Electrochemical Determination of Nitric Oxide in Blood Samples

[a]M. RIEVAJ, [b]J. LIETAVA, and [a]D. BUSTIN

[a] *Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-812 37 Bratislava*
e-mail: bustin@cvt.stuba.sk

[b] *2nd Department of Internal Medicine, Comenius University Hospital, SK-813 69 Bratislava*

Received 2 September 2003

A simple, sensitive, and specific electroanalytical method using a chemically modified ultramicroelectrode sensor was applied for nitric oxide determination in blood samples. The sensor fabricated by electrodeposition of *p*-type semiconducting polymeric films on a carbon fibre and Nafion deposition in the final step of its preparation was selective to NO. The Nafion coating eliminated the diffusion and adhesion of anionic species oxidizable at the microelectrode surface. One-electron oxidation current of differential pulse voltammetry peak was used for the quantitative measurement of NO in the samples. The method was applied to NO analysis in real blood samples.

Nitric oxide belongs to the most intensively studied small molecules because of its important bioregulatory properties. It plays many physiological roles in a cardiovascular system. One of them is the role of NO in maintaining of the normal blood pressure by vasodilatory responses [1, 2]. Because of the relatively short half-life period the measurement of NO content in biological systems is still a challenging analytical problem.

Different methods for NO determination were used. Some of them were indirect – based on the chemical detection of the decomposed products of NO removed from biological system. Other methods, such as chemiluminescence and EPR are direct methods specific for NO determination [3]. They cannot, however, be applied to monitor NO *in vivo*. Electrochemical methods have great potentiality because they are simple, effective and can easily be performed *in vivo* [4]. That is why there has been an explosive interest in the development of sensors specific for NO determination. These sensors fall into three categories. The first one is based on the direct oxidation of NO at the electrodes modified with gas-permeable or anionic membranes [5, 6]. The second is on the basis of the oxidation of NO on the electrodes modified with an electropolymerized film of a monomer, such as metalloporphyrin [7], metallophthylocyanin [8] or *o*-aminobenzaldehydeethylenediamine nickel [9]. The third is based on the electrocatalytic reduction of NO at chemically modified electrodes [10]. All these sensors were found to show high sensitivity and selectivity and were promising for *in vivo* measurements of NO.

In this report the electroanalytical method using a carbon fibre microelectrode coated with an electrodeposited *p*-type semiconducting porphyrin film was applied for the quantitative measurement of NO in the samples of the arterial blood. The influence of the medical treatment and the physical strain was studied on a group of patients suffering from blood pressure disorder.

EXPERIMENTAL

All chemicals were of reagent grade purity used without any further purification. All solutions were prepared in 0.1 mol dm^{-3} phosphate buffer of pH 7.4.

Voltammetric experiments were performed using potentiostat/galvanostat (Model 273A, EG&G PAR, Princeton, USA) interfaced to IBM AT-80486 computer with custom data acquisition and control electrochemical software made available by the manufacturer. A 3-electrode arrangement was used with an NO-sensor, saturated calomel reference electrode (SCE), and a platinum wire as a counter electrode. The pH measurements were performed with Metrohm pH-meter.

The porphyrin-based microsensor was prepared by the procedure reported in [11]. The base for the construction of the microsensor was the carbon fibre (Amoco Performance Products) 7 μm in diameter. An array of several fibres was inserted into a glass capillary so that *ca.* 5 mm protruded out of its pulled end. A copper wire was covered with a layer of the conductive silver epoxy resin (AI Technology) and inserted into an opposite end of the glass capillary. It was kept at an increased temperature of 80 °C for 30 min for hardening. Then the tip of the glass capillary was sealed with bee's wax.

The polymeric porphyrin film was obtained by electrodeposition on a carbon fibre from the monomeric tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin (TMHPP) with nickel(II) as the central atom. The deposition of the polyNiTMHPP was done from the deaerated (10 min purging with N_2) solution of 0.1 mol dm^{-3} NaOH containing monomeric porphyrin by repeated scan cyclic voltammetry in the potential region from −0.2 to +1.2 V *vs.* SCE with a scan rate 100 mV s^{-1} (20 cycles) until steady cyclic voltammetry responses were obtained.

The final step of the fabrication of the sensor was the dipping of the fibre in a Nafion solution (1 mass % in ethyl alcohol) for 15 s. Next was the sensor left to dry for 5 min. The Nafion coating eliminated the diffusion and the adhesion of anionic species that could be oxidizable at the surface of the microelectrode and made sensor to be specific for NO determination serving as its mediator. The sensor was stored in phosphate buffer of pH 7.4. Due to the good mechanical properties it could be used for a long time and large number of samples.

Differential pulse voltammetry (DPV) was used for the sensor current sensitivity determination as well as for the NO content determination in samples. In both cases the polarization of a sensor was done in the potential region from +0.4 to +1.0 V *vs.* SCE with a scan rate 20 mV s^{-1}. Pulses of 25 mV amplitude with 5 Hz frequency were superposed.

RESULTS AND DISCUSSION

It was necessary to determine the current sensitivity of each sensor before its use for NO analysis. One possibility is to use a standard NO solution. Because of the problems with its preparation and stability different procedure was used. In such case the current sensitivity of a sensor could be determined using another stable analyte solution (*e.g.* NO_2^-). Then it is possible to recalculate the found value of current sensitivity according to the number of electrons exchanged in the redox reaction [12]. The procedure gives satisfactory results for the same working electrode and the same experimental conditions of the electrochemical determination. The determination of the sensor current sensitivity is done before the final coating with the Nafion on a microelectrode surface with the electrodeposited polymeric film. Nafion coating serves as NO mediator and does not influence the active electrode surface for NO determination [11].

The calibration of the microelectrode signal to the concentration of the analyte was done in a series of 5 standard $NaNO_2$ solutions in the range of $c(NaNO_2)$ from 1.0 to 3.0 × 10^{-7} mol dm^{-3} in 0.1 mol dm^{-3} phosphate buffer of pH 7.4. Differential pulse voltammetry was applied in the potential region, scan rate and pulses amplitude are specified in Experimental. DPV peak corresponding to the two-electron oxidation of nitrite was observed at +0.7 V *vs.* SCE. The signal linearly depended on the $NaNO_2$ concentration in the analyzed solutions. In Fig. 1 are given experimental DPV voltammograms registered in the abovementioned series of standard $NaNO_2$ solutions. The current sensitivity was determined for each microelectrode sensor in nA/nmol of the analyte in 1 cm^3 of the solution.

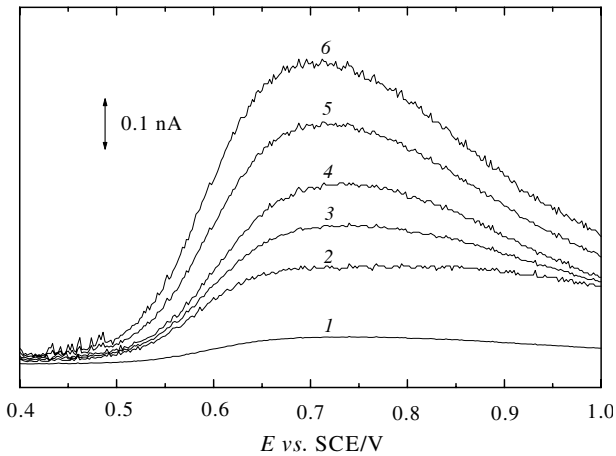

Fig. 1. Experimental DPV voltammograms registered in a series of standard $NaNO_2$ solutions in 0.1 mol dm^{-3} phosphate buffer of pH 7.4 on a porphyrin-based microsensor. *1.* Blank; *2.* $c(NaNO_2)$ = 1.0 × 10^{-7} mol dm^{-3}; *3.* $c(NaNO_2)$ = 1.5 × 10^{-7} mol dm^{-3}; *4.* $c(NaNO_2)$ = 2.0 × 10^{-7} mol dm^{-3}; *5.* $c(NaNO_2)$ = 2.5 × 10^{-7} mol dm^{-3}; *6.* $c(NaNO_2)$ = 3.0 × 10^{-7} mol dm^{-3}.

To confirm the applied procedure the current sensitivities of some sensors were determined also in standard NO solution and compared with the above-described one. No significant difference was found in current sensitivity values determined by both procedures.

Differential pulse voltammetry was also applied for NO determination in blood. In case of NO the evaluated DPV peak corresponded to the one-electron oxidation of NO. It was observed at +0.75 V *vs.* SCE. The concentration of NO in blood varies in the range from 10^{-6} to 10^{-7} mol dm^{-3}. Generally the detection limit of the DPV method is much lower. The sensitivity of the DPV method with the microelectrode NO-biosensor is sufficient for NO determination in the concentration region expected in real blood samples.

Possible $NaNO_2$ interference was investigated in experiments in which nitrite was added to the analyzed sample solution. It was found that the addition of nitrite at the concentration ten times higher than NO did influence neither the height nor the potential of the NO peak. On the basis of this experiment it could be confirmed that Nafion coating prevents the nitrite interference and the peak current is exclusively due to NO.

The interference of some other substances which

may be present in the blood samples must be taken into consideration. NO like other simple diatomic molecules such as O_2 and CO may react with hemoproteins and metalloenzymes [11]. Therefore, the sample in which NO is determined should not contain other electroactive substances coexisting in the biological fluid such as ascorbate, uric acid, and nitrite in large excess. Under usual conditions the abovementioned substances are present at much lower concentrations to interfere with NO determination. Nevertheless, under certain physiological conditions, *e.g.* when the patient is in intense medical treatment the level of some of them (catecholamine, dopamine, epinephrine, 5-hydroxytryptamine) might be higher and the DPV technique cannot be employed [11].

At the testing of sensors with different polymeric films in the electrodeposition process the demetalled porphyrin film was formed on the sensor. It was supposed that nickel central atom within the porphyrin environment is not essential for the detection of NO. Other polymeric films might have (what concerns specificity and sensitivity of NO determination) even more suitable properties. This was the reason for testing another microelectrode sensor – carbon fibre covered with tetraaminophthalocyanine polymeric film. The similar procedure of polymeric film formation – voltammetric electrodeposition – as in case of monomeric tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin with nickel(II) was applied for monomeric tetraaminophthalocyanine with cobalt(II) (polyCoTAPc). The deposition of the polyCoTAPc was done from the deaerated (10 min purging with N_2) solution of 3 mass % acetic acid containing CoTAPc monomer by consecutive cyclic voltammetry under the same conditions as described in Experimental for formation of polyNiTMHPP. The sensitivities of the microsensors were compared. As seen in Fig. 2 the shape and the height of DPV signals were very close. The microsensor the modification of which was based on polyCoTAPc Nafion did not provide any advantage to those based on polyNiTAPc Nafion. This experiment only confirmed that generally microsensors based on polyMTAPc Nafion films serve as mediators for the electrochemical detection of NO. At the unmodified microelectrodes DPV responses of NO exhibit significantly (at least 50 times) smaller and broad peaks in comparison with well developed sharp peaks observed at the modified microsensors.

At the analysis of real samples immediately after sampling with the special type of the capillary syringe the samples of blood for NO analysis were airtightly sealed and stored at the ice-melting temperature (0 °C). Voltammetric determination (*in situ ex vivo*) started several minutes after sampling to eliminate possible decrease of NO concentration with the time of storage.

The sample from capillary was in the environment free of oxygen (in the stream of nitrogen) put into

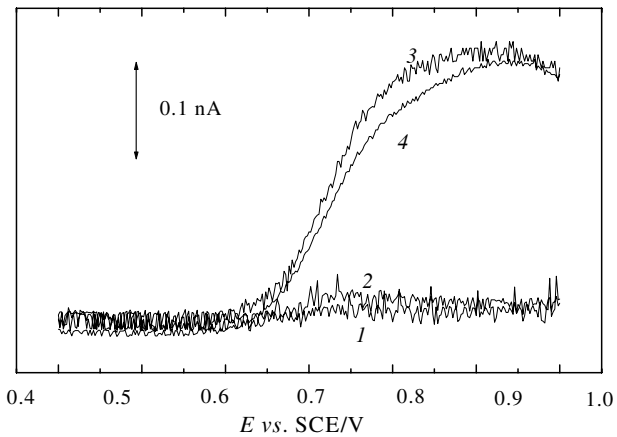

Fig. 2. Experimental DPV voltammograms registered in analysis of NO content in the sample of femoral artery blood using sensors of the close sensitivity (1.05 ± 0.02) nA/nmol NO in 1 cm^3 with surfaces of different modification. *1.* Blank; *2.* unmodified microelectrode; *3.* polyNiTAPc Nafion; *4.* polyCoTAPc Nafion.

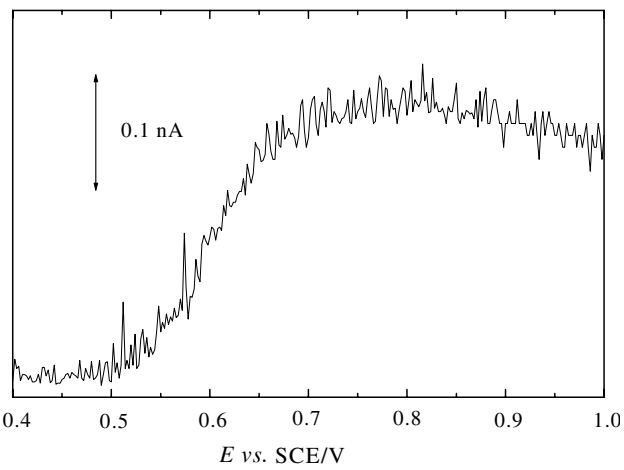

Fig. 3. Experimental DPV voltammogram registered in analysis of NO content in the sample of femoral artery blood. The sensitivity of the applied microelectrode NO-biosensor was (1.05 ± 0.02) nA/nmol NO in 1 cm^3.

low-volume vial (0.4 cm^3) and the electrodes were immersed into it. Polarization of the microelectrode NO-biosensor started and DPV signal was registered. An instrument PAR was set to "auto" mode in which the sensitivity was automatically adjusted to the optimal extent. Fig. 3 presents a typical example of the registered experimental DPV curve for *in situ ex vivo* measurement of NO in a real femoral artery blood sample. The height of the registered DPV peak was measured and after dividing the current sensitivity of the applied microsensor the content of NO in the sample was calculated. In the case of the analysis of the sample the DPV curve of which is depicted in Fig. 3 the measured height of the peak was 0.235 nA which after correction with respect to the sensor sensitivity

Table 1. NO Content and Confidence Interval (95 % Probability Level) in the Real Blood Samples

Patient	c(NO) / nmol dm^{-3}	δ_r/%	Patient	c(NO) / nmol dm^{-3}	δ_r/%
1	241 ± 17	7	*3*	208 ± 21	10
2	235 ± 19	8	*4*	238 ± 19	8

Table 2. NO Content in the Blood of 15 Patients

Patient	c(NO) / nmol dm^{-3}	Patient	c(NO) / nmol dm^{-3}	Patient	c(NO) / nmol dm^{-3}	Patient	c(NO) / nmol dm^{-3}
1^a	230	5^a	240	9^a	240	13^a	215
1^b	210	5^b	215	9^b	215	13^b	215
1^c	220	5^c	230	9^c	245	13^c	255
2^a	215	6^a	225	10^a	235	14^a	225
2^b	205	6^b	220	10^b	225	14^b	220
2^c	230	6^c	230	10^c	245	14^c	250
3^a	220	7^a	240	11^a	230	15^a	220
3^b	205	7^b	225	11^b	215	15^b	210
3^c	210	7^c	230	11^c	245	15^c	250
4^a	230	8^a	240	12^a	225		
4^b	225	8^b	225	12^b	215		
4^c	260	8^c	250	12^c	235		

a) Before any treatment. *b*) After 30 min of the physical exercising. *c*) After 3-week cure in spa. (The relative standard deviation of DPV determination was lower than 10 %.)

corresponds to the NO concentration c = 225 nmol dm^{-3}. Registered experimental curves could be processed by the electrochemical software ECHEM (supplied with PAR). It allows smoothing of experimental curves by elimination of random noise signals. Nevertheless, significant improvement of the accuracy of the determined values was not achieved.

All experimental curves and determined values of NO content given in tables are stored in computer and are at disposal for further comparison or processing.

The determined values of NO content in real blood samples may be influenced by some errors. These come out of the irregularities between the sampling and voltammetric analysis as are a time delay, changes of the temperature of the blood sample during NO determination or intake of the air oxygen to the analyzed sample in vial. These irregularities could be to some extent overcome by keeping an equal procedure from the sampling to the voltammetric NO determination that could minimize this kind of error. An exact procedure was applied to find out the accuracy of the overall process of NO determination. Table 1 summarizes the results of 5 parallel NO determinations in the real samples of 4 patients femoral artery blood. The samples were taken of each patient keeping the exact overall procedure in the half-hour intervals. As it can be seen from the obtained data, the confidence interval at the probability level 95 % is acceptable taking into account the complex matrix and a rather complicated overall procedure of NO determination.

While studying the physiological role of NO in some cases it is satisfactory to know the relative value (or the change) of the determined NO in the blood of a patient during medical treatment. This was the case of our study – to find out the relative change of NO content in blood of a group of patients suffering from cardiovascular diseases accompanied with blood pressure irregularities during their medical treatment. The content of NO in the sample of blood of each patient of the studied group was determined before any treatment after entering the hospital for therapy. Then two parallel studies were done. The aim of the first study was to find out if the physical strain influences the level of NO in blood of patient. The content of NO in blood of patient was determined before and after the half of an hour exercising on a stationary bicycle. The aim of the second study was to find out if the 3-week balneal stay of patients in spa would have some influence on the change of the level of NO in their blood. The results of the study are given in Table 2.

The results of the first study were a surprise. It was expected that physical strain would increase NO level in blood. It was concluded that other factors might have influence on NO content in blood. One of them is that during the physical exercising the blood is in a greater extent saturated with oxygen by intensive breathing. The increased level of oxygen may influence the level of NO content in blood. The oxygen is known to be the scavenger of NO. Of course, some other explanations may also come into consideration. More study is necessary to come to the relevant conclusions.

On the other hand, the results of the second study were in agreement with expectation. After the 3-week balm therapy in spa NO content in blood has increased in most of the patients of the studied group.

DPV method using microelectrode NO-biosensors proved to be an appropriate method for NO analysis in biological samples of the arterial blood. Especially promising would be the application of this method for *in vivo* determination of NO. This procedure would exclude some sources of errors such as time delay between the sampling and voltammetric analysis, changes of the temperature of the blood sample during NO determination or intake of the air oxygen to the analyzed sample in a not quite safely sealed voltammetric vial.

All of these possible sources of errors could be excluded by performing of the *in vivo* NO determinations. Such measurements were until now done only with animals when microelectrode NO-biosensor in catheter was inserted into their cardiovascular system. Because of a risk of infection these experiments are restricted to special clinical laboratories and are beyond our possibilities.

Acknowledgements. The authors gratefully acknowledge financial support from the Slovak Grant Agency VEGA (Project No. 1/9129/02).

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