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Biosensors Based on a Light-Addressable Potentiometric Sensor (LAPS) for Analysis in Both Aqueous Solutions and Organic Solvents

Elena G. Kondrat'eva,*[,1] Tatiana A. Osipova,[2] Vladimir I. Sklyar,[2] and Anatolyi N. Reshetilov[3]

[1]Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr.33, Moscow, 117071, Russia, E-mail: kondr@enzyme.chem.msu.su; [2]Department of Chemistry, Moscow State University, Moscow, 119899, Russia; and [3]Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Puschino, Moscow oblast, 142292, Russia

Abstract

Characteristics of a light-addressable potentiometric sensor (LAPS) based on silicon with Ta_2O_5 dielectric are reported. The pH sensitivity obtained is 45 mV/pH unit in the pH range from 4.01 to 7.5 (for measurements conducted in citrate buffer solutions) and 55 mV/pH unit over the pH range from 7.5 to 8.9 (for measurements conducted in Tris-HCl buffer solutions). The kinetic characteristics (K_m, V_m, pH-profile) of different enzymes, i.e., glucose oxidase, α-chymotrypsin, butyrylcholinesterase, and urease, have been measured under homogeneous conditions. The values of the Michaelis constant obtained are very close to analogous data described in the literature. Biosensors were prepared by immobilization of the enzymes on a pH-sensitive matrix. Two methods of immobilization were used: incorporation into a hydrophilic matrix of bovine serum albumin (BSA) and incorporation into a hydrophobic matrix of modified polyethylenimine (PEI). It is demonstrated that LAPS can be used for recording the enzymatic reactions in organic media (mixture of the solvents).

Index Entries: Light-addressable potentiometric sensor (LAPS); biosensors; immobilization; polymer matrix.

Introduction

The light-addressable potentiometric sensor has been recently introduced into biotechnological practice. The LAPS-based biosensors for detecting glucose *(1,2)*, urea *(3)*, pesticides *(4)*, and DNA *(5)* are described

*Author to whom all correspondence and reprint requests should be addressed.

in the literature. LAPS is a pH-sensitive electrode with enhanced signal stability and high chemical sensitivity to protons. This allows pH changes induced by enzymatic reactions to be detected with sufficient accuracy both in homogeneous media *(6,7)* and in immobilized matrices on LAPS surface (*8*).

Our survey of the literature showed that none of the currently available biosensors combine a semiconductor transducer with enzyme-containing polymer membranes for detecting substances in organic media. However, in addition to stabilization of enzymatic activity, a hydrophobic enzyme-containing polymer matrix formed on the surface of a sensor also allows organic substances with poor solubility in aqueous solutions to be concentrated within the hydrophobic matrix.

The goal of this work was to examine the chemical and pH sensitivity of silicon sensor that contains Ta_2O_5 as a pH-sensitive dielectric and to develop a technique for potentiometric assay of different class enzymes (proteolysis and redox). The enzymes were tested both in the native state and after immobilization in hydrophilic or hydrophobic matrices. The study of the enzymes immobilized in aprotonic matrices provides an opportunity for the LAPS-mediated monitoring of enzymatic activity in nonelectrolyte media and for assessing the effects of a hydrophobic environment on enzymatic activity.

Materials and Methods

Glucose oxidase from *Penicillium vitale* (Bakpreparat, Ukraine; activity of 92–96 thousand U/g), α-chymotrypsin, butyrylcholinesterase, and urease (Sigma) were used. Enzyme substrates: β-D-glucose (Reakhim, Russia), *N*-acetyl-L-tyrosine ethyl ester-ATEE (Reanal, Hungary), acetylcholine iodide (ACh), and urea (Sigma) were used. Salts for preparing buffer solutions were of analytical grade. pH of solutions were measured in independent experiments, with the aid of an ion meter I-120.1, which was preliminary calibrated using standard buffer solutions (Radiometer, Denmark). Kinetic measurements were performed at 20°C in a stirred cell. An Ag/AgCl electrode was used as a reference electrode. A bias potential ranging from 0 to 3 V was applied to the reference electrode to bring the working point of the voltage–current characteristic of LAPS to its linear portion. During kinetic measurements, the bias potential was maintained at a constant level. The biochemical reaction of interest induces a change in the pH value of the medium and corresponding change in the photoelectric current, which is an output signal of the biosensor. The output signal was amplified and recorded by a recording amplifier. In this work we utilized sensors prepared from *n*-type silicon slices of the (100) crystallographic orientation. LAPS schematics and operation were demonstrated in detail in ref. *9*. Kinetic constants, K_m and V_m, were calculated from double-reciprocal plots by standard methods.

Bovine serum albumin (BSA) and glutaric aldehyde used in polymer matrices to immobilize enzymes were from Serva (Germany) and Merck

(Germany), respectively. Polyethylenimine (PEI) was consecutively modified with cetyl bromide and ethyl bromide as described in ref. *10*. For immobilization, glucose oxidase and BSA were separately (i.e., in different beakers) dissolved in 1 m*M* Tris-HCl buffer (pH 7.2) containing 0.2 *M* NaCl to final concentrations of 12 and 90 mg/mL, respectively. The enzyme and BSA solutions were then mixed together, and 8% solution of glutaric aldehyde was added to the resulting mixture immediately before immobilization at a volume ratio of 1:6 (relative to the total volume of the mixture). A 5-μL droplet of the mixture was applied to the sensitive element of the pH electrode. At room temperature, gel polymerization was completed within 1 h. The electrode was soaked in the buffer solution for 2 h before use.

Enzymes were also immobilized in reversed polymer micelles of modified PEI. A required amount of enzyme (1.1 m*M* for glucose oxidase and 0.16 m*M* for α-chymotrypsin, respectively) was dissolved in a buffer solution and added to 1 mL of toluene-*n*-butanol mixture (2:1 by volume) containing modified PEI (5 mg). The resulting mixture was thoroughly stirred for 10–30 s until a homogeneous solution was obtained. Two microliters of this solution of reversed polymer micelles and enzyme solution in buffer were applied to the sensitive element of the pH electrode. Under atmospheric air at room temperature, polymerization was complete within 10–15 min. The polymer membrane obtained was coated with a protective cover of 0.1% triacetate cellulose (8 μL of chloroform solution). The electrode was allowed to dry at room temperature for 10–15 min. The electrode was soaked in a buffer solution for 2 h before use. The amount of the enzyme immobilized in the whole volume of the polymer matrix was equal to the amount of enzyme added to the measuring cell in measurements in homogeneous media.

Operating stability was defined as the number of records of the same concentration of substrate that did not change the initial activity of the enzyme.

Results and Discussion

Chemical Sensitivity of Silicon Sensors with Ta_2O_5 Dielectric

The LAPS systems described to date are based on sensors containing Si_3N_4 as the pH-sensitive dielectric *(7,11,12)*. The authors thought it important to investigate the pH sensitivity of LAPS transducers coated with a Ta_2O_5 dielectric. It is a known fact that Ta_2O_5 is highly selective with respect to protons. It is widely used as a gate dielectric in pH-sensitive field-effect transistors owing to a small hysteresis of this dielectric *(13,14)*.

We examined the pH sensitivity of the sensors at pH from 4.1 to 9.0. Solutions with pH 4.1–7.5 were based on 0.1 *M* citrate buffer, while those with pH 7.5–9.0 utilized 0.1 Tris-HCl buffer. The pH sensitivity, α_{pH}, was estimated as the ratio of the change in the bias to the change in solution pH: $\alpha_{pH} = \Delta U_b/\Delta pH$. The change in the bias was determined for the point in current-voltage curves (CVC), $U_{b50\%}$, which corresponded to 50% of the saturation current.

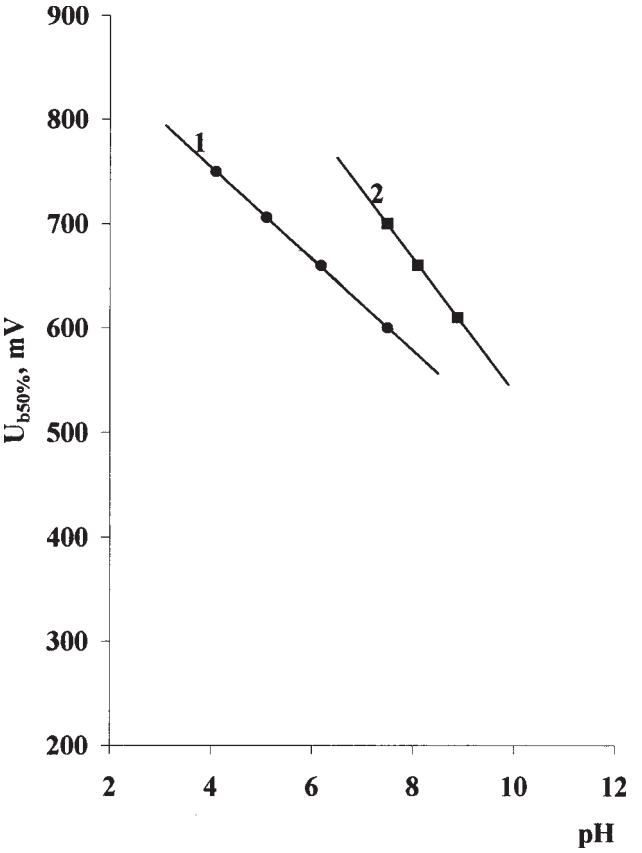

Fig. 1. The dependence of $U_{b50\%}$ on pH values of: (1) citrate buffer and (2) Tris-HCl buffer (pH-sensitivity of the sensor).

The dependence of $U_{b50\%}$ on pH of buffer solutions is shown in Fig. 1. As can be seen, in the citrate buffers with pH 4.1–7.5, the pH sensitivity is virtually constant and is equal to 45 mV/pH unit. In the Tris-HCl buffers with pH 7.5–9.0, the pH sensitivity is also constant and is equal to 55 mV/pH unit.

Note that switching from citrate buffers to Tris-HCl buffers disrupts the pH dependence of $U_{b50\%}$ and makes it shift in the direction of higher values of $U_{b50\%}$. To our minds, this could be caused by nonspecific chemical sensitivity of the system.

Dependence of LAPS Signals on Ionic Strength and Ionic Composition of Electrolytes

Treating the device that we developed as the basis for future biosensors, we have explored the dependence of the sensor readings on ionic strength and the ionic composition of environment. This problem is important because the biochemical reactions and introduction of probes not only can bring about changes in pH, but also in the ionic composition of the environment in the receptor of the biosensor.

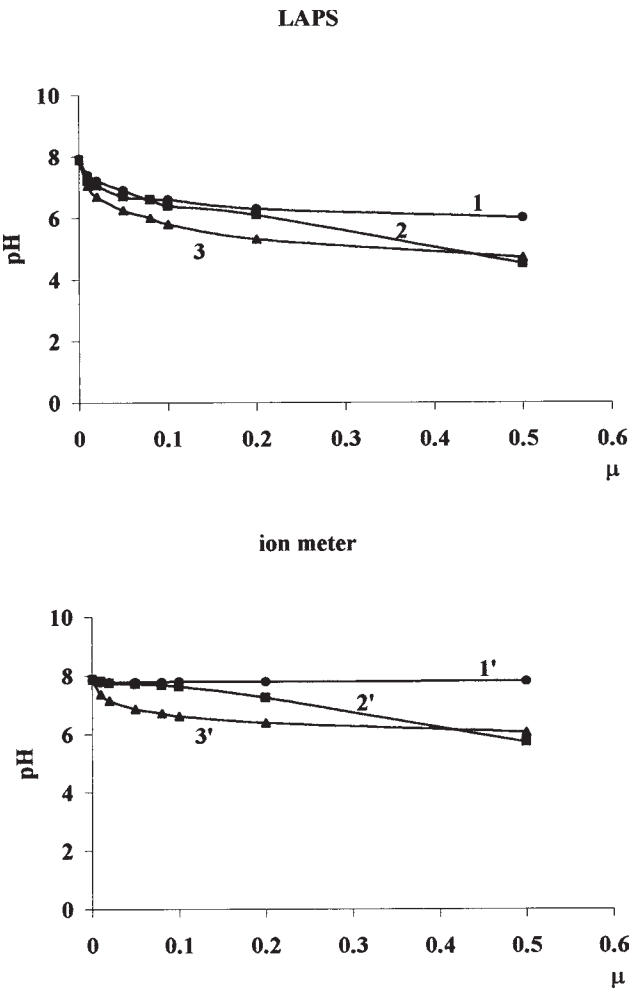

Fig. 2. Dependence of pH values on the ionic strength, μ, of univalent cations K^+, Na^+, and NH_4^+ as measured with the aid of: (1–3) LAPS and (1'–3') an ion meter. The notation used: (1,1') K^+, (2,2') Na^+, and (3,3') NH_4^+.

In order to perform the estimation, on the basis of 1 m*M* solutions of Tris-HCl buffer (pH 8.0), we prepared solutions of various salts (NaCl, KCl, NH_4Cl) with the ion concentration increasing from 1 to 500 m*M*. The pH values of each solution were measured with the aid of LAPS and standard ion meter of the I-120 type. The results show (Fig. 2) that the effective values of pH obtained with the aid of LAPS diminish as the ionic strength grows. Moreover, these values depend on the anion type: they decrease in the series K^+, Na^+, and NH_4^+. A comparative analysis of these data and the dependencies obtained with the aid of an ion meter show that pH values did decrease in the case of NaCl and NH_4Cl solutions. This means that LAPS was instrumental in a qualitatively correct estimation of the pH magnitude, and no influence of the Na^+ and NH_4^+ cations on the LAPS

signal was observed. From the pH dependencies for KCl solutions one can see that LAPS records a decrease in the effective value of pH, provided the pH value measured with I-120 does not change as the salt concentration grows. This fact is apparently attributable to nonspecificity of the measurement.

This sensor with a Ta_2O_5 dielectric was employed for measuring and comparing kinetic characteristics of native enzymes to the characteristics of the enzymes immobilized in polymer matrices. The procedure of the LAPS measurement of the catalytic activity of enzymes was developed and described in Materials and Methods and in ref. *15*.

Evaluation of Rate Constants in Homogeneous Media and of Immobilized Enzyme

The dependence of the initial reaction rate V_0 on the substrate concentration (S_0) in native enzymes is of typical hyperbolic shape. Therefore, this reaction fits the Michaelis–Menten equation (Fig. 3). The kinetic constants of native enzymes listed in Table 1 are in agreement with the data obtained using other methods *(16–18)*.

Enzyme immobilization in a BSA gel with glutaric aldehyde is quite a common method. In this work we also employed this method for enzyme immobilization. Kinetic parameters of immobilized glucose oxidase showed that immobilization caused an approximately threefold decrease in the enzymatic activity (Fig. 4). However, the stability of the immobilized membrane preparations was rather high: about 90% of the initial activity of the immobilized enzyme was retained after two weeks of storage (Fig. 5). The results of our experiments showed that hydrolytic enzymes induced proteolysis of the BSA matrix in some cases (α-chymotrypsin, for example).

Since the method of immobilization described above failed to solve the problem, we studied theoretically a number of polymers of different structure that have been used by various researchers to immobilize enzymes. In our opinion, enzyme incorporation into reversed micelles is promising. This method was suggested and described in detail in ref. *10*. A polymer matrix of modified PEI was chosen due to the ability of the polymer to produce reversed micelles and entrap aqueous solutions of enzymes of high catalytic activity inside the micelles and the capacity of polymer micelles to retain their structure in both aqueous solutions and organic solvents.

The results of a comparative study of native enzymes and enzymes immobilized in a membrane of modified PEI are shown in Fig. 4 for glucose oxidase and Table 1 for other enzymes. The K_m and V_m values of the glucose oxidation reaction catalyzed by immobilized glucose oxidase were 6 m*M* and 13.5 m*M*/min, respectively. The same kinetic parameters of immobilized α-chymotrypsin were 3 m*M* and 10 m*M*/min, respectively. Kinetic characteristics of immobilized enzymes were measured by the same method as in homogeneous solution.

The operating stability of immobilized enzymes was comparatively high: 25 or 20 reaction cycles (without any change in the initial enzymatic

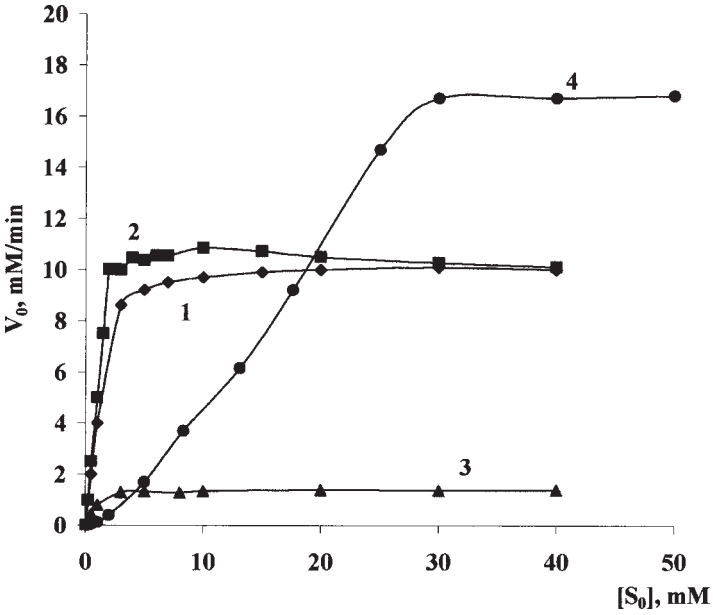

Fig. 3. Dependence of the initial reaction rate V_0 on substrate concentration (S_0) measured with LAPS in a homogeneous medium: (1) glucose oxidation catalyzed by glucose oxidase (pH 6.9, 20°C); (2) hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) catalyzed by α-chymotrypsin (pH 7.5, 20°C); hydrolysis of acetylcholine iodide (ACh) catalyzed by butyrylcholinesterase (pH 8.0, 20°C); hydrolysis of urea catalyzed by urease (pH 7.0, 20°C).

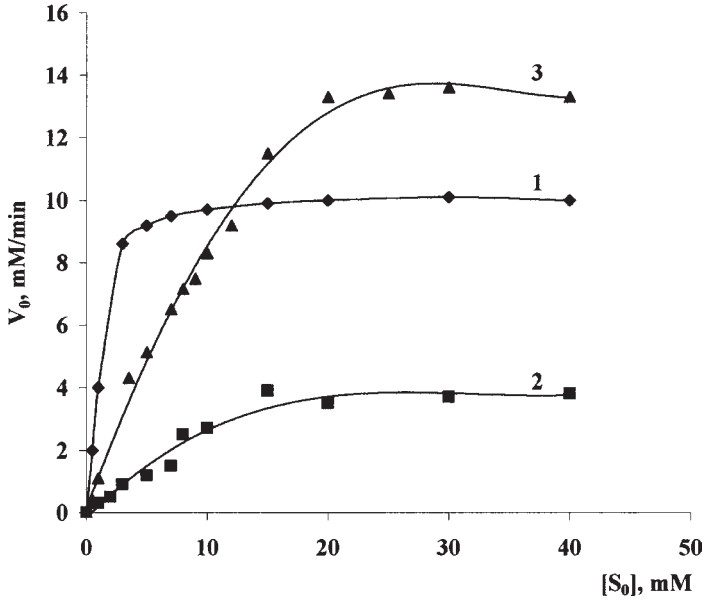

Fig. 4. Dependence of the initial rate of glucose oxidation in presence of glucose oxidase on the initial glucose concentration (S_0) (pH 7.0, 20°C): (1) native enzyme; (2) enzyme immobilized in a matrix of BSA and glutaric aldehyde; (3) enzyme immobilized in a matrix of modified PEI.

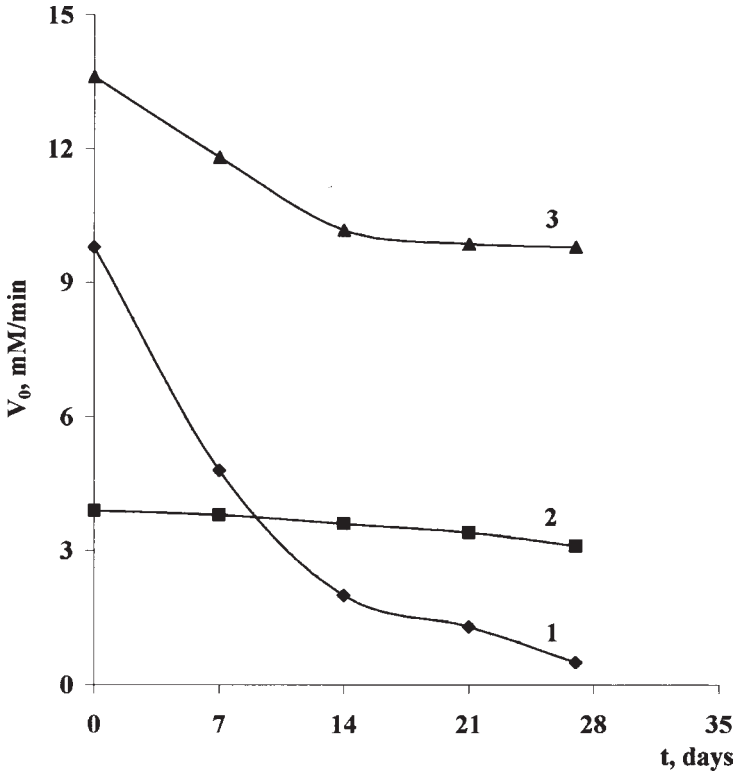

Fig. 5. Dependence of the initial rate of the glucose oxidation on time of storage of glucose oxidase in the buffer (pH 7.0, 20°C): (1) native enzyme; (2) enzyme immobilized in a matrix of BSA; (3) enzyme immobilized in a matrix of modified PEI.

Table 1
Kinetic Parameters of Native and Immobilized Enzymes as Measured with the Aid of LAPS

[E]	K_m, m*M*	V_m, m*M*/min	V_m/K_m, min^{-1}	pH-optimum	Interval pH
GOD (native enzyme)	1.0	10.0	10.0	6.5	5.0–9.0
GOD (immobilized in BSA)	8.0	2.8	0.4	7.2	4.0–8.5
GOD (immobilized in CE-PEI)	6.0	14.0	2.3	7.0	4.0–9.0
α-Chymotrypsin (native enzyme)	0.55	11.0	20.0	7.5	6.0–8.5
α-Chymotrypsin (immobilized in CE-PEI)	3.1	11.0	3.6	7.7	4.0–9.0
Butyrylcholin-esterase	0.95	1.3	1.4	8.0	5.9–10
Urease	14.0	16.0	1.1	7.0	4.0–9.0

activity) for glucose oxidase or α-chymotrypsin, respectively. If the number of the reaction cycles exceeded the threshold level of the operating stability, the activity of the immobilized enzymes decreased exponentially. The activity of the immobilized enzymes was shown to recover to the initial level after one day of incubation of the enzyme-containing sensor in a buffer solution. In our opinion, such a reversible inactivation of an immobilized enzyme can be caused by accumulation of excessive reaction products in the buffer solution adjacent to the enzyme-containing matrix as a result of reaction cycling.

The minimal concentrations of analyte substrates detectable by immobilized enzymes were 0.5 and 0.25 m*M* for glucose and ATEE, respectively. The activity of the enzymes immobilized in polymer membranes and stored for two weeks in a buffer solution at room temperature was 80 and 70% of the initial level for glucose oxidase and α-chymotrypsin, respectively (Fig. 5).

The pH dependence of the activity of native and immobilized enzymes showed that incorporation into a PEI matrix slightly broadened the pH-profiles of the immobilized enzymes. This can be explained by conservation of optimal pH inside the membrane micelles *(10)*.

Our experiments demonstrated definite advantages of biosensors based on modified PEI. This method of immobilization of enzymes in a matrix of modified PEI meets several requirements simultaneously: it conserves a high catalytic activity of enzyme and provides the enzyme stabilization in a water-insoluble polymer matrix, which allows monitoring of both aqueous and nonaqueous media.

The mixture of toluene and *n*-butanol (2:1) was selected as organic media for recording the enzymatic reactions. Ions $LiClO_4$ (50 m*M*) was used for conductivity in the organic media. α-Chymotrypsin was used in this experiment as solution-reversed polymer micelles of modified PEI, containing solution of enzyme in buffer. The substrate concentration (ATEE) was 5 m*M* in organic mixture. The initial rate hydrolysis of ATEE was 10 m*M*/min, and it was equal to the initial rate of hydrolysis of ATEE in aqueous buffer solution by immobilized enzyme in a matrix of modified PEI. This experiment shows for the first time the potential of LAPS for analysis of organic substances in nonaqueous media.

Conclusions

The results of this work demonstrate the possibility of the combined use of LAPS and enzyme-containing polymer matrices for measuring kinetic characteristics of enzymatic reactions in both aqueous solutions and organic solvents. Using the example of two enzyme-containing matrices of different chemical structure (hydrophilic or hydrophobic), a comparative study of the biosensor parameters, conditions of use, and characteristics of enzymes immobilized in aprotonic matrices was performed. The effect of the hydrophobic environment on enzymatic activity was also studied.

Moreover, our experiments show that the proposed method of evaluation of kinetic characteristics of enzymatic reactions in homogeneous media allows LAPS to be used in addition to the common methods (Clark electrode, spectrophotometry, etc.) as an analytical device in combination with specific classes of enzymes.

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