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Nitric oxide determination by amperometric carbon fiber microelectrode

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Abstract

Nitric oxide (NO) amperometric microsensor was prepared by the modification of bare carbon fiber electrode by Nafion and cellulose acetate (CA). Detection limit, response time, reproducibility and influence of some possible interferences (nitrite, nitrate, arginine) were tested and evaluated. This sensor was used for in vitro determination of NO release from fresh porcine aorta induced by calcium ionophore A23187 (CI). © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide; NO sensor; Electrode; Electrochemical; Carbon fiber; Endothelium

1. Introduction

Since the late 1980s, when the importance of nitric oxide (NO) in smooth muscle vasodilation has been discovered [1,2], the research connected with NO produced in the biological systems is in the center of great interest of many scientists. NO is an endogenously synthesised (from arginine by nitric oxide synthase, NOS) free radical playing an important role in many physiological and pathophysiological functions, e.g. regulates vascular tone, acts as a neurotransmitter and has an antimicrobial activity. The need of its real time determination flows from the requirements both basic physiological research and pharmacological monitoring of potential selective NOS effectors/inhibitors.

The measurement of NO is possible to carry out by indirect or direct methods. Indirect methods are based on the monitoring of concentration of compounds concerned with the change of NO concentration, e.g. determination of L-citrulline [3], nitrite/nitrate as the oxidation products of NO [4], EPR after the formation of complex NO with haemoglobin [5], or chemiluminescence after the reaction of NO with hydrogen peroxide or ozone [6]. For real-time measurements of NO in biological sample, the following factors have to be considered: (i) low NO concentration (nM up to μM), (ii) fleeting presence of NO (up to few seconds), (iii) complexity of such matrix and (iv) limited dimension of biological samples. The main drawbacks of indirect methods are discontinuity and not local measurement, which cause their use for the local NO measurements in real time in biological sample to be very restrict or impossible. Those goals should be presently attainable only by direct-amperometric method using microelectrodes with required parameters (fast response, small diameter, sensitivity to low concentrations). The usage of that type of electrochemical microsensors allows the observation of the dynamics and stabilization of NO production in real time, and permits highly localized measurements with small biological system in the vicinity of a target place.

It exists several published and few commercially available NO microelectrodes. NO can be oxidized at about +0.7–+0.9 V (vs. SCE) at variously modified C, Pt or Au electrodes. The electrodes were covered by electropolymerised films (Ni-porphyrin [7,8], *o*-phenylenediamine [9], poly(4,4′-dihydroxybenzophenone) [10]) and/or by polymeric films (Nafion [7,8,11], cellulose acetate [10,11], polydimethylsiloxane [12], polystyrene [13]) to increase sensitivity to NO and to exclude interfering ions. Electrode with immobilized nanoparticles of palladium [14] was used for NO detection at +700 mV (vs. SCE) with linear response from nanomolar to millimolar. NO can also be reduced, e.g. at surface-modified gold electrode with immobilized cytochrome *c*′ at −220 mV [15]. NO biosensor was based on the inhibition/activation effect of this radical [16]. With increased interest in direct NO, determination is connected in searching for many different ways on how to effect its electrochemical detection [17,18].

One of these ways is the use of modified carbon fiber microelectrodes [8]. Small diameter of the carbon fiber

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(usually less than 10 μm) enables sufficient spatial resolution and measurements in small biological systems. In this paper, NO microsensor is presented based on the carbon fibers covered by Nafion and/or acetate cellulose membrane. To test its ability to measure NO in biological sample in vitro, NO release from fresh porcine aorta induced by calcium ionophore A23187 was monitored.

2. Experimental

Arginine and Nafion®117 solution were obtained from Fluka (Buchs, Switzerland). Arginine, calcium ionophore A23187 (CI) and cellulose acetate (CA) were purchased from Sigma (St. Louis, MO, USA). Other reagents were commercially available as analytical grade.

Carbon fiber microelectrodes were prepared from carbon fibers (Amoco Performance Products, Greenville, SC, USA, 6 μm diameter). A bunch of them (4–10) was inserted into a glass capillary (1 mm i.d., approximately 6-cm length), inside the capillary was connected with a bare copper wire by conductive glue (Electropol, Techlab ÚPOSAV, Bratislava, Slovakia) to insure good electrical contact, and the tip of the glass capillary was sealed with beeswax. The carbon fibers were shortened to a length of 2–4 mm and the rest of the beeswax was removed in the flame and electrode was rinsed with distilled water. The electrode was then modified with CA (immersed in 1% solution in acetone (55%)-cyclohexanone (45%) for 5 s) and/or with Nafion (immersed once or twice in 5% solution in alcohol for 5 s).

NO measurements were performed by two-electrode system (prepared carbon fiber microelectrode and saturated calomel reference electrode, SCE, at 870 mV vs. SCE) connected with potentiostat PST-3 (FEI STU Bratislava, Slovakia) and recorder TZ 4620 (Laboratorní přístroje Praha, Czech Republic) in a vessel equipped with magnetic stirring. Current-time response curves were recorded. The height of the recorded wave (current increase) corresponding to the concentration of analyte was evaluated. Recording of sensor parameters and calibration plots were carried out at room temperature in 0.1 M phosphate buffer pH 7.4 (5 ml). The portions of saturated standard NO solution (2 mM) were added with Hamilton's syringe. A piece of fresh porcine aorta was put into the vessel with Krebs–Henseleit's buffer (10 ml) doped with 0.1 mM arginine at in vitro measurements. These measurements were carried out at 37 °C and non-stirring condition. The active tip of NO microelectrode was placed on the aorta endothelium. The release of NO was stimulated by the addition of CI solution in ethanol (2 mM).

3. Results and discussion

For the preparation of each electrode, a bunch of carbon fibers (4–10) was used. The preparation of electrode based on the single fiber was unsuccessful because of the problems with carbon fiber fragility related to its small diameter (6 μm). The use of a bunch of carbon fibers led to the preparation of usable sensors, however, the increase of noise sometimes appeared because of fibers flattering. In spite of the fact that normally carbon fiber microelectrodes present have acceptable reproducibility and good repeatability of measurements, reproducibility of fabrication of our NO carbon fiber microelectrodes was not in some cases so excellent (differences in sensitivity of two parallel prepared electrodes were 5–20%, exceptionally up to 100%) probably due to possible insufficient removing of beeswax from the carbon fibers during microelectrode preparation. However, because each electrode was calibrated before the measurement, the results are not influenced by this.

Nitrite is the most problematic interference for NO determination in biological solutions. For that reason, the electrochemical behaviour of both compounds at different potentials using bare carbon fiber electrode was studied. In comparison with maximum relative responses of electrode to NO and nitrite at 950 mV vs. SCE (100%), no significant difference in the decrease of relative electrode responses between them at lower potentials was observed. The same results were achieved with Nafion-coated microelectrode. Both for this reason and to achieve suitable sensitivity, the working potential of 870 mV vs. SCE was chosen.

The bare sensor was sensitive to nitrite, dopamine, ascorbic acid and CI, no response to nitrate and arginine was observed. To decrease the response to large molecules, electrodes were modified with CA. Dip coating in 1% CA for 5 s produced on the electrode surface a sufficient barrier against used CI (M.W. 523.6), no response after coating was measured. Responses to the other tested interferences decreased as follows: for nitrite to about 35%, for NO to 60%, for dopamine to 12% and for ascorbic acid to 17% of their initial value. This is in spite of the described increase of sensitivity to NO after this coating step [11]. Because small molecules (NO) are highly permeable through CA layer, this decrease is probably caused by the change of electrochemical properties of electrode active surface after coating with CA. The decrease of sensitivity for dopamine and ascorbic acid is related to their size, for nitrite (also for ascorbic acid), it can be due to negative charge of CA.

The bare and CA modified electrodes were coated with Nafion to decrease response to nitrite and other anions by dipping the electrode in the 5% solution once or twice for 5 s. Nafion produces a thin anionic film that repels or retards charged species (e.g. nitrite). The electrode response decreased after one coating to about 35% of its initial value for nitrite and to 60% for NO, after double coating to about 13% for nitrite and to 35% for NO. The decrease of NO sensitivity is higher than reported [11,19,20], what can be again caused by the changes on the surface of carbon fiber microelectrode. Anyway, the nitrite response decreased by a factor of 8 and NO response only by a factor of 3. Dopamine and ascorbic acid sensitivities decreased after double Nafion coatings to 12%, respectively, 13% of their initial responses. This find-

ing is particular because Nafion is for dopamine that is highly permeable. This decrease likely again results from electrode surface changing.

Calibration plots for NO sensor modified with CA and Nafion in aerated and deaerated solution are shown in Fig. 1. In deaerated solution, no decrease and in the aerated one, only slow NO concentration decrease during calibration due to NO oxidation in solution was observed (not shown).

The time of stabilization of initial signal was about 10 min and was shorter in deaerated medium, this was caused by the presence of some species on the electrode surface able to be oxidized in aerated solution. Response time to addition of NO standard was about 3–4 s. The detection limit was defined as a signal-to-noise ratio of 3 and was about 1 μM.

The electrode was used for in vitro measurement of NO release from endothelium of porcine aorta. NO release was induced by CI, invoking stimulation of constitutive nitric oxide synthase (cNOS) included in vein endothelium [21]. Fig. 2a shows an amperogram (current vs. time) after the addition of 40 μl 2 mM CI. CI was injected as close as possible to the active tip of electrode placed on aorta endothelium about 60 min after a pig was killed. About 10 s after injection, a rapid increase of current was observed. The maximal peak value was observed after 1–2 min after CI addition, measured current was equivalent to local NO concentration up to approximately 10 μM. Because the experiment was carried out at a non-stirring condition, the local concentration of CI was decreasing in time, proportional to diffusion of CI away from the electrode. Consequential decrease of current equal to local NO concentration was observed. A new background current level was reached about 3 min after CI addition. Probably, while concentration of CI on endothelium surface lowered below certain value, stimulation of cNOS was not sufficient and production of NO was stopped or restricted. After a few minutes, the next addition of CI caused similar response. Fig. 2b shows the amperogram recorded 140 min after the death of an animal. The course of response is similar but the measured currents are lower, 200 min after the death of an animal is the response unmeasured. These results indicate a progressive degradation of enzyme cNOS in dead animal tissue.

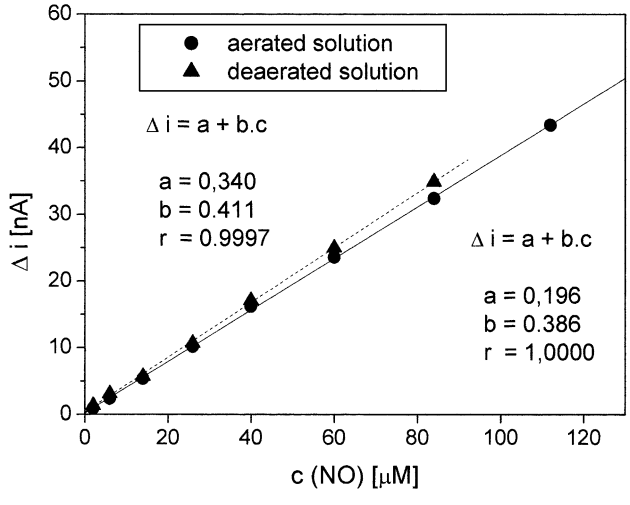

Fig. 1. NO calibration plots at cellulose acetate and Nafion modified carbon fiber electrode carried out in aerated and deaerated (N_2) phosphate buffer solution 0.1 M, pH 7.4, working potential +870 mV vs. SCE.

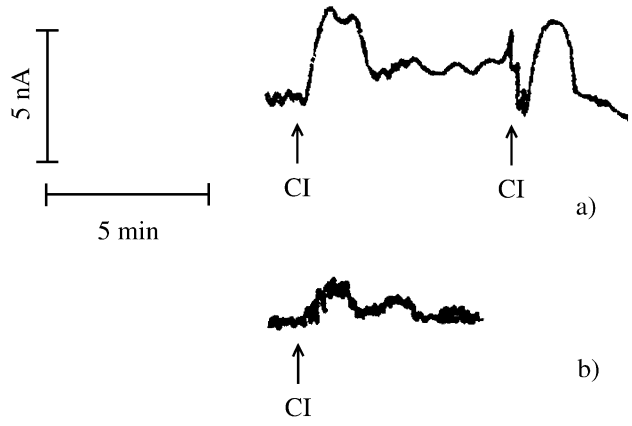

Fig. 2. Amperogram showing NO release from porcine aorta endothelium stimulated by additions of calcium ionophore A23187 (CI), measured by cellulose acetate and Nafion modified carbon fiber electrode 60 min (a) and 140 min (b) after the death of an animal. Conditions: Krebs–Henseleit's buffer doped with 0.1 mM arginine, working potential +870 mV vs. SCE, at 37 °C, non-stirring.

4. Conclusions

Evaluated parameters (detection limit, response time and influence of interferences) have shown that demonstrated very simple NO amperometric carbon fiber microelectrode modified only by cellulose acetate and Nafion membranes enables to measure NO concentration at micromolar level (the lowest measured concentration is 1 μM). Nafion and cellulose acetate membranes form barrier against possible interferences, but influence of nitrite was not fully removed. The preparation of the sensors is fast, easy and not expensive. The microelectrode has been successfully used for local in vitro measurement of NO release from fresh aorta endothelium. However, for future in vitro and in vivo applications in biological systems at nanomolar level, the oxidation efficiency of NO at the electrode surface as well as the reproducibility of preparation has to be improved and also a biocompatibility has to be counted.

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