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Flow-through sensor array applied to cytotoxicity assessment in cell cultures for drug-testing purposes

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ABSTRACT

The viability of cells cultured in microsystems for drug screening purposes is usually tested with a variety of colorimetric/fluorescent methods. In this work we propose an alternative way of assessing cell viability—flow-through sensor array that can be connected in series with cell microbioreactors as compatible detection system. It is shown, that the presented device is capable of cytotoxic effect detection and estimation of cell viability after treatment with 1,4-dioxane and 5-fluorouracil, which proves that it can be used for truly non-invasive, fast, reliable, continuous cell culture monitoring in microscale.

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1. Introduction

Currently cell cultures play a fundamental role in many fields of research, for example in biotechnology and pharmaceutical industries. The number of research associated with cell culture and survival analysis of cells is constantly increasing, which is connected with discover and manufacture of new drugs, as well as the development of regenerative medicine. Cell cultures can be also used for studying biological processes.

Development and implementation of new drugs is a long and costly process. Any drug must be tested through several stages in order to produce a product that is effective and safe. This process consists of: identifying a specific drug target, which responds to the disease; product characterization; pharmacodynamic and pharmacokinetic evaluations, preclinical toxicology testing and investigative new drug (IND) application; bioanalytical testing and clinical trials. Cell cultures play important role in drug research at a cellular level during preclinical testing. These tests provide crucial information with respect to dosing and safety criteria and allow proceeding to the next step of clinical trials. The use of cell culture to test the usefulness of new drugs is easier due to the fact that the cells provide a quick response and representative to the substances which were used. It is important also that it limits the amount of tests on live animals, in accordance with the principle of 3R (replacement, reduction and refinement), assuming the search of alternative methods to replace animals in the experimental tests (Andersson and Van den Berg, 2004; Bhadriraju and Chen, 2002). Therefore the cell cultures of animal or human origin are very often used for studying new compounds, which is an alternative, more economic and ethical method of *in vitro* drug testing during preclinical studies. New drugs are tested on many types of cells, mostly on tumor cells (Torisawa et al., 2005; Shackleton, 2010).

The use of microchips (called *lab-on-a-chip*) for testing of drugs is a good solution that gives the opportunity of not only cost and time-consuming bordering studies, but also to reduce the amount of animal used for experiments. The microsystems allow for investigation of the cellular response to specific drug compounds in the well-defined cellular microenvironment, which is needed to maintain the phenotypic properties of the tested cells. Microtechnology assures the integration of biomaterials, tissue engineering and the cell culture for the development of novel cell culture platforms for drug research (Dittrich and Manz, 2006; Wegil et al., 2003; Wu et al., 2010; Zguris et al., 2005).

There are many examples of microfluidic systems dedicated for cell analysis described in the literature. Microsystem components should primarily be biocompatible, non-toxic to cells, and they should also allow for microstructure fabrication. Many materials are used, ranging from silicon, through glass and ceramics, to various types of polymers (*e.g.* polydimethylsiloxane—PDMS) (Gomez, 2008; Kang et al., 2008; Komen et al., 2008; Li et al., 2004; Velve-Casquillas et al., 2010; Vozzi et al., 2003; Zhang et al., 2009).

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Transparent materials allow for optical investigation of cells and subcellular structures, moreover, they enable to utilize the majority of standard cell viability tests and cytotoxicity assays based on exogenous fluorophores or transfected fluorescent reporters. However, potential cytotoxicity of fluorophores and photobleaching are essential problems of fluorescence-based methods. Moreover, these assays cannot be used continuously, on the same sample; therefore real-time monitoring becomes problematic (Starly and Choubey, 2008). Additionally, the use of fluorophores in microfluidic system is complicated, due to necessity of quick and uniform penetration of the cell culture. Therefore there is a great need for non-invasive detection methods enabling to estimate cell biochemical response to toxic stimuli, which could be fully integrated with microscale cell bioreactors. Five main factors changing during cell growth and proliferation can be used to control cell metabolism: glucose, lactate, and oxygen concentration, pH, and heat. For this purpose several devices were already proposed, among which most of them are based on ISFETs, Clarc electrodes, impedance spectroscopy, etc. (Adami et al., 2013; Burdallo and Jiménez-Jorquera, 2009; Eklund et al., 2006; Krommenhoek et al., 2007, 2008; Lorenzelli et al., 2003; Martinoia et al., 2001; Thedinga et al., 2007).

In this work we propose miniaturized, potentiometric flow-through sensor array for real-time monitoring of toxic effects in cell cultures, which is compatible with cell microbioreactors. The developed system was applied in viability study of A549 cells treated with 1,4-dioxane and 5-fluorouracil and the results of sensor array measurements coupled with Partial Least Squares (PLS) analysis were correlated with standard cytotoxicity tests.

2. Experimental

2.1. Sensor array

An integrated array of solid-state Au microelectrodes was fabricated on double-side coppered glass wool soaked with epoxy resin, using printed circuit board (PCB) technology (Ciosek et al., 2009a). Final pattern of 16 microelectrodes with conductive paths was obtained by electrochemical deposition of Ni and Au layer on Cu surface. The last stage was the hot-press bonding of the obtained structure with another layer of the epoxy-glass (without Cu cover), insulating the electric paths. The holes drilled in the insulating layer created compartments to be filled with membrane solutions. Details of the fabrication of the integrated array can be found elsewhere (Toczyłowska-Maminska et al., 2008).

Sensor array consisted of 7 types of membrane electrodes: four ion selective (K^+-, Na^+-, H^+-, Ca^{2+}-selective) and 3 partially selective (selective towards amines, “cation-selective”, and “anion selective”). There were two electrodes of each type. Two electrodes were left without membrane to serve as red-ox sensors. The membranes contained appropriate ionophores, 20–50 mol% (versus ionophore) lipophilic salt, 61–66 wt% plasticizer, and 31–33 wt% high-molecular-weight PVC (see Table S1 in Supplementary Data).

Preparation and deposition were described in detail previously (Ciosek et al., 2004a, 2004b). Three types of polymer matrix were tested (polyurethane, polyurethane: polyvinyl chloride 1:1, and polyacrylate). Miniaturized silver/silver chloride electrode with a double junction was applied as reference electrode. All measurements were carried out in the cells of the following type: Ag, AgCl; KCl 3M | CH_3COOLi 1M | sample solution ‖ membrane ‖ Au. Potentiometric multiplexer (EMF 16 Interface, Lawson Labs Inc., Malvern, USA) was used for EMF measurements.

2.2. Microflow system for sensor array measurements

Microflow system was fabricated with the use of few polymeric layers which formed holder for the integrated PCB sensor array. Design and 3D visualizations of the proposed system were performed with AutoCAD (Autodesk).

The microchannel was made in the microsystem housing in order to bring solutions to the cavities of the electrodes. Laws governing fluid flow in micro scale differ from the ones used in macroscopic devices and one of the basic characteristic of microfluidics is laminar flow. Because of the volume/surface ratio viscosity has to be taken into account. Omitting the effects based on mutual influence of molecules is no longer possible. Thus it is important to aid the design of a microfluidic system with means of advance modeling and simulation programs. In our work all models and simulations were performed with COMSOL Multiphysics (COMSOL Group) using Incompressible Navier-Stokes equations. It was assumed that fluid flowing in the channel is water, pressure in the inlet is 20 Pa, pressure in the outlet is 0 and temperature equals 25 °C. It was also presumed that liquid does not move in the vicinity of the wall and that the entire device is made from PMMA. To take into account the interaction between the liquid and the wall the Plane Strain model was used. Models mesh was defined as Extra fine.

After modeling of the flow, appropriate architecture of the channel was chosen. The microchannel was made using the method of micro-milling by means of a micro-milling machine controlled numerically (Minitech Mini-Mill 3 Pro) which allows for high precision and repeatability. The step motors controlling the stage employ micro-stepping, and a XYZ resolution of around 1 μm is achieved. The repeatability of the system, including the spindle run-out, has been experimentally found to be less than 5 μm when working on areas a few centimeters across, which is the dimension typically used for microfluidic devices. A first phase consisted in performing a project of a microchannel in AutoCAD, which with the help of CAM software was converted to G-code controlling the operation of a micro-milling machine. The performed microchannel has a rectangular cross section and it was 800 μm wide and 500 μm deep.

2.3. Cell culturing and cytotoxicity tests

A549 cells (human lung adenocarcinoma epithelial cell line; American Type Culture Collection) were cultured in MEME medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mmol L-glutamine (Sigma), 100 U mL^{-1} penicillin (Sigma), and 100 μg mL^{-1} streptomycin (Sigma), and grown until confluent, following which cells were subcultured by trypsinization. The cells were seeded in black-walled 96-well plates at a density of 1×10^4/well. After 24 h, growing cells attached to the bottom were treated with different concentrations of prepared test compounds (1,4-dioxane—as a model toxic reagent, and 5-fluorouracil—5-FU) in a complete medium for 24 h. After the incubation with the reagent, the medium was removed and the cells were treated with 50 μl of Calceine-AM (CAM, 2 μM) for 30 min at 37 °C. After cells' staining and washing in PBS the fresh culture medium was added and green fluorescence was measured with the fluorescence plate reader Varian (Cary Eclipse) at 485 nm (excitation) and 538 nm (emission) wavelength.

Compounds concentrations was ranged from 0 to 20% v/v of 1,4-dioxane and from 0 to 300 μM of 5-FU. 1,4-dioxane was diluted with the medium for further experiments. 5-FU was dissolved in 100% dimethylsulfoxide (DMSO; Sigma) and then diluted with the medium for further experiments. The final concentration of DMSO was maintained at 0.2% which had no effect on cellular growth and survival.

2.4. Data analysis

Chemical images of investigated samples–cell culture media, were obtained using steady-state responses of 16 sensors (2 electrodes of

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