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Potentiometric tungsten electrodes with enzymes immobilised by the sol-gel technique

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The application of sol-gel transitions in development of potentiometric enzyme electrodes with urease, glucose oxidase and tyrosinase is described. Urease and glucose oxidase electrodes showed features of typical potentiometric electrodes with the response modified by the change of pH. The slopes of calibration curves in semilogarithmic coordinates were lower than the Nernstian values and the dynamic ranges were narrow for both electrodes. The tyrosinase electrode shows response to mono- and *o*-diphenols. The response of this electrode is not connected with the pH changes. The mechanism of the response of the tyrosinase electrode is discussed; it is proposed that the potential changes reflect the redox state of the enzyme. The electrode responds to reactant concentration (phenol, catechol and some others) even at concentrations 2×10^{-6} M. Such a high sensitivity makes the electrode a promising candidate for application in detecting traces of phenols.

Key words: *sol-gel; potentiometric enzyme electrode; urease; tyrosinase; glucose oxidase*

1. Introduction

The method of the sol-gel entrapment of enzymes and other biologically active molecules is a widely used technique of immobilisation for construction of biosensors, because of its simplicity and flexibility as well as low temperature of the process. The method allows us also to immobilise large amounts of biological materials with a good retention of their activity and low leakage from the gel. Since 1990, when Braun et al. [1] reported for the first time immobilisation of the enzymes in silica gel obtained by the sol-gel method, it has been widely used in various types of biosensors (electrochemical and optical) [2–5].

Enzymatic reactions in which acids or bases are produced can be traced by appropriate pH sensors. pH transducers typically used in the construction of biosensors are

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potentiometric electrodes: glass [6], metal–metal oxide [7–9] and chemically modified graphite [10, 11]. Other types of transducers with potentiometric response to pH used in biosensors are miniature semiconductor devices like field effect transistors (FET) [12, 13] or light addressable photodiodes (LAPs) [14]. Although the sol-gel method is widely used to immobilise enzymes in biosensors, its application in potentiometric biosensors is rare [15–17].

We developed potentiometric urea electrodes based on the tungsten–tungsten oxide system [8, 9]. We have also been involved in the application of the sol-gel method to the construction of amperometric enzyme electrodes based on enzymes of different types [18]. The aim of this work is to immobilise enzymes on the surface of tungsten –tungsten oxide pH-metric electrode in silica gel obtained by the sol-gel method. As the models, such enzymes as urease, glucose oxidase and tyrosinase were chosen. Tyrosinase, which oxidises phenols with consumption of two hydrogen ions leading to a local increase of pH [19, 20], was used in the construction of the enzyme FET selective for monophenols [20].

2. Experimental

2.1. Reagents

As the gel precursor, tetramethoxysilane (TMOS) (99+%, Aldrich) was used. It was chosen because the preliminary experiments with gels obtained from tetraethoxysilane showed lack of adhesion to the electrode surface. The enzymes used in experiments were: urease from Jack bean (type III, glycerol solution, 680 U/ml, Sigma), glucose oxidase from *Aspergillus niger* (solution, 5370 U/ml, Serva) and tyrosinase from mushroom (solid, 6050 U/mg, Sigma). Urease and glucose oxidase were used as delivered, tyrosinase was dissolved in 0.1 M phosphate buffer, pH 7 (1 mg per 1 ml). All other reagents were of analytical grade. Twice-distilled water was used throughout.

2.2. Apparatus

Potentiometric measurements were done with a microcomputer pH/oxygen meter CPO-551 (Elmetron, Zabrze, Poland). Saturated calomel electrode (SCE) OH-962 P (Radelkis, Budapest, Hungary) was used as a reference electrode. The measurements were done at the ambient room temperature. A magnetic stirrer BMM 21 maintained a constant stirring rate of the solutions.

2.3. Preparation of electrodes and enzyme immobilisation

Tungsten electrodes were prepared from tungsten rods, 3 mm in diameter and 70 mm long. After polishing, the electrodes were flame oxidised [8]. Then the central

part of the electrode was covered with lacquer leaving 1.5 cm of free surface at each end. Prior to use, the electrodes were soaked in water overnight.

The stock solution of the silica sol was prepared as follows: 5 ml of TMOS, 1 ml of water and 0.05 ml of 0.1 M HCl [21] were stirred vigorously at room temperature until a transparent homogenous solution was obtained (15 min) and stored in a refrigerator.

The casting solution was prepared by mixing 0.5 ml of the sol solution with 0.1 M phosphate buffer, pH 7, and the enzyme solution: 0.25 ml of buffer with 0.25 ml of urease solution, or 0.5 ml of buffer with 0.05 ml of glucose oxidase or tyrosinase were added. The casting solutions were used immediately after mixing and the electrodes were covered with the gel layer by dip coating. After formation of hydrogel (about 1 min), the electrode was dipped in 0.005 M phosphate buffer, pH 6.

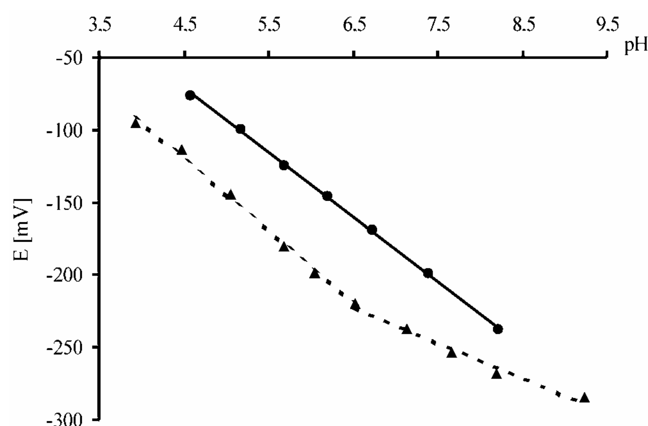


Fig. 1. pH response of the tungsten–tungsten oxide electrode:
● – uncovered electrode; ▲ – covered electrode

The electrodes were tested in a series of buffers for their pH response before and after covering with silica gel (Fig. 1).

2.4. Measurement procedure

The substrate water solutions of the concentration of 0.1 M were prepared freshly every day except glucose. Solution of this substrate was prepared one day before use to allow mutarotation. Solutions of gallic acid were adjusted to pH 6 by addition of NaOH.

The enzyme electrode and reference SCE connected with a voltmeter were dipped in 50 ml of 0.005 M phosphate buffer, pH 6, (if not stated otherwise), stirred with magnetic stirrer (at the rate of 500 rpm) at ambient temperature. In the case of urease, buffers were added with 0.002 M EDTA to avoid inactivation of the enzyme by heavy metal ions. When the potential of the electrode became stable, the desired amount of

0.1 M solution of the substrate was added. The electrode potential was measured and collected every 1 s by a PC computer until no changes were observed during 30 s (typically 5 min were enough for the whole measurement, in the case of urease this time was longer). From the obtained dependencies of the electrode potential vs. time (Fig. 2), the differences of initial and final potentials ΔE (steady-state method) and initial rates of potential change dE/dt (kinetic method) were found.

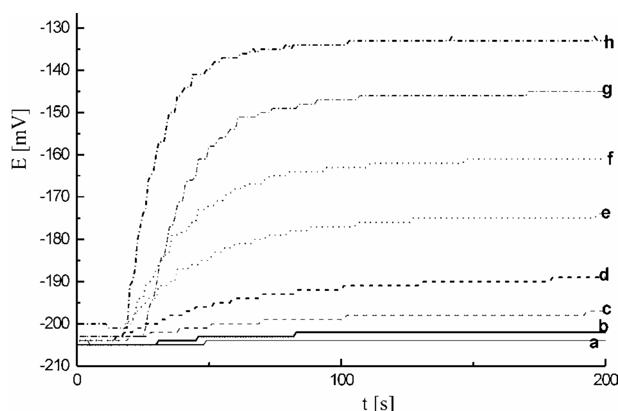


Fig. 2. Example of the potentiometric enzyme electrode response: enzyme–glucose electrode at pH 6; volume of added 0.1 M glucose solution: a) 10 μl, b) 25 μl, c) 50 μl, d) 100 μl, e) 250 μl, f) 500 μl, g) 1000 μl, h) 2500 μl

Calibrations curves of the electrodes were plotted as the dependencies of the electrode response (ΔE or dE/dt) on decimal logarithm of the substrate concentration. From these curves, dynamic range (linear parts of the calibration curves) and the slopes of the linear parts were read.

3. Results and discussion

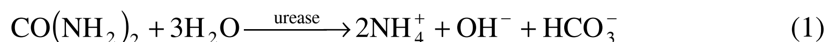
3.1. pH response of the electrode

Figure 1 shows the dependence of the electrode potential on pH. For a bare tungsten–tungsten oxide electrode its potential decreases linearly with the increase of pH. The slope of the straight line is –44.5 mV/decade (r = 0.999). For the electrode covered with silica gel, the potential of the electrode is lower than for the bare electrode. Its potential also decreases with increasing pH but one can recognise two regions with different slopes: first at the pH range from 4 to 6.5 (slope –49.9 mV/decade, r = 0.997), the other – between 6.5 and 9.5 (slope –24.3 mV/decade, r = 0.989). In each case, the slope is lower than the Nernstian value –59 mV/decade. The deviations from linearity for the electrode covered with silica gel and the sub-Nernstian values of

the slopes are probably connected with the reaction of neutralisation of hydrogen ions with silanol groups on the surface of silica [15].

The enzymes studied in this work are the catalysts of the following reactions:

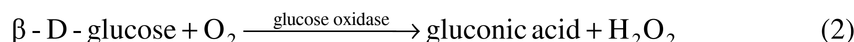
• Urease:



$$CO(NH_2)_2 + 3H_2O \xrightarrow{\text{urease}} 2NH_4^+ + OH^- + HCO_3^- \qquad (1)$$

The products of this reaction form a self-buffering mixture with pK_a = 8.83 [22]. When the pH of the environment of the reaction is less than pK_a value, the pH increases. Thus the potential of the urease electrode would decrease.

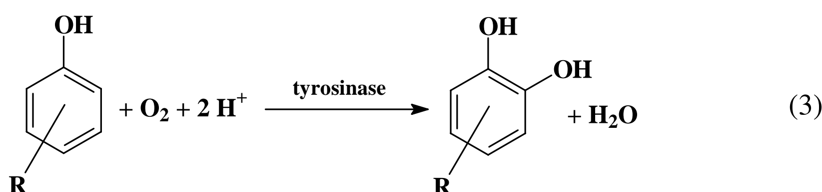
• Glucose oxidase:

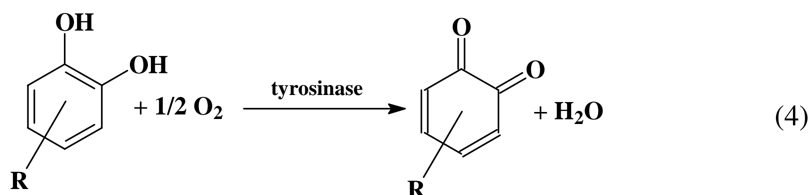


$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \qquad (2)$$

As the product of this reaction is an acid, the potential of the glucose oxidase electrode is predicted to increase after addition of the substrate.

• Tyrosinase:





Tyrosinase oxidises differently substituted mono- and *o*-diphenols with different rates of reactions (3) and (4). In reaction (3), the pH value increases, while in reaction (4) it remains constant. Thus by immobilisation of tyrosinase on a pH-transducer, a biosensor selective for phenols can be constructed [20].

3.2. Urease electrode

As predicted, after addition of urea the potential of the electrode decreases. The rate of the decrease is low and thus it cannot be the measure of the changes in urea concentration. Thus to obtain calibration curves for urea only the steady-state method was used. Figure 3 shows the calibration curves of the electrode with urease immobilised in silica gel.

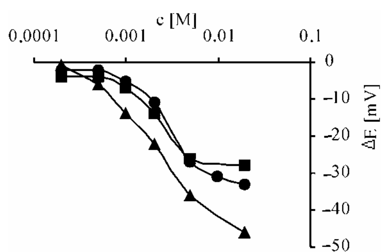


Fig. 3. Calibration curves for the urease electrode in 0.005 M buffers (steady -state method): ● – acetate, pH 5; ■ – phosphate, pH 6; ▲ – alanine, pH 6

The dynamic range of this sensor in acetate and phosphate buffers is very narrow; only about one decade. The result is similar to the results obtained with the tungsten–tungsten oxide electrode with urease covalently bound to the surface [9] and entrapped in gelatine gel [8]. The response time is rather long – about 6–7 min. Only when alanine buffer was used, the dynamic range of the electrode was broader (2×10^{-4}–2×10^{-2} M of urea) with the slope of –24.2 V/decade ($r = 0.991$), which is much less than for the tungsten electrode with urease covalently bound [9].

These preliminary measurements indicate that entrapment of urease in silica gel on the surface of the tungsten–tungsten oxide electrode is not a promising way to construct the urease electrode with properties allowing its practical use. But the results of Ogura et al. [15] and Pandey and Singh [17] indicate that using a different pH sensor would improve the properties of the urea sensor.

3.3. Glucose oxidase electrode

Figure 2 shows typical changes of the glucose oxidase electrode after addition of glucose. As predicted, the electrode potential increases after addition of the substrate. The increase of the potential is quick and the response time of the electrode is shorter than 3 min. Only at a very low concentration of the buffer and at high concentration of the substrate it is longer (about 4 min).

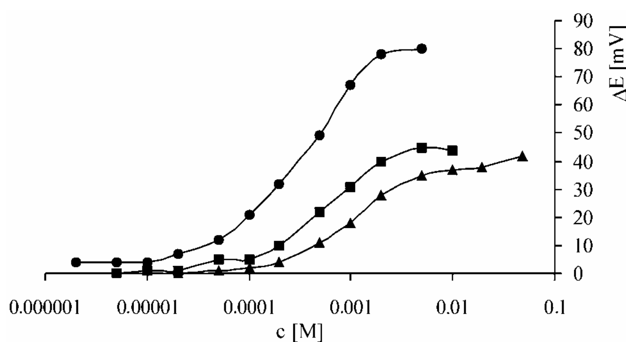


Fig. 4. Calibration curves for the glucose oxidase electrode at pH 6 (steady-state method) at different concentrations of the buffer: ● – 0,001 M; ■ – 0,005 M; ▲ – 0,02 M

Figure 4 shows the calibrations curves of the glucose oxidase electrode at pH 6 at different concentrations of the buffer. When the buffer concentration increases, the dynamic range of the curve is moving to higher substrate concentrations and the slope

of the linear part of calibration curve is decreasing (Table 1). This is a typical behaviour of the pH-based potentiometric enzyme electrodes, connected with increasing buffer capacity at higher concentrations [8–10]. The kinetic response of the electrode is practically independent of the buffer concentration.

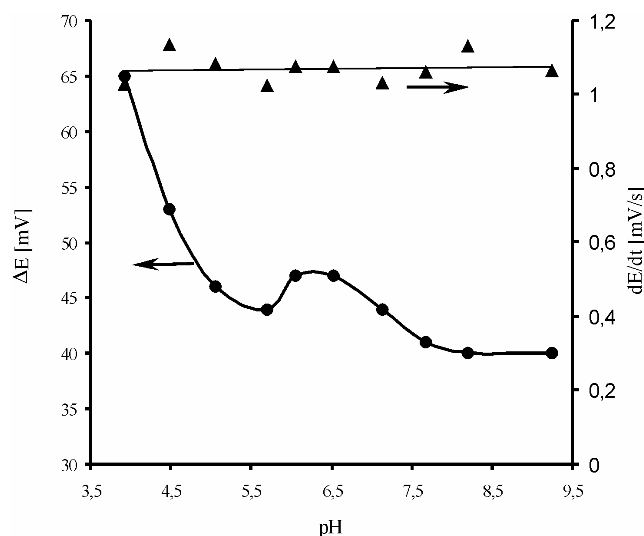


Fig. 5. Dependence of the glucose oxidase electrode response on pH (0.005 M buffers, for pH less then 5.5 – acetate, higher – phosphate, glucose concentration 5×10^{-4} M): ● – steady-state method; ▲ – kinetic method

Table 1. Characteristics of glucose oxidase electrode (steady-state method)

pH	Buffer concentration [M]	Dynamic range [M]	Slope [mV/decade]	*r*
5	0.005	10^{-4}–5×10^{-3}	46.3	0.998
6	0.001	5×10^{-5} – 2×10^{-3}	42.6	0.995
	0.005	10^{-4}–5×10^{-3}	37.7	0.995
	0.02	2×10^{-4} – 5×10^{-3}	23.2	0.994
7	0.005	10^{-3}–2×10^{-2}	31.2	0.998

The response of the glucose oxidase electrode depends on pH (Fig. 5 and Table 1). Figure 5 shows the variation of the electrode response at different pH values. It can be seen that the response in the steady-state method depends significantly on pH with local maximum at pH about 6 and sharp increase for decreasing pH values. The local maximum well corresponds with the optimum value of pH of a free enzyme [23] and those immobilised on platinum in bovine serum albumin [24]. The increase of the response with decreasing pH for the values less than 5.5 when acetate buffers were used is most probably connected with decreasing buffer capacity. In the kinetic method, the electrode response is practically independent of pH. When the rates of the

initial changes of the electrode potential are taken into account as the glucose concentration measures, the beginning of the linear part of the calibration curves passes to higher values and the slope of the linear part is practically the same for pH 5, 6 and 7. The slopes of calibration curves in the steady-state method are less than the Nernstian value for all pH values tested and all buffer concentrations (Table 1).

The results obtained indicate that the glucose electrode potential is altered by the hydrogen ion concentration (pH). The sub-Nerstian slopes of the calibration curves in the steady-state method and independence of the rate of the potential changes (kinetic method) of pH and buffer capacity indicate, however, that there could also be other sources of potentiometric response like red-ox state of the enzyme [24] or reactions of hydrogen peroxide produced in reaction (2) [25].

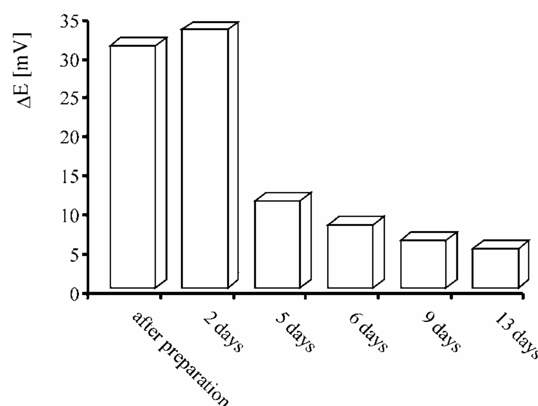


Fig. 6. Stability of the glucose oxidase electrode (tested for 0.005 M phosphate buffer, pH 6, glucose concentration 5×10^{-4} M)

The potentiometric glucose oxidase electrode is very unstable (Fig. 6). Glucose oxidase is a very stable enzyme as its molecule is a glycoprotein [23] and many of the biosensors for glucose are also very stable allowing practical use. But some data indicate that glucose oxidase immobilised in silica gel is unstable [18, 26]. This instability is partially caused by the leakage of the enzyme from the silica gel [18].

3.4. Tyrosinase electrode

For the tyrosinase electrode, the results were surprising. The predicted response was: a decrease of the electrode potential for phenol as substrate and no change for *o*-diphenol (catechol). The electrode studied showed an increase of the potential after addition of both substrates. The example of the electrode response for catechol is shown in Fig. 7.

The potential increase is quick and very significant even for very low concentrations of catechol (compare curve a in Fig. 7 – $c_{\text{catechol}} = 2\times10^{-6}$ M). For an amperometric electrode constructed on the basis of oxygen electrode there would be practically no response for such a concentration [18]. For higher concentrations of the substrate, after initial quick increase of the electrode potential a slow decrease is observed

(curves f, g and h in Fig. 7). Thus, in the case of tyrosinase electrode, ΔE was calculated as a response in the steady-state method i.e., as a difference between initial value and the maximum one after addition of the substrate. Also the initial rate of the potential increase *dE/dt* is lower for high concentrations of the substrate. This effect is connected with the properties of tyrosinase which undergoes a suicide auto-inhibition by the substrate [27, 28]. This inactivation is irreversible and because of that many biosensors with tyrosinase have very low operational stability [20, 29].

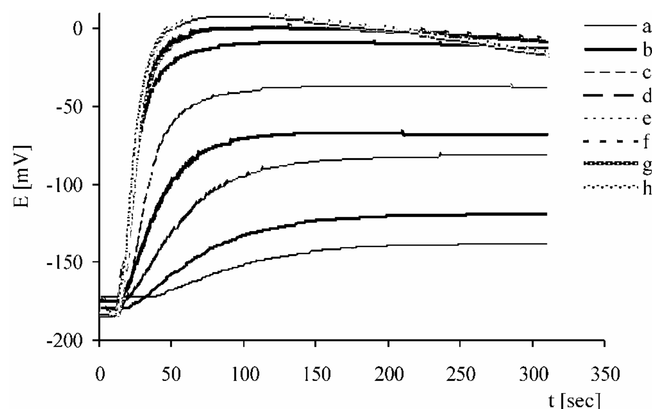


Fig. 7. Potentiometric tyrosinase electrode response to catechol at pH 6; volume of 0.1 M substrate solution added:
a) 1 μl, b) 2.5 μl, c) 5 μl, d) 10 μl, e) 25 μl, f) 50 μl, g) 100 μl, h) 250 μl

The influence of the buffer pH and concentration was tested for a model substrate – catechol. For the catechol concentration equal to 2×10^{-5} M, the maximum response in a steady-state and kinetic methods was observed at pH = 6 (Fig. 8).

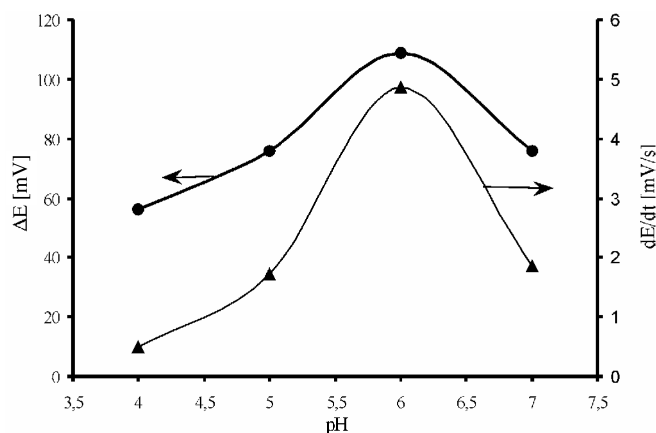


Fig. 8. Dependence of the tyrosinase electrode response on pH (0.005 M buffers, for pH 4 and 5 – acetate, higher – phosphate, catechol concentration 2×10^{-5} M):
● – steady-state method; ▲ – kinetic method

This optimum of the sensor response is in good agreement with the results obtained by other authors [20, 30, 31]. The slopes of linear parts of the calibration curves vary slightly with pH with a minimum at pH 5 (Table 2). The response time of the electrode to catechol is practically about 3 min, only at pH 4 it is longer. The inhibition effect of the substrate is enhanced at higher pH. At pH 7 this effect is visible for catechol concentration 5×10^{-5} M (compare the results in Fig. 7 at pH 6). At pH 4 for all catechol concentrations tested no effect of auto-inhibition was evident. The buffer concentration was tested at pH 6 (Table 2) and practically no influence was found.

At pH 6, the tyrosinase electrode was tested for catechol in a buffer containing 10% of isopropanol. This was done because tyrosinase activity can be modulated by addition of organic solvents [32]. Addition of isopropanol causes the increase of the electrode response, especially of dE/dt. Also the slope of the linear part of the calibration curve in the steady-state method is increased and is higher than the Nernstian value (Table 2). These results indicate that addition of isopropanol activates tyrosinase.

Table 2. Characteristics of tyrosinase electrode (steady-state method) for catechol as a substrate

pH	Buffer concentration [M]	Dynamic range [M]	Slope [mV/decade]	*r*
4	0.005	5×10^{-6}–2×10^{-4}	60.2	0.992
5	0.005	2×10^{-6}–10^{-4}	51.1	0.992
6	0.001	2×10^{-6}–10^{-4}	62.6	0.988
	0.005	2×10^{-6}–10^{-4}	58.4	0.992
	0.02	2×10^{-6}–10^{-4}	56.5	0.997
6	0.005 with 10% of isopropanol	2×10^{-6}–10^{-4}	79.3	0.999
7	0.005	2×10^{-6}–5×10^{-5}	57.9	0.992

The source of the tyrosinase electrode response is unknown. It is of course not the change of pH because the results are completely different to those obtained for pH sensitive FET with immobilised tyrosinase [19, 20]. It was checked that similar response was obtained when tyrosinase was immobilised in albumin gel on the surface of tungsten–tungsten oxide electrode. When tyrosinase is immobilised on the surface of a glass pH-metric electrode in silica gel, no response to catechol was observed.

There could be two other sources of the potentiometric response of the electrode. The first – the potential of the electrode is altered by the local oxygen concentration near the electrode surface. Preliminary experiments indicate that the tungsten –tungsten oxide electrode is also sensitive to oxygen. The second – the potential variation is caused by the red-ox state of the enzyme. Tyrosinase belongs to the class of copper enzymes. The active centre of tyrosinase contains two copper atoms (Cu^{2+}) which are reduced to Cu^{+} when the substrate molecule (monophenol or *o*-diphenol) is oxidised, and reduced form of the enzyme is formed [28]. In the next step, the enzyme is reoxidised by oxygen. A similar copper-containing enzyme – ascorbate oxidase was used to construct the potentiometric electrode for ascorbate [33]. The potential varia-

tion of such an electrode was attributed by the authors to a change of the electron density on the electrode surface caused by the reduction of Cu^{2+} to Cu^{+} by the substrate. The characteristics of that electrode was also similar to that of the tyrosinase electrode studied in this work: very low concentrations detected and similar dynamic ranges, no influence of the buffer concentration. Because of that it is supposed that the source of potential variation for tyrosinase electrode is most probably the red-ox state of the enzyme.

Tyrosinase oxidises variously substituted mono- and *o*-diphenols at different rates. Thus the biosensors with immobilised tyrosinase show the response to many substrates [18]. The selectivity spectrum of a biosensor could depend on the source of the enzyme, method of immobilisation, transducer and composition of environment in which it is used [18, 20, 34]. The studied electrode was tested for various substrates. The results are collected in Table 3.

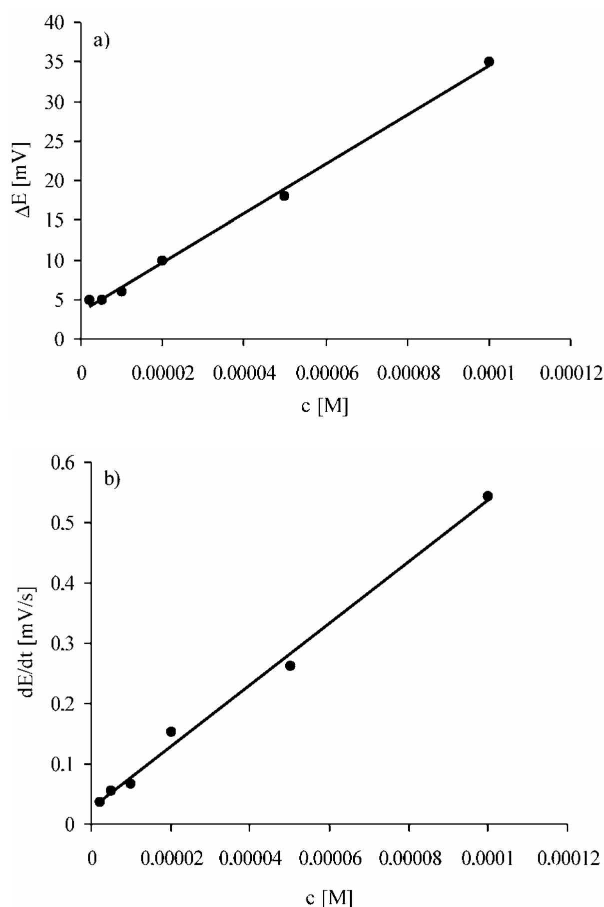


Fig. 9. Calibration curve of the tyrosinase electrode for phenol in 0.005 M phosphate buffer, pH 6: a) steady-state method – equation of the straight line is $\Delta E = 3010390c + 3.5$ ($r = 0.998$); b) kinetic method – equation of the straight line is $dE/dt = 5114c + 0.027$ ($r = 0.997$)

Table 3. Analytical characteristics of tyrosinase electrode at pH 6 (0.005 M phosphate buffer) for different substrates

Substrate	Dynamic range [M]	Slope [mV/decade]	*r*
Catechol	2×10^{-6}–10^{-4}	58.4	0.992
Phenol	2×10^{-5}–10^{-3}	40.7	0.990
p-Cresol	2×10^{-6}–10^{-4}	24.5	0.982
m-Cresol	5×10^{-6}–5×10^{-4}	28.2	0.989
p-Chlorophenol	2×10^{-6}–10^{-4}	18.7	0.999
Pirogallol	2×10^{-4}–10^{-3}	13.4	0.996
Gallic acid	10^{-4}–10^{-2}	14.8	0.985
Dopamine	10^{-5}–5×10^{-4}	91.9	0.990

As can be seen from Table 3, the potentiometric tyrosinase electrode is most sensitive to dopamine, catechol and phenol. For other substrates tested, the sensitivity is much lower. For pirogallol and gallic acid the dynamic range is shifted to higher concentrations. There were no response for *o*-cresol, *o*-chlorophenol, *o*-aminophenol, *p*-aminophenol and tyrosine.

If the response of the electrode to phenol is plotted against concentration (Fig. 9), the straight line is obtained for both methods: the steady-state- and the kinetic one, and the fit is better than for the logarithmic dependence (see Table 3).

These results suggest that tyrosinase electrode based on metal–metal oxide with enzyme immobilised in silica gel could be used to assay traces of phenol, for example, in water. Such an application needs an optimisation of the biosensor construction and operation conditions. Because of properties of tyrosinase (autoinhibition) such a biosensor ought to be used rather as a disposable one.

4. Conclusions

The results presented in this work indicate that entrapment of the enzymes in silica gel obtained by the sol-gel method is suitable in construction of potentiometric biosensors. The layer of silica gel changes the potentiometric response of the tungsten –tungsten oxide electrode to pH. The urease and glucose oxidase electrodes showed behaviour as predicted but their properties are too poor for practical use. The results indicate also that glucose oxidase is instable in silica gel.

The most promising results were obtained for tyrosinase electrode. It is very sensitive to phenol, catechol and dopamine. The direction of changes of the electrode potential in this case is completely different to the predicted one, indicating that the the electrode potential does not depend on the concentration of hydrogen ions. A reasonable explanation is that the potential of the electrode reflects the red-ox state of the active centre of tyrosinase.

A practical use of tyrosinase electrode needs an improvement of its construction and optimisation of the operation conditions.

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