ISSN 1061-9348, Journal of Analytical Chemistry, 2019, Vol. 74, No. 1, pp. 41–57. © Pleiades Publishing, Ltd., 2019.
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REVIEWS

Optical Sensors on the Basis of a Polyelectrolyte Peroxidase–Chitosan Complex for the Determination of Biologically Active Compounds

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Received April 2, 2018; in final form, July 16, 2018

Abstract—The review summarizes authors' works of the for the last 15 year covering the possibilities, advantages, and prospects for the use of optical sensors based on peroxidase for the determination of a wide range of biologically active compounds, i.e., phenolic compounds and hydroperoxides of different structures, phenothiazines, catecholamines, and their metabolites, for the quality control of drugs, foodstuffs, biomedical research, and clinical diagnostics.

Keywords: optical sensors, horseradish root peroxidase, indicator reactions, spectrophotometry, fluorescence, phenolic compounds, hydroperoxides, phenothiazine, catecholamines and their metabolites, analysis, drugs, foodstuff, biological fluids

DOI: 10.1134/S106193481901012X

One of urgent problems of present-day analytical chemistry is the development of simple, reliable, and sensitive sensors for the quality control of medicinal and cosmetic preparations, foodstuffs, biochemical monitoring, and clinical diagnostics. Prospects for the use of sensors in the practice of chemical analysis are determined by a number of their advantages in comparison with traditional analytical procedures, i.e., rapidity and economic feasibility of analysis, and also technological and methodological simplicity and no need in the attraction of highly skilled personnel. The inclusion of biological components—enzymes, antibodies, DNA— in such analytical means ensures an increase in the sensitivity and selectivity of analysis and expands its possibilities because of the high selectivity of biochemical interactions.

Research into the development of enzymatic sensors for the determination of biologically active compounds in environmental and food samples, pharmaceutical preparations, and body fluids have been actively conducted in the world in recent 20–25 years. The majority of the known enzymatic sensors are traditionally based on the electrochemical registration of an analytical signal, and such biosensors allow the successful determination of organic compounds from many classes, whereas the development and evolution of biosensors with optical (spectrophotometric, fluorimetric, and chemiluminescence detection) has received less attention.

Sensors are developed using enzymes catalyzing the transformation of either only one compound (examples of such highly specific enzymes are scanty) or a group of compounds with similar properties. Enzymes catalyzing transformations of compounds differing in properties and structure, for example, reductant and oxidant substrates, are of special interest.

In this regard, horseradish root peroxidase occupies a special niche in biochemical methods of analysis. It is a well-studied highly active commercial enzyme, catalyzing transformations of different groups of organic substances, whose determination is of considerable interest. In particular, peroxidase reductant substrates are phenolic compounds of different structures and oxidant substrates may be organic peroxides [1–3]. Among the first group are many major ecotoxicants and natural and industrial antioxidants; the second compounds may be quality markers of food and medicinal and cosmetology products and also important technical and pharmaceutical raw materials. In addition, both peroxidase reductant and oxidant substrates are the most important markers of various socially important diseases. The determination of a whole class of compounds listed above is an important task of chemical analysis.

The main requirement for any chemical sensor is that the response of the sensing layer must be proportional to the concentration of only the compound to be determined (or several compounds in the group deter-

mination). The matrix components of the analyzed sample must not affect the results of measurements of the analytical signal. However, real samples, which are of the most analytical interest, usually have matrixes of complex composition (for example, biological fluids), also insoluble in water (many cosmetic and drug preparations, foodstuffs). The components of these matrixes can not only bring errors into the results of measurements, but in some cases also considerably complicate the analytical procedure. For this reason, the analysis of real samples often includes additional sample preparation using toxic or aggressive organic solvents, filtering or separation, which significantly increases errors of the results of measurements and the duration of analysis, and also complicates its procedure.

According to the authors, a promising approach to solving the above problems is the development of solid-phase sensors based on the formation and measurement of an optical (spectrophotometric or fluorescence) signal directly on the sensor surface in its sensing layer rather than in the test solution. There is good reason to believe that the introduction of such indicator systems and approaches, expanding the possibilities of enzymatic methods, will strengthen the positions of optical biosensors among the modern analytical means.

This review is devoted to the problem of the improvement of the existing and the development of new optical sensors based on immobilized peroxidase for the determination of biologically active compounds.

PEROXIDASE IN A SELF-ASSEMBLING COMPLEX WITH CHITOSAN AS A BASIS FOR THE BIORECOGNIZING LAYER OF AN OPTICAL SENSOR

As was noted above, horseradish root peroxidase is an enzyme most often used in chemical analysis [2, 3]. However, the expansion of its analytical applications is limited by insufficient specificity and sensitivity to a number of substrates and effectors, and also by the low stability of the native enzyme and the low efficiency of biocatalysis in organic media in the determination of biologically active compounds in samples poorly soluble or insoluble in water [4].

Within the three last decades, interests of researchers in the search for approaches to the implementation of enzymatic catalysis in organic solvents steadily grew [5–8]. It should be noted that, in nonpolar (hydrophobic) solvents, which almost do not affect the structure of proteins, enzymes usually retain catalytically active conformations [9, 10] and are stable [11, 12]. In the presence of polar organic solvents, biocatalysts partially or completely lose their catalytic activity because of the denaturation of the protein globula [13, 14]. Among the approaches recommended for increasing the stability of biocatalysts to this type of inactivation, we can note the following: immobilization of enzymes on solid supports [15], change of amino-acid residues of the protein [16, 17], its covalent modification by low-molecular reagents [18, 19] and polymers [20], and the molecular imprinting of enzymes with ligands insoluble in water followed by lyophilization [21]. However, none of these methods of the stabilization of biocatalysts ensures the retention of their high catalytic activity in a wide concentration range of polar organic solvents.

A promising approach (previously not used in chemical analysis) to solving the specified problems is the incorporation of biocatalysts into self-assembling polyelectrolyte structures. An advantage of enzyme–polyelectrolyte complexes formed by nonspecific electrostatic interactions is a possibility of modeling the physical and chemical properties of the complex and the kinetic parameters of reactions catalyzed by it by varying the nature and molecular weight of polymers and changing their ratio in the reaction mixture and the conditions of complex formation (nature of buffer solutions, pH, ionic strength, etc.) in the course of immobilization [22]. Therefore, there are possibilities for the development of highly active and stable enzymatic systems with the specified properties (sensitivity and selectivity) for solving specific analytical problems.

It should be noted that the method of enzyme incorporation into a polyelectrolyte complex differs by the simplicity of the used procedures, ensures the uniform distribution of the biocatalyst in volume of the support, and gives stable immobilized preparations with well reproducible characteristics [7, 21]. In addition, such systems are easily obtained and in some cases are optically transparent and convenient for the further use in chemical analysis. The formation of polyelectrolyte complexes between polyions today is the simplest method of the formation of structures of nano (water-soluble conjugates, nanoparticles) and microscopic (physical gels, films) sizes [22]. Their subsequent immobilization on solid supports (optical glasses, test strips, plates, etc.) leads to a considerable improvement of the mechanical properties of enzyme preparations and forms a basis for the design of biosensors [23–25].

Choice of polyelectrolyte nature for the formation of peroxidase complexes. A promising method of the formation of noncovalent polyelectrolyte–enzyme complexes is the use of natural polysaccharides. An increased interest in this type of polymers is determined by their high biological activity and the ability of formation of interpolyelectrolyte complexes with proteins. By varying the chemical nature of polysaccharides and using their different derivatives, one can obtain matrixes with characteristics optimum for peroxidase, including recognizing ability necessary for the further application of its immobilized prepara-

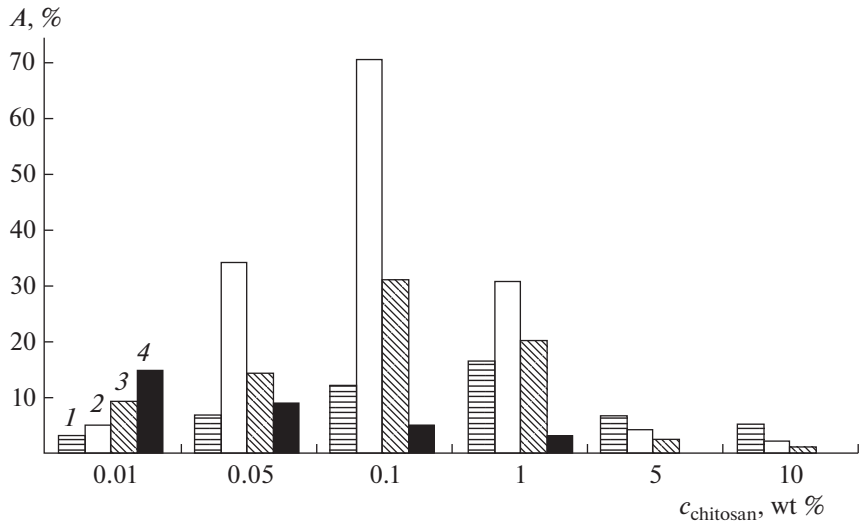

Fig. 1. Degree of peroxidase activation (*And*, %) as a function of the molecular weight (MW, kDa) and concentration of chitosan ($c_{chitosan}$, wt. %) in the reaction mixture (0.05 M phthalate buffer solution, pH 5.0). MW of chitosan, kDa: (*1*) 5; (*2*) 10; (*3*) 25; (*4*) 150.

tions, in particular, in the composition of biosensors. In the study of the effect of polyelectrolyte nature on the physical and chemical properties of peroxidase, we tested natural polysaccharides and their synthetic derivatives of three different types: anionic, i.e., carboxymethylcellulose and calcium alginate; nonionic, i.e., agarose, β-cyclodextrin and starch; and also cationic, i.e., chitosan [26]. The activity and stability of peroxidase in their presence was controlled by the rate of the indicator reaction of the oxidation *o*-dianisidine by hydrogen peroxide [7, 27]. Despite different nature and types of polysaccharides, all of them accelerated (by $22 \pm 4\%$) the rate of the peroxidase transformation of the organic substrate. It is extremely important for the further analytical application of enzyme preparations that peroxidase in the presence of chitosan [7] was the most stable. Chitosan is a polysaccharide possessing high affinity to proteins, especially to enzymes, and a good sorption capacity. It is often used as a substrate for the immobilization of biomolecules by different methods: covalent and noncovalent binding, encapsulation, inclusion into gels, etc. This is due to its properties, such as biocompatibility; low toxicity; chemical inertness; ability to form films, gels, and membranes; mechanical durability; high permeability with respect to water; and hydrophilicity. Chitosan is a promising matrix for the development of optical sensors, because it does not absorb in the near UV and visible spectral regions [27, 28]. In addition, the presence of reactive amino groups in the polymer, as it will be shown below, ensures the regulation of the sensitivity and selectivity of peroxidase to a number of organic substrates, for example phenols of different structures [28].

The effect of chitosans with different molecular weights on the catalytic activity of peroxidase (degree of deacylation 85%) was studied using the same indicator reaction of the oxidation of *o*-dianisidine by hydrogen peroxide. It was found that all chitosans, both low-molecular (average molecular weights (MW) 5, 10, and 25 kDa) and high-molecular (average MW 150 kDa) in different concentrations in the reaction mixture (0.01–10 and 0.001–0.05 wt %, respectively) have an activating effect on peroxidase, the strength of which depends both on the molecular weight of the polymer and on its concentration (0.1 nM). The most activating effect on peroxidase has a 0.1% (by weight) solution of chitosan with the average MW 10 kDa (Fig. 1) [7].

The activating effect of chitosans on peroxidase is, obviously, due to the formation of a self-assembling complex {enzyme–polysaccharide}, more catalytically active than the native biocatalyst. A similar increase in the activity of such enzymes as xanthokidnase and chitinase through the formation of their polyelectrolyte complexes with chitosan was noted in [29].

The existence of electrostatic interactions between peroxidase and chitosan (on an example of a polymer with MW 150 kDa) was proved by us in the study of the effect of potassium chloride (0.001–0.1 M) on the rate of the peroxidase oxidation of *o*-dianisidine in the presence of the polysaccharide (it is known that strong electrolytes break electrostatic interactions between polyions). It was shown that the strength of the activating effect of chitosan on peroxidase decreases with an increase in the concentration of KCl in the reaction mixture. In the absence of the polysaccharide, potas-

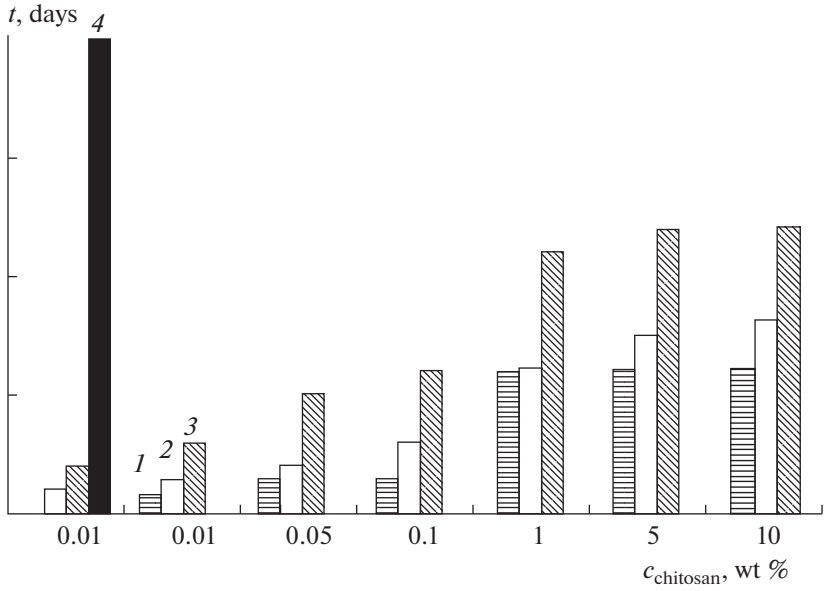

Fig. 2. Duration (t, days) of retention of not less than 50% activity (of the initial) of peroxidase preparations immobilized on polyurethane foam in the presence of chitosans with different molecular weights and concentrations in the mixture for immobilization. MW of chitosan, kDa: *1* – 5, 2 – 10, 3 – 25, 4 – 150.

sium chloride in the studied concentration range did not affect the rate of the indicator reaction [7].

Effect of chitosans with different molecular weights on the stability of peroxidase. To study the stability of immobilized peroxidase preparations, its polyelectrolyte complexes with chitosans with average MW 5, 10, 25, and 150 kDa were previously attached by physical adsorption on a solid adsorbent [7] and then stored in a tight packing at 4°C. The activity of the obtained enzyme preparations was controlled until it remained at the level not lower than 50% of the initial one. The stability of polyelectrolyte complexes with low-molecular chitosans increased with an increase in MW of the polysaccharide. The complex in the presence of 10 wt % of chitosan with MW 25 kDa demonstrated the best stability (activity at a level of 50% of the initial preparation was retained within 120 days). However, the maximum stability was shown by the polyelectrolyte complex formed by high-molecular (MW 150 kDa) chitosan with the concentration 0.001 wt %: it retained not less than 50% of the initial activity within 550 days (Fig. 2).

Because the stability of the biocatalyst in the complex is one of the most important analytical characteristics of sensors, the further research was done with chitosan of the average MW 150 kDa.

The effects pH, ionic strength, and nature of buffer solution on the catalytic activity of the complex {peroxidase–chitosan} were studied in detail under the optimum conditions of the formation of noncovalent complexes as a result of electrostatic interactions between the polyelectrolytes. Such studies were necessary, first of all, in the cases when the pK_a of the polymer (pK_a of chitosan is 6.3–6.5, it dissolves only in the protonated form [29, 30]) and the isoelectric point of the enzyme (pI of peroxidase is 7.2 [31]) differed insignificantly.

The catalytic activity of peroxidase in the reaction of the oxidation of *o*-dianisidine in the presence of 0.0005–0.01 wt % of high-molecular chitosan attained a maximum in a 0.05 M phthalate buffer solution at pH 5.9–6.2 [7]. Therefore, the activity of the complex was maximum in the pH range in which, judging by the values of pK_a of chitosan and the electroneutrality point of peroxidase, we could expect the greatest number of deprotonated chitosan groups and also a sufficient number of protonated peroxidase groups necessary for the formation of a polyelectrolyte complex. Of not lesser importance is the concentration of chitosan in the system. The complex {enzyme–polysacharide} shows the highest catalytic activity (is twice as high as for the native enzyme) in a 0.05 M phthalate buffer solution at the concentration of high-molecular chitosan in the reaction 0.006–0.009 wt %.

In the study of the effect of the nature of a buffer solution on the properties and conditions of complex formation, phosphate and citrate buffer solutions with pH 5.9–6.2 were used in addition to the 0.05 M phthalate solution. In the citrate buffer solution, chitosan in different concentrations (0.0005–0.01 wt %) in the reaction mixture did not change the catalytic activity of peroxidase. In the phosphate buffer solution, chitosan activated peroxidase, but weaker than in the phthalate solution. The change in the character and strength of the effect of polysaccharide on the

enzyme can be due to either a significant increase in the ionic strength of the buffer solution, or a change in the anion charge, or the simultaneous effect of these two factors [7].

To reveal the reasons for the effect of the nature of the buffer solution on the conditions of complex formation, diagrams of species distribution of phthalic, phosphoric, and citric acids were studied and the values of the ionic strength of the corresponding buffer solutions were calculated at pH 5.9. At this pH value, the ionic strength increases in the following series of 0.05 M buffer solutions: phosphate (I = 0.03 M), phthalate (I = 0.09 M), citrate (I = 0.18 M). The ionic strength at which the degree of peroxidase activation with chitosan is more than 60% in a 0.05 M citrate buffer solution is only 1.5-fold lower than that in a 0.2 M phthalate buffer solution with I = 0.27 M. Therefore, the nature of the buffer solution slightly affects the ionic strength of the solution, which could determine the conditions of {peroxidase–chitosan} complex formation.

It should be noted that, according to distribution diagrams, about 7% of phosphoric and 80% of citric acid at pH 5.9 occur as doubly charged ions; for phthalic acid at the given pH such ions were not found. According to the data of [32], the presence of doubly charged acid anions favors the linking of chitosan molecules, which occurs at pH 5.9 in the citrate buffer solution. In the phosphate buffer solution, two processes compete, i.e. reaction of peroxidase with chitosan and a chitosan–chitosan interaction. In the first case, a possibility of complex formation between the protein and polysaccharide molecules is excluded; in the second case, the concentration of the polyelectrolyte complex in the system is low.

Particle size distribution of the {peroxidase–chitosan} complex. In the study of the uniformity of the obtained particles of the polysaccharide complex and their average hydrodynamic radius by photon correlation spectroscopy, it was found that, under the optimum conditions of complex formation, peroxidase particles form with chitosan an ordered structure with an average particle size of 22 ± 3 nm [4]. Such high uniformity of the obtained particles at the nanolevel points to the prospects of their further use for preparing homogeneous and reproduced biosensitive films as a basis of optical sensors.

Effect of dimethyl sulfoxide on the catalytic activity of the {peroxidase–chitosan} complex. In mixtures of polar organic solvents, enzymes often lose their catalytic activity because of the denaturation of the biomolecule, the degree of which depends both on the polarity of the organic solvent and on its concentration in the reaction medium [33, 34]. Protein complexes with polyelectrolytes are stable in the presence of organic solvents [35–39].

We studied the activity and stability of the {peroxidase–chitosan} complex in the medium of a polar organic solvent, dimethyl sulfoxide (DMSO). The choice of DMSO was due to its quite frequent application in medical practice for the targeted transport of medicinal substances through skin cover and cell membranes. As a result, it is a constituent of many pharmaceutical preparations. In addition, DMSO is used for sample preparation to analysis by biochemical methods. For example, it is used as an extractant of physiologically active compounds from cell membranes of different structure, biological fluids and other biosamples and as a solvent of pharmaceutical preparations, drugs, etc. [40]. In addition, this solvent mixes with water in any ratio, which allows the investigation of its effect on the activity of peroxidase in a wide concentration range in the reaction mixture.

We found that, at the concentration of DMSO 30 vol % in the reaction of the oxidation *o*-dianisidine, the {peroxidase–chitosan} complex is twice as active as the native enzyme; at the concentration of organic solvent 60 vol %, their catalytic activities are equal; and, in the presence of 70 vol % of DMSO, only the complex possesses catalytic properties [7].

A comparison of the stability of native peroxidase and that in the complex with chitosan in the presence of 30 vol % of DMSO showed that the enzyme retains one half of its initial activity in the medium of the organic solvent within 7 and 28 h, respectively.

Formation of optically transparent films based on the {peroxidase–chitosan} complex. To develop sensors on the basis of the {peroxidase–chitosan} complex with the spectrophotometric and fluorimetric registration of an analytical signal, we developed a simple technology of the formation of biorecognizing films on optical glasses and in cells of a polystyrene plate. In a comparative study of the transmission of films after their storage in an aqueous solution (0.05 M phosphate buffer solution with pH 6.5) for an hour and in an aqueous–organic medium (in the presence of 30 vol % of DMSO), we found that the films stored in the presence of an organic solvent were more transparent (T = 96 ± 2%, n = 5) than those stored in an aqueous solution (T = 81 ± 4%, n = 5). To study the morphology and topology of the films obtained from various media, we used atomic force microscopy (AFM). Figure 3 testifies that the films after storage in a 30% (by volume) DMSO solution are more uniform and smooth than the films obtained from the aqueous solution (fluctuations by height in 1 μm^2 of the surface were 30–35 and 140–150 nm, respectively). The thickness of optical films, equal to 5 μm, was found by optical spectroscopy [4, 41].

Therefore, we have shown that the inclusion of peroxidase in a complex with chitosan gives highly active and stable enzyme preparations in water and water–organic media, the application of which opens wide prospects for the development of optical sensors and devices working both in aqueous and aqueous–organic media.

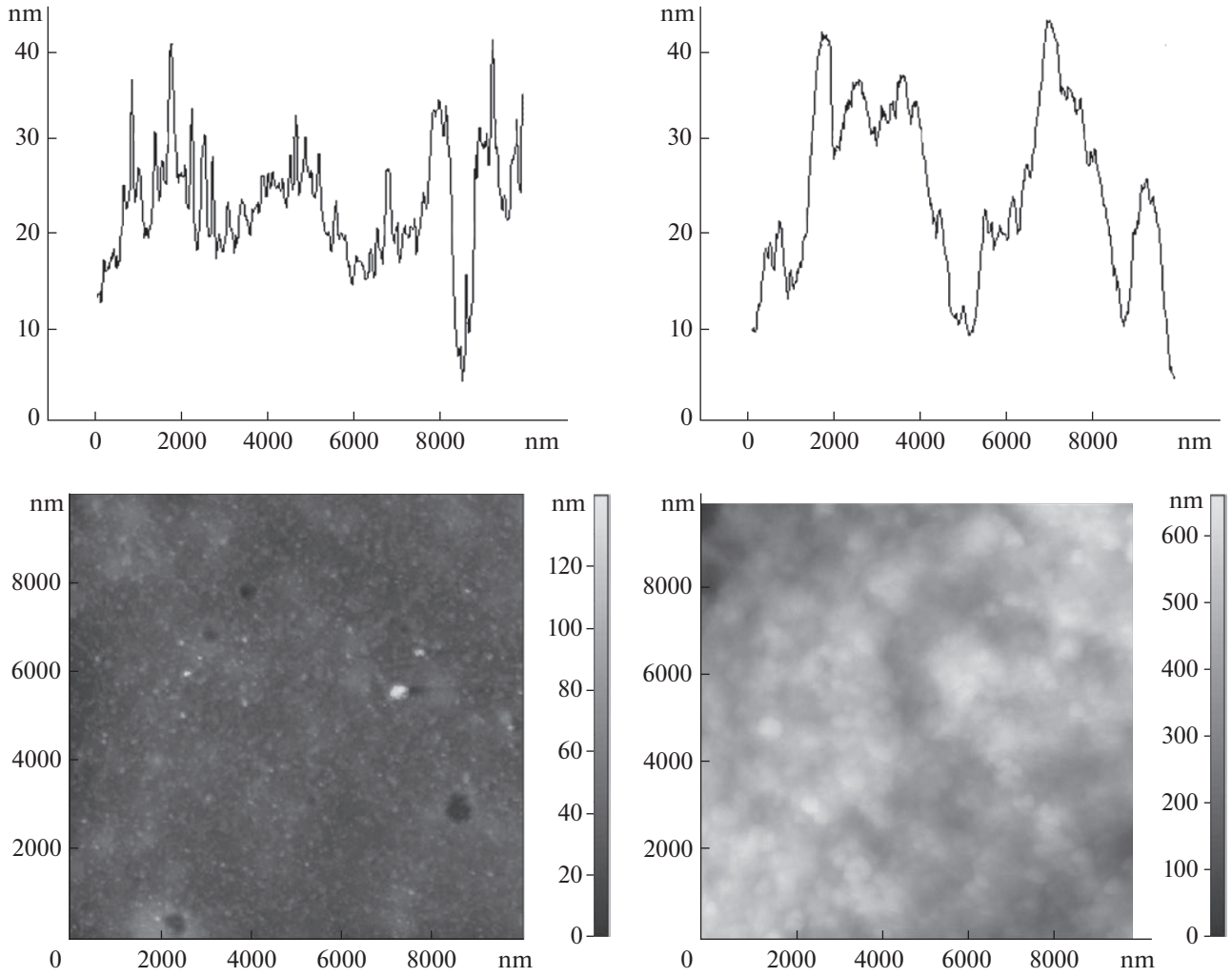

Fig. 3. AFM profiles and surface images of a biosensitive layer based on the self-assembling complex {peroxidase–chitosan} after storage in a 0.05 M phosphate buffer solution of pH 6.5 (on the right) and in the medium water (0.05 M phosphate buffer solution of pH 6.5)–DMSO (30 vol %) (on the left).

DESIGN OF SOLID-PHASE SPECTROPHOTOMETRIC AND FLUORIMETRIC SENSORS, SCHEMES OF REGISTRATION OF AN ANALYTICAL SIGNAL

The use of solid-phase optical sensors based on the formation and measurement of an analytical signal outside the solution expands the analytical possibilities of sensors through the determination of biologically active compounds in samples based complex matrixes (turbid, colored, insoluble in water, of unknown composition) at their minimum sample preparation. However, it should be noted that the sensitivity, selectivity, and especially the rapidity of solid-phase sensors described in the literature are insufficient for fulfilling many analytical tasks. An approach based on the formation and measurement of an analytical signal on a solid surface has not been previously used in the development of spectrophotometric and fluorescence sensors for the determination of phenolic compounds, hydroperoxides, phenothiazine, catecholamines, and their metabolites.

As the simplest design of a solid-phase sensor based on the formation and measurement of an analytical optical signal directly on the sensor surface (in its sensing layer) rather in the test solution, we proposed a plate adapted to cell departments of standard spectrophotometers and fluorimeters by its sizes. The surface of the plate was coated by a sensing layer containing the enzyme and the components of an indicator reaction immobilized in chitosan, so that the indicator compound remained in this sensing layer after the performance of the reaction [4, 28, 41]. The mixture was applied by the drop method and uniformly distributed over the plate surface located on a strictly horizontal surface and dried in air at room temperature [4, 28].

To carry out an indicator reaction, the sensor was immersed in a solution containing the analyte, stored

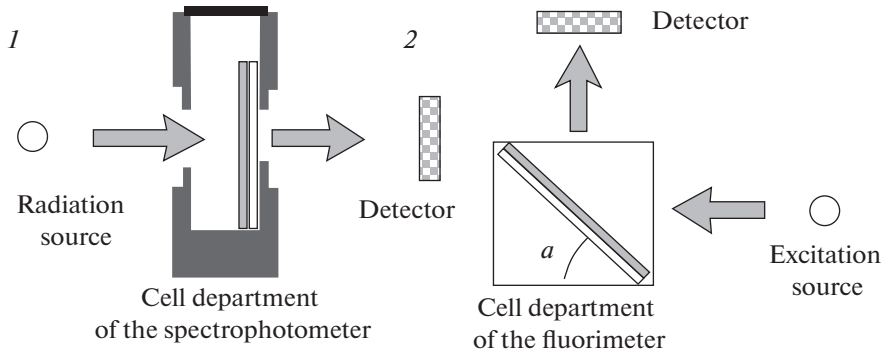

Fig. 4. Schemes of measurement of (*1*) absorbance (1) and (*2*) fluorescence of the sensing layers of a solid-phase optical biosensor.

in it for a necessary time (batch version of the indicator reaction), taken outside, and dried in air at room temperature [4, 28, 41].

The analytical signal was registered by the absorption (spectrophotometric detection) or reflection (fluorimetric detection) sensor directly in the sensing layer on the surface of the plate (substrate). The substrate was installed on the frontal surface of the cell department of the spectrophotometer or in the cell department of the fluorimeter before a reflecting mirror, respectively (Fig. 4). Such design allowed a combination of two methods of measurement of an analytical signal when necessary. In using of a reflecting surface in the fluorimetric signal registration, the sensitivity of the determination significantly increased, because a larger part of the excited radiation arrived at the detector.

However, a serious problem in the registration of the fluorescence signal using solid-phase sensors of the proposed design is the high value of the background signal in the region 300–500 nm (λ_{ex} = 300–400 nm and λ_{em} = 400–500 nm). The presence of an intense background signal makes the measurement of the useful analytical signal actually impossible. To identify the nature of the background signal, reduce its value, and also determine the working region of the spectrum (and, therefore, to choose suitable indicator systems), we used an approach consisting in changing the angle of reflection of the exciting radiation. It was found that the ratio of the fluorescence intensity of the background to the fluorescence intensity of the indicator pyronin B attained a minimum at the angle 75°. The recommended width of entrance and exit slits was 5.0 nm [31].

Thus, we proposed new designs and devices for registering an analytical signal on a solid surface taking into account their further use in universal commercial spectrophotometers and fluorimeters.

SPECTROPHOTOMETRIC SENSORS FOR THE DETERMINATION OF BIOLOGICALLY ACTIVE COMPOUNDS AND THEIR APPLICATION TO CHEMICAL ANALYSIS

Determination of a number of phenolic compounds and peroxides of different structures. The presence of reactive amino groups in the chitosan are responsible for its ability to enter some reactions characteristic for amines. For example, it is known [42] that chitosan reacts with quinones. Some methods of the practical application of such interaction were proposed, including the removal of phenolic compounds from sewage waters after their enzymatic oxidation and the preparation of modified polymers on the basis of chitosan. However, this approach has still not find application in the analytical practice. At the same time, the adduct of chitosan and quinone strongly absorbs light in the UV and visible spectrum regions and, therefore, this reaction can be used for the determination of phenolic compounds after their preliminary oxidation, i.e., an optical biosensor for the determination of phenolic compounds, which does not require additional chromogenic substances can be developed. The operation of the proposed sensor is based on two consecutive processes: the enzymatic oxidation of a phenolic compound with the formation of a quinone product and the interaction of the quinone product with chitosan amino groups with the formation of a strongly absorbing adduct [4, 41]. The last process was investigated in detail and described in the works by Payne with coauthors [42, 43], who supposed that the reaction of the formation of the chitosan–quinone adduct proceeds by the Michael addition mechanism [44].

In developing a spectrophotometric biosensor, the model of a water-soluble phenolic compound was hydroquinone, which was a peroxidase substrate. Hydroquinone is used as an antioxidant in the production of polymers and as a skin-whitening component in some pharmaceutical preparations for external use.

Table 1. Analytical characteristics of the spectrophotometric sensor for the determination of different phenolic compounds and hydroperoxides

Analyte	Analytical range, μM*	Limit of detection, μM	Sensitivity, M^{-1}	Organic solvent, vol %
Phenolic compounds				
Phenol	20–100	13	2×10^3	–
Hydroquinone	20–200	3	7×10^3	–
Pyrocatechol	20–250	7	3×10^3	–
Quercetin	10–150	3	12×10^3	DMSO (10)
Rutin	10–150	10	4×10^3	DMSO (10)
Esculetin	10–200	10	4×10^3	DMSO (10)
Hydroperoxides				
Hydrogen peroxide	100–1000	45	25×10^2	–
2-Butanol peroxide	50–1000	32	12×10^2	DMSO (20)
Benzoyl peroxide	50–250	45	9×10^2	DMSO (20)
tert-Butyl hydroperoxide	150–2500	60	10×10^2	Acetone (50)

* In all cases RSD did not exceed 8%.

Under the optimum conditions of the formation of a biorecognizing matrix on the surface of an optical glass and the registration of an analytical signal, hydroquinone can be determined with the following characteristics: analytical range 20–200 μM, RSD = 2% at the concentration of hydroquinone 20 μM (n = 4), limit of detection 3 μM (Table 1) [4].

It should be noted that adducts of the products of enzymatic oxidation of some phenolic compounds and chitosan absorb light in UV or visible spectrum regions at 300 nm or shorter wavelengths [4, 41] However, in most cases their effect on the determination of hydroquinone at 345 nm was low and, if necessary, could be eliminated by chemometric methods. The absorbance of a chitosan film at 345 nm is low and well reproducible. Other substances present in the system also do not affect 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 thus incorporated into the film, whose absorbance is the analytical signal.

The biorecognizing layer is characterized by high stability (it was studied at the concentration of hydroquinone 100 μM). The plate with hydroquinone before use can be stored in a refrigerator within at least 3 months. The initial response of the biosensor was taken equal to 100%. The response of the biosensor after 5 days of storage at room temperature (25°C) was 69 ± 7% (n = 4, P = 0.95), and after 90 days of storage of the same sensor at 4°C it was 98 ± 8% (n = 4, P = 0.95). Moreover, the adduct used for the determination was also highly stable in time, and its absorbance virtually did not change on the storage of the biosensor within, at least, 6 weeks after the determination. An important feature of the developed biosensor is that the analytical signal was the absorbance of the plate rather than of the reaction solution. This ensures the analysis of emulsions and opaque solutions without the preliminary separation of the matrix. If any particles of a sample (for example, cream) remained on the plate surface, they could be washed from the film with water. As the analyte was covalently bound with chitosan, it could not be washed from the film. Moreover, for the optical biosensor developed by us, the value of absorbance at the analyte concentration 20–200 μM was found in the range 0.4–1.6; therefore, there was no need in using expensive highly sensitive devices. This favorably distinguishes the proposed sensor from the sensor of a similar design developed by Abdullah with coauthors [41], whose response in the presence of much higher analyte concentrations (500–9000 μM pyrocatechol) changed in the range 0.01–0.06 absorbance units, respectively.

Samples for testing procedures for the determination of hydroquinone are often pharmaceutical ointments and gels for skin whitening. It is known that the long use of hydroquinone-containing products is hazardous to health; therefore, the use of hydroquinone is limited or forbidden in many countries [45]. In addition, such samples are of interest from the viewpoint of sample preparation to analysis. To demonstrate the efficiency of a sensor in the analysis of pharmaceutical products, it was used for the determination of hydroquinone in a cream for skin depigmentation ("Achromin" from Alen Mak, Bulgaria), in which it was the only active component (Table 2). The result 1.9 ± 0.1% (n = 4, P = 0.95) obtained by the method of standard additions using the proposed biosensor did not differ from the result obtained by HPLC with amper-

Table 2. Determination of phenolic compounds and organic peroxides in real samples using the spectrophotometric sensor

Sample/ sample preparation	Analyte/concomitant components according to the label claim	Found using a biosensor	Claimed by the manufacturer
Cream "Achromin"/suspending in water, alcohol, or acetonitrile	Hydroquinone/paraffin, protegin X, glycerol, lanoline, sodium chloride, eutanol G, hydroquinone, vitkonol APM, parsol MCX, rose fragrance, sodium metabisulfite, trilon BD, trilon B, lactic acid	1.9 ± 0.1%	1.9%
Vitamins "Askorutin"/tablets suspended in DMSO	Rutin/ascorbic acid, sucrose; potato starch; calcium stearate; talc	0.051 ± 0.003 г/tablet	0.05 g/tablet
Injectable powder "Korvitin"/sample dissolution in DMSO	Quercetin/polyvinylpyrrolidone, sodium hydroxide	0.052 ± 0.004 g/0.5 g	0.05 g/0.5g
Gel "Basiron"/suspending in water	Benzoyl peroxide/acrylate copolymer, poloxamer 182, carbomer 940 (carbopol 980), glycerol, sodium edetate, sodium dioctyl sulfosuccinate, propylene glycol, silica colloidal anhydrous, sodium hydroxide	5.0 ± 0.4%	5%

ometric detection (1.9 ± 0.1%, $n = 3$, $P = 0.95$) and corresponded to the value declared by the producer (1.9%). It should be noted that use of the sensor proposed by us in the analysis of a real sample for the concentration of hydroquinone was much more convenient than the analysis by HPLCs and spectrophotometry in the UV region, as it was not necessary to make the sample transparent; the homogenization of a sample weighed portion in water or DMSO was sufficient.

The response of the sensor to phenolic compounds decreased in the series quercetin, hydroquinone, catechol, rutin, esculetin, phenol. For phenols such as resorcinol, pyrogallol, and phloroglucinol, no response of the biosensor was observed. This is, probably, because the products of the oxidation of these phenolic compounds do not react with chitosan to form Michael's adducts, because, as we know, all of the studied phenols are oxidized with hydrogen peroxide in the presence of peroxidase [1–3]. The use of two consecutive reactions as an analytical system ensures the elimination of the interfering effect of many components of nonphenolic structure (except for quinones), which can present in real samples.

In developing procedures for the determination of organic hydroperoxides, the indicator (chromogenic) substance was pyrocatechol. Determination was conducted under the conditions optimum for the determination of phenolic compounds at the concentration of pyrocatechol 1 mM. The sensitivity of the biosensor to organic hydroperoxides decreased in the series 2-butanone peroxide, benzoyl peroxide, *tert*-butyl peroxide. The analytical characteristics of the developed procedures are summarized in Table 1.

The analytical possibilities of the spectrophotometric sensor were tested in the determination of nine phenolic compounds of different structures and also of organic hydroperoxides. In the determination of phenolic compounds (quercetin, rutin, esculetin) and organic peroxides (2-butanon peroxide and benzoyl peroxide) poorly soluble in aqueous solutions, the reaction was conducted in the presence of DMSO (10 and 20 vol %, respectively) (Table 2) [46].

Determination of phenothiazines in aqueous–organic media. Phenothiazines are a class of artificially synthesized heterocyclic compounds used in medicine as tranquilizers, antidepressants, neuroleptics, anti-allergens, etc. [47]. The chemical structures of this group of preparations are based on a heterocyclic system, which consists of a six-membered thiazine heterocycle with two condensed benzene rings [48].

Because of their amphiphilic nature, phenothiazine derivatives, when entering an organism, interact with cell membranes and are distributed between two phases, aqueous solution and hydrophobic lipid bilayer (cell membrane) [49]. For the effective targeted transport of phenothiazine derivatives into cells (for example, chloropromazine into erythrocyte cells), polar organic solvent DMSO is also added to the composition of pharmaceutical preparations as the effective carrier through membrane structures. The same solvent is used for the extraction of phenothiazine from biological fluids, tissues, and also for the dissolution of drugs in the preparation of various samples to analysis [50]. The determination of phenothiazine in a biological matrix is usually preceded by complex sample preparation, i.e., the extraction and preconcentra-

Table 3. Performance characteristics of procedures for the determination of phenothiazines by their activating effect on the reaction of *o*-dianisidine oxidation catalyzed (I) by native enzyme and (II) by the {peroxidase–chitosan} complex in aqueous solutions and in the presence of 30% (by volume) DMSO ($n = 5$)

Analyte		Analytical range, μM	RSD at c_{min} ($n = 5$, %)
0.05 M phthalate buffer solution of pH 5.8			
Promazine	I	0.03–0.2	0.03
	II	0.02–01	0.05
Chloropromazine	I	0.07–0.5	0.07
	II	0.05–0.4	0.04
Trifluoroperazine	I	0.1–1	0.05
	II	0.09–0.7	0.08
0.05 M phthalate buffer solution of pH 5.8 + DMSO (30 vol %)			
Promazine	I	0.1–0.8	0.07
	II	0.02–0.1	0.05
Chloropromazine	I	0.5–2	0.06
	II	0.05–0.4	0.04
Trifluoroperazine	I	–	–
	II	0.09–0.7	0.08

tion of phenothiazine. The need in the stage of sample preparation is connected, first, with the low concentration of preparations in biological samples (of the order ppm or ppb), and, second, with the ability of phenothiazine to bind with protein molecules present at the analyzed samples.

The most widespread and convenient method of the extraction of phenothiazines from the matrix is their liquid–liquid extraction with a suitable organic solvent that does not mix with water [51]. The solvent for the extraction of phenothiazine from blood plasma is most often a mixture of a nonpolar (hexane) solvent with a small volume (5 vol %) of a polar (including DMSO) solvent [52, 53].

The developed sensor was used for the determination of the following phenothiazines: chloropromazine (or aminazine), promazine, and trifluoroperazine (or triftazine). These preparations are commercially available, widely used in medical practice as drugs; the mechanism of their peroxidase oxidation was partially studied and described in [54, 55].

It was found earlier [47] that promazine, chloropromazine, and trifluoroperazine in a neutral medium accelerate the peroxidase oxidation of peroxidase substrates, such as nicotinamide adenine dinucleotide (NADN) and the ascorbate ion. The same effect of the so-called "substrate–substrate" activation we also found in the reaction of *o*-dianisidine oxidation. An increase in rate of *o*-dianisidine oxidation linearly with the concentration of phenothiazine in the reaction mixture was used as a basis for the determination of promazine, chloropromazine, and trifluoroperazine in aqueous solutions in the presence of 30 vol % DMSO (Table 3) [4].

As can be seen in Table 3, the performance characteristics of procedures for the determination of phenothiazine in aqueous–organic solution in the presence of a {peroxidase–chitosan} complex are much better than those of procedures in the presence of the native biocatalyst. The sensitivity coefficient of the determination of promazine and chloropromazine in an aqueous–organic medium in the presence of the complex is higher by 6 and 11.5 times, respectively, in comparison with the identical parameters of similar procedures in the absence of chitosan. The lower boundaries of the analytical ranges for phenothiazines in the aqueous–organic medium in the presence of the complex are tenfold lower than those in using native peroxidase in aqueous solutions. It should be noted that, in the absence of chitosan, we could not develop a procedure for the determination of the most hardly oxidized peroxidase substrate, trifluoroperazine, in an aqueous–organic medium. The proposed procedure for the determination of promazine was applied to the analysis of organic extracts from venous blood plasma of humans with hexane–DMSO.

FLUORESCENCE SENSORS FOR THE DETERMINATION OF BIOLOGICALLY ACTIVE COMPOUNDS AND THEIR APPLICATION TO CHEMICAL ANALYSIS

Two types of new indicator systems for the determination of phenolic compounds and peroxides of dif-

ferent structures were proposed including the following processes: (1) interaction of the products of the enzymatic oxidation of phenols with chitosan, labeled by a fluorescent tag, accompanied by the reduction of fluorescence intensity; (2) peroxidase derivatization of catecholamines and their metabolites with aromatic diamines, phenylenediamine or ethylenediamine, with the formation of fluorescent derivatives [28].

A fluorescence sensor for the determination of biologically active compounds based on chitosan labeled by rhodamine B isothiocyanate. A widespread approach to the development of fluorescence sensors consists in the introduction of compounds possessing strong intrinsic fluorescence into their sensing layer followed by the determination of the components of the indicator system by the quenching of fluorescence [56]. The biosensitive layer in this case is a mixture of chitosan, labeled by a fluorescent tag (rhodamine B isothiocyanate), with peroxidase. In the enzymatic oxidation of phenolic compounds, the fluorescence intensity of the biosensitive layer decreases linearly with the analyte concentration.

Determination of phenolic compounds. The optimum working conditions of the sensor on the basis of peroxidase in a complex with chitosan labeled by rhodamine B isothiocyanate (concentrations of chitosan, fluorescent tag, enzyme, and hydrogen peroxide; film thickness; concentration and pH of the buffer solution; and excitation and emission wave lengths) were presented in [28]. Procedures for the determination of pyrocatechol, resorcinol, hydroquinone, dopamine, and adrenaline (Table 4) were developed using this system.

The response of the sensing layer of the sensor was additive at analyte concentrations belonging to the linearity ranges of the calibration graphs. As the sensitivity coefficients of the determination differed in values, the information obtained in the analysis of a sample simultaneously containing a phenol mixture allowed drawing a conclusion only about their total concentration in the sample.

Thus, the developed biosensors based on chitosan labeled with a fluorescent tag can be used for the analysis of samples containing only one phenolic compound (for example, pharmaceutical and cosmetic preparations), or for an assessment of the total concentration of phenolic compounds in the analyzed sample (for example, in industrial and sewage waters), and also in an analysis using an array of sensors based on different indicator reactions. The analysis of more complex mixtures (biological fluids) requires the development of more sensitive, selective, and stable indicator systems.

Determination of peroxides of different structures. The fluorescence response of a biosensitive layer based on peroxidase in a complex with chitosan labeled by rhodamine B isothiocyanate is proportional to the concentration of not only the reductant, a phenolic compound, but also of the oxidant, peroxide. Therefore its application to the determination of peroxides of different structures, including organic ones, seems promising. The reductant substrate was pyrocatechol (phenolic compound with the highest determination sensitivity coefficient) at the concentration 2.5 μM, corresponding to the midpoint of the linearity range. The performance characteristics of procedures for the determination of a number of peroxides are summarized in Table 4.

Table 4. Performance characteristics of the determination of dihydroxyphenol, catecholamines, and hydroperoxides in a water medium using a biosensor based on chitosan labeled by a fluorescent tag

Analyte	Linearity range, μM	c_{min}, μM	RSD (at c_{min}, $n = 4$, $P = 0.95$, %)
Phenolic compounds			
Pyrocatechol	0.5–5.0	0.10	4
Resorcinol	0.5–7.5	0.15	3
Hydroquinone	0.5–7.5	0.20	3
Dopamine	5.0–50.0	2.0	5
Adrenaline	5.0–50.0	1.5	6
Hydroperoxides			
Hydrogen peroxide	100–1000	45	8
Urea peroxide	25–250	10	8
2-Butanone peroxide	100–1000	40	8
Benzoyl peroxide	50–750	45	7
tert-Butyl hydroperoxide	250–25000	230	7

By the sensitivity and reproducibility of the results of determination of organic hydroperoxides, the described fluorescence biosensor is comparable with the known analogs, including the solid-phase spectrophotometric biosensor based on chitosan (being a little superior to the last one). A significant advantage of the solid-phase fluorescence biosensor in comparison with the spectrophotometric analog consists in the shorter duration of analysis at room temperature (10–15 min. instead of 24 h) [4, 28, 41].

To increase of the sensitivity of the determination of hydroperoxides, the indicator reaction was conducted in the medium of direct and inverse surfactant micelles, which should favor an increase of the activity of the enzyme because of a change of its conformation and the accessibility of the active site. In addition, in micellar media, the analytical signal might increase because of an increase in the solubility of poorly soluble analytes. The introduction of surfactant molecules into the fluorophore can be associated with an increase in fluorescence intensity as a result of the fluorophore inclusion into a micelle and the reduction of number of its rotation degrees of freedom, which reduces the percentage of nonradiative transitions. As

the effect of the biosensitive layer based on labeled chitosan is due to the quenching of its intrinsic fluorescence, the introduction of surfactant molecules can both increase and decrease the sensitivity of the determination (because of the existence of two competing processes in the system). Therefore, it is necessary to distinguish the contribution of each process to the formation of the analytical signal. For this reason, a possibility of using micellar media simultaneously for the fluorescence and spectrophotometric sensors described above was studied [28, 41].

It was found that carrying out the reaction in micellar media is accompanied by a significant increase in the intensity of the intrinsic fluorescence of the chitosan film (also in the blank experiment) in comparison with the nonmicellar system (by two times for micelles based on sodium dodecyl sulfate, **SDS**). The reproducibility of the results of measurements is this case was considerably worse (RSD ~ 20–30% at $n = 4$, $P = 0.95$). The performance characteristics of the procedures for the determination of peroxides in the medium of direct surfactant micelles using a spectrophotometric biosensor revealed the following regularities: the sensitivity of the determination of all peroxides, except for *tert*-butyl hydroperoxide, increased in the series media cetyltrimethylammonium bromide (CTAB) < nonmicellar medium < TWIN 80 < SDS, and the increase in sensitivity was regularly accompanied by the narrowing of the linearity range [41].

Therefore, the analytical characteristics of the fluorescence biosensor proposed by us on the basis of chitosan labeled by rhodamine B isothiocyanate are comparable with the characteristics of sensors described in the literature [57–60]. A considerable advantage of the proposed sensor is that the procedure of the formation and measurement of the analytical signal used in its development ensures the analysis of inhomogeneous and opaque media. However, the attained sensitivity and selectivity of the determination are insufficient for fulfilling some important analytical tasks, for example, the analysis of biological fluids and tissues for the concentration of catecholamines, such as dopamine, adrenaline, and their metabolites (homovanillic and vanillylmandelic acids).

A FLUORESCENCE SENSOR FOR THE DETERMINATION OF BIOLOGICALLY ACTIVE COMPOUNDS BASED ON THE INTERACTION OF THE PRODUCTS OF ENZYMATIC OXIDATION OF PHENOLIC COMPOUNDS WITH DERIVATIZING AGENTS

An approach based on measurements of the fluorescence of catecholamine derivatives seems promising for the determination of catecholamines (dopamine, adrenaline) and their metabolites (homovanillic and vanillylmandelic acids). The derivatizing agents ensuring the sensitive and selective determination of phenolic compounds by the fluorescence of their derivatives are aromatic and aliphatic amines.

The reaction of the derivatization of catecholamines and their metabolites by aromatic and aliphatic amines is carried out according to the following scheme: at the first stage, the analyte is oxidized to a corresponding quinone with an open chain, which then enters an intermolecular Michael addition reaction. In an alkaline medium, the obtained Michael adduct is instantly reduced to a hydroxyindol, which is then oxidized with the formation of a corresponding closed-chain *o*-quinone. The final oxidation product reacts with the derivatizing agents to form benzoxazole or quinoxaline derivatives. The identity of the products of the enzymatic and nonenzymatic derivatization was proved [61] by spectrophotometry and liquid chromatography–mass spectrometry on an example of the reaction of adrenaline with benzylamine.

Determination of phenolic compounds. In the development of fluorescence procedures for the determination of phenolic compounds in the sensor version, the derivatizing agents were *o*-phenylenediamine (*o*-PDA) and ethylenediamine (EDA), whose derivatizates fluoresce in the region 500–600 nm. The fluorescence intensity is proportional to the concentration of phenolic compounds. The spectra of fluorescence excitation are symmetrical, and the positions of the fluorescence maxima are independent on the wavelength of exciting radiation [28].

To obtain the maximum amount of the fluorescent adduct in the biosensitive layer, conditions of the formation of an analytical signal (concentrations of enzyme, hydrogen peroxide, and derivatizing agent; reaction time, pH and concentration of the buffer solution) were optimized. In using immobilized *o*-PDA, the model phenolic compounds were elementary isomeric dihydroxyphenols, i.e., pyrocatechol, catecholamines (dopamine and adrenaline), and their metabolites (homovanillic and vanillylmandelic acids). In using EDA, the researchers were limited by the development of procedures for the determination of pyrocatechol, dopamine, and adrenaline (Table 5).

The sensitivity and reproducibility of the results of measurements, and also the rapidity of the analysis significantly increased in going from signal measurement in solution to its measurement on the surface, and also in the replacement of EDA by *o*-PDA. Thus, a biosensor based on *o*-PDA ensures the determination of catecholamines and their metabolites at the nanomolar concentration level, which is sufficient for the determination of these compounds in biological fluids of humans. The time necessary for the achievement of the 90% value of the analytical signal did not exceed 30 s. The biosensor on the basis of EDA ensures the determination of phenolic compounds at the micromolar concentration level, and the duration of the reaction is from 2 to 15 min. The response of the sensing layer of biosensors based on *o*-PDA and EDA

Table 5. Performance characteristics of the determination of phenolic compounds using a fluorescence biosensor based on derivatization with *o*-phenylenediamine and ethylenediamine

Analyte	Linearity range, nM	c_{min}, nM	RSD (at c_{min}, $n = 5$, $P = 0.95$, %)
Derivatizing agent *o*-phenylenediamine			
Pyrocatechol	50–1000	20	3
Dopamine	50–500	18	3
Adrenaline	10–2500	5	4
Homovanillic acid	10–250	5	3
Vanillylmandelic acid	5–100	3	3
Derivatizing agent ethylenediamine			
Pyrocatechol	500–5000	180	4
Dopamine	250–5000	75	6
Adrenaline	250–1000	150	8

remains at the level of 90–95% of the initial value within two days at 25°C and for not less than 4 weeks at –20°C. In going from measurements of the analytical signal in solution to the use of biosensors, the linearity range narrows, which is indicative of the preconcentration of the products of the oxidation of phenolic compounds in the polymer film. As in the case of the solid-phase fluorescence biosensor based on labeled chitosan, described earlier, an increase in the volume of the reaction system was accompanied by a decrease of the sensitivity of determination and the expansion of the analytical range.

On an example of an analysis of mixtures simultaneously containing pairs dopamine–adrenaline, homovanillic acid–adrenaline, and also vanillylmandelic acid–adrenaline, it was found that, both in solution and in using a biosensor, adrenaline was much superior to the other model phenolic compounds in the rate of formation of its fluorescent derivatives in the reaction of derivatization with *o*-PDA, because of which the value of the analytical signal in the above systems was not additive. Therefore, the determination of dopamine and homovanillic and vanillylmandelic acids in the presence of adrenaline was possible only at their multiple (100- to 1000-fold) excess. In the simultaneous introduction of dopamine and homovanillic acid, dopamine and vanillylmandelic acid, and also homovanillic and vanillylmandelic acids into the reaction system, the response of the sensor was additive. It should be noted that, at pH 10 (optimum for determination of homovanillic acid), the rate of reaction of the enzymatic derivatization of vanillylmandelic acid with *o*-PDA is so low that even a double excess vanillylmandelic acid does not interfere with the determination of homovanillic acid. The concentration of homovanillic acid in biological fluids normally exceeds the concentration of vanillylmandelic acid. Therefore, taking into account a possible dilution of the analyzed sample, and also the required and reached sensitivity of the determination of catecholamines and their metabolites, the biosensor based on *o*-PDA can be used for the individual determination of homovanillic acid in blood and urine (at pH 10) and also for the simultaneous determination of homovanillic and vanillylmandelic acids in urine (at pH 9.5).

Thus, the fluorescence biosensor developed by us on the basis of the reaction of peroxidase derivatization with *o*-PDA is superior to the analogs described in the literature in the sensitivity of determination of phenolic compounds and comparable to them in the selectivity of the determination and reproducibility of the results of measurements, and also in the stability of the analytical signal [60, 61]. The attained sensitivity and selectivity of the determination, and also the short response time (30 c) ensure the use of the proposed sensor for the individual determination of catecholamines and their metabolites in pharmaceutical preparations and for the determination of homovanillic and vanillylmandelic acids in biological fluids.

Determination of peroxides. The biosensors described above were also used for the development of procedures for determination of peroxides of different structures. The derivatizing agent was *o*-PDA, because the biosensor on its basis had the best performance characteristics than the biosensor based on EDA. The performance characteristics of the determination of by

Table 6. Performance characteristics of the determination of peroxides in water and micellar media using a fluorescence biosensor based on derivatization with *o*-phenylenediamine

Analyte	Linearity range, μM	c_{min}, μM	RSD (at c_{min}, $n = 4$, $P = 0.95$, %)
Hydrogen peroxide (water)	25–500	10	3
Hydrogen peroxide (CTAB)	100–2500	80	15
Hydrogen peroxide (TWIN)	50–500	30	15
Urea peroxide	25–500	15	5
2-Butanone peroxide	100–1000	80	6
Benzoyl peroxide	50–500	30	7
tert-Butyl hydroperoxide	50–2500	40	13

the fluorescence biosensor on a basis *o*-PDA are summarized in Table 6.

It should be noted that the works [62–69] devoted to optical sensors for the determination of hydrogen peroxide and organic peroxides are rather scanty. The known optical sensors allow the determination of peroxides mainly at the micro- and millimole level, i.e., are comparable (in some cases superior [62, 63]) with spectrophotometric and fluorescence sensors on the basis of the {peroxidase–chitosan} polyelectrolyte complex in sensitivity. Note than only single optical sensors (according to the published data) were tested in the analysis of real samples: optically transparent media (rain water and solution for the disinfection of contact lenses) that did not require additional sample preparation [67, 68]. An important advantage of the registration of an analytical signal directly in the biorecognizing layer of sensors proposed by us is a possibility of analyzing colored solutions and also samples insoluble in aqueous and micellar media (solutions, suspensions of different density and polarity) without preliminary sample preparation.

USE OF FLUORESCENCE BIOSENSORS IN THE ANALYSIS OF REAL SAMPLES

The fluorescence biosensors based on fluorescence quenching (labeled with chitosan) and the enhancement of the signal (derivatization with *o*-PDA) and also the spectrophotometric system based on the complex {peroxidase–chitosan} were tested in the determination of phenolic compounds (hydroquinone, dopamine, adrenaline) and organic peroxides (benzoyl peroxide, urea peroxide) in the composition of cosmetic and medicinal preparations of different nature (soluble and insoluble in water). It is necessary to emphasize that sample preparation was limited to the homogenization (water-insoluble preparations) or dilution (injection solutions) of the analyzed sample in water. The results of the determination of phenolic compounds in pharmaceutical preparations well agreed with the concentrations specified by the producer (Table 7).

The fluorescence biosensor based on *o*-PDA was applied to the determination of the products of the metabolism of catecholamines (homovanillic and vanillylmandelic acids) in urine. The analysis was performed by the method of standard additions under the conditions optimum for the determination of homovanillic acid (its selective determination) and vanillylmandelic acid (determination of the total concentration of homovanillic and vanillylmandelic acids). The results of the determination of homovanillic acid well agreed with the data obtained in the determination of this compound by HPLC with amperometric detection [28].

* * *

Therefore, our research into the development and sophistication of the available optical (spectrophotometric and fluorescence) sensors allowed us to propose original approaches to the immobilization of the peroxidase enzyme widely used in analysis, which ensure its activity and stability on storage and in the course of catalytic processes under the conditions and in the media unfavorable for proteins. New types of design and devices for registering an analytical signal of a solid phase (biosensitive layer) were developed on the basis of universal commercial spectrophotometers and fluorimeters. New indicator systems for the determination of phenolic compounds, i.e., phenothiazine, catecholamines, and their metabolites; peroxides of different structures were proposed for the quality control of drugs, biomedical research, and clinical diagnostics. An advantage of the developed optical sensors is the simplicity of their production and operation. The sensitivity, selectivity and rapidity of the proposed

Table 7. Determination of hydroquinone, dopamine, adrenaline, benzoyl peroxide, and urea peroxide in pharmaceutical and medicinal preparations in a (I) water medium and in the (II) medium of direct surfactant micelles)

Type of biosensor	Pharmaceutical preparation	Analyte	Found, %		Claimed by the manufacturer, %
			I	II	
Spectrophotometric sensor	Achromin	Hydroquinone	2.1 ± 0.2	–	1.9
	Basiron	Benzoyl peroxide	4.8 ± 0.7	4.8 ± 0.7	5.0
	Peroxyderm		2.8 ± 0.4	2.5 ± 0.3	2.5
	Splat Exreem White	Urea peroxide	0.09 ± 0.01	0.09 ± 0.01	0.1
Fluorescence sensor based on chitosan with a fluorescent tag	Achromin	Hydroquinone	2.1 ± 0.2	–	1.9
	Dopamine – Ferrein	Dopamine	0.53 ± 0.04		0.5
	Adrenaline hydrochloride	Adrenaline	0.1 ± 0.01		0.1
	Xylocaine – Adrenaline		$(5.4 \pm 0.6) \times 10^{-4}$		5×10^{-4}
	Basiron	Benzoyl peroxide	4.7 ± 0.6		5.0
	Peroxyderm		2.5 ± 0.4		2.5
	Splat Exreem White	Urea peroxide	0.11 ± 0.02		0.1
Fluorescence sensor based on derivatization with *o*-PDA	Dopamine – Ferrein	Dopamine	0.54 ± 0.07		0.5
	Adrenaline hydrochloride	Adrenaline	0.11 ± 0.02		0.1
	Xylocaine – Adrenaline		$(4.9 \pm 0.6) \times 10^{-4}$		5×10^{-4}
	Basiron	Benzoyl peroxide	4.7 ± 0.4		5.0
	Peroxyderm		2.4 ± 0.4		2.5
	Splat Exreem White	Urea peroxide	0.11 ± 0.01		0.1

devices are sufficient for the determination of the above analytes in pharmaceutical products, and, in some cases, in biological fluids of humans. The formation and measurement of an analytical signal outside the solution, directly in the sensing layer of a sensor, ensures the analysis of opaque and turbid media and, therefore, samples based on matrixes of complex composition without additional sample preparation. The last advantage favorably distinguishes the proposed biosensors from the analogs described in the literature based on the formation and measurement of an analytical signal in solutions.

ACKNOWLEDGMENTS

We are grateful to A.V. Kireiko, P.V. Rodionov, and L.I. Malinina for participation in the problem formulation, performance of experiments, and discussion of the results of studies.

This work was supported by the Russian Science Foundation, project no. 14-23-00012.

REFERENCES

1. Zakharova, G.S., Uporov, V.I., and Tishkov, V.I., *Usp. Biol. Khim.*, 2011, vol. 51, p. 37.
2. That T. Ngo, *Anal. Lett.*, 2010, vol. 43, nos. 10–11, p. 1572.
3. Shivakumar, A., *Int. J. Clin. Nutr. Diet*, 2017, vol. 3, no. 2, p. 1.
4. Veselova, I.A., Malinina, L.I., Rodionov, P.V., and Shekhovtsova, T.N., *Talanta*, 2012, vol. 102, p. 101.
5. *Enzyme Stabilization and Immobilization. Methods and Protocols*, Minteer, Sh.D., Ed., Ser.: *Methods in Molecular Biology*, vol. 679, Heidelberg: Springer, 2010.
6. Veselova, I.A., Kireiko, A.V., and Shekhovtsova, T.N., *Appl. Biochem. Microbiol.*, 2009, vol. 45, no. 2, p. 125.
7. Klibanov, A.M., *Nature*, 2001, vol. 409, p. 241.
8. Martinek, K., Levashov, A.V., Khmelnitsky, Y.L., Klyachko, N.L., and Berezin, I.V., *Science*, 1982, vol. 218, p. 889.
9. Ramirez-Corredores, M. and Borole, A., *Biocatalysis in Oil Refining*, London: Elsevier, 2007.

10. Fitzpatrick, P.A., Steinmetz, A.C.U., Ringe, D., and Klibanov, A.M., *Proc. Natl. Acad. Sci. U. S. A.*, 1993, vol. 90, p. 8653.
11. Yennawar, H.P., Yennawar, N.H., and Farber, G.K., *J. Am. Chem. Soc.*, 1995, vol. 117, p. 577.
12. Mozhaev, V.V., Poltevsky, K.G., Slepnev, V.I., Badun, G.A., and Levashov, A.V., *FEBS Lett.*, 1991, vol. 292, p. 159.
13. Wu, X.J., Choi, M.M.F., and Wu, X.M., *Analyst*, 2004, vol. 129, p. 1143.
14. Khmelnitsky, Yu.L., Mozhaev, V.V., Belova, A.B., Sergeeva, M.V., and Martinek, K., *Eur. J. Biochem.*, 1991, vol. 198, p. 31.
15. Zaks, A. and Klibanov, A.M., *J. Biol. Chem.*, 1988, vol. 263, p. 3194.
16. Vakurov, A.V., Gladilin, A.K., Levashov, A.V., and Khmelnitsky, Y.L., *Biotechnol. Lett.*, 1994, vol. 16, p. 175.
17. Dordick, J.S., *Biotechnol. Prog.*, 1992, vol. 8, p. 259.
18. Sears, P., Schuster, M., Wang, P., Witte, K., and Wong, C.-H., *J. Am. Chem. Soc.*, 1994, vol. 116, p. 6521.
19. Khmelnitsky, Yu.L., Belova, A.B., Levashov, A.V., and Mozhaev, V.V., *FEBS Lett.*, 1991, vol. 284, p. 267.
20. Kudryashova, E.V., Artemova, T.M., Vinogradov, A.A., Gladilin, A.K., Mozhaev, V.V., and Levashov, A.V., *Protein Eng.*, 2003, vol. 16, p. 303.
21. Stepankova, V., Bidmanova, S., Koudelakova, T., Prokop, Z., and Chaloupkova, R., *ACS Catal.*, 2013, vol. 3, no. 12, p. 2823.
22. Rich, J.O., Mozhaev, V.V., Dordick, J.S., Clark, D.S., and Khmelnitsky, Yu.L., *J. Am. Chem. Soc.*, 2002, vol. 124, p. 5254.
23. Lukacheva, L.V., Zakemovskaya, A.A., Karyakina, E.E., Zorov, I.N., Sinitsyn, A.P., Sukhacheva, M.V., Netrusov, A.I., and Karyakin, A.A., *J. Anal. Chem.*, 2007, vol. 62, no. 4, p. 388.
24. Karyakin, A.A., Kotel'nikova, E.A., Lukacheva, L.V., and Karyakina, E.E., *Anal. Chem.*, 2002, no. 74, p. 1593.
25. Kazakova, L.I., Shabarchina, L.I., Anastasova, S., Pavlov, A.M., Vadgama, P., Skirtach, A.G., and Sukhorukov, G.B., *Anal. Bioanal. Chem.*, 2013, vol. 405, p. 1559.
26. Chaniotakis, N.A., *Anal. Bioanal. Chem.*, 2004, vol. 378, p. 89.
27. Veselova, I.A. and Shekhovtsova, T.N., *Anal. Chim. Acta*, 1999, vol. 392, p. 151.
28. Byeon, J.H., *Sci. Rep.*, 2016, vol. 6, 27006.
29. Rodionov, P.V., Veselova, I.A., and Shekhovtsova, T.N., *Anal. Bioanal. Chem.*, 2014, vol. 406, p. 1531.
30. Zikakis, J.P., *Chitin, Chitosan and Related Enzymes*, Orlando: Academic, 1984.
31. *Stability and Stabilization of Biocatalysts*, Balesteros, A., Plou, F.J., Iborra, J.L., and Holling, P.I., Eds., Ser.: *Progress of Biotechnology*, vol. 15, Amsterdam: Elsevier, 1998.
32. Decher, G., *Science*, 1997, vol. 277, p. 1232.
33. *Chitosan for Biomaterials*, Jayakumar, R., Prabaharan, M., and Muzzarelly, R., Eds., Ser.: *Advances in Polymer Science*, vol. 243, Heidelberg: Springer, 2011.
34. Khmelnitsky, Yu.L., Levashov, A.V., Klyachko, N.L., and Martinek, K., *Enzyme Microb. Technol.*, 1988, vol. 10, p. 710.
35. Halling, P.J., *Enzyme Microb. Technol.*, 1994, vol. 16, p. 178.
36. Levitsky, V., Lozano, P., Gladilin, A., and Iborra, J.L., *Prog. Biotechnol.*, 1998, vol. 5, p. 417.
37. Mozhaev, V.V., Kudryashova, E.V., Efremova, N.V., and Topchieva, I.N., *Biotechnol. Tech.*, 1996, vol. 10, p. 849.
38. Kudryashova, E.V., Gladilin, A.K., Vakurov, A.V., Heitz, F., Levashov, A.V., and Mozhaev, V.V., *Biotechnol. Bioeng.*, 1997, vol. 55, p. 267.
39. Shipovskov, S. and Levashov, A., *Biotechnol. Bioeng.*, 2003, vol. 84, p. 258.
40. Xu, K., Griebenov, K., and Klibanov, A.M., *Biotechnol. Bioeng.*, 1997, vol. 56, p. 485.
41. Griebenov, K. and Klibanov, A.M., *Biotechnol. Bioeng.*, 1997, vol. 53, p. 351.
42. Oleinik, L.I., Veselova, I.A., Rodionov, P.V., Budashov, I.A., and Shekhovtsova, T.N., *Zavod. Lab., Diagn. Mater.*, 2011, vol. 77, no. 4, p. 23.
43. Payne, G.F., Chaubal, M.V., and Barbari, T.A., *Polymer*, 1996, vol. 37, p. 4643.
44. Kumar, G., Bristow, J.F., Smith, P.J., and Payne, G.F., *Polymer*, 2000, vol. 41, p. 2157.
45. Abdullah, J., Ahmad, M., Karuppiah, N., Heng, L.Y., and Sidek, H., *Talanta*, 2006, vol. 40, p. 527.
46. Erdem, A., Pabuccuoglu, A., Meric, B., Kerman, K., and Osnoz, M., *Turk. J. Med. Sci.*, 2000, vol. 30, p. 349.
47. Rodionov, P.V., Alieva, E.A., Sergeeva, E.A., Pavlova, M.E., Veselova, I.A., and Shekhovtsova, T.N., *J. Anal. Chem.*, 2016, vol.71, no. 9, p. 932.
48. Rogozhina, T.V. and Rogozhin, V.V., *Issledovano Rossii*, 2002, p. 767.
49. Madej, K., Kala, M., and Wozniakievicz, M., *Chromatographia*, 2005, vol. 62, p. 533.
50. Pola, A., Michalak, K., Burliga, A., Motohashi, N., and Kawase, M., *Eur. J. Pharm. Sci.*, 2004, vol. 21, p. 421.
51. Gordeliy, V.I., Kiselev, M.A., Pole, A.V., and Teixeira, J., *Biophys. J.*, 1998, vol. 75, p. 2343.
52. Escribano, J., García-Cánovas, F., García-Carmona, F., and Losano, J.A., *Biochim. Biophys. Acta*, 1985, vol. 831, p. 313.
53. *Phenothiazines: Advances in Research and Application*, Ashton Action, Q.J., Ed., Atlanta: Schlolarly Edition, 2012.
54. Yamini, Y. and Faraji, M., *J. Pharm. Anal.*, 2014, vol. 4, p. 279.
55. Nakano, M., Sugoika, K., Nakano, H., Takyu, C., and Inaba, H., *Biochem. Biophys. Res. Commun.*, 1985, vol. 3, p. 952.
56. Kelder, P.P., de Mol, N.J., Fischer, M.J.E., and Janssen, L.H.M., *Biochim. Biophys. Acta*, 1994, vol. 1205, p. 230.

57. Ma, O., Lavertu, M., Sun, J., Nguyen, S., Buschmann, M.D., Winnik, F.M., and Hoemann, C.D., *Carbohydr. Res.*, 2008, vol. 72, p. 616.
58. Drexhage, K.H., *J. Res.*, 1976, vol. 80, p. 421.
59. Park, S.A., Jang, E., Koh, W.G., and Kim, B., *Sens. Actuators, B*, 2010, vol. 150, p. 120.
60. Rodionov, P.V., Veselova, I.A., and Shekhovtsova, T.N., *J. Anal. Chem.*, 2013, vol. 68, no. 11, p. 931.
61. Veselova, I.A., Malinina, L.I., Barsukova, M.E., Tokareva, A.I., Buslova, T.S., Pirogov, A.V., and Shekhovtsova, T.N., *Talanta*, 2017, vol. 171, p. 108.
62. Demiyanova, A.S. and Sakharov, I.Yu., *Analyst*, 2015, vol. 140, p. 2964.
63. Vdovenko, M.M., Demiyanova, A.S., Kopylov, K.E., and Sakharov, I.Yu., *Talanta*, 2014, vol. 125, p. 361.
64. Sakuragawa, A., Taniai, T., and Okutani, T., *Anal. Chim. Acta*, 1998, vol. 374, p. 191.
65. Rubtsova, M.Y., Kovba, G.V., and Egorov, A.M., *Biosens. Bioelectron.*, 1998, vol. 12, p. 75.
66. Collaudin, A.B. and Blum, L.J., *Sens. Actuators, B*, 1997, vols. 38–39, p. 189.
67. Porsh, H.E. and Wolfbeis, O.S., *Microchim. Acta*, 1989, vol. 1, p. 41.
68. Lobnik, A. and Cajlakovic, M., *Sens. Actuators, B*, 2001, vol. 74, p. 194.
69. Hanko, M., Bruns, N., Tiller, J.C., and Heinze, J., *Anal. Bioanal. Chem.*, 2006, vol. 386, p. 1273.

Translated by E. Rykova

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