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Meldola's Blue - doped titania sol-gel sensor for NADH determination

Research Article

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Abstract: Titania layers obtained by a sol-gel technique doped with redox mediator, Meldola's Blue, were employed for construction of a new NADH senor. Optimization of preparation process as well as experimental conditions affecting the response of the sensor were examined. Under optimal conditions NADH could be determined in the wide linear range from 90 to 2300 μM with detection limit 12 μM and a high sensitivity 12.5 nA μM^{-1}. The usefulness of developed sensor was preliminarily checked in determination of NADH forming during enzymatic oxidation of ethanol catalyzed by alcohol dehydrogenase (ADH).

Keywords: *Sol-gel technique • Titania • NADH sensor • Meldola's Blue*

1. Introduction

In recent years an increasing interest has been observed in the development of small, portable and inexpensive devices that can be used for analytical purposes. Enzymatic biosensors, especially based on amperometric transducers are capable of fulfilling these requirements. Many enzymes used demand nicotine adenine dinucleotide (NAD^+) as a cofactor. In such biosensors analytical signal is obtained as an oxidation current proportional to the amount of reduced form of cofactor (NADH) generated during enzymatic reaction of analyte consumption. Near 300 dehydrogenases are known which require nicotinamide coenzymes as co-reactants and many different matrices were proposed for immobilization of these enzymes [1].

Immobilization of bioelement into matrix plays a crucial role for biosensor application and stability. Sol-gel glasses are known to have very promising properties as a sensor matrix, because matrices can be prepared under ambient temperature and can retain the catalytic activity of bioreceptors [2,3]. By changing experimental conditions of sol-gel process, gel structures of different characteristics can be obtained. Compared with other immobilization matrices, sol-gel layers can entrap large amount of enzymes, they are thermal and chemical stable. Moreover, during sol-gel process additional substances, such as redox mediators, *e.g.* Os complexes [4], cobalt (II) phthalocyanine [5], poly(nile blue A) [6], and Meldola's Blue (MB) [7], can be easily incorporated into sol structure. Entrapped mediators allowed oxidation or reduction of interesting species at lower potentials.

It is well know that direct NADH oxidation at conventional electrodes, such as platinum, gold, and carbon, requires large overpotential (in most cases about 1 V) that would lead to the oxidation of other electroactive species present in the media. Furthermore, adsorption of NADH oxidation products at unmodified electrode surface cause its fouling manifested by decreasing of oxidation current [8]. Therefore, modification of electrode surface seems to be necessary in NADH sensor construction.

This work proposes a new NADH sensor with redox mediator immobilized in titania gel matrix. Titania gel was prepared by the sol-gel method. Optimization of construction as well as measuring conditions of proposed NADH sensor were performed. The dependence on sensor response was investigated in terms of mediator concentration in titania gel, pH of supporting electrolyte, and polarization potential of working electrode. The usefulness of developed sensor was preliminarily checked for determination of NADH forming during enzymatic conversion of ethanol to aldehyde catalyzed by ADH (Scheme 1).

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2. Experimental Procedure

2.1 Chemicals and reagents

Saccharomyces cerevisiae alcohol dehydrogenase (316 U mg^{-1}), β-NADH, β-NAD, titanium(IV) isopropoxide and Meldola's Blue (MB) were purchased from Sigma (USA); ethanol (96%), 2-propanol, L-(+)-ascorbic acid, H_3PO_4 (85%), KH_2PO_4, $Na_4P_2O_7 \cdot 10H_2O$, and glucose (all analytical grade) were from POCh (Poland); NH_{3aq} (25%), HCl (35%), were bought from LACH-NER (Czech Republic); acetone (HPLC grade), 100% acetic acid (Suprapur) and $Na_2HPO_4 \cdot 2H_2O$ were from Merck (Germany). 0.3 μm alumina (Buehler Micropolish, USA) was used for polishing surface of working electrode. Solutions were prepared using double-distilled water and, if necessary, deaerated using high purity nitrogen (99.996%).

Buffer solutions were prepared by mixing appropriate volumes of 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 (pH range from 5.0 to 7.5) or by mixing 0.1 M $Na_4P_2O_7$ with 0.85 M solution of H_3PO_4 (pH from 8.0 to 9.0).

2.2 Apparatus

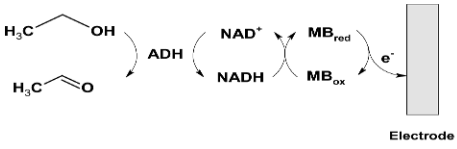
EMU multimeter (WUT, Poland) was employed for electrochemical measurements. A three-electrode system, consisted of graphite working electrode (WE), saturated silver/silver chloride reference electrode (RE) (Mtm-anko, Poland) and platinum auxiliary electrode (AE), was used. Ultrasonic bath Sonic 3 (POLSONIC, Poland) was used in sonification procedure and combined glass electrode ERH-11 (HYDROMED, Poland) together with pH-meter CP-501 (Elmetron, Poland) were used for pH measurements.

2.3 Preparation of titania sol

Titania sol was prepared by acid hydrolysis and further polycondensation of titanium(IV) isopropoxide [3,9]. 250 μL of precursor was slowly added to the mixture of isopropanol (2.5 mL), 100% acetic acid (10 μL) and hydrochloric acid (1:9 v/v water solution). Thus obtained mixture was slowly added (7 drops per minute) to 3 mL of cold water acidified with 10 μL of 100% acetic acid. Obtained stable sol was stored in refrigerator.

2.4 Preparation of modified carbon electrodes

WE was prepared by placing spectral carbon rod (d = 6 mm, ZWE, Poland) into Teflon body. Surface of graphite was firstly polished onto glass plate using alumina suspension and rinsed with double distilled water [8,9]. Then the electrode was sonificated in: double distilled water (5 min), ethanol to disinfect of graphite surface (10 min); 1:1 (v/v) mixture of concentrated HNO_3 - water to activate of electrode surface and formation of different functional groups (5 min); 0.15 M ammonia solution for removal of acid traces (5 min), saturated ascorbic acid solution for reduction of some oxygen-containing functional groups (5 min, significant reduction of background current was achieved in this way); acetone to remove of insoluble in water impurities (5 min) and again in double distilled water (5 min). Cleanness of electrode surface was checked in deaerated 0.1 M phosphate buffer solution (pH 7.0) voltammetrically between -500 to 1000 mV with a scan rate 0.5 V s^{-1}. If no peaks were observed in this potential range the electrode was used in further experiments.



Scheme 1. Mechanism of mediated bioelectrocatalytic determination of ethanol.

Titania sol was mixed 1:1 (v/v) with 0.1 M phosphate buffer solution (pH 7.0) containing appropriate amount of mediator (MB). 20 μL of thus obtained mixture (in two portions of 10 μL) was placed on the top of carbon electrode. Modified electrode was dried above saturated KH_2PO_4 solution diluted with water (1:1, v/v) for 12 h in refrigerator. Drying of matrix layer above such KH_2PO_4 solution provided the proper humidity and prevented cracking and shrinking of sol.

For preparation of modified electrodes without mediator the same procedure was applied, but the sol was mixed with a pure buffer solution.

2.5 Evaluation of sensor response

Analytical characteristic of sensor response was performed using three-electrode system (see p. 2.2) in, if not stated otherwise, 0.1 M phosphate buffer solution (pH 7.0) containing reduced form of NAD^+.

2.6 Test of sensor usefulness. Determination of ethanol

The performance of developed sensor was preliminary tested by detection of NADH formed during enzymatic oxidation of ethanol catalyzed by alcohol dehydrogenase (ADH). Experiments were carried out "in drop" using 200 μL of 0.1 M phosphate buffer (pH 8.0) to which different amounts of ethanol were added. The NAD^+ and ADH concentrations in buffer solution were 15 mM and 10 mg mL^{-1}, respectively. NAD^+ was presented in excess compared to ethanol content. WE polarization potential was set at –50 mV.

3. Results and Discussion

3.1 Morphology of MB/titania sol-gel films

The morphology of titania sol-gel film doped with Meldola's Blue was characterized by SEM. On the SEM (Fig. 1) we can see three-dimensional micro-porous structure of composite film. The aggregates of titania sol-gel matrix on electrode surface are well distributed and pores are observed. Therefore the substrates can easily diffused into and out of matrix layer.

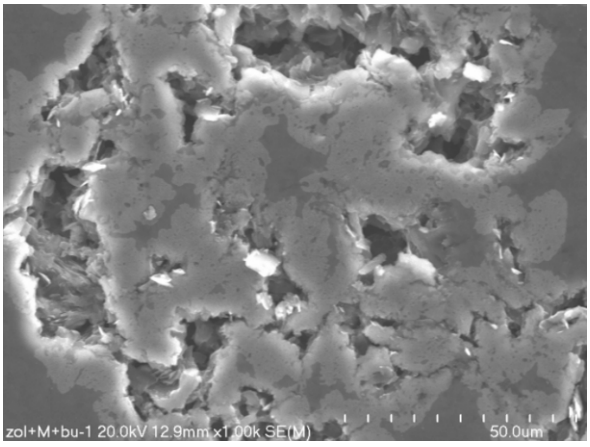


Figure 1. Scanning electron micrograph of graphite electrode coated with MB-doped titania sol-gel film.

3.2 Influence of electrode modification on NADH oxidation potential

Cyclic voltammetry was used to evaluate the influence of electrode modification on NADH oxidation potential using 1 mM NADH solution in 0.1 M phosphate buffer, pH 7.0 (Fig. 2). During experiments it was observed that modification of the electrode surface by titania layer increased active surface of electrode. This was manifested by rise of charging current. Moreover, this additional layer slightly shifted NADH oxidation potential to anodic direction (from 600 mV to about 650 mV; Figs. 2b,c). That phenomenon could be explained by decrease of rate constant of an electrode reaction connected with presence of sol-gel layer. Incorporation of mediator into titania layer significantly changed voltammetric curves (Figs. 2d,e). In buffer solution peaks corresponded to MB redox processes were noticed (Fig. 2d). After addition of NADH increase of MB anodic wave was observed (Fig. 2e). From this it could be concluded, that MB acted as a mediator in the NADH oxidation process. Moreover, the oxidation/reduction process for MB immobilized into titania layer was highly reversible – cathodic and anodic peak potentials differed by about 66 mV, and $i_{p,a}/i_{p,c}$ rate was close to unity. The small pre-peak on cathodic scan, which potential was almost the same as anodic peak, was probably connected with adsorption processes. The broad peak, which could be noticed for MB-containing electrode at 650 mV (Fig. 2e), may indicate that traces of analyte, despite the matrix layer, could be still oxidized directly at the surface of the graphite electrode.

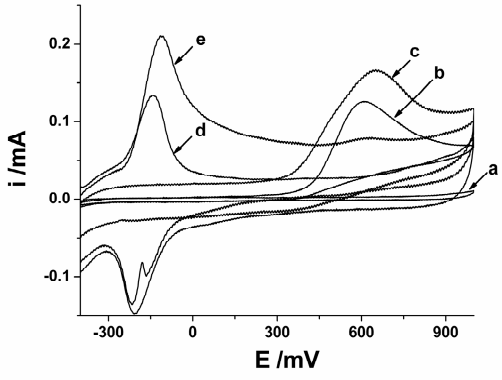


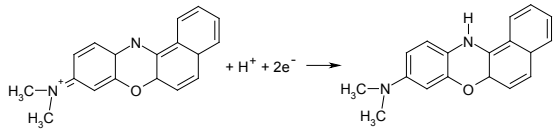
Figure 2. Influence of electrode modification on NADH oxidation potential. Cyclic voltammograms of a bare graphite electrode in buffer solution (a) and in 1 mM NADH solution (b); an electrode covered with titania layer in 1 mM NADH solution (c); electrodes with mediator (0.25 mM MB) incorporated in titania layer: in buffer (d) and in 1 mM NADH solution (e). Background electrolyte: 0.1 M phosphate buffer, pH 7.0. Scan rate 0.5 V s^{-1}.

3.3 Optimization of sensor preparation

Several parameters affecting the response of the sensor, *i.e.*, mediator concentration in gel, working electrode polarization potential and pH of supporting electrolyte were optimized.

3.3.1 Mediator concentration in titania layer

Influence of Meldola's Blue concentration in gel layer on sensor response was studied for the following concentrations of mediator: 5×10^{-3}, 5×10^{-4}, 2.5×10^{-4}, 5×10^{-5}, and 5×10^{-6} M. Cyclic voltammograms in 0.1 M phosphate buffer (pH 7.0) between –500 and 1000 mV were recorded (scan rate 0.5 V s^{-1}) using thus modified electrodes. For two higher concentrations, mediator leaching from titania layer was observed. The process was manifested by color changes of buffer solution used for electrodes storage. For lower values of MB concentrations, *i.e.*, 5×10^{-5} and 5×10^{-6} M peaks corresponding to mediator's redox reactions were very small. Therefore the concentration 2.5×10^{-4} M was chosen for further studies. For this MB concentration mediator's leaching from matrix layer was not revealed (which was confirmed spectrophotometrically). Moreover, almost linear dependence of anodic current vs. square root of scan rate was observed (see Fig. 3) which suggests, that the overall oxidation of NADH at the electrode was diffusion controlled.



Scheme 2. Two-electron reduction of oxidized form of Meldola's Blue.

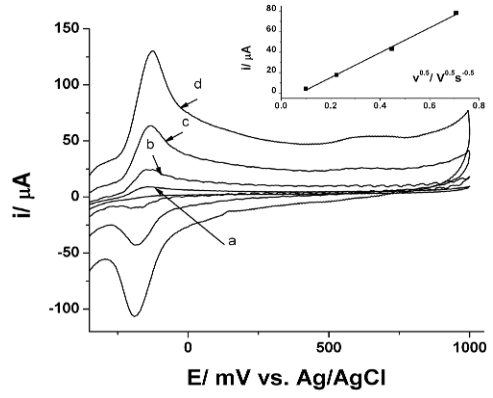


Figure 3. Influence of scan rate (a – 0.01, b – 0.05, c – 0.10 and d – 0.5 V s^{-1}) on voltammograms recorded in 0.5 mM NADH solution of pH 7.0. Inset: anodic peak current as a $v^{0.5}$ function. MB concentration in titania layer was 2.5×10^{-4} M.

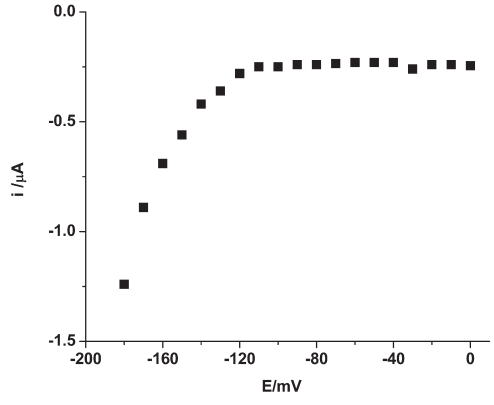


Figure 4. Background current as a function of WE polarization potential. Supporting electrolyte was 0.1 M phosphate buffer (pH 7.0) and MB concentration in titania layer was 0.25 mM.

3.3.2 Influence of WE polarization potential

For chosen optimal mediator's concentration, residual current after 30 s of polarization in 0.1 M phosphate buffer solution (pH 7.0) at WE potentials between 0 and -180 mV was recorded (Fig. 4). The shape of the curve measured in solution of supporting electrolyte results from the fact that Meldola's Blue was incorporated into matrix layer in oxidized form. For the potentials more cathodic then –100 mV, increase of background current was observed, due to reduction of oxidized form of mediator. For more anodic potentials the reduction process was less significant and current decrease was noticed.

3.3.3 Influence of pH

Evaluation of influence of background electrolyte pH on voltammetric response of MB immobilized in titania layer was performed in pH range from 5.0 to pH 9.0 with interval 0.5 pH unit (Fig. 5A). Obtained results suggested that hydrogen ions are involved in the redox process. Moreover, from the slope of cathodic or anodic peak potential *vs*. pH (about 37 mV/pH, Fig. 5B) it can be concluded that two electrons are needed in this process (Scheme 2).

The influence of pH of supporting electrolyte on NADH oxidation current at polarization potential of –50 mV was checked in 1 mM NADH solutions. In accordance with results (data not shown), supporting electrolyte at pH 7.0 would be the optimal choice.

3.4 Analytical characterization of NADH sensor

Analytical characteristic of developed sensor was performed at optimal values of MB concentration (0.25 mM), WE polarization potential (-50 mV) and pH of supporting electrolyte (7.0).

3.4.1 Response time

For seven different electrodes chronoamperometric responses were recorded in 0.4 mM NADH solution. From these studies the mean value of $t_{95\%}$ was 10 s.

3.4.2 Sensor calibration

Typical chronoamperometric response of sensor for successive additions of NADH solution is presented in Fig. 6. Based on these results linear calibration curve was obtained in range from 90 to 2300 µM. However, significant variation in sensors sensitivity was observed. That phenomenon could be explained by differences in real active electrode surface and thickness of sol-gel layer. During preparation of modified electrodes, it was observed that the sol had tendency to accumulate in the centre of the electrode surface. For five modified electrodes the sensitivity varied from 10.2 nA μM^{-1} to 16.0 nA μM^{-1} (10.2, 11.0, 11.5, 13.8 and 16.0). Nevertheless, the calibration ranges were similar.

3.4.3 Detection limit

Because of variations in sensor sensitivity the detection limit was calculated using mean sensitivity obtained for seven different sensors: $\bar{a} = 12.5$ nA μM^{-1} or, taking into account geometrical surface of electrode (34.7 nA μM^{-1}

cm^{-2}) according to equation: $LOD = 3 \cdot S_x \cdot \bar{a}^{-1}$. Standard deviation ($S_x$, n = 10) obtained for blank (*i.e.*, buffer solution) was 51 nA and the LOD was 12 μM.

3.4.4 Repeatability and reproducibility

Five different modified electrodes were used to check repeatability and reproducibility of analytical response of developed NADH sensor. These electrodes were firstly calibrated in NADH solutions (198, 392 and 582 μM) and then 10 measurements in 392 μM NADH solution were performed. Between each measurement electrode was moved from experimental cell and thoroughly washed with distilled water. The repeatability of developed sensor was satisfied, relative standard deviations (RSD) were 1.24%, 1.52%, 1.64%, 2.05%, 2.09%. The mean value of NADH concentration obtained for these five sensors, 401 μM (RSD 5.4%), agrees well with its true concentration.

3.4.5 Operational stability

Based on sensor response obtained for repetitive measurements in the same solution of NADH, decrease in oxidation current after few measurements could be observed. Practically no change in response current was noticed after 7 measurements but additional 8 repetitions caused current decrease average 12%. Graphic expression of that dependence is presented in Fig. 7. It was possible to recover initial sensor sensitivity when the modified electrode was stored in 0.1 M phosphate buffer (pH 7.0) overnight. NADH oxidation products, which were adsorbed during measurements on titania layer and which could be responsible for signal reduction, were apparently removed from the electrode surface during this period.

3.4.6 Interference effects

The main difficulty in the analysis of real samples is the presence of substances that give additional signal to those obtained for pure analyte. In our studies three potential interferences which are usually met in biological (*e.g.* blood, urine) and food (*e.g.* alcoholic beverages) were selected: acetone, glucose and ascorbic acid. For each of them the sensor response was recorded for the concentration ranges covering typical values for biological material [6]. The analysis were performed in following ranges: acetone – from 1 to 91 mM, glucose – from 9.90 to 476.4 mM and L-(+)-ascorbic acid – from 1 to 9.09 mM. No interferences were noticed for acetone and glucose. For L-(+)-ascorbic acid additional signal of its oxidation to dehydroascorbic acid was observed. In order to overcome that problem, the membrane the polycarbonate membrane coated with the negatively charged hydrogel layer could be employed [10].

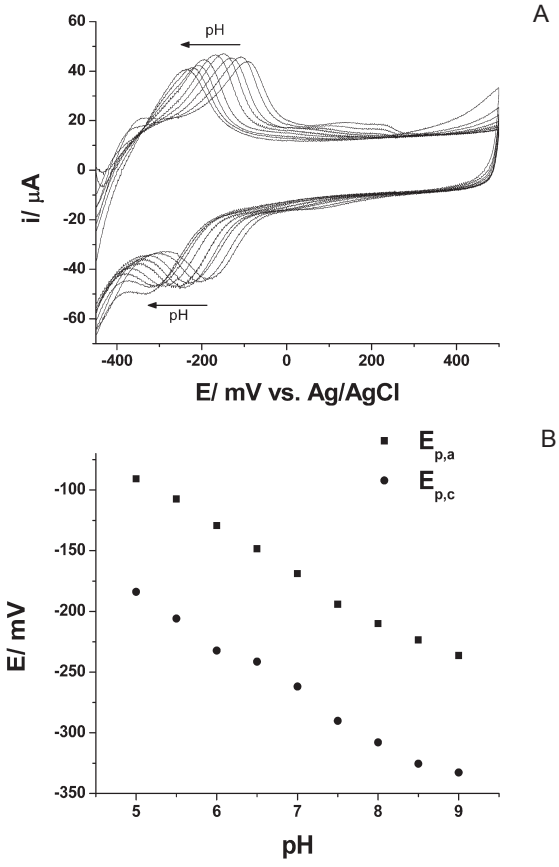


Figure 5. Influence of background electrolyte pH on MB voltammetric curves (A) and cathodic ($E_{p,c}$) and anodic ($E_{p,a}$) peak potentials as a function of pH (B). Scan rate was 0.5 V s^{-1}. MB concentration in titania layer was 0.25 mM.

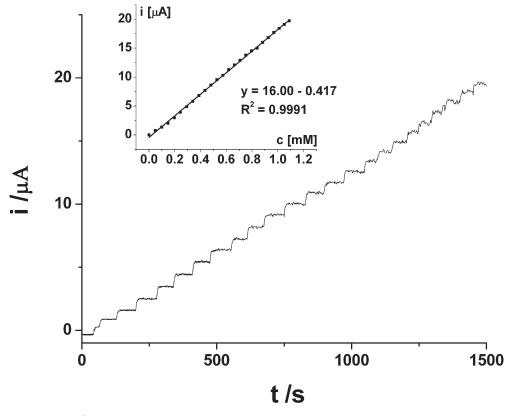


Figure 6. Chronoamperometric response for successive additions of NADH solution for sensor working in optimal conditions. Supporting electrolyte was 0.1 M phosphate buffer (pH 7.0) and MB concentration in titania layer was 0.25 mM. Inset: exemplary calibration curve for NADH.

Table 1. Comparison of analytical characteristics of different MB-based NADH sensors.

Matrix	LOD/μM	Linear range/μM	Sensitivity	Ref.
MB incorporated into ZnO	10	50 - 300	-	11
MB in SiO_2/TiO_2/graphite composite	8	18 - 7290	0.286 nA μM^{-1} cm^{-2}	12
MB in SiO_2/NiO composite	-	10-750	48 nA μM^{-1}	13
MB modified ceramic carbon electrode	-	up to 10 mM	-	14
MB adsorbed on CNT	0.048	up to 500	7.354 nA μM^{-1} cm^{-2}	1
MB in epoxy-graphite composite	-	120 - 280	1.8 nA μM^{-1}	15
MB adsorbed on epoxy-graphite composite	-	9 - 280	0.04 nA μM^{-1}	15
MB electropolymerized onto epoxy-graphite composite	-	10 - 40	0.5 nA μM^{-1}	15
MB electropolimerized onto SPE	-	20 – 500[a]	0.400 nA μM^{-1} cm^{-2}	16
MB in polysulfone-graphite composite	-	20 – 500[a]	4.204 nA μM^{-1} cm^{-2}	16
MB electropolimerized onto SPE	2.5	8 - 500	3.713 nA μM^{-1}	17
MB in CNT-sol-gel composite	6	50-1830	0,465 nA μM^{-1} cm^{-2}	18
MB in titania layer	12	90 – 2300[a]	34.7 nA μM^{-1} cm^{-2} 12.5 nA μM^{-1}	this work

CNT – carbon nanotubes, SPE – screen-printed electrode; [a] evaluated range

3.5 Determination of ethanol

The possibility of ethanol determination based on electrochemical detection of NADH forming during enzymatic process was examined. Due to low enzymatic activity of ADH at pH 7.0 (which was checked by additional spectrophotometric measurements; data not shown), phosphate buffer solution of higher pH (8.0) was used in these experiments. In tested ethanol concentration range, 92 – 458 mM, linear calibration curve was obtained.

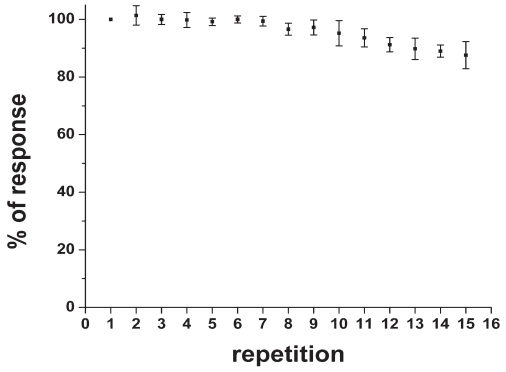


Figure 7. Operational stability of the biosensor in 0.392 mM solution of NADH. Mean values of response +/- SD obtained for five different biosensors.

4. Conclusions

A new NADH sensor was constructed by immobilization of redox mediator, Meldola's Blue, on the surface of graphite electrode using titania layer obtained by sol-gel process. The influence of some parameters, such as mediator concentration in titania layer, pH of supporting electrolyte, and WE polarization potential, on analytical performance of sensor was evaluated. Obtained results suggested that titania sol-gel film can be successfully employed in construction of sensor used for determination of NADH formed during enzymatic reaction. In comparison with other NADH sensors based on Meldola's Blue [11-18] (see Table 1), the sensitivity of developed sensor is substantially higher then that of almost all sensors (except [13]), indicating that MB entrapped in titania gel remained its excellent redox mediator properties. Moreover, the present sensor, in comparison with other, showed quite wide linear range. However, further research will be focused on an improvement of operational stability of NADH sensor. The developed sensor could be used for determination of ethanol based on its conversion to aldehyde catalyzed by alcohol dehydrogenase. Experiments will be performed with construction of ethanol biosensor based on ADH incorporated into titania layer doped with Meldola's Blue.

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