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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

30 min similarly to the method described above. For optical microscopy analysis analysis of viability of immobilized cells, prepared agarose matrix was stained with acridine orange/ethidium bromide and remelted at 37 °C to liberate cells. Green fluorescent cells were considered viable whereas orange and red fluorescent ones were referred to apoptotic and necrotic cells [28].

3. Results and discussion

3.1. Detection of cellular metabolites on modified electrodes

We used carbon electrodes modified with a thick film of multilayered CNTs as electrochemical sensors. As shown earlier [29,30], the modified electrodes (MEs) exhibit enhanced sensitivity of voltammetric detection of various biomolecules compared with conventional carbon electrodes. The sensitivity of MEs is due to their pore nanostructured surface which provides high effective area for adsorption and electrochemical oxidation of redox species [29,30].

These sensors were applied for studying electrochemical properties of different human cells suspended in PBS buffer. We found that suspensions of both adherent cells (HeLa, HEK 293, and MCF7) and blood cells on the surface of ME generated up to three well-defined oxidation peaks at the potentials of +0.82, +1.05, and +1.17 V further denoted as peaks 1, 2, and 3, respectively. Fig. 1 shows representative voltammograms of human cells on ME. Under experimental conditions viable HeLa cells produced peaks 1 and 2 whereas blood mononuclears produced all three peaks (1–3) on ME. Some voltammograms of studied cells also contained a pre-peak at the potential of +0.75 V (Fig. 1).

Registered peaks were attributed to certain redox components which were released from living cells into the buffer solution and adsorbed on ME. The reason is that the electrochemical signal was time-dependent but remained unchanged after removal of cells from analyzed solution by means of centrifugation. Similarly, washing of MEs after contact with cell suspensions slightly affected this signal as if detected cellular components were strongly adsorbed on ME.

In contrast to ME, unmodified glassy carbon electrode did not allow the detection of adequate voltammetric response from human cells at the same conditions. Altogether, the results show that cell-generated voltammograms (Fig. 1) arise from adsorbed cellular metabolites rather than whole cells attached to ME. This is in agreement with our earlier studies demonstrated that pre-oxidized CNTs used in this study intensively adsorb biomolecules of aromatic nature, e.g. purine derivatives, as a result of hydrophobic interactions [31,32].

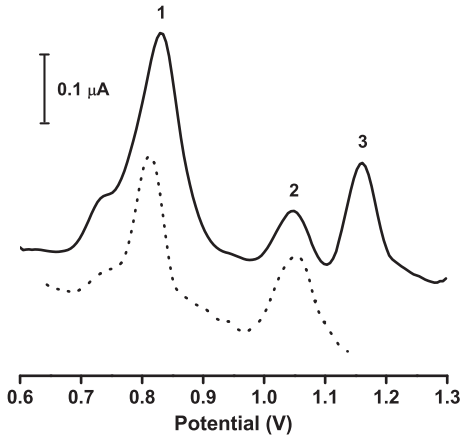

Fig. 1. Representative square-wave voltammograms produced by viable human blood mononuclears (—) and HeLa cells (···) on modified electrode. Cell suspensions in PBS (1×10^6/mL) were incubated on the surface of ME to adsorb cellular metabolites. Measurements were performed at frequency 10 Hz, amplitude 10 mV, step potential 5 mV in 0.1 M NaCl + 0.01 M sodium acetate (pH 5.0) supporting electrolyte.

Such metabolites can be normally secreted from mammalian cells involving an active transport [33] and probably by a passive diffusion out of cells into buffer solution.

In order to ascertain the nature of the redox active metabolites we compared potential values of cell generated peaks and some biomolecules on ME (Table 1). Several biomolecules were found to be oxidized near the potential of peak 1 (+0.8 V), e.g. amino acids and peptides including glutathione. Another key metabolite detected at this potential is NAD(P)H (Table 1). It also exhibits second oxidation step at +1.2 V which apparently corresponds to adenine nucleotide component. Notably, this second step of NAD(P)H oxidation was not observed on conventional carbon electrodes [34,35] confirming superior sensitivity of ME towards hardly detectable biomolecules.

All the above biomolecules exhibit antioxidative activity in vivo including NAD(P)H [36], amino acids [37] and particularly glutathione, a key intracellular antioxidant [38]. Such an activity correlates with relatively low oxidation potential of these biomolecules on ME. Therefore, we referred the cellular metabolites detected at peak 1 potential as antioxidants.

Peaks 2 and 3 of the cells at the potentials of +1.05 V and +1.2 V (Fig. 1) were respectively attributed to guanine and adenine nucleotides which exhibited very similar oxidation potentials on ME (Table 1). One can assume that these peaks reflect intracellular levels of GTP and ATP, abundant macroergic compounds in cells. In our study, both GTP and ATP exhibited unexpectedly high electrochemical activity on ME unlike the other types of electrodes.

Thus, the results show that MEs allow simultaneous and sensitive detection of different types of cellular metabolites. Three registered peaks (Fig. 1) seem to represent cumulative signals each derived from a group of metabolites with similar redox properties. We hypothesize that these signals reflect the presence of both antioxidant and macroergic molecules in living cells and may characterize energetic status and metabolic activity of the cell. An advantage of using MEs is rapid, enzyme-free, and at the same time function-selective analysis of cell metabolites that is achieved by modifying electrodes with metabolite-sensitive CNT layer.

3.2. Assessment of energetic status of human cells

Intracellular concentration of adenine nucleotides, and especially ATP, is a relevant biochemical parameter which indicates an intensity of metabolic processes in norm and pathology. In particular, ATP level in human cells was proved to be a useful diagnostic marker for the assessment of cell viability [39], induction of apoptosis [40], myocardial cell injury [41] and sperm function [42]. Therefore, we tested MEs for the detection of cellular ATP and energetic status of human cells.

We found that electrochemical oxidation of ATP pre-adsorbed on ME was characterized by linear signal vs. concentration relationship in the concentration range 0.05–15 mM: $I\ (\mu A) = 0.03 \pm 0.15 + 0.6 \pm 0.03 \times C(mM)$; $R = 0.99$. The results indicate relatively broad linear range and high reproducibility of the analysis of ATP. For assessment of total ATP concentration in human cells with the use of ME the cells were subjected to lysis prior to analysis by means of brief sonication or hypoosmotic shock. Both methods resulted in 4–10-fold increase of metabolite peaks compared with intact human cells in suspension

Table 1
Oxidation potential (E) of some biomolecules adsorbed on modified electrode.

Biomolecule	E (V)	Cell generated peak
Tyrosine	+0.79	Peak 1
Cysteine	+0.82	Peak 1
Glutatione	+0.81	Peak 1
Proteins (albumin, hemoglobin)	+0.80	Peak 1
NAD(P)H	+0.80/1.20	Peak 1/peak 3
GTP	+1.06	Peak 2
ATP	+1.20	Peak 3

due to release of metabolites from cytoplasm and organelles into solution. Further, we used hypoosmotic lysis of the cells to quantify intracellular level of metabolites with the aid of modified sensors.

Addition of 0.5 mM ATP to the lysate of HeLa cells resulted in an amplification of peak 3 at the potential of +1.2 V. The resulting oxidation peak (1.12 ± 0.10 μA) was almost equal to a sum of peak heights generated by ATP (0.65 ± 0.03 μA) and the cell lysate alone (0.42 ± 0.04 мкА) at the same concentrations. This shows the possibility of ATP quantification in cell lysates using MEs. Regarding the fact that other adenine nucleotides may contribute to peak 3 along with ATP we studied how this peak height correlated with ATP level as well as viability of human cells.

In order to induce changes in cell viability and ATP content HeLa cells were treated with anthracycline antibiotic doxorubicin which intercalates into the DNA and inhibits topoisomerase II causing cell damage and induction of apoptosis [43]. According to proliferative MTS assay we found two doxorubicin concentrations: up non-toxic one (2 μM) and the concentration which induced 50% inhibition of cell proliferation (9 μM). After culturing HeLa cells with doxorubicin at these concentrations we registered peak 3 associated with ATP as well as peak 1 attributed to antioxidants (Fig. 2).

Results show that cells treated with 9 μM doxorubicin produced almost 3 times lower current for peak 3 indicating highly reduced level of adenine nucleotides in cells apparently due to induction of apoptosis which is accompanied with a depletion of energetic molecules in cells [19,40]. Moreover, this signal decreased by almost 22% even at non-toxic doxorubicin concentration of 2 μM (Fig. 2) as if MEs were more sensitive towards alteration in cell viability than conventional MTS assay. Notably, at this concentration we observed almost 2-fold increase of peak 1 compared to control (Fig. 2) that is apparently explained by a stimulation of synthesis of antioxidants such as glutathione. Over-production of glutathione was shown to be a form of resistance of cancer cells to chemotherapy [44].

Similar bi-directional effect of doxorubicin on electrochemical signal of antioxidants and adenine nucleotides was observed for blood mononucleares during 2–4-h incubation of the cells with the antibiotic (data not shown). While the decrease of adenine nucleotide peak is attributed to cytotoxicity of doxorubicin, the increase of antioxidant peak can be explained by indirect stimulation of the synthesis of glutathione which may protect human lymphocytes upon oxidative damage [45].

We also studied a correlation between the electrochemical signal of HeLa cells with intracellular ATP level using conventional ATP bioluminescent assay (Fig. 3). To induce depletion of intracellular ATP, the cells were subjected to short-time heating. We found that such treatment caused a drastic drop of the signal of adenine nucleotides (peak 3) in cells along with the decrease of intracellular concentration of ATP (Fig. 3). According to flow cytometry with annexin V–propidium iodide staining, 15-min heating resulted in the appearance of about 13% of the apoptotic cells and 38% of nectrotic cells (data not shown) which are characterized by reduced level of ATP.

Altogether, the results show that modified sensors are useful tool for the analysis of metabolic activity of mammalian cells under different conditions. In particular, these sensors can be used for the direct detection of both antioxidants and energetic molecules in less than 5 μL of cell suspension or lysate, thus allowing simple evaluation of changes in cell viability and antioxidant capacity at an early stage. The correlation between electrochemical signal of adenine nucleotides and ATP concentration in human cells (Fig. 3) supports the possibility of using MEs for the analysis of intracellular ATP level. The latter analysis commonly requires relatively unhandy and expensive luciferase-based luminescent assay [18].

The developed sensors are of particular interest for rapid assessment of different cellular samples, e.g. in the field of cell transplantology as well as in medical screening of disorders accompanied with a change in cell energetic/antioxidant status. We address possible applications of the sensors to existing studies of metabolic profiles, and especially ATP content in human cells in different diseases [41,42,46,47].

3.3. Monitoring of metabolic activity of living cells in agarose matrix

A promising approach in cell biosensor design is an implementation of monitoring of metabolic activity of mammalian cells at different conditions. Such monitoring requires an immobilization of the cells with preserving their viability and functional activity. Continuous analysis of live mammalian cells adhered on a flat surface of transducers was carried out using surface plasmon resonance [48] and impedimetric biosensors [49]. There is a challenge of developing three-dimensional biosensors which are based on biocompatible matrices for living cells.

We studied a possibility to monitor metabolic activity of HeLa cells entrapped in a polymer matrix on the surface of ME. The cells were immobilized in the gel of agarose with melting temperature of 37 °C used as a model matrix. For biosensor fabrication, we mixed cell suspension with melted agarose and allowed the mixture to form drop-like gel on the surface of ME at room temperature (Fig. 4, inset). The resulting agarose gel did not cause a blocking effect on electrochemical reactions on ME allowing detection of cellular metabolites with the use of composite biosensor.

According to fluorescent microscopy more than 95% of HeLa cells were viable in agarose gel at least during the 3-h incubation of the

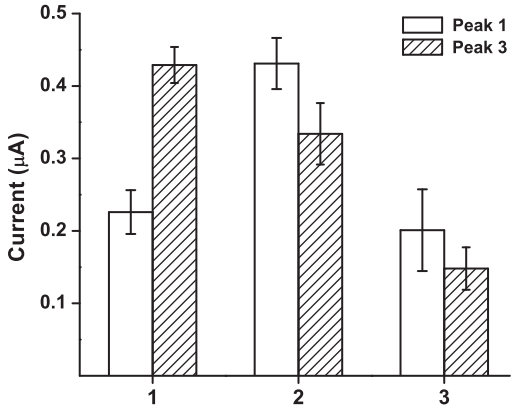

Fig. 2. Electrochemical signal of lysates of HeLa cells cultured in the presence of doxorubicin. 1 — control (untreated cells); 2 — cells treated with 2 μM doxorubicin; 3 — cells treated with 9 μM doxorubicin. The cells were lysed at the concentration of 0.5×10^6/mL.

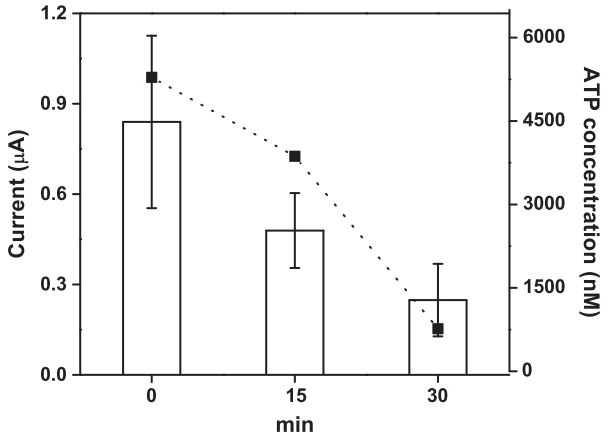

Fig. 3. Effect of heat shock of HeLa cells on electrochemical signal of intracellular adenine nucleotides (columns) and ATP concentration determined by bioluminescent luciferase assay (dot line). The cell suspension (1×10^6/mL) was heated at 45 °C for 0, 15, and 30 min and lysed prior to analysis.

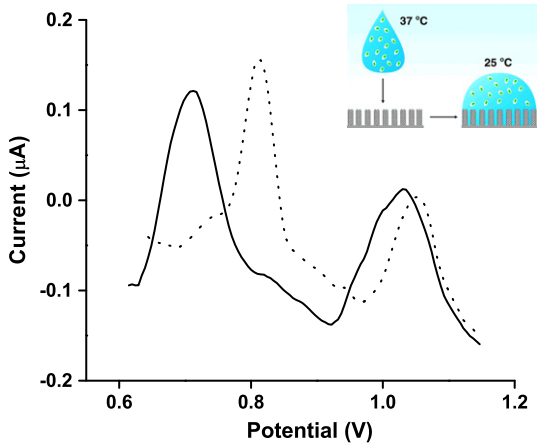

Fig. 4. Electrochemical detection of metabolites released from agarose gel entrapped HeLa cells with the use of composite biosensor. Biosensor response (—); the cells in suspension (···). Inset: scheme of biosensor fabrication by incorporating cells into low melting point agarose gel on the surface of modified electrode.

biosensor in ambient atmosphere. Incorporated cells exhibited metabolic activity detected by an appearance of well-defined electrochemical peaks from metabolites. These peaks were somewhat broadened and shifted towards lower potentials compared with the signal produced by intact cells in suspension (Fig. 4) due to matrix effect. Note that under experimental conditions intact HeLa cells generated mainly peaks 1 and 2 but not peak 3 which normally appeared on voltammograms after cell lysis.

Prolongation of biosensor incubation was accompanied with a gradual increase of its response indicating that released cellular metabolites accumulated in agarose gel and diffused to the surface of ME. Both peaks approached a maximum at 4 h of incubation that coincided in time with dramatic decrease in the number of viable cells in the matrix (Fig. 5) as the conditions were not optimal to support cell growth.

Altogether, our results for the first time provide proof of concept for electrochemical monitoring of metabolic activity of mammalian cells embedded in the polymeric matrix with the use of metabolite-sensitive electrodes. The proposed approach can be of interest for certain biomedical applications, e.g. testing of cytocompatibility of

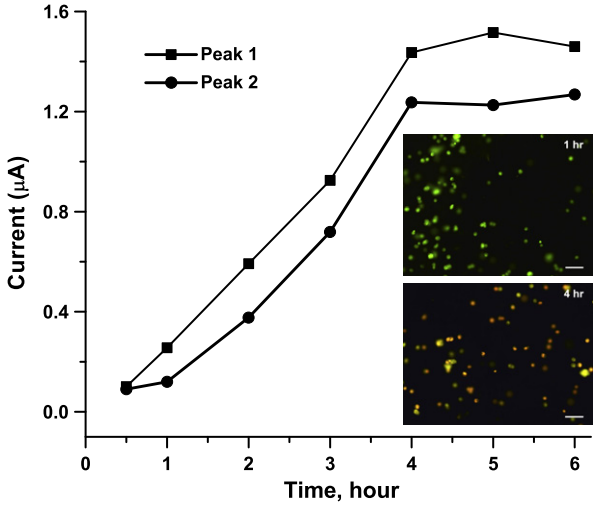

Fig. 5. Continuous monitoring of metabolic activity of HeLa cells incorporated in composite biosensor in ambient atmosphere. Inset: fluorescent microphotographs of the cells in agarose gel matrix incubated for 1 and 4 h (acridine orange/ethidium bromide staining). Cell concentration 1×10^6/mL. Scale bar 100 μM.

hydrogel-based materials and design of novel cell biosensors for continuous detection of cellular metabolite release under different conditions.

4. Conclusions

We designed novel carbon nanotube-modified voltammetric sensors which allow for simple pre-sorption and sensitive detection of cellular metabolites differing in their redox potentials. The sensor response was shown to be associated with key metabolites such as antioxidants and macroergic molecules and well correlated with viability and ATP level for human cells. The sensors represent a promising tool for different biomedical applications including diagnostics of metabolic disorders, rapid assessment of transplanted cells as well as toxicological screening of drugs. An important background for such applications is that developed sensors can be implemented in a format of inexpensive disposable screen-printed electrodes. In our study, we proposed a novel approach which is based on coupling modified sensor with polysaccharide matrix for electrochemical monitoring of metabolic activity of encapsulated cells. This approach can be extended to a microarray format for efficient analysis of cytocompatibility of soft polymeric materials.

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