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Experiments
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AMPEROMETRIC NITRIC OXIDE SENSOR BASED ON MWCNT CHROMIUM(III) OXIDE NANOCOMPOSITE

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Abstract. Multiwalled carbon nanotube and Chromium (III) oxide nanocomposite was used as a mediator in glassy carbon electrodes to improve the performance of electrode for the detection of nitric oxide in comparison with unmodified electrodes. The sensor could be operated as a detector in a flow injection analytical setup under physiological conditions (pH 7.5, 0.1 M phosphate buffer) with an operating potential from 800 mV (vs. Ag/AgCl), a flow rate of the carrier of 0.4 mL/min and an injection volume of 200 µL. The amperometric response of the sensor showed good linearity up to 200 µM with a sensitivity of about 21.6 nA/µM. The relative standard deviation for the repeatability of measurements for 100 µM NO was 3.9% (n = 10 measurements) and the reproducibility was 12% (n = 5 sensors). The effect of all investigated interferences (nitrite, ascorbic acid, dopamine and L-arginine) was not fatal and at 800 mV the signal ratio of NO/NO_2^- is around 10. The new sensor was successfully applied to the determination of NO in mice liver homogenisate after addition of L-arginine.

*Keywords***:** nitric oxide; electrochemical sensor; nanocomposite; MWCNT; chromium(III) oxide

Nitric oxide is implicated in a wide range of physiological and pathophysiological effects (Davis et al., 2001; Lancaster, 1996). In controlled lower concentration it is used for pulmonary vasodilatation and lung disease of prematurity (Weinberger et al., 2001). In lower respiratory tract nitric oxide is produced by various cells and is detectable in the exhaled air of normal human subjects and an exhaled nitric oxide test can help with the diagnosis and treatment of asthma (Kharitonov et al., 1994).

Determination of NO is not a simple task because of its high reactivity; the half-life of nitric oxide in physiological conditions is around 5 seconds; therefore the required analytical methods for its spatial detection have to have rapid response time (Hakim et al., 1996). Due to its short half-life because of the unpaired electron, a direct determination can be made using electrochemical sensors/biosensors because they do not suffer from different components which may be present in samples (Hetrick & Schoenfisch, 2009)**.** Electrochemical sensors can measure NO directly in real time also in vivo, because they can be implanted as micro-sized probes (Zhang, 2004). Most of the investigations are focused on electrochemical sensors because of their easy applicability and low cost. Nitric oxide can be detected with chemically modified electrodes at lower potentials than with unmodified ones which make them useful in different media without significant interference from the sample matrix. Different modifiers were investigated on their ability to decrease the overpotential of nitric oxide on different electrode materials, such as nickel phthalocyanine which has a catalytic effect on the NO oxidation (Allen et al., 2000; Trevin et al., 1996). Decreasing the overpotential is important because at higher potentials possible interferents present in the sample may be oxidized as well. The product of NO oxidation are nitrite and nitrate ions, from which the former may interfere with the determination of NO (Bedioui & Griveau, 2013). Using different polymers such as o-phenylenediamine (Friedemann et al., 1996), poly(thionine) and Nafion (Chen et al., 2006) the selectivity of sensors was increased and could be employed for NO monitoring in rat kidneys. Because of its catalytic effect on the oxidation of NO, a nickel hexacyanoferrate-modified electrode could be operated at a potential of +0.40 V vs. Ag/AgCl (Krylov & Lisdat, 2007), and an indium hexacyanoferrate analogue at +0.75V vs. SCE (Casero et al., 2003) and copper hexacyanoferrate (Berisha et al., 2014). Also, RuO_2 (Peiris, 2009) and Cr_2O_3 (Berisha et al., 2015) were found suitable modification for the quantification of NO. The study presented here is the optimization of working parameters for the sensor based on a modified glassy carbon electrode with MWCNT/chromium (III) oxide nanocomposite for the determination of nitric oxide. The new sensor was successfully applied to the determination of NO in homogenisated mice liver using a flow injection analysis (FIA) system.

Materials and methods

All chemicals used were of analytical reagent grade. MWCNT, Sulfanilamide, N-(1-naphthyl)-ethylenediamine were obtained from Sigma Aldrich and chromium (III) nitrate was purchased from Fluka. Phosphate buffer solution (PBS) was prepared by mixing aqueous solutions of sodium dihydrogen phosphate (0.1 mol/L) and disodium hydrogen phosphate (0.1 mol/L) until the required pH was achieved. A nitric oxide stock solution was prepared by bubbling NO produced by the reaction of a saturated aqueous solution of sodium nitrite with 2 M sulfuric acid through a 4 M potassium hydroxide solution and finally collecting it in phosphate buffer solution (0.1M, pH 7.5). All the apparatus for NO gas production first was deaerated with nitrogen gas for 30 minutes. The standard stock solution was freshly prepared before use. The concentration of NO in the stock solution was determined using sulfanilamide and N-(1-naphthyl)-ethylenediamine as described in (Nims et al., 1995). Sodium nitrite and nitrate stock solution (0.1 M) were freshly prepared before use.

Nanocomposite preparation

Chromium nitrate was mixed with multiwall carbon nanotubes in the ball mill for 15 minutes. The mixture was let in the oven for 4 h at 220°C on fresh air. Treated nanotubes mixture was mixed with nafion solution to prepare a film for modification of glassy carbon electrode.

Apparatus

For cyclic voltammetry and hydrodynamic amperometry, a potentiostat Autolab PSTAT 10 with software GPES version 4.9 and a potentiostat PalmSens with software PSTrace were used. The electrochemical cell consisted of a glassy carbon electrode (unmodified or modified with chromium (III) oxide) as the working electrode, an Ag/AgCl/3 M KCl reference electrode (Metrohm 6.0733.100), and a platinum wire as the counter electrode. Nitrogen was used for degassing the solutions. A magnetic stirrer provided convection of the solution. All potentials mentioned in this paper are referred to the Ag/AgCl reference electrode.

The flow injection system was assembled from a potentiostat (PalmSens and the corresponding software, PSTrace) as the detector, a high performance liquid chromatographic pump (ICI 1100 HPLC Pump), a sample injection valve (5020 Rheodyne, Cotati, CA, USA), and a thin layer electrochemical detector (LC 4C, BAS, West Lafayette, Indiana, USA) with a flow-through cell (spacer thickness 0.19 mm; CC-5, BAS). The working electrode was a glassy carbon electrode (unmodified or modified with chromium (III) oxide), the reference electrode an Ag/AgCl (3 M NaCl) electrode, and the counter electrode was the steel back plate of the cell. All potentials mentioned in this paper are referred to the Ag/AgCl reference electrode.

Preparation of working electrode

Unmodified glassy carbon electrode was polished in alumina slurry before use and was checked for its surface electroactivity with potassium ferrocyanid. The modified glassy carbon with nafion/MWCNT was prepared by mixing 3 mg MWCNT or nanocomposite MWCNT-Cr_2O_3 with 30 μL nafion and 1.50 mL ethanol. This mixture was let on ultrasonic bath for 10 min. and was injected two times 10 μL on glassy carbon electrode surface. The electrode was dried on air.

Procedures

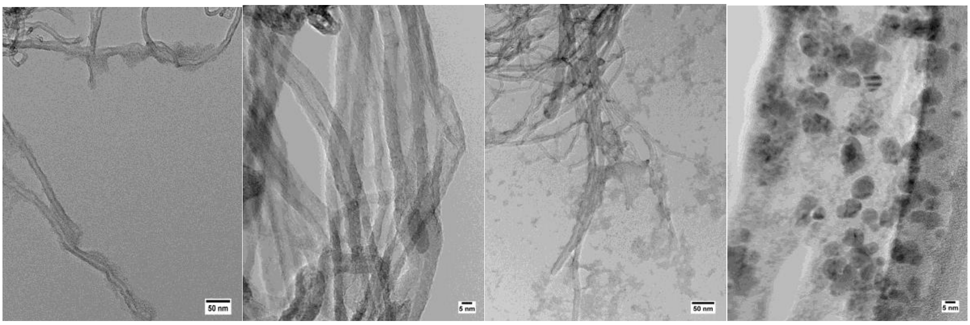
Cyclic voltammograms were scanned between 0 mV and +1200 mV with a scan rate of 50 mV/s, unless stated otherwise. In hydrodynamic amperometry measurements in batch system, also in flow injection analysis mode were made by injection of NO solution (freshly prepared) at different operating potentials from +550 mV to +900 mV.

Sample measurements

The measurements of nitric oxide produced by Nitric Oxide Synthase in mice organs (liver, kidney, heart and brain) were made with the new sensor in flow injection system. After the centrifugation of homogenized mice organs, the supernatant was diluted 1:9 with phosphate buffer. The L-Arginine solution was added on diluted supernatant and was let for 5 min. Spectrophotometric method was used as reference method for measurement of the NO concentration in the stock solution and mice liver homogenized. Methods is based on determination of an azo dye formed from the analyte and an amine coupled to an activated aromate. The calculation for NO concentration were made based on molar absorptivity 12500 $M^{-1}cm^{-1}$and absorbance measurements at λ= 496 nm. The measurement for NO at 496 nm is specific for media of neutral pH.

Results and discussion

The nitric oxide electrochemical sensor was designed on the basis of glassy carbon (GC) electrode modified with nanocomposite of MWCNT-chromium (III) oxide/Nafion. The electrochemical behavior of the modified glassy carbon (GC) electrode towards nitric (II) oxide was studied primarily by cyclic voltammetry and hydrodynamic amperometry, quantitations were done with a flow injection system. The modifier effect was studied in three different pH media 4.5, 7.5 and 9.5 in cyclic voltammetry. The electrode showed a better response on neutral pH because of more clarified modifier effect and oxidation peak of nitric oxide. In Fig. 1 are shown TEM images of nanocomposite prepared as described before, where can be seen the nanoparticles of chromium oxide in the MWCNT.



A B C D

Figure 1. TEM images of unmodified MWCNT (A,B) and modified MWCNT/ Cr_2O_3 (C,D)

Cyclic voltammetry

In Fig. 2 are compared cyclic voltammograms to show the effect of MWCNT-Cr_2O_3 after modification of glassy carbon (GC) electrode. The current is increased after modification of electrode and resulting in appear of reduction peak around 0.30 V of chromium (VI) to chromium (III). Oxidation peak is not observed in cyclic voltammetry but the current is going to increase going in oxidation direction after potential 0.60 V, and it happened because of chromium (III) oxidation but more slowly rate.

Electrode response to nitric oxide (II) after modification with nanocomposite MWCNT-Cr_2O_3-Nafion exhibits oxidation of NO with a current increase after 0.70 V (Fig. 3). The electrochemical oxidation peak of NO and Chromium (III) are not observed but the wide oxidation peak can be attributed to the chemical interaction of NO with chromium (III/VI). At the modified electrode the voltammogram in anodic direction exhibits oxidation after 0.70 V which is ascribed to oxidation of Cr(III) to Cr(VI) and its chemical interaction with NO near the electrode by oxidizing it to nitrite ion and going to more positive potential is reached final step of nitric (II) oxide oxidation producing nitrate ion.

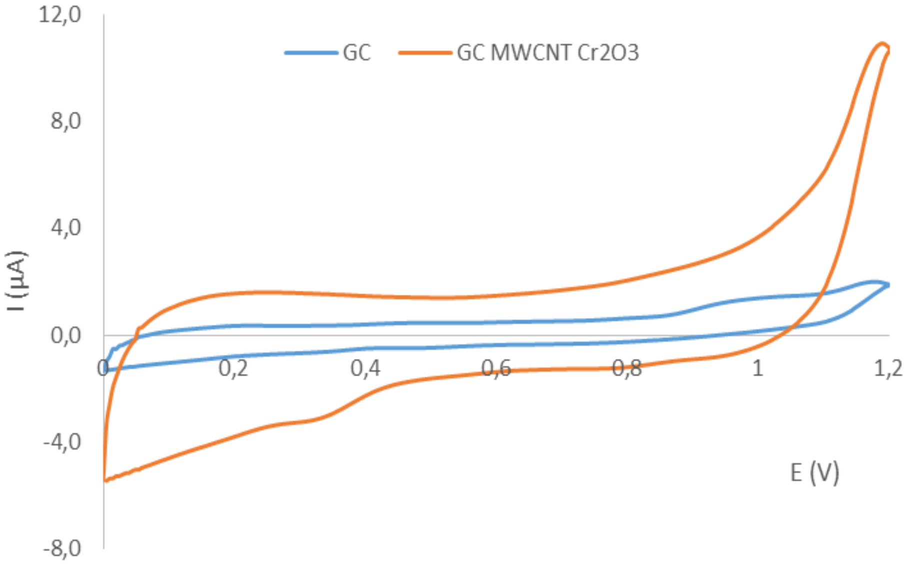


Figure 2. Cyclic voltammograms of GC and modified GC with MWCNT-Cr_2O_3 in phosphate buffer 0.1 M, pH 7.5, scan rate 50 mV/s, Einit. = 0.0 V, Efinal = 1.20 V

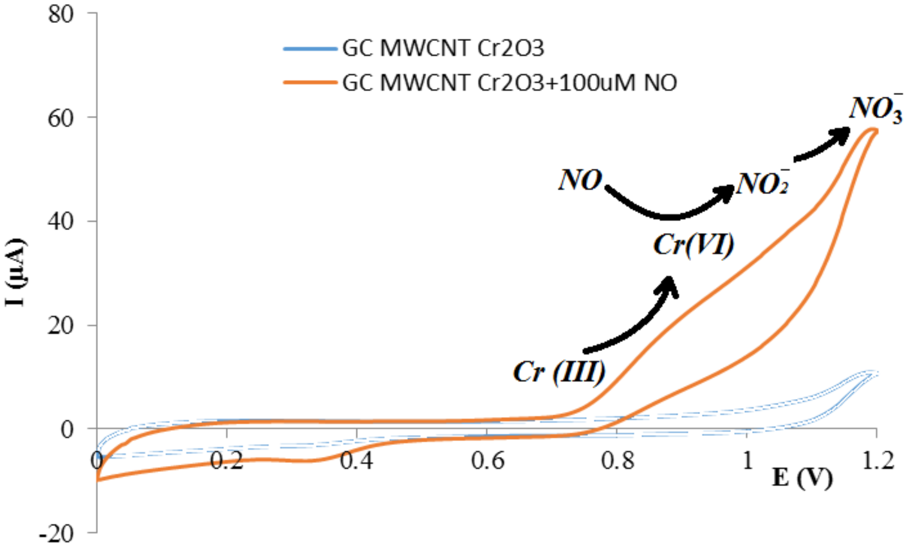


Figure 3. Cyclic voltammograms of GC and modified GC with MWCNT-Cr_2O_3 before and after the addition of 100 mmol/L NO, scan rate 50 mV/s, Einit. = 0.0 V, Efinal = 1.20 V; phosphate buffer 0.1 M, pH 7.5

Hydrodynamic amperometry

The electrochemical mechanism of nitric oxide on modified and unmodified electrode was studied in hydrodynamic amperometry in positive operating potential due to its oxidation observed in cyclic voltammetry. Signal responses are detectable with positive operation potentials; the more positive the latter the higher the current. The sensor was tested also for nitrite response in the same conditions as with nitric oxide in neutral pH.

Operating potential

The sensor can operate in the positive potential range, near the potential of chromium (III) oxidation around 700 mV in which region the mediator effect seems to be most dominant on the nitric oxide detection. Based on results (Fig. 4) for different operating potentials, the more favorable potential is 700 mV because of lower background current and modified electrode shows a better response to nitric oxide compared to unmodified electrode around three times higher. In the same operating potential electrode shows a response to nitrite ions around 70 times lower for the same concentration at operating potential 700 mV. Going to more positive potential electrode response to nitrite ions was increased and go higher than nitric oxide current and it has happened because the oxidation peak of nitrite ion is around 1.0 V.

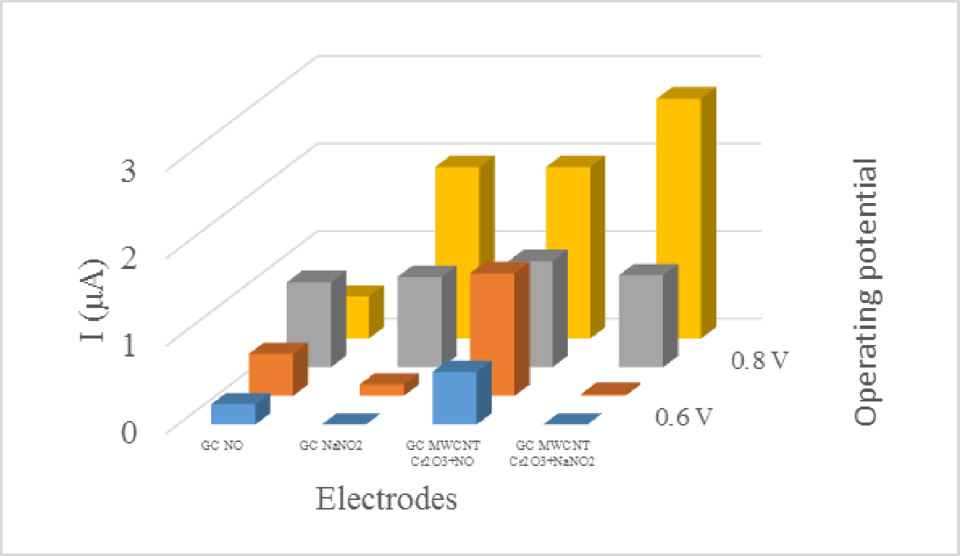


Figure 4. GC and modified GC electrode response at different operating potential in hydrodynamic amperometry

Based on earlier studies the same reaction mechanism is proposed where chromium (VI) is reduced by NO and electrochemically is turned back to chromium (III).

Flow injection analysis

The dependence of the peak currents on the operating potential is shown in Fig. 5. Suitable operating potentials for the sensor for nitric oxide were examined by injecting 50 µM NO at different potentials. Signal responses are detectable with positive operation potentials; the more positive the latter the higher the current. The sensor can operate in the positive potential range, near the potential of chromium (III) oxidation around 700 mV in which region the mediator effect seems to be most dominant on the nitric oxide detection. Based on the results for different operating potentials, the more favorable potential is 800 mV. The latter parameter is closely related to the lifetime of the sensor; if it's lower, the lifetime is longer. Going to more positive potentials, the sensor sensitivity becomes higher. For reasons displayed above already it is good to have high responses at relatively low operating potentials.

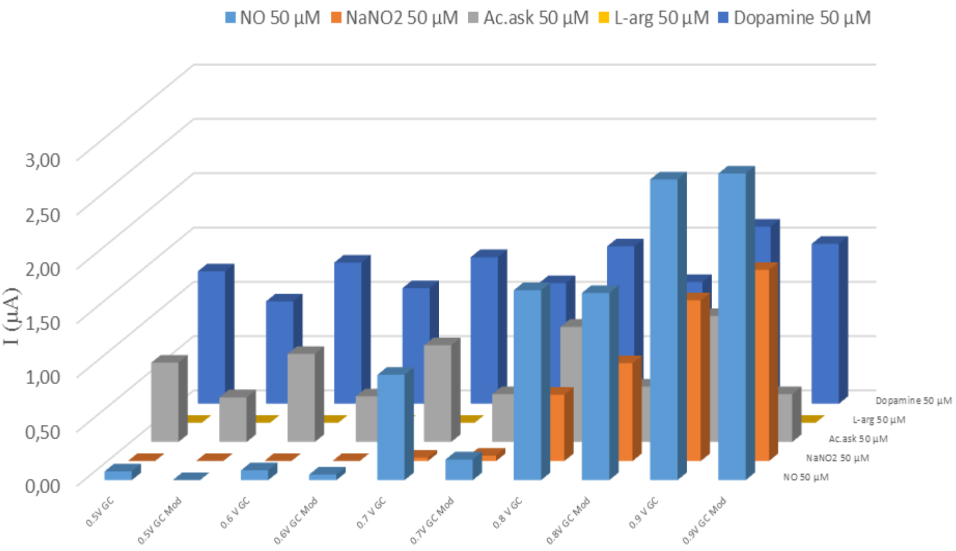


Figure 5. GC and modified GC electrode response at different operating potential in FIA in different operating potential, carrier phosphate buffer pH 7.5, flow rate 0.40 mL/min. and injection volume 200 µL

Unmodified electrode show same response compared to modified electrode but background current decrease after modification and decrease of electrode response to other interferents as ascorbic acid and dopamine, promised more on its application for NO measurements in biological fluids. Electrode response after modification in operating potential 800 mV was same as with unmodified, and it is expected because the electrode surface is covered with nanocomposite of multi-walled carbon nanotube/chromium (III) oxide/nafion. Using nafion as entrapment of nanocomposite material shows better results in neutral pH and electrode live was around six hours measurement at 0.80 V with the addition of analyte and RDS% was less than 25% after. Using anionic polymer also ensured the lower interference from other anionic analytes which are mostly present in real samples such as biological fluids. L-Arginine also was tested as possible interferent because it is a substrate of NO-Synthase producing NO and electrode didn't show response until 100 µM L-Arginine.

Selecting operating potential 0.80 V was taken attention to electrode response also to dopamine and ascorbic acid which are present in biological fluids but their concentration is lower than tested concentration.

Calibration curve and statistics

The calibration curve for NO is shown in Figure 6, obtained by flow injection analysis. A quasi-linear relation between concentration and signal could be obtained for NO concentrations up to 200 µM with a sensitivity of 21.6 nA·L·µmol^{-1} (Equation 1) and a correlation factor R^2=0.996.

$$I(nA) = 21.6 * C(\mu M) + 294.5 \qquad (1)$$

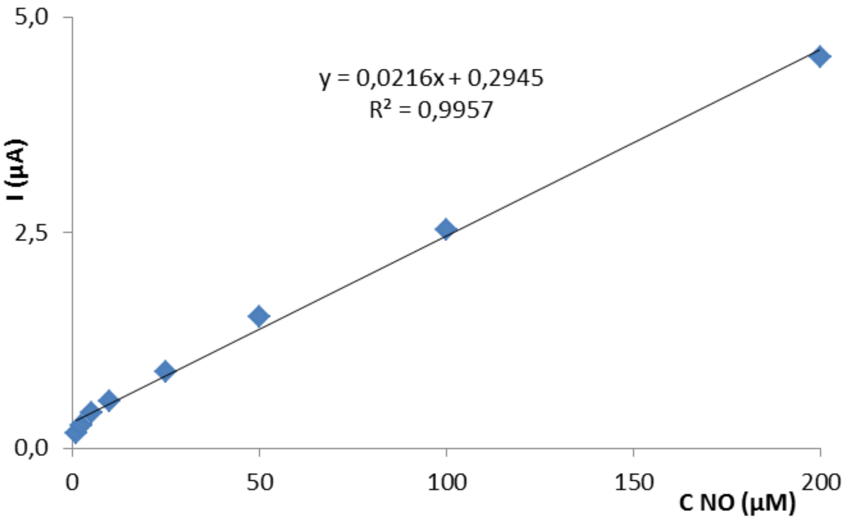


Figure 6. Calibration curve for NO at a glassy carbon modified with MWCNT/ Cr_2O_3/nafion, operating potential 0.80 V, flow rate 0.40 mL/min., injection volume 200 mL, carrier phosphate buffer 0.1M pH 7.5.

The detection limit (3σ) estimated from the standard deviation of 2.5 µM nitric oxide FIA peaks is LOD 0.48 µM. The relative standard deviation for the repeatability of measurements for 100 µM NO was 5.1 % (n = 5 measurements), and the corresponding reproducibility was 12% (n=5 sensors).

Table 1. Determined nitric oxide in rat organs extracts

	Electrochemical sensor (µM)	**Reference method (µM)**
Liver	27.5±2.5	31.0±2.3
Kidney	25.0±2.8	28.0±2.4
Heart	5.2±0.8	6.1±1.4
Brain	10.2±1.8	9.6±1.9

Finally, the sensor was tested in biological fluids, measuring nitric oxide as a product of the enzymatic reaction of NO Synthase contained in rat organs homogenate after addition of L-Arginine. Sacrificed rat weight was 44.6 g and weight of organs were liver 3.262 g, kidney 0.695, heart 0.266 and brain 0.477g. After homogenization for 5 min. with 1500 rpm was centrifuged for 15 min. at 5000 rpm and the supernatant was used in a further experiment. 9.8 mL of phosphate buffer 0.1M was mixed with 200 µL supernatant and 100 µL L-Arginine. After 1 min. mixing solution was taken through nylon filter 0.45µm and injected in the flow injection system. Reference method was used to measure nitric oxide concentration in the same solution with Sulfanilamide and N-(1-naphthyl)-ethylenediamine at 496 nm and blank solution was made without L-Arginine. The results for a rat organs extract sample are presented in Table 1. Results between the method employing the new biosensor and the reference on measuring nitric oxide concentration in organs sample extracts are in very good agreement (Table 1).

Conclusion

The work presented here has clearly demonstrated that using a simple method for nanocomposite preparation of MWCNT with chromium nanoparticles improved electrode response to nitric oxide. The suggested reaction mechanism assumes the reduction of chromium (VI) to chromium (III) by NO, which in turn is oxidized electrochemically at 800 mV. The modified electrodes have a long life time, good stability and high sensitivity which can be exploited for the determination of nitric oxide up to 200 µM. Glassy carbon electrode has been modified with MWCNT/Cr_2O_3-Nafion film and could be used for the detection of nitric oxide at physiological pH (7.5) and operation potential (800 mV) as an amperometric detector in flow injection analysis. The influence of possible interferents on the determination of nitric oxide has been estimated. The new sensor has been successfully applied to the determination of nitric oxide in extracts of rat organs.

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