Scientific paper

Influence of Nafion in Titania Sol-gel Matrix on Analytical Characteristic of Amperometric Phenol Biosensor Based on Tyrosinase

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Abstract

For detection of phenolic compounds a simple amperometric biosensor based on tyrosinase immobilized in titania sol-gel/Nafion composite was employed. Titania sol-gel was mixed with Nafion (v/v) in ratios 1:1 (TiO_2/NF-1/1) and 2:1 (TiO_2/NF-2/1), v/v. Morphology of immobilization composites was characterized by scanning electron microscopy. Analytical performance of biosensors with Nafion and based only on titania sol-gel (TiO_2) was compared. Apart from sensitivity, linear range and detection limit also repeatability, reproducibility and storage stability were evaluated. The biosensor based on titania sol mixed with Nafion in ratio 1:1 (v/v) exhibited the best analytical parameters in terms of sensitivity: 2.84 μA L μmol^{-1}, corresponding LOD, 0.056 μmol L^{-1}, and the long-term stability within 20 days: it retained 80% of initial activity.

Keywords: biosensor, titania gel, Nafion, phenol, tyrosinase

1. Introduction

Phenol and substituted phenols are important contaminants in ground and surface water. They are highly toxic, carcinogenic and allergenic. Therefore there is an interest in developing a simple, sensitive and accurate device for determination of phenolic compounds. Spectrophotometric and chromatographic methods have been usually applied for these purpose.[1] Those methods are expensive, time consuming, include complicated sample pretreatment and are inadequate for in-situ monitoring. Electrochemical methods, particularly amperometric biosensors, proved to be suitable for phenols determination because of effectiveness, good selectivity, low cost and potential for miniaturization and automation.

Immobilization of active biomolecules into a matrix plays a crucial role for biosensor application and stability. Sol-gel materials are an interesting and versatile way to prepare modified electrodes, because of the ability to form film with tunable porosity, thermal stability and chemical inertness. Sol-gel glasses are attractive methods for immobilization of biomolecules, e.g. enzymes, because matrices can be prepared under ambient conditions and can retain the catalytic activity of enzymes. Sol-gel films have been widely employed to development of electrochemical and optical biosensors in the last decade.[2]

A general difficulty for many biosensors is the lack of the operational and storage stability, and is presently an important problem to solve in biosensors area. Nafion, perfluorinated sulfonate ionomer due to its easy fabrication, thermal and chemical stability, mechanical strength and ability to resist interferences from anions is often employed to modify electrodes.[3–7] In Nafion hydrophilic clusters are surrounded partially by hydrophobic PTFE regions.[8] It has been reported that Nafion membrane significantly decreased cofactors leakage and simultaneously enhanced the sensor stability.[9] Upadhyay *et al.* described that Nafion not only promoted the electron transfer rate, it also reduced the brittleness of the matrix layer.[4]

The biosensors most sensitive to phenols are those based on tyrosinase, copper containing polyphenol oxidase. Tyrosinase catalyses the oxidation of monophenols by molecular oxygen to form *o*-diphenols (catechols), which are subsequently oxidized to *o*-quinones. Then quinones can be electrochemically reduced to diphenols on electrode surface. The detection of phenolic com-

pounds thus relies on monitoring the catecholic products.

For detection of phenolic compounds we developed an amperometric biosensor based on tyrosinase immobilized in titania sol-gel.[2] The biosensor exhibited high sensitivities towards phenolic compounds, however, the stability of biosensor's response was unsatisfactory. In order to improve storage stability, a simple modification was made by incorporation of Nafion into immobilizing composite. In developed biosensors tyrosinase was entrapped on the surface of carbon electrode in titania gel mixed with Nafion (v/v) in ratios 1:1 and 2:1, TiO_2/NF-1/1 and TiO_2/NF-2/1, respectively. Morphology of matrix layers was characterized by scanning electron microscopy. Analytical performance of biosensors with Nafion and based only on titania sol-gel (TiO_2) was compared. Apart from sensitivity, linear range and detection limit, also repeatability, reproducibility and storage stability of biosensors were evaluated.

2. Experimental

2. 1. Chemicals

Tyrosinase (E.C. 1.14.18.1, 5370 U/mg) from mushrooms and 5% (w/v) Nafion were purchased from Sigma-Aldrich; graphite electrodes (diameter: 0.6 cm) were from ZEW Raciborz (Poland); titanium isopropoxide was from Fluka Chimie (Switzerland); paraffin (used for impregnation of electrodes), disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate and acetone were obtained from Merck; ethanol and L-(+)-ascorbic acid were purchased from POCh (Poland), nitric acid and ammonia were from Lach-Ner (Czech Republic), catechol was obtained from BDH Chemicals (UK). All chemicals were of analytical grade and were used as received. Solutions were prepared in ultra-pure water.

2. 2. Apparatus and Measurements

A vortex (IKA, Germany) was employed to obtain homogenous mixture of titania sol, Nafion and tyrosinase solution during biosensors fabrication process.

Voltammetric and amperometric measurements were performed using an EMU/O mulitmeter (Poland) in thermostatic cabinet Pol-Eko-Aparatura (Poland). A conventional three-electrode system was employed consisting of the enzyme electrode as a working electrode, a platinum wire as a counter electrode and Ag/AgCl (3 mol L^{-1}) reference electrode. Phosphate buffer solution (pH = 6.0; 0.1 mol L^{-1}) was used as supporting electrolyte. For voltammetric measurements the buffer solution was purged from oxygen by bubbling with laboratory-grade nitrogen (99.99%). Amperometric experiments were performed under constant stirring with magnetic bar and under free access of air. The enzyme electrode worked at a potential 0.0 V vs. Ag/AgCl. Because it was not necessary to activate the sensor before measurements, additional voltammetric cycles before measurements were not conducted. All experiments were performed at the temperature of 25 °C. Exemplary cyclic voltammograms of TiO_2/NF-1/1 recorded in phosphate buffer solution and in 10 μmol L^{-1} catechol solution are presented in Fig. 1.

2. 3. Construction of the Biosensor

A graphite rod was impregnated in paraffin and placed into a Teflon holder with copper wire as a current lead.[2,10] The working surface of electrode was polished with emery paper, then with -alumina powder (0.5 μm) and finally rinsed with ultra-pure water. Next, the electrode was successively sonicated in following media: ultra-pure water, ethanol, nitric acid (1:1), ammonia water, saturated solution of ascorbic acid and acetone. Electrodes were rinsed with ultra-pure water after each sonication and finally dried at room temperature.

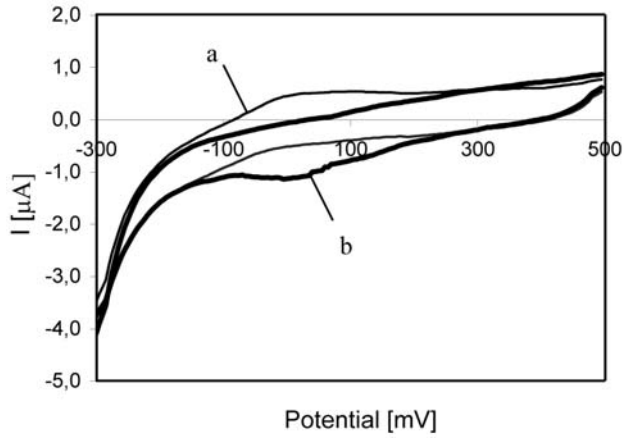

Figure 1. Cyclic voltammograms of TiO_2/NF-1/1 biosensor in phosphate buffer solution (a) and in 10 μmol L^{-1} catechol solution; scan rate: 62.5 mV/s.

In order to prepare titania sol, titanium isopropoxide (250 μL) was added to propan-2-ol (2.5 mL) formerly acidified with concentrated HCl (10 μL) and CH_3COOH (20 μL), and next stirred. The prepared solution of precursor was then slowly instilled into cold water (3 mL), under constant intensive stirring.

The prepared titania sol was shaken with Nafion (in small portions) in ratios 1:1 and 2:1 (v/v). Then the solution of tyrosinase in phosphate buffer (pH = 7.0, 0.1 mol L^{-1}) was prepared, and shaken with titania sol with (or without) Nafion. 20 μL of prepared mixture was deposited on the surface of a pretreated electrode in portion of 10 μL. After each portion of sol had been added, the surface of electrode was dried in air for ca.10 min. Finally the electrode was allowed to dry over saturated disodium

phosphate solution for 20 h at 4 °C. Loading of enzyme was 50 μg of tyrosinase per electrode.

The biosensors were stored at 4 °C in phosphate buffer, (pH = 6.0, 0.1 mol L^{-1}), their active surface touching the surface of solution. Before measurements electrodes were immersed in buffer at room temperature for 15 min. When not in use biosensors were stored in buffer solution (pH 6.0, 0.1 mol L^{-1}).

3. Results and Discussion

3.1. Surface Morphologies of Developed Electrodes

Scanning electron microscopy (SEM) was used to characterize and compare the morphology of modified biosensor. The SEM imagine of titania gel layer (Fig. 2a) showed rather flat surface with irregular patches. After incorporation of Nafion a more varied surface was observed. As illustrated in Fig. 2b, surface of TiO_2/NF-1/1 was more rough and pitted. For TiO_2/NF-2/1 composite spheres of different sizes (diameter up to 10 μm) on the surface were appeared (Fig 2c). During fabrication of biosensor it was noticed, that for that composition of matrix a gelation proceeded very fast. It is known that the sol-gel preparation strongly influenced by several parameters, including pH.[11] Nafion is strong acidic character therefore a run of sol-gel process depends on ionomer content. It is likely that in that conditions gelation process run so fast that obtainment of uniform sol was not possible, spheres revealed on electrode surface were probably of Nafion.

3. 2. Analytical Characteristic of Developed Biosensors

The analytical characteristics obtained for biosensors TiO_2, TiO_2/NF-1/1 and TiO_2/NF-2/1 were evaluated in term of response time, sensitivity, linear range and detection limit.

It was observed that developed biosensors exhibited short response time (t_{95}) in solution of catechol (0.11 μmol L^{-1}): 5–6 seconds for biosensor based on pure titania sol and 8–10 seconds for Nafion biosensors. In Figure 3 an example of chronoamperometric response of TiO_2/NF-1/1 biosensor recorded in phosphate buffer solution at pH = 6 and 2,45 μmol L^{-1} catechol solution is presented.

Effect of matrix composition on analytical parameters toward catechol is given in Table 1 where comparison of linear ranges, detection limits and sensitivities of studied electrodes was shown. Exemplary calibration curves are presented in Fig. 4. The linear concentration range was obtained as log I (μA) = a + $b$logc (μmol L^{-1}) in accordance with Liu *et al.*[12] Limit of detection was calculated according to the formula $3s_b/b$, where s_b is the standard deviation of blank measurements (n=10) and b is slope of the calibration curve.[2] The widest linear range, 0.11– 15.09 μmol L^{-1}, was obtained for unmodified biosensor (based on pure titania sol). Biosensors with ionomer had slightly narrower linear range: 0.22–14.04 μmol L^{-1} and 0.44–15.09 μmol L^{-1} for TiO_2/NF-1/1 and TiO_2/NF-2/1 sensors, respectively. Addition of Nafion to sensor matrix caused increasing of biosensor sensitivity toward catechol; the highest value, 2.84 μA L μmol^{-1}, was observed for TiO_2/NF–1/1, while for electrodes without Nafion the mean sensitivity was 1.52 μA L μmol^{-1}. For that composition of matrix the lowest detection limit for catechol determination: 0.056 μmol L^{-1} was obtained. That phenomenon could be explained by the surface morphology of composite. More rough surface observed for that composition of matrix (see Fig. 2b) made possible faster diffusion of substrates into the matrix layer, to the active center of enzyme,

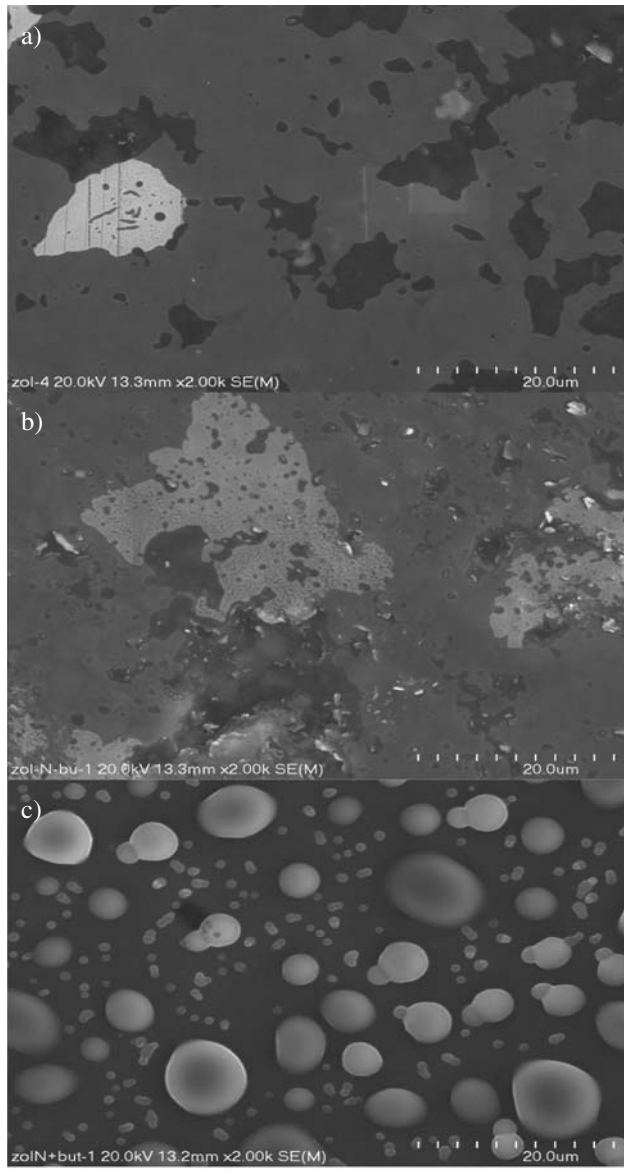

Figure 2. SEM imagines of TiO_2 (a), TiO_2/NF-1/1 (b) and TiO_2/NF-2/1 (c) composite film.

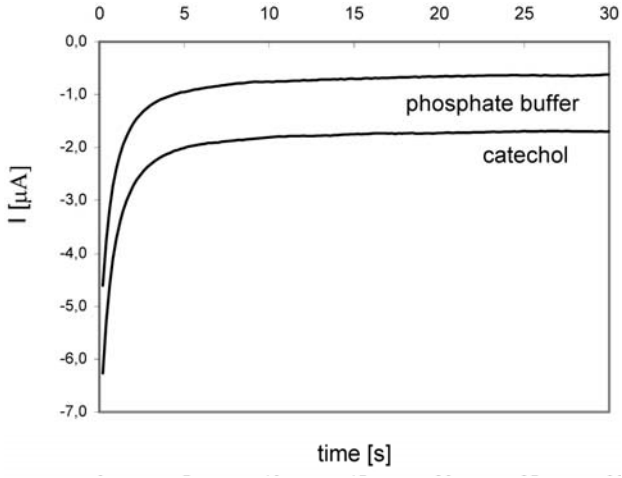

Figure 3. Exemplary chronoamperometric response of TiO_2/NF-1/1 biosensor in phosphate buffer solution at pH = 6 and 2,45 µmol L^{-1} catechol solution; potential 0.0 mV vs. Ag/AgCl.

compared to layer without Nafion. It could also be connected with biocompatibility of ionomer. Nafion has hydrophobic fluorocarbon backbone and hydrophilic cation-exchange site, thus rendering moderate hydrophobicity.[13]

It is feasible that the content of ionomer in TiO_2/NF-1/1 biosensor matrix ensured the proper hydrophilicity for high activity of enzyme. On the other hand the structure of composite with smaller contents of ionomer, TiO_2/NF-2/1 (see Fig. 2c) could slightly make worse availability of enzyme for an analyte because of Nafion spheres. Therefore that biosensor showed lower sensitivity, 2.58 µA L $µmol^{-1}$ in comparison with biosensor TiO_2/NF-1/1, but still significantly higher than biosensor without ionomer (1.52 µA L $µmol^{-1}$).

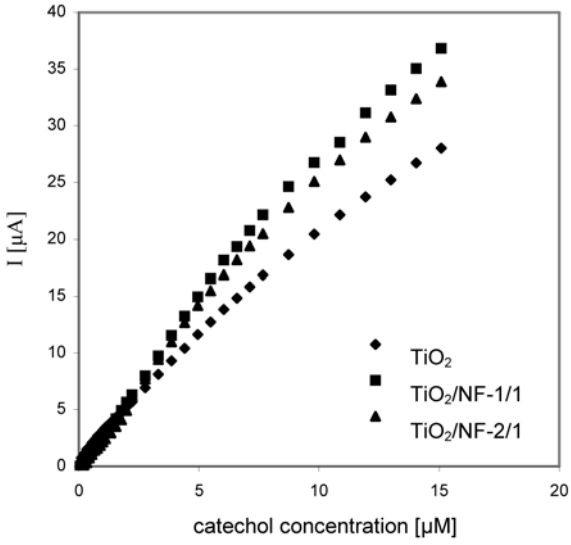

Figure 4. Calibration curves for catechol obtained for biosensors without and with Nafion.

The repeatability of proposed sensors was estimated in 0.89 µmol L^{-1} solution of catechol. It was evaluated for five sensors of each matrix composition. For electrodes with pure titania sol mean value of relative standard deviation (RSD) was 1.85%, while for TiO_2/NF-1/1 and TiO_2/NF-2/1 was 2.15% and 2.74%, respectively. It was checked if short storage of biosensors in buffer solution between series of measurements could influence the repeatability. It was found that immersion of bioelectrodes for 3 minutes in phosphate buffer solution (pH = 6.0, 1 mol L^{-1}) improved their performance: mean RSD were 0.90%, 1.75% and 1.76% for sensors without ionomer, for TiO_2/NF-1/1 and TiO_2/NF-2/1, respectively. It is likely that traces of products of enzymatic reaction which were accumulated on the matrix layer and which could be responsible for worse signal repeatability were removed from the electrode surface to buffer solution during that short period of time.

In order to compare the reproducibility, series of five biosensors of different matrix composition were prepared at different time. Based on amperometric results for 0.89 µmol L^{-1} catechol solution RSD were calculated: 5.78% for biosensors without ionomer, 7.62% and 8.17% for TiO_2/NF-1/1 and TiO_2/NF-2/1 biosensors, respectively.

3. 3. Long-Term Stability of Developed Biosensors

The long-term storage stability was evaluated by monitoring of biosensors response current in 1.1 µmol L^{-1} catechol solution. Figure 5A presents the comparison of the relative stability of electrodes response in 3, 8 and 12 weeks after electrodes fabrication. The results led to the conclusion that the sensors activity decreased gradually and depended on matrix composition. After 12 weeks of storage the best stability was noticed for sensor without Nafion, it retained 44% of initial activity. Biosensor with ionomer TiO_2/NF-1/1 maintained only 28% of initial re-

Table 1. Effect of matrix composition of tested biosensors on analytical characteristics toward catechol.

Parameter	Biosensor without Nafion (TiO_2)	Biosensors with Nafion	
		TiO_2/NF-1/1	TiO_2/NF-2/1
Sensitivity* [µA L $µmol^{-1}$]	1.52	2.84	2.58
Linear range [µmol L^{-1}]	0.11–15.09	0.22–11.93	0.44–11.93
LOD [µmol L^{-1}]	0.065	0.056	0.069

* mean value for five electrodes

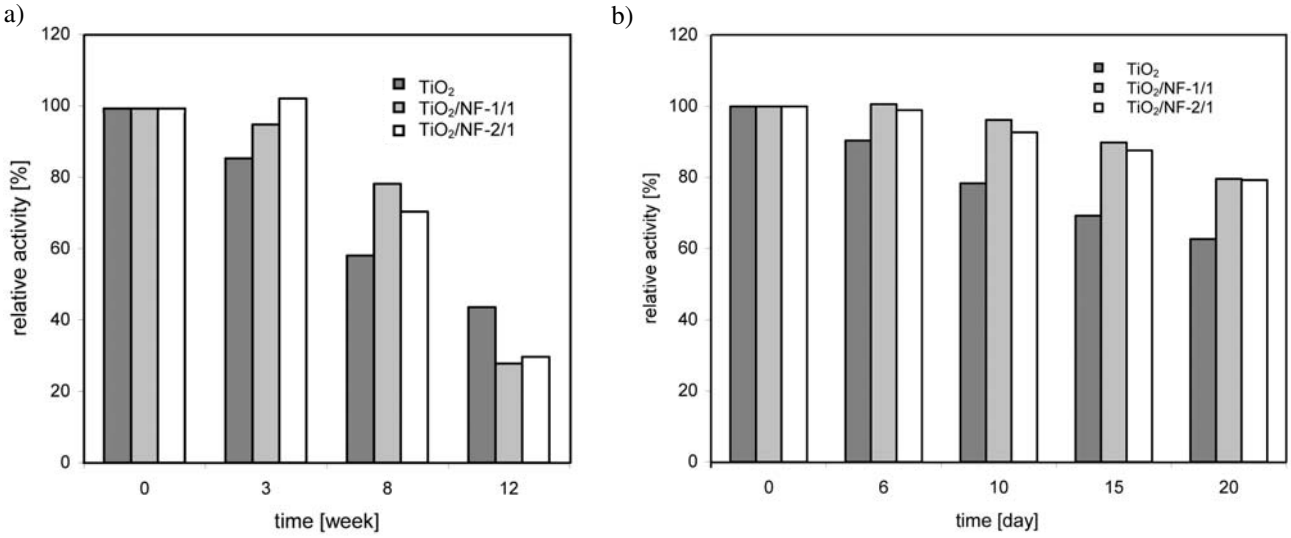

Figure 5. Comparison of long-term storage stability for biosensor with Nafion and without ionomer within 12 weeks (a) and 20 days (b).

sponse. However, for a shorter period of time performance of Nafion biosensors was better than observed for sensor without ionomer. In order to check that phenomenon successive amperometric measurements were carried out once per 5 days starting from day of sensor fabrication. It was found that after 20 days relative stability of Nafion biosensors was significantly higher than that observed for electrodes based on pure titania sol (see Fig. 5B), ca. 80% and 60%, respectively. It is apparent that addition of Nafion into matrix improved the biosensor stability within 20 days. On the other hand, within long period of time it promote leaching of enzyme from immobilizing layer.

4. Conclusion

The biosensors based on tyrosinase entrapped in titania sol-gel and in titania sol-gel/Nafion composites were presented and their functioning was compared. The reported data reveals that addition of Nafion into matrix influenced the analytical performance of biosensors. The biggest impact was observed on sensitivity, limit of detection and long term stability. Among studied sensors: TiO_2, TiO_2/NF-1/1 and TiO_2/NF-2/1, the biosensor based on titania sol mixed with Nafion in ratio 1:1 (v/v) exhibited the best analytical parameters in terms of sensitivity: 2.84 μA L μmol^{-1}, corresponding LOD, 0.056 μmol L^{-1} and the long-term stability within 20 days: it retained 80% of initial activity. The biosensor manifested very good repeatability with RSD of 2.15% and satisfied reproducibility with RSD of 7.6%. In comparison with other amperometric tyrosinase biosensors based on sol-gel/Nafion composite, the sensitivity of proposed biosensor for catechol (2.84 μA L μmol^{-1}) is very good (see Table 2). It is significantly higher than sensitivities reported for biosensor based on tyrosinase entrapped in silicate/Nafion composite film, 0.2 μA L μmol^{-1} [13] for carbon nanotube/titania/Nafion tyrosinase biosensor, 0.647 μA L μmol^{-1},[14] and for

Table 2. Comparison of analytical characteristic toward catechol for amperometric biosensors based on tyrosinase immobilized in sol-gel/Nafion matrix.

Electrode	Sensitivity [μA L μmol^{-1}]	Linear range [μmol L^{-1}]	LOD [μmol L^{-1}]	Long-term stability	Ref.
Tyrosinase/silicate/ Nafion composite film	0.200	1–100	0.35	Retained 74% of initial activity after 14 days of storage	13
Tyrosinase/ CNT/ titania/Nafion composite film	0.647	0.1–50	0.087	Retained 89% of initial activity after 14 days of storage	14
Tyrosinase/ CNT/sol-gel derived ZnO /Nafion	1.89	0.03–32	0.03	Retained 88% of initial activity after 14 days of storage	15
Tyrosinase/titania sol-gel/ Nafion (1:1 v/v) matrix	2.84	0.11–15.09	0.056	Retained 90% of initial activity after 15 days of storage and 80% (measurements were carriedout once per 5 days)	This study

* CNT – carbon nanotubes

bioelectrode with tyrosinase immobilized in carbon nanotubes/sol-gel-derived zinc oxide/Nafion film.[15] Moreover, LOD obtained for developed biosensor was lower than reported by Kim,[13] and Lee.[14] Long-term stability of proposed biosensor was slightly better comparing with other sol-gel derived tyrosinase biosensors recently described in the literature.

It is worth to mention that the proposed biosensor, exhibiting good analytical performance, was fabricated by simply modification of titania sol/gel matrix with Nafion only, without any nanomaterials, like carbon nanotubes.

6. References

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Povzetek

Za detekcijo fenolnih spojin smo uporabili enostaven amperometrični biosenzor na osnovi tirozinaze imobilizirane na sol-gel TiO_2/Nafionski kompozit. Sol-gel TiO_2 je bil zmešan z Nafionom v volumskih razmerjih 1:1 (TiO_2/NF-1/1) in 2:1 (TiO_2/NF-2/1). Morfologijo imobiliziranih kompozitov smo spremljali z uporabo vrstičnega elektronskega mikroskopa. Primerjali smo analitsko učinkovitost biosenzorjev, kjer je bil prisoten Nafion, in kjer je bil prisoten samo sol-gel TiO_2. Poleg občutljivosti, linearnega območja in meje zaznavnosti, smo ovrednotilitudi ponovljivosti meritev in stabilnost pri shranjevanju. Biosenzor, ki je vseboval sol-gel TiO_2/Nafionski kompozit v volumskem razmerju 1:1, je izkazal najboljše analitske parametre: občutljivost 2,84 μA L μmol^{-1}, mejo zaznavnosti 0.056 μmol L^{-1} in obstojnost z 80 % prvotne aktivnosti po dvajsetih dneh.