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Assessment of metabolic activity of human cells in solution and in polymer matrix with the use of metabolite-sensitive sensors

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ABSTRACT

We developed metabolite-sensitive electrochemical sensors on the basis of electrodes modified with a thick film of carbon nanotubes. Modified electrodes provide efficient pre-adsorption of cellular metabolites and their sensitive detection using anodic square-wave voltammetry. On the electrode surface both adhered and non-adhered human cells produce three oxidation peaks at the potentials of +0.82, +1.05, and +1.17 V attributed to three groups of cellular metabolites: amino acid-derived antioxidants including glutathione, guanine nucleotides, and also adenine nucleotides including ATP. The electrochemical response was well correlated with cell viability, intracellular ATP level and induction of apoptosis, as determined by independent assays. Developed sensors allow for robust and cost-effective assessment of ATP in cells in contrast to enzyme-based electrodes and conventional bioluminescent assay. Results can be used for rapid analysis of human cells for the purpose of medical diagnostics, transplantology, and toxicological screening. Additionally, we combined modified electrodes with human cells entrapped in agarose matrix. The resulting biosensor allowed for electrochemical monitoring of metabolic activity and death of cells within polymeric matrix that is of interest for tissue engineering applications.

1. Introduction

Biochemical analysis of mammalian cells is carried out in various biomedical studies [1,2]. Developmental and pathological processes in living cells are accompanied with a change in composition of plasma membrane components and intracellular metabolites [3]. Studying biochemical parameters of living cells is of particular interest in medical diagnostics of many human disorders [1] as well as for drug and toxicant screening [4].

Fluorescent probes/labeled antibodies are routinely used for the detection of individual components in fixed and live cell samples with the aid of optical microscopy [5]. Since flow cytometry has been introduced for quantitative fluorescent analysis it became an essential technique for identification and assessment of isolated cells [6,7].

Other modern techniques involve chromatographic separation of cell metabolites followed by their high-precision determination by means of mass spectrometry. Combined chromatography/mass spectrometry is an irreplaceable tool for metabolome research when informative analysis of metabolite profiles is required [1,8]. However, limitations of this tool, e.g. high cost, laboriousness, inapplicability to live cell assays, complicate its introduction to some biomedical applications including routine clinical diagnostics.

Cellular metabolites can be detected without separation by the use of spectroscopic techniques such as Raman spectroscopy. This technique allows for direct assessment of biochemical composition of living cells by Raman spectra under physiological conditions. Changes in Raman spectra provide valuable information about normal and pathological cellular processes [9,10]. However, Raman analysis of biological matter requires qualified interpretation of acquired data involving relatively complex mathematical methods.

During the last decade many efforts have been made to develop more handy tools for rapid assessment of key cellular metabolites. A promising platform for solving this problem is electrochemical sensors, especially voltammetric ones, which are characterized by selectivity, time efficiency and low cost of an analysis [11]. Electrochemical sensors can be used for direct detection of a wide range of biomolecules which are oxidized or reduced on the surface of electrodes. In certain cases the electrode is coupled with enzyme(s) to perform highly selective quantification of a metabolite of interest in biological sample [12]. To date, different electrochemical assays have been proposed for detection of catecholamines and nitric oxide on chemically modified electrodes [13–15] and also glutamate with the use of glutamate oxidase based sensors [16,17].

A reliable parameter of energetic status, viability and induction of apoptosis of human cells is intracellular ATP level [18–20]. Whereas some existing studies show the possibility of electrochemical detection of some unidentified metabolites in cells [21–23] they do not consider ATP and other adenine nucleotides which were shown to be oxidized with high overpotential at about +1.2 V [24]. For

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detection of ATP in biological samples platinum microelectrodes were modified with glucose oxidase and hexokinase [25] or glycerol kinase and glycerol-3-phosphate oxidase [26]. These bi-enzyme electrode configurations improve determination of ATP by converting it to more readily detectable products but apparently should result in increase of the cost and decrease of robustness of the analysis.

To overcome these limitations, we developed enzyme-free electrochemical sensor for simultaneous assessment of ATP and other reliable metabolites related to antioxidants and energetic molecules in human cell samples. The sensor based on metabolite-sensitive carbon nanotube modified electrode was applied for effective detection of alterations in energetic status and viability of human cells in the form of suspensions and lysates. We also demonstrate for the first time that this sensor can be implemented for electrochemical monitoring of metabolic activity of the cells entrapped in polysaccharide matrix.

2. Materials and methods

2.1. Reagents

Multi-layered carbon nanotubes (Sigma-Aldrich) were produced by CVD (purity >90%, inner diameter 1–3 nm, outer diameter 3–10 nm, length 0.1–10 μm, specific surface area 300–400 m^2/g). Rabbit immunoglobulins, bovine serum albumin were purchased from Sigma-Aldrich. Nicotinamide adenine dinucleotide, nucleoside triphosphates, amino acids, glutathione, and hemoglobin were produced by Serva-Heidelberg. Doxorubicin hydrochloride was purchased from Ferane (Russia).

Cell culture reagents were purchased from Paneco (Russia). Trypan blue, acridine orange and ethidium bromide were produced by Serva-Heidelberg. FITC annexin V apoptosis/necrosis detection kit was purchased from BD Biosciences.

2.2. Cell isolation and culturing

Human-derived cells (HeLa, cervical cancer cells; MCF7, breast adenocarcinoma cells; HEK 293, embryonic kidney cells; and skin fibroblasts) were cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin. After reaching confluency, the cells were collected from a culture flask by treating with 0.05% trypsin. Dissociated cells were washed by means of repetitive centrifugation at ×200 g in PBS and calculated on hemocytometer. Viability of collected cells was verified by staining with 0.1 mg/mL trypan blue or 5 μg/mL acridine orange/ethidium bromide mixture on a fluorescent microscope AxioObserver A1 (Carl Zeiss).

Human blood cells were isolated from peripheral blood of healthy donor according to conventional protocol [27]. Briefly, 10 mL of blood was mixed with EDTA (final concentration 0.27%) as a coagulant in a plastic tube. The mixture was kept for 60 min at room temperature to separate settled erythrocytes from blood plasma containing leukocyte fraction. Mononuclear cells were isolated by means of centrifugation of blood plasma in density gradient of ficoll-paque (1.077 g/mL) at ×400 *g* for 40 min. Cell containing layer was collected, then mixed with equal volume of 0.02% EDTA solution and additionally centrifuged at ×400 *g* for 5 min to remove platelets. Mononuclear cell pellet was resuspended in PBS buffer for subsequent analysis.

2.3. Proliferation and ATP assays

HeLa cells were seeded into 96-well plate at the density of 2000 cells per well and allowed to adhere overnight. The cells were cultured for 2 days in the presence of doxorubicin at different concentrations. Cell viability was analyzed with a proliferation assay with tetrazolium compound MTS (Promega). Absorbance of metabolically reduced MTS (formazan product) was measured at 490 nm using a microplate reader Stat Fax 2100 (Awareness Technology). The experiments were carried out in triplicates.

For determination of ATP in cell samples we used bioluminescent assay kit (Lumtek, Russia) according to the manufacturer's protocol. Briefly, the lysates of treated cells were mixed with a freshly prepared solution of firefly luciferase (2000 U/mL) followed by luminescence measurement at 570 nm on a chemiluminometer CHEMILUM-12 (Russia). ATP concentration in the samples was calculated using standard ATP solution (10 nM).

For flow cytometry analysis, treated human cells (1×10^6 cells/mL) were mixed with annexin V–propidium iodide mixture and incubated in the binding buffer according to the manufacturer's protocol (BD Biosciences). The analysis was performed on a BD FACSCalibur cytometer (BD Biosciences). The numbers of counted fluorescence events were >1000.

2.4. Cell sample preparation

For electrochemical study both cultured and blood human cells were transferred into PBS and suspended at defined concentrations. To release intracellular metabolites the cells were lysed by means of 30 s sonication on ice using Sonopuls HD 2200 sonicator (Bandelin). Alternatively, the cells were lysed by resuspending them in deionized water. To prevent a destruction of metabolites, cell lysates were immediately frozen at −80 °C and thawed straight before the analysis.

2.5. Sensor fabrication

Pristine carbon nanotubes (CNTs) were pre-treated in a mixture of concentrated nitric and sulfuric acids (1:3, v/v) under ultrasonic agitation at frequency of 22 kHz and power of 70 W. Oxidized CNTs were precipitated and washed 3 times by repeated centrifugation in deionized water. The precipitate was resuspended in water resulting in stable black-colored suspension of CNTs.

Two microliter aliquot of CNT suspension (~250 μg/mL) was cast onto the working surface of polished disk glassy carbon electrode (GCE) 1.5 mm in diameter (geometric area 1.8 mm^2) followed by solvent evaporation. The procedure resulted in modified electrodes with uniform and reproducible surface and good electrochemical performance.

2.6. Electrochemical measurements

We used three-electrode cell consisted of modified GCE (working electrode), silver-silver chloride reference electrode and nickel plate as a counter electrode. An aliquot (5 μL) of analyzed solution of a biomolecule in millimolar concentration or cell suspension was placed onto the surface of CNT-modified electrode and incubated for 5 min to allow the adsorption of analytes onto CNTs. The electrode with adsorbate was rinsed and transferred into supporting electrolyte (0.01 M sodium acetate in 0.1 M NaCl, pH 5.0). Square-wave voltammograms of adsorbed analytes were recorded on Autolab PGSTAT12 potentiostat–galvanostat (EcoChemie) in the potential range from +0.1 to +1.3 V and treated with GPES 4.9 software (EcoChemie). Electrochemical data were presented as mean value ± SD of oxidation peak height from three independent measurements.

2.7. Assessment of cells in agarose matrix

To prepare agarose matrix we used low melting point agarose (Fisher BioReagents). Intact HeLa cells were gently mixed with 2% melted agarose at 37 °C in PBS at final concentration of 1×10^6 cells in 1 mL of the solution. Five microliter aliquot of the mixture was cast onto the working surface of CNT-modified electrode. After brief incubation at room temperature, stable droplet-like agarose gel was formed on the electrode. Electrochemical signal of metabolites released by the cells immobilized in agarose gel was measured every