

# Direct Measurement of Nitric Oxide in the Cardiovascular System

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## Summary

Nitric oxide generated from L-arginine is a messenger for cell- to-cell communication. Abnormalities in nitric oxide release have been implicated in diseases ranging from hypertension and atherosclerosis to septic shock and rheumatoid arthritis. We report here the *in vivo* and *in vitro* measurements of nitric oxide in the cardiovascular system using a porphyrinic sensor specific for NO. The sensor has a detection limit  $10^{-9}$  M, response time of 0.1–10 ms and diameter of 1–20  $\mu\text{m}$ . Protected by an intravenous catheter or Swan-Ganz catheter, the sensor can be implanted into tissues as well as into the blood stream. Nitric oxide concentrations were measured directly in the heart and also in veins and arteries, ranging in diameter from 100  $\mu\text{m}$  to 5 mm. Nitric oxide production was induced by the action of different physical agents (shear stress, stretching) as well as various chemical substances agonists (bradykinin, acetylcholine, ATP).

## Key words

Nitric oxide determination – Porphyrinic sensor – Heart – Arteries – Veins – Blood

## Introduction

We have described electrochemical methods for the measurement of nitric oxide in single cells and in tissues of vascular system. These recently developed methods for nitric oxide determination provide several advantages that are not available using analytical spectroscopic methods. Most important is the capability afforded by the use of ultramicroelectrodes for direct *in situ* measurements of NO• in single cells near the source of NO• synthesis.

Electrochemical methods currently available for nitric oxide detection are based on the electrochemical oxidation of NO• on solid electrodes (Kiechle *et al.* 1993, Archer 1993). If the current generated during NO• oxidation is linearly proportional to the concentration, the oxidation current can be used as an analytical signal. This current can be measured in either an amperometric or a voltametric mode, both methods providing a quantitative signal. In the amperometric mode, current is measured while the potential, at which nitric oxide is oxidized, is applied and kept constant. In the voltametric mode, the current is measured while the potential is linearly scanned

through the region that includes nitric oxide oxidation. Although the amperometric method is faster than the voltammetric method, voltammetry provides not only quantitative but also qualitative information that can prove that the current measured is in fact due to NO• oxidation.

Generally, the oxidation of nitric oxide on solid electrodes proceeds *via* a two-step "EC mechanism" with step (1), an electrochemical reaction, followed by step (2), a chemical reaction. The first electrochemical step is a one-electron transfer from an NO• molecule to the electrode resulting in the formation of a nitrous cation.



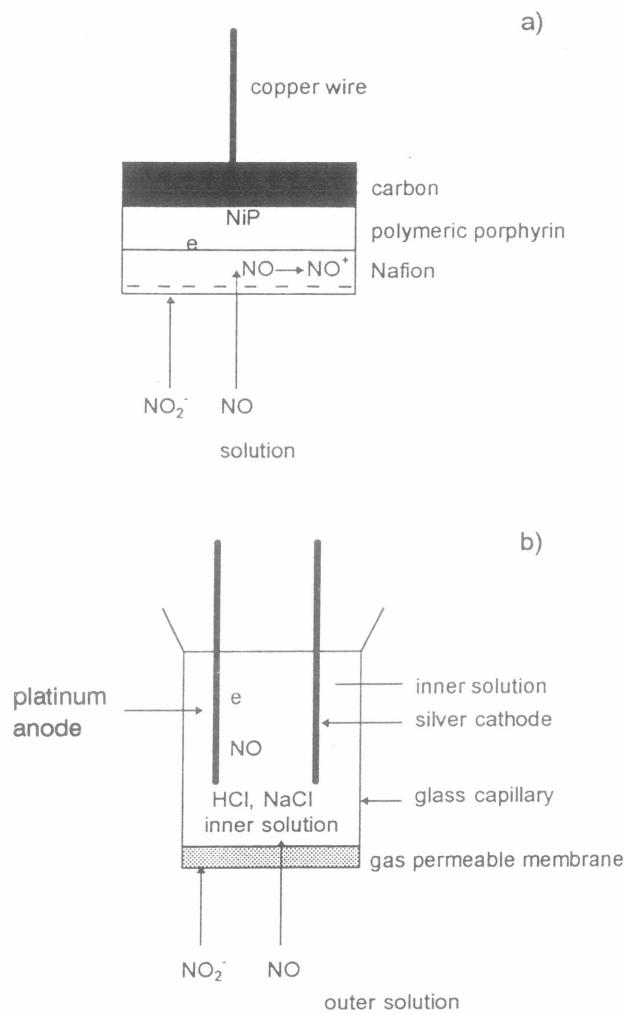
NO<sup>+</sup> is a relatively strong Lewis acid and in the presence of OH<sup>-</sup> is converted to nitrite (NO<sub>2</sub><sup>-</sup>):



The rate of the chemical reaction (2) increases with increasing pH. Since the oxidation potential of nitrite in aqueous solution is only 60–80 mV more positive than that of NO•, oxidation of NO• on solid electrodes with a scanned potential results in the transfer of two additional electrons. Thus nitrite is ultimately oxidized

to nitrate,  $\text{NO}_3^-$ , the final product of electrochemical oxidation of  $\text{NO}^\bullet$ .

Figure 1 shows sensors that have been developed for the electrochemical measurement of nitric oxide. One is based on the electrochemical oxidation of nitric oxide on a conductive polymeric porphyrin (porphyrinic sensor) (Malinski and Taka 1992). The other is based on an oxygen probe (Clark electrode) and operates in the amperometric mode (Turner *et al.* 1987). In the porphyrinic sensor, nitric oxide is oxidized on a polymeric metalloporphyrin (*n*-type semiconductor) on which the oxidation reaction occurs at 630 mV (versus SCE), 270 mV lower than the potential required for comparable metal or carbon electrodes. The current efficiency (analytical signal) for the reaction is high, even at the physiological pH of 7.4.



**Fig. 1**

Schematic diagram of porphyrinic sensor (a) and Clark type probe for determination of nitric oxide (b).

The Clark probe consists of a platinum wire as the working electrode (anode) and a silver wire as the counter electrode (cathode). The electrodes are

mounted in a capillary tube with a sodium chloride/hydrochloric acid solution separated from the analytic solution by a gas-permeable membrane. A constant potential of 0.9 V is applied and the current (analytical signal) is measured from the electrochemical oxidation of nitric oxide on the platinum anode.

## Methods

### Porphyrinic sensor

The microsensors are produced by threading a carbon fibre (diameter 7 μm, Amoco) through the pulled end of a capillary tube with 1 cm left protruding. Non-conductive epoxy is put at the glass/fibre interface. After the epoxy cement drawn into the tip of the capillary has cured, the carbon fibre is sealed in place. The carbon fibre is then sharpened by gradual burning (propane air micro burner 1300–1400 °C). The sharpened fibre is immersed in melted wax-rosin (5:1) at a controlled temperature for 5–15 s, and after cooling is sharpened again. The flame temperature and the distance of the fibre from the flame need to be carefully controlled. The resulting electrode is a slim cylinder with a small diameter (0.5–2 μm) rather than a short taper, a geometry that aids in implantation and increases the active surface area. The tip (length 2–6 μm) is the only part the carbon fibre where electrochemical processes can occur. For the sensor to be implanted into a cell, this length must be shorter than the cell diameter. The unsharpened end of the fibre is attached to a copper wire lead with silver epoxy cement.

Monomeric nickel(II) tetrakis (3-methoxy-4-hydrophenyl) porphyrin (Ni(II)TMHPP) is synthesized according to a procedure described previously (Malinski *et al.* 1991). The polymeric film of Ni(II)TMHPP is deposited on a single carbon fibre electrode from a solution of  $5 \times 10^{-4}$  M monomeric Ni(II)TMHPP using cyclic scanning of the potential between 0.2 to 1.0 V (versus a saturated calomel electrode, SCE) with a scan rate of 100 mV/s for 10–15 scans. Dip coating the dried polymeric porphyrin/carbon fibre tip (3 times for 5 s) in 1% Nafion in alcohol (Aldrich), processes a thin anionic film that repels or retards charged ionic species while allowing the small neutral and hydrophobic  $\text{NO}^\bullet$  molecule access to the underlying catalytic surface. The sensor is interference-free from the following readily oxidizable secretory products at concentrations of at least two orders of magnitude greater than their expected physiological concentrations: epinephrine, norepinephrine, serotonin, dopamine, ascorbate, acetylcholine, glutamate, glucose, the  $\text{NO}^\bullet$  decay product  $\text{NO}_2^-$ , and peptides containing tryptophan, tyrosine or cysteine. The porphyrinic sensor has a response time of 0.1 ms at micromolar  $\text{NO}^\bullet$ .

concentrations and 10 ms at the detection limit of 5 nM.

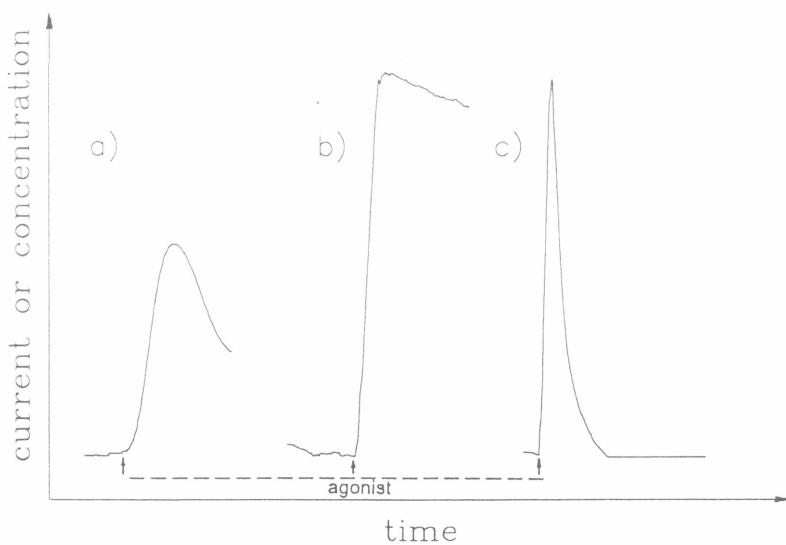
For *in vivo* and *in vitro* measurement of NO<sup>•</sup> in tissues and the blood, a catheter-protected porphyrinic NO<sup>•</sup> sensor is constructed from the needle of a 22-gauge 1-inch long intravenous catheter/needle unit (Angiocath, Becton Dickinson) truncated and polished flat to be 5 mm shorter than a 20-gauge catheter (Malinski *et al.* 1994, Pinsky *et al.* 1994). A bundle of seven carbon fibres (each 7 µm in diameter, protruding 5 mm, 12 Ωcm, Amoco) is mounted inside the hollow truncated 22-gauge needle with conducting epoxy cement. After curing, the exterior of the truncated needle is coated with non-conductive epoxy (2-TON, Devcon) and allowed to cure again. The protruding 5 mm carbon fibre bundle is made more sensitive and selective for NO<sup>•</sup> by covering it with polymeric porphyrin and Nafion, before calibration with NO<sup>•</sup>. To implant the porphyrinic NO<sup>•</sup> sensor, ventricular tissue is pierced with a standard 20-gauge angiocatheter needle (clad with its catheter containing four 400 µm ventilation holes near the tip), and subsequently advanced to the desired location. Intracavitory contact of the catheter tip is withdrawn by 2–4 mm. The catheter's position is secured and the placement needle is removed and quickly replaced with the truncated 22-gauge porphyrinic NO<sup>•</sup> sensor.

At an operation potential of 0.63–0.65 V, the sensor does not respond to other gases such as oxygen, carbon dioxide and carbon monoxide. The sensor is too slow to respond to superoxide, which is scavenged in

the biological environment in fast reactions with other molecules including nitric oxide ( $K = 3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) (Hogg *et al.* 1992).

#### *Response and calibration of the porphyrinic sensor*

Differential pulse voltammetry (DPV) and differential pulse amperometry (DPA) can be used to monitor analytical signals, where current is linearly proportional to NO<sup>•</sup> concentration. In DPV, a potential modulated with rectangular pulses (amplitude 1–40 mV) is linearly scanned from 0.4 to 0.8 V. The resulting voltamogram (alternating current versus voltage plot) contains a peak due to NO<sup>•</sup> oxidation. The maximum current of the peak should be observed at a potential of 0.63 to 0.67 V (at 40 mV pulse amplitude), the characteristic potential for NO<sup>•</sup> oxidation on the porphyrinic/Nafion sensor. DPV is used primarily to verify that the current measured is due to NO<sup>•</sup> oxidation. In differential pulse amperometric measurements, a potential of 0.63 to 0.67 V modulated with rectangular pulses is kept constant and a plot of an alternating current versus time is recorded. The amperometric method (with a response time better than 10 ms) provides rapid quantitative measurement of minute changes of NO<sup>•</sup> concentration. DPV also provides quantitative information but requires approximately 5–40 seconds for the voltamogram to be recorded, and is therefore used mainly for qualitative analysis.



**Fig. 2**  
Amperograms (current-time curves) showing porphyrinic sensor response for nitric oxide in homogeneous static solution (a), in homogeneous laminar flow solution (b), and heterogeneous laminar flow solution (c).

Many other electroanalytical techniques including normal pulse voltammetry, square wave voltammetry, fast scan voltammetry, and coulometry can be used to measure nitric oxide with a porphyrinic sensor. Amperograms and voltamograms can be recorded with two- or three-electrode systems. However, differential pulse voltamograms should always be recorded in three-electrode systems in order to obtain accurate and reproducible values of peak potentials. The three-

electrode systems consist of a NO<sup>•</sup> sensor working electrode, a platinum wire (0.25 mm) counter electrode and a silver/silver chloride or SCE as the reference electrode. The reference electrode is omitted from the two-electrode system. The porphyrinic sensor can be connected to any fast response potentiostat for amperometric or coulometric measurements, or to a voltametric analyzer (a potentiostat and waveform generator) for voltammetric measurements. An



instrumental current sensitivity of 100 pA/inch of recorder display will be sufficient for most of the measurements using a multifibre sensor. For single fibre experiments, at least ten times greater sensitivity is required, readily achieved by adding a low noise current-sensitive preamplifier to the potentiostat. The sensor can be calibrated with saturated NO<sup>•</sup> solution at 2 mM as a standard, or by preparing a calibration curve using the standard addition method. A typical amperometric response of the sensor under different flow conditions is shown in Figure 2. In static solutions where mass transport of NO<sup>•</sup> to the electrode is only due to a diffusion process, a linear increase in current reaching a plateau is followed by a slow decrease in the current due to NO<sup>•</sup> oxidation (Fig. 2a). In a flowing or stirred homogeneous NO<sup>•</sup> solution, a rapid increase in current reaching a plateau occurs after the release of NO<sup>•</sup> (Fig. 2b). In heterogeneous flowing solutions with high localized concentrations of NO<sup>•</sup> such as the blood, the amperometric signal is observed in the form of a peak (Fig. 2c).

#### Clark probe for NO<sup>•</sup> detection

The Clark probe, originally designed for the detection of oxygen, is a glass pipette with its opening sealed by a gas permeable membrane of thick rubber. Only a low molecular weight gas can readily diffuse into the glass pipette through the membrane, and be oxidized or reduced at the surface of the metal electrode (working electrode). In the Clark probe for oxygen detection, a working electrode (platinum) is polarized with a potential -0.8 V (versus a silver counter/reference electrode), and a current due to the reduction of oxygen is reported. For the detection of nitric oxide with the Clark probe, only the polarization of the electrodes must be changed. In order to oxidize nitric oxide on the platinum electrode, the polarization has to be reversed to 0.8 V (versus the silver electrode) instead of -0.8 V for oxygen detection. As the electrolyte in the Clark probe for NO<sup>•</sup> detection, a mixture of NaCl and HCl (1 M) can be used.

## Results and Discussion

### Single cell measurements with a porphyrinic sensor

By the use of a manual or automated computer controlled micromanipulator with 0.2 μm x-y-z resolution, the porphyrinic sensor can be implanted into a single cell, or placed on the surface of the cell membrane, or kept at a controlled distance (0.2–0.5 μm) from a cell membrane surface (Malinski *et al.* 1993, Balligand *et al.* 1994). The highest concentration of NO<sup>•</sup> release from the cell containing nitric oxide synthase (cNOS) is on the cell membrane. For measurements of NO<sup>•</sup> on the cell membrane, a bundle of 2–5 carbon fibres mounted on an L-shaped

capillary is effective. The cell can be grown on any type of solid support such as a glass or plastic plate. An injection of the agonist near the cell to stimulate NO<sup>•</sup> release requires application by a picopipette or femtopipette. Depending on the forces applied during the injection, an initial release of NO<sup>•</sup> may be due to shear stress.

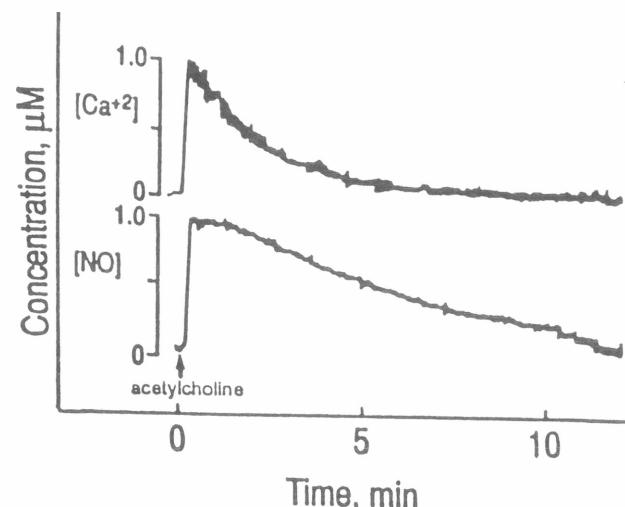


Fig. 3

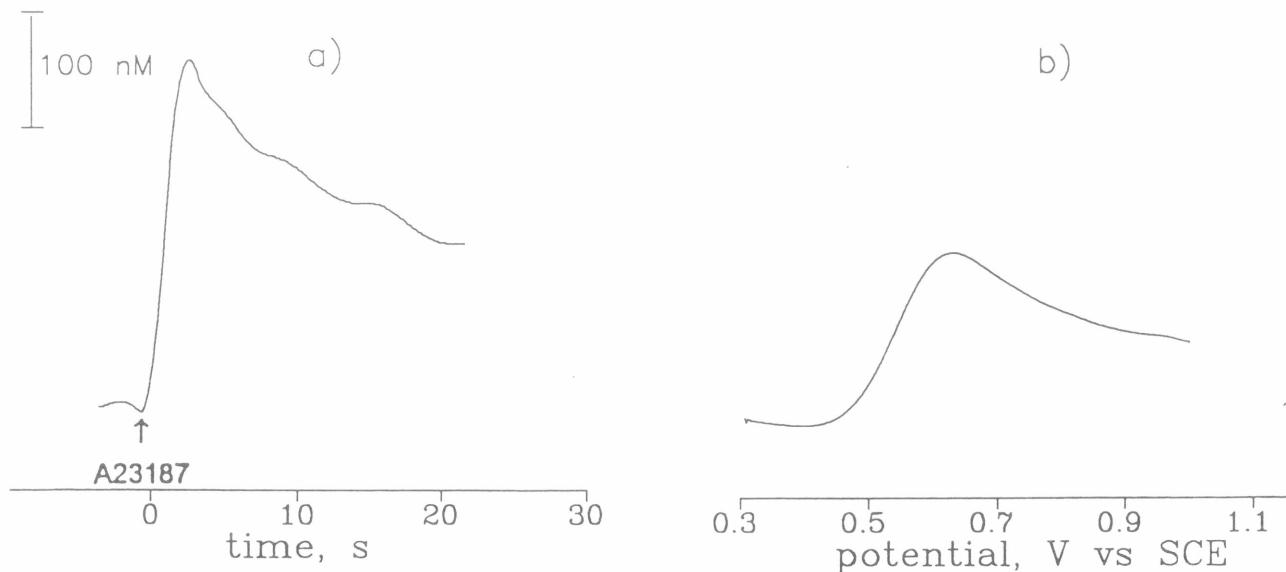
Time-course of nitric oxide production and calcium flux measured simultaneously in single endothelial cells.

Figure 3 shows a typical amperometric response of the porphyrinic sensor to NO<sup>•</sup> release from single endothelial cells. In this experiment with rabbit aortic endothelium exposed to acetylcholine, the sensor detected NO<sup>•</sup> at the cell membrane surface within 5 s. The surface NO<sup>•</sup> concentration reached a maximum of  $950 \pm 70$  nM within 9 s. Simultaneously with the NO<sup>•</sup> measurements, intracellular calcium ion ( $[Ca^{2+}]_i$ ) measurements were performed with a fura-2-acetoxymethyl ester (fura-2AM) and fluorescence imaging system. The intracellular calcium increased at a rate of  $130 \pm 10$  nM/s within 3 s after the addition of acetylcholine.

Intracellular calcium remained near maximum levels ( $0.90 \pm 0.07$  μM) for 10–45 s after the application of bradykinin, followed by a decline and return to basal levels within 360 s. Because cell surface NO<sup>•</sup> levels represent NO<sup>•</sup> synthase activation as well as subsequent diffusion both within the cytosol and cell membrane, NO<sup>•</sup> detected at the cellular surface would be expected to outlast the calcium transient. The rapid release of NO<sup>•</sup> was observed after injection of 10 nanomoles of acetylcholine with a nanoinjector. This maximum NO<sup>•</sup> concentration will be the same if the measurement of NO<sup>•</sup> is performed on the membrane of single isolated cell, or on the membrane of single cells in the group of similar cells, or cells in a tissue. However, the duration of the plateau will be much

different and will depend on the number of neighbouring cells. For an isolated cell, the duration of the plateau will be shorter due to the rapid depletion of  $\text{NO}^\bullet$  and the higher concentration gradient. The membrane of the cell is a storage reservoir for  $\text{NO}^\bullet$ , and a small membrane volume can develop a relatively

high concentration within a short period when  $\text{NO}^\bullet$  is released by the  $\text{NO}^\bullet$ -producing enzyme. From an analytical viewpoint, the detection of  $\text{NO}^\bullet$  at the site of the highest concentration, the surface of the cell membrane, is most convenient and accurate method of measurement of endogenous  $\text{NO}^\bullet$ .

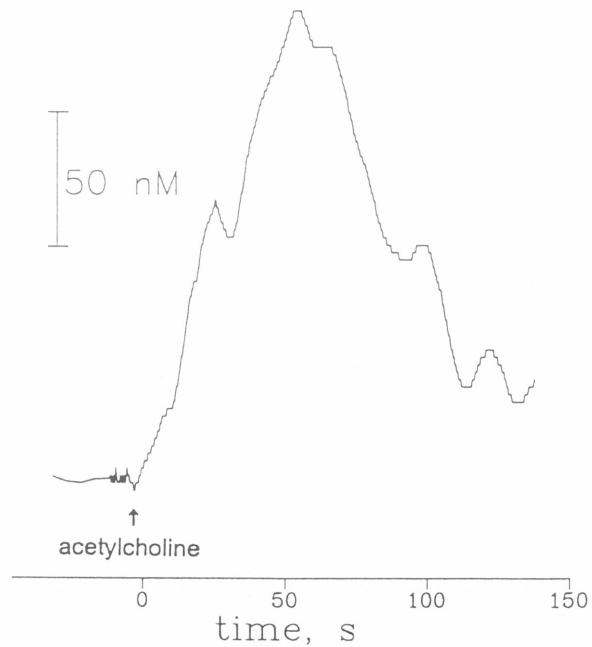


**Fig. 4**

Amperogram (a) and differential pulse voltamogram (b) of nitric oxide release from endothelial cells of rabbit mesenteric resistance artery due to action of acetylcholine (a); differential pulse voltamogram was recorded after 10 s from the injection of acetylcholine; potential scan rate 25 mV/s and pulse amplitude 40 mV.

#### Measurements with a porphyrinic sensor in tissues

Figure 4a shows a typical amperogram of nitric oxide in a rat mesenteric resistance artery of a 15-week-old rat. A multifibre porphyrinic sensor (20 fibres, diameter 35  $\mu\text{m}$ ) was inserted in the lumen of a resistance artery. 20  $\mu\text{l}$  of a 1 mM calcium ionophore A23187 solution were then injected to reach a final concentration of 10  $\mu\text{M}$  in the organ chamber. Immediately after injection of the calcium ionophore, a rapid increase of  $\text{NO}^\bullet$  concentration was observed. The rate of the concentration increase was 160 nM/s and the peak concentration was reached 2.2 s after injection of the calcium ionophore. Figure 4b shows a differential pulse voltamogram recorded 10 s after injection of calcium ionophore with the potential scanned from 0.3–1.00 V, using a scan rate of 25 mV/s. A peak potential at 650 mV indicates that the electrochemical process is due to oxidation of nitric oxide, and the peak current indicates that the average  $\text{NO}^\bullet$  concentration during the time of voltamogram measurement is 130 nM.



**Fig. 5**

Amperogram of nitric oxide release measured *in vivo* in the rabbit ear (auricular vein) with a porphyrinic sensor protected by an intravenous catheter.

### *Measurements in vivo with a porphyrinic sensor*

Figure 5 presents a typical amperometric curve obtained for *in vivo* measurements of NO<sup>•</sup> after the administration of acetylcholine. The porphyrinic sensor was stored in a physiological saline solution spiked with 1000 μM/ml of heparin for 15 min before implantation into the rabbit auricular vein in the left ear. Thirteen seconds after intravenous injection of acetylcholine (1 ml, 10 pM), a peak due to NO<sup>•</sup> release was observed which reached a maximum concentration of 320 nM. This response represents a significant increase of NO<sup>•</sup> concentration from its basal concentration in the blood (3–5 nM). For *in vivo* measurements, this sensor was calibrated using the flow system and a dextran (m.w. 70,000)/saline solution with viscosity similar to that of the blood (3.5 cP).

### Conclusion

Although considerable effort may be required to establish conditions for obtaining a stable, high

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sensitivity, miniature porphyrinic sensor, the electrochemical approach can lead to the detection of low NO<sup>•</sup> ( $10^{-8}$  to  $10^{-9}$  M) concentrations in a biological environment with short (millisecond) response time to nitric oxide. A wide spectrum of information about the kinetics and dynamics of nitric oxide release can be obtained that cannot be achieved by other methods. The sensor permits extensive exploration of the reactivities of NO<sup>•</sup> with redox molecules, as well as analysis of NO<sup>•</sup> production *in vitro* or *in vivo*. The method is ideally suited for the characterization of labile systems for which conditions must be met for preparation of spectroscopic samples.

The Clark probe can be used for the measurement of NO<sup>•</sup> in synthetic homogeneous solutions containing high concentrations of nitric oxide, i.e. studies of NO<sup>•</sup> release from drugs. However, the Clark probe has very limited application for measurement of NO<sup>•</sup> from biological samples due to the high temperature coefficient, slow response time, interferences, baseline drift and their relatively large dimension.