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RESEARCH PAPER

A solid-phase fluorescent biosensor for the determination of phenolic compounds and peroxides in samples with complex matrices

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Abstract A solid-phase fluorescent biosensor for the determination of phenolic compounds (simple substituted phenols and catecholamines) and peroxides has been developed. The biosensor has a simple construction and the analytical signal is measured directly in a biosensitive layer {peroxidase-chitosan} on the sensor surface. This approach allowed analyzing samples with complex matrices (including water-insoluble samples and nontransparent solutions) without their preliminary pretreatment. Two novel fluorescent indicator reactions for the determination of the above-mentioned analytes in wide concentration ranges (from nmol l^{-1} to mm l^{-1}) which provided an analytical signal registration on a solid phase were proposed. The developed sensor was applied successfully for the analysis of urine, cosmetics, pharmaceuticals preparations, etc.

Keywords Solid-phase fluorescence biosensor · Phenols · Catecholamines · Peroxides

Introduction

Chemical sensors are the devices which transform chemical information into an analytically useful signal. These devices are considered to be an alternative to the conventional techniques of analysis due to their simplified measurement procedure, high stability, and reproducibility of the obtained results. Ideal chemical sensors should response to the analyte without a sample matrix disturbance, avoiding the necessity of its pretreatment. However, this ideal situation is not always achieved in practice. Very often those real samples, which are of the greatest analytical interest, are accommodated within extremely complex matrices (for example blood fractions, urine, tissue, cosmetics, pharmaceutical preparations, and etc.). The components of these matrices may not only give false positive or negative results of the measurement, but can also impede completely the analysis procedure. Thus analysis of real samples usually requires their pre-treatment (filtration, separation, and derivatization), which can significantly prolong the analysis procedure and affect its sensitivity and selectivity. For this reason, there has been a growing interest in searching for approaches making the analysis procedure simpler by the simplification of a sample preparation step.

A wide application in the field of clinical, industrial, and environmental analysis is found by electrochemical sensors. These sensors are known to be simple in manufacturing and operation; they have relatively low response times and detection limits. The peculiarity of electrochemical sensors consists in the initial pre-concentration and further oxidation (or reduction) of an analyte directly on the electrode surface. For this reason, the analysis employing electrochemical sensors does not require very often any sample treatment (excepting dilution) even in the cases of samples with complex matrices [1–3].

Optical sensors have significant advantages in comparison with electrochemical ones: wide dynamic range, electrical passiveness, indifference to electromagnetic interference, ability to send, and receive optical signals over the long distances without distortion. However, the optical biosensors, described in literature, are based on the analytical signal measurement in the solution (in absorption [4] or reflection [5–9], steady-state [4–6] or flow-thought [7–9] modes). This technique does not allow analyzing non-transparent or water-insoluble samples and samples with complex matrices without a cumbersome and time-consuming sample preparation. As a result, the analysis procedures are rather complicated. In particular, the

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previously constructed optical sensors for phenols determination were applied predominantly in pre-filtered industrial waters and water-soluble drugs analysis [6–9]. Thereby, the design of the optical sensors is one of the key factors, which restricts the range of the analyzed samples. So the next step in the improvement of these devices consists in principally different construction.

A promising approach to overcome the above mentioned limitation is the development of the solid-phase optical sensors, which are based on the analytical signal measurement directly in the sensitive layer on the sensor surface. In turn, this construction implies immobilization of the components of the indicator reaction in the same sensitive layer. Thus the correct choice of the matrix for their immobilization is an important aspect of the design of the solid-phase optical sensors. Natural polysaccharide chitosan is widely applied as a matrix in optical biosensors production due to its excellent film-forming properties, high biocompatibility, and ability to form interpolyelectrolyte complexes with enzymes [4]. In recent years, our research group has developed a spectrophotometric solid-phase biosensor based on the optically transparent films {enzyme-chitosan} deposited on a glass plate for the determination of isomeric hydroxyphenolic compounds and flavonoids [10]. The biosensor performance is based on the following subsequent reactions: a phenolic compound oxidation and interaction of the formed quinonic product with amino groups of chitosan yielding a strongly light absorbing adduct. Since there was no need to register the absorption of the solution itself; the biosensor allowed analyzing non-transparent or non-homogeneous samples. However, its sensitivity (the detection limit in the range of 3–13 μM) and especially response time (24 h) were not sufficient to solve many analytical problems. According to the literature data the fluorescent sensors are known to be characterized by higher sensitivity than the spectrophotometric ones. Thus a promising way to improve the sensitivity, selectivity and perhaps rapidity of the analysis is to use fluorescent detection.

Therefore, the aim of the present investigation was to develop the solid-phase fluorescent biosensor construction, to propose novel indicator systems for the determination of phenolic compounds (simple substituted phenols and catecholamines) and peroxides, which provide the measurement of the analytical signal in the biosensitive layer directly; and to demonstrate the analytical possibilities of the biosensors in the analysis of real-world samples including water-insoluble and nontransparent samples without preliminary pretreatment.

Experimental

Reagents

Chitosan, horseradish peroxidase (HRP), laccase from *Trametes Versicolor*, mushroom tyrosinase, rhodamine B (RB), rhodamine B isothiocyanate (RBITC), catechol (CH), resorcinol (RS), hydroquinone (HQ), dopamine hydrochloride (DA), epinephrine hydrochloride (EP), homovanillic acid (HVA), vanillylmandelic acid (VMA), benzylamine (BA), 1,2-diphenylethylenediamine (DPE), *o*-phenylenediamine (PDA), ethylenediamine (EDA), urea hydrogen peroxide, tert-butylhydroperoxide, and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were purchased from Sigma (USA). Hydrogen peroxide and the components of phosphate and pyrophosphate buffer solutions were obtained from Merck (Germany). Benzoyl peroxide and 2-butanone peroxide were produced by Fluka (Switzerland). Ethanol, dimethyl sulfoxide (DMSO), acetic acid, potassium hydroxide, and the components of ammonium buffer solution were supplied by Chimmed (Russia). All chemicals were used without any additional purification.

Apparatus

Deionized water with specific electroconductivity 18.2 MOhm cm^{-1} was prepared employing Millipore Simplicity (France). pH meter was acquired from Mettler Toledo (USA). The dialysis sacks 1215-10 TZ with the pore size 12–14 kDa were purchased from Biolot (Russia). All absorption measurements were carried out using Shimadzu UV-mini 1240 UV–vis spectrophotometer (Japan). All fluorescence measurements were performed by Shimadzu RFPC-5301 fluorescence spectrophotometer (Japan). Xe lamp was used as an excitation source. In the case of the measurements employing the optical biosensor, a small 3-mm wide mirror plate arranged in the fluorimeter cuvette cell was used as a reflecting surface. Glass slides (supporting material) were obtained from Menzel (Germany).

Evaluation of the background signal

To reveal the origin of the background signal, a clean reflecting surface (mirror plate) was placed in the fluorimeter cuvette cell. Its fluorescence spectra were recorded in the wavelength range of 300–600 nm. The excitation was varied in 25 nm intervals in the wavelength range of 300–575 nm. The excitation and emission slits were set at 1.5 nm.

To estimate the analytical signal/background signal ratio, a glass slide, covered with a film {chitosan-RB}, was placed into a fluorimeter cuvette cell in front of the reflecting surface at different incidence angles of the excitation source. The fluorescence spectra were recorded in the wavelength range of 350–600 nm (λ_{ex}=350 nm), and the ratio $I_{575\ nm}/I_{425\ nm}$ was calculated.

Fluorescence quenching in the solution

The required amounts of RB, a phenolic compound, HRP, and hydrogen peroxide were introduced to phosphate buffer

solution. The reaction mixture was then removed to a cuvette and placed into a fluorimeter cuvette cell. The fluorescence spectra of the solutions were recorded in the wavelength range of 500–600 nm (λ_{ex}=550 nm), respectively. RB was dissolved in ethanol.

Peroxidase derivatization of catecholamines (CAs) and their metabolites with aromatic and aliphatic amines

The required amounts of a derivatization agent, a phenolic compound, HRP, and hydrogen peroxide were introduced to a buffer solution. CAs were derivatized with BA, DPE, and PDA (or EDA) in the basic media in 0.5 M glycine–KOH, 0.1 M CAPS–KOH, and 0.1 M ammonium buffer solutions, respectively. The reaction mixture was then removed to a cuvette and placed into a fluorimeter cuvette cell. The fluorescence spectra of the solutions were recorded in the wavelength range of 350–500 nm (λ_{ex}=350 nm) and 450–600 nm (λ_{ex}=400–450 nm) in the cases of BA (or DED) and PDA (or EDA) use, respectively.

RBITC-labeled chitosan synthesis

Chitosan solution (1 %, wt·v^{-1}) was prepared as described in [10] by dissolving 40 g of a chitosan powder in 40 mL of acetic acid (0.5 %, v·v^{-1}). The viscous chitosan solution was stirred overnight at a room temperature. RBITC-labeled chitosan was prepared according to the procedure reported in [11]. RBITC was dissolved in ethanol. The polymer solution was mixed with the required amount of the fluorescent label and left overnight in a darkness at a room temperature. The obtained pink solution was dialyzed for 24 h on stirring. The resulting label concentration in the polymer was determined spectrophotometrically ($\varepsilon_{550}=10.3\times10^4 M^{-1}\cdot sm^{-1}$ [12]).

Preparation of the bio-sensitive layer of the indirect biosensor

A homogeneous {enzyme/RBITC-labeled chitosan} mixture was prepared using the required amounts of an enzyme and labeled chitosan solutions. The stock solution was pipetted onto a horizontally arranged glass plate (14×38 mm), spread over it and left to air-dry (Fig. 1). The obtained biosensor slides were kept at 4 °C in a dark place.

Preparation of the bio-sensitive layer of the direct sensor

The homogeneous {chitosan-derivatization agent-HRP}, {chitosan-derivatization agent}, and {chitosan-HRP} mixtures were prepared using the required amounts of chitosan, the derivatization agents, and HRP. To form single-layer film on the biosensor surface the solution {chitosan-derivatization agent-HRP} was pipetted onto a horizontally arranged glass plate (14×38 mm), spread over it and left to air-dry. To form

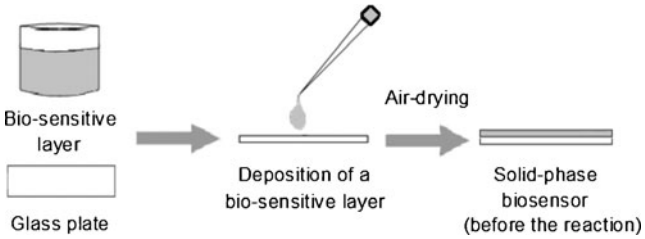

Fig. 1 The optical biosensor production

double-layer film the glass plate was initially covered with the solution {chitosan-derivatization agent} and after air-drying was covered with the solution {chitosan-HRP} and left to air-dry again. The obtained biosensor slides were kept at 4 and −20 °C in the cases of immobilized EDA and PDA, respectively.

Measurement procedure employing the optical biosensor

To carry out the analysis, a glass slide, covered with the bio-sensitive film, was immersed into the reaction medium, containing phosphate (indirect biosensor) or pyrophosphate (direct biosensor) buffer solution, the required concentrations of hydrogen peroxide, and a phenolic compound (Fig. 2). After being exposed in the tested solution the biosensor was air-dried and then placed into a fluorimeter cuvette cell (in front of the reflecting surface, Fig. 3). Fluorescence spectra were recorded in a reflection mode in the wavelength range of 500–600 (λ_{ex}=550 nm, indirect biosensor) or 400–600 nm (λ_{ex}=400–450 nm, direct biosensor). The analytical signal was measured as an emission intensity of the bio-sensitive layer. Excitation and emission slits were set at 5 nm. The incidence angle of the excitation wave was 75°.

Kinetic study of the enzymatic oxidation of catechol

The required amounts of CH, HRP, and hydrogen peroxide (or organic peroxide) were introduced into 0.05 M phosphate buffer solution, pH 7.5. The reaction mixture was then removed to a cuvette and placed into a spectrophotometer cuvette cell. The kinetic curves of the reaction media were recorded at 390 nm. The Mikhaelis constant (K_m), maximum velocity (V_m), the rate constant (k_{cat}), and the constant of a substrate specificity (k_{cat}/K_m) were calculated according to Lainuiver–Berk.

Reproducibility study

The relative standard deviation (RSD, %) of the results was calculated according to the formula RSD=$\{s/x_{average}\}\cdot100$, where s—standard deviation of the results (n=4, P=0.95) and $x_{average}$—mean.

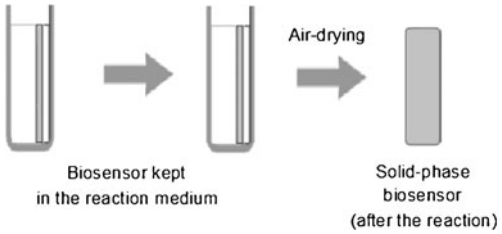

Fig. 2 The analysis procedure using optical biosensor

Pharmaceuticals and cosmetics analysis

The skin ointments (Achromin, Bulgaria; Basirone, France), shampoo (Peroxiderm, Poland) and tooth paste (Splat Extreem, Russia) samples were weighted and suspended in water. Injection samples of catecholamines (Dopamine, Russia; Epinephrine, Russia; Xylocaine-Epinephrine, Sweden) were used without any additional preparation. An aliquot of the obtained suspension (or the injection solution) was introduced into the reaction system. The content of the determined compounds in the analyzed samples was calculated by the method of the standard additions.

Urine analysis

In the case of analysis employing the biosensor, urine sample was used without any additional preparation. In the case of analysis of the solution, urine sample was dissolved in concentrated hydrochloric acid. An aliquot of the analyzed solution was introduced into the reaction system. Selective determination of HVA was carried out under the conditions, optimal for the determination of HVA. The determination of total content of HVA and VMA was carried out under the conditions, optimal for the individual determination of VMA. The content of the determined compounds in the analyzed samples was calculated by the method of the standard additions.

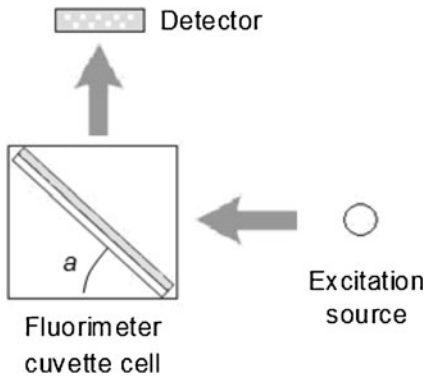

Fig. 3 The analytical signal measurement, using the optical biosensor; *a*—the incidence angle of the excitation wave

Results and discussions

Biosensor construction and analytes

The proposed biosensor has a simple construction: a small (14×40 mm) glass plate is covered with the optically transparent bio-sensitive film formed by the water-insoluble polymer chitosan containing immobilized components of the indicator reaction (an enzyme and a fluorescent reagent). The procedures of the analytical signal measurement are presented in Experimental. In this investigation phenols and peroxides were chosen as model analytes, since a considerable number of medicals and biological liquids contain them as the active components, thus their determination in these samples is of the great analytical interest.

Detection of the solid-phase analytical signal

The main problem in the case of the selection of the indicator system was an extremely high value of the background signal in the emission wavelengths range of 300–500 nm. This background signal was associated with the emission of the Xe lamp. Its intensity and shape depended upon an excitation wave. Thus the proposed construction of the solid-phase fluorescent sensor demanded the analytical signal measurement in the long-wavelength region of the spectrum (higher than 500–550 nm). In order to clarify the optimal conditions of the fluorescence measurement we recorded the fluorescence spectra of the films {chitosan-fluorescent dye}. We were interested in those fluorescent dyes, which provided covering a wide spectral range, including the region of the background signal (λ_{em}=400–500 nm) and the sensor working wavelength range (λ_{em}=500–600 nm). Rhodamines, in particular RB, met this requirement, since they are known to have an absorption band near 350 nm (excepting band at 500–550 nm) and fluorescence band near 580 nm [13]. For this reason RB was chosen as a fluorescent dye. It was found out that in contradiction to the background signal the shape of RB fluorescence maxima didn't depend upon an excitation wave (Fig. 4). Since the common value of a background signal was high even in the chosen working wavelength range, an incidence angle of the excitation wave was changed to decrease it. The incidence angles of 10°, 15°, 30°, 45°, 60°, 75°, and 80° were examined. The maximum ratio of the useful analytical signal ($I_{575\ nm}$) to a background signal ($I_{425\ nm}$) was observed when the incidence angle of the excitation wave was 75°. Thus this angle was chosen for all further studies.

Systems for the detection of the solid-phase analytical signal

The response of optical sensors is based on two principles: optical signal quenching (indirect determination) or its enhancement (direct determination). As a rule, the response of

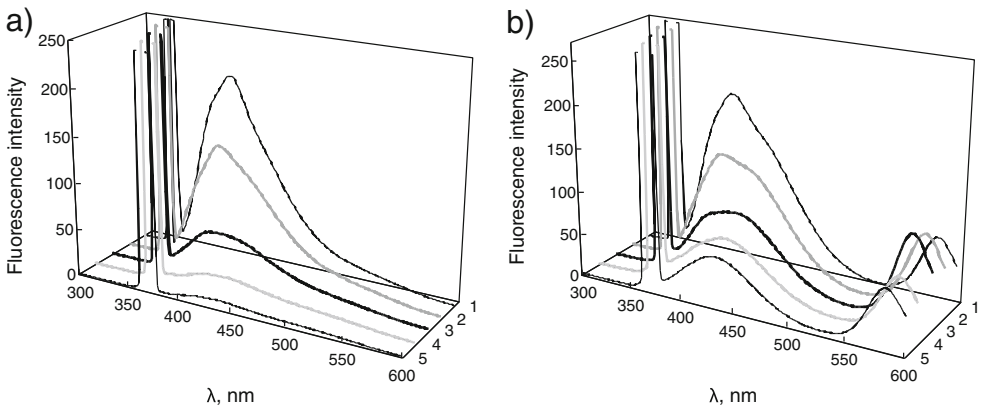

Fig. 4 Fluorescence spectra of chitosan (**a**) and {chito san-RB} (**b**) films recorded at different excitation wavelengths: (*1*) λex=330, (*2*) λex=340, (*3*) λex=350, (*4*) λex=360, and (*5*) λex=370 nm; excitation and emission slits set at 5 nm, incidence angles of the excitation wave 75°

indirect optical sensors is additive towards tested analytes within their linear concentration range. Indirect sensors are characterized by medium sensitivity (μmol l^{-1}–mmol l^{-1}) and wider substrate specificity than their direct analogs. For these reasons, indirect sensors can be used in the analysis of the real samples, containing only one analyte or for the evaluation of the total content of a group of analytes in the sample (cosmetics and pharmaceuticals analysis). Direct sensors are more suitable for the selective determination of one analyte in the presence of another one (biological liquids analysis). Basing on the above mentioned actual analytical problems two novel indicator reactions were proposed in this investigation to develop the solid phase fluorescent biosensor: the interaction between chitosan labeled with the fluorescent label and the products of the enzymatic oxidation of phenolic compounds (fluorescence quenching, indirect determination) and the enzymatic derivatization of phenolic compounds with aromatic and aliphatic amines (fluorescence enhancement, direct determination).

Indirect determination of phenolic compounds and peroxides using RBITC-label chitosan

Fluorescent labeling is a process of a covalent attaching of a fluorophore to another molecule (a polymer, antibody, enzyme, etc.). In this study a novel analytical system based on fluorescence quenching of labeled chitosan in the presence of oxidation products of phenolic compounds was proposed. The proposed construction of the sensor demanded the analytical signal measurement in the long-wavelength region of the spectrum (greater than 500–550 nm). Thus it was necessary to use such label-molecules, which had the emission maxima in the above-mentioned region. A fluorescent reagent RBITC met this requirement, as it is characterized by a long excitation and emission wavelengths (λ_{ex}~550 nm, λ_{em}~580 nm). It's isothyocyanate group reacts readily with amino groups of chitosan to form a fluorescent derivative which emits fluorescence with high intensity and stability [12].

A possible quenching mechanism was previously reported by S.A. Park et al. [14]. The enzyme immobilized in a bio-sensitive layer catalyzed a phenolic compound oxidation with hydrogen peroxide (in the case of HRP) or oxygen (in the cases of laccase and tyrosinase). The resulting quinonic products acted as the electron acceptors. The electron in the excited state was transferred from the electron donor (fluorescent label) to the electron acceptor reducing the initial fluorescence intensity of the bio-sensitive layer. This fluorescence quenching was proportional to the concentration of an analyte. The same quenching effects were observed in a water solution when a phenolic compound was oxidized by hydrogen peroxide in the presence of HRP and RB, and there was no fluorescence quenching in the absence of an oxidized product. Since phenols and quinones could be physically absorbed by a chitosan film through hydrogen bonding [15, 16], we supposed that a decrease of the initial fluorescence intensity in the presence of the oxidation products was conditioned by the formation of their non-fluorescent complexes in the bio-sensitive layer.

Optimization of the indirect biosensor response

The following parameters were varied in turn at the fixed wavelengths (λ_{ex}=550 nm, λ_{em}=575 nm) to characterize and optimize the response of the indirect fluorescent biosensor: concentrations of chitosan and a fluorescent label, a volume of a film-forming mixture, an activity of the enzyme, a concentration of hydrogen peroxide (in the case of HRP biosensor), a concentration and pH of a buffer solution.

The series of chitosan solutions with different concentrations (0.25 %–1.0 % wt·v^{-1}) were prepared. Homogeneous chitosan solution with a higher concentration could not be prepared due to its low solubility in 0.5 % (v·v^{-1}) acetic acid. In turn more concentrated acetic acid affected the enzyme activity. The higher was chitosan concentration, the more RBITC molecules it could bind, and consequently the higher was the initial fluorescence intensity and the fluorescence quenching of the bio-sensitive layer after carrying out the

reaction. The fluorescence quenching was maximal at the chitosan concentration 1 % (wt·v^{-1}), which was used for labeling and the enzyme immobilization.

The effect of RBITC concentration in the bio-sensitive layer on the response of the indirect biosensor was studied covering the range from 1 to 25 ng of the label per sm^2 of the surface. Labeled chitosan with a higher RBITC concentration could not be prepared, since no more label molecules could be chemically bounded with 1 % (wt·v^{-1}) chitosan solution. The fluorescence quenching was maximal and constant at the concentration of RBITC equaled to 25 ng·sm^{-2}.

The effect of the enzyme loading in a chitosan film on the indirect biosensor response was studied. The analytical signal value increased until some maximal value with the increase of the enzyme activity. This situation has also been reported by J. Abdullah et al. [4] and in our previous works [10]; it can be explained by the saturation of the enzyme in the bio-sensitive layer. The optimal activities of HRP in the presence of chitosan and CAs, and laccase, and tyrosinase in the presence of catechol were found to be 0.1, 0.25, 0.1, and 2.5 units, respectively.

A concentration of hydrogen peroxide was also critical for HRP sensor response: the fluorescence quenching in the case of catechol oxidation was maximum at 100 μM hydrogen peroxide. Further increase in the peroxide concentration decreased the sensor response, probably due to the inactivation of the enzyme in the presence of high H_2O_2 concentrations [17]. The optimal concentrations of hydrogen peroxide in the reactions of dopamine and epinephrine oxidation were found to be 350 and 250 μM, respectively. Since laccase and tyrosinase are known to be oxidases, which catalyze phenolic compounds oxidation with oxygen, it was assumed that the concentration of dissolved oxygen in the buffer solution was constant and did not change at a room temperature.

To study the effect of soaking time, the sensor was immersed into the reaction media for various periods of time. The HRP sensor did not practically change its response in the reaction of CH (and its isomers) and CAs oxidation after 10 and 15 min, respectively. Thus these soaking times were chosen as optimal for HRP biosensor performance. In the cases of laccase and tyrosinase sensors (in the presence of catechol), 15 min was found to be the optimal soaking time.

Phosphate buffer solution was chosen as a reaction media, since it is widely used in the enzymatic processes involving peroxidases. The response of the bio-sensitive layer did not depend on the concentration of phosphate buffer solution in the range of 10–75 mM. Thus 50 mM phosphate buffer solution was used for all subsequent studies. The dependence of the biosensor response on pH was studied in its range 6–8.5. The optimum response of the biosensor corresponded to pH 7–7.5, which was close to the reported pK_a of chitosan [18] and within the range of the maximal enzymes activity [19–21]. Therefore, pH 7.5 was chosen for the experiments.

Analytical performance of the indirect biosensor

Analytical performance of the biosensor based on RBITC-labeled chitosan was evaluated using five phenolic compounds and five peroxides of different structure. The sensitivity of the HRP sensor towards phenolic compounds decreased in the sequence: CH>RS>HQ>EP>DA. These data don't agree with the earlier results [22–24], where fluorescence quenching had the reverse sequence in the case of phenols determination in water solution (not on the surface). The observed discrepancy may be attributed to the different adsorption characteristics of chitosan towards different quinones. There are no literature data on the comparison of physical sorption of isomeric phenolic compounds and quinones on chitosan, and no information on physical sorption of quinones on other sorbents. However, the adsorption capacity of phenolic compounds on other sorbents, in particular on activated carbons, is known to diminish in the sequences: CH>RS [25] and CH>HQ [26]. Since phenols and quinones are engaged in physical adsorption by means of the same interactions (hydrogen bonding), the obtained results are in a good agreement with the chosen model of the analytical signal formation.

The response of HRP sensor towards the studied peroxides decreased in the sequence: urea hydrogen peroxide>hydrogen peroxide>2-butanone peroxide>benzoyl peroxide>*tert*-butyl hydroperoxide. The kinetic study of the peroxidase oxidation of catechol indicated that the obtained sequence was in a good

Table 1 Analytical characteristics of the HRP, laccase (*), and tyrosinase (**) sensors based on RBITC-labeled chitosan for the determination of different phenolic compounds and peroxides

Phenolic compound	Linearity, μM	Detection limit, μM	RSD, % (n=4, P=0.95)
CH	0.5–5	0.10	4
RS	0.5–7.5	0.15	3
HQ		0.20	3
DA	5–50	2	5
EP		1.5	6
CH*	0.5–5	0.10	3
RS*	0.5–7.5	0.15	4
HQ*		0.20	4
CH**	0.5–5	0.10	5
RS**	–		
HQ**	–		
Hydrogen peroxide	10–50	7	8
Urea hydrogen peroxide	10–75	5	6
2-Butanone peroxide	10–100	7	5
Benzoyl peroxide	25–100	10	6
tert-Butyl hydroperoxide	250–1,000	100	10

agreement with the substrate specificity of HRP towards tested peroxides.

The analytical parameters of the developed procedures are summarized in Table 1. The proposed biosensor provides the determination of isomeric dihydroxyphenols, CAs, and peroxides in the range of 0.5–7.5, 5.0–50.0, and 10–1,000 μM, respectively. Thus its sensitivity is comparable with the sensitivity of the fluorescent sensors based on the analytical signal measurement in a solution [4–9]. Besides it exhibits apparent advantages over our earlier developed solid-phase spectrophotometric biosensors [10] in terms of sensitivity and response time. Thus under optimal conditions $t_{90\%}$ (time necessary for 90 % of the total signal change to occur) could be achieved within 15 min (instead of 24 h).

Biosensors, containing different enzymes are characterized by the comparable response characteristics. At the same time, tyrosinase biosensor gives no response to the presence of RS and HQ. Unfortunately due to the effect of substrate–substrate activation [27] (when one of peroxidase reducing substrate is oxidized more rapidly in the presence of another phenolic substrate), the substitution of one enzyme by another one, in particular peroxidase by tyrosinase, did not allow us to determine selectively CH in the samples, containing CH and RS or CH and HQ simultaneously. However, the biosensor response is additive towards tested phenolic compounds and peroxides within their linear concentration range. Thus the achieved sensitivity and selectivity provide the analysis of samples containing only one phenolic compound, or the estimation of the total content of phenolic compounds in the sample.

The reproducibility of the biosensors response did not exceed 10 % ($n=4$). The flow-thought fluorescent sensors for the determination of different phenols, described in the literature, are characterized by a better reproducibility [8, 9]. However, they are based on the fluorescence measurement in the solution, not on the sensor surface, as it is in our case. The spectrophotometric biosensors based on the analytical signal measurement in the solution [4, 6] and the earlier developed solid-phase spectrophotometric biosensor [10] demonstrated similar or worse reproducibility.

The proposed fluorescent sensor could be stored at least for 2 weeks after the reaction, when stored dry at 4 °C in darkness and retained 90–95 % of its initial fluorescence intensity.

Commonly beside the active ingredient (phenolic compound or peroxide) medical formulations and cosmetics contain significant amount (equal or 10 times less) of sodium metabisulfite (an antioxidant additive) or glycerol (to increase the viscosity of a preparation). The experimental results revealed, that no significant interference (less than 5 %) was caused by 100-fold of both additives.

Thus the developed indirect fluorescent biosensor provides rapid (in comparison with the solid-phase optical analogues) determination of phenolic compounds and peroxides on the μM–mM level of their concentrations (see calibration curves in the Electronic Supplementary Material Fig. S1–S5). The achieved sensitivity and selectivity of such sensor make possible analyzing cosmetics, pharmaceutical preparations but are not sufficient to determine the above indicated analytes in biological liquids. The adaptation of more sensitive and selective indicator systems (in particular direct ones) in sensor technologies should overcome this limitation.

Direct determination of CAs and their metabolites using the enzymatic derivatization with aromatic and aliphatic amines

The determination of some phenolic compounds such as DA, EP, and their metabolites, HVA and VMA, in biological materials at nanomolar concentrations is an actual analytical problem. Some of these phenolic compounds possess their own fluorescence. However, fluorescent signals of CAs and their metabolites are not suitable for the quantification of their traces in real samples, because the emission wavelengths are weak and short (λ_{em}~320 nm), and they are obstructed by those of endogenous compounds in real samples (blood, urine, etc.). Analytical characteristics of the above described indirect biosensor are also not sufficient to solve this problem [28].

Nowadays the procedure based on the derivatization of CAs and their metabolites with BA [29] or DPE [30] producing highly fluorescent derivatives is the most selective (the wavelengths of fluorescent CAs derivatives are red-shifted, λ_{em}~470–490 nm) and sensitive one for their determination in biological liquids.

The reactions known for CAs derivatization are carried out in the presence of inorganic oxidizer $K_3[Fe(CN)_6]$ in methanol media at 50 °C during 60 min, and such procedure is not convenient for biosensor proceeding.

We found that efficiency of CAs derivatization by BA and DPE was improved significantly using the catalytic system {peroxidase-H_2O_2} instead of inorganic oxidizer. The formation of the same fluorescent derivatives was confirmed by MC method (Fig. 5). Opposite to $K_3[Fe(CN)_6]$, the biocatalytic system provides more intensive fluorescence of the produced derivatives for the shorter time and does not require high temperature for the incubation of the reaction system in the presence of toxic solvents. Such parameters as pH and the concentration of the buffer solution, temperature, nature of a solvent, concentrations of the reactants were optimized.

The optimal activity of HRP and concentration of H_2O_2 were found to be 10 units and 100 μM, respectively. Fluorescence enhancement was maximal and constant after 2–5 min after the start of the reaction at the concentrations of BA and PDE equaled to 33 and 3 mM, respectively. The reaction was carried out in alkaline media (CAPS–KOH buffer solution, pH 11, and glycine–KOH buffer solution, pH 8, in the cases of BA and PDE).

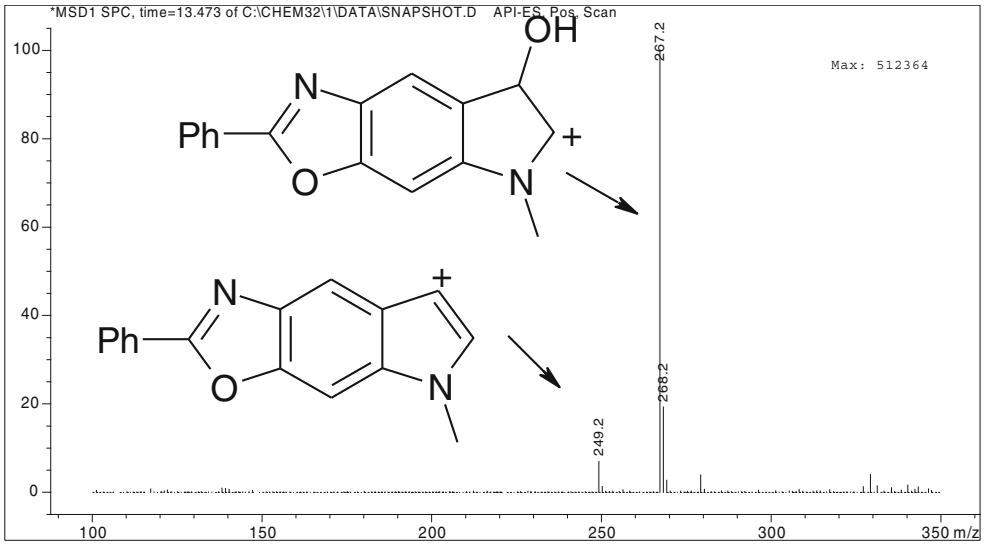

Fig. 5 Mass-spectra of the product of enzymatic derivatization product of AD by BA

Moreover other amines, such as PDA and EDA may be used for the interaction with CAs producing the fluorescent derivatives with shift of the analytical signal in longer wave spectral range (λ_{em}~500–600 nm). Opposite to BA, PDA and EDA the derivatization reactions based on PDA require smaller amounts of the enzyme (0.1–0.25 units) and derivatization agent (250 μM).

The proposed techniques make possible rapid and selective determination of the analytes at nM–μM concentrations. The proposed and studied indicator reaction can be used for the determination of traces of simple phenolic compounds in samples. Solid-phase fluorescent sensor for the direct determination of phenolic compounds based on their peroxidase catalyzed derivatization.

The proposed indicator system was adopted to develop a sensitive sensor technique for the determination of catecholamines and their metabolites. Fluorescence spectra of the derivatization products of EP with BA in the solution and in the biosensitive layer on the surface are presented in Fig. 6. It was found that in both cases fluorescence enhancement was proportional to the EP concentration. However in the case of the analytical signal measurement on the biosensor surface not only the fluorescence intensity, but also the shape of the corresponding maxima, depended upon the excitation wave. Since it was measured at the wavelengths less than 500 nm (λ_{em}~450 nm), the fluorescent signal on the surface could be caused not only by useful analytical signal, but also by the background signal. On the contrary in the case of PDA and EDA (λ_{em}~500–550 nm) no deviation from the laws of fluorescence was observed.

The following parameters were varied in turn at the fixed wavelengths to characterize and optimize the response of the direct fluorescent biosensor based on PDA and EDA: a volume of a film-forming mixture, concentrations of the reactants, and a concentration and pH of the buffer solution. In order to provide better retention of the derivatization agent in the chitosan film, the bio-sensitive layer consisted of two layers. The internal layer contained the immobilized derivatization agent, the external-immobilized enzyme. The optimal conditions for the determination of DA, EP, HVA and HMA

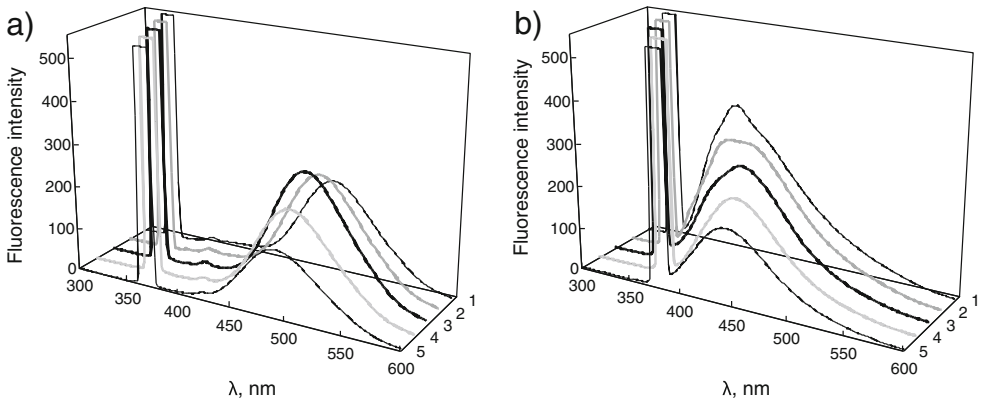

Fig. 6 Fluorescence spectra of epinephrine—BA derivatization adducts recorded in the solution (**a**) and on the surface (**b**) at different excitation wavelengths: (*1*) λ_{ex}=330, (*2*) λ_{ex}=340, (*3*) λ_{ex}=350, (*4*) λ_{ex}=360, and (*5*) λ_{ex}=370 nm

Table 2 Analytical characteristics of the direct biosensors for the determination of different phenolic compounds and peroxides

Phenolic compound (derivatization agent)	Linearity, 10^{-8} M	Detection limit, nM	RSD, % ($n=4$, $P=0.95$)
DA (PDA)	25–200	70	3
EP (PDA)	10–100	50	4
HVA (PDA)	1–10	5	3
VMA (PDA)	0.5–7.5	3	3
DA (EDA)	5–25	2.5	5
EP (EDA)	2.5–25	1.5	8
Hydrogen peroxide (PDA)	25–100	10	3
Urea hydrogen peroxide (PDA)		15	5
2-Butanone peroxide (PDA)	100–1,000	90	6
Benzoyl peroxide (PDA)	50–500	30	7
tert-Butyl hydroperoxide (PDA)		40	10

employing the solid-phase biosensors and in the solution almost did not differ. It should be mentioned, that the derivatization reaction involving PDA was more rapid (30 s) than the reaction in the presence of EDA (2–5 min). The optimum response of the biosensors in the presence of EP and HVA corresponded to pH 10 and 9.5 in the cases of DA and VMA, respectively.

Analytical performance of the direct biosensor

The analytical performance of the biosensor based on PDA was evaluated using seven phenolic compounds and five peroxides of different structure. The sensitivity of the biosensor towards phenolic compounds decreased in the sequence: VMA>HVA>EP>DA>CH. As it could be expected, the biosensor gave no response in the presence of hydroquinone and resorcinol, since only those phenols which are oxidized producing *o*-quinones are engaged in the derivatization with aromatic and aliphatic amines. The response of the PDA biosensor towards peroxides decreased in the sequence, similar to that observed in the case of the indirect biosensor. The analytical performance of the biosensor based on EDA was evaluated using catechol, dopamine and epinephrine only. The analytical parameters of the procedures are summarized in Table 2.

The biosensor based on PDA provides the determination of catecholamines, their metabolites and peroxides in the ranges of 0.01–2, 10–100, and 10–1,000 μM, respectively (see calibration curves in the Electronic Supplementary Material Fig. S6–S9). Under optimal conditions $t_{90\%}$ could be achieved within 30 s. The reproducibility of the biosensors response of the did not exceed 10 % ($n=4$). It should be mentioned that the sensitivity, reproducibility and rapidity of the determination decreased dramatically in the case of EDA biosensor. The biosensors should be stored dry for 4 weeks at least after the reaction, at 4 °C and −20 °C in the cases of immobilized EDA and PDA, respectively.

Analyzing solutions containing DA and EP, HVA and EP, VMA and EP we found that the reactivity of EP was so high in comparison with other catecholamines; that the analytical signal was not additive in such mixtures. So the determination of DA, HVA and VMA in the presence of EP is possible only in the case of their multiple excess (10–1,000 times) in the reaction medium. The analytical signal was additive in the analysis of the solutions containing DA and HMA, DA and VMA,

Table 3 The application of the direct and indirect fluorescent biosensors for the determination of phenols and organic peroxides in pharmaceuticals and cosmetics ($n=4$, $P=0.95$)

Analyte	Sample	Fluorescent biosensor	Determined concentration	Producer's data
HQ	Water-insoluble skin bleaching ointment	Indirect	(2.1±0.2) %	1.9 %
DA	Injection samples, water mixable matrix	Indirect Direct	(0.53±0.04) % (0.54±0.07) %	0.5 %
EP [(1)]		Indirect Direct	(0.10±0.01) % (0.11±0.02) %	0.1 %
EP [(2)]		Indirect Direct	(0.00054±0.00006) % (0.00049±0.00004) %	0.0005 %
Benzoyl peroxide	Water-insoluble skin bleaching ointment	Indirect Direct	(4.7±0.6) % (4.7±0.4) %	5 %
	Shampoo, non-water mixable matrix	Indirect Direct	(2.5±0.4) % (2.4±0.4) %	2.5 %
Urea hydrogen peroxide	Tooth paste, non-water mixable matrix	Indirect Direct	(0.1±0.02) % (0.1±0.01) %	0.1 %

EP in «Epinephrine»[(1)] and «Xylocaine-Epinephrine»[(2)] injections

and HVA and VMA. Under the conditions optimal for the determination of HVA, the velocity of the enzymatic derivatization of VMA with PDA was so low that even in the case of its double excess VMA did not interfere the determination of HVA. Thus the achieved sensitivity and selectivity of the analysis employing the direct biosensor based on PDA make possible the individual determination of HVA and VMA in biological liquids.

Real sample analysis

The proposed solid-phase fluorescent biosensors were applied for the determination of HQ, DA, EP, benzoyl peroxide, and urea hydrogen peroxide in pharmaceuticals and cosmetics (Table 3). In all the cases, the obtained results were in a good agreement with the values declared by the producers. The reaction of the enzymatic derivatization of catecholamines and their metabolites with PDA was applied for the determination of HVA and VMA in urine. The determination was carried out in two variants: in the solution (the selective determination of HVA with BA) and employing solid-phase fluorescent biosensor (the selective determination of HVA and total determination of HVA and VMA with PDA). The found concentrations of HVA and VMA were 41±2 and 10±2 μM, respectively. The obtained results were in a good agreement with each other and the standard contents of CAs metabolites in urine.

Conclusions

In the result of our investigation the construction of the solid-phase fluorescent biosensor for the determination of simple substituted phenolic compounds (phenols and catecholamines), and peroxide was developed. The procedure of the analytical signal registration directly in the bio-sensitive layer {peroxidase-chitosan} on the sensor surface was submitted. This biosensor provided the analysis of samples with complex matrices (including water-insoluble samples and nontransparent solutions) without their preliminary pretreatment. Two novel fluorescent indicator reactions for the indirect (based on the interaction of the phenolic compounds with RBITC-labeled chitosan) and direct (based on peroxidase catalyzed derivatization of the phenolic compounds) determination of the mentioned analytes in the wide concentration ranges (from nmol l^{-1} to mm l^{-1}) for the solid-phase registration of the analytical signal were introduced. Analytical characteristics of the proposed biosensors were comparable with the analytical characteristics of optical sensors described in literature (see Electronic Supplementary Material Table S1). The analytical possibilities of the proposed biosensor were demonstrated successfully in the analysis of urine, cosmetics, pharmaceutical preparations, and etc.

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