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Research paper

A novel mediatorless biosensor based on flavocytochrome b_2 immobilized onto gold nanoclusters for non-invasive *L*-lactate analysis of human liquids

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ABSTRACT

Lactic acid (*L*-lactate) is among the most important analytes since it is a universal metabolite of nearly all the living organisms. The determination of the *L*-lactate level is used in clinical diagnostics of hypoxia, lactic acidosis, some acute heart diseases, in drug toxicity tests as well as in sports medicine, while monitoring the athletic performance to evaluate the best training equipment and regimes.

The measurement of metabolites in fluids other than blood is becoming increasingly significant because of major advantages of non-invasive analysis (safety, rapidity, and accuracy). In sensor technology, the most efficient means for the non-invasive analysis of human fluids seems to be the third generation (mediatorless) amperometric biosensors, as they do not require any exogenous cofactors, toxic electron transfer mediators or the use of high working potential.

The aim of this work is the construction of a novel non-invasive electrochemical biosensor of the third generation for *L*-lactate based on yeast *L*-lactate:cytochrome *c* oxidoreductase (EC 1.1.2.3; flavocytochrome b_2, FC b_2) immobilized onto gold nanoclusters (nAu). FC b_2 is a tetramer with four identical subunits, each consisting of FMN- and heme-binding domains. The main properties of FC b_2 of thermotolerant yeast *Ogataea (Hansenula) polymorpha* are the selectivity for *L*-lactate, its high stability and ability to direct electron transfer. The developed FC b_2-nAu modified bioelectrodes are characterized by improved parameters compared to the enzyme electrodes, obtained without the use of nAu. The constructed bioelectrodes were adapted for direct non-invasive analysis of human saliva and sweat samples using the amperometric microcomputer-based analyzer system. The obtained results of the real samples analysis have a high correlation ($0.7 < R < 1$) with the reference approaches.

1. Introduction

Lactate, a key metabolite of the anaerobic glycolytic pathway, plays an important role in medicine, in the nutrition sector as well as in food quality control. The level of *L*-lactate content in human blood is an important clinical indicator of hypoxia, acidosis [1], heart attack [2], drug toxicity [3], and serves as a marker for the evaluation of the optimal sportsmen's training [4]. *L*-lactate is also an important biomarker for different types of cancer due to Warburg phenomenon [5]. Moreover, as a lactate threshold can be increased greatly along with training, the monitoring of the athletic performance with the purpose of evaluating the best training equipment and regimes is quite relevant [6]. Therefore, reliable determination of *L*-lactate is important in clinical diagnostics and sports medicine.

The measurement of metabolites in fluids other than blood (saliva, urine, and sweat) is becoming increasingly significant because of major advantages of non-invasive analysis. A non-invasive method of *L*-lactate assay has eliminated the risks of handling blood and feeling any pain of puncturing. The advantages of this analysis are the simplicity of sample collection and the possibility of more frequent collection of samples with much less stress for the patient. A tight correlation between the *L*-lactate concen-

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tration in sweat and blood was confirmed many years ago and can form the basis of correct non-invasive analytical approach [7].

Amperometric biosensors offer a sensitive and selective means of monitoring organic analytes like *L*-lactate. Up to now, there have been a number of described *L*-lactate-selective biosensors of the first and second generations, using mainly lactate oxidase [8,9], NAD^+-dependent lactate dehydrogenase [10,11], or flavocytochrome b_2 [12,13]. However, biosensors of the third generation for *L*-lactate, based on the direct electronic coupling of the biorecognition elements to the electrode without the assistance of intermediate substances such as substrates/products or artificial mediators [14,15], have not been described yet. In spite of this fact, these properties make sensors very promising devices in the development of efficient non-invasive direct analysis of human fluids.

The first reports on direct electron transfer between the redox protein and the electrode were provided by Eddowes and Hill [16], and Yeh and Kuwana [17], who independently discovered the ability of cytochrome *c* to directly transfer electrons to gold or tin-doped indium oxide electrodes. Subsequently, it was followed by the discovery that larger redox proteins such as laccase and peroxidase are also able to undergo the direct electron transfer [18,19]. Since that time, direct electron transfer has been reported for catalase [20], cytochrome P450 [21], hydrogenases [22], bilirubin oxidase [23], ascorbate oxidase [24], succinate dehydrogenase [25] fumarate reductase [26], alcohol dehydrogenase [27], fructose dehydrogenase [28] cellobiose dehydrogenase [29] and flavocytochrome b_2 [30]. Most of the enzymes, which are capable of directing electron transfer, contain metals such as iron or copper and have easily accessible active centers. The important factors for efficient electron transfer are the orientation of the enzyme structure on the electrode [31] distance and the driving force between an active center of the enzyme and the electrode [32]. However, high selectivity of many enzymes requires deep embedding of the enzyme prosthetic group in the protein structure and thus prevents such enzymes from direct electron transfer to the electrode due to the fact that the distance between the redox centres of such enzymes and electrode exceeds the distance across which electrons can be efficiently transferred [33]. This limitation can be surmounted by chemical [34] or genetic [35] modification of proteins or reconstitution of the apo-enzymes with relay-cofactor units, immobilized on the electrode surface [36,37]. The direct contact between enzyme redox center and the electrode can also be improved by the incorporation of nanomaterials [38–40].

Recent progress in nanobiotechnology allows using nanomolecular approaches for improvement of bioanalytical parameters of the sensors – selectivity, response time, miniaturization of the biorecognition unit [41,42].

This study is focused on designing a new prototype of non-invasive mediatorless *L*-lactate-selective biosensor based on *L*-lactate-cytochrome *c*-oxidoreductase (EC 1.1.2.3; flavocytochrome b_2, FC b_2). FC b_2 is a tetramer with four identical subunits, each consisting of FMN- and heme-binding domains. The main properties of FC b_2 are absolute selectivity to *L*-lactate, the ability to direct electron transfer and high stability, which fit the requirements for biosensors. The electrodes modified by enzyme binding gold nanoclusters were adapted for amperometric analyzer system and the approbation of the sensor prototype on the real samples of saliva and sweat was described.

2. Materials and methods

2.1. Reagents

L-lactic acid sodium salt, *L*(+)-lactic acid, Triton X-100, H_2O_2, $HAuCl_4$, and EDTA were obtained from Sigma-Aldrich (Buchs, Switzerland). *D*(+)-glucose monohydrate was purchased from J.T. Baker (Deventer, The Netherlands). $(NH_4)_2SO_4$, Na_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$ were obtained from Merck (Darmstadt, Germany). E-Toyopearl 650 M was obtained from Toyo Soda (Tokyo, Japan). The cathodic electrodeposition paint "GY 83-0270 0005" was from BASF Farben und Lacke (Munster, Germany). All chemicals were of analytical grade and all solutions were prepared using triply-distilled water.

2.2. Purification of flavocytochrome b_2 (FC b_2)

L-lactate:cytochrome *c* oxidoreductase (flavocytochrome b_2) (EC 1.1.2.3) was isolated and purified from the recombinant cells of the thermotolerant yeast *Ogataea polymorpha* "tr 1" (*gcr1 catX/prAOX_CYB2*) [43]. The enzyme was isolated by ion-exchange chromatography on DEAE-Toyopearl cellulose 650 M. The enzyme was purified to the specific activity of 22 U mg^{-1} and stored as a suspension in 70%-saturated ammonium sulphate, pH 7.8 at +4 °C before usage.

2.3. Modification of the surface of gold planar microelectrode by gold nanoclusters

In order to improve a direct electron transfer from FC b_2 to an electrode surface, the working surface of the electrode was modified by gold nanoclusters (nAu). The nAu was obtained directly on the electrode surface by reduction of the $HAuCl_4$ solution to Au^0. The reaction was performed using 30% H_2O_2 according to Panda and Chattopadhyay [45]. The procedure was as follows: 4 μl 2 mM $HAuCl_4$ was dropped on the top of the working electrode; after drying, 4–5 μl 30% H_2O_2 was added. Prior the modification of the surface, the planar gold electrodes were cleaned by 70% ethanol solution. The modification of electrode surface by the nAu was accompanied with changing the polished surface structure to scabrous and color from yellow (gold) to high-colored orange (nanoclusters).

2.4. Scanning electron microanalysis

A Scanning Electron Microscope (SEM-microanalyser REMMA-102-02, Sumy, Ukraine) was used for morphological analyses of the electrodes surface. The special cover film on the samples with a Butvar solution B-98 (Sigma, St. Louis, MO, USA) in 1.5% chloroform was formed using an ultrasound method. The distance from the last lens of the microscope to the sample (WD) ranged from 20.1 mm to 26.1 mm; the accelerator voltage was in the range from 20 kV; zooms were from 2500.

2.5. Atomic force microscopy

The size and structure of nAu particles were studied by atomic force microscope Solver P47-PRO (NT-MDT, Russia).

The tested sample was analyzed in air using the tapping mode with a resonance frequency of 160 kHz, scan rate of 1 Hz/s and resolution of 256 × 256 pixels.

2.6. Apparatus and techniques for biosensor construction

Amperometric biosensors were evaluated using constant-potential amperometry in a three-electrode configuration using 4 mm diameter planar gold electrodes DRP-C220AT from "DropSens" (Llanera (Asturias) Spain). Amperometric measurements were carried out using a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) connected to a personal computer and performed in a batch mode under continuous stirring in a standard 4 ml electrochemical cell at 25 °C.