scrubbed with solid KOH and 10% KOH in water before dissolution in the deoxygenated water. The resulting solutions were calculated to be 1.93 mmol dm^{-3} in NO at 25 °C.[18] Calibration solutions were obtained by adding aliquots of NO solution to nitrogen-blanketed, deoxygenated PBS (pH 7.4) and were found to be stable for at least 1 h.

The electrochemical behaviour was characterised using cyclic voltammetry. The $[Fe^{III}(salphen)]^+$ produced a quasi-reversible voltammogram, in 0.1 mol dm^{-3} H_2SO_4, at a scan rate of 1 V s^{-1}. The anodic and cathodic peak currents, at 0.76 and 0.20 V respectively, were proportional to the scan rate indicating successful electrode modification.[19] A NO oxidation peak occurred when aliquots of aqueous NO solution were added to PBS (pH 7.4). The peak position was 0.95 V at the scan rate of 50 mV s^{-1} (compared with 1.2 V on bare carbon).[1] The NO peak height increased linearly with subsequent additions of aqueous NO solution.

Differential pulse voltammetry (DPV) was performed at a sweep rate of 12.5 mV s^{-1} and 2.5 pulses s^{-1} (pulse height 100 mV, pulse width 80 ms). A NO oxidation peak was observed at 0.75 V when aliquots of NO solution were added to PBS (pH 7.4) (see Fig. 1). The NO peak height was proportional to concentration in the range 0–200 μmol dm^{-3}. Above 200 μmol dm^{-3} the current response tailed off.

The detection limit (defined as the concentration at three times the standard deviation of the intercept of the linear fit[20]) of a 1.0 mm diameter electrode (3 : 2 graphite : epoxy) was 190 nmol dm^{-3} and the sensitivity was 295 nA $(\mu mol\ dm^{-3})^{-1}$ (DPV) [Fig. 1 (insert)]. No interference was found from physiological concentrations of nitrite, nitrate, hydrogen peroxide, serotonin or dopamine on the bare carbon electrode, which indicated that only a thin membrane of Nafion™ was needed to facilitate biocompatibility. Ascorbate was detected, but only above expected physiological concentrations with DPV and was clearly resolved from the NO peak.

Preliminary *in vivo* evaluation of the electrodes was in anaesthetized (Hypnorm) white male Sprague–Dawley rat muscle and liver. Nafion™ coated and uncoated electrodes were used. An endogenous NO peak was found at the expected

potential of 0.75 V, which increased when authentic NO-saturated saline was added at the electrode site (Fig. 2). A second prominent peak at 0.12 V was clearly visible. Subsequent investigations of ascorbate solutions (0.1 mmol dm^{-3}) in PBS (pH 7.4) with an electrode which had been implanted in the rat identified this peak as due to ascorbate. The *in vivo* ascorbate peak (not shown) decreased on local addition of NO solution.

The NO current was found to increase with L-arginine, the biological precursor of NO, and decrease with L-ω-*N*-monomethyl arginine (L-NMMA), a nitric oxide synthase inhibitor.[22] The variation in NO concentration was synchronous with expected variations in blood pressure.

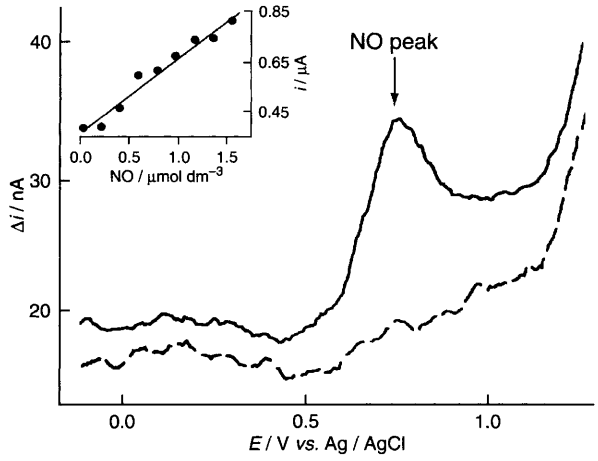

Fig. 1 Differential pulse voltammogram (DPV) before and after addition of saturated NO solution to PBS pH 7.4 (dashed and full lines respectively). Final concentration of NO = 176 μmol dm^{-3}. Potential of NO peak = 0.75 V; Δi refers to the differential current. Insert: calibration curve over the expected physiological concentration range of NO.[21]

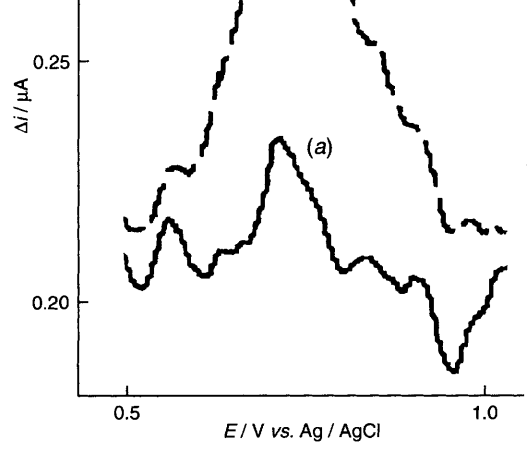

Fig. 2 *In vivo* DPV showing NO peaks measured in rat muscle. The endogenous NO peak [(*a*), —], increased dramatically when saturated NO saline was added to the electrode site to produce a large NO peak [(*b*), - - - -].

The electrodes showed no deterioration in performance after exposure to living tissue. No marked improvement in the electrode performance *in vivo* was found in electrodes coated with Nafion.™ Further *in vivo* studies are required to evaluate the importance of the Nafion™ for, although it reduces interference and increases biocompatibility, it also reduces the sensitivity of the electrode. In conclusion, a novel sensitive NO electrode has been developed which employs a chemically modified $[Fe^{III}(salphen)]^+$–graphite–epoxy electrode. Preliminary *in vivo* experiments demonstrate that the electrode is capable of detecting endogenous NO in complex biological matrices. We anticipate that this electrode will be of great utility for research into the functions of NO in physiological systems.

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