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Properties and analytical applications of the self-assembled complex {peroxidase–chitosan}

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ABSTRACT

A novel promising approach to the improvement of analytical properties of horseradish peroxidase based on its inclusion into self-assembled structures of chitosan is discussed. It is shown that the reasonable choice of a polyelectrolyte, a detailed investigation of its interaction with the enzyme and the conditions of the {peroxidase–polyelectrolyte} complex formation allow for stabilizing the biocatalyst in aqueous and aqueous-organic media without a substantial loss in its activity and developing corresponding analytical procedures and biosensors. The latter provides highly selective determination of a number of organic compounds and sensitive determination of heavy metal ions that becomes possible due to the specific interactions of the analytes with the polymer matrix. Besides, the application of the proposed analytical systems and biosensors provides the expansion of the range of the compounds, and poorly water soluble and slowly oxidized substrates of peroxidase as well, which could be determined and real samples which could be analyzed by enzymatic methods. Analytical performance of the developed spectrophotometric indicator procedures and biosensors based on the self-assembled complex {peroxidase–chitosan} is demonstrated in the determination of metal ions (Hg(II), Cd(II), and Pb(II)), phenothiazines (promazine, chloropromazine, and trifluoroperazine), phenolic compounds (phenol, hydroquinone, catechol, pyrogallol, quercetin, rutin, and esculetin), organic peroxides (*tert*-butyl peroxide, 2-butanone peroxide, and benzoyl peroxide) in various samples, including water-insoluble matrices.

1. Introduction

Horseradish peroxidase is one of the most popular enzymes in analytical chemistry. However, its insufficient specificity and sensitivity towards some substrates and effectors limit its further introduction into clinical analysis, medical diagnostics, food quality control, etc. In addition, the analytical possibilities of peroxidase are limited by its low stability as well as insufficient efficiency of the catalysis in aqueous-organic and organic media observed when determining bioactive compounds in samples which are poorly soluble or insoluble in water.

A promising approach to the improvement of the analytical properties of the enzymes, including peroxidase, is their inclusion into self-assembling structures of polyelectrolytes. An attractive feature of the {enzyme–polyelectrolyte} complexes which are formed due to non-specific electrostatic interactions is the possibility to vary their physicochemical properties and the kinetic parameters of the reactions which are catalyzed by the complexes by changing the nature and molecular weight of the polymers, varying their ratio in the immobilization premixture and the conditions used for the complex formation (nature of buffer solutions, pH, ionic strengths, etc.) [1,2]. These advantages enable the creation of highly active and stable new-generation biosensors with the desired properties (sensitivity and selectivity) which can be used for solving certain analytical problems.

There has been a growing interest in the search of the approaches providing enzymatic catalysis in the media of organic solvents over the past 30 years [3–6]. It should be noted that in the systems containing non-polar (hydrophobic) solvents which hardly influence the protein structure, the enzymes usually preserve the catalytically active conformation [7,8] and stability [9,10]. In the presence of polar organic solvents the biocatalysts lose their catalytic activity partially or completely due to the denaturation of the protein globule [11,12]. The approaches recommended for the improvement of the biocatalyst stability towards this type of inactivation include immobilization of enzymes on solid supports [13], replacement of amino acid residues of the protein [14,15], its covalent modification with low-molecular components [16,17] and polymers [18], and molecular imprinting of enzymes with water-insoluble ligands followed by freeze drying [19]. However, none of the enzyme stabilization procedures provide their high catalytic activity

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in a wide range of concentrations of polar organic solvents. The formation of protein-polyelectrolyte complexes seems to be one of the most efficient and promising approaches to enzyme stabilization in aqueous-organic media although such procedure has only single examples of its application in electrochemical sensors [20,21]. Enzyme-polyelectrolyte complexes exist in rather wide ranges of pH and ionic strength; they are stable due to cooperative (multi-point) type of interaction [22,23]. The conformations of the free enzyme and the enzyme included into a complex with a polymer differ only under denaturating conditions—in aqueous-organic media with high concentrations of the organic solvent (over 30%v/v) [23]. In this case the polyelectrolyte stabilizes the conformation of the protein which has existed under the conditions of the complex formation. Due to this ability of the polyelectrolytes, the formation of their complexes with the enzymes is one of the most efficient and general approaches providing stabilization of the latter [23]. It should be noted that the inclusion of enzymes into polyelectrolyte complexes is simple, provides uniform distribution of the biocatalyst over the carrier volume and thus allows producing stable immobilized preparations with reproducible parameters [24]. In addition, such systems are easily formed, optically transparent and convenient for further application in chemical analysis. At the moment, the formation of polyelectrolyte complexes between polyions is one of the simplest ways for the formation of both nano- (water-soluble conjugates, nanoparticles) and microsized (physical gels and film) structures [25]. Subsequent attachment of these structures on solid supports (glass slides, test stripes, etc.) significantly improves the mechanical properties of the enzyme preparations and provides the base for biosensor construction [26].

The reasons listed above demonstrate the importance of basic research in this area.

It is known that natural polysaccharides are promising components for the formation of non-covalent polyelectrolyte–enzyme complexes. A special interest in this type of polymers is associated with their high biologic activity observed as their biocide effect on pathogenic microorganisms and the ability to form interpolyelectrolyte complexes with proteins [27]. The proper selection of the chemical nature of polysaccharides and using their derivatives allows obtaining the matrices which have optimal characteristics for the immobilization of the enzyme. In some cases, such matrices have recognition ability as well, such property being useful for the further application of these preparations of immobilized enzymes, for example, for biosensor construction.

Therefore, *the aim of the present investigation* was the selection of the optimal polysaccharide for the formation of self-assembling structures including peroxidase; investigation of the catalytic activity and stability of {peroxidase–polyelectrolyte} complex in aqueous and aqueous-organic media, and its possible application in chemical analysis.

2. Experimental

2.1. Reagents

Horseradish peroxidase, chitosan with molecular weight (MW) of 150 kDa, carboxymethyl cellulose, sodium alginate, agarose, β-cyclodextrin, starch, phenothiazines (promazine, chloropromazine, and trifluoroperazine), *o*-dianisidine, phenol, hydroquinone, catechol, pyrogallol, quercetin, rutin, and esculetin were purchased from Sigma. The gel of calcium alginate was obtained by adding 3 mM calcium chloride to the solution of sodium alginate. Solutions of agarose, β-cyclodextrin, starch with different contents of the polymers were prepared by dissolving them in water. Carboxymethyl cellulose was dissolved in hot water (85 °C). Chitosan (150 kDa) solution was prepared by dissolving its powder in 1% v/v acetic acid. Water-soluble chitosans with molecular weights 5, 10 and 25 kDa were produced by the Center of Bioengineering of Russian Academy of Sciences (Moscow, Russia). Hydrogen peroxide and the components of phthalate and phosphate buffer solutions were obtained from Merck. *Tert*-butyl peroxide, 2-butanone peroxide, and benzoyl peroxide were purchased from Fluka. Dimethyl sulfoxide (DMSO) was supplied by Chimmed, Russia. Chemicals were used without any further purification. Deionized water (18.2 MOhm/cm) was used in all experiments.

2.2. Apparatus

The study of the dispersion of the particles of {peroxidase–chitosan} complex in aqueous medium and determination of their hydrodynamic size were carried out using dynamic light scattering (Malvern Instruments Zetasizer). Atomic force microscopy (AFM) images were obtained using NT-MTD scanning probe microscope. The transparency of {peroxidase-chitosan} films kept in water and water-DMSO media were studied using UVmini1240 UV–vis spectrophotometer (Shimadzu). The pH values were measured by K-20 potentiometer (Mettler Toledo). All absorption measurements were carried out using Shimadzu UVmini1240 UV–vis spectrophotometer.

2.3. Preparation of a biosensitive layer based on the self-assembled complex {peroxidase–chitosan} in the wells of an immunochemical polystyrene plate or on a glass slide

Chitosan solution (1% wt/v) was prepared by dissolving 0.4 g of chitosan powder in 40 mL of water containing 0.5% v/v acetic acid (chemically pure). The viscous chitosan solution was stirred overnight at room temperature. A homogeneous enzyme/chitosan mixture was prepared using the required amounts of the enzyme and 1% wt/v chitosan solutions. The stock solution was pipetted into wells of an immunochemical polystyrene plate or onto a clean glass slide, 14 × 38 mm (and spread over it), and left to air-dry. The obtained biosensor slides and immunochemical polystyrene plates were kept at 4 °C before use.

2.4. Measurement procedure

The catalytic activity of native peroxidase in the absence and presence of polysaccharides (including chitosan) was monitored spectrophotometrically at 460 nm since this wavelength corresponded to the maximal absorbance of the product of the oxidation of *o*-dianisidine, the reducing substrate of peroxidase [26]. Since daily fluctuations in the room temperature by several degrees did not affect significantly the stability and activity of peroxidase, all measurements were performed at room temperature without thermostating. The degree of peroxidase activation (A, %) in the presence of chitosan was calculated according to the formula $A,\% = \{(\tan \alpha_{polysaccharides}/\tan \alpha_0) - 1\} \cdot 100$, where $\tan \alpha_{polysaccharides}$ and $\tan \alpha$ were the slope ($\tan\alpha$) of the kinetic curves in absorbance(A)–time (t, s) coordinates in the presence and in the absence of polysaccharides, respectively.

The stability of {peroxidase–chitosan} complex immobilized on polyurethane foam (i.e., the stability of the enzyme included into the polyelectrolyte complex during its storage) was estimated by the change of the rate of the peroxidase-catalyzed oxidation of *o*-dianisidine by hydrogen peroxide on a polyurethane foam tablet. The reaction rate was monitored visually by the appearance of the brown product of *o*-dianisidine oxidation on the polyurethane foam tablet [26].

The reaction, which was used to control the activity of the self-assembled {peroxidase–chitosan} complex in aqueous and aqueous–DMSO media and the determination of phenothiazines, was carried out by adding 12 μl of 0.05 M phthalate buffer solution, 6 μl of 5 mM *o*-dianisidine, 6 μl of 0.05–50 μM phenothiazines (if necessary) and hydrogen peroxide solutions into a well of a polystyrene plate. The catalytic activities of native peroxidase in the absence and presence of chitosan were monitored using Multiskan ascent photometer (ThermoLab System) at 450 nm, which corresponded to the maximal absorption of *o*-dianisidine oxidation product ($\varepsilon=(1.2\pm0.2)\ 10^4\ M^{-1}cm^{-1}$).

To carry out the analysis, the glass slide, covered with {peroxidase–chitosan} film, was immersed into the container with the phosphate buffer solution (and DMSO, in some cases), required concentrations of hydrogen peroxide (in some experiments 2-butanone, benzoyl, or *tert*-butyl peroxide or the sample) and the phenolic compound (or the sample). After having been exposed to the reaction mixture and kept overnight, the slide was air-dried and its absorption spectrum was measured in the range from 200 to 700 nm. The analytical signal was the absorption of the biosensitive layer after the analysis procedure minus the absorption of the initial film before the reaction at 345 nm (if the phenolic compound was phenol, hydroquinone, catechol, pyrogallol, phloroglucinol, esculetin, or pyrocatechol) or 280–300 nm (quercetin or rutin).

2.5. The real sample preparation

Skin ointment and cream samples were weighed and suspended in water. An aliquot of the obtained suspension was introduced into the reaction system. A food supplement or a pharmaceutical preparation was dissolved in DMSO and a portion of a resultant solution/suspension was introduced into the reaction system. Organic (heptane-DMSO) extracts from human blood serum containing chloropromazine were supplied by an official clinical laboratory (Moscow, Russia). An analyte concentration in a sample was determined using the standard addition method.

3. Results and discussions

3.1. Formation and properties of the self-assembled complex {peroxidase–polysaccharide} selection of the polysaccharide for the formation of the self-assembled complex with peroxidase

Water-soluble complexes of cationic peroxidase with anionic, non-ionic, and cationic polyelectrolytes can be obtained due to different types of interactions i.e. electrostatic (Coulomb), Van der Waals, and hydrophobic interactions, respectively. In the case of cationic polymer the association of the enzyme with the polyelectrolyte has been attributed to salt linkage as well [28]. We studied the possibility of the regulation of the activity and stability of peroxidase through the enzyme–polyelectrolyte interaction. Natural and semi-natural polysaccharides of three different types were chosen as possible components of the complex: carboxymethyl cellulose and calcium alginate as the anionic species, non-ionic agarose, β-cyclodextrin, starch and cationic chitosan. The activity and stability of peroxidase (1 nM) in the presence of polysaccharides were controlled using the oxidation of *o*-dianisidine by hydrogen peroxide as the indicator reaction. For this reaction the optimal conditions were determined as *o*-dianisidine concentration -0.05 M, H_2O_2 -0.1 M, phthalate buffer solution 0.05 M and pH 5.0 [26].

It should be noted that despite the different nature and type of the polysaccharides, all of them enhanced (for (22 ± 4)%) the rate of the peroxidase-catalyzed transformation of the organic reducing substrate under the stated conditions. This fact may be explained by some stabilization of low concentrations of the enzyme in aqueous solution that leads to the insignificant increase in peroxidase activity [23].

The stability of the catalytic activity of the enzyme depends on the type of the polymer. The complexes of peroxidase with the anionic and non-ionic polysaccharides (which were formed due to electrostatic and Van der Waals interactions) lost more than 50% of their initial activity after 3 and 6 months, respectively. At the same time, peroxidase adsorbed onto the surface of a solid support (polyurethane foam) in the presence of the polymer with the same charge-chitosan seemed to be the most stable: it kept 50% of its initial activity for 18 months (such effect was very important for further analytical application of the enzyme preparations).

Moreover, in the result of the detailed optimization of the conditions for the self-assembled complex formation it has been established that the catalytic activity of peroxidase in the presence of 0.006–0.009% wt/v chitosan (150 kD) is two times higher than that of the native enzyme [2]. Actually, the activity of the complex was maximal in the pH range 5.9–6.2 (0.05 M phthalate buffer solution). These pH values are close to the isoelectric points of peroxidase (7.2 [29]) and chitosan (6.3–6.5 [30]), and both macromolecules have weak charges. It is known that even fully protonated chitosan tends to form aggregates with a protein as a result of hydrogen bonds through amino groups and hydrophobic interactions. This hydrophobic behavior is based on the presence of the main polysaccharide backbones and N-acetyl groups at C2 position [31]. On the basis of this speculation it has been proposed that the complex between peroxidase and chitosan is formed due to hydrogen bonds and hydrophobic interaction between their macromolecules; this fact was confirmed by the addition of potassium chloride (as an electrolyte) to the {peroxidase–chitosan} complex solutions [28]. The significant decrease in the degree of chitosan activating effect on peroxidase in the reaction of *o*-dianisidine oxidation was observed in the presence of the critical concentration of potassium chloride (0.08–0.1 M). In the absence of the polysaccharide, potassium chloride did not influence the rate of the indicator reaction in the considered concentration range [2].

Such results evidence that the peroxidase-activating effect of chitosan is associated with the formation of the self-assembled complex. The nature of the interactions between the protein and polysaccharide in this case is similar to the interactions in living organisms [28]. Peroxidase associated with chitosan has such high catalytic activity and stability due to the insignificant changes in its native conformation.

It should be noted that chitosans with the low-molecular weights (MW 5, 10, and 25 kDa) allowed achieving higher peroxidase-activating effect, but the best reproducibility of the results and stability of the enzyme preparations were significantly better in the presence of the polymer with MW 150 kDa [2].

Taking into account the fact that these immobilized enzyme preparations were intended for the development of biosensors and test devices where the stability of the biocatalyst was one of the most important parameters, chitosan with MW 150 kDa was chosen for further investigations.

3.1.1. Size distribution of the particles of {peroxidase–chitosan} self-assembled complex

The dispersity of the resultant particles of the polyelectrolyte complex formed by peroxidase and chitosan and their average hydrodynamic radius were studied by means of light scattering. It was established that under the optimal conditions of the complex

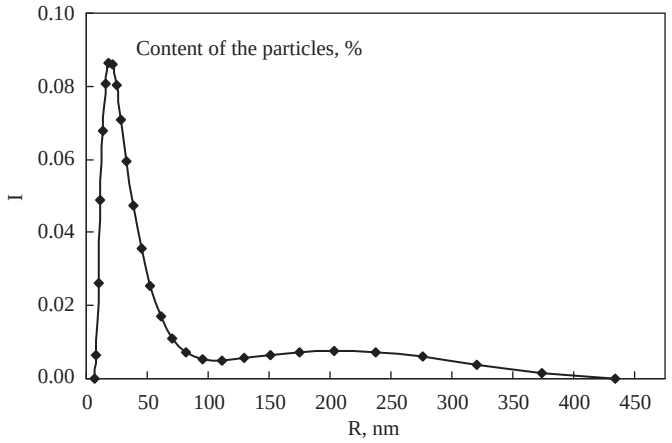

Fig. 1. Size distribution of particles of self-assembled {peroxidase–chitosan} complex in 0.05 M phthalate buffer (pH 5.9).

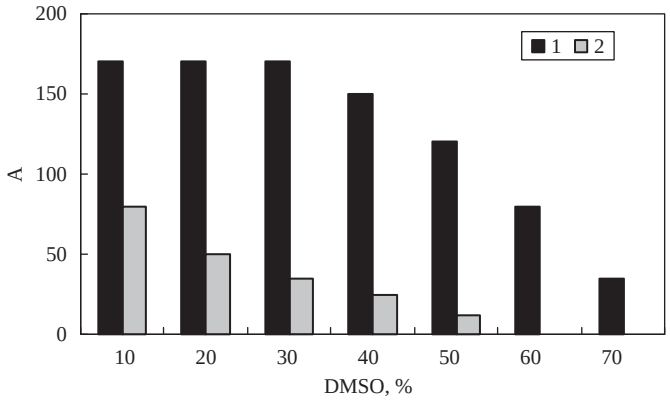

Fig. 2. The catalytic activity of the self-assembled {peroxidase–chitosan} complex (1) and native peroxidase (2) in aqueous (0.05 M phthalate buffer, pH 5.9) -DMSO media.

formation its particles formed an organized structure, their average size being (22 ± 3) nm (Fig. 1). The high monodispersity of these particles enables their further application for the production of homogeneous and reproducible biosensitive films which can be used as the basic element of optical sensors (with excellent analytical performance) for the determination of bioactive compounds.

3.1.2. *Influence of dimethyl sulfoxide on the catalytic activity of {peroxidase–chitosan} complex*

Enzymes often lose their catalytic activity in the mixtures of polar organic solvents due to denaturation of the biomolecule which depends both on the polarity of the organic solvent and its concentration in the reaction medium [32,33]. At the same time, as literature data evidence (for example, [34–38]), complexes formed between proteins and polyelectrolytes are stable in the presence of organic solvents.

We have studied the activity and stability of the complex {peroxidase–chitosan} in the medium of polar organic solvent dimethyl sulfoxide. DMSO has been chosen because it is widely used in medical practice for the targeted transport of pharmaceutical substances through skin and cell membranes and, as a result, it is a component of many pharmaceutical preparations. In addition, DMSO is used for sample treatment in biochemical analytical procedures. For example, it is used as an extractant of physiologically active compounds from the cell membranes of different structure, biological liquids and other biosamples, as a solvent for pharmaceutical preparations, etc. [39]. At the same time, this solvent mixes with water in any ratios enabling the investigation of its influence on peroxidase activity in a wide range of its concentrations in the reaction mixture. In earlier studies concerned with the influence of DMSO on horseradish peroxidase, it was shown that as little as 0.66% v/v of this solvent reduced the activity of the enzyme in the reaction of aromatic substrates oxidation; the residual activity of peroxidase in 20% v/v DMSO did not exceed 20% of its activity in aqueous solution [39]. The studies of Klibanov et al. demonstrated that DMSO changed significantly the secondary structure of the protein molecule of the enzyme [40,41]. For the reasons listed above, it was important to investigate the approaches aimed to preserve the catalytic activity and stability of horseradish peroxidase in the media containing this organic solvent.

The influence of different DMSO concentrations on the rate of *o*-dianisidine oxidation with hydrogen peroxide catalyzed by native peroxidase and {peroxidase–chitosan} complex was studied. It was established that the complex in the system containing 30% v/v DMSO was two times more active than the native enzyme in aqueous solution; if the organic solvent concentration was 60% v/v, the complex was as active as the native biocatalyst in aqueous solution and it still had some catalytic activity in the presence of 70% v/v DMSO (Fig. 2).

When investigating the stability of native peroxidase and peroxidase included into the polyelectrolyte complex with chitosan in the presence of 30% v/v DMSO, we revealed that the enzyme preserved half of the initial activity in the medium of organic solvent after 7 and 28 h, respectively.

Therefore, our investigation demonstrated that the polyelectrolyte complex {peroxidase–chitosan} was a promising catalyst for enzymatic reactions in aqueous-organic media. In particular, it could be used for the development of the procedures for the determination of peroxidase substrates in the presence of polar organic solvent.

3.1.3. *Formation of optically transparent films based on {peroxidase–chitosan} complex*

Chitosan is a suitable matrix for the development of optical biosensors. The films based on this polymer are optically transparent and do not absorb light in visible and near-UV spectrum region [42]. In addition, as reported below, the presence of reactive amino groups in the polymer molecule enables managing the sensitivity and selectivity of peroxidase towards a number of its inhibitors (heavy metal ions) and some organic substrates (phenolic compounds of different structure). To develop the sensors based on {peroxidase–chitosan} complex with the spectrophotometric registration of the analytical signal, we employed a simple technology (see the Experimental section) for the formation of biorecognizing films on the surface of optical glass slides and in the wells of polystyrene plates for immunochemical analysis. The comparative study of the transparency of the films soaked for 1 h in an aqueous solution (0.05 M phosphate buffer solution, pH 6.5) and aqueous-organic medium (30% v/v DMSO) demonstrated that the films kept in aqueous-organic solution were more transparent (transmission $T=(96 \pm 2)$ %, $n=5$) than the ones kept in aqueous solution ($T=(81 \pm 4)$%, $n=5$). To study the morphology and topology of the films after preparation and immersion in different media, we used atomic force microscopy. The images presented in Figs. 3 and 4 evidenced that the films kept in 30% DMSO solution were more uniform and smoother than the ones kept in aqueous solution (the height fluctuations

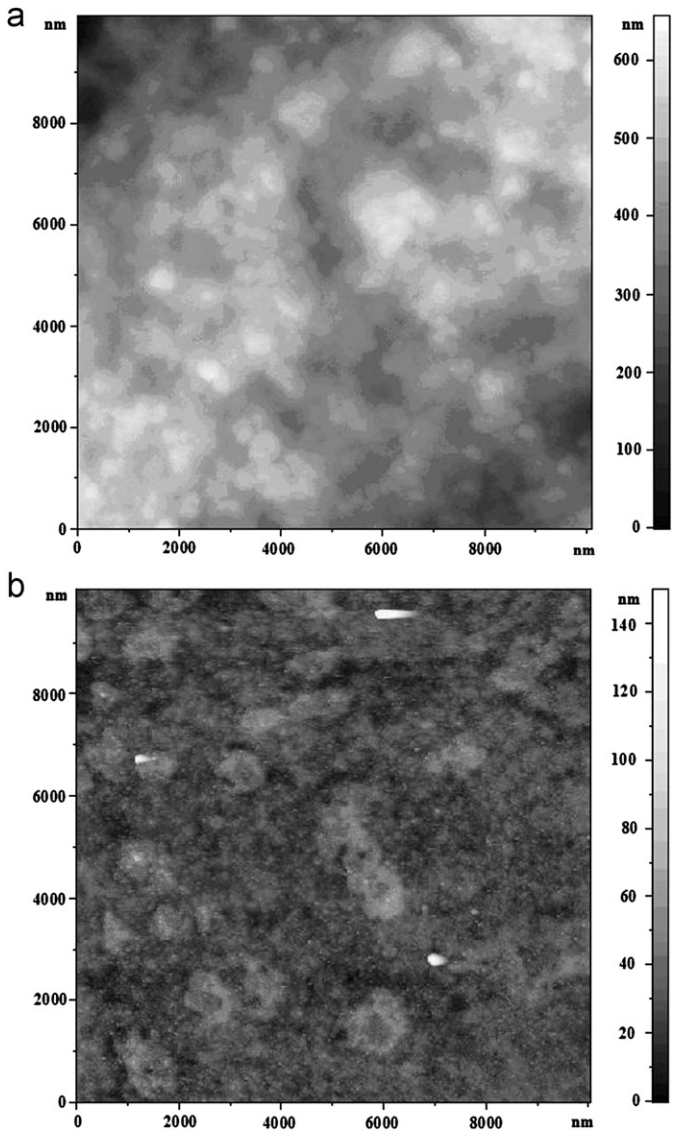

Fig. 3. AFM-images of morphology of self-assembled {peroxidase–chitosan} films exposed to water (a) and aqueous-DMSO media (b).

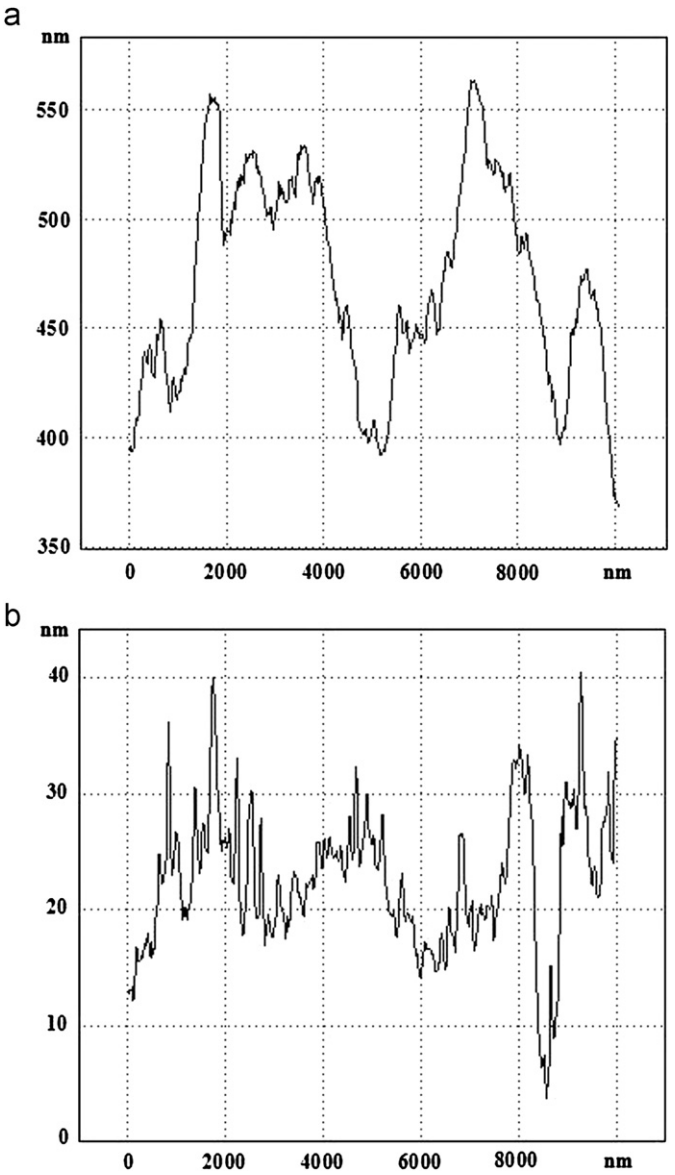

Fig. 4. AFM-images of topography of self-assembled{peroxidase–chitosan} films exposed to water (a) and aqueous-DMSO media (b).

were 30–35 and 140–150 nm over 1 μm^2 area). According to the results of optical microscopy, the thickness of the optical films was 5 μm.

Therefore, the described study showed that the inclusion of peroxidase into the self-assembled complex with chitosan allowed obtaining highly active enzyme preparations, stable in aqueous and aqueous-organic solutions. Their application can enable the development of new enzymatic analytical procedures, highly sensitive and selective optical biosensors and test-devices which can operate both in aqueous and aqueous-organic media.

3.2. Application of {peroxidase–chitosan} complex in chemical analysis

This section describes the results of the application of the polyelectrolyte complex for the development of the procedures for the determination of some metal ions (peroxidase inhibitors) as well as the enzyme's substrates (phenothiazines, phenolic compounds, and organic peroxides) and provides some examples of real-world sample analysis, including water-insoluble samples.

3.2.1. Visual test-determination of Cd(II), Pb(II), and Hg(II) ions

In our earlier studies we used the ability of chitosan to absorb metal ions including Cd(II), Pb(II), and Hg(II) as well as the high stability and catalytic activity of {peroxidase–chitosan} complex immobilized on polyurethane foam for the development of visual test procedures for the determination of the listed toxicants—peroxidase inhibitors [26,43]. The indicator reaction used was the oxidation of *o*-dianisidine by hydrogen peroxide. The reaction rate was registered visually by the time of the appearance of the red color of the reaction product, the time value increasing proportionally to the metal ion concentration [43]. It should be noted that the developed test procedures for the determination of Cd(II), Pb(II), and Hg(II) had unique sensitivity among the test methods (LOD were 50, 10, and 1 ng/L). Moreover, the sensitivity of the developed test procedures was one or two orders higher than the sensitivity of the determination of these analytes involving native enzymes [43]. Obviously, it was associated with the effect of a metal ion preconcentration in the chitosan matrix. This assumption was supported by the coincidence of the optimal conditions (first of all,

pH of the solution) for the determination of each of the metal ions and their recovery with chitosan [44,45]. The developed test procedures for the metal ions determination were successfully applied for the analysis of a wide range of real-world samples [26,43].

3.2.2. Determination of phenothiazines in aqueous-organic media

The possibility of using the effects of the enhancement of the stability and keeping rather high catalytic activity of peroxidase in aqueous-organic media employing its inclusion into the complex with chitosan was demonstrated by developing procedures for the determination of phenothiazines. Phenothiazines are a class of heterocyclic compounds which are used in medicine as tranquilizers, antidepressants, neuroleptic and antiallergic agents, etc. [46]. The chemical structure of this group of substances involves a heterocyclic system consisting of the six-member heterocycle of thiazine condensed with two nuclei of benzene [47]. Due to their amphiphilic nature, the derivatives of phenothiazine group interact with the cell membrane in organisms and are distributed between the phases of aqueous solution and hydrophobic lipid bilayer (cell membrane) [48]. The efficient transfer of phenothiazine derivatives into cells (for example, chloropromazine into erythrocytes) is provided by the addition of DMSO into the pharmaceutical preparations, this solvent working as a transporter of the compounds through the membrane structures. This solvent is also used for the extraction of phenothiazines from biological liquids and tissues [49]. The determination of phenothiazines in biological matrix is usually preceded by a complicated sample treatment procedure involving extraction and preconcentration. The necessity of such sample treatment is associated with low concentrations of phenothiazines in biological samples (ppm or ppb level) and their ability to bind with protein molecules present in the tested samples. The most wide spread and convenient method of phenothiazine recovery from the matrix is their liquid–liquid extraction into a suitable organic solvent immiscible with water [50]. As it has been revealed by the analysis of literature, the most popular solvent for the extraction of phenothiazines from blood plasma is a mixture of non-polar (hexane) and polar solvent, including DMSO [51,52].

We have developed enzymatic procedures for the determination of such phenolthiazines as chloropromazine (or aminazine), promazine, and trifluoroperazine (or triphthazine). These compounds are commercially available and widely used in medical practice as pharmaceutical preparations. The mechanism of their peroxidase-catalyzed oxidation is partially investigated and described in literature [53,54].

Promazine, chloropromazine, and trifluoroperazine were earlier found [53] to activate peroxidase-catalyzed oxidation of such peroxidase substrates as NADH and ascorbate ion in neutral medium. We observed this effect (the so-called 'substrate–substrate' activation) in the reaction of *o*-dianisidine oxidation. Since the increase in the rate of *o*-dianisidine oxidation in the presence of phenothiazines was proportional to their concentration in the reaction mixture; this phenomenon was used in the enzymatic procedures for the determination of these phenothiazines in aqueous solutions and in the presence of 30 vol% DMSO (see Table 1). The experimental procedure and the optimal conditions are described in the experimental section.

As Table 1 shows that the procedures for the determination of phenothiazines in aqueous-organic solution in the presence of the polyelectrolyte complex have better analytical parameters than the procedures in the presence of the native biocatalyst. The sensitivity coefficient of the determination of promazine and chloropromazine in aqueous-organic media in the presence of the complex is six-times and 11.5-times higher than the same parameter of the procedures in the absence of chitosan. In the presence of the polyelectrolyte complex the quantification limits of phenothiazines in aqueous-organic and aqueous media coincide; these values are by an order lower than those for the procedures involving native peroxidase in aqueous-organic solutions. In the absence of chitosan we did not manage to develop a procedure for the determination of trifluoroperazine, the substrate of peroxidase most resistant to oxidation among the considered compounds. Let us also note that in aqueous solution the procedures for phenothiazine determination in the presence of the polyelectrolyte/enzyme complex have better metrological parameters (Table 1) than the ones involving native enzyme. The proposed procedure for chloropromazine determination was applied to the analysis of organic extracts (hexane-DMSO, 1–5% v/v) of plasma of human dark blood: (3.2 ± 0.4) μM were found. The results of phenothiazine determination in the indicated sample were confirmed with the official spectrophotometric procedure: the results were (3 ± 1) μM.

Table 1
Analytical parameters of the procedures for the determination of phenothiazines using their activating effect in the reaction of *o*-dianisidine oxidation catalyzed by native enzyme and {peroxidase–chitosan} complex in aqueous solutions (I and II) and in the presence of 30% v/v DMSO (III and IV, respectively) ($n=5$).

Phenothiazine		Applicable concentration range (mM)	Calibration curve equation	RSD (%)
Promazine	(I)	0.03–0.2	$y^{*}=0.72\ x^{**}+0.09$	3
	(II, IV)	0.02–0.1	$y=1.19\ x+0.19$	5
	(III)	0.1–0.8	$y^{*}=0.20\ x^{**}+0.03$	7
Chloropromazine	(I)	0.07–0.5	$y=0.29\ x+0.10$	7
	(II, IV)	0.05–0.4	$y=0.46\ x+0.19$	4
	(III)	0.5–2	$y=0.04\ x+0.04$	6
Trifluoroperazine	(I)	0.1–1	$y=0.14\ x+0.09$	5
	(II, IV)	0.09–0.7	$y=0.23\ x+0.18$	8
	(III)	–	–	–

* *y*—absorbance at 450 nm registered using a microplate spectrophotometer in 5 min after the reaction start.
** *x*—phenothiazine concentration (mM).

3.2.3. Determination of a number of phenolic compounds and organic peroxides using optical biosensor based on {peroxidase–chitosan} complex in aqueous and aqueous-organic media

Due to the presence of reactive amino groups in the molecule, chitosan can participate in some reactions which are typical for amines, for example, it is known to react with quinones [55].

Several approaches were proposed to the practical application of this reaction (including the elimination of phenolic compounds from waste waters after their enzymatic oxidation and production of chitosan-based modified polymers) [55,56] but to our knowledge this process has not been applied in analytical chemistry so far. At the same time, the adduct of chitosan and quinone strongly absorbs light in UV and visible spectrum region and, therefore, this reaction can be used for the determination of phenolic compounds after their preliminary oxidation. It implies that it is possible to create an optical biosensor based on the self-assembled complex {peroxidase–chitosan} which does not require any additional chromogenic reagents. The operation of the proposed biosensor is based on the following sequence of reactions:

i. enzymatic oxidation of the phenolic compounds yielding a quinone product;

ii. interaction of the produced quinone product with amino groups of chitosan resulting in the formation of a light-absorbing

adduct. This process was thoroughly investigated and described in the studies of Payne et al. [55,56], who assumed that the reaction of the formation of chitosan–quinone adduct proceeded as Michael-type addition [55].

In this study the model water-soluble phenolic compound for the development of the biosensor is hydroquinone which is known to be a substrate of peroxidase. Hydroquinone is used as an antioxidant in a polymer production and as a skin-whitening agent in some pharmaceutical preparations for external use.

The response of the biosensor (film absorption at 345 nm, this wavelength value corresponds to the absorption of the adduct formed by chitosan and hydroquinone oxidation product) was measured using a spectrophotometer. Optical glass slides were used as the support for the biosensitive layer. A simple procedure providing the formation of even and transparent films of {peroxidase–chitosan} complex on a glass surface is described in the experimental section.

The conditions chosen for the formation of the films based on the self-assembled complex and registration of the analytical signal were as follows: 2 mg of chitosan, 0.05 nmol of the enzyme, 0.05 M phosphate buffer solution, pH 6.5, the reaction mixture volume −5 mL, hydrogen peroxide concentration −1 mM. These conditions provided hydroquinone determination with the analytical characteristics presented in Table 2.

It should be noted that the adducts of the products of the enzymatic oxidation of some phenolic compounds and chitosan absorb light in UV region at 300 nm or below [55]. However, in majority of the cases their influence on hydroquinone determination at 345 nm is not significant and can be eliminated by means of chemometrical methods if it is necessary for the analysis of the sample. Concerning the possible influence of the components of the indicator system, we should note that the absorption of the chitosan film is small at 345 nm and is well reproducible. The other components present in the system also do not influence the analytical signal: the product of the enzymatic oxidation of hydroquinone (quinone) is the only compound in the system which interacts with chitosan and is incorporated into the film, the absorbance of which is used as the response.

The biosensor has a high stability (it was examined for hydroquinone concentration 100 μM) and it can be kept in a fridge (4 °C) at least for a month before use. If the initial response of the biosensor was taken as 100%, the response of the biosensor after 5 days of the storage at room temperature (25 °C) was $(69 \pm 7)\%$ ($n=4$, $P=0.95$), while after 30 days of storage of the sensor in a fridge (4 °C) the response was $(98 \pm 8)\%$ ($n=4$, $P=0.95$). Moreover, the adduct used for the signal registration was also highly stable in time and its absorbance hardly changed after the storage of the sensor during 6 weeks. It is a unique feature of the developed biosensor that the analytical signal is measured as the absorbance of the slide with the film rather than the absorbance of the reaction solution. As a result, it is possible to analyze emulsions and non-transparent solutions without preliminary separation of the matrix. If there are any particles of the sample (for example, an ointment) left on the slide surface, they can be washed away from the slide surface with water. Since the analyte is attached to chitosan with a covalent bond, it cannot leach from the film. Moreover, for the analyte concentrations lying in 20–200 μM range the absorbance values vary from 0.4 to 1.6, and, consequently, the application of the developed biosensor does not require using highly sensitive equipment. It is an advantage of the proposed biosensor over the biosensor of a similar construction proposed by Abdullah et al. [57], the response of which towards much higher concentrations of the analyte (500–9000 μM of catechol) varies in the range 0.01–0.06 units of absorbance.

Hydroquinone-containing pharmaceutical skin-bleaching ointment and gels are popular real samples for the estimation of the performance of the procedures for hydroquinone determination. Long-term application of hydroquinone-containing products is reported to be dangerous for health and for this reason hydroquinone application is limited or prohibited in many countries [58]. In addition, such samples are interesting in terms of their treatment before the analysis due to their water-insoluble and non-transparent matrices which are traditionally complicated for spectrophotometric registration of an analytical signal. Therefore, the efficiency of the biosensor for the analysis of pharmaceutical products was demonstrated by determining hydroquinone in skin whitening cream ('Achromin' produced by Alen Mak, Bulgaria), where it was the single active component. The result of the analysis obtained by the standard addition method using the proposed biosensor $(1.9 \pm 0.1)\%$ ($n=4$, $P=0.95$) did not differ from the result obtained by HPLC analysis $(1.9 \pm 0.1)\%$ ($n=3$, $P=0.95$) and was in accordance with the value reported by the producer (1.9%). We should note that the analysis of the real sample using the biosensor was much more convenient as compared to the analysis by means of HPLC and UV-spectrophotometry because there was no necessity to provide the transparency of the sample for hydroquinone determination. Homogenization of the sample in water or DMSO was sufficient for the analysis procedure.

Analytical performance of the optical biosensor based on {peroxidase–chitosan} complex was evaluated for nine phenolic compounds of different structure and some organic peroxides. When determining phenolic compounds (quercetin, rutin, and esculetin) and organic peroxides (2-butanon peroxide and benzoyl peroxide) which were poorly soluble in aqueous solutions, we carried out the indicator reaction in the presence of 10 and 20% v/v DMSO, respectively (Table 2).

The response of the biosensor towards phenolic compounds decreased in the sequence: quercetin > hydroquinone > catechol > rutin > esculetin > phenol. The biosensor did not respond towards such phenols as resorcinol, pyrogallol, and phloroglucinol. Obviously, such situation could be explained by the absence of an interaction between the products of oxidation of these phenolic compounds and chitosan resulting in the formation of Michael-type adduct, since all phenolic compounds considered in this study are known to undergo oxidation by hydrogen peroxide in the presence of peroxidase. The analytical system consisting of two subsequent reactions allowed eliminating the interference of many admixtures of non-phenolic structure (except for quinones) which could be present in real samples.

Catechol was used as the indicator (chromogenic) compound for the development of the procedures for the determination of organic peroxides. The determination was carried out under the optimal conditions for the determination of phenolic compounds;

Table 2
Analytical characteristics of the optical biosensor for the determination of different phenolic compounds and peroxides.

Analyte	Applicable concentration range (Mm)*	Detection limit (μM)	Sensitivity ($\times 10^{-3}$ M^{-1})	DMSO (vol%)
Phenol	20–100	13.3	1.8	–
Hydroquinone	20–200	3.3	6.8	–
Catechol	20–250	7.4	3.2	–
Quercetin	10–150	3.1	12.5	10
Rutin	10–150	10.0	3.9	10
Esculetin	10–200	10.8	3.6	10
2-Butanone peroxide	50–1000	31.7	1.2	20
Benzoyl peroxide	50–250	45.1	0.86	20

* In all cases RSD of the results of the determination did not exceed 8%.

Table 3
The application of the proposed biosensor in the real-world samples analysis ($n=5$, $P=0.95$).

Analyte	Sample	Special features	Determined concentration	Producer's data
Hydroquinone	Skin-bleaching ointment	Oily non-water mixable matrix	(1.9 ± 0.1)%	1.9%
Quercetin	Pharmaceutical preparation	–	(0.052 ± 0.004) g/0.5 g	0.05 g/0.5 g
Rutin	Food supplement	Water insoluble sample, some solid particles remain after dissolution in DMSO	(0.051 ± 0.003) g/tablet	0.05 g/tablet
Benzoyl peroxide	Cream for acne treatment	Oily non-water mixable matrix	(5.0 ± 0.4)%	5.0%

catechol concentration was 1 mM. The sensitivity of the biosensor towards organic peroxides decreased in the following sequence: 2-butanone peroxide > benzoyl peroxide > *tert*-butyl peroxide. It should be noted that even in the presence of high concentrations of the latter (5 mM) the absorbance of the biorecognizing layer of the sensor was low and did not exceed 0.2 units of absorbance. For this reason, we did not manage to develop a procedure for the determination of *tert*-butyl peroxide. The analytical parameters of the developed procedures are presented in Table 2.

The procedures for the determination of quercetin, rutin, and benzoyl peroxide were applied for the analysis of real-world samples (see Table 3). In all cases the approaches to the sample pretreatment, measurement of the analytical signal, etc. were similar to those of the procedure for the determination of hydroquinone.

4. Conclusions

Our study demonstrated the prospects of the application of the self-assembled complex {peroxidase–chitosan} for chemical analysis. It was shown that the detailed investigation of the interaction of the enzyme with polyelectrolytes, sensible choice of their nature as well as the conditions for the complex formation allowed developing biosensors and analytical systems of a new generation with certain properties (sensitivity and selectivity). The results of this study provided an increase in the activity of the biocatalyst and its stability in aqueous-organic media; improvement of the selectivity of the determination of a number of organic compounds and sensitivity of the determination of inorganic compounds due to specific interactions of the analytes with the polymer matrix; expansion of the range of compounds, including the poorly soluble and slowly oxidized substrates of peroxidase, which could be determined and tested samples which could be analyzed by enzymatic methods.

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