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A novel conductometric biosensor based on hexokinase for determination of adenosine triphosphate

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ABSTRACT

The paper presents a simple and inexpensive reusable biosensor for determination of the concentration of adenosine-5′-triphosphate (ATP) in aqueous samples. The biosensor is based on a conductometric transducer which contains two pairs of gold interdigitated electrodes. An enzyme hexokinase was immobilized onto one pair of electrodes, and bovine serum albumin-onto another pair (thus, a differential mode of measurement was used). Conditions of hexokinase immobilization on the transducer by cross-linking via glutaraldehyde were optimized. Influence of experimental conditions (concentration of magnesium ions, ionic strength and concentration of the working buffer) on the biosensor work was studied. The reproducibility of biosensor responses and operational stability of the biosensor were checked during one week. Dry storage at −18 °C was shown to be the best conditions to store the biosensor. The biosensor was successfully applied for measurements of ATP concentration in pharmaceutical samples. The proposed biosensor may be used in future for determination of ATP and/or glucose in water samples.

1. Introduction

Adenosine-5′-triphosphate (ATP) is an organic molecule (nucleoside triphosphate) which consists of adenine, ribose, and three phosphoric acid residues. ATP serves as a temporary carrier of energy in all living cells, so it is a common substance in any organism. In the cells, new ATP molecules are synthesized during decomposition of organic matter. Energy stored in ATP is utilized for numerous processes of biosynthesis. ATP is also a source of energy for the function of cell membrane proteins, an important precursor of the second messenger-cyclic adenosine monophosphate, an allosteric regulator of a number of cell processes, etc. [1,2].

Determination of ATP concentration is promising for estimation of the energetic state of cells and tissues. Also, ATP determination may be useful in medicine for studying the biological processes, in which it is involved, namely, the regulation of muscle contraction and platelet aggregation, maintenance of vascular tone, neurotransmission and regulation of the nervous system [3,4]. The determination of ATP concentration in human blood is promising for the diagnosis of various diseases [5]. The creation of kinase inhibitors may include an evaluation of the amount of ATP used by kinases in the presence of inhibitors and their absence.

Modern standard methods of precise determination of ATP concentration, such as spectrophotometry [6] and liquid chromatography [7], require qualified personnel and sophisticated expensive equipment, need complex pretreatment of samples for analysis [8,9]. Fluorescent, bio- and chemiluminescent methods are free from the above drawbacks; however, often they do not correspond with the demands of ATP monitoring [10]. Radioisotope methods of ATP analysis are highly accurate, but potentially dangerous [11]. Therefore, at present the development of easy-to-use, accurate, fast, selective and low-cost method for determination of ATP concentration in biotechnology and research is an actual challenge.

Today there are several laboratory prototypes of biosensors for ATP determination. They are based on pH-sensitive field effect transistors [12], amperometric glassy carbon electrodes [13], amperometric platinum microelectrodes [14], which are usually coated with the enzymes. A common drawback of these biosensors is quite complex structure of electrodes, which increases their cost and reduces the possibility of mass production. Moreover, often two-enzyme systems are used as biorecognition

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elements of biosensors, what increases overall complexity of the biosensors. Recently, sensors based on photo detection of ATP binding with different receptor molecules were developed [15–17]. However, the measurement procedure in these cases is quite complicated.

An alternative is an application of conductometric biosensors based on planar transducers. These biosensors are advantageous because of simple structure of transducers, low-cost manufacture and fast measurement procedure [18]. Also they do not need a reference or other additional electrodes, and their response time is quite fast. On the other hand, the conductometric transducers are sensitive to all charged substances, including ATP, which presents significant difficulties in measurement of real biological samples. This is the reason why conductometric biosensors are inferior to amperometric and potentiometric biosensors. To the best of our knowledge, no conductometric biosensor for ATP determination has yet been described.

The study was aimed at the development of an original conductometric hexokinase-based biosensor that would be simpler in structure and usage comparing with the existing ATP biosensors. To prevent an influence of charged particles, a differential two-step procedure of measurement was supposed.

2. Materials and methods

2.1. Materials

Enzyme hexokinase (HEX, EC 2.7.1.1) from *Saccharomyces cerevisiae* with activity 30.6 U/mg (Sigma-Aldrich, Germany) was used for creation of biorecognition elements of biosensors. Bovine serum albumin (BSA, fraction V), glucose, ATP (disodium salt hydrate, grade 1, $\geq$99%), glycerol, HEPES, magnesium chloride, and 50% aqueous solution of glutaraldehyde (GA) have been purchased from Sigma-Aldrich Chemie (Germany). All other chemicals were of p.a. grade.

2.2. Design of conductometric transducers

Biosensors were based on planar conductometric transducers. They were manufactured in V. Lashkaryov Institute of Semiconductor Physics of National Academy of Science of Ukraine (Kyiv, Ukraine) in accordance with our recommendations. Each transducer was 5×30 mm^2 in size and contained two pairs of gold interdigitated electrodes deposited onto a ceramic support. The sensitive area of each electrode pair was about 1.0×1.5 mm^2. 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 microphotographs of these transducers can be found in [19].

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 another pair. Details about the differential mode can be found in Section 2.4.

2.3. Preparation of bioselective elements

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. Glycerol was added to the solutions to stabilize HEX and BSA during storage at −18 °C and to prevent early drying of the solutions on the transducer surface. In case of solution for bioselective membrane, BSA was added to stabilize HEX in solution; also BSA and HEX formed intermolecular bindings during immobilization. These solutions were mixed with 0.5% aqueous solution of glutaraldehyde (cross-linking agent) in a ratio of 1:1 and immediately deposited onto the sensitive regions of transducer (approximately 100 nL of the mixture onto each region). Afterwards, the created biosensors were dried for 30 min in air at room temperature. During this time, GA formed covalent bonds between amino groups of enzymes and BSA. Then the biosensors were immersed in the working buffer for 10 min in order to stop immobilization and to wash out unbound components.

2.4. Measurement procedure

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. 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 measurements were carried out in a 2 ml plastic cell filled with 5 mM HEPES buffer, pH 7.4, under magnetic stirring.

All experiments were repeated three times. The data in the figures is either mean of three repeated results of the experiment or mean ± standard deviation (SD).

3. Results and discussion

3.1. Working principle of the biosensor

The operation of conductometric biosensor for ATP determination is based on the enzymatic reaction in bioselective membrane:

$$\text{D–glucose} + \text{ATP} \xrightarrow{\text{HEX}} \text{D–glucose–6–phosphate} + \text{ADP} \quad (1)$$

During the Eq. (1), the local concentration of ions in the working membrane increases, thus, the conductivity of the solution in the near-electrode region changes, which is registered by a conductometric transducer. This change of conductivity is directly proportional to a concentration of glucose and ATP in the working cell. It is important that this reaction is catalyzed by a single enzyme, what is an advantage of the proposed biosensor over existing amperometric and potentiometric ones which require two enzymes to operate. This reaction itself neither generate electroactive products nor change solution pH, and can be registered directly only by conductometry.

A typical procedure for measuring the ATP concentration is as follows (Fig. 1A). Initially, ATP or ATP-containing sample is added to the working cell. Since ATP is a charged substance, the biosensor generates a nonspecific signal (a peak at 50 s in Fig. 1A). 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 model glucose solution is added to the working cell, and the Eq. (1) takes place in the enzyme membrane. This reaction results in the appearance of two new charged substances (ADP and phosphorylated glucose), thus the