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Développement de biocapteurs pour la détermination de substances biologiquement actives

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Résumé

Les biocapteurs sont des moyens d'analyse en plein essor à la fois rapides, sélectifs et peu coûteux applicables à des domaines extrêmement variés (environnement, santé, agroalimentaire,…). Dans ce type d'outil, un élément sensible de nature biologique (anticorps, enzyme, microorganisme, ADN…) doté d'un pouvoir de reconnaissance pour un analyte ou un groupe d'analytes est associé à un transducteur pouvant être de type électrochimique, optique ou thermique.

Dans ce travail, nous nous sommes intéressés au développement de trois biocapteurs pour la détection de substances biologiquement actives. Le premier permet la détermination simultanée de l'adénosine triphosphate (ATP) et du glucose par ampérométrie, le deuxième celle de la créatine kinase, et le troisième est un biocapteur conductimétique pour la quantification de l'ATP. Dans les deux premiers biocapteurs, deux enzymes (l'hexokinase et la glucose oxydase) sont immobilisées à la surface de microélectrodes constituées d'un disque de platine. Le troisième biocapteur est basé sur l'immobilisation de l'hexokinase sur des microélectrodes interdigitées en or. L'immobilisation est réalisée dans tous les cas par co-réticulation des enzymes en présence d'albumine de sérum bovin à l'aide de glutaraldehyde. Les caractéristiques analytiques des biocapteurs ont été déterminées et différentes procédures ont été développées pour l'analyse d'échantillons réels. Les biocapteurs ont pu être appliqués avec succès à la quantification de l'ATP, du glucose et de la créatine kinase dans des préparations pharmaceutiques et du sérum sanguin.

Mots-clés: Biocapteurs, ampérométrie, conductimétrie, enzymes immobilisées, glucose oxydase, hexokinase, adénosine triphosphate, glucose, créatine kinase.

Abstract

Biosensors are rapid, selective and inexpensive devices that combine a biological recognition element, the so-called bioreceptor (e.g. enzymes, antibodies, DNA or microorganisms) to a physical transducer (e.g. electrochemical, optical, thermal or piezoelectrical). They can be used to detect one specific analyte or one family of analytes for a wide range of applications (e.g. environment, food, health).

In this work, the detection of biologically active substances was targeted. A biosensor system for simultaneous determination of adenosine triphosphate (ATP) and glucose, a biosensor for creatine kinase analysis, and a novel conductometric biosensor for ATP determination were developed. In the first two biosensors, two enzymes (hexokinase and glucose oxidase) were immobilized at the surface of platinum disc microelectrodes for amperometric detection. The third biosensor was based on hexokinase immobilized onto gold interdigitated microelectrodes for conductometric detection. In all cases, the enzymes were co-immobilized with bovine serum albumin by cross-linking using glutaraldehyde. Analytical characteristics of the biosensors were determined and different procedures were developed for real samples analysis. The biosensors could be successfully applied to the determination of ATP, glucose, and creatine kinase in pharmaceutical samples and blood serum.

Keywords: biosensors, amperometry, conductometry, immobilized enzymes, glucose oxidase, hexokinase, adenosine triphosphate, glucose, creatine kinase.

Résumé (détaillé)

L'adénosine-5'-triphosphate (ATP) est une molécule organique qui est le principal fournisseur d'énergie aux cellules. Elle est synthétisée par phosphorylation de l'adénosine-5'-diphosphate (ADP) via les principales voies métaboliques cellulaires - la glycolyse et la phosphorylation oxydative. L'ATP est également produite lors de nombreuses réactions enzymatiques. Les molécules d'ATP formées sont impliquées (directement ou indirectement) dans la plupart des processus cellulaires, tels que la synthèse de composés, le transport, la régulation, etc. En outre, l'ATP participe à de nombreuses réactions physiologiques telles que la transmission de l'influx nerveux, la thromborégulation, la régulation du système immunitaire et la motilité gastro-intestinale. Ainsi, la surveillance de la concentration en ATP, en particulier dans les échantillons biologiques, ainsi que in vivo dans les tissus et les cellules, est très importante.

Les méthodes existant pour la détermination de l'ATP comprennent des tests luciférase, des techniques spectrophotométriques ou chromatographiques. Ces méthodes sont sensibles, assez sélectives et simples d'utilisation. Cependant, elles nécessitent un équipement volumineux et coûteux, et il est difficile (voire impossible) de les utiliser en temps réel ou pour des mesures in vivo. Le développement de biocapteurs à ATP sensibles peut aider à remédier à ces inconvénients.

Les biocapteurs représentent un groupe varié de dispositifs d'analyse qui se développent rapidement depuis les 30 dernières années. Les progrès récents réalisés dans le domaine de la microélectronique, la science des matériaux, la biochimie et la synthèse chimique a permis le développement de biocapteurs de sensibilité accrue vis-à-vis d'une très large gamme de substances organiques et inorganiques. Les nouvelles tendances dans le domaine des biocapteurs se concentrent maintenant sur l'application de nanostructures, la création de systèmes complexes de biocapteurs pour la détection multianalyte, incorporant microélectronique et microfluidique dans des dispositifs uniques de type laboratoire sur puce. D'énormes efforts ont été et restent consacrés au développement de biocapteurs visant des applications cliniques, mais les biocapteurs destinés à la surveillance écologique sont également en amélioration constante.

Les biocapteurs électrochimiques sont les plus anciens et leur développement a été l'objet d'un très grand nombre de travaux. Ils peuvent être classés selon quatre catégories : les biocapteurs ampérométriques, impédimétriques, potentiométriques et conductométriques,

l'ampérométrie représentant la technique de détection électrochimique la plus couramment utilisée.

Dans ce travail, nous avons développé différents biocapteurs électrochimiques pour la détermination de l'ATP, le glucose, et de la créatine kinase. La détection de ces substances dans les échantillons biologiques et cliniques est importante pour la recherche et à des fins médicales. Un système composé de deux biocapteurs ampérométriques a été proposé, le premier permettant la détection du glucose et le second celle de l'ATP. Il fournit une détermination précise de l'ATP et plus d'informations sur l'échantillon par rapport à un seul biocapteur à ATP. Ces biocapteurs ampérométriques sont basés sur des électrodes à disque de platine sur lesquels des enzymes sont immobilisées (glucose oxydase, GOx, dans le premier cas, et mélange GOx/hexokinase (HEX), dans le second). Nous avons par ailleurs développé le premier biocapteur conductimétrique sensible à l'ATP offrant à la fois simplicité et faible coût. Enfin, nous proposons un biocapteur ampérométrique réutilisable pour la détermination rapide et simple de la créatine kinase. Les caractéristiques analytiques des biocapteurs ont été étudiées et des procédures ont été développées pour l'analyse d'échantillons réels. Afin de montrer la potentialité de ces biocapteurs, ils ont été utilisés pour la mesure des concentrations d'ATP, de glucose et de créatine kinase dans des échantillons pharmaceutiques et des échantillons de sérum sanguin.

La combinaison des deux biocapteurs ampérométriques a permis une détermination précise et simultanée des concentrations de glucose et d'ATP dans des échantillons aqueux. La concentration optimale des enzymes dans la biomembrane a été déterminée comme étant de l'ordre de 2 à 2,5 %. Les conditions optimales de réticulation des enzymes sur les électrodes ont été sélectionnées, à savoir, la concentration de l'agent de réticulation glutaraldéhyde - 0,2%, le temps d'immobilisation - 40 min. L'exactitude de mesure du système de biocapteurs a été améliorée par le dépôt d'un film de poly(phénylènediamine) sur la surface des électrodes. Il a été démontré que la sensibilité du biocapteur vis-à-vis de substances électroactives interférantes telles que les acides ascorbique et urique devient très faible après le dépôt du film.

Il a également été montré qu'il est possible de modifier la limite et la gamme de détermination de l'ATP par variation des concentrations en glucose et en Mg^{2+}. La réponse du système de biocapteur est stable et reproductible sur une journée. L'écart type relatif des réponses au glucose est de 3-6%, à l'ATP de 8-12%. Le temps de réponse du biocapteur est d'environ 5 min. Après analyse, les biocapteurs peuvent être lavés dans un tampon de travail et réutilisés plusieurs fois. Le système de biocapteur a montré une bonne stabilité au stockage

s'il est conservé à -18 ° C, le stockage de 50 jours a entraîné une diminution de la réponse à l'ATP de 43% et n'a provoqué aucune diminution de la réponse au glucose. Nous avons montré la possibilité de mesures simultanées des concentrations d'ATP et de glucose dans des préparations pharmaceutiques. Dans la suite de ce travail, nous envisageons de développer des systèmes encore plus complexes de multi-biocapteurs pour la détermination simultanée de plusieurs autres substances telles que le lactate, le glutamate, la choline et d'autres.

Dans la deuxième partie de ce travail, un biocapteur enzymatique ampérométrique pour la détermination de l'activité de la créatine kinase (CK) dans le sérum sanguin a été développé. La créatine kinase est une enzyme de marquage biologique, et l'augmentation de sa concentration dans le sang est due à certaines maladies cardiaques et musculaires. Le biocapteur est basé sur le biocapteur de l'ATP qui a été utilisé auparavant comme une partie du système de biocapteur, mais la procédure d'analyse et de composition de travail de la solution tampon sont différentes. La détection de la CK nécessite l'ajout de deux substrats (ADP et phosphate de créatine). Les concentrations optimales d'ADP et de phosphate de créatine ont été déterminées comme étant égales à 1 mM et 10 mM, respectivement. Le facteur de dilution optimal de l'échantillon a par ailleurs été déterminé. Le biocapteur a été caractérisé par une bonne reproductibilité des réponses pendant la mesure. L'écart-type relatif obtenu à partir de 15 mesures varie entre 7 et 18% aux différentes concentrations de CK évaluées.

La détermination de l'activité en CK a été réalisée dans des échantillons de sérum sanguin dopés. Les résultats obtenus à partir des biocapteurs sont en assez bon accord avec les valeurs de CK calculées, l'erreur se situant dans la fourchette 20-25%. Le biocapteur pourrait convenir pour des mesures rapides de l'activité en CK dans le sérum sanguin de patients atteints d'infarctus aigu du myocarde ou d'autres blessures musculaires. La petite taille du dispositif ainsi que la simplicité de préparation des biocapteurs et les conditions de mesure sont des avantages par rapport aux méthodes traditionnelles de détermination de la CK. Le principal inconvénient du biocapteur proposé est une influence du glucose sur la sensibilité du capteur biologique. Il est donc nécessaire de choisir entre la détermination approximative de l'activité de CK pendant une mesure et une détermination précise au cours de deux mesures, la première mesure donnant la concentration en glucose nécessaire pour la sélection du facteur de dilution optimal de l'échantillon au cours de la deuxième mesure.

Le mode opératoire décrit pour la détermination de l'activité de la CK peut être adapté pour la détermination d'autres kinases à des fins médicales ou de recherche. Il convient de

noter que le procédé proposé pourrait être utilisé pour évaluer le taux de production de l'ATP ou d'une dégradation dans la cellule de travail, ce qui peut être utile à des fins de recherche.

La troisième partie du travail a été consacrée à l'élaboration d'un biocapteur conductimétrique pour la détermination de l'ATP. Les transducteurs utilisés pour l'élaboration de biocapteurs conductimétriques sont généralement simples et peu chers comparés avec ceux utilisés pour les détections potentiométrique ou optique, ou pour d'autres types de biocapteurs. En outre, la détection conductimétrique ne nécessite pas l'emploi d'une électrode de référence complexe. Dans ce contexte, l'objectif était de développer un biocapteur conductimétrique à ATP plus simple et moins cher en comparaison avec d'autres biocapteurs. Une procédure a été élaborée afin d'éviter toute interférence de substances chargées, ce qui constitue un gros problème pour les biocapteurs conductométriques.

Le biocapteur proposé est basé sur l'enzyme HEX, immobilisée sur la surface du capteur conductimétrique. La meilleure immobilisation de l'enzyme a été obtenue par réticulation à l'aide de glutaraldéhyde pendant 30 min en présence d'albumine de sérum bovin (BSA). Les caractéristiques du biocapteur dépendent de la composition du tampon de travail. Le tampon optimal est l'HEPES 5 mM, pH 7,4, contenant 3 mM d'ions magnésium. L'examen de la relation entre la sensibilité du biocapteur à l'ATP et la concentration en glucose a montré que la concentration optimale de glucose est de 0,2 mM. La limite de détection obtenue pour l'ATP est de 15 μM. L'écart type relatif obtenu à partir de 10 mesures consécutives est de 10,3%. Le biocapteur développé reste stable pendant au moins une semaine lorsqu'il est utilisé une fois par jour. Il peut être appliqué pour la détermination de la concentration en ATP dans des préparations pharmaceutiques ou d'autres échantillons aqueux. L'avantage principal du biocapteur proposé est son faible coût (environ 1 USD, ou même encore moins si l'on envisage une production de masse). Le système de mesure est par ailleurs moins coûteux que le potentiostat nécessaire aux mesures ampérométriques ou impédimétriques.

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ABBREVIATIONS

A	**AA**	Ascorbic acid
	ATP	Adenosine-5'-triphosphate
	ADP	Adenosine-5'-diphosphate
B	**BSA**	Bovine serum albumin
C	**CK**	Creatine kinase
	CTP	Cytidine-5'-triphosphate
F	**FET**	Field-effect transistor
G	**GA**	Glutaraldehyde
	GCE	Glassy carbon electrode
	GOx	Glucose oxidase
	GPDH	Glucose-6-phosphate dehydrogenase
	GTP	Guanosine-5'-triphosphate
H	**HEPES**	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
	HEX	Hexokinase
	HPLC	High-performance liquid chromatography
I	**ISFET**	Ion-selective field-effect transistor
L	**LOD**	Limit of detection
P	**PPD**	Poly(phenylenediamine)
S	**SH**	Salicylate hydroxylase
	SPE	Screen printed electrode
U	**UTP**	Uridine-5’-triphosphate

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INTRODUCTION

GENERAL INTRODUCTION

Adenosine-5'-triphosphate (ATP) is an organic molecule which 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. The resulting ATP molecules are involved (directly or indirectly) almost in all cellular processes such as synthesis of compounds, transport, movements, regulation, etc. Furthermore, ATP takes part in many physiological reactions such as transmission of nerve impulses, thromboregulation, regulation of immune system and gastrointestinal motility. Thus, 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.

Existing methods for ATP determination include luciferase assays, spectrophotometry, and chromatographic techniques. These methods are sensitive, quite selective and convenient. However, they require large and expensive equipment, and it is hard (often impossible) to use them for real-time or *in vivo* measurements of ATP concentrations. Development of ATP-sensitive biosensors can help to overcome these disadvantages.

Biosensors represent a diverse group of analytical devices that have been rapidly developing since the last 30 years. The first biosensors were designed half a century ago as simple devices for the fast determination of substances in liquid samples. However, extensive development of biosensors actually started in 1980s, when pioneer research groups proposed a new generation of effective analytical devices combining transducers and biomaterials of various types. Since then, the number of papers describing biosensors has exploded. Progress in microelectronics, materials science, biochemistry and chemical synthesis has resulted in the development of biosensors with enhanced sensitivity to a very wide range of organic and inorganic substances. New trends in the biosensor field are now focused on the application of nanostructures, the creation of complex biosensor systems for multianalyte detection, embedding microelectronics and microfluidics into a single lab-on-a-chip device. Huge efforts are devoted to biosensors targeting clinical applications, but biosensors for ecological monitoring are also under constant improvement.

Electrochemical biosensors are the oldest and the most successful group of biosensors. They are divided into four classes, i.e., amperometric, impedimetric, potentiometric and conductometric biosensors, amperometric biosensors representing the largest class.

In the present work, we developed different electrochemical biosensors for the determination of ATP, glucose, and creatine kinase. Detection of these substances in biological and clinical samples is important for research and medical purposes. We propose a two-biosensor system for ATP and glucose detection, which provides more accurate determination of ATP and gives more information about the sample in comparison with a single ATP biosensor. For the first time we describe a conductometric ATP-sensitive biosensor, which offers simplicity and low cost advantages. Finally, we propose a reusable amperometric biosensor for rapid and simple determination of creatine kinase.

The present manuscript will be organized in five chapters. The first chapter will be dedicated to a bibliographic review of biosensors as analytical devices (classification, new trends, and applications). Biological roles of ATP and creatine kinase, importance of their determination in biological samples and modern methods of determination will be also discussed.

The second chapter will describe materials, methods and devices used in this work.

In the third chapter, a biosensor system for simultaneous determination of ATP and glucose will be proposed. Analytical characteristics of the system will be studied, and procedure for simultaneous determination of ATP and glucose will be described.

The fourth chapter will report the development of a biosensor for the determination of creatine kinase. This biosensor is based on an ATP biosensor applied to the detection of ATP produced by creatine kinase. A procedure for the evaluation of creatine kinase level will be developed and applied to the measurements of creatine kinase in blood serum samples.

The fifth chapter will focus on the elaboration of a conductometric ATP biosensor and its application to ATP determination in pharmaceutical samples.

The last section of this manuscript will summarize the results obtained in this work and discuss about the perspectives.

CHAPTER I: BIBLIOGRAPHIC STUDY

I.1. Generalities about Biosensors

Analytical chemistry is a branch of chemistry that aims at the detection and quantification of substances in samples. Development of analytical chemistry started simultaneously with emerging of modern chemistry in Middle-Ages. First, chemists created simple separation methods (like precipitation or distillation) combined with non-instrumental detection of reaction with human senses (visual change of color or appearance of a precipitate). These methods allowed qualitative or semi-quantitative determination of some substances. Since then, analytical chemistry developed dozens of different techniques, from quite simple to very complicated methods. Nowadays, analytical chemistry is still a quickly growing field because it is very useful for chemical, physical and biological studies. Practical applications like toxins determination or quality control are also very important [1].

Basis of modern analytical analysis is high-tech equipment like high-performance liquid chromatography (HPLC), spectroscopes or mass spectrometers. Capabilities of these techniques are very large. However, their back side is high price and complexity. Alternative methods of analysis are developing, which are less powerful but much cheaper and easy to use. Sensors, including biosensors, belong to these alternative methods of analysis.

The first biosensor was created more than 50 years ago, in 1962 [2]. This biosensor was based on a pH-sensitive electrode with immobilized glucose oxidase for the measurement of glucose concentration in human blood. After this pioneer work, quick development of glucose biosensors was carried out in many laboratories, what resulted in a number of commercial glucometers available nowadays.

Intensive studies of different types of biosensors started in 1980-s in Europe and United States. Since then, biosensor development is still going on, spreading all over the world. This may be attributed to the fact that biosensors combine low price, fast response time and operational simplicity with the specificity and sensitivity of a biomaterial.

Modern biosensor development aims at miniaturizing the devices and increasing their sensitivity by incorporating nanomaterials.

I.1.1. Working Principles of Biosensors

Accordingly to the classical definition of International Union of Pure and Applied Chemistry, “a biosensor is an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element.” [3]. A biosensor consists of biomaterial acting as a receptor

immobilized in intimate contact with a physicochemical transducer which converts the biological recognition event between the receptor and analyte of interest into an exploitable signal. Biosensor is considered to be a variant of chemical sensor with a biochemical receptor mechanism instead of a chemical one.

As presented in Fig. 1, several events occur during the analysis of an analyte by the biosensor:

1) biomaterial comes into contact with the sample (liquid or gaseous) containing the target analyte. The latter reacts with the biomaterial (i.e. the substance binds to the biomaterial or biomaterial catalyses a chemical reaction involving this substance),

2) this process induces some changes in physical or chemical properties of the near-transducer solution (e.g., pH is changed) or transducer surface changes its characteristics (e.g., optical properties are modified),

3) these changes directly depend on the concentration of the target substance and are measured by the transducer which is connected to the recording device. The recording device gives information to a computer and the result is shown on a display. In some commercial products, the recording device and the display are embedded into the transducer, so all the analysis can be done at home without additional equipment,

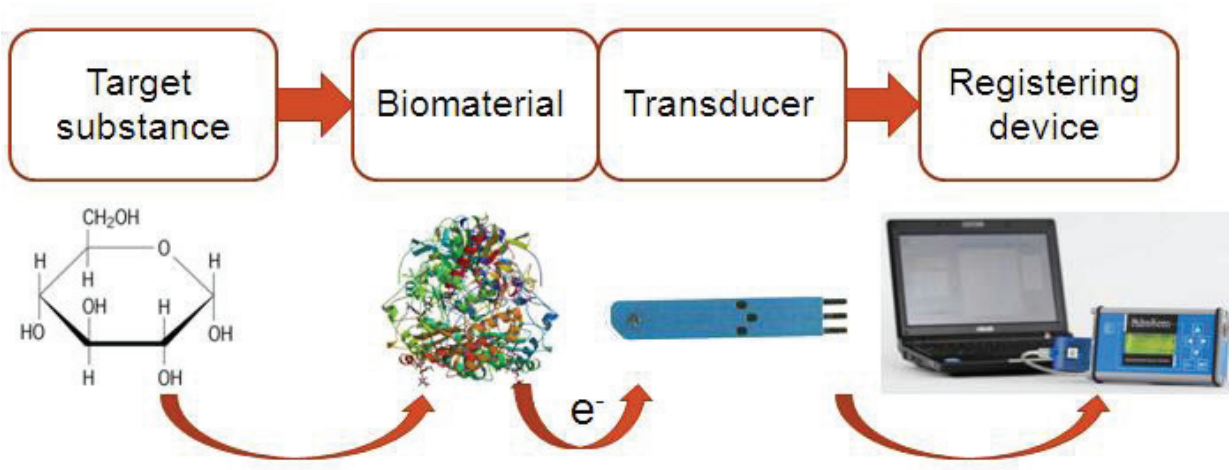

Figure 1. General working principle of a biosensor (top) and principle of the amperometric glucose biosensor (bottom).

I.1.2. Types of Biosensors

There are two main classifications of biosensors. Biosensors are usually classified according to the type of biomaterial or the type of transducer (Fig. 2) [4].

The biorecognition element of a biosensor can have catalytic activity or just act as a receptor. Purified enzymes are most widely used as biocatalysts, and enzyme biosensors are the largest group of biosensors, which is due to the wide range of enzyme-catalyzed chemical reactions, the high activity, good selectivity and stability of many enzymes. Immobilized tissues, cells (including bacteria) or even cell organelles can also serve as sensitive elements. Such biomaterial is much cheaper than enzymes, but generally offers lower selectivity and sensitivity as well as slower response time. Alternatively, non-catalytic receptor molecules can be used, including antibodies, cellular receptors, nucleic acids, biomimetics, etc. Biosensors based on antibodies offer excellent selectivity and sensitivity, but this biosensors are single-use because of practically irreversible interaction between antibody and antigen. DNA-based biosensors are very promising since they can detect genetic disorders, viruses, bacteria or even differentiate several strains of bacteria [5, 6].

Biosensors may be also classified according to the type of transduction (physical property) used for the detection of the interaction between biomaterials and target substances. The transducer can be optical, piezoelectric, thermal, and electrochemical. Electrochemical transducers are widely used in biosensors. These transducers are divided into amperometric [7], potentiometric [8], conductometric [9], and impedimetric [10] biosensor subgroups.

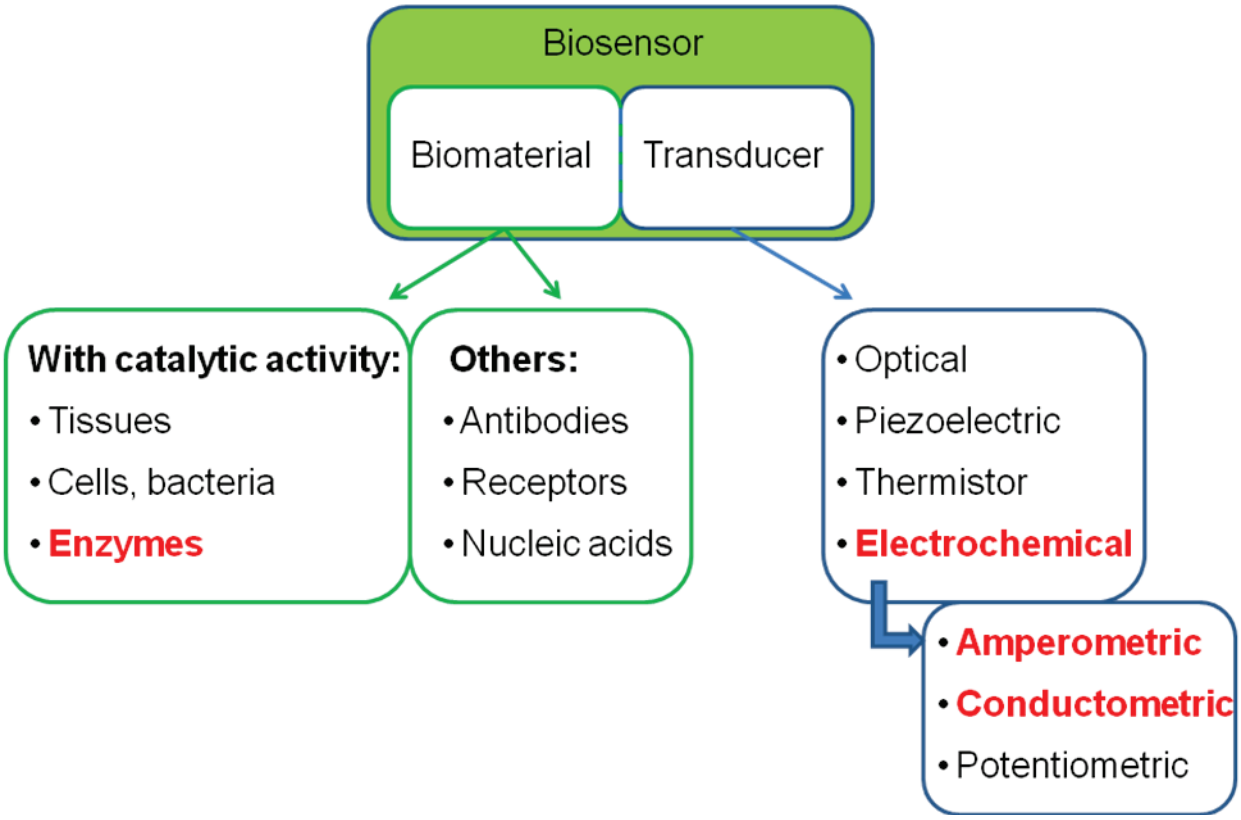

Figure 2. Schematic classification of biosensors.

In electrochemical biosensors, an electrode is used as signal transducer. The electrode can be sensitive to changes in pH (ISFETs), conductance (conductometric biosensors), impedance (impedimetric biosensors), or can measure redox reactions that take place at the electrode surface (amperometric biosensors).

Amperometric biosensors are the oldest and the most popular ones. Impedimetric biosensors are also widely used. Other types of electrochemical biosensors (conductometric and potentiometric) are much less popular due to their limitations.

What makes electrochemical biosensors so popular? They have the classical advantages common to all biosensors: low-cost, relatively fast and simple procedure of analysis, small size of measuring setup. A wide range of biochemical reactions can be detected by electrochemical transducers. Success of electrochemical biosensors is also connected with rapid development of screen printed electrodes, which are versatile, easy to miniaturize, low-cost, and sensitive [11, 12]. Finally, electrochemical biosensors have been successfully applied to the analysis of real samples, including blood serum, milk, juices, and waste waters, showing the real efficiency of this type of biosensors.

Among the other types of biosensors, the first place belongs to optical biosensors. Surface plasmon resonance biosensors increase popularity nowadays. Their typical applications involve real-time detection and studies of biological binding events, and usage as a detection method in polymerase chain reaction [13]. Thermal and colorimetric biosensors are still under development in some laboratories. Other types of biosensors are more rarely used.

I.1.3. Current Trends in Electrochemical Biosensor Development

The field of electrochemical biosensors is quite old, but active research still continues. Current studies are focusing on the following objectives.

Integration of Nanostructures

Integration of nanostructures into biosensors is expected to improve their analytical characteristics, i.e. sensitivity, linear working range, and stability. Such nanostructures as carbon nanotubes, conductive polymers, and different nanoparticles are used to increase electron transfer between biorecognition element and transducer. This is a common approach for development of the third generation of electrochemical enzyme biosensors, which are

interference free due to low operation potential and direct electron transfer from enzymes to electrode [14].

Magnetic beads have been recently studied as supports and/or carriers for biomaterial. They have particular characteristics that make them useful in biosensor configurations: they are biocompatible, they have large surface area, they can be easily functionalized, and their location and transport can be controlled by a magnetic field [15].

Furthermore, novel methods of enzyme immobilization can be developed by using electrospun nanofibers. Such fibers can be easily produced by electrospinning and they are expected to increase the surface area of electrode, and thus increase enzyme loading. Application of the fibers can increase biocompatibility of the electrode surface. Furthermore, the fibers can conduct electrons between enzymes and electrodes, thus enabling direct electron transfer. Finally, the fibers can incorporate other nanostructures such as carbon nanotubes and gold nanoparticles for further enhancement of the biosensor work [16-18].

Development of Multiplexed Biosensor Systems

Biosensor systems with several sensitive elements (biosensor arrays and multibiosensors) can simultaneously detect concentrations of multiple analytes present in one sample. Such determination is important for research and clinical applications since it gives more information about the sample in comparison with a single biosensor [19].

Furthermore, development of microelectrode arrays is promising because they enhance temporal resolution, have rapid mass transfer, high current densities and high signal-to-noise ratio, and thus arrays improve sensitivity and accuracy of the biosensors [20].

Microfluidics

Perspective approach is integration of biosensors into fluid-handling and/or sample processing devices. Microfluidic platforms are considered as a promising approach for integration of sensing unit (biosensor) and structures for sample preparation (i.e. sample preconcentration or separation). This allows integration of one or more laboratory functions on a single substrate, and analysis becomes more automatic. Furthermore, size of such systems can be decreased up to several millimeters, what decreases the amount of materials required for production and thus decreases overall costs of the system. Such systems can be rapidly produced in large quantities by several lithography techniques. Due to the small size, microfluidic systems are portative and require a small volume of sample. Time of analysis can be decreased down to several seconds. Next step of the biosensor research can be integration

of biosensors into microelectromechanical systems to obtain implantable multipurpose devices. However, now the production of such systems is difficult and requires expensive equipment. [21, 22]

Development of On-Site Analyzers

On-site (*in situ*) analyzers are supposed to be portable, user-friendly and powerful enough to perform analysis outside the analytical laboratory, for example they can be used at home, at market, in hospital room, or at some natural site. Point-of-care analyzers are an example of such devices developed for medical applications. For example, home-used biosensors for the control of glucose level are already widely used, and biosensors for other substances will certainly be commercialized in the near future. Point-of-care analyzers will accelerate disease diagnosis and facilitate treatment, reduce diagnostics cost, and thus improve quality of live. Furthermore, due to their low cost and easy application, such devices will make healthcare affordable to developing and underdeveloped countries. Other promising on-site analyzers are being developed for the determination of toxic compounds is aqueous samples and different products; these analyzers are often based on enzyme inhibition and can determine different toxicants – from neurotoxins to heavy metal ions. Direct determination of toxicants is also possible by using enzymes which react specifically with some toxicants [22, 23].

Creation of Wearable Biosensors

Creation of wearable sensors and biosensors is a perspective field of research. For example, such biosensors can be integrated into clothes and perform real-time detection of substances like toxicants and explosives. If environment is dangerous, the biosensor will signal to its wearer. On the other hand, direct placement of biosensors in combination with physical sensors on the epidermis (i.e., like tattoo) gives broad opportunities for real-time monitoring of physiological parameters, such as glucose concentration, pH level, heart rate, etc. First prototypes of such systems are already developed [24-27].

Development of Fully-Printable Biosensors

Biosensor printing is considered as a very promising technique for future biosensors. Development of printing technologies allows printing of transducer itself, battery, display, and other required parts on a single sheet of plastic (e.g., polyethylene terephthalate). However, some electronics cannot be printed and should be integrated into a tiny, inexpensive

silicon chip. Such printing can dramatically decrease the price of biosensors and facilitate their mass production [28].

Development of Large Biosensor Systems

In parallel to the small, cheap, and simplified biosensor devices mentioned above, it is to be noticed that several companies are now creating high-throughput biosensor-based machines capable of rapid, accurate and convenient measurement of complex biological interactions and components. These machines may consist of several dozens of biosensors, have high level of automation and quick sample processing, and have a size and price comparable with HPLC setup. These systems are intended for usage at diagnostic and clinical laboratories as alternative to traditional sophisticated analytical equipment like spectrophotometers and HPLC. The biosensor analyzers compete with traditional methods of analysis not only in price of analysis but also in capabilities and analytical characteristics. However, development of such machines is done in large companies rather that in small research groups. For example, Biacore™ 4000 system is designed for large-scale drug discovery. It is capable of 60 hours unattended operation with parallel analysis of up to 16 targets or 4800 affinity interactions in 24 h. Biacore™ Q system offers qualitative and quantitative analysis of food products (targets include biotin, folic acid, pantothenic acid, and vitamin B_{12}). This system is fully automated, from sample injection to data analysis and report generation, and time of analysis is within minutes. Nova Biomedical developed Stat Profile Prime system that consists of chemosensors and biosensors for the detection of 10 parameters: pH, partial pressures of CO_2 and O_2, concentrations of Na^+, K^+, Cl^-, Ca^{2+}, glucose, lactate and hematocrit. The system requires only 100 µl of blood sample for the whole analysis and can analyze 45 samples in 1 hour [28-30].

I.1.4. Amperometric Biosensors

Amperometric biosensors represent the largest and the oldest group of electrochemical biosensors. During an amperometric measurement, a constant or varying potential is applied between the working electrode and the auxiliary (counter) electrode, while the current is monitored. This current is related to the concentration of the target substance. Usually a three-electrode cell, containing a working electrode, an auxiliary electrode and a reference electrode, is used for measurements. A two-electrode setup (without a reference electrode) can be also used.

Amperometric biosensors offer many advantages, among which:

1) the simple structure of working electrode which typically consist of a piece of noble metal, carbon boron-doped diamond, or glassy carbon,
2) the fast response,
3) the wide range of chemical reactions that can be detected,
4) the existence of well-developed methods to avoid interferences, e.g., application of permselective membranes to prevent access of interfering compounds to the electrode surface, or decrease of working potential, e.g., by incorporation of nanomaterials as mediators.

A major disadvantage of amperometric biosensors is the necessity to use a complex reference electrode, which is required to apply the correct working potential to the working electrode [31, 32].

Enzymes and other catalytic biomaterials are used as biorecognition elements in this type of biosensors. Glucose oxidase (GOx) is the most widely used enzyme in amperometric biosensors, firstly because of the importance of glucose biosensors for people with diabetes, and because of the excellent characteristics of GOx. On the one hand, GOx offers high selectivity toward glucose, excellent thermal and pH stability, high turnover rate, thus biosensors based on glucose oxidase have good analytical characteristics. On the other hand, GOx is a quite cheap enzyme. Glucose concentration in blood should be regularly measured in case of millions of people who suffer from diabetes. Glucose biosensors meet needs of those people and thus it is understandable that these biosensors represent the largest group of commercial biosensors. Interest to development and improvement of glucose biosensors is high [28, 33].

GOx belongs to the oxidoreductases (EC 1), which are widely used for biosensor elaboration. Chemical reactions catalyzed by these enzymes involve oxidation or reduction of substrates, and these events can be directly registered by amperometric transducers. Besides GOx, examples of other oxidoreductases used in amperometric biosensors include: d-fructose dehydrogenase (determination of fructose) [34], glutamate dehydrogenase (glutamate) [35], l-lysine-α-oxidase (lysine) [36], and many others. All mentioned biosensors are based on a direct detection of the substrates, i.e. target substance is a substrate of the enzyme, and the biosensor signal is directly proportional to the substrate concentration. However, biosensors based on enzyme inhibition are also developed, e.g., biosensor based on hydroxyphenylpyruvate dioxygenase for the detection of herbicides [37].

Furthermore, hydrolases (EC 3) are also used in amperometric biosensors. E.g., organophosphorus hydrolase catalyzes the hydrolysis of some pesticides, and electroactive species are generated [38].

Reactions catalyzed by other classes of enzymes cannot be directly detected by amperometric transducers. However, such enzymes are used multi-enzyme biosensors, where several enzymatic reactions occur, but only one is detected by the transducer [39-41]. For example, acetylcholinesterase/choline oxidase bienzymatic system is used for direct detection of acetylcholine and inhibitory analysis of different toxic compounds which are inhibitors of acetylcholinesterase. Acetylcholinesterase catalyses hydrolysis of acetylcholine to choline (this reaction is not detected by amperometry), and choline oxidase catalyzes oxidation of choline followed by production of hydrogen peroxide.

I.1.5. Conductometric Biosensors

Conductometric biosensors rely on the measurement of conductivity of the near-electrode solution. Usually they are based on interdigitated electrodes made from noble metals like gold or platinum [42].

Conductometric biosensors have following advantages:

1) simple and cheap conductometric transducers, especially in comparison with potentiometric, optical and other biosensor types,
2) no need in complex reference electrode or any other additional electrodes,
3) chemical reactions which cause conductivity changes can be directly detected.

The main disadvantage of conductometric biosensors is the sensitivity of the transducers to all charged substances, making measurement of real samples quite difficult. Differential measuring mode is usually applied to circumvent this issue [43]. Another problem is significant dependence of biosensor response on ionic strength and concentration of the working buffer.

Biorecognition elements of conductometric biosensors usually include enzymes. These enzymes catalyze chemical reactions that cause changes in conductivity of the near-electrode layer of solution. Such enzymes can be found in all enzyme classes. Examples of enzymes used in conductometric biosensors include: glycoside hydrolases [44], proteases [45], amidohydrolases [46], esterases [47], kinases [48], phosphatases [49], oxidases [50, 51].

Immobilized tissues or cells may be also used [52]. Antibody-based conductometric biosensors are more scarcely proposed, but possible [53].

I.2. Adenosine Triphosphate

I.2.1. Chemical Properties of ATP

Adenosine-5'-triphosphate (ATP) is an organic molecule which is composed from three structural parts: adenine (nitrogen-containing cyclic compound), ribose (sugar), and three phosphate groups (Fig. 3).

Under neutral pH, phosphate groups of ATP are negatively charged, and this charge is compensated by two sodium ions. Thus ATP is usually used as a disodium salt. In biological tissues, ATP charge is compensated by Mg^{2+} ions.

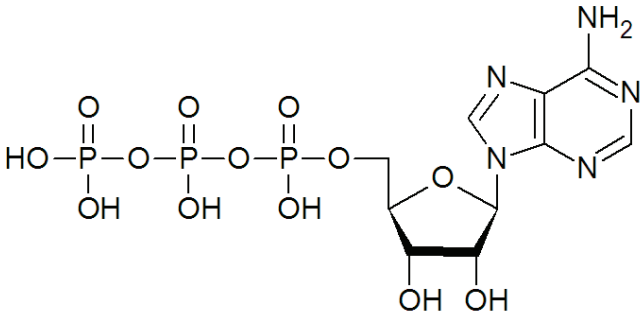

Figure 3. Chemical formula of ATP.

I.2.2. Biological Role of ATP

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 (glycolysis and the citric acid cycle/oxidative phosphorylation). In the fasting or pathological states, ATP is formed by the decomposition of fatty acids (β-oxidation) or amino acids. The resulting ATP molecules are used in the synthesis of almost all necessary cell compounds such as proteins, nucleotides, nucleic acids, fatty acids. In fact, ATP is involved almost in all cellular reactions (directly or indirectly), and in many physiological reactions [54, 55]. ATP is involved in transmission of nerve impulses in central nervous system. ATP is a neurotransmitter and a cotransmitter, and it is released in synapses [56]. Furthermore, ATP is involved into thromboregulation, serving as a signaling molecule in the cascade which controls clotting and regulation of immune system during asthma attacks [57, 58]. ATP is thought to have a significant influence in gastrointestinal motility by regulating serotonin (5-HT) release [59, 60].

Despite the wide usage of ATP in human organism, levels of ATP in blood plasma are very low. Typical values reported in the literature range from 120 to 1250 nM, This wide dispersion of results may be attributed to analytical issues, mainly coming from sample treatment [61]. In case of injuries, cells become damaged and release large amounts of ATP into the blood. However, the released ATP is rapidly degraded within few minutes [62].

Small concentrations of ATP (i.e., 2–6 nM for healthy patients and 7–11 nM for patients with overactive bladder syndrome) are also present in urine [63].

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. Determination of ATP is used to evaluate cell viability and proliferation, cytotoxicity, apoptosis, and energy transmission. Additionally, the determination of ATP concentration can be effective in the drug-design, including those based on kinase inhibitors [64-66]. Furthermore, in food industry ATP determination can be used for assessment of bacterial contamination of food and beverages [67].

I.2.3. Classical Methods of ATP Determination

Luciferase assays

The oldest and the most frequently used method of ATP determination is the luciferase method. Luciferase enzyme is found in some organisms like firebugs and it is responsible for light emission by the organisms. Luciferase produces light according to the following reaction:

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{Luciferase, } Mg^{2+}} \text{AMP} + \text{PP} + \text{oxyluciferin} + CO_2 + h\nu \qquad (1)$$

As seen, luciferase splits ATP to AMP and pyrophosphate (PP), simultaneously oxidates luciferin and emits light. The emission spectrum is located in the 470–700 nm region with a maximum at 562 nm. Luciferase is very selective to ATP and the intensity of produced light is directly proportional to the ATP concentration. This reaction was discovered in 1884 [68] and first applied for ATP determination in 1947 [69]. Nowadays, this reaction is applied in modern luminometry and dozens of other bioluminescent assays that are rapid, selective, sensitive, and therefore widespread. The most sensitive techniques have a detection limit of ATP in the fmol range [70]. The disadvantage of this method is that it is hardly applicable to real-time and *in vivo* measurements for several reasons. First, sensitive cameras should be used which makes difficult miniaturization of the measuring device. Second, luciferin and

luciferase should be added to sample, for example injected into tissue. Another problem is that activity of luciferase is influenced by the composition of working solution. Luciferase is sensitive to concentrations of different cations (like Cu^{2+}, Zn^{2+}, Ca^{2+}) and anions (like CO_3^{2-}, PO_4^{3-}, SO_4^{2-}), thus treatment steps have to be added for the ATP determination in complex samples. In addition, luciferase requires Mg^{2+} as a cofactor [71].

Spectrophotometry

ATP molecule is able to absorb light in the UV region and is a fluorophore. UV adsorption can be is used for the determination of ATP concentration by UV spectrophotometry at 260 nm [70]. This method is quite simple and does not require the addition of reagents, but it is not selective. Other adenine-containing molecules have similar peak of adsorption. To circumvent this issue, separation of the substances by HPLC is performed before detection.

ATP may be also determined using fluorescence. The reported methods are based either on fluorescence quenching of rare earth metal complexes by ATP or on fluorescence enhancement mechanisms, e.g., the fluorescence enhancement of rare earth elements (Eu or Tb) with doxycycline, norfloxacin, oxytetracycline, and phenanthroline [70]. More recently, other strategies of fluorescence enhancements involving the formation of complex between ATP and aptamers [72] or aggregation of a cationic polythiophene derivative, have been proposed [73].

Liquid chromatography

Liquid chromatography-based techniques are often used for high-precision determination of the ATP concentration. Beyond doubt chromatography (and especially HPLC) is a very powerful, sensitive, selective and quite quick method of analysis. Other advantage of this method is the possibility to detect various substances simultaneously. To improve the separation capacities of HPLC, ultraperformance liquid chromatography (UPLC) has been recently proposed for ATP determination [74]. The detection is classically performed by UV absorption or fluorescence emission spectrometry. In spite of their advantages, chromatographic methods require skilled personnel and sophisticated expensive equipment. Sometimes sample pretreatment should be made prior to the analysis. In addition, chromatography cannot be used for real time and *in vivo* measurements of ATP concentration [70, 75].

Microdialysis

Microdialysis is a method for obtaining liquid samples from a living organism without significantly damaging the organism. Microdialysis is used to measure the concentrations of ATP or other purine molecules in living tissues such as nervous tissue. This method is based on a small probe (tube or catheter) that is implanted into the tissue. Small molecules and water from tissue diffuse inside the probe and form dialysate. Then the dialysate flows through the tube and is collected in the outer side of the probe and is analyzed by an appropriate analytical method. An advantage of the microdialysis is the possibility to determine multiple substances in each sample of dialysate by applying different techniques. However, the temporal resolution of microdialysis is not very good since the production of dialysate is quite slow [76-78].

I.2.4. Electrochemical Biosensors for ATP Determination

Biosensors are promising devices for ATP determination. As compared to traditional methods, ATP biosensors offer the following advantages:

1) the cost of measuring setup is quite low (1000 to 5000 Euros),
2) it may be portative and easily transported for on-site measurements,
3) it is more easy to use sensors than HPLC or other high-tech methods,
4) enzyme biosensors can be used for real-time measurement of ATP concentration. For example, the biosensors can determine gradual increase or decrease of ATP concentration. This is useful in biochemical research for studying kinetics of different reactions,
5) microbiosensors can be implanted into tissues to measure ATP concentrations *in vivo*.

However, some disadvantages should be mentioned:

1) many biosensors, especially non-enzymatic, are sensitive not only to ATP but also to structurally similar molecules like adenosine diphosphate (ADP), guanosine triphosphate (GTP), uridine triphosphate (UTP), or cytidine triphosphate (CTP),
2) most enzyme-based biosensors require constant concentration of either glycerol or glucose because these biosensors are based on two-enzyme systems. Thus, changes in glycerol or glucose concentration can decrease the accuracy of measurement method,

3) different ions, as Mg^{2+}, may influence enzymes activity. Thus, it is difficult to accurately measure concentration of ATP in samples of complex composition, as it is also the case for luciferase-based assays,
4) electrochemical biosensors suffer from interference, coming from electroactive substances like ascorbic acid or dopamine. Chemical reactions which involve these substances can cause non-specific signals and interfere measurements. Despite effective methods to avoid interference exist (e.g., application of permselective membranes or decrease of working potential), a control sensor without enzyme is generally measured in parallel to control non-specific reactions.

Today, there are a number of laboratory prototypes of biosensors for ATP determination, which are based on aptamers [79-81], fluorescent probes [82], various enzymatic reactions, etc. Below we will describe only electrochemical enzyme biosensors, since they are close to the biosensors we propose. Several potentiometric and amperometric enzyme biosensors have been reported, but, to the best of our knowledge, no conductometric biosensor has been proposed for the moment.

Potentiometric biosensors

Only a few ATP-sensitive biosensors have been developed by using ISFETs with immobilized enzymes. In one work, H^+-ATPase (a proton pump) was immobilized via a polyvinylbutyral resin on a pH-sensitive field effect transistor [83]. Linear range of ATP determination was 0.2–1.0 mM, and the biosensor lost 90% of activity after 18 days. Response time was 1-1.5 min. In a more recent work, apyrase (ATP-diphosphohydrolase) was immobilized onto a Ta_2O_5 ISFET gate surface [84]. When the enzyme layer selectively catalyses the dephosphorylation of ATP, protons are accumulated at the gate because the enzymatic reaction produces H^+ as a by-product. The linear range of ATP determination was similar to that of the previous biosensor (0.2–1 mM), and response time was 5 min. The main disadvantages of these potentiometric ATP biosensors are their low sensitivity to ATP and the dependence of their response to the buffer capacity of the medium. Moreover, the biosensors were not applied to real samples analysis.

Using another approach, Katsu and Yamanaka developed a potentiometric ATP biosensor was based on a choline-sensitive membrane electrode and choline kinase enzyme [85]. The choline-sensitive electrode was based on a poly(vinyl chloride)-based membrane containing sodium tetrakis[3,5-bis(2-methoxyhexafluoro-2-propyl)phenyl]borate as an ion

exchanger and 2-fluoro-2′-nitrodiphenyl ether as a membrane solvent. The biosensor produced signal proportional to choline concentration. In the presence of ATP, choline was phosphorylated by choline kinase and, therefore, the signal of biosensor decreased. Limit of ATP detection was 10 μM. This biosensor was used successfully to determine the intracellular ATP content of human red blood cells.

The most sensitive ATP potentiometric biosensor reported in the literature is based on a planar bilayer lipid membrane embedded with Na^+,K^+-ATPase (Na^+ pump). The planar bilayer lipid membranes are formed across a small circular aperture bored through a thin Teflon film by the folding of two monolayers spread on the air/water interface. The Na^+,K^+-ATPase is further incorporated into the lipid membrane. The proposed biosensor was found to respond potentiometrically toward ATP (membrane potential increased with increase in the ATP concentration). Limit of ATP detection was 6.3 μM. Working range was from 6.3 μM to 1 mM. The biosensor was sensitive to UTP and GTP, but not to CTP and ADP due to the enzyme selectivity. The biosensor required addition of Na^+ and K^+ since they are transported by Na^+,K^+-ATPase. The design and characteristics of the biosensor are quite interesting, but its elaboration is difficult and storage stability is likely to be poor [86].

Amperometric biosensors

Most of the electrochemical biosensors recently reported for ATP determination are based on amperometric transducers, which are miniaturizable to be used for *in vivo* measurements. Also these biosensors do not highly depend on buffer capacity and ionic strength of solution in comparison with conductometric and potentiometric biosensors. This makes measurement of real samples much easier. There is no enzyme of “ATP oxidase” type that could be used for direct oxidation of ATP. Thus bienzymatic systems based on glycerol kinase-glycerol-3-phosphate oxidase or glucose oxidase-hexokinase are typically used.

ATP detection by the first enzyme system relies on the following reactions:

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP} \quad (2)$$

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow{\text{glycerol-3-phosphate oxidase}} \text{DHAP} + H_2O_2 \quad (3)$$

In a first step, glycerol kinase phosphorylates glycerol by using ATP, and in the second step glycerol-3-phosphate is oxidized by glycerol-3-phosphate oxidase and hydrogen peroxide and dihydroxyacetone phosphate (DHAP) are produced. The detection is based on the oxidation of H_2O_2 at the electrode, and the biosensor response is directly proportional to ATP concentration. However, the biosensor response also depends on glycerol and glycerol-3-phosphate concentrations. To determine ATP concentration accurately, glycerol-3-phosphate should be absent in samples, and glycerol should be provided at a level that saturates glycerol kinase (>0.5 mM). In this case, the reaction becomes solely dependent on ambient ATP concentrations. Microbiosensors based on this enzymatic reactions cascade were successfully applied for ATP detection is living tissues [78, 87]. The linear range of ATP determination was 200 nM–50 μM, and response time was 10 s. Interestingly, authors applied differential measuring mode (with a control sensor) to remove signals coming from interfering compounds.

This bienzymatic system was used to create commercially available ATP microbiosensors by Sarissa Biomedical Ltd. [88]. The microbiosensors are based on Pt/Ir wire (25 and 50 μm in diameter) and carbon fiber (7 μm in diameter). The electrodes are covered with a permselective membrane to decrease interferences from substances such as ascorbate, urate, dopamine and serotonine. Characteristics of the microbiosensors are well-suited for the determination of low concentrations of ATP (limit of ATP detection is 200 nM). The response is linear in the 0.2 μM to 50 μM range, and a good selectivity (no interference from UTP, ADP and adenosine) is observed. Permselective layer on the electrodes is used to prevent oxidation of electroactive compounds. The biosensors can be stored in a dry state at 2-8 ºC for 6 months. However, the price of the microbiosensors is quite high, i.e., minimum 650.00 $ USD for 5 microbiosensors (price for January 2012).

Recently, graphene-modified Pt microelectrodes with immobilized glycerol kinase and glycerol-3-phosphate oxidase were applied for real-time ATP determination in plant tissues. In this case, a Nafion membrane was used to decrease non-selective signals [89].

An alternative bienzymatic system is glucose oxidase-hexokinase. In this case, the catalyzed reactions are the following:

$$\text{D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad (4)$$

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{D-glucose-6-phosphate} + \text{ADP} \quad (5)$$

The biosensor response is due to the oxidation of H_2O_2 produced during the oxidation of glucose by glucose oxidase. If ATP is present in the measured sample, hexokinase converts glucose to glucose-6-phosphate, which is not a substrate for glucose oxidase. Thus, the biosensor response decreases proportionally to ATP concentration. The main issue comes from the dependence of the biosensor on glucose concentration, which should be stable during measurement.

The reported biosensors based on this system are based on Pt electrodes [90, 91], glassy carbon electrodes [92] and Pt microelectrodes [93, 94]. The highest sensitivity to ATP was achieved for the biosensor based on glassy carbon electrodes [92]. Its range of ATP determination was 0.5-20 μM, and during storage in 0.1 M pH 7.4 PBS buffer solution at 4°C the biosensors lost 35% of activity after 22 days. The biosensors based on Pt electrodes had wider range of ATP determination (50-500 μM [90] and 10-200 μM [91]) but poorer storage stability (60% loss of activity after 2 weeks). To prevent oxidation of electroactive species, surface of the electrodes could be covered with poly(m-phenylendiamine) membrane, which allowed diffusion to electrode and oxidation of hydrogen peroxide, but prevented other molecules (e.g., ascorbic, aspartic and uric acids, dopamine and acetaminophen) from passing through [93]. ATP concentration in real samples (human erythrocyte hemolisate [90] and gastrointestinal tissues [94]) was evaluated, showing that the ATP biosensors based on glucose oxidase-hexokinase system are quite efficient. A summary table for comparison of different electrochemical ATP biosensors in given Chapter III, page 52. The table is given in the experimental part because we wanted to add there characteristics of the developed in this work biosensor.

I.3. Creatine Kinase

I.3.1. Biological Role of CK

Creatine kinase (CK) is an important intracellular enzyme that is present in animal tissues. Other names of CK are creatine phosphokinase and phospho-creatine kinase. EC number is 2.7.3.2. CK is found mostly in the muscles (first of all in the skeletal muscles and myocard) and in brain, but CK is also present in other tissues which require large amount of energy.

CK provides additional supply of energy for cell functioning and maintains stable ATP/ADP ratios. Thus, CK regulates energy homeostasis. As was described earlier, the main temporary energy carrier inside cells is ATP. For example, energy stored in ATP is required

for muscle contraction. During active muscular work, reserves of ATP in the muscle cells become depleted, and then CK synthesizes new ATP molecules by transferring phosphate group from creatine phosphate to ADP. Thus, CK supplies additional energy to muscles that is stored in the form of creatine phosphate. During a rest period, CK catalyzes a reverse reaction and renews stockpile of creatine phosphate [95, 96]. Usually CK concentration is expressed through the unit of activity per volume (U/ml), common methods of CK determination measure CK activity and not the amount of CK itself.

This enzyme is well-studied, and nowadays determination of CK is important not only for research but also for clinical diagnostics, as CK concentration in blood serum is a biomarker of certain diseases. CK level in the blood serum of healthy people is very low: 0.038-0.174 U/ml in the male serum and 0.026-0.14 U/ml in the female serum. CK concentration in child serum is several times higher. After intensive physical work or muscular injuries, CK is released into blood from the muscle cells and CK activity in serum increases up to 2.0 U/ml or 39-185 ng/ml or even higher [97-100]. Such increase can be caused by different inflammation processes, myocardial infarction and muscular dystrophy. Furthermore, CK activity in blood serum increases in the case of hypothyroidism. Usually, CK serves as a biomarker of acute myocardial infarction (AMI) and in the modern clinical diagnostics CK activity (or concentration) is determined for confirmation of other symptoms of this disease. CK level in blood starts to increase immediately after AMI and becomes abnormal after 4-6 hours; maximal CK concentration is observed after 18-24 hours after AMI. Level of CK increase is used for assessment of AMI severeness in order to choose an appropriate treatment [101]. Increase in CK levels (myocardial band isoenzyme) after percutaneous coronary intervention is associated with significantly higher risk of late mortality. Monitoring cardiac enzymes after the intervention may help predict the long term clinical outcome [102]. In addition, CK determination in blood serum is useful for assessment of training status and physical qualities of athletes [103].

I.3.2. Classical Methods of CK Determination

Classical methods of CK determination can be divided into two categories: immunological methods and spectrophotometric methods.

The most precise and selective methods of biomarker determination (i.e., CK) are immunological methods [99, 104]. They are based on binding of CK with a specific antibody and further detection of this interaction by using chemoluminescence or enzyme

immunoassay. Electrochemical detection of CK-antibody binding has been also described [105]. These methods determine the mass concentration of CK (in ng/mL). Thus, activity of CK is not important and CK determination can be carried out in samples that were stored inappropriately or during long periods. The possibility of separate determination of different isoforms of CK is another advantage of immunological methods. This is useful for finding the source tissue from which CK was released into blood. Disadvantages of these methods are high price, requirement of animals for antibody production, impossibility of multiple usage of main reaction components.

Medical laboratories usually use much simpler and cheaper spectrophotometric methods of CK determination. These methods assess CK activity by measuring the rate of formation of the products of CK-catalyzed reaction [106, 107]. An enzymatic system based on hexokinase and glucose-6-phosphate dehydrogenase is generally used (Fig. 4). CK synthesizes ATP that is used by hexokinase for glucose-6-phosphate synthesis. The latter is oxidized by glucose-6-phosphate dehydrogenase and NADH is formed. NADH is registered spectrophotometrically at 340 nm. The amount of NADH is proportional to CK activity in the sample. Such methods cannot distinguish between different isoforms of CK and total activity of all isoforms is registered. However, in most cases it is enough for diagnostic purposes.

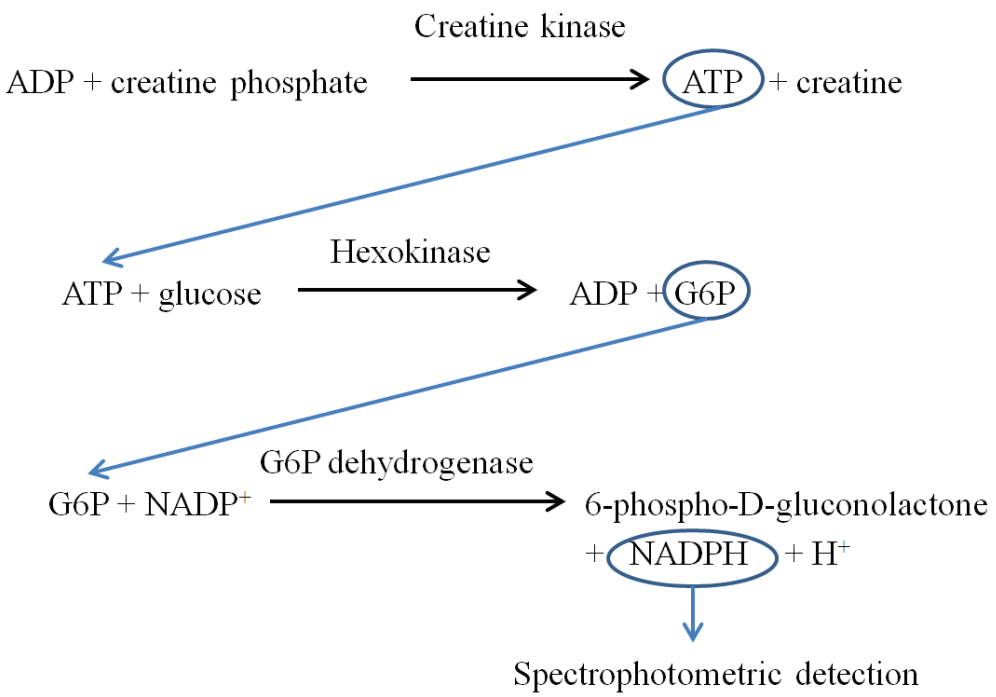

Figure 4. Reactions, which are a basis for spectrophotometric determination of CK activity.

I.3.3. Biosensors for CK Determination

Electrochemical biosensors are perspective alternative to immunological and spectrophotometric methods of CK determination. The biosensors are potentially cheap, simple in production and application, and need no sophisticated equipment or large space to operate [108]. Single biosensor can be used for multiple measurements of different samples. Furthermore, the biosensors can be combined into multibiosensor systems for simultaneous determination of different components of a sample. However, only a few biosensors for CK determination have been described up to date.

The first biosensor was proposed in 1986; it was amperometric and was based on graphite disc electrodes, and glucose oxidase-hexokinase enzyme system (see reactions 4 and 5) was used to determine the rate of ATP production by CK [98]. This work, for the first time, demonstrated the possibility to determine CK by using an ATP-sensitive biosensor. However, the authors did not propose a procedure for CK determination in real samples.

Later on, disposable amperometric ATP-sensitive biosensors based on glycerol kinase and glycerol phosphate oxidase (reactions 2 and 3) were developed for CK determination [97, 109].

Several years later, Moreira et al. proposed an electrochemical biosensor CK determination working on a completely different principle. The dephosphorylation of creatine phosphate, immobilized onto the surface of an amperometric transducer, was induced by CK addition and resulted in changes in electrochemical characteristics of the electrode surface, which could be evidenced using electrochemical impedance spectroscopy and square wave voltammetry. The biosensor exhibited high sensitivity and selectivity to CK. It was successfully applied to CK analysis in synthetic blood. However, the biosensor could not be reused and the fabrication process was complex, as well as the analytical procedure [99].

I.4. Conclusion

The presented bibliographic study gives a brief overview of the modern field of biosensors and of the attractive characteristics of these new analytical devices for the determination of a large variety of molecules, including biologically active substances, such as ATP and CK. Electrochemical biosensors appear as an appealing perspective alternative to classical methods used for these substances, due to their low cost, relatively fast and simple procedure of analysis, small size of measuring setup, possibility to make real-time and *in vivo* measurements.

The most frequently reported electrochemical biosensors for ATP are amperometric and use two-enzyme systems based on glycerol kinase-glycerol-3-phosphate oxidase or glucose oxidase-hexokinase for biorecognition. 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 the first goal of this work. Results will be presented in Chapter III.

The second goal of this work was to develop a new biosensor for CK determination. Most of previously reported enzyme biosensors for CK detection are completely disposable, so they could be used to measure only one sample. Other biosensors require addition of enzymes to the working cell before measurement. This results in larger amounts of enzymes needed for one measurement. The aim of our work was to develop a reusable amperometric biosensor for rapid and simple determination of CK activity in blood serum. In our case, enzymes were immobilized onto the surface of the electrode and were used for multiple measurements. These results will be presented in Chapter IV.

The last chapter of this manuscript will be devoted to the development of an original conductometric hexokinase-based biosensor that would be simpler in structure and usage comparing with the existing amperometric and potentiometric ATP biosensors. To prevent an influence of charged particles, a differential two-step procedure of measurement was proposed.

CHAPTER II: MATERIALS AND METHODS

II.1. Reagents

Hexokinase (HEX, EC 2.7.1.1, activity: 30.6 U/mg) from *Saccharomyces cerevisiae* (Sigma-Aldrich, Germany) and glucose oxidase (GOx, EC 1.1.3.4, activity: 272 U/mg) from *Aspergillus niger* (Genzyme, UK) were used as biorecognition elements of biosensors. Creatine kinase (CK, EC 2.7.3.2, activity: 32 U/mg) from rabbit muscle was obtained from Serva Feinbiochemica (Heidelberg/New York). Bovine serum albumin (BSA, fraction V, ≥96%), glucose (≥99.5%), ATP (disodium salt hydrate, grade 1, ≥99%), ADP (sodium salt, ≥95%), creatine phosphate (≥98%), magnesium chloride (≥99%), glycerol (≥99%), ascorbic acid (≥99%), *m*-phenylenediamine (≥99%), HEPES (≥99.5%) and 50% aqueous solution of glutaraldehyde (GA) have been purchased from Sigma-Aldrich Chemie (Germany and France). All other chemicals were of p.a. grade.

Blood serum samples were obtained from Kiev municipal scientific and practical center of nephrology and hemodialysis (Ukraine). The samples were supplied undiluted and were stored at -18°C. Dilution of the samples was performed directly in the working cell during biosensor measurements by addition of aliquots of samples to the working buffer.

II.2. Transducers

II.2.1. Amperometric Transducers

In this work, homemade platinum disc electrodes were used as amperometric transducers (Fig. 5).

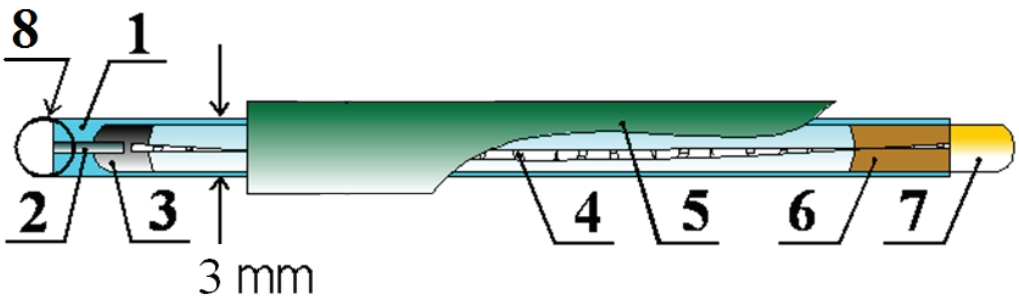

Figure 5. Scheme of the working amperometric electrode.
1 – glass tube; 2 – platinum wire; 3 – Wood's alloy; 4 – copper wire;
5 – protective coating; 6 – epoxy; 7 – contact pad, 8 – sensitive region.

Platinum wire (0.4 mm in diameter and 3 mm long) was sealed in the end of a glass capillary with an outer diameter of 3.5 mm. The 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 was placed at the other end of the copper wire for connecting the electrode to the measuring setup. The working surface of platinum electrodes was obtained by grinding using alumina powder (particle size 0.1 and 0.05 μm) and treated with distilled water and ethanol prior to immobilization of the bioselective element.

II.2.2. Conductometric Transducers

Biosensors were based on planar conductometric transducers. They were manufactured in the V. Lashkaryov Institute of Semiconductor Physics of National Academy of Science of Ukraine (Kyiv, Ukraine) in accordance with our recommendations. Each transducer was 5 x 30 mm in size and contained two pairs of gold interdigitated electrodes deposited onto a ceramic support. The sensitive area of each electrode pair was about 1.0 x 1.5 mm. The width of each digit as well as interdigital space was 20 μm. The deposition of gold onto ceramic surface was done by vacuum sputtering.

A photograph and a scheme of these transducers are presented in Fig. 6.

Transducers were intended to operate in a differential mode of measurements: biorecognition element (enzyme) was placed on one pair of electrodes, and reference element (BSA membrane) – on the other pair. Details about the differential mode can be found in the section II.6.3.

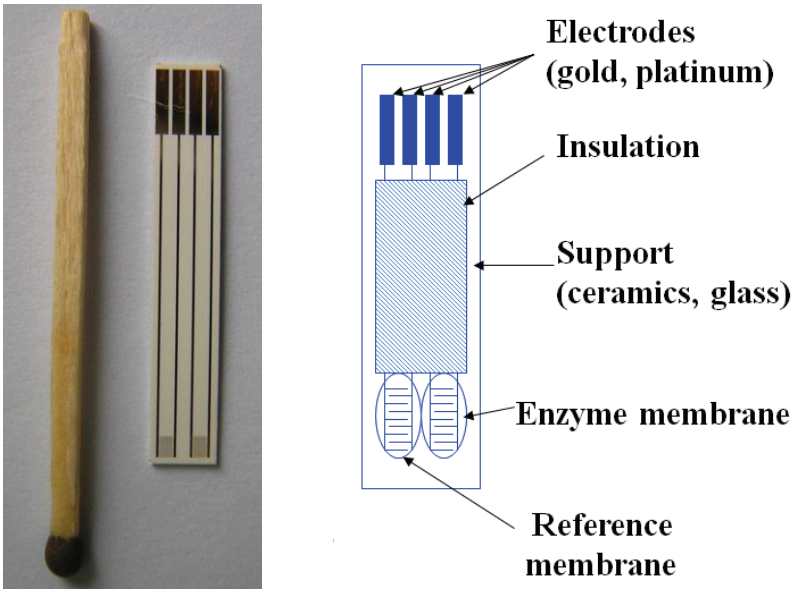

Figure 6. A photograph (on the left) and a scheme (on the right) of the conductometric transducer used in this work.

II.3. Experimental Setups

II.3.1. Setup for Amperometric Measurements

The biosensor system (two amperometric biosensors, the first one being sensitive to glucose and the second one – to glucose and ATP) was placed in a 5 ml electrochemical cell containing an auxiliary electrode (platinum wire) and a reference electrode (saturated Ag/AgCl) that were connected to a PalmSens potentiostat (Palm Instruments BV, Netherlands). Usage of the 8-channel device (from the same producer), connected to the potentiostat, allows simultaneous monitoring of signals from 8 working electrodes. The overall view of the measurement setup is shown on Fig. 7, and scheme of the biosensor system is shown on Fig. 8. To decrease background noise, measuring cell can be placed into Faraday cage or other equipment for blocking external electric fields. This is useful for measuring very small substrate concentrations that generate very weak currents (below 1 nA).

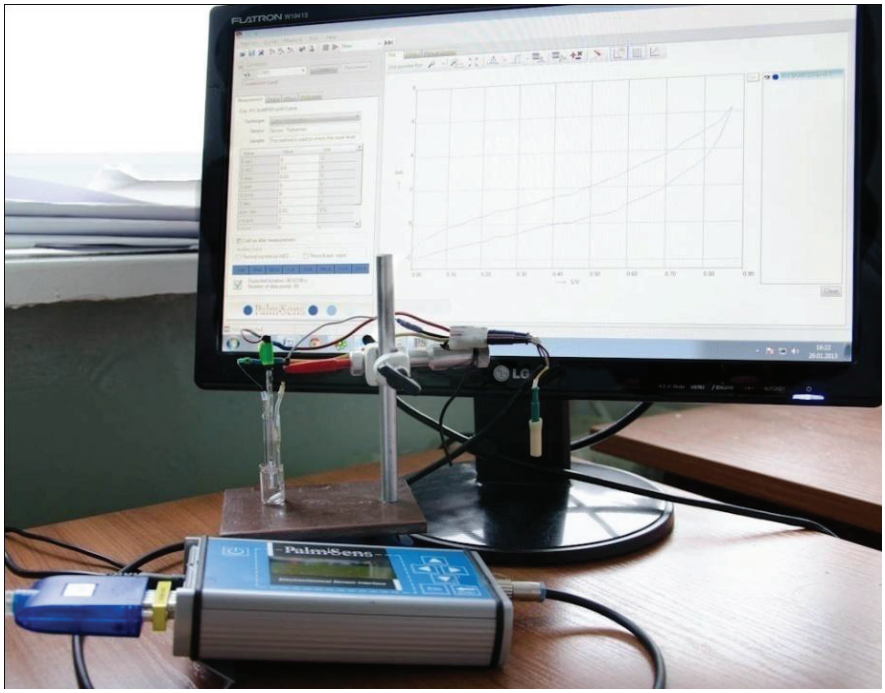

Figure 7. Photograph of the working area, presenting the PalmSens potentiostat, the electrochemical cell with conventional three electrode system, and the computer used during the amperometric experiments.

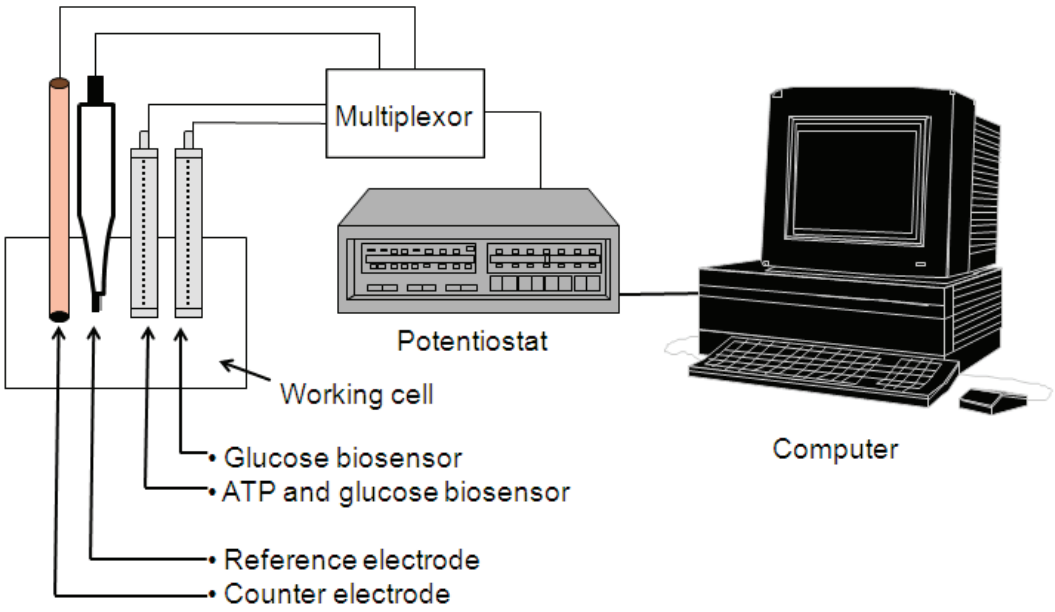

Figure 8. Scheme of a biosensor system.

II.3.2. Setup for Conductometric Measurements

Conductometric transducers were connected to the portative device for conductometric measurements (9.5 cm * 2.5 cm * 13.5 cm) that was made in Institute of Electrodynamics of National Academy of Sciences of Ukraine (Kiev, Ukraine). This device applied sinusoidal potential with frequency of 36.5 kHz and amplitude of 14 mV allowed avoiding such effects as faradaic processes, double-layer charging and polarization of the microelectrodes. In this device limit of the frequency was 36.5 kHz, but is should be noted that the biosensor sensitivity can be further improved by increasing the frequency to 100 kHz by using other generator. The nonspecific changes in the output signal induced by the fluctuations of ion concentrations, medium pH, etc. were decreased due to usage of differential mode of measurement: conductivity of solution measured by reference pair of electrodes was subtracted from the conductivity measured by pair of electrodes with biorecognition element.

The overall view of the measurement setup is shown on Fig. 9, and its scheme is shown on Fig. 10.

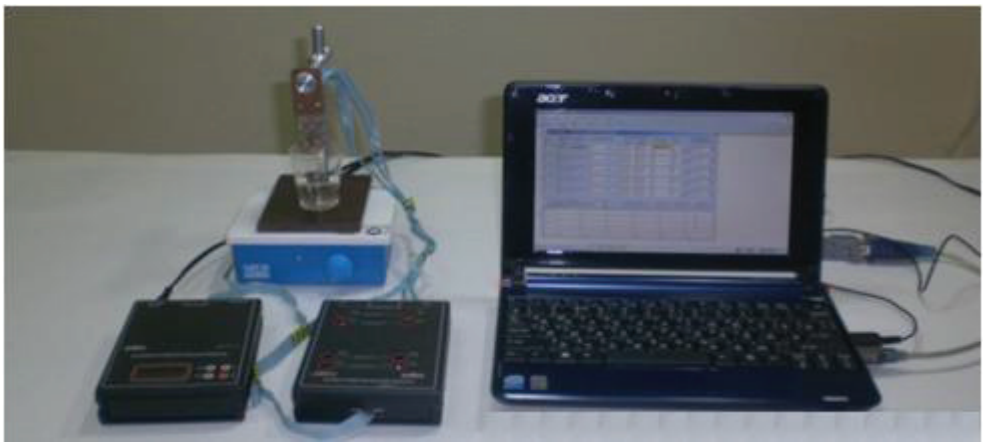

Figure 9. Photograph of the working area, presenting the conductometric device, the working cell with the transducer, and the computer.

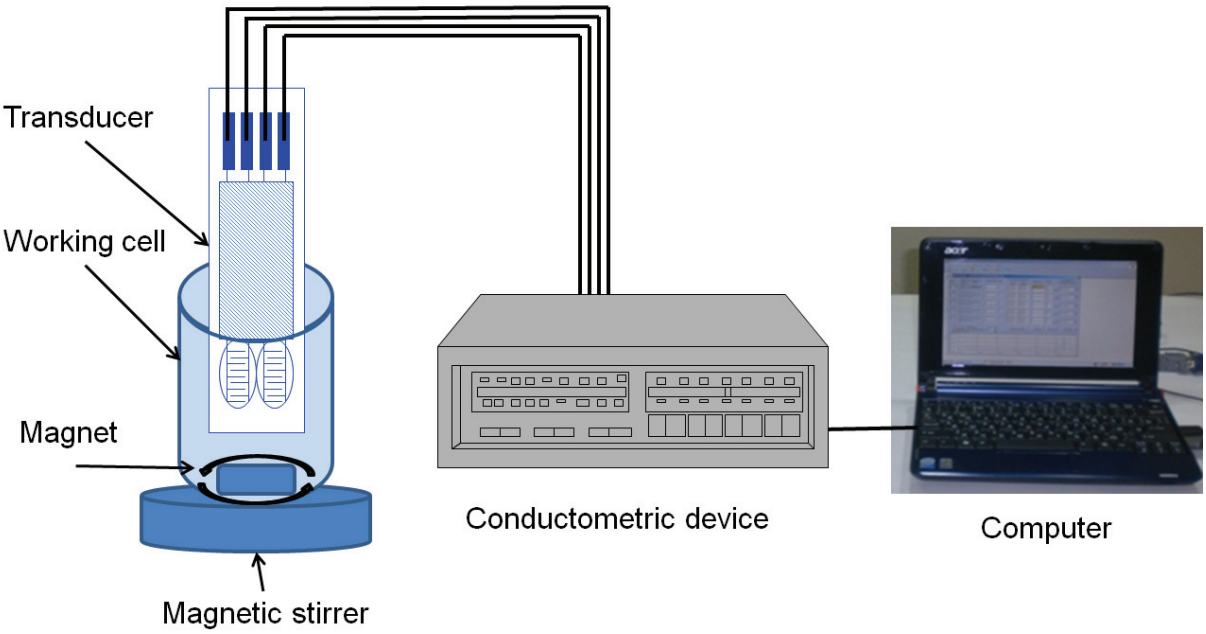

Figure 10. Scheme of a setup for conductometric measurements.

II.4. Immobilization of Biorecognition Elements

II.4.1. Immobilization of Biorecognition Elements in Amperometric Biosensors

Biorecognition layers were obtained by chemical crosslinking of enzymes and auxiliary substances on the surface of amperometric transducer. The initial solution for preparing the working membrane of the biosensor for determination of ATP and glucose contained 5% GOx (hereafter – w/w), 5% HEX, 3% BSA in 20 mM phosphate buffer, pH 6.5, mixed with 10% glycerol; the solution for glucose biosensor consisted of 4.5% GOx and 3% BSA in the same buffer containing 10% glycerol. Glycerol was added to the solutions to stabilize the enzymes during storage at -18°C and to prevent early drying of the solution on

the transducer surface. BSA stabilizes the enzymes and participates in the formation of intermolecular bindings. These solutions were mixed with 0.4% aqueous solution of glutaraldehyde (crosslinking agent) in a ratio of 1:1 (v:v) and immediately deposited onto the transducer working surface (approximately 100 nL of the mixture onto each electrode) by using an Eppendorf micropipette. Afterwards, the created biosensors were dried for 40 min in air at room temperature. During this time, GA formed covalent bonds between amino groups of enzymes and BSA. Then immersed in the working buffer for 10 min in order to stop immobilization and wash out unbound components. The biosensors were used immediately after preparation.

II.4.2. Immobilization of Biorecognition Elements in Conductometric Biosensors

Hexokinase was immobilized by the following procedure. The initial solution for preparing the bioselective membrane of the biosensor contained 10% HEX (hereafter – w/w), 5% BSA and 10% glycerol in 20 mM phosphate buffer, pH 6.5; the solution for a reference membrane consisted of 15% BSA and 10% glycerol in the same buffer. Subsequent procedure of enzyme immobilization was the same as in the case of amperometric biosensors (described earlier). The difference is that 0.5% aqueous solution of glutaraldehyde was used, and time of biosensor drying was 30 min. HEX/BSA solution was deposited on the one pair of electrodes, while BSA solution for a reference membrane was deposited onto the second pair of electrodes.

II.5. Measurement Conditions

II.5.1. Measurement Conditions for Amperometric Biosensor System

The amperometric measurements were carried out at room temperature in an open measuring cell containing 3.5 mL of buffer solution at 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 of Mg^{2+} (magnesium ions were necessary for the work of HEX) 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 mM and 500 mM glucose and 50 mM ATP). Biosensor responses were calculated after reaching steady-state.

II.5.2. Measurement Conditions for CK biosensor

The amperometric measurements were carried out at room temperature in an open measuring cell containing 3.5 mL of buffer solution at permanent stirring by a magnetic stirrer and at constant potential of 0.6 V versus Ag/AgCl reference electrode. Ten mM HEPES, pH 7.4, with 10 mM Mg^{2+} was used as a working buffer in all experiments (magnesium ions were necessary for the work of HEX and CK). The substrates concentrations in the working cell were obtained by the addition of aliquots of stock solutions (500 mM glucose, 50 mM ATP, 117 mM ADP, 300 mM creatine phosphate). Stock solution of CK contained 1 mg/ml of CK and 1 mg/ml of BSA in a working buffer (BSA was needed to prevent inactivation of CK) and was stored at -20°C. The values of biosensor responses to glucose and ATP were calculated after reaching steady state, response to CK was measured as a rate of signal decrease (see below).

II.5.3. Measurement Conditions for Conductometric Biosensors

The conductometric measurements were carried out in an open measuring cell containing 2 mL of buffer solution, under magnetic stirring. Five mM HEPES buffer, pH 7.4, with 3 mM of Mg^{2+} was used as a working buffer in all experiments. Biosensor responses were calculated after reaching steady-state.

CHAPTER III: AMPEROMETRIC BIOSENSOR SYSTEM FOR SIMULTANEOUS DETERMINATION OF ATP AND GLUCOSE

III.1. Introduction

In the first chapter we have showed that most of electrochemical ATP-sensitive biosensors are based on two-enzyme systems – glucose oxidase-hexokinase or glycerol kinase-glycerol-3-phosphate oxidase. The large drawback of these biosensors is the sensitivity not only to ATP but also to glucose or glycerol (depending on the enzymatic system used). A promising approach for avoiding this drawback is the usage of two independent biosensors, one of which is sensitive only to glucose, while the second biosensor is sensitive to ATP and glucose. Creation of such biosensor system was a goal of the work presented in this chapter.

III.2. Results and Discussion

III.2.1. The Principle of Biosensor System Operation

The proposed biosensor system includes two biosensors, the first one contains GOx and HEX sensing elements and is sensitive to glucose and ATP (called further GOx/HEX biosensor), whereas the second one contains only GOx and is sensitive only to glucose (called further GOx biosensor). Most of the work was devoted to the study of the two-enzyme biosensor, as it is the more complex one.

The work of GOx/HEX biosensor 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:

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOx}} \text{D-gluconic acid} + H_2O_2 \qquad (6)$$

$$H_2O_2 \xrightarrow{+\,600\ \text{mV, Pt}} 2H^+ + 2e^- + O_2 \qquad (7)$$

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HEX}} \text{D-glucose-6-phosphate} + \text{ADP} \qquad (8)$$

In the presense of glucose and absence of ATP, only the reactions (6) and (7) take place on the electrode surface: glucose oxidase oxidizes glucose, and produces hydrogen peroxide. The latter is oxidized on the surface on the transducer, generating current that is registered as amperometric signal. In this case, the biosensor response is directly proportional to glucose concentration. After addition of ATP to the working cell, HEX phosphorylates

glucose by transferring a phosphate group from ATP (reaction 8), this reaction reduces the local concentration of glucose and, consequently, the biosensor response to glucose decreases in proportion to the ATP concentration. To demonstrate visually these events, in Fig. 11 a typical biosensor response to glucose and ATP is shown.

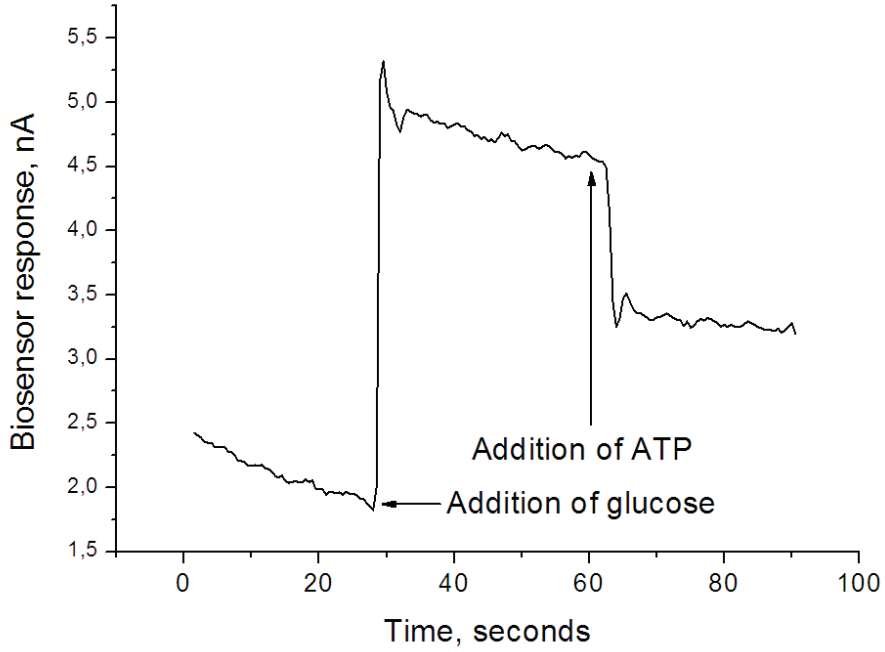

Figure 11. Example of the GOX/HEX biosensor response to subsequent additions of glucose (75 μM) and ATP (50 μM). Measurements were carried out in 25 mM phosphate buffer with 2 mM Mg^{2+}, pH 7.4, at constant potential of 0.6 V vs Ag/AgCl reference electrode.

In the case of GOx biosensor, only reactions (6) and (7) occur, thus the biosensor is sensitive only to glucose. This biosensor is used for determination of glucose concentration in glucose/ATP mixtures, since ATP biosensor in sensitive to both substances and response of this biosensor is proportional to both ATP and glucose. More detailed information about simultaneous usage of these two biosensors is presented in section III.2.9.

III.2.2. Optimization of Enzymes Concentration in the Biomembrane

The biomembrane composition, namely the amount and ratio of GOx and HEX enzymes, is an important parameter which will strongly affect the biosensors operation, and consequently the responses. Therefore, the first phase of the work aimed at selecting optimal amount of these enzymes in the biorecognition layer of both biosensors.

Initially, we determined the optimal GOx concentration in the biomembrane of the GOx biosensor. For this purpose, the working membrane was prepared according to the procedure described in section 'Immobilization procedure' using initial enzyme solutions with different GOX concentrations - from 1.2% to 20%. These solutions were mixed with 0.4% GA solution in a ratio of 1:1 (v:v). The mixture was immediately deposited on the surface of amperometric transducer and immobilized during 30 min. Thus, the mass fraction of GOx in the biorecognition membrane ranged from 0.6% to 10%. Then the sensitivity of the obtained GOx biosensors was tested. As shown in Fig. 12 (curve 1), an increase in GOx concentration up to 2-2.5% resulted in an increase of the biosensor response. Further increase in the GOx concentration from 2.5% to 10% did not change biosensor response significantly. Therefore, GOx concentration of 2% was used in further experiments. In this part of work 4 mM glucose was added to the working cell, but in the next experiments we used lower concentrations of glucose (i.e. 100 μM) to increase the sensitivity to ATP. We consider that lower concentration of GOx (i.e. 1%) would be enough to detect small glucose concentrations, but we decided to use the same composition of bioselective element during whole work.

Next, we determined the optimal concentration of HEX in the bioselective membrane of the GOx/HEX biosensor following the same procedure. The biosensor sensitivity was tested at various HEX concentrations in the range of 1% to 8% and at constant GOx concentration (2%). The highest responses to ATP were observed at HEX concentration in the 2-3% range (Fig.12, curve 2). A decrease in the biosensor response to ATP at higher 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 small amount of ATP molecules reach deeper layers, close to the electrode surface, which are more important for the biosensor response. Another probable reason is a decrease of HEX activity due to aggregation of proteins which can occur at high concentrations of the enzymes in biomembrane. Therefore, in further work we used the biorecognition elements with the HEX concentration of 2.5%.

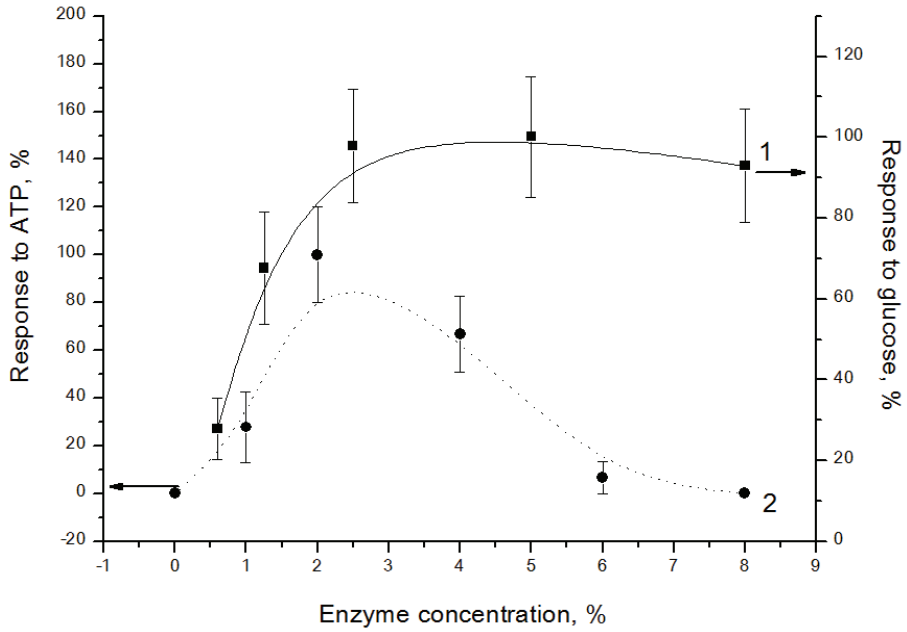

Figure 12. Dependence of biosensor response to 4 mM glucose (1) and 0.1 mM ATP (2) on concentrations of GOx (1) and HEX (2) in biorecognition element. The highest biosensor response was taken as 100%. When selecting HEX concentration, GOx concentration in biomembranes was 2%. Measurements were carried out in 25 mM phosphate buffer with 2 mM Mg^{2+}, pH 7.4, at constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the average value of four biosensors ± standard deviation.

For comparison, another approach for the constitution of the bi-enzymatic layer was tested. Instead of casting a mixture of the enzymes onto the electrode, a 2.5 % GOx solution was dropped onto the electrode surface to constitute the first layer (close to the electrode) and a 2.5 % HEX solution (with 0.2 % of GA) was further deposited the same way to produce the second layer (see the procedure of preparation of the working membrane of biosensor for glucose determination described in section 'II.4.1. Immobilization of Biorecognition Elements…'). Before adding the second layer, the biosensor was washed, dried and its sensitivity to glucose was evaluated. We expected that HEX in the outer layer of biomembrane would consume glucose (in the presence of ATP) and thus glucose will not diffuse to GOx located in the near-electrode layer, what would lead to significant decrease of the biosensor response and higher sensitivity to ATP in comparison with co-immobilization of GOx and HEX in a single layer.

After the addition of HEX, the shape of calibration curves obtained for glucose determination did not change, but the baseline noise significantly increased. Thus, it became

impossible to register the responses to small concentrations of ATP. The bi-enzymatic monolayer method of enzymes immobilization was consequently chosen for further experiments.

III.2.3. Optimization of Cross-linking Conditions

Immobilization of the biorecognition element is a key factor which directly affects biosensors analytical characteristics. In this work, GOx and HEX enzymes were immobilized onto the transducers surface via cross-linking with GA in presence of BSA. GA concentration and cross-linking time were optimized to favor the formation of a robust biomembrane but to limit enzymes release or deactivation.

For that GA concentrations of 1%, 0.2% and 0.1% were tested. No significant difference in biosensor responses to glucose was observed. On the contrary, the biosensor responses to ATP varied with GA concentration. They were very small for 1% GA, while quite sufficient for 0.2% and 0.1% GA. This may be attributed to the lowest stability of HEX compare to that of GOx. For 0.1% GA, we observed a gradual decrease in responses upon reuse, probably due to enzymes release from the biomembrane. So, 0.2% GA was selected for further experiments.

The influence of cross-linking time on biosensors response was also investigated in the 20 to 50 min domain. The use of a 20-min reaction time was accompanied by a gradual decrease in biosensor responses to ATP, which could be assigned to unstable enzyme immobilization. At the highest time tested (50-min), the biosensor responses to ATP were lower than in all other cases, probably due to a decrease in HEX activity. The optimal immobilization time was therefore in the 30-40 min range.

III.2.4. Influence of Glucose Concentration on ATP Determination

As described previously, ATP determination by the bi-enzymatic GOx/HEX biosensor is based on the competition between GOx 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 background noise also increases in proportion to the amount of glucose. For example, at glucose concentration of 2 mM, the limit of ATP detection was 100 μM (calculated as an ATP concentration at which the biosensor response is 3 times higher than background noise), and the linear range of ATP determination

was 0.1 – 2.5 mM (operating range extended to 4 mM). At higher glucose concentrations, it was not possible to measure ATP because the background noise was too high. Table 1 presents the evolution of the biosensor characteristics for ATP detection for glucose concentrations ranging from 10 μM to 250 μM (Table 1). The highest absolute value of the biosensor response recorded for 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 by decreasing glucose concentration. For example, at 10 μM glucose concentration, the biosensor response to ATP 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 limit of detection (LOD) for ATP was observed for 25 μM of glucose. Increasing glucose concentration had also a significant effect of the linear range of ATP determination. For example, at 100 μM glucose, the ATP linear measurement range was 20-200 μM, whereas it was150-400 μM for 250 μM glucose. These results demonstrate that we can significantly modify the biosensor ability to detect ATP, changing 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 (GOx biosensor), and then figured out how the GOx/HEX biosensor, which is sensitive to both glucose and ATP, will respond to ATP.

Table 1. Comparison of the characteristics of GOx/HEX 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

III.2.5. Selection of Magnesium Concentration

The presence of Mg^{2+} ions in the working buffer is necessary for HEX catalytic activity since magnesium stabilizes ATP molecule and activates HEX. To select the optimal

Mg^{2+}concentration, we tested the biosensor at Mg^{2+} concentrations ranging from 0.5 mM to 3 mM. It should be noticed that disodium salt of ATP was used as a substrate. In case of magnesium salt, results could be different. The biosensor responses to glucose practically did not depend on magnesium concentration, because GOx does not require magnesium for catalysis. The biosensor responses to ATP, as expected, increased with increasing amount of magnesium (Fig. 13). We observed an increase in biosensor responses to ATP directly proportional to the magnesium concentration in the 0.5 mM-2 mM range. The 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 form of magnesium nitrate).

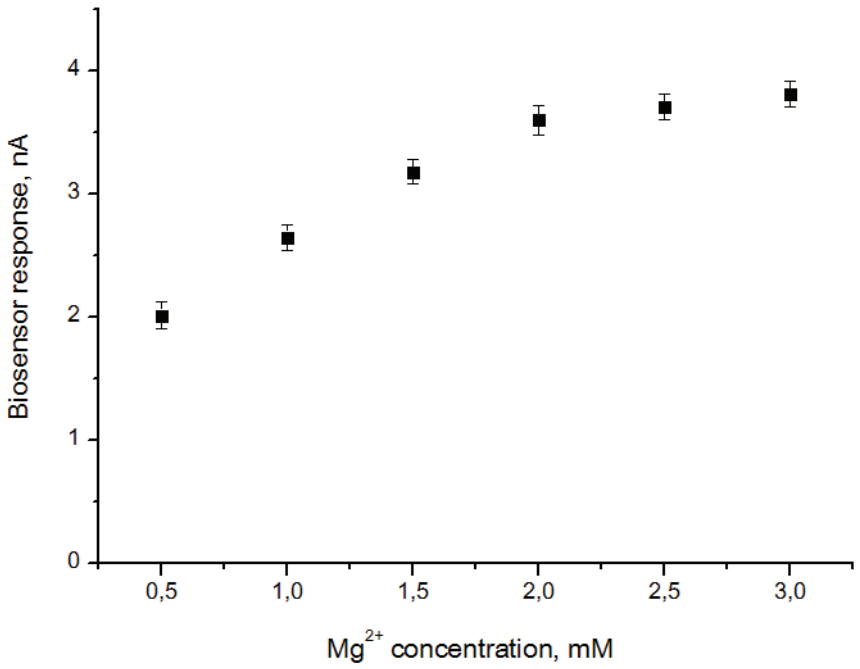

Figure 13. Influence of Mg^{2+} concentration in working buffer on GOx/HEX biosensor response to ATP. Glucose concentration: 100 μM, ATP concentration: 100 μM. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, at constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the mean of three biosensors responses ± standard deviation.

III.2.6. Use of Poly-phenylenediamine Film to Improve the Selectivity of GOX/HEX Biosensor

The biosensor selectivity is an important factor when working with real biological fluids, so the research on selectivity towards 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 [110]. We deposited a poly(m-phenylenediamine) (PPD) film onto the sensitive part of the amperometric transducer by the method described in the literature [111]. This film is porous, the size of the pores being 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 5 mM solution of *meta*-phenylenediamine, and then obtained 4 - 5 cyclic voltammograms (*meta*-phenylenediamine isomer was chosen according to previous studies [112]). 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 trends of cyclic voltammograms coincided with those given in the original source [111]. Afterwards, the biomembranes based on GOx and HEX enzymes were immobilized onto the PPD film.

To confirm the improvement of selectivity, 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, significant responses to these substances were observed, which could interfere during ATP 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). The reproducibility of the biosensor responses to glucose and ATP was also 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 in a dry state at -18 °C. In 50 days, the responses increased by only 10%, indicating retention of properties of PPD membrane.

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

III.2.7. Analytical Characteristics of the GOx/HEX Biosensor for ATP Determination

Analytical characteristics of the ATP biosensor were further determined setting the glucose concentration in working buffer at 50 μM. The LOD for ATP was 5 μM. The biosensor response was proportional to ATP concentration in the 15 μM - 90 μM range (Fig. 14). The average biosensor sensitivity to ATP was 240 nA/(mm^2*mM). Calibration curves of the biosensor for the determination of glucose and ATP are shown in Fig. 14. Linear parts of these calibration curves are described by the equations I = -0.27 + 32 * C (ATP determination; R^2 = 0.988) and I = -0.5 + 80 * C (glucose determination; R^2 = 0.993), where I is a current when the response reaches the steady-state value (nA), C - concentration of ATP or glucose (mM). Linear range of glucose determination was from 12 μM to 2 mM (LOD - 1 μM), and sensitivity to glucose was about 130 nA/mM. Notably, determination of ATP by the biosensor strongly depended on the composition of working solution, 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.

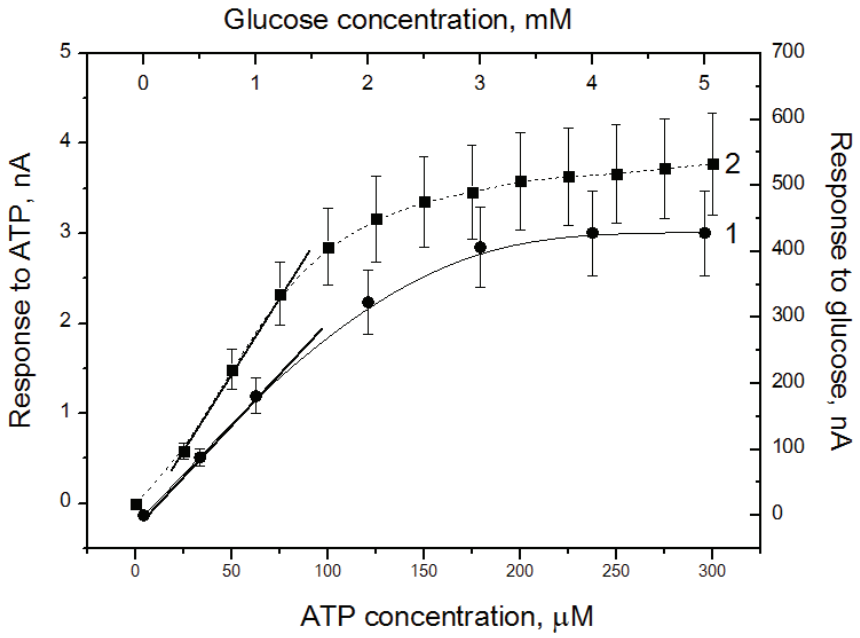

Figure 14. Typical calibration curves of GOX/HEX biosensor for the determination of glucose (1) and ATP (2). Calibration curve for ATP determination was plotted at glucose concentration of 50 μM. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+}, constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the mean of five biosensors ± standard deviation.

The possibility to use the biosensors for a long time and perform plentiful measurements is a very important characteristic. However, partial leaching of the components from the bioselective membrane often occurs during biosensor operation. It may be due to the diffusion of weakly bound membrane components at vigorous stirring of buffer in the working cell. Additionally, some decrease in the enzyme activity can take place during the membrane operation and storage. This can result in the decrease in biosensor responses, and thus to the degradation of their sensitivity. Thus, we studied the reproducibility of biosensor responses 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 (100 μM) was 4%, to ATP (50 μM) - 10%. The lowest reproducibility observed for ATP measurement may be attributed to the fact that ATP signal is obtained from a complex system involving two

enzymes (GOx and HEX), two substrates (glucose and ATP) and a competitive enzymatic process whereas the response to glucose relies only on one enzyme-substrate reaction.

Storage stability of the GOx/HEX biosensor was also investigated. Responses to glucose and ATP of the freshly prepared biosensors were checked, and then the biosensors were stored dry at +4 °C or -18 °C. Every two days the biosensors were defrosted and the responses were obtained again. As seen in Fig. 15, in two weeks the responses to glucose decreased by 20-25% (storage at +4°C) and by 10-15% (storage at -18°C), whereas the responses to ATP decreased by 65-75% and 45-55%, respectively. Greater loss of sensitivity to ATP suggests that HEX is much less stable enzyme than GOx, and it is the HEX stability that restricts the duration of ATP biosensor storage. The same results were obtained during study of thermal stability of biosensor based on immobilized GOx and HEX [93]. This coincides with the data obtained in the study on enzymes immobilization – during immobilization HEX lost its activity faster than GOx (see section III.2.2. Selection of conditions of enzyme immobilization).

Since the storage at -18 ° C showed better results, we examined the biosensors under this condition for longer period. We did not check their activity every few days, so the biorecognition element lost its activity more slowly. The responses to glucose did not reduce at all after 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 5 μM.

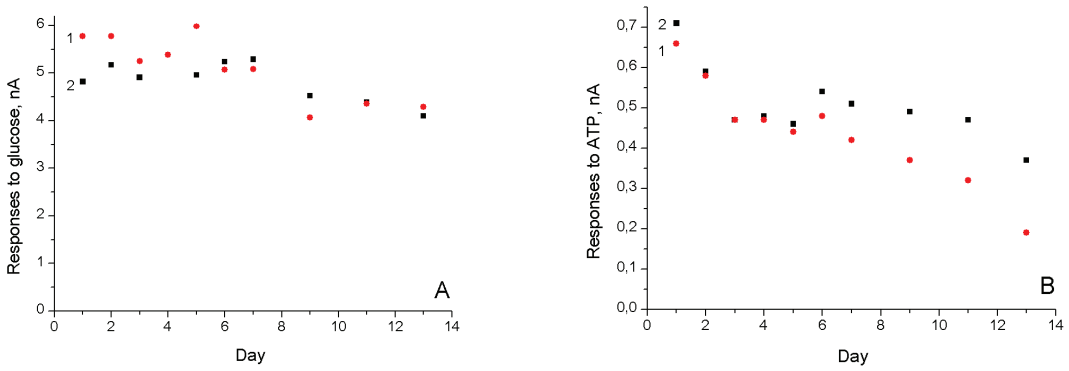

Figure 15. Responses to 50 μM glucose (A) and 25 μM ATP (B) of the biosensor based on GOx and HEX stored dry for two weeks at temperatures of +4 °C (1) and -18 °C (2). Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+}, constant potential of 0.6 V vs Ag/AgCl reference electrode.

Table 3. Characteristics of some electrochemical biosensors based on aptamers and enzymes already reported for ATP determination. FET: field-effect transistor; GCE: glassy carbon electrode; SPE: screen printed electrode; AA: ascorbic acid; SH: salicylate hydroxylase; GPDH: glucose-6-phosphate dehydrogenase; *: microelectrodes, **: incubation with sample, ***: LOD and linear range depend on glucose concentration.

Bioreceptor	Transducer	Response time (min)	Linear/working range (μM)	LOD (μM)	Selectivity, interferences	Application to real samples	Ref.
Aptamers	Au	2h**	0.001-1	0.00025	No interference CTP, GTP, UTP	Spiked human serum samples	113
		30**	0.001- 100	0.00054	No interference CTP, GTP, UTP	Spiked urine samples	114
		3.5h**	0.1 pM - 100 nM	30 fM	Low interference CTP, GTP, UTP	Spiked fetal bovine serum	80
		1.5h**	0.005-1	0.0014	No interference adenosine, ADP, CTP, UTP, GTP	Spiked human serum sample	115
	polycry-stalline Au	No data	1-111	1	No data	No data	116
	graphene FET	No data	0-50	10 pM	No data	No data	81
	GCE	2h**	10 pM - 10 nM	6.7 pM	No interference CTP, GTP, UTP	Spiked human serum samples	117
	graphene oxide-GCE	30**	10 pM - 100 nM	10 pM	No interference UTP Low interference GTP,CTP	Cell lysate (HeLa cells)	118
dual-labeled aptamers complex	GCE	45**	0.01-2000	0.0003	PPD membrane, no interference from Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_3^-, HPO_4^{2-}, glucose, dopamine, AA, CTP, GTP and UTP	Cell lysate (HeLa, MCF-7, and PC-3 cells)	119
Enzymes H^+-ATPase	ISFET	1-1.5	200-1000	200	Dependence on buffer capacity	No data	83

EF_0F_1-H^+-ATPase	Au	No data	2500-6000	2500	No data	No data	120
Apyrase	ISFET	5	200-1000	200	Dependence on buffer capacity	No data	84
Choline kinase	Choline-sensitive	1-2	10-75	4	No interference ADP, AMP, Na^+, K^+	Cell lysate (human erythrocytes)	85
GK + GPO	Pt/Ir*	0.2	0.2-50	0.2	Selective vs. UTP, ADP and adenosine. Permselective layer vs. electroactive compounds	No data	88
	graphene-Pt*	0.3	1.3-12	0.0001	Nafion membrane, no interference from AA	*Ceratopteris* fern spores and *Zea mays* roots	89
SH, GPDH, and HEX	SPE Pt	2	5-4000	3	Interference from electroactive compounds	No data	121
	Clark	2	100-8000	30	Teflon membrane, no interference from electroactive compounds	No data	121
GOx + HEX ***	Oxygen-sensitive	2	200-1800	200	No data	No data	122
	GCE	0.3	0.5-20	0.2	No data	No data	92
	Pt disk	0.1-1.3	0.01-0.2	0.01	No data	No data	91
	Pt*	5	0.25-4	0.01	No interference from AA, dopamine, serotonin, ADP, AMP	Gastrointestinal tissues (*in vitro* and *in vivo*)	94
	Pt	2-3	50-500	10	No data	Cell lysate (human erythrocytes)	90
	Pt disk	0.2-0.3	15 – 90	5	PPD membrane, no interference from electroactive compounds	Pharmaceuticals	This work

As seen in Table 3, the linear range of ATP amperometric biosensors already reported in the literature is highly variable, rendering the comparison with our biosensor quite difficult. Aptamer-based biosensors are far more sensitive but the proposed biosensor offers shorter response time and the possibility of a real-time ATP determination, while aptamer-based biosensors require a long incubation with the sample.

It is also interesting to compare the developed ATP biosensor with a microbiosensor (25 μm Pt wire) based on the same biorecognition element [93]. The microbiosensor was a bit more sensitive to ATP (LOD 2.5 μM) with almost the same response time (15-20 s). Dependence of the microbiosensor on Mg^{2+} concentration was similar. Range of ATP determination was almost the same in both cases. This indicates that the GOx/HEX based enzymatic system can be used from microelectrode to usual Pt disc electrode without significant changes in the biosensor characteristics.

III.2.8. 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 in biological samples, it is difficult to determine their individual concentrations. To overcome this weakness, an additional auxiliary biosensor, sensitive only to glucose, may be used. The proposed biosensor uses GOx as bioselective element.

The ultimate goal of this part of our work was then to develop a biosensor for the determination of glucose and couple the quantitative information drawn from the GOx and GOx/HEX biosensors (i.e. glucose and ATP concentrations, respectively) for the quantification of both substances in real samples. In this system including two biosensors, the bienzymatic biosensor is more important and complex, this is why we considered the working conditions, determined in previous parts for the biosensor based on GOx and HEX, as suitable for the GOx-based biosensor. Under these conditions, the GOx-based biosensor response was plotted vs glucose concentration.

Fig. 16 represents the average calibration curve measured from 5 different biosensors. The curve is linear in the 10 μM - 2 mM range ($I = - 6.87 + 138 * C$, where I – the current when the response reaches the steady-state value (nA), C - concentration of glucose (mM), $R^2 = 0.994$). LOD was 1 μM for glucose. These results are very close to the characteristics of the GOx/HEX biosensor for the detection of glucose.

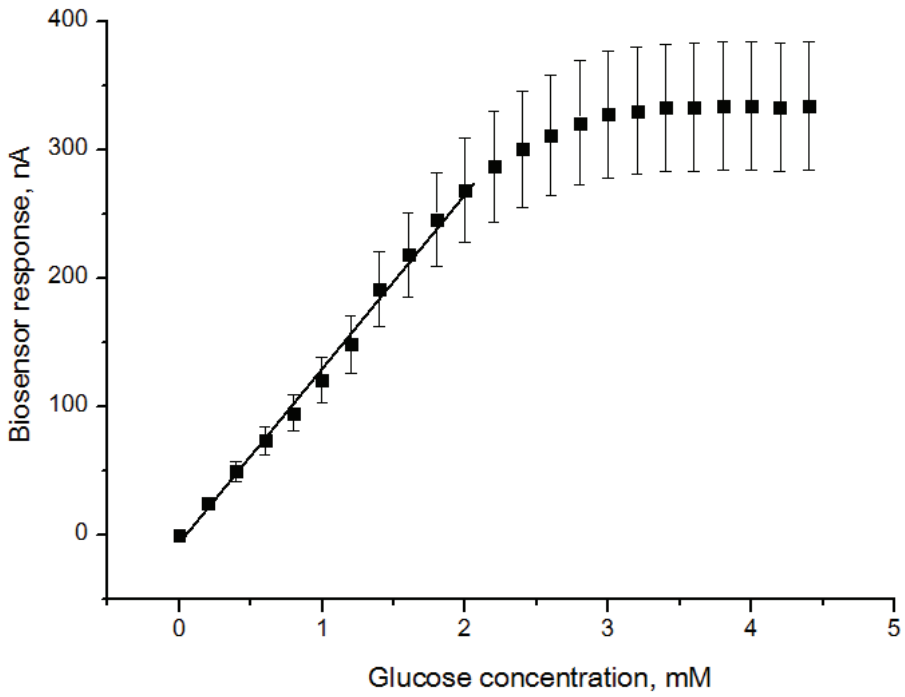

Figure 16. An average calibration curve (n=5) of GOx-based biosensor for glucose determination. Measurements were carried out in 25 mM HEPES buffer, pH 7.4, with 2 mM Mg^{2+}, the constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the mean of five biosensors ± standard deviation.

III.2.9. Measurements of Glucose and ATP in Pharmaceutical Samples

To evaluate the capacity of the biosensor system to be used for the simultaneous determination of glucose and ATP in real samples, the two biosensors (i.e., the first one based on GOx and HEX, the second one based on GOx only) were placed in the same measuring cell and connected to the same measuring setup via multiplexer. After obtaining the baseline, a test sample was added to the working solution and we measured the responses of both biosensors. The response of the second (GOx) biosensor is proportional to glucose concentration in the sample, and glucose concentration can be determined by the previously obtained calibration curve (Fig. 16). From the glucose calibration curve of the GOx/HEX biosensor (Fig. 14, curve 1), it is possible to calculate which would be the response of the biosensor to glucose in the absence of ATP. By the difference between the calculated and actual values of responses of the first biosensor to sample addition, the response to ATP can be calculated and ATP concentration determined from the calibration curve (Fig. 14, curve 2). Thus, using these two biosensors we can analyze the samples containing both glucose and ATP.

Tests samples were pharmaceutical solutions containing ATP (nominal concentration 10 mg/mL or 18 mM) produced by "Darnitsa" (Kiev, Ukraine) and glucose (nominal concentration 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 GOx/GEK biosensor and then received several responses to a sample (233-fold dilution); ATP concentration was calculated from the calibration curve. In case of the standard additions method, we obtained the response to glucose (100 μM), then (without washing) added ATP model solution (final concentration 40 μM), then added aliquot from the pharmaceutical vial (900-fold dilution of the sample), and finally - 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 15-20 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. The curve biosensor response (in nA) vs added ATP concentration after sample addition was linear and ATP concentration in the working cell was deduced from the intersection of the regression line with the abscissa axis. Results are given in Table 4. They completely coincided (within the measurement error) with a nominal ATP concentration.

Table 4. Results of biosensor measurement of ATP in vial. The nominal concentration of ATP in the vial - 18 mM. Measurements were repeated three times and an average value ± standard deviation is given.

Measurement method	Number of a vial			
	1	2	3	4
Calibration curve, mM	16.8 ± 0.9	19.5 ± 0.7	17.9 ± 0.5	17.3 ± 0.6
Standard addition, mM	16.3 ± 1	19.8 ± 2.3	15.9 ± 0.2	17.7 ± 1.2

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. Aliquotes of these solutions were added to the working cell and responses of the biosensor system were measured using procedure described in section 'Biosensor for glucose determination'. The results of measurements are given in Table 5; deviation from the nominal values usually did not exceed 15%. The measurement error was bigger than in case of separate glucose and ATP samples, which can be explained by more complicated measurement procedure using two biosensors. However, these results demonstrate that proposed biosensor system is suitable for reliable measurements in samples containing both ATP and glucose, which was the main goal of this work.

Table 5. Results of biosensor measurement of glucose-ATP mixtures. Biosensor measurements were repeated three times and the average value ± standard deviation is given.

	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	3	1.5
Measured concentration	2.68 ± 0.1	4.16 ± 0.4	11.7 ± 0.4	8.8 ± 0.8	2.9 ± 0.1	1.6 ± 0.2
Recovery, %	108 ± 4	84 ± 8	117 ± 4	88 ± 8	97 ± 3	106 ± 13

III.3. Conclusions and Perspectives

The biosensor system for simultaneous determination of ATP and glucose was developed. The proposed system consisted of two biosensors: one - sensitive to glucose, another - 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 crosslinking 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 an 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 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%, to ATP - 8-12%. Such deviation is typical for enzymatic ATP biosensors. The biosensor system showed good storage stability if stored at -18 °C, 50-day storage resulted in a decrease of responses to ATP by 43% and did not cause any drop in responses to glucose. Such storage stability is better if compared with most of described enzyme-based ATP biosensors, but it is worse if compared with aptamer-based biosensors which can be stored for 4-6 month without loss of sensitivity. We showed the possibility of simultaneous measurements of ATP and glucose concentrations in pharmaceutical vials. The biosensor system can be further used for determination of glucose and ATP in aqueous samples. Also, the system can be included into more complicated multibiosensors and biosensor arrays for simultaneous determination of many substances like lactate, glutamate, choline, and others.

CHAPTER IV: CREATION OF A BIOSENSOR FOR DETERMINATION OF CREATINE KINASE ACTIVITY

IV.1. Introduction

The aim of the work presented in this chapter was to develop a reusable amperometric biosensor for rapid and simple determination of CK activity in blood serum. For that, we used the GOx/HEX biosensor proposed in the previous chapter and optimized its working conditions.

IV.2. Results and Discussion

IV.2.1. Biosensor Principle and Operation

The determination of CK activity by the proposed biosensor is based on the measurement of the rate of ATP production following the introduction of CK and its substrates, adenosine diphosphate (ADP) and creatine phosphate, into the electrochemical measurement cell containing the GOx/HEX biosensor. According to reaction 9, ATP molecules are produced by phosphorylation of ADP catalyzed by CK enzyme. Almost all kits commercialized for the assessment of CK activity use the reaction 9.

$$\text{ADP + creatine phosphate} \xrightarrow{\text{Creatine kinase}} \text{ATP + creatine} \qquad (9)$$

Experimentally, CK determination is performed by successive additions of creatine phosphate, ADP, glucose, ATP and finally CK. As seen in Fig. 17, recorded for 10 mM creatine phosphate, 5 mM ADP, 300 μM ATP and 0.13 U/mL CK, the biosensor does not react to creatine phosphate and ADP additions. After addition of glucose, a biosensor response is observed. After addition of ATP to the working cell, HEX starts to phosphorylate glucose (reaction 8 in page 41) and the biosensor response to glucose decreases. This decrease demonstrates that immobilized HEX is still active (addition of ATP is not necessary for CK determination and it was done only to demonstrate the biosensor sensitivity to ATP). After addition of CK, the biosensor response starts to decrease and steady state is reached in 20 min in those conditions. CK activity is directly related to the initial slope of the response to CK addition. This latter can be calculated from the 100 first seconds of the response (further expressed in the figures as “Decrease of response in nA/100s”). Further decrease is not observed because ATP concentration becomes too high (more that 1 mM) and the biosensor response to ATP is maximal (HEX works at maximum rate). It should be mentioned that CK also catalyzes the reverse reaction forming ADP and creatine phosphate from ATP and creatine, but this reaction is favored only at pH 9.0 [123].

The proposed procedure enables measuring CK activity only because CK substrates only are added to the solution. However, it should be easily modified for the determination of other kinases like protein kinase A or casein kinase by adding corresponding substrates.

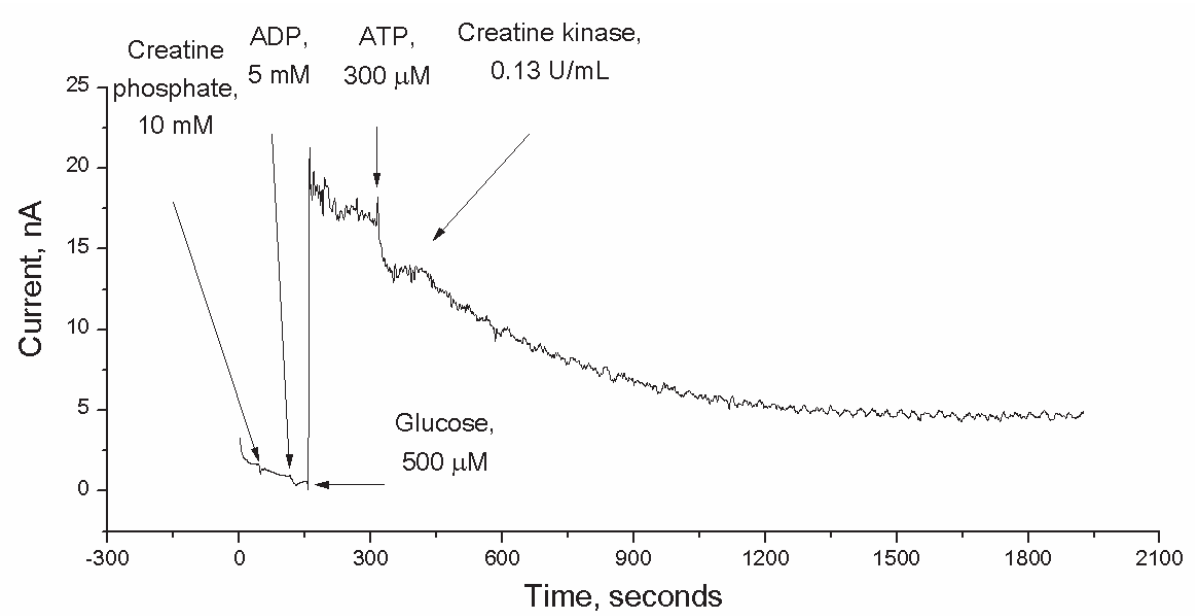

Figure 17. Typical responses of the biosensor to consecutive additions of substrates and CK (concentrations are given on the figure). Measurements were carried out in 10 mM HEPES buffer, pH 7.4, with 10 mM Mg^{2+}, the constant potential of 0.6 V vs Ag/AgCl reference electrode.

To control that the decrease in biosensor response observed in the presence of ADP and creatine phosphate is really due to the production of ATP catalyzed by CK, and not by non-specific adsorption or other non-selective processes, a control experiment was carried out in the same conditions except that creatine phosphate was not added. No decrease in biosensor response was noticed after CK addition, which was also the case if ADP was absent.

IV.2.2. Optimization of ADP and Creatine Phosphate Concentrations

The first step of the biosensor optimization process was dedicated to the definition of CK substrates concentrations to be used to achieve the highest sensitivity of the response to CK ma. To start with, we used ADP and creatine phosphate concentrations published in the first paper describing the application of ATP biosensor to CK measurements [98]. In this work, we investigated the effect of lowerconcentrations of ADP and creatine phosphate on the

biosensor response at three CK concentrations in the working cell: 0.013 U/mL, 0.038 U/mL, and 0.1 U/mL. Using lower substrate concentrations will participate to decrease the analytical costs.

At first, the effect of ADP concentration was investigated in the 0.1 to 5 mM range at a constant 10 mM concentration of creatine phosphate. It was found that the best sensitivity to CK addition was achieved at 1 mM of ADP, and higher ADP concentrations led to a decrease in the sensitivity (Fig. 18, A). This unexpected effect can be explained by HEX inhibition at higher concentrations of ADP [124, 125].

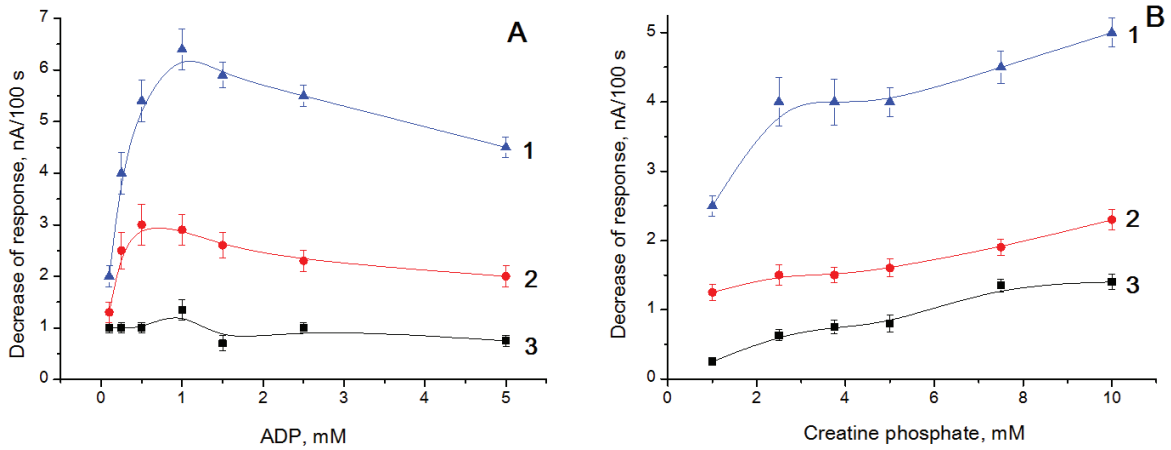

Figure 18. Influence of concentrations of ADP (A) and creatine phosphate (B) on CK activity (1 – 0.1 U/mL, 2 – 0.013 U/mL, 3 – 0.038 U/mL, in the working cell). Creatine phosphate concentration during ADP selection was 10 mM. ADP concentration during creatine phosphate selection was 1 mM. Measurements were carried out in 10 mM HEPES buffer, pH 7.4, with 10 mM Mg^{2+}, the constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the mean of three biosensors responses ± standard deviation.

In subsequent experiments, CK activity was determined at 1 mM of ADP, varying creatine phosphate concentration between 1 and 10 mM. As seen in Fig. 18, B, an increase of creatine phosphate concentration caused an increase in the biosensor sensitivity to CK addition. 10 mM of creatine phosphate was therefore used in further experiments. We did not use concentrations above 10 mM because this is enough for the detection of small CK activities found in real samples.

IV.2.3. Calibration Curves at Different Glucose Concentrations

In the next stage of the work, the calibration curves for CK determination were obtained by recording the decrease of response following CK injection (= initial slope in nA/100s) vs CK activity (in U/ml) at different glucose concentrations. As was already mentioned in the previous chapter, the biosensor sensitivity to ATP depends on glucose concentrations, so the calibration curves were built at glucose concentrations that is susceptible to be found in the working cell in the case of 20-fold dilution of blood serum (Fig. 19).

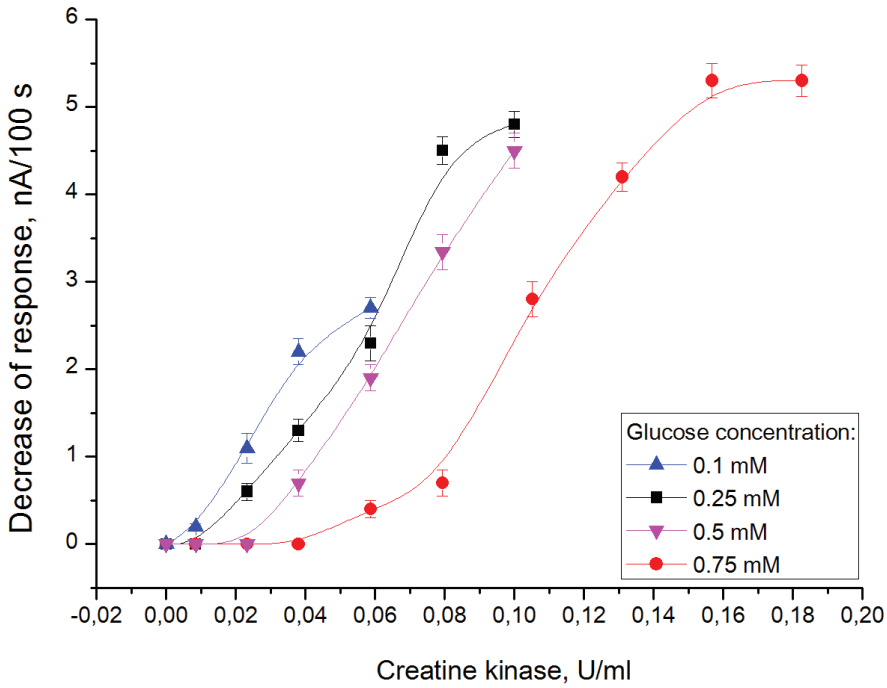

Figure 19. Calibration curves for creatine kinase determination, obtained at different concentrations of glucose in the working cell. Measurements were carried out in 10 mM HEPES buffer, pH 7.4, with 10 mM Mg^{2+}, 1 mM ADP, 10 mM creatine phosphate, at the constant potential of 0.6 V vs Ag/AgCl reference electrode. Data is presented as the mean of three biosensor responses ± standard deviation.

As seen in the figure, the range of CK determination changes at higher concentrations of glucose. At glucose concentrations between 0.1 mM and 0.25 mM, it was possible to measure CK activities in the 0.01 – 0.1 U/ml range. In the case of glucose concentration of 0.75 mM, the the range of possible CK measurements shifted to 0.06 – 0.16 U/ml. The sensitivity was enough to measure increased levels of CK in the case of 20-fold dilution. This is a significant drawback of the proposed biosensor, because in case of healthy people the

biosensor will only provide information that the CK level is normal and could not measure CK activity accurately. However, if the level of CK is normal (below 0.17 U/ml), then sample dilution should be decreased in order to increase the amount of CK in the working cell. It should be mentioned that previously developed biosensors also could not measure CK activity below 0.01 U/ml [97, 98].

IV.2.4. Reproducibility of CK Activity Measurements

The reproducibility of biosensor responses to glucose and ATP (Fig. 20, A), and to CK activity (Fig. 20, B) was further investigated. Measurements were carried out for one day, with 15-min intervals. No significant decrease of the responses could be detected for several hours of continuous operation, indicating that enzymes are properly immobilized and remain active. The relative standard deviations calculated from the 15 measurements of three biosensors recorded the same day were 2% for glucose, 5-7% for ATP, and 7-18% for different concentrations of CK. Higher errors measured for ATP compared to glucose may be explained, as previously, by the fact that signals are obtained from a two enzyme-competitive process instead of a single enzyme process. Similarly, higher deviations of CK responses are caused by involvement of one more enzyme and two more substrates.

Long-term stability of the biosensors was also investigated. The biosensors were stored for one month in dry state at -20°C and their responses to glucose and ATP decreased by 20-30%. Thus, the biosensors can be used after storage but recalibration is needed.

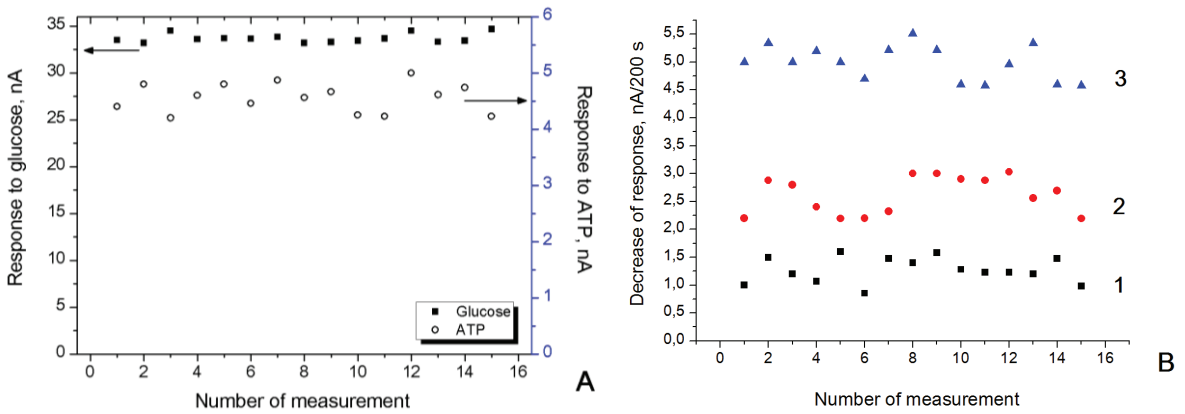

Figure 20. Reproducibility of biosensor responses to glucose and ATP (A), and to different concentrations of CK (B): 0.013 U/ml (B, 1), 0.038 U/ml (B, 2), 0.1 U/ml (B, 3). Glucose concentration – 0.25 mM, ATP – 0.1 mM. Measurements were carried out in 10 mM HEPES buffer, pH 7.4, with 10 mM Mg^{2+}, 1 mM ADP, 10 mM creatine phosphate, at the constant potential of 0.6 V vs Ag/AgCl reference electrode.

IV.2.5. Application of the Biosensor to the Analysis of CK Activity in Blood Serum

Procedure

As already emphasized in the previous chapter, GOx/HEX biosensor is sensitive to two substrates – glucose and ATP. Glucose is always present in blood serum samples. Normal glucose concentration range is 4-7 mM (5.5 mM on average) but it can increase up to 15-20 mM in case of diseases. As the linear range of glucose determination using the GOx/HEX biosensor does not exceed 1.5 mM (Fig. 14, curve 1) serum samples should be diluted 10-20 times in order to obtain a measurable glucose concentration in the working cell. The biosensor is also sensitive to ATP and a calibration curve may be also determined (Fig.14, curve 2) but ATP concentration in blood (1 μM) is below the biosensor limit of detection and ATP will not affect glucose measurements. The calibration curve will enable calculating the rate of ATP production by the kinase.

The addition of two substrates (ADP and creatine phosphate) is needed for CK determination. Their concentrations in blood serum are quite low. Thus, both substrates should be added to the working cell after obtaining the biosensor response to the serum sample. This will activate CK, ATP will be produced, and biosensor response to glucose will decrease proportionally to ATP production. After evaluation of the response decrease, it will be possible to determine CK activity in the sample. Glucose concentration will be determined by comparison of the biosensor response (before addition of the CK substrates) with a previously obtained calibration curve. Using this procedure, it will be possible to measure both glucose and CK in one run.

Selection of Optimal Dilution of the Serum Sample

The sensitivity of the biosensor to ATP (and to CK) depends on glucose concentration: the higher glucose concentration, the lower sensitivity to ATP. To measure CK activity with sufficient accuracy, it is therefore necessary to record the calibration curve for CK determination at a certain glucose concentration (for example, 250 μM) and perform two measurements of the sample. During the first measurement, glucose concentration will be determined, and then the dilution of the sample will be calculated in order to obtain 250 μM of glucose in the working cell. The second measurement will be done using this dilution of the sample. After ADP and creatine phosphate addition, it will be possible to determine CK activity by using previously obtained calibration curve.

However, for clinical needs it is not necessary to accurately determine CK concentration in blood. It is important to know if CK level is increased and how much it is increased. This will make possible to interpret symptoms of a disease. A simple one-step procedure can be used instead of the procedure described above in order to see if the CK level is increased. In case of normal glucose level, 20-fold dilution of the sample during measurement will result in 200-350 μM of glucose in the working cell. Sensitivity to ATP will not be much different, so CK activity can be determined quite accurately. However, for patient with diabetes, glucose concentration in the measurement cell after 20-times dilution of blood serum will be too high, so higher dilution of the sample will be necessary. In order to select the optimal sample dilution, we used a test sample prepared in deionized water and containing only glucose (5.0 mM) and CK (0.35 U/ml, twice bigger concentration than normal for males). This sample was added to the working cell after 6.7 to 50- fold dilutions, and then ADP and creatine phosphate were added. Table 6 gathers biosensor variation of current (due to glucose) following sample addition as well as decrease in response for 100 s observed following substrates addition. The latter was expressed in nA and percent of response to glucose. The biosensor registered CK activity at all dilutions, the response was very small for the 50-times dilution. Twenty-fold dilution was considered as optimal because the decrease of the response is sufficient and interferences from electroactive compounds of the serum sample will be decreased by dilution. Furthermore, if glucose concentration in the sample is different (e.g., 3 mM or 15 mM), 20-fold dilution will give 0.2 or 0.75 mM of glucose in the working cell and CK determination will still be possible, as seen from the table.

The biosensor operates at quite high working potential (+0.6 V) that is needed for direct oxidation of H_2O_2. However, other electroactive substances present in serum samples can be oxidized on the surface of working electrode and produce non-selective signal. A small response was detected by measuring clean working electrodes after addition of the serum samples. The value was quite high, representing about 15% of the response recorded for the same samples using the GOx/HEX modified electrodes. However, this non-selective reaction did not cause gradual decrease of biosensor response and thus did not interfere with CK determination. So, we did not try to avoid these reactions by application of permselective membranes or lower working potential.

Table 6. Selection of optimal dilution of the serum samples for biosensor analysis of CK activity. The sample contained 5 mM of glucose and 0.35 U/ml of CK. Measurements were carried out in 10 mM HEPES buffer, pH 7.4, with 10 mM Mg^{2+}, 10 mM creatine phosphate, and 5 mM ADP, at the constant potential of 0.6 V vs Ag/AgCl reference electrode.

Dilution of a sample	Glucose concentration in the cell, μM	CK activity in the cell, U/mL	Biosensor response to glucose, nA	Decrease of response, nA/100 s	Decrease of response, %/100 s
x6.7	750	0.052	67 ± 17	3.6 ± 1	5.3 ± 0.9
x10	500	0.035	46 ± 14	2.9 ± 0.6	6.4 ± 0.6
x20	250	0.017	24.3 ± 4.7	1.7 ± 0.5	6.7 ± 0.4
x50	100	0.007	10.3 ± 2	0.5 ± 0.1	5.0 ± 0.4

Analysis of Spiked Blood Serum Samples

In order to evaluate the capacity of the biosensor to quantify CK activity in real samples, we further measured CK activity in spiked blood serum samples. Raw samples were obtained from Kiev municipal scientific and practical center of nephrology and hemodialysis (Ukraine). CK lost almost all activity when the serum samples were stored frozen. No decrease in biosensor response was observed after addition of the serum to the working cell during measurements (this fact also suggests that the serum components do not inhibit biorecognition elements of the biosensor). Mayo Medical Laboratories (USA) recommends storing blood serum with CK not more than 1 week in a refrigerated state or 28 days in a frozen state [107]. Indeed, even in model solutions we observed quick loss of CK activity (during 1-2 weeks) and it was necessary to prepare fresh solutions of CK from the dry enzyme.

Serum samples, coming from different patients, were spiked with different CK concentrations (0.12 U/ml, 0.26 U/ml, 0.65 U/ml, 0.76 U/ml and 1.3 U/ml). The chosen CK concentrations cover CK range found in serum of healthy (0.026-0.174 U/ml) and ill (0.2-2.0 U/ml) people. Then, optimal conditions defined previously were applied. After successive additions of sample, ADP and creatine phosphate, glucose concentrations and CK activities were measured using adequate calibration curves. As seen in Table 7, biosensor results are in relatively good agreement with calculated CK concentrations, but systematically lower by 11 to 25%.

Table 7. Biosensor measurements of different samples of blood serum with added CK. All samples were obtained from different people.

# of sample	Added CK in the serum, U/ml	Measured CK, U/ml	Recovery, %
1	0.12	0.10 ± 0.01	83 ± 10
2	0.26	0.23 ± 0.03	89 ± 13
3	0.65	0.52 ± 0.05	80 ± 10
4	0.76	0.57 ± 0.06	75 ± 11
5	1.3	1.07 ± 0.12	82 ± 11

Furthermore, the biosensor was examined during continuous measurements of blood serum. It was noticed that after two-hour incubation of the biosensor in 20-fold diluted serum the responses to glucose fell to 90% of the initial value. This result may be attributed to clogging of the enzyme membrane of the biosensor with components of the serum. However, during the measurements of the serum samples with washing of the biosensor between measurements, the decrease of response was not so high. The biosensor could measure about 7-10 samples without significant changes in its analytical characteristics, and then the biosensor should be recalibrated.

IV.3. Conclusions

An amperometric enzyme biosensor was proposed for the determination of creatine kinase activity in blood serum. Optimal values for sample dilution, ADP and creatine phosphate concentrations were first determined. Biosensor was characterized by sufficient reproducibility of responses during measurements of the main analytes (glucose, ATP and CK). The determination of CK activity in spiked blood serum samples was then performed. The biosensor could be suitable for express measurements of CK activity in blood serum of patients with acute myocardial infarction or other muscular injuries. Small size of measuring device and simple procedure of biosensor preparation and application are advantages over traditional methods of CK determination. The main disadvantage of the proposed biosensor is an influence of glucose on the biosensor sensitivity, so it is necessary to choose between approximate determination of CK activity during one measurement and accurate determination during two measurements (the first measurement gives glucose concentration needed for the selection of the optimal sample dilution during the second measurement).

Accuracy of CK activity measurement was about 80-85% if glucose concentration was known before experiment. Otherwise, biosensor accuracy decreased to about 30-50%, what was still enough in most cases to distinguish samples from healthy and ill people.

The described procedure of determination of CK activity can be adapted for the determination of other kinases for medical or research purposes. It should be noticed that the proposed method can be used to assess the rate of ATP production or degradation in the working cell what can be useful for research purposes. For example, at CK concentration 0.038 U/ml, the rate of ATP synthesis was 20 μM/min. In the current work it was not important, thus such calculations were not presented.

CHAPTER V: DEVELOPMENT OF A CONDUCTOMETRIC ATP BIOSENSOR

V.1. Introduction

The third part of the work was devoted to the elaboration of a conductometric biosensor for the determination of ATP. Conductometric transducers are simple and cheap and this was the first time that a conductometric biosensor was proposed for ATP detection. Different elaboration and working parameters affecting biosensor response were optimized and the applicability of the developed biosensor to pharmaceutical samples was assessed.

V.2. Results and Discussion

V.2.1. Biosensor Principle and Operation

The determination of ATP by the proposed conductometric biosensor is based on the measurement of local conductivity changes induced by the phosphorylation of glucose by ATP catalyzed by HEX immobilized at the surface of gold interdigitated electrodes. This reaction has been already described in chapter III and used for ATP and CK activity measurement using the two formerly developed biosensors (page 41 reaction 8).

During this reaction, the local concentration of ions in the working membrane increases, thus, the conductivity of the solution in the near-electrode region changes, which can be detected using conductometric transducers. This change in conductivity is directly proportional to glucose and ATP concentrations in the working cell. One important advantage of the proposed method, compared to other existing amperometric and potentiometric biosensors which require two enzymes to operate, is that it relies only on one enzymatic reactionones. This reaction itself, neither generates electroactive products, nor changes the solution pH, and can be detected directly by conductometry.

Experimentally, ATP or ATP-containing sample is first added to the working cell. Since ATP is a charged substance, the biosensor generates a nonspecific signal (a peak at 50 s in Fig. 21, A). This signal is compensated by using the differential measuring mode if working and reference membranes are identical by their morphology and thickness (the peak disappears and the biosensor signal returns to baseline). After stabilization of the signal, a glucose solution of known concentration is added to the working cell, and phosphorylation of glucose takes place in the enzyme membrane. This reaction results in the appearance of two new charged substances (ADP and phosphorylated glucose), thus the solution conductivity changes and the biosensor signal is generated. Since glucose is not charged, its addition does not cause any non-specific response, and the signal generated is exclusively due to the enzymatic reaction. Thus, the two-step procedure of addition of substances allows avoiding

one of the main disadvantages of conductometric biosensors, i.e., sensitivity to any charged substances. The analysis time is about 5 min, which is quite acceptable. After measurements, the biosensor and the working cell are washed from substrates during 5 min using the working buffer, and the biosensor can be used again.

As a control, the inverse experiment was carried out (Fig. 21, B), i.e. first glucose and then ATP were added to the working cell. As seen, there was no response to glucose, whereas the response after ATP addition was identical to the response obtained after glucose addition in the previous experiment.

Overall, experiments in Fig. 21 demonstrate that separate addition of glucose or ATP to the working cell does not generate any signal. The biosensor response is generated only when both ATP and glucose are introduced the cell. Furthermore, charged substances like ADP or ascorbic acid that can be present in real samples will not interfere measurements because the biosensor response depends only on concentrations of glucose and ATP, while possible non-specific response to ions (including ATP) is compensated by differential measurement mode.

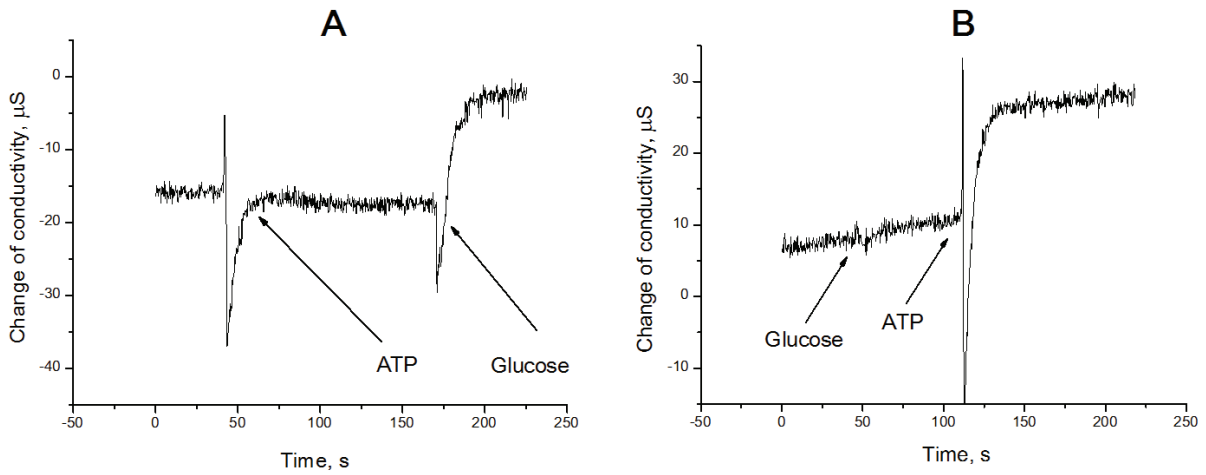

Figure 21. A: typical biosensor responses during measurements of the ATP concentration (first ATP or sample is added, and then glucose). B: control experiment, first glucose is added, and then ATP. Concentration of ATP was 100 µM, glucose – 200 µM.

V.2.2. Optimization of Hexokinase Immobilization

In our previous work, conducted on the amperometric ATP biosensor based on HEX and GOx, we successfully used a co-immobilization process involving cross-linking of the

two enzymes by GA in presence of BSA and glycerol. Here, we used the same approach but only one enzyme (HEX) was immobilized at the surface of the conductometric transducers.

To optimize GA concentration, different HEX/BSA ratios were tested. Stock solutions containing 3% – 15% HEX, and 0% – 5% BSA were used. These solutions were mixed with 0.5% or 0.8% GA (1:1 v:v) and then the biosensors were allowed to dry at room temperature for 15-30 min in order to complete cross-linking process. The highest biosensor responses were observed for 10% HEX, 5% BSA - 0.5% GA and 30 min cross-linking time.

Different GA concentrations were also investigated (from 0.3 to 0.9% before mixing with the enzyme solution). Very small responses to glucose and ATP were observed when using the 0.7% and 0.9% GA concentrations, which was due to a significant decrease in the HEX activity after immobilization. Biosensor responses were high and almost identical for 0.3% and 0.5% GA concentrations. 0.5% GA was therefore used for immobilization in further experiments.

V.2.3. Influence of Working Buffer Composition on Biosensor Response

Working buffer composition may influence the biosensor response since it affects enzyme activity. In the next stage of the work, parameters such as Mg^{2+} concentration, buffer capacity and ionic strength were therefore investigated.

Mg^{2+} concentration in working buffer varied from 1 mM to 4 mM. In the experiment, aliquots of concentrated magnesium chloride solution were initially added to the working buffer, and then 100 μM of ATP was added. After stabilization, 200 μM of glucose were injected in 30 s and steady-state biosensor responses were measured. As seen in Fig. 22, the biosensor response increased with magnesium concentration up to 3 mM and remained constant afterwards. This result is consistent with the optimal magnesium concentration determined for the amperometric biosensor in chapter III, since HEX concentration in the biomembrane was twice lower in this case. 3 mM Mg^{2+} was chosen as optimal concentration in further experiments.

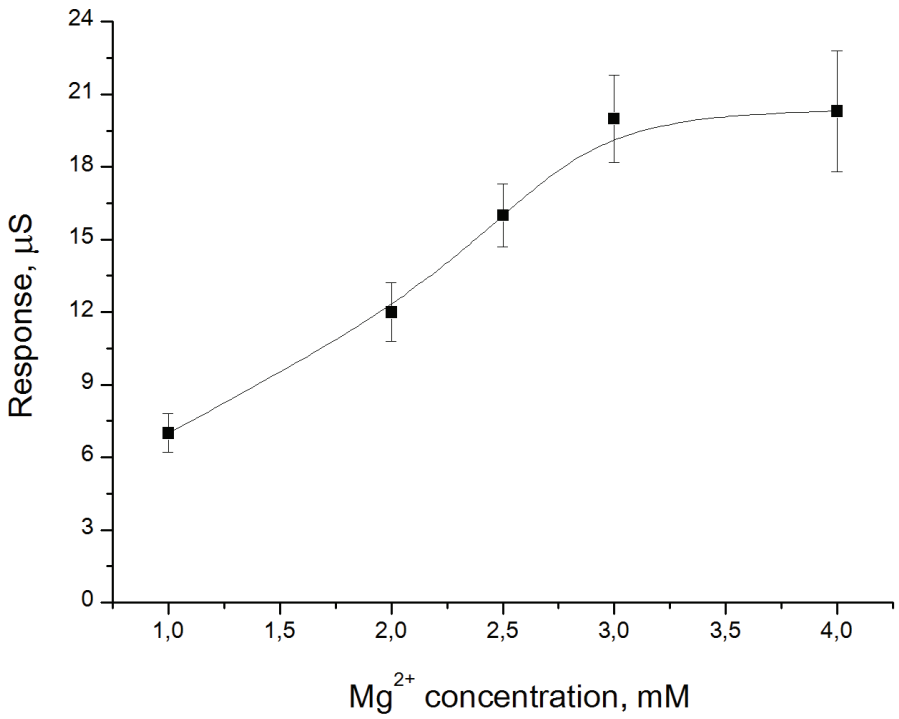

Figure 22. Influence of magnesium ions concentration in working buffer on biosensor response. Concentration of ATP was 100 µM ATP, glucose – 200 µM. Data is presented as the mean of three biosensors ± standard deviation.

Working buffer capacity is also a parameter strongly affecting the performance of conductometric biosensors. As seen in Fig. 23, the highest biosensor response was observed at the minimal buffer concentration tested (1 mM), and the response further decreased exponentially with increasing concentration to achieve nearly zero at the maximal concentration evaluated (15 mM). Such dependence is typical for conductometric biosensors. 5 mM HEPES buffer was finally selected for further experiments as it was a good compromise between the low concentration required to achieve the highest sensitivity to ATP, and the high buffering capacity necessary to keep the pH value stable after sample addition, whatever the sample pH could be.

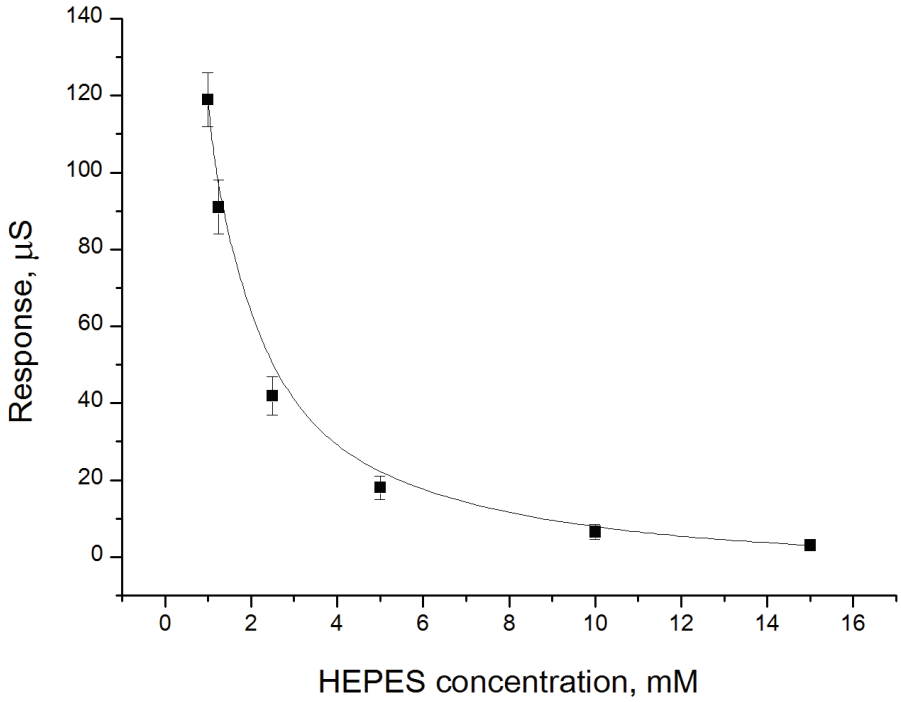

Figure 23. Influence of working buffer concentration on biosensor response. Buffer pH: 7.4. ATP concentration in buffer: 100 µM, glucose concentration: 200 µM. Data is presented as the mean of three biosensors ± standard deviation.

The biosensor sensitivity may be also strongly affected by electrolyte ionic strength. , To vary this parameter, potassium chloride was added in the measurement cell in order to achieve final concentrations in the 1 to 20 mM range. As seen in Fig. 24, no significant variation of the biosensor response was observed in this domain. However, higher KCl concentrations decreased the reproducibility of the signal and increased the background noise, and thus limit of ATP detection increased.

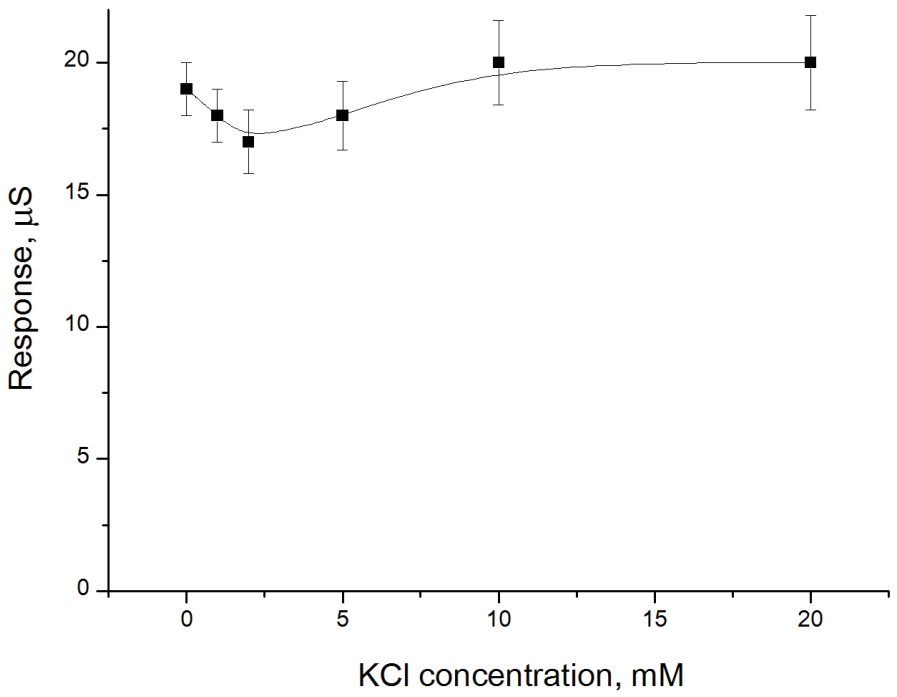

Figure 24. Influence of KCl concentration on biosensor response. ATP concentration: 100 µM, glucose concentration: 200 µM. Data is presented as the mean of three biosensors ± standard deviation.

V.2.4. Analytical Characteristics of the Biosensor

The biosensor response depends on both ATP and glucose concentrations. In a first series of experiments, ATP (25-300 µM) was added to the solution, then the glucose concentration was gradually increased from 0 to 600 µM and the biosensor response was measured. The corresponding response curves obtained for glucose at the different ATP concentrations are shown in Fig. 25. As seen in the figure, the maximal responses were observed at 200 µM glucose, whatever ATP concentration, and further increase in glucose concentration did not lead to a significant increase of the response value. Thus, 200 µM glucose can be considered as suitable for ATP determination, at least for ATP concentrations that do not exceed 300 µM. We did not investigated ATP concentrations over 300 µM as the real samples targeted are not expected to contain more than 300 µM after dilution. .

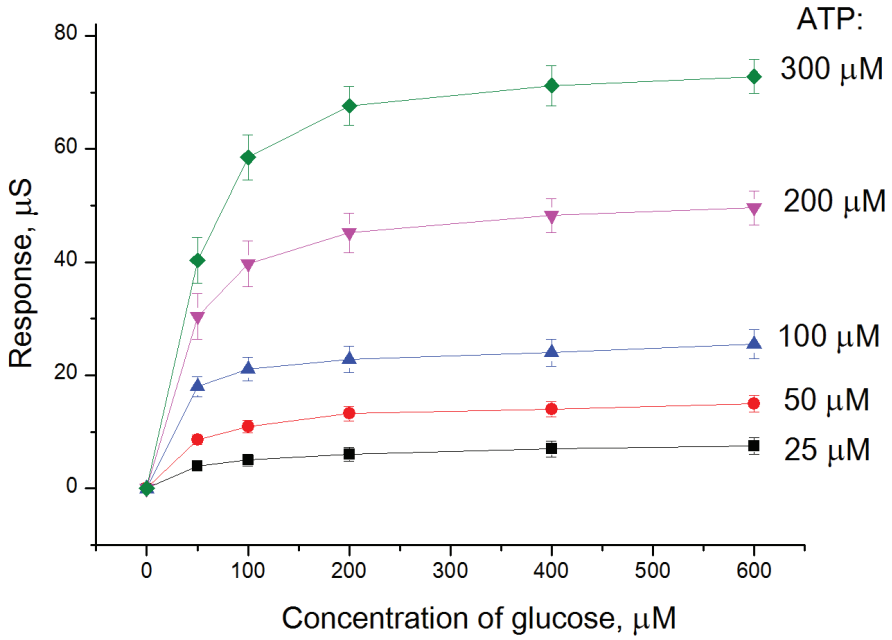

Figure 25. Influence of glucose and ATP concentrations on the biosensor response curves. Data is presented as the mean of three biosensor responses ± standard deviation.

When using 5 mM HEPES, pH 7.4 containing 3 mM Mg^{2+}, and injecting 200 μM glucose, the limit of ATP detection was 15 μM (S/N = 3). The typical calibration curve of the biosensor for ATP determination is shown in Fig. 26. This curve is described by the equation $\sigma = 0.69 + 0.22 * C$ ($R^2 = 0.99$), where σ is the solution conductivity (steady-state response, μS), C is the ATP concentration (μM).

These characteristics can be varied by changing the working solution composition, depending on the task to be solved.

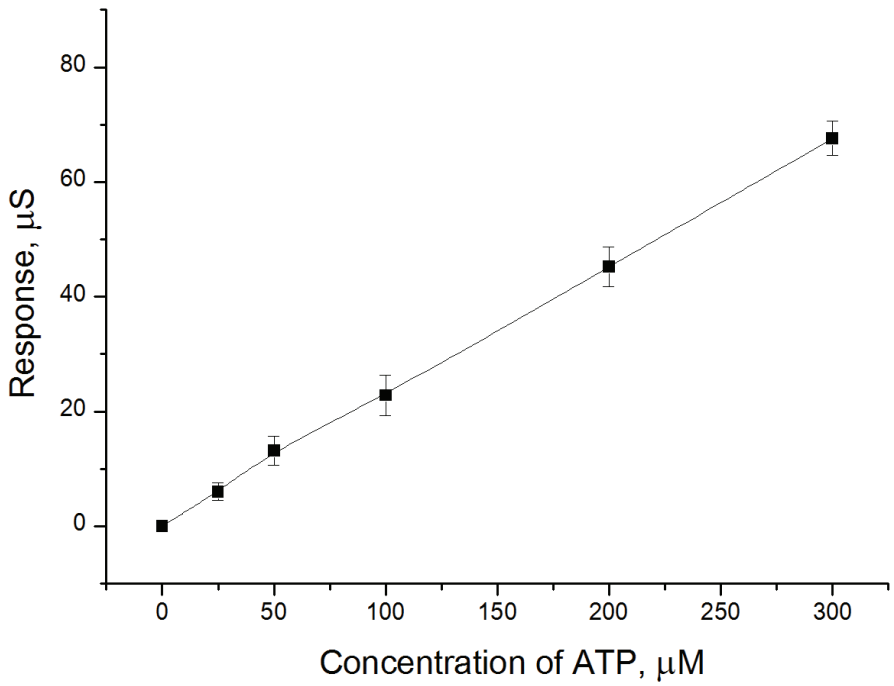

Figure 26. Typical calibration curve of HEX-based conductometric biosensor for the determination of ATP concentration. Glucose concentration: 200 µM. Working buffer: 5 mM HEPES, pH 7.4, 3 mM of Mg^{2+}. Data is presented as the mean of three biosensor responses ± standard deviation.

V.2.5. Reproducibility of Biosensor Responses

The proposed biosensor is reusable, and thus the next step of the work aimed at investigating the reproducibility of biosensor response to ATP upon several hours of continuous operation. One complete determination of ATP lasted 5-7 min. The biosensor was washed from substrates during the 10-min intervals between measurements, repeatedly changing working buffer.

The results obtained are presented in Fig. 27. The biosensor response values did not reduce noticeably during 10 measurements. The relative standard deviation, calculated from the 10 points, was 10.3%.

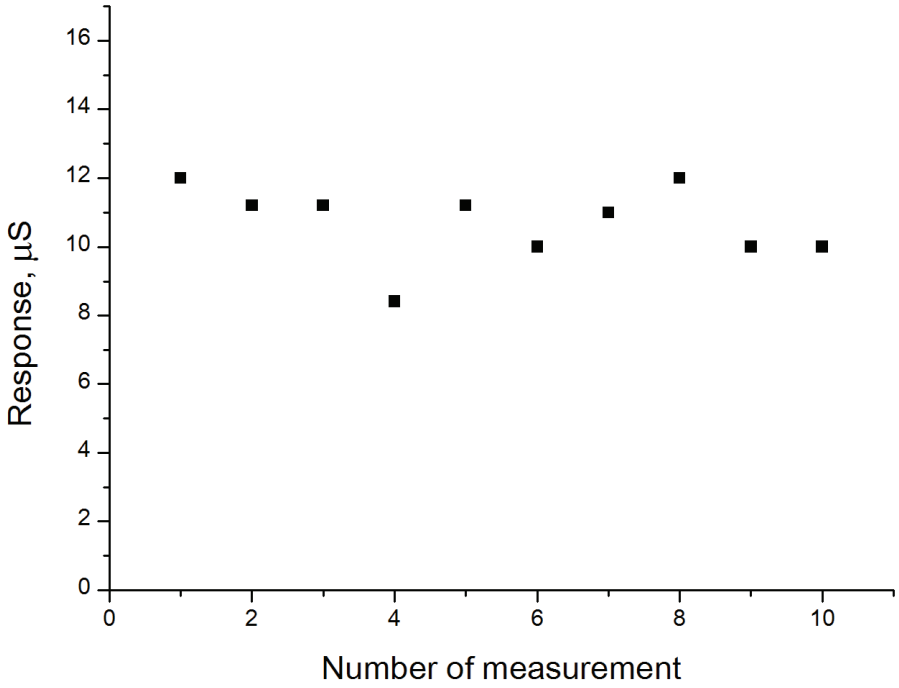

Figure 27. Reproducibility of biosensor response to ATP upon several hours of operation. Concentration of ATP: 100 µM, glucose: 200 µM.

V.2.6. Operational Stability of Biosensors

In the next phase of our work, we investigated the operational stability of the biosensor developed. During one working day, five or six responses to glucose and ATP (0.2 mM and 0.1 mM, respectively) were measured. Afterwards, the biosensor was stored in the working buffer at 4 °C until next use. On the next day, the responses to the same concentration of glucose and ATP were measured. The total period of the biosensor operation and storage was 6 days.

As shown in Fig. 28, a gradual decrease of the response was observed, which was accompanied by some deterioration of reproducibility. Nevertheless, the biosensors remained suitable for daily measurements during one week.

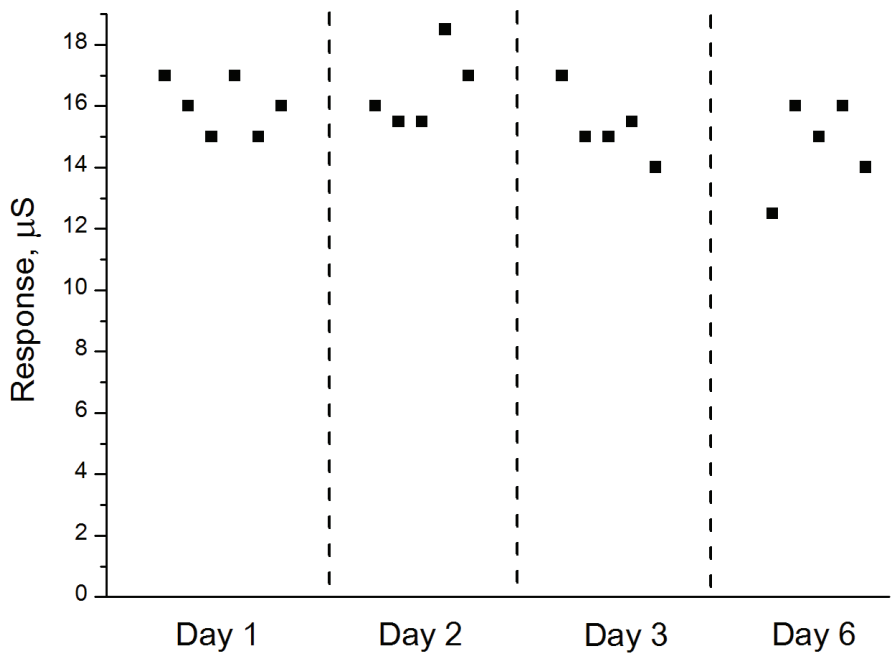

Figure 28. Operational stability of the proposed biosensor. Concentration of ATP: 100 µM, glucose: 200 µM.

V.2.7. Long-term Storage of Biosensors

In the next series of experiments, responses to 0.1 mM ATP and 0.2 mM glucose were measured using several freshly prepared biosensors. Then, the biosensors were stored under various conditions. After 11-day storage, the biosensors response to the same substrate concentrations was measured again.

Three modes of storage were investigated: dry storage at +4 °C and -18 °C, storage in the working buffer at +4 °C (only sensitive transducer parts coated with the enzyme and BSA were immersed into the buffer). No response could be observed after dry storage at +4 °C. After storage in buffer at +4 °C, the biosensor responses decreased by 10 to 15%. The best results were finally obtained by storing the biosensors at -18°C, the responses decreasing only by 7 to 12%.After one month storage in the same conditions, the biosensor responses decreased by 30%, indicating a lower stability of HEX compared to other enzymes as glucose oxidase or urease.

V.2.8. Measurement of Pharmaceutical Samples

In order to test the capability of the developed biosensor to measure ATP in real samples, the pharmaceutical samples containing ATP and already described in chapter III, were used. First, a calibration curve for ATP determination was built from ATP standard

solutions. Then, an aliquot of the sample was added to the working cell, and glucose was added after the stabilization of the baseline was reached. The value of biosensor response was compared with the calibration curve. The sample added to the working cell was small, enabling a direct dilution of the sample in the working cell.73-fold, 91-fold and 114-fold dilutions were used, indicated in Table 8.

Five mM of ATP was also added to one sample in order to achieve a total concentration of ATP of 23.15 mM. This sample was also measured by the biosensor. As shown in Table 8, recovery values were very good, ranging from 92 to 121 %.

The repeatability of ATP determination was also determined for one sample. For this purpose, aliquots of the sample were added 6 times to the working cell (with washing between additions), and responses of the biosensor were recorded (Fig. 29). Relative standard deviation of biosensor responses was 13.7%.

Table 8. Comparison of nominal or calculated ATP concentrations with ATP measured by the HEX conductometric biosensor in pharmaceutical samples. Working buffer: 5 mM HEPES, pH 7.4, 3 mM of Mg^{2+}. Glucose concentration: 200 μM.

Nominal ATP concentration in a sample (mM)	ATP concentration, measured by the biosensor (mM)	Recovery (%)	Sample dilution in the working cell
18.15	22.00 ± 3.10	121 ± 14	91
18.15	17.06 ± 2.38	94 ± 14	91
18.15	19.42 ± 2.64	107 ± 13	73
18.15	18.15 ± 2.49	100 ± 14	73
23.15	21.30 ± 2.92	92 ± 15	114

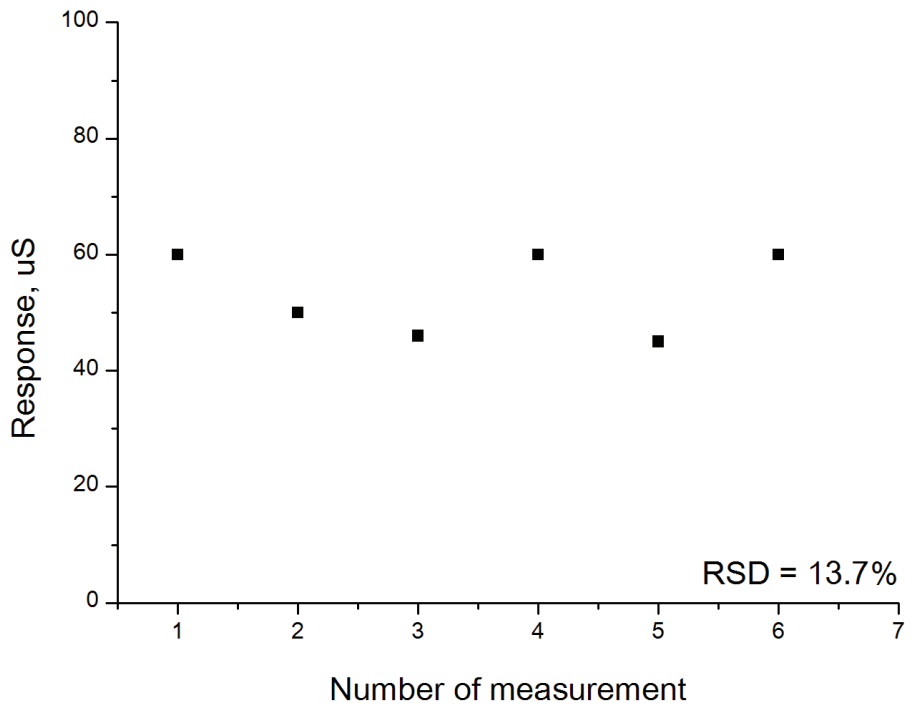

Figure 29. Repeatability of biosensor responses to ATP in one pharmaceutical sample (91-fold dilution). Working buffer: 5 mM HEPES, pH 7.4, 3 mM of Mg^{2+}. Concentration of glucose: 400 μM.

V.3. Conclusions and Perspectives

A reusable conductometric biosensor for the determination of ATP and glucose has been developed. The best immobilization of HEX was obtained as a result of HEX and BSA cross-linking by glutaraldehyde for 30 min. The biosensor characteristics depended on the composition of working buffer. 5 mM HEPES, pH 7.4, with 3 mM magnesium ions was found to be the optimal buffer solution. The investigation of relationship between the biosensor sensitivity to ATP and concentration of glucose showed that the optimum glucose concentration was 0.2 mM. In these conditions, the limit of ATP detection was 15 μM. The relative standard deviation of 10 consecutive measurements of biosensor responses to glucose and ATP was 10.3%. The biosensor developed remained suitable for daily measurements during at least one week. It can be applied for determination of ATP concentration in pharmaceutical vials or other aqueous samples. The biosensor can be also used for the determination of glucose, but the conditions and characteristics of such determination were outside the scope of the present work. Point-by-point comparison of the developed in this work amperometric and conductometric ATP biosensors is presented in Table 9. The advantage of the conductometric biosensor is that the transducer is very simple and its price is

about 1 USD. One enzyme is used in bioelement of conductometric biosensor instead of two enzymes in amperometric biosensor. Moreover the conductometric measurement setup is far less costful than potentiostat for amperometric or impedance measurements.

Table 9. Comparison of the amperometric and conductometric ATP biosensors developed in this work.

	Amperometric biosensor	**Conductometric biosensor**
Biorecognition element	glucose oxidase + hexokinase	hexokinase
Time of analysis	~5 min	~5 min
LOD	5 μM	15 μM
Linear range	15 – 80 μM	15 – 300 μM
Selectivity to interferents	Good	Medium, depends on quality of transducer
Need of additional electrodes	yes	no
Price of transducer	10-20 Euros	1-5 Euros
Price of device	~2000 Euros	~1000 Euros

GENERAL CONCLUSION AND PERSPECTIVES

GENERAL CONCLUSION AND PERSPECTIVES

In the present dissertation, we reported the successful development of a biosensor system for simultaneous determination of adenosine triphosphate and glucose, a biosensor for creatine kinase determination, and a novel conductometric biosensor for adenosine triphosphate determination. Analytical characteristics of the biosensors were studied and different procedures for real samples analysis were developed. In order to show the practical application of the biosensors, they were used for the measurements of concentrations of ATP, glucose, and CK in pharmaceutical and spiked blood serum samples.

The combination of two different amperometric biosensors into a biosensor system allowed precise and simultaneous determination of glucose and ATP concentrations in aqueous samples. The proposed system consisted of two biosensors: the first one sensitive to glucose, the second one to both glucose and ATP. Utilization of two biosensors allowed increasing the accuracy of ATP determination. The biosensors were based on platinum disc electrodes with immobilized enzymes. The biorecognition element of the glucose biosensor contained GOx, while in case of glucose and ATP biosensor a bienzymatic system GOx/HEX was utilized. 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 crosslinking agent glutaraldehyde was 0.2% and immobilization time was 40 min. The accuracy of the biosensor system was improved by deposition of a poly(*m*-phenylenediamine) film onto the electrodes surface. It was demonstrated that the biosensor sensitivity to electroactive interfering substances like ascorbic and uric acids became very low after deposition of the film.

It was also shown that the operational characteristics of the biosensor can be modified by changing the composition of working solution. Thus, it was possible to change the limit and range of ATP determination by adapting glucose and Mg^{2+} concentration. The biosensor system responses during the day were stable and reproducible over one day of successive measurements. The relative standard deviation of responses to glucose was 3-6%, to ATP - 8-12%. Such deviation is typical for enzymatic ATP biosensors. Storage stability of the biosensor system was better if compared with most of described enzyme-based ATP biosensors, but it is worse if compared with aptamer-based biosensors which can be stored for 4-6 month without loss of sensitivity. Biosensors time of analysis was about 5 min with a response time within 20-30 s, what is much quicker than most of biosensors, especially aptamer-based ones. After analysis, the biosensor system could be washed from substrates in

a working buffer and reused several times. The biosensor system showed good storage stability if stored at -18 °C, 50-day storage resulting 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.

The biosensor system can be further used for determination of glucose and ATP concentrations in other types of aqueous solutions like biotechnological, biological, and pharmaceutical samples. The biosensor system has is not significantly dependent on the buffer capacity and ionic strength of the sample, and application of permselective membrane gives possibility to avoid almost all interference from electroactive substances which can be present in biological samples. These characteristics give possibility to determine ATP and glucose in pharmaceuticals and in complex biological or medical samples without their pretreatment. The system can be also included into more complicated multibiosensors and biosensor arrays for simultaneous determination of many substances like lactate, glutamate, choline, and others. Another perspective work is improvement of sensitivity of the ATP biosensor, for example by application of nanostructures. Now LOD of ATP is enough for most of biological samples, but in some samples like blood serum ATP concentration is still too low for determination.

In the second part of the work, an amperometric enzyme biosensor for determination of creatine kinase (CK) activity in blood serum was developed. CK is a biomarker enzyme, and increase of its concentration in blood is caused by certain heart and muscle diseases. The biosensor was based on the ATP biosensor that was used previously as a part of the biosensor system, but procedure of analysis and working buffer composition were different. CK requires two substrates (ADP and creatine phosphate) for reaction, thus the biosensor work at different concentrations of these substances was studied. Optimal concentrations of ADP and creatine phosphate were determined as 1 mM and 10 mM, correspondingly. Optimal sample dilution was also studied. The biosensor was characterized by sufficient reproducibility of responses during measurements of CK. Relative standard deviation of 15 responses was 7-18% for different concentrations of CK.

The determination of CK activity in spiked blood serum samples was performed. Biosensor results were consistent with calculated CK concentrations, with an error of 20-25%. The biosensor is suitable for express measurements of CK activity in blood serum of patients with acute myocardial infarction or other muscular injuries. The small size of the measuring device and the simple procedure of biosensor preparation and application are significant advantages over traditional methods of CK determination. Comparing with other

electrochemical CK biosensors, out biosensor is reusable while other biosensors are mostly disposable, with almost the same sensitivity. Developed immunosensors are more sensitive than the proposed biosensor and can distinguish between isoforms of CK. On the other hand, antibodies for them are expensive and require usage of animals for production. Also immunosensors cannot be reused. The main disadvantage of the proposed biosensor is the influence of glucose on the biosensor sensitivity, so it is necessary to choose between approximate determination of CK activity during one measurement and accurate determination during two measurements (the first measurement gives glucose concentration needed for selection of the optimal sample dilution during the second measurement).

The described procedure for the determination of CK activity can be adapted for the determination of other kinases for medical or research purposes. It should be noticed that the proposed method could be used for the assessment of ATP rate of production or degradation in the working cell what can be useful for research purposes, for example for screening of kinase inhibitors.

The third part of the work was devoted to the elaboration of a conductometric biosensor for ATP determination. For the first time we have created a conductometric ATP biosensor and demonstrated that the hexokinase reaction can be detected by conductometry. Also we elaborated a procedure for the detection of ATP by this biosensor in order to avoid interference from charged substances, which is a huge problem of conductometric biosensors. The biosensor is based on HEX, immobilized onto the surface of conductometric transducers. The best immobilization of HEX was obtained as a result of HEX and BSA cross-linking by glutaraldehyde for 30 min. The biosensor characteristics depended on the composition of working buffer. A 5 mM HEPES buffer solution, pH 7.4, containing 3 mM magnesium ions was found to be optimal. The investigation of the relationship between the biosensor sensitivity to ATP and glucose concentration showed that the optimum glucose concentration was 0.2 mM. Limit of ATP detection was 15 μM. The relative standard deviation of 10 consecutive measurements of biosensor responses to glucose and ATP was 10.3%. The biosensor developed remained suitable for daily measurements during at least one week. It was successfully applied to the determination of ATP concentration in pharmaceutical vials. Generally, analytical characteristics of the biosensor were similar to other enzymatic ATP biosensors with a bit higher limit of ATP detection. Also amperometric and potentiometric biosensors do not depend on buffer concentration so much as the conductometric one. One advantage of the proposed biosensor comes from the simplicity of the transducer: its price is about 1 USD or even less in case of mass production, and there is no need of reference

electrode. Another advantage is a price of measuring setup, since conductometric device usually costs much less than potentiostat for amperometric or impedance measurements.

Comparing developed amperometric and conductometric ATP biosensors, it can be concluded that the amperometric biosensor is more sensitive and has lower standard deviation of responses. On the other hand, the conductometric biosensor has wider linear range of ATP determination and it is more simple and cheap. The biosensor can be used for determination of ATP in pharmaceutical samples, since the biosensor sensitivity is enough and the biosensor is not influenced significantly by the ionic strength. Such determination is useful for both pharmaceutical process control and quality assurance of pharmaceuticals. Also the biosensor can be utilized for analysis of biological samples with low buffer capacity.

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