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Microbiosensor based on glucose oxidase and hexokinase co-immobilised on platinum microelectrode for selective ATP detection

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ABSTRACT

ATP determination is of great importance since this compound is involved in a number of vital biological processes. To monitor ATP concentration levels, we have developed a microbiosensor based on cylindrical platinum microelectrode, covered with a layer of poly-m-phenylendiamine (PPD), and layer of co-immobilised glucose oxidase and hexokinase. Conditions for biosensor measurement of ATP (pH, Mg^{2+} and substrates concentration) *in vitro* and microbiosensor characteristics such as sensitivity, selectivity, reproducibility, storage stability were studied and optimized. Under optimal conditions the microbiosensor can measure ATP concentrations down to a 2.5 μM detection limit with response time about 15 s. Interferences by electroactive compounds like biogenic amines and their metabolites, ascorbic acid, uric acid and L-cystein are rejected in general by the PPD layer. The microbiosensor developed is insensitive to ATP analogues (or substances with similar structure), such as ADP, AMP, GTP and UTP, too. It can be used for ATP analysis *in vitro* in the reactions consuming or producing macroergic triphosphate molecules to study kinetics of the process and in drug design concerning development of inhibitors specific to target kinases and others target enzymes.

1. Introduction

Adenosine-5′-triphosphate (ATP), a well known macroergic substance in all living organisms, plays a key role in the energy turnover of a cell. Its additional functions (the regulation of muscle contraction and platelet aggregation [1], vascular tone and neurotransmission [2]) have been revealed as a result of extensive research in several branches of biology and biomedicine over the last decades.

ATP determination is of great importance since this compound is involved in a number of vital biological processes. The variation in ATP concentration can exert strong modulatory effects in central nervous system of mammalian. ATP can influence transmitter release, synaptic plasticity, neurone–glia interactions, nociception, sleep–wake cycles, respiratory and locomotor rhythms, anxiety, depression, aggression and addiction (see Ref. [3] and corresponding references from this paper). Moreover, ATP detection can be efficiently used in food industry as a marker of micro-fungal contamination and in drug design at development of inhibitors for specific kinases and others enzymes.

Consequently, there is an actual demand for ATP assays. ATP concentration is usually analyzed using spectrophotometry [4], liquid chromatography [5], fluorescence [6], chemiluminescence [7], bioluminescence [8] methods, potentiometric [9–11] and amperometric [12–18] biosensors. Among these techniques, biosensors seem to be the most promising tools owing to their characteristics (Table 1).

With the help of ATP-biosensors, different biochemical processes can be studied and visualised directly. An analysis of the characteristics presented in Table 1 shows that ATP potentiometric biosensors demonstrated rather low sensitivity to ATP and strong dependence on buffer capacity. These disadvantages do not permit to use them for ATP measurement in real biological fluid samples at its micromolar physiological concentrations. Concerning amperometric biosensors, there are two main approaches to their fabrication: application of the bi-enzyme system based on (1) glycerol oxidase and glycerol kinase (GO/GK) or glycerol kinase and glycerol-3-phosphate oxidase (GK/G-3-PO), and (2) glucose oxidase and hexokinase (GOD/HK). Both approaches give rather good results regarding sensitivity of the developed microbiosensors, however, measurement selectivity and stability presented in Table 1 are not satisfactory enough (unless shown at all). We preferred the development of the GOD/HK microbiosensor since in this case the system needs glucose for ATP detection, which exists practically in all biological samples. So, second approach is used in our work to develop

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Table 1
The main characteristics of developed ATP biosensors.

Enzymatic system	Type of transducer and dimensions	Time of response and sensitivity	Linear dynamic range	Selectivity, interferences	Stability (residual enzyme activity)	Ref.
H^+–ATPase	ISFET	1–1.5 min	0.2–1.0 mM	Dependence on buffer capacity	10% after 18 day	[9]
Choline kinase	Choline-sensitive membrane electrode	1–2 min, 50 mV/100 μM	10–75 μM	No interferences from ADP, AMP, Na, K	No data presented	[10]
Apyrase (ATP dihydrolyase)	ISFET	5 min, 80 V M^{-1}	0.2–1 mM	Dependence on buffer capacity	No data presented	[11]
GOD + HK	Oxygen electrode	2 min	0.2–3.0 mM	Not presented	Not presented	[12]
GOD + HK	Pt electrode, size was not presented	2–3 min, cannot be calculated	50–500 μM	Not presented	30% after 1 week	[13]
GOD + HK	Pt disk electrode Ø 1 mm	5–80 s, 1 A M^{-1} cm^{-2} [a]	10–200 nM	Not presented	40% after 2 weeks	[14]
GOD + HK	Pt disk electrode Ø 25 μm	150 ms, 100 μA M^{-1} cm^{-2}	0–1 mM	Not presented	40% after 2 weeks	[14]
GK + G3POx	Pt/Ir electrode (90/10), Ø 25–100 μm	≤10 s, 250 mA M^{-1} cm^{-2}	200 nM–50 μM	Low[b], AA, urate, AcPh, 5HT	Not presented	[15]
GOD + HK	Glassy carbon electrode, Ø 3 mm	≤15 s, 85 mA M^{-1} cm^{-2}	0.5–20 μM	Not presented	65% after 22 days	[16]
EF_0F_1–H^+–ATPase	Au electrode, 2 mm × 2 mm	50 mA M^{-1} cm^{-2}	2.5–6.0 mM	Not presented	Not presented	[17]
Commercial product, no information about enzyme system	Pt/Ir electrode (90/10), *L* = 2 mm or 0.5 mm, Ø 50 μm	≤10 s, 0.5 mA M^{-1}	0.5–50 μM	Selective vs. UTP, ADP and adenosine, no information concerning electroactive compounds	Shelf-life (dry) ≥6 months	[18]

[a] It seems for us that density of current obtained by the authors [14] is too high and not possible. Authors presented erratum [19] concerning only last Fig. 7 in the paper [14], but it seems for us that all data have to be recalculated.
[b] Measurement in differential mode with null sensor; shortening: G3Pox–glycerol-3-phosphate oxidase; AA, ascorbate; AcPh, acetaminophen; 5HT, serotonine.

the amperometric microbiosensor for ATP measurement in different biological samples. We apply the bi-enzyme system with GOD/HK co-immobilised on the surface of platinum microelectrode preliminarily covered by poly-m-phenylendiamine (PPD) that allows to create microbiosensors with improved main characteristics, i.e. highly sensitive, with good selectivity, reproducibility and long-term stability. Such microbiosensor developed can be used for detection of low micromolar concentration of ATP in small tens microlitre volumes.

2. Experimental

2.1. Reagents

The following chemicals and preparations were used: glucose oxidase (GOD) from *Aspergillus niger*, EC 1.1.3.4 (190 U mg^{-1} solid), hexokinase (HK) from *Saccharomyces cerevisiae*, EC 2.7.1.1 (35.8 U mg^{-1} solid), apyrase, grade I from potato (7.7 U mg^{-1} solid), bovine serum albumin (BSA), glutaraldehyde (GA) (50% (w/v) aqueous solution), hydrogen peroxide (HP) (3% (w/v) aqueous solution), glucose, magnesium acetate tetrahydrate, sodium and potassium chlorides, m-phenylenediamine (PD), adenosine-5′-triphosphate (ATP), guanosine-5′-triphosphate (GTP) and uracil-5′-triphosphate (UTP). All these chemicals were purchased from Sigma–Aldrich SARL (France). Working HEPES buffer 0.025 M, pH 7.4, was prepared from HEPES [N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonicacid] (free acid) and deionised water and then was adjusted to needing pH by NaOH.

2.2. Microelectrode preparation

Microelectrodes constructed from platinum wire (25 μm in diameter; 90% Pt/10% Ir wire (Goodfellow, Huntington, UK) were developed and manufactured by the authors [20]. The electrodes were then washed 30 min in 0.5 M KOH and 20 min in ethanol.

Poly (phenylenediamine) layer was subsequently deposited on the electrode surface from a quiescent solution of 0.1 M phosphate buffer (pH 7.0) containing 0.1 M PD. The film was grown electrochemically on the platinum surface at a constant potential (+0.7 V) for 45 min. Before use, PD solutions were deoxygenated by bubbling with argon for 15 min. The modified electrode surface was thoroughly washed with distilled water prior to bioselective membrane creation.

2.3. Bioselective-membrane preparation

The bioselective enzymatic membranes of ATP and glucose sensitive microbiosensors were prepared by using a GOD–HK–BSA mixture prepared in a 0.1 M phosphate buffer at pH 7.0. The final concentration of each enzyme was of 40 mg ml^{-1} while that of BSA was of 20 mg ml^{-1}. For glucose only sensitive biosensor, the same buffer solution was used with the final concentration of GOD 40 mg ml^{-1} and BSA 60 mg ml^{-1}. In cases of both microbiosensors creation glycerol was also added to each mixture (3% w/v) in order to stabilize the enzymes during their immobilization, to prevent early membrane drying and to improve membrane adhesion to the transducer surface. GA was used as a cross-linker (final concentration at 0.2% w/v). The mixture containing GA was deposited on the sensitive part of the transducer and afterwards cured for 1 h at room temperature. Before use microbiosensors were washed by working buffer.

2.4. Measurement procedure

Measurements were performed at room temperature using a model solution (25 mM HEPES buffer, pH 7.4, with 127 mM NaCl and 2.7 mM KCl and necessary concentrations of Mg^{2+} ions). The required substrates concentrations were obtained by the addition of specific volumes of stock solutions.

Amperometric measurements were performed in open and closed cells filled with working buffer comprising the following conventional three-electrode amperometric system: the platinum working electrode (microbiosensor), an Ag/AgCl reference and a platinum auxiliary electrode. The PalmSens potentiostat equipped with a CH-8 multiplexer devise (Palm Instruments BV, the Netherlands) was used in all experiments. The measurements for the ATP biosensor were carried out at a constant potential of +0.6 V.

3. Results and discussion

The bioselective element of the amperometric microbiosensor developed consists of two co-immobilized enzymes (GOD and HK). The basic enzymatic reactions are:

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

$$H_2O_2 \xrightarrow{+600\,\text{mV}} 2H^+ + O_2 + 2e^- \quad (2)$$

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

As can be seen, this biosensor is sensitive to both the substrates, glucose and ATP, owing to competition between GOD and HK towards substrate glucose. If only glucose is injected in the analyzed medium, reactions (1) and (2) take place, and the electrochemical response proportional within certain range to glucose concentration is generated by the microbiosensor, its value being taken as 100%. When ATP is injected, the reaction (3) takes place resulting in a decrease of the response obtained to glucose injection in the ATP absence, the reduction value being proportional to the ATP concentration in the solution tested.

Therefore, determination of ATP concentration requires the glucose concentration to be kept constant which is possible only under *in vitro* conditions. In the case of biological samples containing unknown glucose concentration the measurements cannot be performed by a single bi-enzyme biosensor since in this case the response depends on both substrates, glucose and ATP. It is though realizable if two microbiosensors are used, one of which is GOD-, another GOD/HK-based. In such set, the first device gives information on glucose concentration, the second shows a difference between the responses to glucose and ATP, thus, the ATP concentration can be evaluated using the data simultaneously obtained from two microbiosensors.

Firstly, the characteristics of the developed bi-enzyme microbiosensor were studied *in vitro* in model solutions. The typical responses of GOD/HK-based microbiosensor to glucose and ATP are presented in Fig. 1. As can be seen, the response to glucose injection was obtained in 20–25 s, while the corresponding responses to ATP were obtained in 15–20 s. The kinetics of generating responses is evidently almost similar in both cases. Addition of apyrase (enzyme hydrolyzing ATP to phosphate and ADP and AMP) in measuring cell shows that ATP responses of microbiosensor directly depend on the ATP concentration.

The calibration curve (Fig. 1B) calculated for ATP measurements from data presented in Fig. 1A shows a possibility to measure ATP starting from 2.5 μM concentration at least.

At the next stage, pH-dependence of the responses of bi-enzyme microbiosensor to glucose and ATP injection was studied. The investigation was carried out in a complex multicomponent buffer (5 mM Tris, 5 mM KH_2PO_4, 5 mM citric acid, 5 mM sodium tetraborate) characterized by identical buffer capacity within a wide pH range (from 5 to 9) [21]. The reason to use such complex buffer in these experiments was to avoid an influence of the buffer composition on different enzymes functioning at various pH values. The results obtained testify the low pH-dependence of the sensor response to glucose with the optimum near pH 7.0 (Fig. 2, curve 1) while in ATP case pH-dependence was more evident and the bell-shaped curve had optimum at pH 7.5–8.0 (Fig. 2, curve 2). This is in very good accordance with the information on pH-dependence of free hexokinase (see Catalog Sigma 2008, Sigma is producer of preparation used in this work). We were afraid that hexokinase co-immobilised with GOD can change pH optimum in comparison to free hexokinase. As the biosensor is supposed for analyses of biological liquids, the 25 mM HEPES buffer, pH 7.4, was utilized in further *in vitro* experiments.

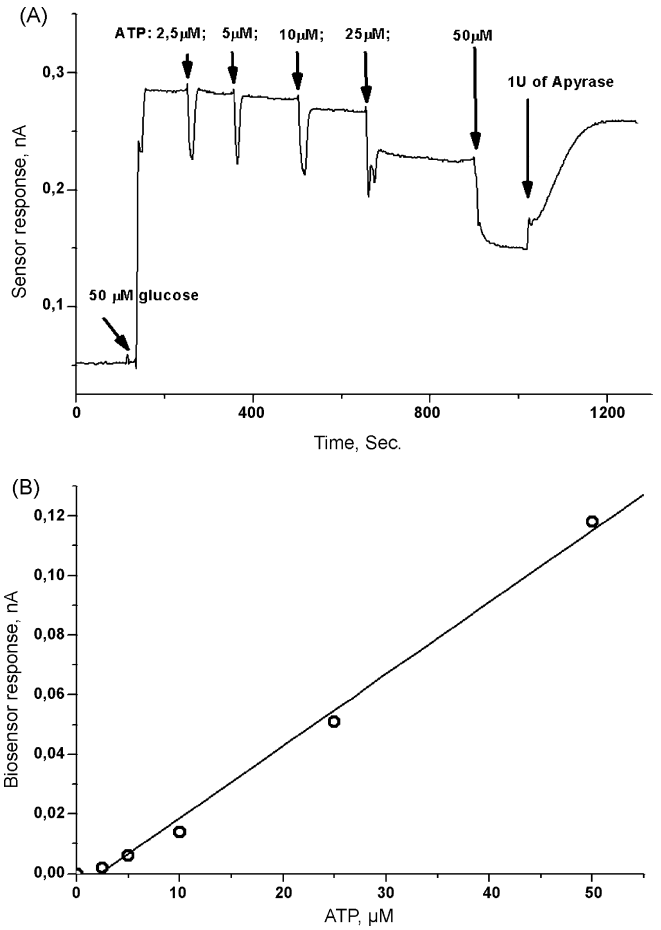

Fig. 1. Typical responses of bi-enzyme microbiosensor to glucose and ATP injections in the measuring cell (A) and calibration curve calculated for ATP measurements (B) (final concentrations of glucose and ATP in the cell are marked on the curve). Additionally apyrase (enzyme hydrolyzing ATP to phosphate and ADP and AMP) was added in measuring cell to show that ATP responses of microbiosensor directly dependent on ATP concentration. Measured in 25 mM HEPES buffer, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl reference electrode.

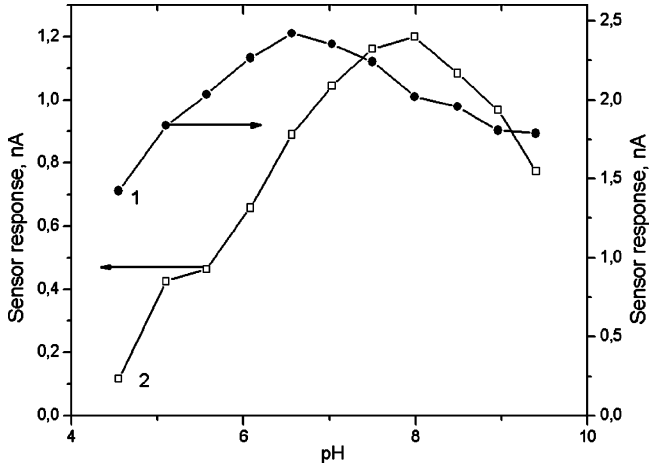

Fig. 2. pH-dependence of microbiosensor responses to 0.5 mM glucose (1) and to 0.5 mM ATP (2). Measurements in polymix buffer at constant potential +0.6 V vs. Ag/AgCl reference electrode.

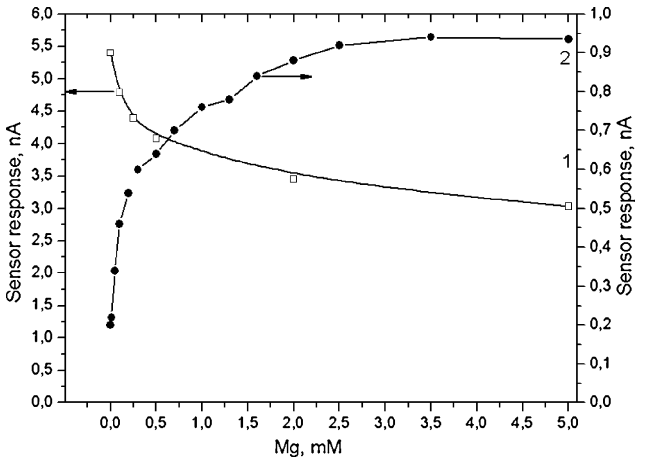

Fig. 3. Mg^{2+}-dependence of bi-enzyme microbiosensor response to 0.5 mM glucose (1) and 0.5 mM ATP (2). Measured in 25 mM HEPES buffer, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl reference electrode.

Since hexokinase is known as an enzyme dependent on the concentration of ions of magnesium which is an enzyme activator (Km = 2.6 mM for free enzyme, see Catalog *Sigma* 2008 and [22,23]), a number of experiments were carried out to ascertain optimal concentration of magnesium ions as regards the bi-enzyme microbiosensor operation. The value of microbiosensor response to ATP appeared to be actually dependent on magnesium concentration (Fig. 3). An increase in Mg^{2+} concentration from 0 to 2 mM resulted in 4.5-fold higher response to injection of 250 μM ATP.

As to the response of bi-enzyme microbiosensor to glucose, an opposite effect was revealed. An increase in Mg^{2+} concentration from 0 to 2 mM resulted in decreasing sensor response to injection of 500 μM glucose. Further rise of concentration of magnesium ions had no remarkable impact on the biosensor responses to ATP and glucose injection. These results were very unexpected and important for optimisation of glucose and ATP analysis. Subsequent *in vitro* experiments on study of microbiosensor characteristics were performed in working buffer with 2 mM Mg^{2+}.

The working buffer with optimal values of pH and magnesium ion concentration was used to study the dependence of microbiosensor responses to ATP and glucose at different concentrations (Fig. 4A and B). The slope of linear part of calibration curves and sensitivity of the microbiosensor for ATP detection were regularly the same within the range of glucose concentrations of 0.1–1.6 mM, though it is noteworthy that the linear range differed remarkably (the higher glucose concentration, the wider linear range). However, increase in glucose concentration to more than 1.6 mM resulted in substantially weaker ATP sensitivity (Fig. 4A, curve 5). It can be caused by the saturation of sensor responses to glucose at the concentrations more than 1.6 mM (Fig. 4B) and, consequently, the absence of response/concentration proportionality. At high glucose concentration and low ATP concentrations, HK consumes small amounts of glucose and this does not influence notably the sensor response to glucose since is at excess concentration. Further increase in ATP concentration results in reducing response to glucose and, correspondingly, in higher ATP sensitivity (see Fig. 4A, curve 5). Therefore, *in vitro* determination of ATP requires that glucose concentration would be within the range of linear dependence of the biosensor response on glucose concentration. At physiological glucose concentration in the sample (3.6–6.4 mM and more), the sample should be diluted and glucose should be controlled by the glucose sensor (the second sensor) prior to bi-enzyme (GOD/HEX) biosensor can be used for measurement of ATP concentration. It is obviously that ATP concentration in diluted samples can be assessed if ATP initial value is sufficiently high. For ATP *in vivo* analysis, the developed bi-enzyme biosensor cannot be used or it should have a wider linear range of glucose measurement covering physiological glucose concentrations. Such microbiosensor is quite feasible by usage of additional polymer membranes in biosensor design [24].

Operational stability and reproducibility are important characteristics of biosensors. The dependence of bi-enzyme microbiosensor responses to ATP and glucose on duration of continuous operation was studied under optimal working conditions, i.e. pH 7.4 and 2 mM Mg^{2+} (Fig. 5). Reproducibility test for one microbiosensor is presented in Fig. 5A, and for series of microbiosensors—in Fig. 5B. It was shown that during 8.5-h continuous work at 25 °C responses of one microbiosensor are very slightly varied in the case of glucose measurement and for ATP analysis. Reproducibility is very high. Variation of sensor response inside of group of 4 microbiosensors is not so high, but quite good (see Fig. 5B).

To compare sensor responses at room and physiological temperatures (22 and 37 °C) and to understand what enzyme in bi-enzyme system (GOD/HK) is more responsible for stability of developed microbiosensor, the study of temperature dependence of sensor response was done (Fig. 6). It was shown, that at increase temperature from 22 to 45 °C sensor responses to glucose and ATP were increased. Increase of temperature from 22 to 37 °C leaded to increasing sensor responses to glucose and ATP in almost 2 times. But at subsequent increasing temperature from 45 to 60 °C the response of bi-enzyme microbiosensor to ATP was decreased almost to zero, whereas response to glucose continued to increase

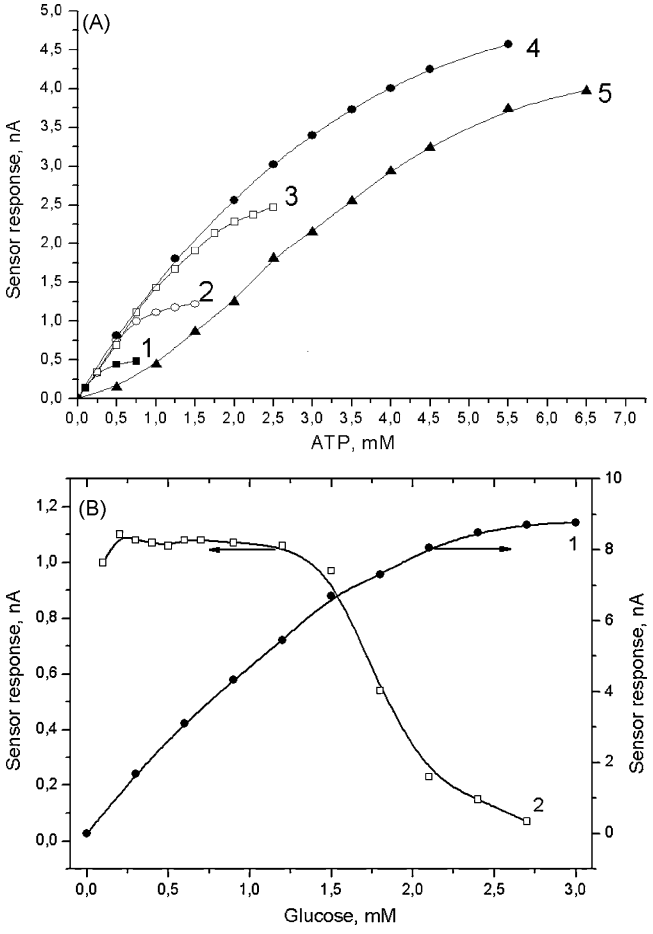

Fig. 4. (A) Calibration curves for ATP biosensor at different glucose concentrations: 1–0.1; 2–0.3; 3–0.8; 4–1.6; 5–2.5 mM. (B) Dependence of microbiosensor responses to glucose (1) and to 0.5 mM ATP (2) at different glucose concentration. Measured in 25 mM HEPES buffer with 2 mM Mg, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl.

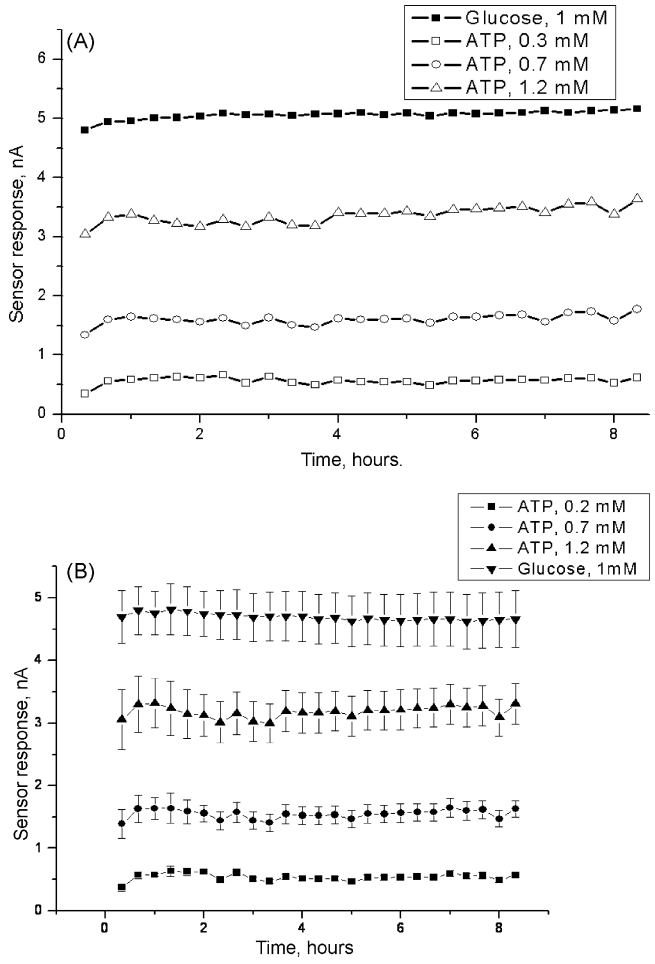

Fig. 5. Reproducibility and operational stability test during 8.5 h for one biosensor sensitive to glucose and ATP (A) and for group ($n = 4$) biosensors with the same constructions (B). Measurements were done in 25 mM HEPES buffer with 2 mM Mg^{2+}, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl reference electrode.

and only after 65 °C it sharply starts to decrease. It is quite clear, that in this bi-enzyme system GOD is more stable enzyme than HK, and to increase general stability of the system it is necessary to stabilize HK.

As to storage stability of the bi-enzyme microbiosensor, the responses to glucose and ATP did not change at least for 3 months while stored in dry at $T = -20$ °C.

Selectivity is also a vitally important characteristic for any biosensor or sensor device. This characteristic is determined by the transducer selectivity, on the one hand, and the enzyme selectivity—on the other. The selectivity of platinum electrodes, used in our case, toward hydrogen peroxide is not high enough unless special approaches are applied. Special surface modification is required because numerous electroactive substances are oxidized on the electrode surface at a potential of +600 mV generating an electrochemical signal [20,25]. That is why the platinum electrode with a PPD film electrodeposited on its surface was used in this work as a transducer. This procedure allowed to obtain a transducer which was almost insensitive to the presence of electroactive substances (ascorbic acid, uric acid, aspartic acid, dopamine, L-cysteine and acetaminophen) at their physiological concentrations in the medium analyzed (Fig. 7). It is remarkable that PPD film is stable enough and selectivity of microbiosensors covered by such films to electroactive interferences remains unaffected during at least 6-h continuous work [20,25].

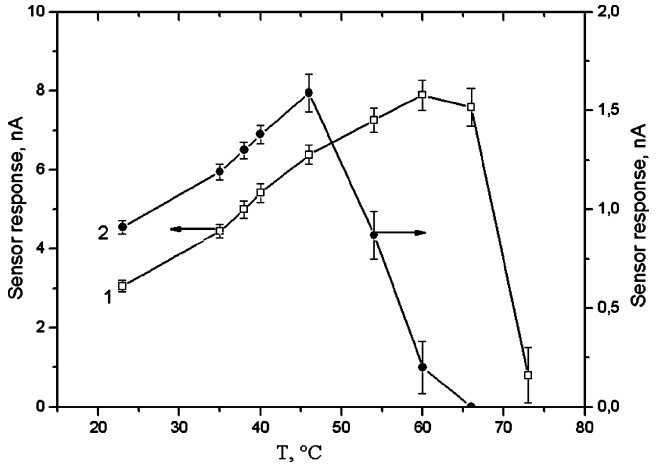

Fig. 6. Temperature dependence of microbiosensor responses to 0.5 mM glucose (1) and to 0.5 mM ATP (2). Measurements in 25 mM HEPES buffer, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl reference electrode.

As for selectivity of the enzymatic system applied, the bi-enzyme microbiosensors were tested with regard to their sensitivity to ATP analogues (or substances with similar structure), such as ADP, AMP, GTP and UTP. Practically no response was obtained at the concentration of the mentioned interfering substances in the tested medium even as high as 500 μM while the response to

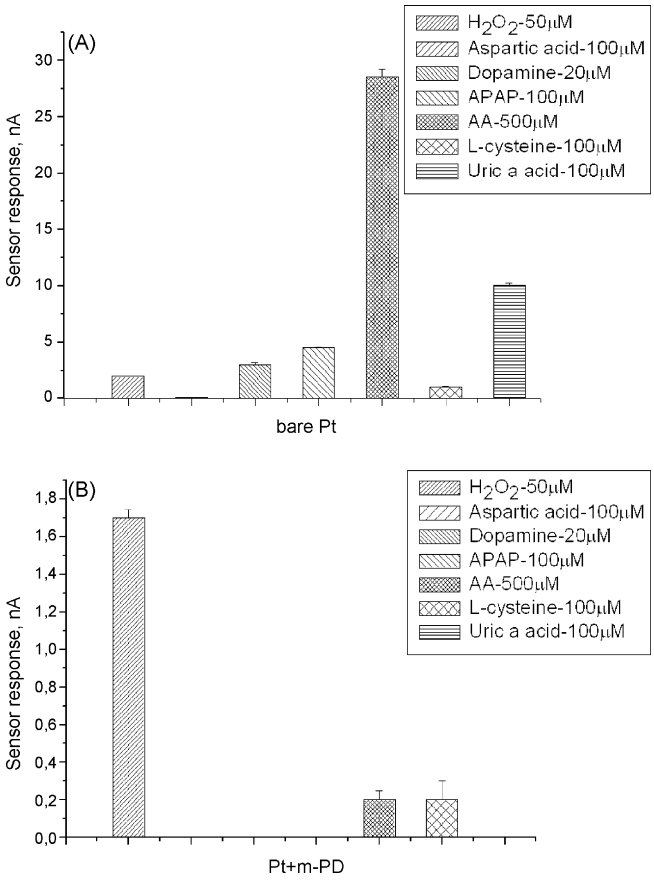

Fig. 7. (A) Responses of bare platinum electrode and (B) one covered with poly-m-phenylendiamine to hydrogen peroxide and different electroactive interfering compounds. Microelectrode covered with poly-m-phenylendiamine demonstrated negligible responses to aspartic and uric acids, dopamine and APAP. Measurements in 25 mM HEPES buffer, pH 7.4, at constant potential +0.6 V vs. Ag/AgCl reference electrode.

Table 2
Influence of interfering analogues of ATP on the glucose and ATP microbiosensors responses. Measurements were done in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs. Ag/AgCl reference electrode.

Responses of glucose microbiosensor (nA)		Responses of bi-enzyme ATP microbiosensor (nA)			
To 0.5 mM glucose without interfering agents[a]	To 0.5 mM glucose + interfering agents[a]	To 0.5 mM glucose without interfering agents[a]	To 0.5 mM ATP + 0.5 mM glucose without interfering agents[a]	To 0.5 mM glucose + interfering agents[a]	To 0.5 mM ATP + 0.5 mM glucose + interfering agents[a]
3.043 ± 0.045	3.029 ± 0.086	3.026 ± 0.045	1.124 ± 0.023	2.943 ± 0.112	1.149 ± 0.048

[a] Interfering agents: 0.5 mM GTP + 0.5 mM UTP.

500 μM ATP remained the same both in the presence of interfering substances and in their absence (Table 2).

Considering the obtained selectivity results a conclusion can be done that the developed bi-enzyme microbiosensor by its characteristics is suitable for ATP analysis in biological samples. Moreover, in the case of biological samples consisting glucose to measure ATP it is necessary to use two biosensors: based on glucose oxidase (glucose biosensor) and based on glucose oxidase and hexokinase (ATP biosensor).

4. Conclusion

The microbiosensor based on cylindrical platinum microelectrode and co-immobilized glucose oxidase and hexokinase have been developed for *in vitro* analysis of ATP concentrations. The microbiosensor can measure ATP concentrations down to the 2.5 μM detection limit with response time about 15–20 s. The applied PPD layer allows rejecting almost completely interferences caused by electroactive compounds like biogenic amines and their metabolites, ascorbic acid, uric acid and L-cystein. Moreover, the microbiosensor developed is insensitive to ATP analogues, such as GTP and UTP too. Thus, the microbiosensor method is potential for *in vitro* study of ATP concentration in low micromolar range in microvolumes to study kinetics of the process of consumption or production of macroergic triphosphate molecules. Moreover, the ATP microbiosensor developed can be efficiently used in food industry as a tool for detection of micro-fungal contamination as well as in drug design at development of inhibitors for specific kinases and others target enzymes.

Acknowledgments

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