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Amperometric Biosensor System for Simultaneous Determination of Adenosine-5′-Triphosphate and Glucose

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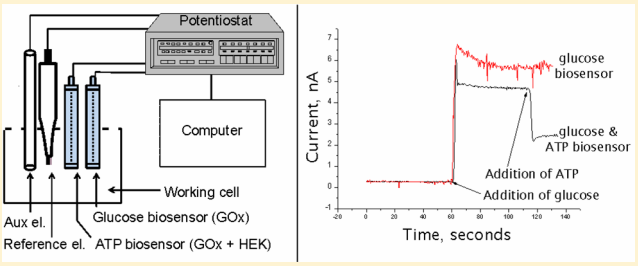
Supporting Information

ABSTRACT: The majority of biosensors for adenosine-5′-triphosphate (ATP) determination are based on cascades of enzymatic reactions; therefore, they are sensitive to glucose or glycerol (depending on the enzymatic system) as well as to ATP. The presence of unknown concentrations of these substances in the sample greatly complicates the determination of ATP. To overcome this disadvantage of known biosensors, we developed a biosensor system consisting of two biosensors: the first one is based on glucose oxidase and is intended for measuring glucose concentration, and the second one is based on glucose oxidase and hexokinase and is sensitive toward both glucose and ATP. Using glucose concentration measured by the first biosensor, we can analyze the total response to glucose and ATP obtained by the second biosensor. Platinum disc electrodes were used as amperometric transducers. The polyphenilenediamine membrane was deposited onto the surface of platinum electrodes to avoid the response to electroactive substances. The effect of glucose concentration on biosensor determination of ATP was studied. The reproducibility of biosensor responses to glucose and ATP during a day was tested (relative standard deviation, RSD, of responses to glucose was 3−6% and to ATP was 8−12%) as well as storage stability of the biosensors (no decrease of glucose responses and 43% drop of ATP responses during 50 days). The measurements of ATP and glucose in pharmaceutical vials (including mixtures of ATP and glucose) were carried out. It was shown that the developed biosensor system can be used for simultaneous analysis of glucose and ATP concentrations in water solutions.



Adenosine-5′-triphosphate (ATP) is a main high-energy compound in the cells of any organism. It is synthesized via phosphorylation of adenosine-5′-diphosphate in the key cellular biochemical pathways, glycolysis and oxidative phosphorylation. Additionally, ATP is formed as a product in many enzymatic reactions. Under normal circumstances, the main source of energy in the cells and organisms, in particular for the ATP synthesis, is the reactions of carbohydrates catabolism. In the fasting state, ATP is formed by the decomposition of fatty acids or amino acids. The resulting ATP molecules are used in the synthesis of almost all necessary cell compounds such as proteins, nucleotides, nucleic acids, and fatty acids. In fact, ATP is involved almost in all cellular reactions (directly or indirectly) and in many physiological reactions.[1,2]

Thus, it is clear that monitoring the concentration of ATP, especially in biochemical, molecular-biological, and biotechnological samples, as well as *in vivo* in tissues and cells, is very important. Additionally, the determination of ATP concentration can be effective in the drug design, including those based on kinase inhibitors.[3]

The oldest method of ATP determination is the luciferase method consisting of the measurement of light emitted by the enzyme luciferase due to ATP splitting.[4] This method is applied in modern luminometry and other bioluminescent assays that are rapid, selective, sensitive, and therefore widespread techniques for ATP determination (i.e., for cellular measurements). The disadvantage of this method is the limitations regarding the real-time and *in vivo* measurements due to the need for sensitive cameras (which impedes miniaturization of a measuring device).

Furthermore, spectrophotometry and liquid chromatography (HPLC) are used for high-precision determination of the ATP concentration. These methods require skilled personnel and sophisticated expensive equipment.[5,6] Another disadvantage of the above methods is the need for complicated sample preparation prior to the analysis.

Biosensors are promising devices for ATP determination because they are sensitive, selective, portative, and quite cheap in comparison with HPLC or spectrophotometric equipment. Furthermore, biosensors can be used by nonprepared personnel. Another possible application of biosensors is real-time measurement of ATP concentration in order to study kinetics of different reactions; in this case, biosensors offer such

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advantages as a small volume of required sample and quick response time. Today, there are a number of laboratory prototypes of biosensors for ATP determination, which are based on various enzymatic reactions.[7−13] In particular, enzymes H^+-ATPase[7] and apyrase (ATP-diphosphohydrolase)[8] were used for the creation of ATP-sensitive biosensors. However, the vast majority of modern biosensors for ATP determination utilize the enzyme systems glycerol kinase-glycerol-3-phosphate oxidase[9] and glucose oxidase-hexokinase.[3,10−13] These biosensors are mostly monosensors, sensitive to ATP and either glycerol or glucose (depending on the enzymatic system used). Potentiometric ATP biosensors have rather low sensitivity to ATP and strong dependence on buffer capacity. Another disadvantage of the majority of biosensors is insufficient storage stability (especially for biosensors based on a glycerol kinase-glycerol-3-phosphate oxidase system) and absence of selectivity studies. Moreover, the procedure of ATP measurement by these biosensors requires constant concentration of glycerol or glucose in solution. Therefore, the change in concentration of one of these substrates automatically causes significant error, because this change cannot be registered by the monosensor when determining the ATP amount (one exception is a very high concentration of glucose or glycerol, when the enzyme will be saturated and the constant concentration of substrate will be unimportant, but in this case, sensitivity of the biosensor to ATP will decrease greatly). For this reason, a promising approach is the creation of a biosensor system able to determine simultaneously both glucose and ATP concentrations by two independent bioselective elements, which was a goal of this work.

MATERIALS AND METHODS

Materials. Enzymes hexokinase (HEX, EC 2.7.1.1) from *Saccharomyces cerevisiae* with activity of 30.6 U/mg (Sigma-Aldrich, Germany) and glucose oxidase (GOD, EC 1.1.3.4) from *Aspergillus niger* with activity of 272 U/mg (Genzyme, UK) were used in biorecognition elements of biosensors. Bovine serum albumin (BSA, fraction V), glucose, ATP (disodium salt hydrate, grade 1, ≥99%), glycerol, ascorbic acid, *m*-phenylenediamine, HEPES, and 50% aqueous solution of glutaraldehyde (GA) were received from Sigma-Aldrich Chemie (Germany). All other chemicals were of p.a. grade.

Design of Amperometric Transducers. In this work, platinum disc electrodes of our own production served as amperometric transducers. Platinum wire, 0.4 mm in diameter and 3 mm long, was sealed at the end of a glass capillary with an outer diameter of 3.5 mm. An open end of the wire served as the transducer working surface. An inner end of the platinum wire was connected to a copper wire, placed inside the capillary, using fusible Wood's alloy. A contact pad for connecting to the measuring setup was placed at the other end of the copper wire. The working surface of platinum electrodes was obtained by grinding using alumina powder (particle size 0.1 and 0.05 μm) and treated with ethanol prior to immobilization of the bioselective element.

Immobilization Procedure. Biorecognition elements of the biosensors were obtained by covalent immobilization of enzymes and auxiliary substances on the surface of an amperometric transducer. The initial solution for preparing the working membrane of the biosensor for determination of ATP and glucose contained 5% GOD (hereafter, w/w), 5% HEX, and 3% BSA in 20 mM phosphate buffer, pH 6.5, mixed with 10% glycerol; the solution for the glucose biosensor consisted of 4−5% GOD and 3% BSA in the same buffer with 10% glycerol. Glycerol was added to the solutions to stabilize the enzymes and to prevent early drying of solution on the transducer surface. BSA stabilized the enzymes and participated in formation of intermolecular bindings. These solutions were mixed with 0.4% aqueous solution of glutaraldehyde (cross-linking agent) in a ratio of 1:1 and immediately deposited onto the transducer working surface (approximately 100 nL of the mixture onto each electrode). Afterward, the created biosensors were dried for 40 min in air at room temperature and then immersed in the working buffer for 10 min in order to stop immobilization and wash out unbound components.

Method of Measurement. The biosensor system (two amperometric biosensors, the first one sensitive to glucose and the second one sensitive to glucose and ATP) was placed in an electrochemical cell with an auxiliary electrode (platinum wire) and a reference electrode (saturated Ag/AgCl) that were connected to the PalmSens potentiostat (Palm Instruments BV, Netherlands). Usage of the 8-channel device (from the same producer), connected to the potentiostat, allowed simultaneous monitoring of signals from 8 working electrodes; however, in our work, we usually used 2−3 working transducers simultaneously. The distance between an auxiliary platinum electrode and all biosensors during measurements was the same, about 5 mm. The measurements were carried out at room temperature in an open 3.5 mL measuring cell with permanent stirring by a magnetic stirrer and at constant potential of 0.6 V versus Ag/AgCl reference electrode. Twenty-five mM HEPES, pH 7.4, with 2 mM Mg^{2+} was used as the working buffer in all experiments (unless otherwise stated). The substrate concentrations in the working cell were obtained by the addition of aliquots of stock solutions (50 and 500 mM glucose and 50 mM ATP). The values of biosensor responses were calculated after reaching steady state. All experiments were carried out in three repetitions.

RESULTS AND DISCUSSION

The proposed biosensor system includes two biosensors, one of which contains a biorecognition element with GOD and HEX and is sensitive to glucose and ATP whereas another biosensor contains a biorecognition element with only GOD and is sensitive only to glucose. We devoted most of the work to the two-enzyme biosensor as it is the more complex part.

Principle of ATP Biosensor Operation. The operation of amperometric biosensor for ATP determination is based on two simultaneous enzymatic reactions and the reaction of hydrogen peroxide oxidation on the working electrode, occurring when applying necessary potential and followed with generation of electrons, which can be directly registered by the amperometric transducer:

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{+600\ \text{mV}} 2H^+ + 2e^- \quad (2)$$

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HEX}} \text{D-glucose-6-phosphate} + \text{ADP} \quad (3)$$

In the presense of glucose and absence of ATP, only the reactions 1 and 2 take place on the electrode surface. In this case, the biosensor response is proportional to the glucose concentration. After addition of ATP to the working cell, in the presence of hexokinase (HEX), the reaction 3 occurs, which

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Table 1. Comparison of Characteristics of Biosensor Responses to ATP at Different Concentrations of Glucose in Tested Solution

biosensor characteristic	glucose concentration, μM				
	10	25	50	100	250
response to 100 μM ATP, nA	0.9 ± 0.3	1.9 ± 0.7	3 ± 1.1	3.6 ± 1.4	3.3 ± 1.8
relative response to 100 μM ATP, % of response to glucose	55 ± 15	48 ± 11	38 ± 11	22 ± 8	7.4 ± 3.5
baseline noise, nA	0.02	0.02	0.11	0.17	0.33
limit of ATP detection, μM	7.2 ± 1.8	3.7 ± 2.2	10 ± 3	16 ± 7	28 ± 8

reduces the local concentration of glucose and, consequently, the biosensor response to glucose decreases in proportion to the ATP concentration.

Selection of Enzymes Amount in Bioselective Elements. The operation of enzyme-based biosensors largely depends on the biorecognition element composition, namely, on the amount and ratio of the enzymes (GOD and HEX). Therefore, the first phase of the work was aimed at selecting an optimal amount of these enzymes in biorecognition elements of both biosensors.

Initially, we determined an optimal GOD concentration in the biomembrane of biosensor for glucose determination (without adding HEX). For this purpose, the working membrane was prepared according to the procedure described in the Immobilization Procedure section using initial enzyme solutions with different GOD concentrations, from 1.2% to 20%. These solutions were mixed with 0.4% GA solution in a ratio of 1:1; the mixture was immediately deposited on the surface of amperometric transducer and immobilized during 30 min. Thus, at the beginning of immobilization, the mass fraction of GOD in the biorecognition element ranged from 0.6% to 10%. Then, the sensitivity of obtained biosensors to glucose was tested. First, an increase in GOD concentration up to 2−2.5% resulted in a higher response value. Afterward, when increasing the GOD concentration from 2.5% to 10%, the response values and shape of calibration curves were virtually identical (Figure S-1, curve 1, Supporting Information). Therefore, in further experiments, we used a GOD concentration of 2−2.5%.

Next, we determined an optimal concentration of HEX in a bioselective element of the biosensor for determination of ATP and glucose (according to a similar procedure). The biosensor sensitivity was tested at various HEX concentrations in the range of 1% to 8% and constant GOD concentration (2%). The highest values of biosensor response to ATP were observed at a HEX concentration of 2−3% (Figure S-1, curve 2, Supporting Information). A decrease in responses to ATP at high HEX concentrations (4−8%) can be explained as follows. In this case, most of ATP molecules take part in the reaction with HEX in the surface layers of biorecognition element and only a small amount of ATP molecules reach deeper layers, close to the electrode surface, which are more important for the biosensor response. Therefore, in further work, we used the biorecognition elements with the HEX concentration of 2.5%.

We also tested two-layer biorecognition elements with GOD in the first layer (close to the electrode) and HEX in the second one. First, we carried out immobilization of 2.5% GOD (according to the procedure of preparation of the working membrane of the biosensor for glucose determination described in the Immobilization Procedure section); then, the biosensor was washed and dried, and its sensitivity to glucose was evaluated. Next, 2.5% HEX (with 0.2% of GA) was deposited as the second layer and dried for 30 min. After this, the shape of calibration curves for the glucose determination did not change, but the baseline noise significantly increased. Thus, the accuracy of ATP determination became worse, and it was impossible to register the responses to small concentrations of ATP; so, we did not use the two-layer method of enzymes immobilization in further work.

Selection of Conditions of Enzyme Immobilization. A very important factor which has a direct effect on the biosensor analytical characteristics is a method of the biorecognition element immobilization. In this work, the enzymes were immobilized onto the transducer surface via covalent binding with GA; during this process, the enzyme activity decreased, depending on the GA concentration and immobilization time. Therefore, the next step was to improve the conditions of immobilization of a bioselective element based on GOD and HEX, specifically to determine the GA concentration and duration of immobilization.

For the selection of optimal GA concentration, we prepared biorecognition elements with final concentrations of 1%, 0.2%, and 0.1% GA. In all cases, the responses to glucose were almost identical, whereas the responses to ATP were very small in the case of 1% GA and quite sufficient at 0.2% and 0.1% GA. This implies that HEX has much less stability than GOD, which is why during immobilization HEX lost its activity faster. However, at 0.1% GA, we observed a gradual decrease in responses during multiuse, due to wash-out of enzymes from the bioselective element. Thus, in further work, we used 0.2% GA.

We also examined the biosensor operation depending on the duration of bioselective element immobilization (20 to 50 min). At a 20 min immobilization, we observed a gradual decrease in biosensor responses to ATP during the work due to unstable enzyme immobilization, and in case of a 50 min immobilization, the responses to ATP were lower than in other cases, probably because of a decrease in the HEX activity. Therefore, the optimum immobilization time was 30−40 min.

Influence of Glucose Concentration on Biosensor Determination of ATP. As described previously, the measurement of ATP concentration by the two-enzyme biosensor is based on the competition between GOD and HEX for glucose. In principle, it is possible to measure ATP at any concentration of glucose, but an increase in glucose concentration leads to a relatively smaller biosensor response to ATP (in % to glucose response) though an absolute value of the response (in nA) increases; the signal noise also increases in proportion to the amount of glucose. For example, at a glucose concentration of 2 mM, the limit of ATP detection was 100 μM, and linear range of ATP determination was 0.1−2.5 mM (operating range extended to 4 mM). At higher glucose concentrations, it was not suitable to measure ATP because the noise of the biosensor response was too big. According to our results, a decrease in glucose concentration results in a lower noise and, correspondingly, in a decrease of the limit of ATP

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detection; additionally, the rate of response to glucose and ATP increases. We were more interested in measuring small concentrations of ATP; thus, more detailed studies were carried out at the glucose concentration from 10 to 250 μM (Table 1). The largest absolute value of biosensor response to 100 μM ATP (3.6 nA) was observed when the glucose concentration was 100 μM. However, the ratio of biosensor responses to ATP and glucose increased at decreasing glucose concentration. For example, at a glucose concentration of 10 μM, the biosensor signal to the ATP addition was more than 50% of the response to glucose, whereas at 250 μM glucose the response to ATP was less than 10% of the response to glucose. The lowest detection limit of ATP was observed at the glucose concentration of 25 μM. Moreover, with increasing concentration of glucose, the linear range of ATP measurement changed. For example, at 100 μM glucose, the ATP linear measurement range was 20−200 μM, whereas at 250 μM glucose it was 150−400 μM ATP. These results demonstrate that we can significantly modify the biosensor ability to detect ATP, changing the glucose concentration in the working solution. On the other hand, if both glucose and ATP were present in the test solution, we first measured glucose concentration with the biosensor, sensitive to glucose, and then figured out how the biosensor, which is sensitive to both glucose and ATP, would respond to ATP.

Selection of Magnesium Concentration. The presence of Mg^{2+} ions in the working buffer is necessary for HEX catalytic activity since magnesium stabilizes the ATP molecule and activates HEX. To select an optimal concentration of Mg^{2+} in the working buffer, we tested the biosensor at Mg^{2+} concentrations from 0.5 to 3 mM. It should be noted that disodium salt of ATP was used as a substrate; in the case of magnesium salt, results could be different. The biosensor responses to glucose practically did not depend on the concentration of magnesium, because GOD does not require magnesium for catalysis. The biosensor responses to ATP, as expected, increased with an increasing amount of magnesium (Figure 1). We observed an increase in biosensor responses to ATP in direct proportion to the magnesium concentration in the range from 0.5 to 2 mM; an addition of more than 2 mM magnesium had no significant effect. Therefore, in further work, we added 2 mM Mg^{2+} to the working buffer (in the form of magnesium nitrate).

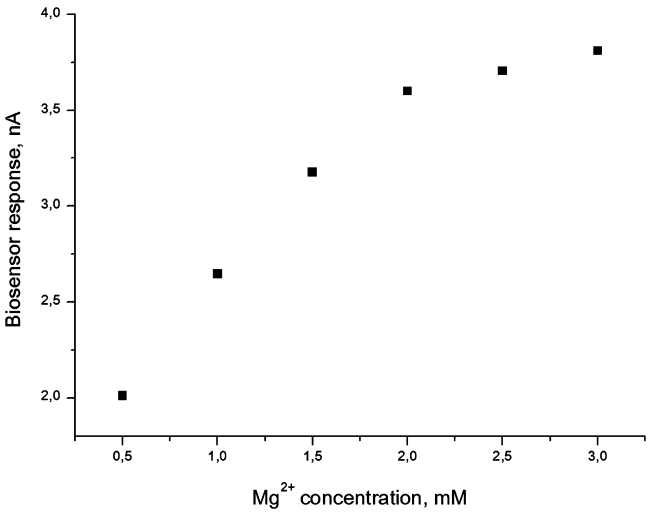

Figure 1. Dependence of biosensor response to ATP on magnesium concentration in working buffer. Glucose concentration, 100 μM; ATP concentration, 100 μM. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, at constant potential of 0.6 V vs Ag/AgCl reference electrode.

Use of Polyphenylenediamine Film for Improving Selectivity of Amperometric Transducer. The biosensor selectivity is an important factor when working with real biological fluids, so the research on selectivity toward possible interfering substances is a mandatory step in the development of biosensors. We used a mediator-free biosensor with relatively high working potential (0.6 V vs Ag/AgCl reference electrode), which makes possible the oxidation of a number of electroactive compounds on the electrode surface. One of the successful approaches to prevent the oxidation of interfering substances is the use of auxiliary semipermeable films that allows selective access of the target substance (in our case, hydrogen peroxide) to the electrode.[14] We deposited a poly phenylenediamine (PPD) film onto a sensitive part of the amperometric transducer by the method described in the literature.[15] This film forms the pores, the size of which is sufficient for hydrogen peroxide to reach the electrode surface, but prevents the access of larger substances. For the formation of PPD membrane, we immersed a three-electrode system with a bare working electrode in a 5 mM solution of *meta*-phenylenediamine and then obtained 4−5 cyclic voltammograms (*meta*-phenylenediamine isomer was chosen according to previous studies[16]). The fourth and fifth voltammograms slightly differed from one another, which indicated the end of the formation of PPD layer on the working electrode. The forms of cyclic voltammograms coincided with those given in the original source.[15] Afterward, the biomembranes based on enzymes GOD and HEX were immobilized onto the PPD film.

To confirm the improvement of selectivity of the modified transducer, we examined the sensitivity of amperometric transducers to possible interfering substances before and after deposition of the PPD film. The interfering substances were dopamine, cysteine, ascorbic acid, uric acid, and paracetamol (*N*-acetyl-*p*-aminophenol). These substances are quite common in biological samples, and their oxidation is possible when applying the potential of 0.6 V to an amperometric transducer. Before the deposition of PPD membrane, considerable responses to these substances were observed, which could be a problem during the measurement in real biological samples. The deposition of PPD membranes resulted in a remarkable decrease or even complete disappearance of the biosensor response to the interfering substances, whereas the transducer sensitivity to hydrogen peroxide remained almost unchanged (Table 2). It was also revealed that the reproducibility of responses to glucose and ATP is significantly improved by using PPD film.

Since the screening effect of PPD membrane may decrease over time due to the gradual destruction of the membrane, we compared the responses to ascorbic acid obtained before and after biosensor storage at −18 °C. In 50 days, the responses increased by only 10%, indicating retention of properties of the PPD membrane.

Analytical Characteristics of Biosensor for ATP Determination. Analytical characteristics of the ATP biosensor were studied in detail (glucose concentration in working buffer was 50 μM). Limit of detection of ATP was 4−6 μM. Linear range of ATP determination lied within 15−100 μM. Sensitivity of the biosensor to ATP was 200−280 nA/

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Table 2. Selectivity of Amperometric Transducer before and after Deposition of PPD Film

electroactive substance	response of amperometric transducer, nA	
	without PPD film	with PPD film
hydrogen peroxide, 50 μM	34.7 ± 2.6	27.6 ± 0.8
dopamine, 20 μM	14.8 ± 1.3	1.2 ± 0.3
cysteine, 100 μM	2.8 ± 0.4	0.02 ± 0.02
paracetamol, 100 μM	7.3 ± 1.2	0
uric acid, 100 μM	10.6 ± 1.8	0
ascorbic acid, 500 μM	33.2 ± 1.7	0.9 ± 0.5

(mm^2·mM). Typical calibration curves of the biosensor for determination of glucose and ATP are shown in Figure 2. Linear parts of these calibration curves are described by the equations $I = -0.27 + 32C$ (ATP determination; $R^2 = 0.988$) and $I = -0.5 + 80C$ (glucose determination; $R^2 = 0.993$), where I is the current when the response reaches the steady-state value (nA) and C is the concentration of ATP or glucose (mM). Notably, determination of ATP by the biosensor strongly depended on the composition of working solution and, in particular, on concentrations of glucose and Mg^{2+} ions; it is possible to alter analytical characteristics of the biosensor by changing these parameters, as described in the previous sections. Presented on Figure 2, calibration curves were obtained for one ATP biosensor; calibration curves of other ATP biosensors had a similar shape but differed in absolute values of response current (±20%).

Furthermore, we studied reproducibility of the responses of the biosensor during several hours of continuous operation. One measurement of glucose and ATP took about 5 min (this time included baseline stabilization, steady-state response to glucose, and steady-state response to ATP); the interval between measurements was about 10 min, and during the intervals, we repeatedly washed the biosensor with the working buffer from substrates. The relative standard deviation of responses to glucose was 3−6% and to ATP was 8−12%. A larger response of deviation in the case of ATP measurement as compared with glucose measurement occurs because the response to ATP is formed due to the competition between two enzymes (GOD and HEX) using two substrates (glucose and ATP) whereas the response to glucose is formed by only one enzyme and one substrate.

The total time of biosensor production was 1 h. This time included preparation of electrode for enzyme immobilization (10 min), immobilization itself (40 min), and washing of the created biosensor after immobilization (10 min). One platinum working electrode costs about 20 US dollars; reagents for 1 immobilization costs less than 2 dollars. Thus, the approximate cost of 1 biosensor is 22 dollars (except the salary of a worker). About 20% of immobilizations failed (biosensor responses were smaller than usual, and biosensors were not used for experiments). This information is true for both ATP and glucose biosensors.

Biosensor for Glucose Determination. Unfortunately, the biosensor for ATP determination, described in the previous part of the work, is sensitive to glucose as well. Therefore, in case of the presence of both substances in the tested sample (which is very probable), it is difficult to determine their individual concentrations. To overcome this limitation, we decided to use an additional auxiliary biosensor, sensitive only to glucose. The bioselective element of this biosensor contained GOD like the bioselective element of the biosensor for ATP determination. Thus, the response of the GOD-based biosensor did not depend on the presence or absence of ATP in the sample and was a starting point for analyzing the responses of the ATP-sensitive biosensor.

The ultimate goal of the study was to develop a biosensor system for the determination of ATP in the presence of glucose. In this system, the two-enzyme biosensor is more important and complex, that is why we considered the working conditions, determined in previous parts for the biosensor based on GOD and HEX, to be suitable for the GOD-based biosensor. Under these conditions, we plotted the calibration curve of the GOD-based biosensor for glucose determination (Figure 3). A linear part of the calibration curve is described by

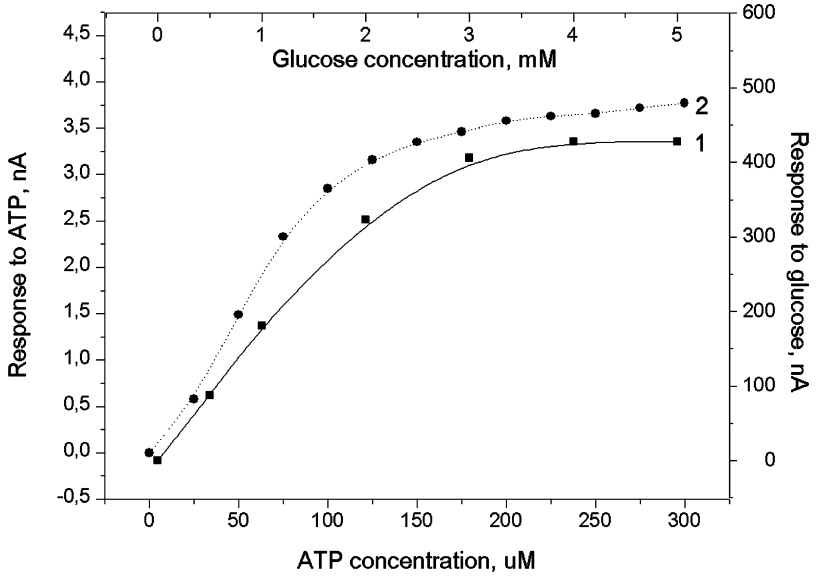

Figure 2. Typical calibration curves of the biosensor based on GOD and HEX for determination of glucose (1) and ATP (2). Calibration curve for ATP determination was plotted at glucose concentration of 50 μM. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+} and a constant potential of 0.6 V vs Ag/AgCl reference electrode.

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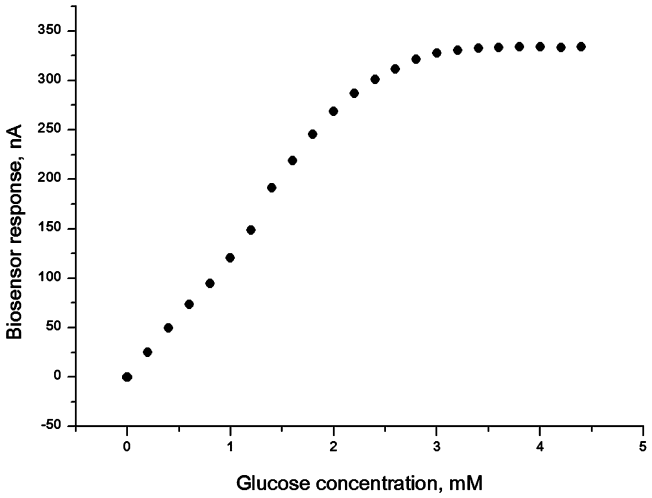

Figure 3. Calibration curve of GOD-based biosensor for glucose determination. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+} and the constant potential of 0.6 V vs Ag/AgCl reference electrode.

the equation $I = -6.87 + 138C$, where I is the current when the response reaches a steady-state value (nA) and C is the concentration of glucose (mM), $R^2 = 0.994$. Calibration curves of other glucose biosensors had a similar shape but differed in absolute values of response current (±15%). The limit of detection of glucose was 1 μM. The linear range of glucose determination was from 10 μM to 2−2.3 mM; sensitivity of the biosensors was 800−1110 nA/(mm^2·mM).

Method of Simultaneous Determination of Glucose and ATP by Using the Biosensor System. For simultaneous measurement of the concentrations of glucose and ATP, we used two biosensors described in this paper: the first one is based on GOD and HEX and the second one is based on GOD only. They were placed in the same measuring cell and connected to the same measuring setup via a multiplexer. After obtaining the baseline, we added the tested sample to the working solution and measured the responses of both biosensors. The response of the second (GOD) biosensor was proportional to glucose concentration in the sample, and we determined glucose concentration by the previously obtained calibration curve (Figure 3). The response of the first (GOD/GEK) biosensor depended on the concentrations of both glucose and ATP in the test solution as a result of competition between the two enzymes for glucose (see the Principle of ATP Biosensor Operation section). From the calibration curve of the first biosensor for glucose determination (Figure 2, curve 1), it was possible to calculate what would be the response of the biosensor to the glucose in the absence of ATP. Using the difference between the calculated and actual values of responses of the first biosensor to sample addition, we calculated the response to ATP and determined the ATP concentration from the calibration curve (Figure 2, curve 2). Thus, using these two biosensors, we can analyze the samples containing both glucose and ATP.

An example of operation of the biosensor system (subsequent additions of 50 μM glucose and 100 μM ATP to the working cell) is shown in Figure 4. It should be noted that changes in ATP or glucose concentrations became evident within seconds; thus, it is possible to measure kinetics of processes that involve ATP production or consumption.

Storage Stability of Biosensor System. Storage stability of the developed biosensor system was investigated. The biosensors were stored dry at temperatures of +4 and −18 °C. It was found that storage stability of GOD for both biosensors was similar; therefore, we showed only the results of storage stability for the two-enzyme biosensor (Figure S-2, Supporting Information). As seen, in 2 weeks, the sensitivity to glucose of the biosensor stored at +4 °C decreased by 20−25% and at

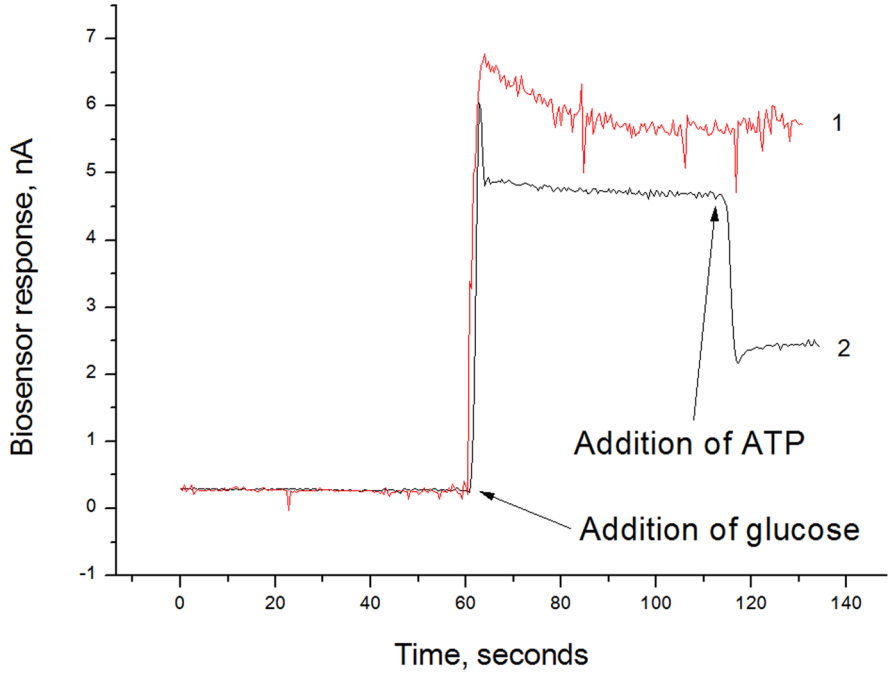

Figure 4. Real responses of the biosensor system to glucose and ATP: (1) biosensor based only on GOD and (2) biosensor based on GOD and HEX. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+} and the constant potential of 0.6 V vs Ag/AgCl reference electrode.

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Table 3. Results of Biosensor Measurement of Glucose-ATP Mixtures[a]

	mixture #1		mixture #2		mixture #3	
	glucose, mM	ATP, mM	glucose, mM	ATP, mM	glucose, mM	ATP, mM
nominal concentration	2.5	5	10	10	1	2.5
measured concentration	2.68 ± 0.1	4.16 ± 0.4	11.7 ± 0.4	8.8 ± 0.8	1.2 ± 0.1	2.7 ± 0.2

[a]Biosensor measurements were repeated three times, and the average value ± standard deviation is given.

−18 °C decreased by 10−15%, whereas the responses to ATP decreased by 65−75% and 45−55%, respectively. This suggests that HEX is a much less stable enzyme than GOD, and it is the HEX stability that restricts the duration of ATP biosensor storage. The same results were obtained during study of termal stability of biosensor based on immobilized GOD and HEX.[3] This coincides with the data obtained in the study on enzymes immobilization: during immobilization, HEX lost its activity faster than GOD (see the Selection of Conditions of Enzyme Immobilization section).

Since the storage at −18 °C showed better results, we examined the biosensors under this condition for a longer period. We did not check their activity every few days, so the biorecognition element lost its activity more slowly. The responses to glucose were not reduced at all after a 50 day storage whereas the responses to ATP in 34 days decreased by 20% and in 50 days by 43%. The detection limit of ATP during storage did not change and amounted to 4−7 μM.

Measurements of Pharmaceutical Samples. To test the developed biosensor system, we purchased pharmaceutical vials with ATP (nominal concentration of 10 mg/mL or 18 mM) produced by "Darnitsa" (Kiev, Ukraine) and glucose (nominal concentration of 5% or 277 mM) produced by "Nikopharm" (Donetsk, Ukraine) for intravenous and intramuscular injection. To demonstrate a possibility of simultaneous determination of ATP and glucose in one sample, we first measured ATP and glucose separately and then mixed the samples with different ATP/glucose ratios.

The concentration of ATP was determined by two methods: by the calibration curve and standard additions. In the first case, we obtained the calibration curve for ATP (with 100 μM glucose in the solution) using the GOD/GEK biosensor and then received several responses to a sample (233-fold dilution); ATP concentration was calculated from the calibration curve. In the case of the standard additions method, we obtained the response to glucose (100 μM), then (without washing) added ATP model solution (final concentration of 40 μM), then added an aliquot from the pharmaceutical vial (900-fold dilution of the sample), and finally added twice 20 μM of ATP model solution. The first addition of the ATP (40 μM) was performed because the linear range of the biosensor started from 15 to 20 μM ATP, and the response to the real sample and the subsequent two responses (to 20 μM of ATP) should be within the linear range of ATP determination. Plotting the results (abscissa, added ATP concentration after sample addition; ordinate, biosensor response in nA), we obtained a straight line, of which the intersection with the abscissa axis corresponded to the ATP concentration in the working cell. The results of measurements are given in Table S-1, Supporting Information; they completely coincided (within the measurement error) with the ATP concentration measured by the luciferase method which is described in the introduction. The ATP concentration determined by both methods was about 18 mM.

The biosensor determination of glucose was conducted by the standard additions method. Glucose concentration was determined to be 275 ± 5 mM, which was identical to the nominal value.

Next, we mixed ATP and glucose from vials to obtain mixtures containing 1−10 mM of glucose and 2.5−10 mM of ATP. Aliquots of these solutions were added to the working cell, and responses of the biosensor system were measured using the procedure described in the Biosensor for Glucose Determination section. The results of measurements are given in Table 3; deviation from the nominal values usually did not exceed 10%. The measurement error was bigger than that in case of separate glucose and ATP samples, which can be explained by the more complicated measurement procedure using two biosensors. However, these results demonstrate that the proposed biosensor system is suitable for reliable measurements in samples containing both ATP and glucose, which was the main goal of this work.

CONCLUSIONS

The biosensor system for simultaneous determination of ATP and glucose was developed. The proposed system consisted of two biosensors: one, sensitive to glucose; another, sensitive to both glucose and ATP. The optimal concentration of enzymes in the biorecognition elements was determined to be 2−2.5%. Optimum conditions of enzyme immobilization were selected, namely, the concentration of cross-linking agent glutaraldehyde, 0.2%; immobilization time, 40 min. To improve the biosensors selectivity, we modified the transducer surface with a poly-*m*-phenilenediamine film, which allowed us to almost avoid the influence of electroactive interfering substances. It was also shown that considering the particular purpose of the biosensor system we could obtain necessary operational characteristics by changing the composition of the sample solution. Thus, it was possible to change the limit and range of ATP determination by variation in the glucose and Mg^{2+} concentration. The study on reproducibility of responses to glucose and ATP demonstrated that during a day the relative standard deviation of responses to glucose was 3−6% and to ATP was 8−12%. The biosensor system showed good storage stability if stored at −18 °C; a 50 day storage resulted in a decrease of responses to ATP by 43% and did not cause any drop in responses to glucose. We showed the possibility of simultaneous measurements of ATP and glucose concentrations in pharmaceutical vials.

ASSOCIATED CONTENT

Supporting Information

Two figures and one table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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