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Synergistic effect of mediator–carbon nanotube composites for dehydrogenases and peroxidases based biosensors

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ABSTRACT

Very sensitive, low cost and reliable NADH and H_2O_2 sensors were realised and used for development of enzyme based biosensors. The active surface of the electrodes was modified with a nanocomposite obtained by modification of SWNT with a proper mediator: Meldola Blue (for NADH) and Prussian Blue (for H_2O_2). Low applied potential of −50 mV *vs.* Ag/AgCl reference electrode proved the synergistic effect of nanocomposite materials towards NADH and H_2O_2 detection. Biosensors for malic acid and alkylphenols have been developed, using mediator-functionalised-SWNT-based electrodes and two different classes of enzymes: NAD^+-dependent dehydrogenases and peroxidases. Immobilization of the enzymes was realised using a series of different procedures — adsorption, Nafion membrane, sol–gel and glutaraldehyde, in order to find the best configuration for a good operational stability. A higher sensitivity comparing with other reported biosensors of about 12.41 mA/M·cm^2 was obtained for L-malic acid biosensor with enzyme immobilised in Nafion membrane. Phenol, 4-t-octylphenol and 4-n-nonylphenol were used as standard compounds for HRP based biosensor. Fast biosensor response and comparable detection limit with HPLC methods were achieved.

1. Introduction

Carbon nanotubes (CNTs) technology has raised new applications in electrochemical sensors and biosensors, due to their high chemical stability, high surface area and relatively high mechanical strength. Recent experiments suggest that carbon nanotubes surfaces show enhanced electron-transfer rates when used as electrodes in electrochemical reactions [1,2]. Functionalized carbon nanotubes offer enormous potential as components for sensors and biosensors construction. Organic and inorganic molecules like dyes can be easily adsorbed on the carbon nanotubes walls improving the solubility of the resulting material and also the catalytic activity towards different analytes [3,4]. Chemically modified carbon nanotubes can easily be fixed on an electrode surface via physical or chemical bonds from the surface of nanotube walls. There have been some important works about the electrocatalysis of CNTs [5,6] and chemical sensors [7,8]. The prospect of these applications has led to successful functionalization of single-walled (SWNT) and multi-walled (MWNT) carbon nanotubes.

Combination of CNTs with Toluidine blue [9,10], azure dye [11], Meldola Blue [12], 5,5′-dihydroxy-4,4′-bitryptamine (DHB) [13], phenothiazine dyes [14–16] or thionine [17] resulted in remarkable improvement of electroactivity of the composite toward NADH oxidation.

The synergistic effect of CNT and redox mediators has been also explored in order to improve the catalytic activity toward H_2O_2 reduction. Doping CNTs with cobalt hexacyanoferrate [18] and nickel hexacyanoferrates [19] nanoparticles greatly improved the sensitivity for glucose and cholesterol determination through synergistic electrocatalytic effect. The combination of PB and CNTs has been used in different configuration, from a simple mixing between mediator and CNT [20–22] to a electrodeposition of PB on the multi-walled carbon nanotube (MWNT) [23,24] or on the MWNT/PVP matrix [25].

The extremely attractive properties of CNTs for the electrochemical detection of NADH and H_2O_2 commented above have led to the development of novel enzyme biosensors where rapid electron transfer at the electrode substrate is required. An amperometric biosensor based on co-immobilization of alcohol dehydrogenase (ADH) and Meldola's Blue (MB) on MWCNTs, exhibited an excellent sensitivity, operational stability and wide linear response range to ethanol [16]. A nanocomposite of poly(Nile blue A) with SWCNTs allowed the preparation of an ADH biosensor for ethanol with a good stability, reproducibility and high biological affinity [26].

Phenol determination employing HRP/CNT-based biosensors have been earlier described [27] where the redox mediator–methylene blue was adsorbed on MWNT. Nanocomposites based on Toluidine blue (TB) adsorbed on MWCNTs was used for detection of hydrogen peroxide in a reagentless biosensor with HRP immobilized in chitosan [28].

This paper describes the use of carbon nanotubes in the development of two different type of amperometric biosensors based on

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dehydrogenases and oxidases. Malic acid and alkylphenols were chosen as model analytes because their determination involves two different classes of redox enzymes: the NADH-dependent dehydrogenases (for malic acid) and the peroxidases (for alkylphenols).

L-malic acid is the major organic acid in most fruits and vegetables. Organic acids contribute greatly to taste, particularly of fruit, with a balance of sugar and acid giving rise to the desirable taste of specific product. Significant increases in L-malic acid concentration have been shown to serve as a primary indicator of fruit maturity [29]. Hence measurement of L-malic acid provides a more objective means of determining the ripeness and hence 'shelf life' of horticultural product than simple appearance and taste.

Biosensors incorporating L-malic acid-specific enzymes coupled to electrochemical transducers have also been developed [30–32]. Simplicity, rapidity, economy, portability and minimal sample size represent the main advantages of these biosensors.

Alkylphenols (APs) represent microbial-degradation products of alkylphenol polyethoxylates (APEs) which are nonionic surfactants. Alkylphenols, especially nonylphenols (NP) and octylphenols (OP) have been reported to bio-accumulate in the lipids of water organisms [33]. Up to now, detection of alkylphenols was carried out using liquid [34,35] and/or gas chromatography coupled to sensitive detection systems such as mass spectrometry (MS) or MS-MS [36,37]. Most of these techniques are time consuming and expensive due to the cleanup and sample pre-treatment methods, and usually require derivatization procedures. In the last years immunochemical methods have been reported for sensitive and selective detection of alkylphenols [38–40].

In our paper the ability of redox mediator Meldola Blue and Prussian Blue to undergo adsorption/precipitation on carbon nanotubes surfaces is explored in order to convert SWNT into mediator-modified carbon nanostructures. The novelty of our work is sustained by the deposition procedure of mediator–CNT composites on the surface of the electrode, without involving any matrix for their immobilization. The nanocomposites modified electrodes were successfully employed to the oxidation of NADH and reduction of H_2O_2 with remarkable analytical parameters (high sensitivity, rapid response).

A biosensor for malic acid detection was developed based on SWNT/MB sensor and malate dehydrogenase (MDH). Horseradish peroxidase (HRP) immobilised on the surface of SWNT/PB sensor was used for detection of phenols (phenol, 4-t-octylphenol and 4-n-nonylphenol).

The stable immobilization of enzymes on the electrode surface with a high retention of its biological activity is a crucial point for development of biosensors. For this purpose, different immobilisation techniques were studied and the optimal procedure was used for biosensor characterisation.

From our knowledge these are the first biosensors for malic acid and alkylphenols based on mediator-modified SWNT reported in the literature.

2. Experimental

2.1. Materials

Single-walled carbon nanotube (0.5–100 μm length), N,N-dimethylformamide (DMF), β-NADH, NAD^+, Meldola Blue (8-dimethylamino-2,3-benzophenoxazin hemizinc chloride salt), L-malic acid, horseradish peroxidase 25 kU (E.C.1.11.1.7, RZ>3), potassium hexacyanoferrate (III), iron (III) chloride, glutaraldehyde 25%, tetramethoxysilane (TMOS), polyethyleneglycol (PEG600) and 4-t-octylphenol were supplied from Sigma-Aldrich. The Nafion 5%, hydrogen peroxide 30% and methyltrimethoxysilane (MTMOS) were provided by Fluka, while the phenol 97%, HCl 37% and 4-n-nonylphenol were provided by Riedel de Haën. Malate dehydrogenase — MAD-211 (E.C.1.1.1.37), 91.1 IU/mg, was supplied by Sorachim, France.

Potassium chloride, sodium hydroxide and phosphate salts used for preparation of buffer solutions were provided by Sigma-Aldrich.

The standard solutions of 5 mM alkylphenols, 4-t-octylphenol and respectively, 4-n-nonylphenol, were prepared in acetonitrile.

2.2. Apparatus and methods

Amperometric measurements and cyclic voltammetry studies were carried out using a BAS 100B Electrochemical Workstation (BAS, West Lafayette, IN USA) and a μAutolab type III computer-controlled potentiostat (Eco Chemie, Utrech, Netherlands).

All experiments were carried out at room temperature, using a 5 mL conventional three-electrode cell. An Ag/AgCl electrode was used as the reference electrode. Working electrode used for NADH sensors was glassy carbon (GC) (BAS, 3 mm diameter) and graphite screen-printed electrode (DropSens ref. 110, 4 mm diameter) for H_2O_2 sensor.

Cyclic voltammetry experiments have been conducted for a scan rate of 100 mV/s in the relevant potential range. The supporting medium used for NADH detection at SWNT or SWNT/MB sensors was 0.1 M phosphate buffer, with 0.1 M KCl, pH 7.5. For MDH based biosensor, the 0.1 M pyrophosphate, containing 0.1 M KCl and 1 mM $MgCl_2$, pH 8 was used as the supporting electrolyte, while for HRP based biosensors all the experiments were performed using 0.05 M phosphate buffer, pH 7.4.

Catalytic effect and stability of SWNT, SWNT/MB and SWNT/PB-based electrodes were studied by cyclic voltammetry and amperometric measurements. All potentials are referred to Ag/AgCl.

2.3. Preparation of the redox mediator modified SWNT based electrodes

The modification of SWNT with Meldola Blue mediator was performed through the adsorption process by using an aqueous solution of MB in a concentration of 10 mM. In 2 mL of mediator solution, 4 mg of SWNT was added and the mixture was stirred for 2 h and sonicated for 5 min. The solid nanocomposite was filtered and washed many times with deionized water and then it was dried at 60 °C for 1 h. This material will be hereafter designated as SWNT/MB.

Modification of GC electrodes surface was performed with a suspension of 2 mg/mL of SWNT/MB in DMF. 2.5 μL of the suspension were deposited on the electrodes surface and then dried at room temperature. Before modification, GC electrodes were polished with 0.1 and 0.05 μm alumina powder, rinsed with water and sonicated in a water bath for 10 min.

The modification of screen-printed electrodes with SWNT and Prussian Blue was accomplished in two steps: 1) SWNT deposition; 2) surface modification with PB. 5 μL of SWNT suspension 2 mg/mL in DMF was placed directly onto the electrode surface. Solvent evaporation was realised at room temperature. PB deposition was realised by direct precipitation of 0.1 M $K_3[Fe(CN)_6]$ and 0.1 M $FeCl_3$ solutions, in the same conditions as described by Gurban et al. [41].

For comparative study, SWNT modified electrodes were prepared. Therefore, the same volumes of simple SWNT suspended in DMF were placed onto the electrode surface in a similar manner as for mediator-modified SWNT.

2.4. Preparation of the modified biosensors

The enzyme L-malate dehydrogenase (MDH) was immobilised on the SWNT/MB modified electrodes by physical adsorption, by entrapment in a Nafion membrane and by encapsulation in a sol–gel matrix.

For physical adsorption, 2.5 μL enzymatic solution (280 IU/mL) was deposited onto the working electrode surface, over the modifying layer of SWNT/MB, dried at room temperature for half an hour, then kept at 4 °C until use. The notification used for these electrodes is SWNT/MB/MDH.

MDH immobilization in Nafion membrane was accomplished with an enzyme solution of 420 IU/mL mixed with 1% Nafion solution (prepared in buffer), in a ratio of 2:1 (v/v). 2.5 μL of mixture was placed onto the working electrode surface, over the SWNT/MB layer, dried at room temperature for half an hour, then kept at 4 °C until use. The biosensors with enzyme immobilized in Nafion was marked SWNT/MB/MDH-Nf.

The sol–gel matrix used for enzyme immobilization was prepared by hydrolysis of two alkoxide precursors: tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMOS). The alkoxide precursors were mixed with 1 mM HCl, distilled water and polyethylene glycol (PEG600) and sonicated for 15 min, in order to hydrolyse the monomers and to obtain the «sol». The ratio between precursors TMOS:MTMOS of 5:15, and the hydrolysis time of the «sol» of 6 h were used according to previous optimised procedure described elsewhere [22]. The MDH solution (560 IU/mL) was mixed with «sol» in a ratio of 50:50 (vol.) and 2.5 μL of this mixture were spread onto the working electrode surface modified with SWNT/MB layer.

The enzyme horseradish peroxidase (HRP) was immobilised on the SWNT/PB modified electrodes by physical adsorption, entrapment in a sol–gel matrix or by cross-linking with glutaraldehyde 1%.

Physical adsorption of HRP was achieved by deposition of 5 μL buffered enzyme solution (3.7 IU) onto the working electrode surface and dried at room temperature. The biosensor was noted as SWNT/PB/HRP.

For HRP immobilisation by cross-linking, the enzyme was dissolved in a solution of glutaraldehyde 1% and BSA 50 mg/mL, and 5 μL from this mixture (7.5 IU) was deposited on the working modified electrodes surface.

Encapsulation of HRP in sol–gel matrices was performed using the sol–gel procedure as described above. The «sol» was mixed with the enzyme solution (5 mg/mL) in a ratio 50:50 (vol%) and 5 μL (3.7 IU) of this mixture was spread onto the working electrode surface modified with SWNT/PB nanocomposite. The biosensor was noted SWNT/PB/HRP-SG.

The storage of all biosensors was accomplished at 4 °C, in dry state.

3. Results and discussions

3.1. Electrochemical properties of the sensors

The mediator-modified-SWNT electrodes were characterized by cyclic voltammetry. For both types of electrodes, voltammetric behaviour was compared with that of SWNT-modified electrodes.

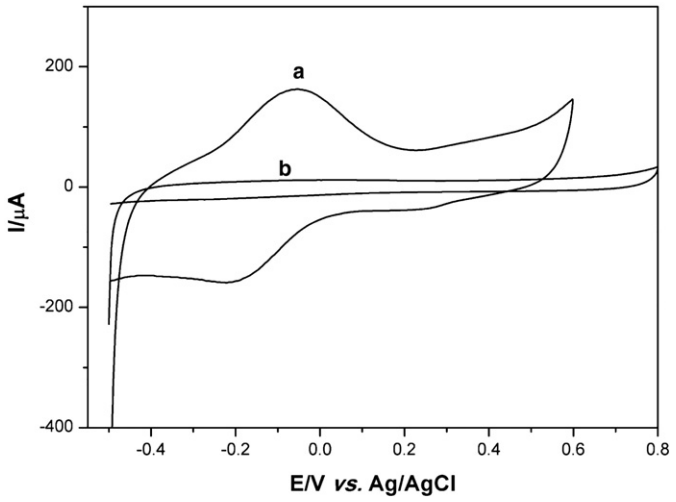

Fig. 1. Cyclic voltammograms in phosphate buffer solution, pH 7.5 performed with (a) SWNT/MB-modified electrode and (b) SWNT modified electrode. Scan rate: 100 mV/s.

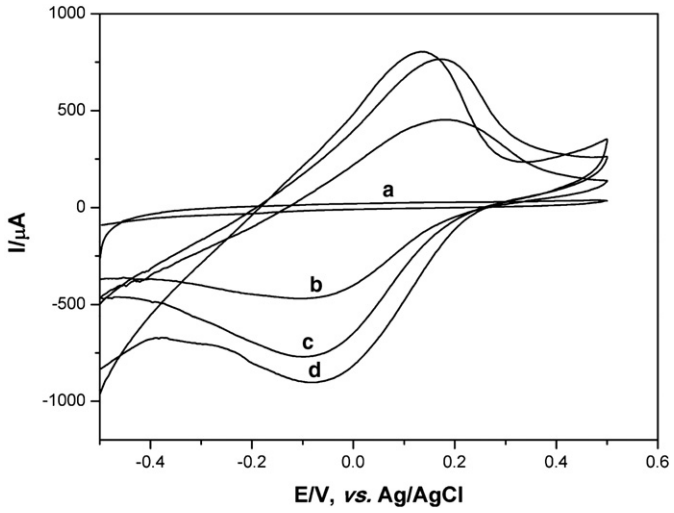

Fig. 2. Cyclic voltammograms recorded at SWNT electrode in (a) buffer and SWNT/PB electrode in (b) phosphate buffer 0.05 M, (c) 1 mM H_2O_2, (d) 5 mM H_2O_2. pH 7.4; Scan rate: 100 mV/s.

As reported in the literature [42] the redox couple of MB appears well defined in the voltammetry. Fig. 1 illustrates that cyclic voltammogram of the SWNT/MB based electrodes (curve a) shows a couple of well defined redox peaks, while the voltammogram for SWNT based electrodes (curve b)) in phosphate buffer does not present any peak. This difference suggests that redox peaks could be attributed to the electrochemical reaction of MB mediator adsorbed onto the SWNT walls. The formal potential of MB calculated as the average value between Epa and Epc, was −150 mV. This value is in accordance with those reported for MB adsorbed on graphite electrode (−175 mV) [43], on zirconium phosphate (−150 mV) [44], or on CNT (−198 mV) [15].

The effectiveness of SWNT and SWNT/PB nanocomposite deposition procedure onto the electrodes was verified by scanning the potential in the range from −0.6 to +0.6 V *vs.* Ag/AgCl. Fig. 2 shows the voltammograms obtained using SWNT-modified electrode (cycle a) and respectively, nanocomposite SWNT/PB modified electrodes (cycle b).

A couple of stable and well-defined redox peaks was obtained for SWNT/PB-based electrode, with the formal potentials of −0.05 V.

3.2. Electrocatalysis of NADH and H_2O_2

The electrocatalytic properties of the mediator-modified-SWNT-based electrodes towards the oxidation of NADH and reduction of H_2O_2 are further presented. Fig. 3 shows voltammetric responses of SWNT and SWNT/MB based electrodes in the presence of NADH, as target analyte (cycles a and b). For unmodified SWNT based electrode, the oxidation peak of NADH is observed at a potential of +0.3 V. Using SWNT/MB electrodes the oxidation occurred at lower potential. The oxidation peak potential of −0.050 V is even lower than the reported potential for NADH oxidation at CNT modified electrodes [45–48]. The figure also shows the voltammetric response of SWNT/MB electrode for different concentration of NADH. For concentrations of 1 and 5 mM NADH (cycles c and b), a significant increase of anodic current was observed, demonstrating the electrochemical reactivity of SWNT/MB towards the oxidation of this compound. The increase of oxidation peak current proves that SWNT/MB composite material can effectively catalyze the oxidation of NADH, which diffuses towards the electrode surface and reacts with the oxidized form of the MB mediator. Thus, the reoxidation of the mediator leads to an augmentation of anodic peak current.

The catalytic effect of SWNT/PB nanocomposite on the H_2O_2 reduction is presented in Fig. 2 cycles c and d. Increase of the hydrogen

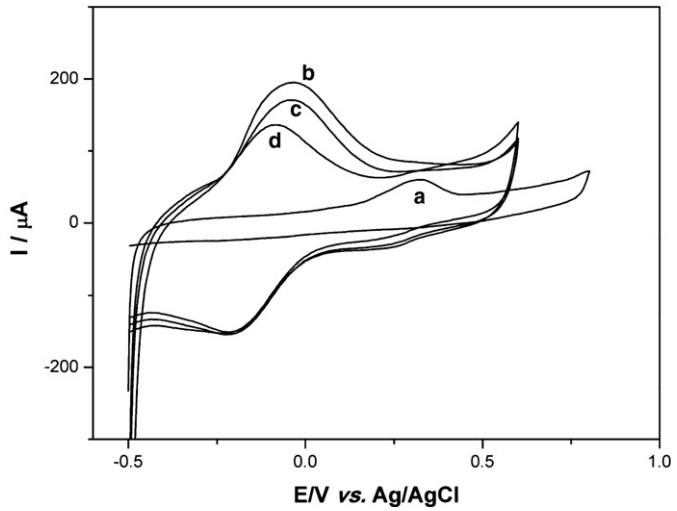

Fig. 3. Cyclic voltammograms of SWNT/MB-based electrodes in buffer solution in the absence (d) and in the presence of 1 mM (c) and 5 mM (b) NADH; (a) SWNT based electrode in the presence of 5 mM NADH. Scan rate: 100 mV/s.

peroxide concentration induces an increase of the reduction current, proving in this way the catalytic properties of SWNT/PB nanocomposite toward H_2O_2 analyte.

3.3. Calibration of mediator-modified-SWNT based sensors

Additionally to cyclic voltammetry experiments, amperometric assays were performed in order to characterize the SWNT/MB- and SWNT/PB-modified electrodes as sensors of NADH, respectively H_2O_2, in terms of sensitivity, linear range, response time and detection limit. The calibration curves of these electrodes were obtained by increasing the addition of analyte, NADH, respectively H_2O_2, in the buffer solution medium.

For SWNT/MB-based electrodes, calibrations were carried out by successive additions of 50 mM NADH in phosphate buffer pH 7.5, applying the potential of −0.05 V *vs.* Ag/AgCl. The calibration curves of SWNT/PB-based electrodes were obtained by addition of H_2O_2 10 mM in 0.05 M phosphate buffer, pH 7.4. The main characteristics of mediator modified-SWNT-based electrodes are summarized in Table 1.

Sensitivity of mediator-modified-SWNT-based electrodes was increased by 2 times, for both sensors and the detection limits were lowered. The potential required for NADH oxidation is with 350 mV lower for the nanocomposite based sensor. An improved selectivity will be assured for dehydrogenase based biosensors developed using this type of detector. SWNT/PB based sensor presents a better response time for H_2O_2 comparing with SWNT sensor. The reproducibility of these sensors was studied by performing calibration curves using 5 sensors in the same experimental conditions.

3.4. Operational stability of mediator-modified-SWNT based sensors

The operational stability of the mediator-modified SWNT-based sensors was evaluated by repetitive amperometric determinations for the same concentration of target analyte, applying the potential of −0.05 V *vs.* Ag/AgCl.

NADH sensor based on SWNT/MB-electrode maintains 94% of initial response after 15 repetitive measurements of 0.05 mM NADH. A slow decrease of the amperometric response could be noticed after 10 determinations. This could be explained by loss of MB from the nanotubes surface during the stirring process and rinsing the electrode between measurements. Overall, the results show a stable electrode toward amperometric determination of NADH, suitable for dehydrogeases based biosensors development.

The operational stability of H_2O_2 sensor was studied by measuring the analytical response to 0.1 mM H_2O_2 at an applied potential of −0.05 V *vs.* Ag/AgCl. An improvement of sensors stability was observed when the single-walled carbon nanotubes were modified with Prussian Blue. The average current for 20 repetitive determinations was 2517 ± 137 nA with RSD = 5.5%. In the case of SWNT modified electrodes, 1508 ± 284 nA was the average current with an RSD of 18.8%.

Table 1
Analytical characteristics of carbon nanotubes modified electrodes ($n = 5$ electrodes).

Analyte	Electrode	Potential *vs.* Ag/AgCl (V)	Sensitivity ($mA \cdot M^{-1}$ cm^{-2})	LOD (3σ) (μM)	Linear range (mM)	R^2	Response time (s)
NADH	SWNT	0.3	65.1 ± 3.5	0.5	0.01–3.10	0.9996	4
	SWNT/MB	−0.05	107.1 ± 1.1	0.4	0.02–2.54	0.9962	4
H_2O_2	SWNT	−0.2	53 ± 14	0.9	0.08–2.52	0.9854	32
	SWNT/PB	−0.05	129.4 ± 3.6	0.4	0.01–1.75	0.9952	13

3.5. Optimization and characterization of enzyme based biosensors

3.5.1. Optimization of immobilization method for biosensors

Biosensors based on enzyme immobilisation by different techniques on the electrodes surface modified with SWNT/mediator nanocomposite were characterised by amperometric studies. A good immobilisation method should provide a satisfactory stability of the biosensors besides a good sensitivity. Consequently, the stability tests were performed for all types of SWNT/MB/MDH and SWNT/PB/HRP biosensors.

The operational stability of SWNT/MB/MDH biosensors prepared according to the experimental section was investigated by repetitive measurements of 0.5 mM L-malic acid, by applying the proper potential *vs.* Ag/AgCl.

The decrease of the biosensor response was about 18% when the enzyme is adsorbed on the electrode surface, and 50% (with a very low sensitivity) when enzyme is encapsulated in the sol–gel matrix. The best results were achieved using Nafion 1% membrane which assures a stability of 95% after 10 repetitive measurements (Fig. 4). Further experiments were performed only for MDH immobilised with Nafion.

The SWNT/PB/HRP biosensors prepared as described in experimental section were tested for a concentration of 50 μM phenol in

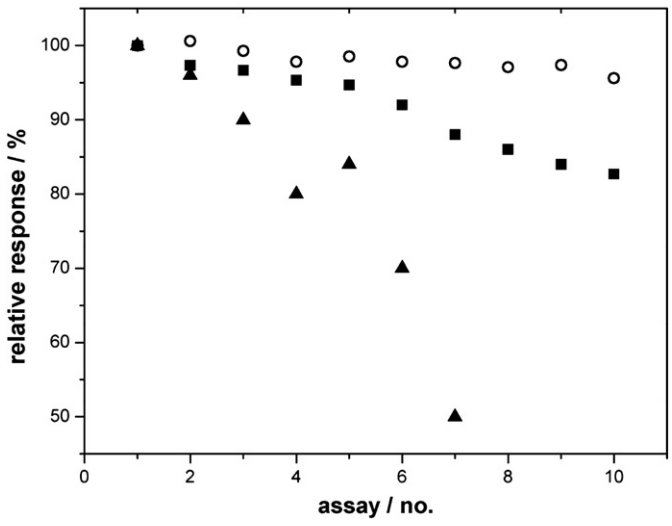

Fig. 4. Operational stability of the MDH based biosensors prepared by different immobilisation methods: adsorption (■ — initial current 750 nA), Nafion 1% membrane (○ — initial current 680 nA) and sol–gel entrapment (▲ — initial current 50 nA). (pH 8, 0.5 mM L-malic acid, 0.7 IU MDH, 2 mM NAD^+, $E = -50$ mV).

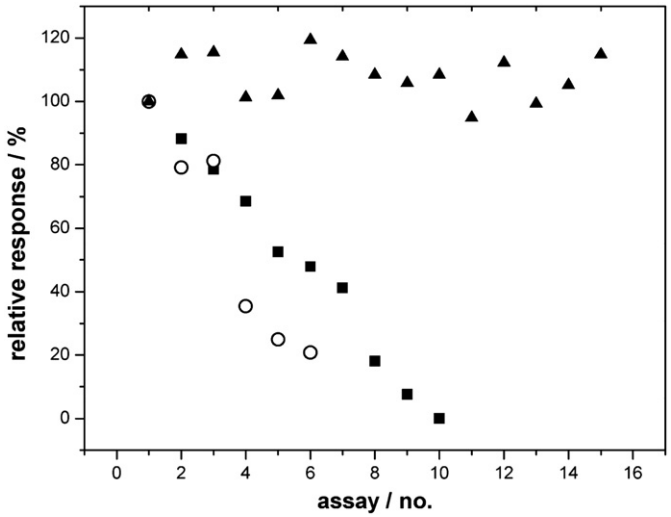

Fig. 5. Operational stability of the HRP based biosensors obtained by different immobilisation methods: glutaraldehyde 1% (■ — initial current 238 nA), adsorption (○ — initial current 48 nA) and sol–gel (▲ — initial current 155 nA). (pH 7.4, 60 μM H_2O_2, 50 μM phenol, $E = -50$ mV).

0.05 M phosphate buffer, pH 7.4, containing 60 μM H_2O_2. The analytical responses decreased to 20% of the initial signal, after only 6 assays, in the case of physical adsorption of HRP. Progressive decreases of the analytical responses were also registered for the biosensors containing the enzyme immobilised using glutaraldehyde (Fig. 5).

An acceptable stability was achieved for biosensors with the HRP enzyme entrapped in sol–gel matrix; for 15 successive assays the average current was 167 ± 11 nA with an RSD of 6.8%. Taking into account all these aspects, the biosensors obtained by entrapment of HRP in sol–gel matrix have been used further for the detection of phenol, and respectively alkylphenols (4-t-octylphenol and 4-n-nonylphenol).

3.5.2. Optimization of the working pH for malic acid biosensor

As mentioned above, the equilibrium of reaction catalyzed by malate dehydrogenase lies very far in the direction of L-malate formation in the neutral pH region. Therefore, it is necessary to shift it in favour of NADH formation, either by removing the reaction products using a second enzyme [49–51] or by using a basic pH [30,32]. Another approach is to use an efficient mediator which consumes the NADH produced in the enzymatic reaction, thus shifting the equilibrium of reaction to the products side.

The optimization of working pH for SWNT/MB/MDH-Nf biosensor was performed by measuring the amperometric response to oxidation of 0.5 mM L-malic acid in pyrophosphate buffer 0.1 M of different pH values. The investigated pH range is between 7 and 9.3. The maximum response was registered for pH 8 and 8.5 ($n = 3$) for medium solution.

The selection between the two pH values for the buffered medium (8 and 8.5) has been done taking into account the stability of the amperometric response of biosensor in each reaction medium. For this experiment, the steady-state current obtained for oxidation of 0.5 mM L-malic acid in pyrophosphate buffer 0.1 M, pH 8, respectively 8.5, was recorded for 30 min.

The decrease of catalytic current is about 13% when reaction takes place in buffer pH 8.5, while for buffer pH 8 the decrease is smaller than 4.5%. pH 8 value represents a compromise between a higher biosensor stability and good enzyme activity, therefore, the optimum pH for SWNT/MB/MDH-Nf biosensor.

3.5.3. Optimization of the H_2O_2 concentration for phenol biosensor

The hydrogen peroxide concentration plays an important role especially in dependence of current intensity by direct or mediated

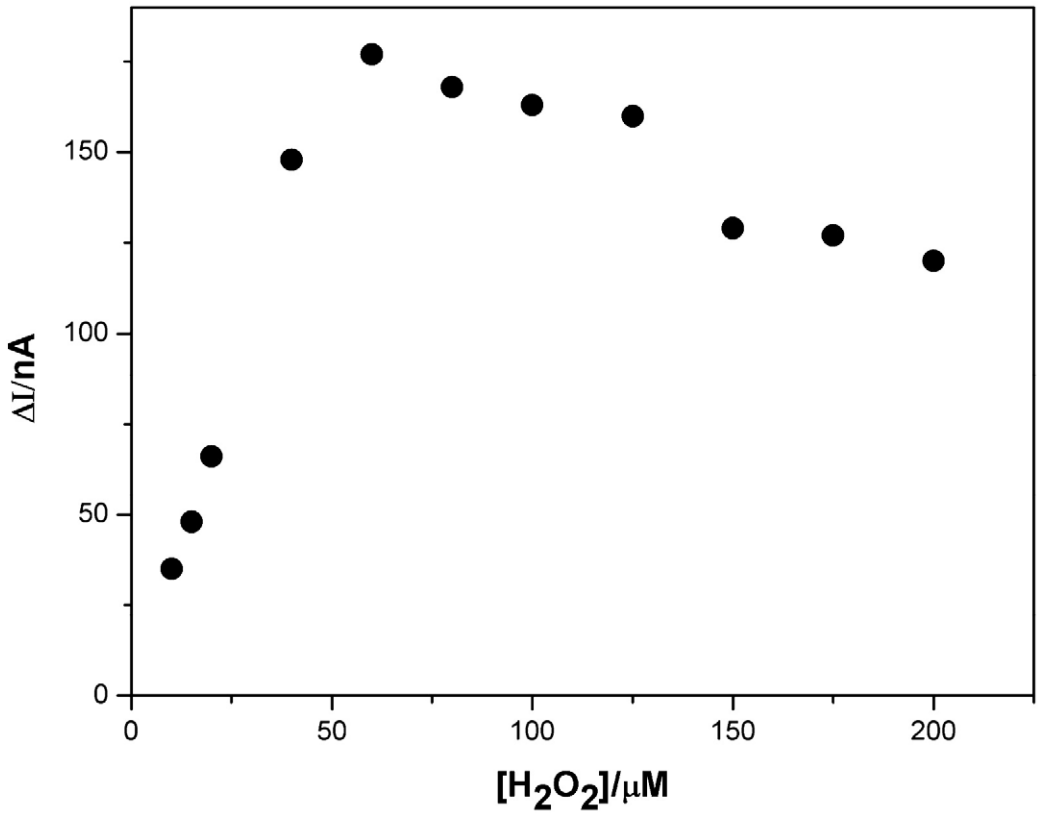

Fig. 6. Dependence of analytical response for SWNT/PB/HRP based biosensors by H_2O_2 concentration (phenol 50 μM, $E = -50$ mV).

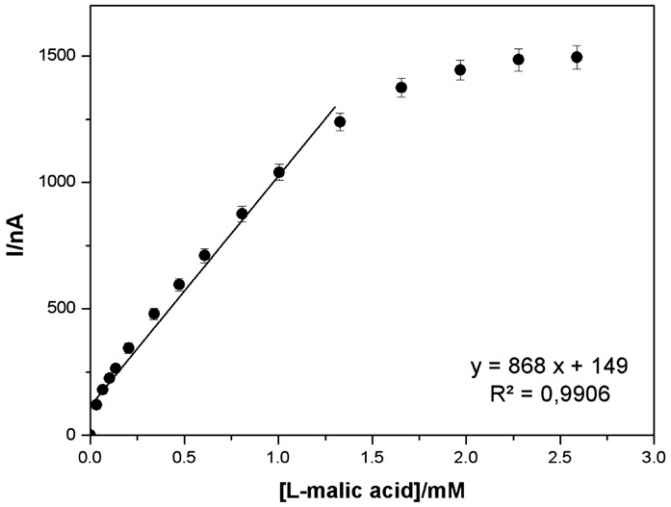

Fig. 7. Calibration curve of SWNT/MB/MDH-Nf for L-malic acid. (pH 8; 0.7 UI MDH, 2 mM NAD^+, $E = -50$ mV).

electronic transfer. Fig. 6 presents the dependence of analytical response for SWNT/PB/HRP based biosensors by H_2O_2 concentration, for a phenol concentration of 50 μM and an applied potential of −0.05 V *vs.* Ag/AgCl. HRP was immobilised by entrapment in sol–gel matrix.

The concentration of H_2O_2 is very important for obtaining a good sensitivity and also to avoid the inactivation of enzyme due to a high substrate concentration. The effect of the hydrogen peroxide was moderate for H_2O_2 concentration up to 60 μM. In order to obtain a good biosensor response the minimum ratio between H_2O_2 and phenol was fixed to 5:6.

3.5.4. Calibration of biosensors

3.5.4.1. Calibration of SWNT/MB/MDH-Nf for L-malic acid. The calibration of SWNT/MB/MDH-Nf biosensor was carried out by successive addition of substrate (L-malic acid) in the reaction medium containing 2 mM NAD^+. Fig. 7 shows the catalytic current measured as a function of total concentration of L-malic acid. A linear variation of the response is observed in the range of 0.034–1.35 mM, with a sensitivity of 868 ± 28 nA/mM. The specific sensitivity is 12.41 ± 0.4 mA/M·cm^2,

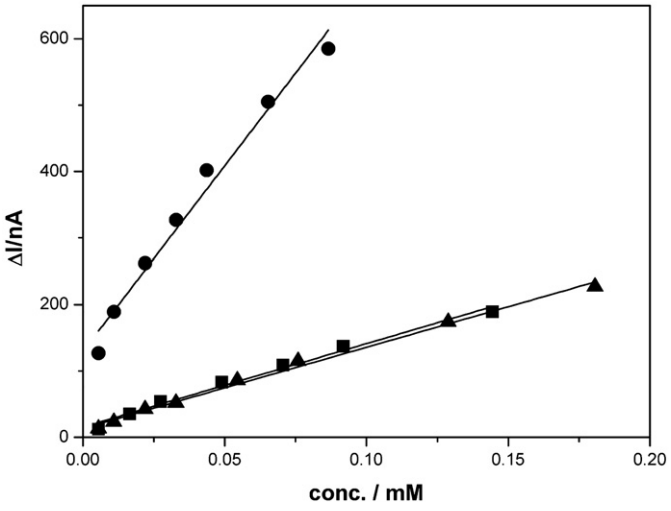

Fig. 8. Calibration of SWNT/PB/HRP-SG based biosensors for phenol (●), 4-t-octylphenol (■) and 4-n-nonylphenol (▲). (pH 7.4, 60 μM H_2O_2, 3.7 IU HRP, $E = -50$ mV).

Table 2
Analytical parameters of biosensors based on SWNT/PB nanocomposite and HRP enzyme ($n = 3$ electrodes).

Immobilisation matrix	Sensitivity (mA·M^{-1} cm^{-2})	RSD % ($n = 3$)	Linear range (mM)	Detection limit (μM)	Response time (s)
Sol–gel	282 ± 2	0.7	0.005–0.086	0.25	6

with a detection limit of 3 μM for a ratio signal/noise = 3. This biosensor presents a higher sensitivity than other biosensors reported in literature for detection of L-malic acid [30,31,52].

3.5.4.2. Calibration of SWNT/PB/HRP-SG biosensor for phenolic compounds. The calibration of SWNT/PB/HRP-SG biosensors was carried out by successive additions of 5 mM phenol, in 0.05 M phosphate buffer, pH 7.4, containing 60 μM H_2O_2 (Fig. 8). Analytical parameters extracted from the linear range of calibration curves accomplished for SWNT/PB/HRP-SG biosensors are given in Table 2.

The detection limit was determined for a ratio signal:noise = 3. The response time of the biosensors was very short, reaching 95% from the maximum response in only 6 s.

Other phenols with a relatively high toxicity such as 4-t-octylphenol and 4-n-nonylphenol were tested and their calibration curves are presented in Fig. 8.

The calibration tests were performed by successive additions of 5 mM alkylphenols in 0.05 M phosphate buffer, pH 7.4, containing 60 μM H_2O_2 and appling a potential of −0.05 V *vs.* Ag/AgCl. Analytical parameters obtained from the calibration curves are given in Table 3.

The detection limits were calculated taking into account the ratio signal/noise to be 3. These values are quite comparable with the detection limits reported in literature for 4-t-octylphenol and respectively 4-n-nonylphenol using chromatographic techniques [53].

3.5.5. Long-term stability of biosensor for L-malic acid

The long-term stability of L-malic acid biosensor based on SWNT/MB composite material was evaluated by measuring the response to 0.5 mM L-malic acid concentration, at every second days, over a period of two weeks. Between measurements, the electrodes have been stored at 4°C. The amperometric response of SWNT/MB/MDH-Nf electrodes is sufficiently stable; the decrease of the anodic current was 12% after two weeks (8 determinations).

The good stability of the biosensor reveals the fact that enzyme immobilization method does not negatively affect their activity. Nafion membrane stabilizes also the SWNT-MB catalytic layer onto the electrode surface.

3.5.6. Stability of biosensor for phenolic compounds

Stability of the SWNT/PB/HRP-SG based biosensors for the detection of 4-t-octylphenol and respectively, 4-n-nonylphenol was studied by measuring the analytical response to injection of 50 μM alchylphenols in 0.05 M phosphate buffer, pH 7.4, containing 60 μM H_2O_2, and applying a potential of −0.05 V *vs.* Ag/AgCl. The average current intensity for 4-t-octylphenol was 333 ± 16 nA, with an RSD of 5%, while for 4-n-nonylphenol the average current was 207 ± 42 nA, with an RSD of 20.3%. A slightly increase of current was observed for both phenols during measurements probably due to the increase of the permeability by hydration of the catalytic layer.

Table 3
Analytical parameters of SWNT/PB/HRP based biosensors for alkylphenols detection ($n = 5$ electrodes).

Analyte	Sensitivity (mA·M^{-1} cm^{-2})	RSD % ($n = 3$)	Linear range (mM)	Detection limit (μM)	Detection limit by HPLC-DAD [53] (μM)
4-t-octylphenol	9.5 ± 1.1	11.4	0.005–0.15	1.51	0.19
4-n-nonylphenol	10.46 ± 0.51	4.8	0.005–0.18	0.48	0.27

4. Conclusions

In this study, we have described the use of nanocomposite materials modified electrodes for the detection of two different analytes. The modification of the SWNT material with two electrochemical mediators, Meldola Blue for NADH detection and Prussian Blue for H_2O_2 detection, provides a significant increase of the sensors sensitivity and decrease of the detection limit. Electrochemical studies demonstrated the synergistic effect of nanocomposite materials used.

Sensitivity of mediator-modified-SWNT-based electrodes has increased by approx. 2 times, for both analytes and the detection limits were lowered. The SWNT/MB-electrodes exhibit a substantial negative shift of the anodic peak potential of NADH compared to SWNT-electrodes (from +0.3 V to −0.05 V).

Different strategies for biocomponent immobilisation were used. The best results for MDH based biosensor were obtained using Nafion membrane. A linear response range was achieved between 0.034 and 1.35 mM L-malic acid. Biosensor shows a good long-term stability.

The HRP based biosensor was first optimised and characterised using phenol as substrate. It was shown that sol–gel matrix was the proper immobilisation method, which can provide superior analytical performances for detection of phenol and further for detection of some alkylphenols. Good sensitivity, fast response and comparable detection limit with recent chromatographic methods reported recommend these biosensors for detection of different alkylphenols.

A new perspective is open by this study for development of future dehydrogenase and peroxidase based biosensors.

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