Analytica Chimica Acta, Volume 659, Issues 1-2, 5 February 2010, Pages 274-279

Accepted Manuscript

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PII: S0003-2670(09)01600-6
DOI: doi:10.1016/j.aca.2009.11.065
Reference: ACA 230313

To appear in: *Analytica Chimica Acta*

Received date: 1-9-2009
Revised date: 26-11-2009
Accepted date: 30-11-2009

Please cite this article as: T.A. Sergeyeva, O.A. Slinchenko, L.A. Gorbach, V.F. Matyushov, O.O. Brovko, L.M. Sergeeva, G.V. Elska, Catalytic molecularly imprinted polymer membranes. Development of the biomimetic sensor for phenols detection, *Analytica Chimica Acta* (2008), doi:10.1016/j.aca.2009.11.065

CATALYTIC MOLECULARLY IMPRINTED POLYMER MEMBRANES. DEVELOPMENT OF THE BIOMIMETIC SENSOR FOR PHENOLS DETECTION.

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Portable biomimetic sensor devices for the express control of phenols content in water were developed. The synthetic binding sites mimicking active site of the enzyme tyrosinase were formed in the structure of free-standing molecularly imprinted polymer membranes. Molecularly-imprinted polymer membranes with the catalytic activity were obtained by co-polymerization of the complex Cu (II)-catechol-urocanic acid ethyl ester with (tri)ethyleneglycoldimethacrylate, and oligourethaneacrylate. Addition of the elastic component oligourethaneacrylate provided formation of the highly cross-linked polymer with the catalytic activity in a form of thin, flexible, and mechanically stable membrane. High accessibility of the artificial catalytic sites for the interaction with the analyzed phenol molecules was achieved due to addition of linear polymer (polyethyleneglycol Mw 20 000) to the initial monomer mixture before the polymerization. As a result typical semi-interpenetrating polymer networks (semi-IPNs) were formed. The cross-linked component of the semi-IPN was represented by the highly cross-linked catalytic molecularly-imprinted polymer, while the linear one was represented by polyethyleneglycol Mw 20 000. Extraction of the linear polymer from the fully-formed semi-IPN resulted in formation of large pores in the membranes' structure. Concentration of phenols in the analyzed samples was detected using universal portable device oxymeter with the oxygen electrode in a close contact with the catalytic molecularly-imprinted polymer membrane as a transducer. The detection limit of phenols detection using the developed sensor system based on polymers-biomimics with the optimized composition comprised 0.063 mM, while the linear range of the sensor comprised 0.063–1 mM. The working characteristics of the portable sensor devices were investigated. Storage stability of sensor systems at room temperature comprised 12 months (87%). As compared to traditional methods of phenols detection the developed sensor system is characterized by simplicity of operation, compactness, and low cost.

K e y w o r d s: phenols, polymers-biomimics, polymer catalysts, molecularly-imprinted polymers, sensor, tyrosinase, environmental monitoring.

1. Introduction

Development of biosensors for detection of various toxic compounds in both environment and food stuffs is one of the most promising areas of modern biotechnology [1–3]. Biosensors are able to provide highly-sensitive, selective, and reliable analysis of toxic molecules. Normally they are characterized by simplicity of operation, low cost, and compactness. One of the general drawbacks of biosensor analysis is the low stability of biomolecules in extreme environments and, therefore, necessity of stabilization of bioselective elements of biosensors. From this point of view, substitution of biomolecules by their synthetic counterparts (molecularly-imprinted polymers) is very attractive [4]. Molecularly-imprinted polymers

(MIPs) are widely used as stationary phases for chromatography and solid-phase extraction [5-6] as well as substitutes of antibodies in immunoassays and biosensors [7-11].
Synthesis of enzyme mimicks able to selective cleavage of analytes of interest using the method of molecular imprinting is of great importance for biosensor technology [12-20]. To date the possibilities of synthesis of molecularly-imprinted polymers able to catalytic cleavage of esters [21-24], p-aminophenol [25-26], glutathione [27], nitrophenols [28] as well as catalytic hydrolysis of carbonates and carbamates [14, 29, 30] and have been demonstrated. During the recent years new formats for MIPs attract significant interest. Application of molecularly imprinted polymer membranes in biosensor technology is becoming increasingly attractive as compared to traditional MIP particles synthesized either by bulk, suspension or precipitation polymerization [31-32].
The aim of the present research is synthesis of molecularly imprinted polymer membranes mimicking natural enzyme tyrosinase, development of biosensors for phenols detection on their basis, as well as optimization of their working parameters.
There are a number of traditional methods of phenols' detection in the environment and food stuffs. Instrumental analytical methods (GC-MS [33], HPLC [34-37], capillary electrophoresis [38], capillary electrochromatography [39], thin-layer chromatography [40, 41] are widely used for routine detection of phenols. As the more effective alternative to the above-mentioned methods, a number of highly-sensitive biosensor devices based on the immobilized mushroom tyrosinase [42-44] and horseradish peroxidase [45], providing the possibility of phenols detection in micromolar range with detection limits for different phenolic compounds 1- 25 μM have been developed. However, stability of biosensors during prolonged storage is insufficient for wide practical application, while necessity of either enzyme stabilization or application of highly stable enzyme mimics is obvious [46, 47].

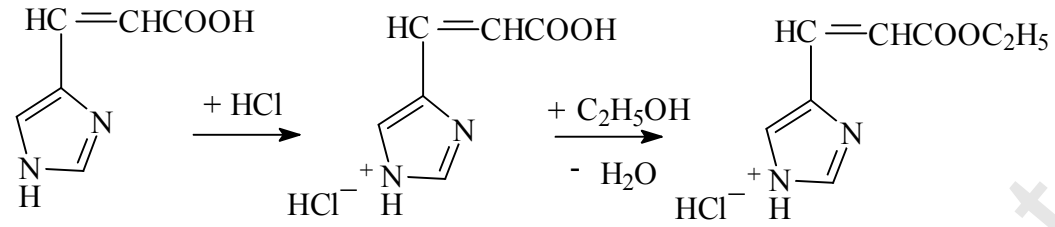
2. Materials and methods.

2.1. Materials.

$CuCl_2$, catechol, (tri)ethyleneglycoldimethacrylate, 2,2"-azobisisobutyronitrile (AIBIN), resorcinol, dimethylformamide, urocanic acid were purchased from Sigma-Aldrich, USA. Urocanic acid ethyl ester was synthesized from urocanic acid by esterification.

2.2. Synthesis of urocanic acid ethyl ester.

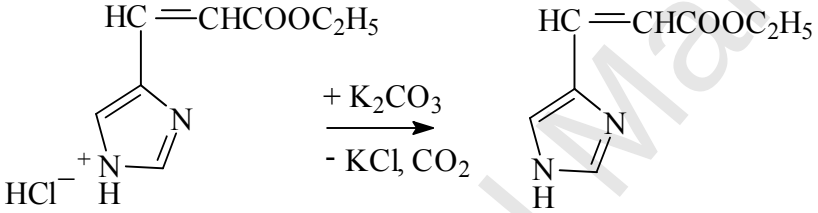
Ethanol was dried over CaO and distilled prior to use. HCl vapours were obtained by slow addition of concentrated HCl to concentrated H_2SO_4 with further bubbling through concentrated H_2SO_4. K_2CO_3 was dried before use by incineration.
Esterification, production of urocanic acid ethyl ester hydrochloride:
0.1 M of urocanic acid were mixed with 4M of ethanol in a reactor with a stirring rod, backflow condenser, and gas-intake pipe. The mixture was saturated with HCl vapors up to pH=1-2 during the mixing and cooling procedure. The mixture was heated up to boiling. That was followed by mixing until the sediment is dissolved (typically for 3 hours). The backflow condenser was changed for a descending one and ethanol (~100 g) was slowly distilled off for 2.5 hours until appearance of the sediment. The distillation was stopped, the reaction mixture was cooled down to the room temperature. Under these conditions precipitation of urocanic acid ethyl ester hydrochloride occurred.
The sediment was filtered, washed with ethanol, dried at room temperature. The urocanic acid ethyl ester hydrochloride (white crystals with the melting point ~ 202-207°C) was obtained.
The reactions take place according to the following schemes:



After re-crystallization from ethanol, the melting point of urocanic acid ethyl ester hydrochloride comprised 205-206°C.

Isolation of urocanic acid ethyl ester from its hydrochloride. The reactor with a stirring rod, dropping funnel, and gas-intake pipe was used for the synthesis. 0.1 M of urocanic ethyl ester hydrochloride was placed in the rector and dissolved in 50 ml distilled water. The dropping funnel was filled with aqueous K_2CO_3 solution and this solution was slowly added to the urocanic acid ethyl ester hydrochloride solution in the reactor during cooling on water bath under constant stirring. The precipitated after this procedure urocanic acid ethyl ester was filtered, washed with H_2O and dried at room temperature.

The reaction takes place according the following scheme:



The product yield at this stage corresponds to the theoretically estimated one, while the melting point comprised ~ 85°C.

The obtained urocanic acid ethyl ester is soluble in organic solvents of moderate polarity (dichloroethane, acetone, alcohols) and polar solvents (dimethylformamide) and insoluble in aliphatic hydrocarbons (hexane) and water.

2.3.Synthesis of molecularly-imprinted polymers.

The synthetic mimics of tyrosinase were synthesized using the method of molecular imprinting. Monomer mixture for the synthesis of catalytic molecularly-imprinted polymer membranes contained 0.0069 g catechol, 0.0619 g urocanic acid ethyl ester, 0.134 g $CuCl_2 \cdot 2H_2O$, 1.25 g dimethylformamide, 1.2012 g mixture of tri(ethyleneglycol)dimethacrylate and oligourethaneacrylate with the ratio 85/15 % (wt), 0.1895 g polyethyleneglycol Mw 20000, 0.1068 g azobisisobutyronitrile. Thermo-initiated radical polymerization was carried out at 80°C for 12 hours between two glass slides. To remove template molecules, non-polymerized compounds, polyethyleneglycol, and Cu (II), molecularly-imprinted polymer membranes were extracted with hot methanol in Soxlet apparatus for 8 h. That was followed by washing with 0.03% aqeous solution of EDTA, dimethylformamide and water. Blank polymeric membranes were synthesized from the same monomer composition, which did not contain catechol. The polymer with the different geometry of the binding site was synthesized with resorcinol as a template molecule and was used as a second control.

2.4. Measurements.

To estimate catalytic properties of the molecularly-imprinted polymer membranes, 60 μm membrane samples (d=12 mm) were fixed near the surface of the oxygen electrode (MERA-ELWRO 5972, Poland) at the distance ≈ 3 mm using a teflon O-ring. The measurements

were carried out at 25°C in a 20 ml electrochemical cell filled with 100 мM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl and 5 mM $CuCl_2$ under constant stirring. The electrochemical cell had no head-space (atmosphere). Dissolved in a working solution O_2 (0.23 mM) was used as a source of oxidant for the reaction. The sensor responses were initiated by injections (5–100 μl) of a catechol stock solution in the same buffer to the reaction vessel. The change in the oxygen concentration during the reaction was monitored by the universal oxymeter MERA-ELWRO 5221 (Poland) and oxygen electrode MERA-ELWRO 5972 (Poland).

Catechol concentration in the analyzed samples of drinking, river, and waste waters were determined also using high performance liquid chromatography (HPLC system LC-20A (SHIMADZU, Japan), UV-detector, λ=284 nm, column SUPELCOSIL LC-18-DB 250mm, t=25-27°C, mobile phase 2% acetic acid in H_2O 70% : acetonitrile 30%).

3. Results and discussion

Synthetic tyrosinase mimics combining ability of natural enzyme to catalytic cleavage of phenols with the stability in extreme environments were synthesized by the method of molecular imprinting.

It is widely known that the active site of tyrosinase is a binuclear copper center capable of oxidizing two molecules of o-diphenol to o-quinone with concomitant reduction of molecular oxygen to two molecules of water (Fig. 1, a) [48, 49]. Therefore, the reaction of catechol oxidation can be monitored by the oxygen electrode due to reduction of molecular oxygen on its surface according to the equation presented in Fig. 1, b. This reaction assumes simultaneous coordination of the two Cu(II) ions by the o-phenolic oxygens with a copper-copper distance 0.36 nm (Fig. 2, a).

Several attempts were made to mimic enzymes able of conversion of o-diphenols to o-quinones [50, 51]. However, they usually involve multi-step organo-metallic synthesis, while the results obtained are quite modest.

Since the method of molecular imprinting can be used for metal imprinting [52, 53] it is attractive for the synthesis of catalytic Cu-containing polymers, mimicking a tyrosinase active site. Taking into account data on the structure of the tyrosinase active sites, it was assumed that catechol molecule, able to coordinate two Cu(II) ions at a distance 0.36–0.4 nm, can be imprinted using urocanic acid ethyl ester, mimicking histidine residues in the natural enzyme (Fig. 2, b). According to the principle of molecular imprinting, catechol extraction from the fully-formed polymeric network would result in formation of cavities where imidazole groups oriented for complexation with Cu(II) and catechol.

Molecularly imprinted polymer membranes with tyrosinase activity were synthesized according to the method developed by our group for the synthesis of MIP membranes with receptor properties [54, 55]. This approach assumes application of additional components-modifiers/elasticators in the traditional monomer mixture for MIP synthesis normally containing template, functional monomer and a cross-linker. Addition of the flexible linear oligomer – oligourethaneacrylate provides synthesis of highly cross-linked molecularly imprinted polymer in a form of thin, flexible, and mechanically stable membranes. MIP membranes were synthesized by radical thermo-initiated co-polymerization of the complex between o-diphenol, Cu(II), and urocanic acid ethyl ester with (tri)ethyleneglycoldimethacrylate, and oligourethaneacrylate. The evidences for the tyrosinase activity of MIPs synthesized with the same monomer composition were demonstrated in our previous work on MIP particles [56]. The ratio template/functional monomer was also optimized earlier [56, 57]. Dimethylformamide used as a porogen for the membrane synthesis was supposed to provide good accessibility of the catalytic sites for the interaction with the analyzed molecules. However, despite of good mechanical properties, the synthesized membranes were characterized with very low catalytic activity. Apparently that was

associated with their insufficient porosity (Fig. 3, a) and, as a result, poor accessibility of the catalytic sites.

To increase porosity of the MIP membranes, the method of molecular imprinting was combined with the principle of synthesis of interpenetrating polymer networks. The effectiveness of such approach was earlier demonstrated [58-60].

A number of MIP membranes with catalytic properties were synthesized according to the principle of semi-interpenetrating polymer networks. The cross-linked component of the semi-IPNs was represented by the highly cross-linked MIP, while the linear semi-IPN component was represented by polyethyleneglycol Mw 20 000. As it was expected, addition of polyethyleneglycol Mw 20 000 resulted in formation of significantly much more porous structures (Fig. 3, b).

Blank polymeric membranes were synthesized using the same mixture of monomers, which did not contain catechol. MIP membranes synthesized with resorcinol as a template molecule were used as a second control.

The catalytic MIP membranes synthesized according to the principle of semi-IPNs were used as selective elements of a portable sensor system for phenols detection. Oxygen electrode modified with porous catalytic MIP membrane was used as a transducer of this system.

Typical calibration curve of the developed sensor system is presented in Fig. 4. It was demonstrated that MIP sensor systems based on the catalytic MIP membranes with the optimized composition allowed one to detect phenols in the aqueous solutions with the detection limit 63 μM, while linear dynamic range comprised 0.063-1 mM (Fig. 4). Blank polymeric membranes synthesized from the same mixture of monomers, but in the absence of the template demonstrated significantly lower sensor responses after addition of catechol to the analyzed sample. Similar situation was observed also for the MIP membranes synthesized with the changed geometry of the catalytic site (these polymeric membranes were synthesized using resorcinol as a template molecule). Their insignificant catalytic activity clearly confirms the imprinting effect: changed geometry of the catalytic sites can not provide conditions for the effective catalysis. Importantly, virtually no catechol oxidation is observed in the working solution in the absence of MIP membranes. On the contrary, modification of the oxygen electrode with the catalytic MIP membranes resulted in immediate initiation of catecol oxidation. That is a clear evidence for tyrosinase activity of the synthesized MIP membranes. This phenomenon was discussed in detail in our previous work [56], where optimization of the catalytic MIP composition based on the analytical data on MIP-based sensor development is given.

Working characteristics of the developed sensor system based on catalytic MIP membranes with tyrosinase activity were also investigated and optimized. Dependencies of the sensor responses on buffer concentration (Fig. 5), NaCl concentration (Fig. 6) and pH of the analyzed solution (Fig. 7) were investigated.

As it was expected, significant changes of the sensor responses were observed in response to the increase in both buffer (Fig. 5) and NaCl concentration (Fig. 6). Apparently, under these conditions (especially at high buffer and NaCl concentrations) an increase in the Cu(II) adsorbtion by the molecularly imprinted polymer membrane occurs, which results in the increase in the catechol oxidation rate. Similar situation is observed in the case of application of the natural enzyme (mushroom tyrosinase) as a selective element of biosensors for o-diphenols detection: the rate of o-diphenols oxidation is increased with the increase in ionic strength of the solution [61].

Investigation of pH-influence of the analyzed solution on catechol oxidation rate by the molecularly-imprinted polymer membranes demonstrated that the pH-dependence had a pronounced maximum at neutral pH values (Fig. 7). Catechol oxidation in solutions with

pH≤5 is almost inhibited (Fig. 7). These results are in a good accordance with the literature data [62], demonstrating that Cu(II) ions are not adsorbed on surfaces modified with amino groups at acidic pHs. Therefore, formation of the complex between Cu(II) and imidazole moieties is inhibited as well as the catalytic oxidation of catechol by molecularly-imprinted polymer membranes with tyrosinase activity. A decrease in values of the sensor responses at both acidic and alkaline pHs can be also associated with swelling of the molecularly-imprinted polymer membranes at these conditions. Swelling of the MIP membranes can change optimal geometry of their catalytic sites necessary for the effective catalysis. Swelling of the polymers in both acidic and alkaline conditions can be associated with the precence of both: carboxygroups of (tri)ethyleneglycoldimethacrylate and imidazole groups of urocanic acid ethyl ester on the surface of MIP membranes. One can expect ionization of carboxygroups at alkaline pH values. In these conditions electrostatic repulsion of the negatively charged polymeric chains occurs. That would facilitate swelling of the polymers in these conditions. Similar situation can be expected also in acidic pHs, where imidazole moieties would be positively-charged. These estimates are in accordance with the literature data on swelling of molecularly-imprinted polymers [63]. However, in acidic conditions catalytic oxidation of catechol is virtually inhibited. Therefore, one can assume that influence of the polymer swelling in these conditions is less significant as compared to complications occurring during formation of the complex between Cu(II) and imidazole moiety of the polymer.

To reveal general selectivity of the developed sensor system, close structural analogues of catechol (phenol, 4-nitrophenol, 1,2,3-trihydroxybenzol, 2-methoxyphenol, m- diphenol, p-diphenol. bisphenol A, 1,2-naphthalenediol, and 1,4-naphthalenediol) were added to the electrochemical cell. In contrast to biosensor devices based on mushroom tyrosinase able to recognition of different phenolic compounds [42-44], the developed sensor system was characterized with high selectivity: it was able of effective catalytic oxidation of o-diphenols, while no sensor responses was observed in the case of their structural analogues (phenol, 4-nitrophenol, and 2-methoxyphenol, m- diphenol, p-diphenol. bisphenol A, 1,2-naphthalenediol, and 1,4-naphthalenediol).

The developed sensor system was used for detection of o-diphenol concentration in both model solutions and real samples of tape, river, and wastewaters. The samples of tape, river, and wastewaters spiked with 1 mM catechol were used for the investigation (Table 1). It was shown that the results of o-diphenol detection by the sensor method were in a good accordance with those obtained by traditional HPLC method (Table 1). Interestingly, 1 mM of catechol was added to all the analysed real samples. However, only 0.4 mM catechol was revealed in the sample 1 by both sensor and HPLC methods. The sample 1 was taken from river Vita (Pyrogiv, Kiev region). The very probable reason for revealing significantly smaller catechol amount by both sensor and HPLC analysis is significant contamination of the real samples of the river water by soil bacteria. It is widely recognized that catechol dioxygenases are key enzymes in the metabolism of aromatic rings by soil bacteria. The decrease in both the sensor responses and HPLC data can be caused by cleavage of the major part of the added to the sample 1 catechol by bacterial catechol dioxygenases. However, it should be noted that the results of the sensor evaluation of this particular sample are in good agreement with those obtained by a traditional instrumental method (HPLC), proving applicability of the developed sensor system for the analysis of real environmental samples.

Stability of the developed sensor systems based on catalytic molecularly-imprinted polymer membranes comprised 12 months (87% of the initial catalytic activity)

As compared to traditional instrumental methods the developed sensor system is simple, compact, and can provide inexpensive express analysis of o-diphenols in aqueous samples. As

compared to the existing biosensor methods of phenols detection, the MIP-membrane-based sensor is highly stable.

4. CONCLUSIONS

For the first time catalytic free-standing molecularly-imprinted polymer membranes mimicking enzyme tyrosinase were synthesized using the combination of the methods of molecular imprinting and semi-interpenetrating polymer networks. An easy-to-use and effective biomimetic sensor system for express o-diphenols detection in aqueous samples was developed on their basis.

AKNOWLEDGEMENT

Financial support from National Academy of Sciences of Ukraine (Program “Sensor systems for medico-ecological and industrial purposes”) is gratefully acknowledged.

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FIGURE CAPTIONS.

Fig. 1. Enzymatic oxidation of catechol to o-quinone in the presence of tyrosinase (a) and reduction of molecular oxygen at the surface of oxygen electrode (b).

Fig. 2. Possible structure of the active site of Streptomyces castaneoglobisporus tyrosinase according to H. Decker et al. [49] (A) and possible structure of a molecularly-imprinted active site with tyrosinase activity (B).

Fig. 3. SEM microfotographs of molecularly-imprinted polymer membranes synthesized with (A) dimethylformamide as a porogen and with a mixture of dimethylformamide and polyethyleneglycol Mw 20 000 according to the principle of semi-IPN formation (B)

Fig. 4. Typical calibration curve for determination of phenols concentration using portable sensor system based catalytic molecularly-imprinted polymer membranes with tyrosinase activity. Measurements were carried out in 100 мM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl and 5 mM $CuCl_2$. 1 – MIP membrane, 2- blank membrane, 3-MIP-resorcinol.

Fig. 5. Dependence of the sensor response of the portable sensor system based on catalytic molecularly imprinted polymer membranes on buffer concentration. Measurements were carried out in Tris-HCl buffer, pH 7.0, containing 150 mM NaCl and 5 mM $CuCl_2$. Sensor responses were initiated by addition 5 mM catechol.

Fig. 6. Dependence of the sensor response of the portable sensor system based on catalytic molecularly imprinted polymer membranes on NaCl concentration. Measurements were carried out in Tris-HCl buffer, pH 7.0, containing 0-200mM NaCl and 5 mM $CuCl_2$. Sensor responses were initiated by addition 5 mM catechol.

Fig. 7. Dependence of the sensor response of the portable sensor system based on catalytic molecularly imprinted polymer membranes on pH of the analyzed solution. Measurements were carried out in 100 mM buffer solutions pH 4-10 (acetate, phosphate, Tris-HCl for acidic, neutral, and alkaline pH values, respectively), containing 150 mM NaCl and 5 mM $CuCl_2$. Sensor responses were initiated by by addition 5 mM catechol.

Table 1.

Determination of catechol in real samples using the developed sensor system based on catalytic molecularly-imprinted polymer membranes and HPLC.

№ sample	Added catechol, мМ	Revealed	
		Sensor method	HPLC method
1	1 mM	0.4±0.05 mM	0.35±0.04 mM
2	1 mM	1.12±0.14 mM	1.19±0.12 mM
3	1 mM	0.8±0.10 mM	1.06±0.11 mM
4	1 mM	0.98 ±0.13 mM	0.97±0.13 mM

1 - river water (river Vita, Pyrogiv, Kiev region),

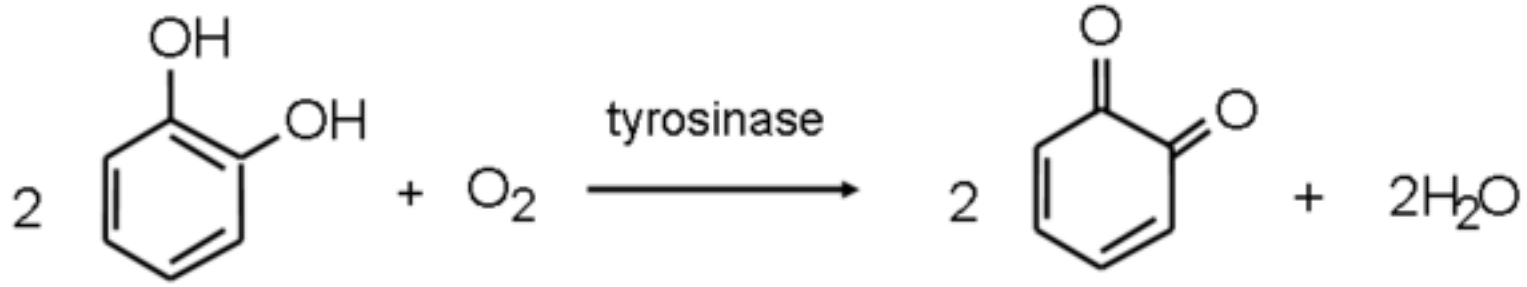
2 - filtrate of the city dump (Pyrogiv, Kiev region),

3 - river water (river Stugna, Vasylkiv, Kiev region),

4 - tape water.

Figure 1

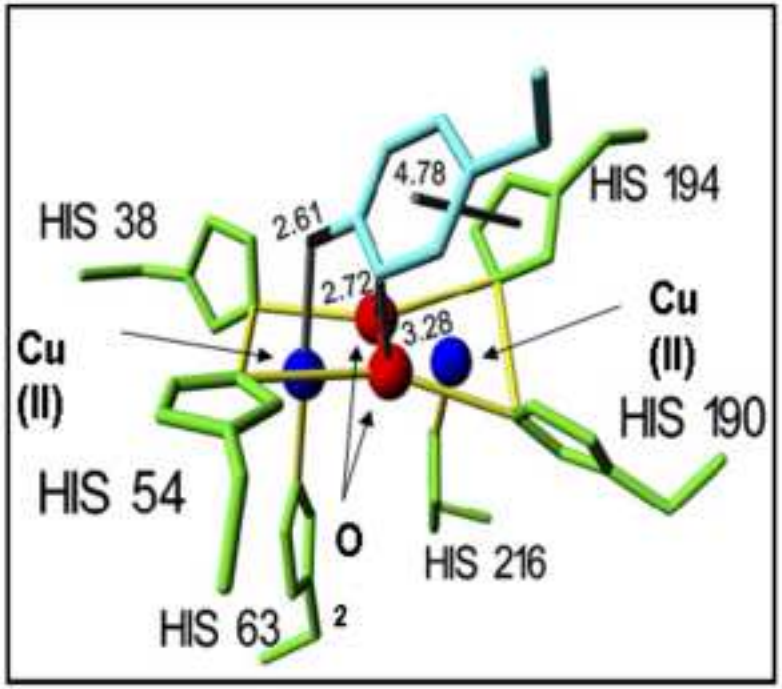
A



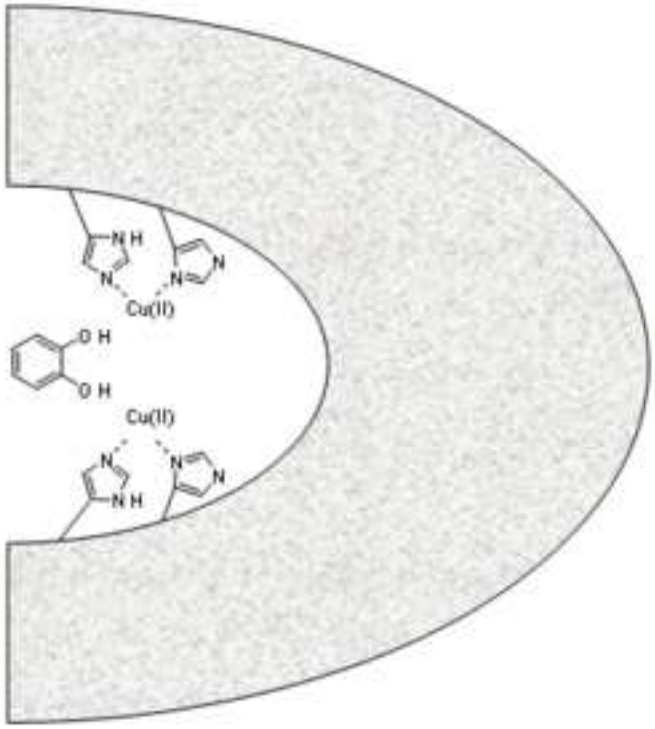
B

$$O_2 + 2\,H_2O + 4e^- \longrightarrow 4\,OH^-$$

Figure 2

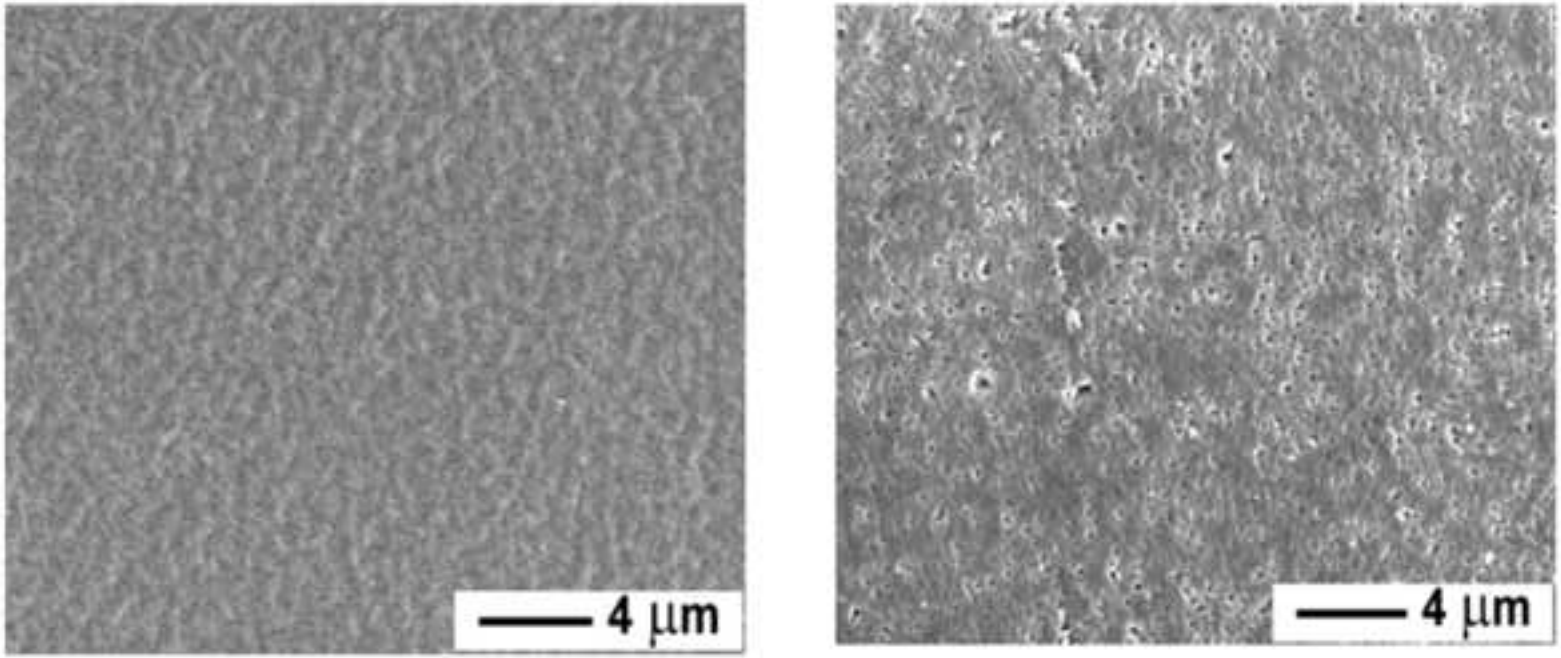

HIS 38
2.61
4.78
HIS 194
2.72
3.28
Cu
(II)
Cu
(II)
HIS 190
HIS 54
O
HIS 216
HIS 63
2

A


Cu(II)
Cu(II)

B

Figure 3



A B

Figure 4

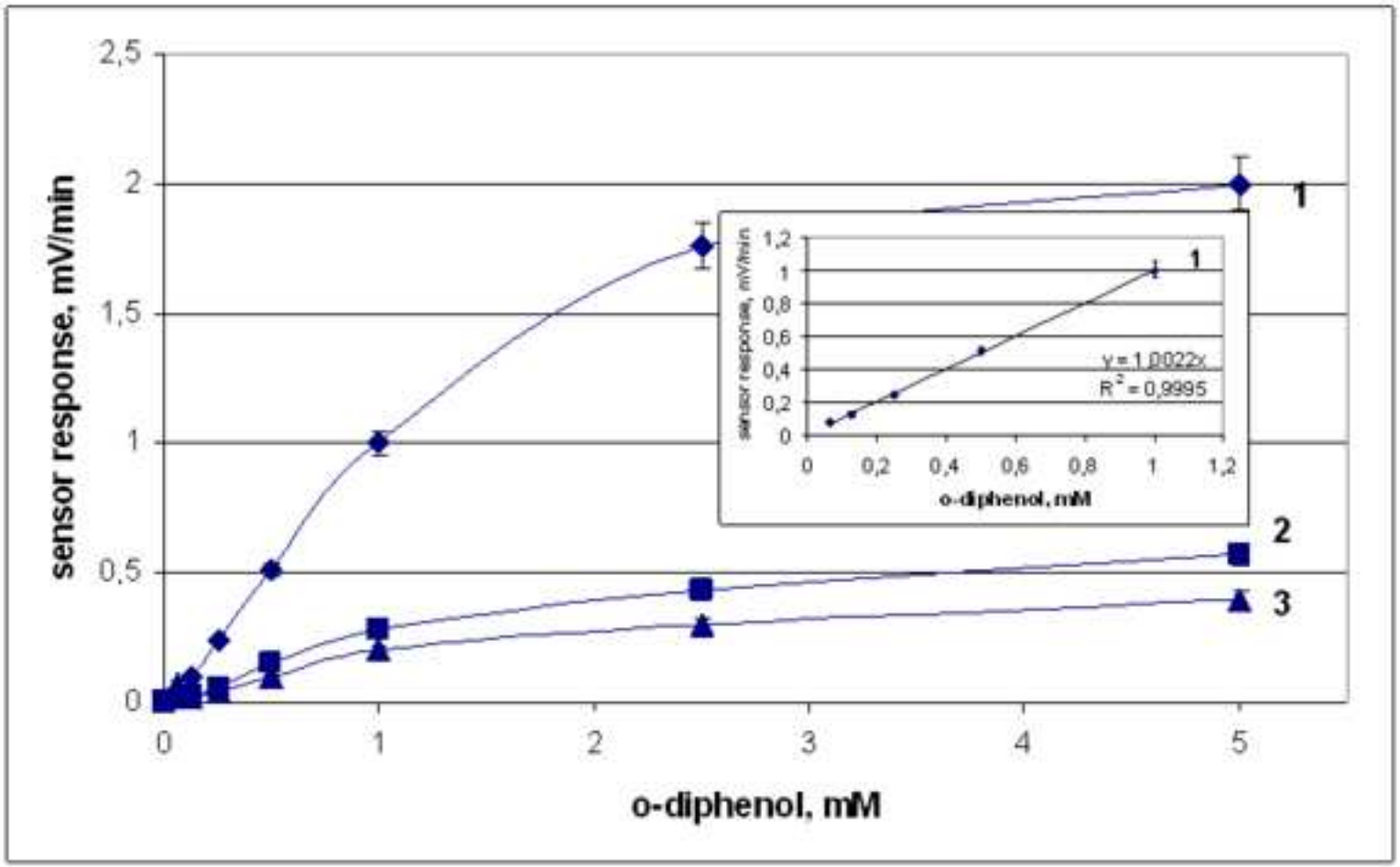


Figure 5

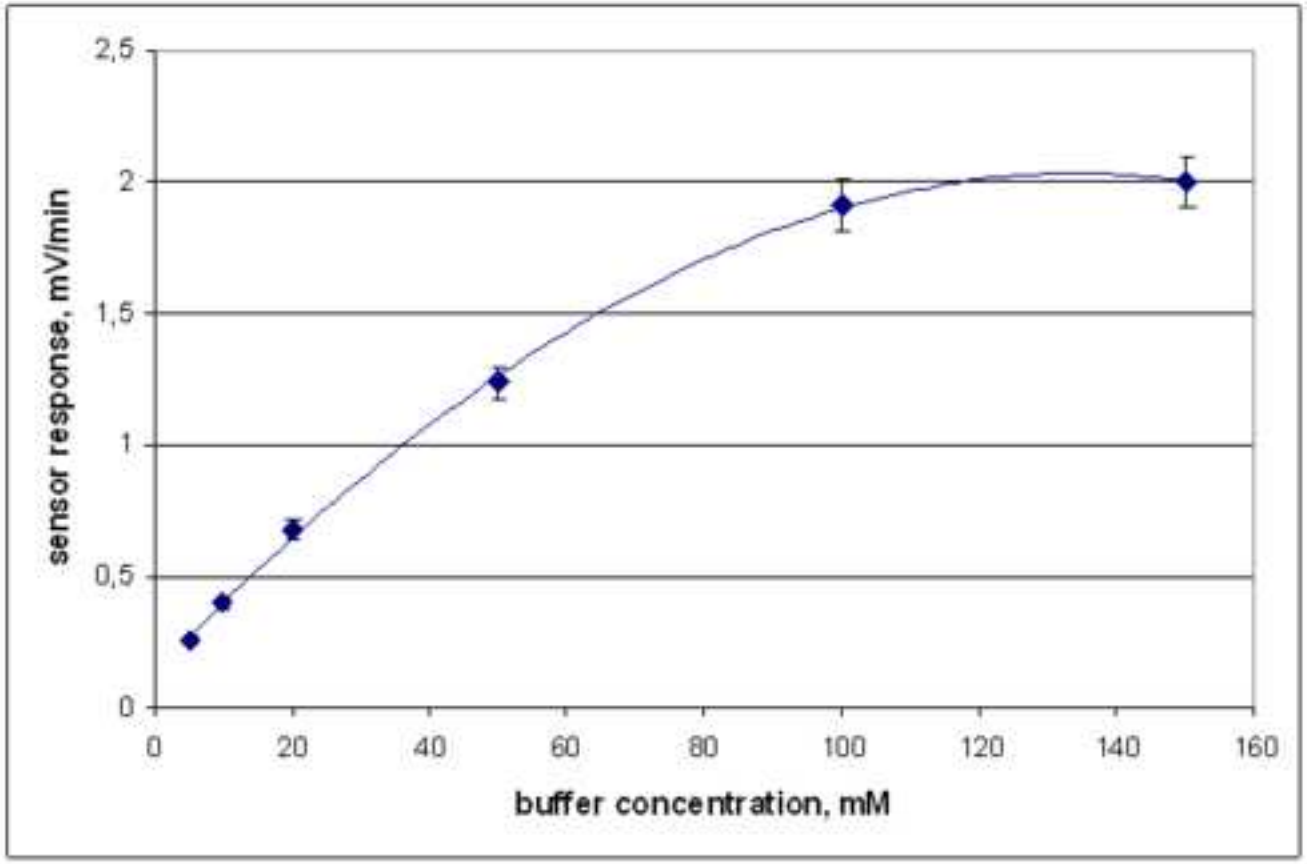

2,5
2
1,5
1
0,5
0
sensor response, mV/min
0
20
40
60
80
100
120
140
160
buffer concentration, mM

Figure 6

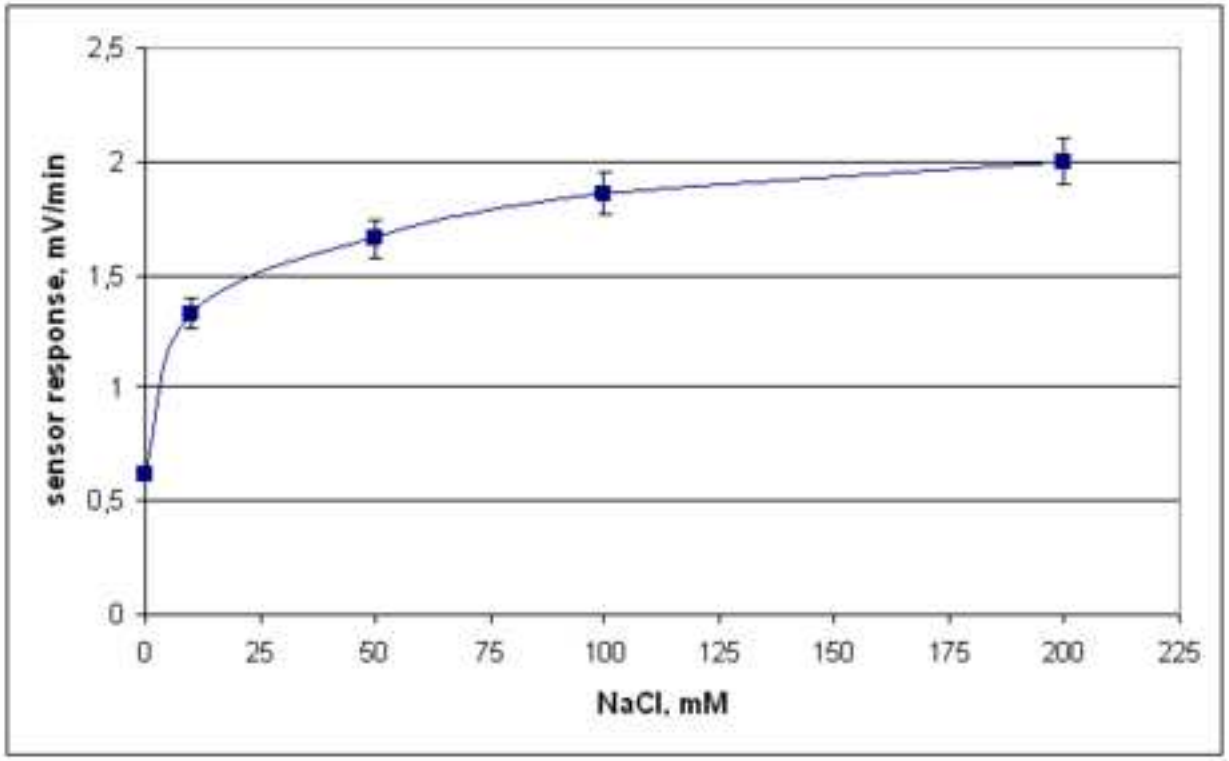

sensor response, mV/min
2,5
2
1,5
1
0,5
0
0
25
50
75
100
125
150
175
200
225
NaCl, mM

Figure 7