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

Jolanta Kochana

Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland

* Corresponding author: E-mail: kochana@chemia.uj.edu.pl; Tel. +48 12 6332014; fax: +48 12 6340515

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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 (TiO₂/NF-1/1) and 2:1 (TiO₂/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 (TiO₂) 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. 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.

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. In Nafion hydrophilic clusters are surrounded partially by hydrophobic PTFE regions. It has been reported that Nafion membrane significantly decreased cofactors leakage and simultaneously enhanced the sensor stability. Upadhyay et al. described that Nafion not only promoted the electron transfer rate, it also reduced the brittleness of the matrix layer.

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.\textsuperscript{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\textsubscript{2}/NF-1/1 and TiO\textsubscript{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\textsubscript{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 Racibor (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 multimeter (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\textsuperscript{-1}) reference electrode. Phosphate buffer solution (pH = 6.0; 0.1 mol L\textsuperscript{-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\textsubscript{2}/NF-1/1 recorded in phosphate buffer solution and in 10 μmol L\textsuperscript{-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.\textsuperscript{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. Solutions were prepared in ultra-pure water.

![Figure 1. Cyclic voltammograms of TiO\textsubscript{2}/NF-1/1 biosensor in phosphate buffer solution (a) and in 10 μmol L\textsuperscript{-1} catechol solution; scan rate: 62.5 mV/s.](image-url)
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 image 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 TiO₂/NF-1/1 was more rough and pitted. For TiO₂/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. 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 TiO₂, TiO₂/NF-1/1 and TiO₂/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 (t₉₅) 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 chronocamperometric response of TiO₂/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 log c (μmol L⁻¹) in accordance with Liu et al. Limit of detection was calculated according to the formula 3σ/b, where σ is the standard deviation of blank measurements (n=10) and b is slope of the calibration curve. 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 TiO₂/NF-1/1 and TiO₂/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 TiO₂/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 images of TiO₂ (a), TiO₂/NF-1/1 (b) and TiO₂/NF-2/1 (c) composite film.
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.\textsuperscript{13} It is feasible that the content of ionomer in TiO\textsubscript{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\textsubscript{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\textsuperscript{-1} in comparison with biosensor TiO\textsubscript{2}/Nf-1/1, but still significantly higher than biosensor without ionomer (1.52 μA L μmol\textsuperscript{-1}).

The repeatability of proposed sensors was estimated in 0.89 μmol L\textsuperscript{-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\textsubscript{2}/Nf-1/1 and TiO\textsubscript{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\textsuperscript{-1}) improved their performance: mean RSD were 0.90%, 1.75% and 1.76% for sensors without ionomer, for TiO\textsubscript{2}/Nf-1/1 and TiO\textsubscript{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\textsuperscript{-1} catechol solution RSD were calculated: 5.78% for biosensors without ionomer, 7.62% and 8.17% for TiO\textsubscript{2}/Nf-1/1 and TiO\textsubscript{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\textsuperscript{-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\textsubscript{2}/Nf-1/1 maintained only 28% of initial re-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biosensor without Nafion (TiO\textsubscript{2})</th>
<th>Biosensors with Nafion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity* [μA L μmol\textsuperscript{-1}]</td>
<td>1.52</td>
<td>2.84</td>
</tr>
<tr>
<td>Linear range [μmol L\textsuperscript{-1}]</td>
<td>0.11–15.09</td>
<td>0.22–11.93</td>
</tr>
<tr>
<td>LOD [μmol L\textsuperscript{-1}]</td>
<td>0.065</td>
<td>0.056</td>
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* mean value for five electrodes

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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$^{-1}$, for carbon nanotube/titania/Nafion composite film, 0.647 μA L μmol$^{-1}$, and for

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

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Sensitivity [μA L μmol$^{-1}$]</th>
<th>Linear range [μmol L$^{-1}$]</th>
<th>LOD [μmol L$^{-1}$]</th>
<th>Long-term stability</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase/silicate/</td>
<td>0.200</td>
<td>1–100</td>
<td>0.35</td>
<td>Retained 74% of initial activity after 14 days of storage</td>
<td>13</td>
</tr>
<tr>
<td>Nafion composite film</td>
<td></td>
<td></td>
<td></td>
<td>Retained 89% of initial activity after 14 days of storage</td>
<td>14</td>
</tr>
<tr>
<td>Tyrosinase/ CNT/</td>
<td>0.647</td>
<td>0.1–50</td>
<td>0.087</td>
<td>Retained 88% of initial activity after 14 days of storage</td>
<td>15</td>
</tr>
<tr>
<td>titania/Nafion</td>
<td></td>
<td></td>
<td></td>
<td>Retained 90% of initial activity after 15 days of storage</td>
<td>This study</td>
</tr>
<tr>
<td>composite film</td>
<td></td>
<td></td>
<td></td>
<td>(measurements were carried out once per 5 days)</td>
<td></td>
</tr>
<tr>
<td>Tyrosinase/ CNT/sol-gel</td>
<td>1.89</td>
<td>0.03–32</td>
<td>0.03</td>
<td></td>
<td></td>
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<tr>
<td>derived ZnO/Nafion</td>
<td></td>
<td></td>
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<tr>
<td>Tyrosinase/titania sol-gel</td>
<td>2.84</td>
<td>0.11–15.09</td>
<td>0.056</td>
<td></td>
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<tr>
<td>/Nafion (1:1 v/v) matrix</td>
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* CNT – carbon nanotubes
bioelectrode with tyrosinase immobilized in carbon nanotubes/sol-gel-derived zinc oxide/Nafion film. Moreover, LOD obtained for developed biosensor was lower than reported by Kim, and Lee. 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


Povzetek