Potentiometric detection of the metabolic activity of human tumor cells

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Monitoring of cellular viability is a key part of toxicological assays *in vitro*. On-line monitoring of metabolic activity would be particularly useful for evaluation of responses to potential therapeutic compounds. Current assays are mostly based on fluorescent dyes and optical detection methods. These methods offer high sensitivity and specificity, however are not suitable for long-term on-line observations. Electrochemical methods can be an alternative for current protocols. Electrochemical detection is low cost and label-free, therefore suitable for long-term cell culture monitoring. In this work investigations on human cancer cells viability will be presented. Cells were cultured as two-dimensional monolayer or three-dimensional spheroids. Different cell culture media were examined. Potentiometric detection was used for continuous monitoring of cell culture as well as end-point investigations. Different growth phases were identified using applied method. Finally, response to an anticancer drug was successfully observed.

Keywords and phrases: cancer, *in vitro* cell culture, potentiometry, cell culture monitoring.

Background

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide [1]. Therefore there is a need for cancer biology research and new anticancer therapies development. Current *in vitro* cellular modes used in cancer research and preclinical trials (such as monolayer) are significantly different from the *in vivo* environment [2]. The simplicity of the monolayer model leads to the ability of automation and standardization of the methods. However, monolayer cultured cells lack essential interactions present *in vivo*, *i.e.* three-dimensional structure, direct cell-to-cell junctions or paracrine signaling, which cause the inability of applying results obtained to the drug effect on living organism [3]. Therefore, the drug screening method more closely mimicking *in vivo* environment is required. The best cellular model for anticancer therapy testing developed so far is a Multicellular Tumor Spheroid (MCTS) [4]. The MCTS presents morphology and physiology similar to tumor *in vivo* with the network of cell-to-cell interactions, three-dimensional structure, presence of natural extracellular matrix and nutrients, metabolites and oxygen gradients [5]. There is an evidence that three-dimensional tumor cell models are more representative for cancer tumor *in vivo* than two-dimensional ones [6]. The tissue structure determines growth rate of a tumor as well as response to anticancer drugs [7].

Number of methods of MCTS formation were described in literature and several found their final applications [8]. However, current methods face a number of limitations [9]. Single cell cultivation on non-adhesive surface, the simplest and most popular MCTS cultivation method leads to obtainment of a large number of spheroids of different sizes [10]. Other methods of mass spheroid formation, such as spinner flask culture or rotary cell culture system provide more homogenous MCTS population, but are cost and energy consuming and spheroids are exposed to a very high, non-physiological shear stress [6]. Moreover, the mentioned techniques do not enable single spheroid observation and analysis, which would be particularly useful for research on cancer tumor physiology [11]. There are also more sophisticated MCTS cultivation methods, such as hanging-drop culture or application of electric, magnetic or acoustic force for cell aggregation enhancement, but still are too costly for a widespread use [12]. Another disadvantage of all widely available MCTS cultivation methods is the periodic (batch) type of cell culture. Moreover, quantitative determination of cellular response in 3D arrangement is still very problematic and challenging [13].

The device developed by our group is a solution for the problems mentioned above. It couples the advantage of three-dimensional culture of MCTS with the possibilities of lab-on-a-chip devices. It is expected, that research

on lab-on-a-chip *in vivo*-like systems will effect in the evolution of methods that can replace animals in different fields of biomedical research [2]. There is a particular focus on development of alternative systems for toxicology and drug screening [14]. Nowadays, over 95% of drug candidates admitted to the pre-clinical trials do not pass tests on animals [15]. Referring to the EU directive on the "Community code relating to medicinal products for human use", all drug candidates' tests "shall be carried out on two species of mammals one of which must be a non-rodent" and the number of animals used should consider "the level of statistical significance" [16]. These combined together give a large number of animals used in pre-clinical tests, which is unwelcome for both ethical and economic reasons. Adding reports on differences between human and non-human physiology and response [17], an emphasis on the research concerning alternative methods for toxicological purpose is fully understandable [18].

The system proposed by our group is presented in Fig. 1. It was already used for a long-term culture of multicellular spheroids of HT-29 human colon carcinoma cells [19–21]. Cells were cultured inside the microdevice for over 25 days and good viability was maintained within this time [19]. It was also observed that cell growth rate decreases inside the micronishes and finally stops. Thus, stable, viable and quiescent tissue was obtained. Similar results were obtained earlier for monolayer cell culture inside lab-on-a-chip devices [22–24]. The possible explanation of this phenomenon is the tissue homeostasis. A living organism is a system of many cells remaining in the equilibrium between life, proliferation and apoptosis [25]. The homeostasis provides a constant number of cells in the body, whereas the cells cultured *in vitro* spread on the whole available surface with high proliferation rates, and need to be passaged to maintain the culture [26]. In artificial conditions, homeostasis is extremely problematic

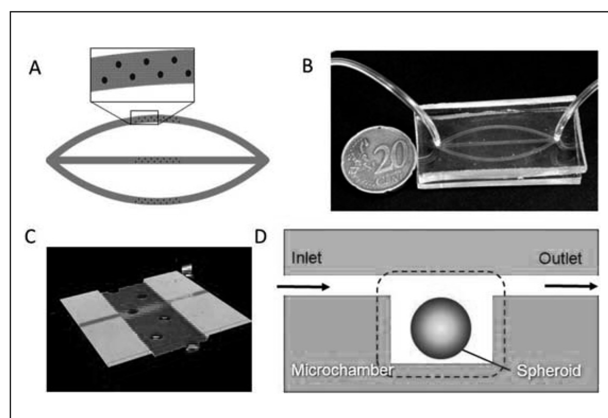

Fig. 1. Microfluidic system for three-dimensional cell culture [19]: (A) design; (B) real photograph; (C) profile of a microchannel with microwells for spheroid formation and (D) scheme of one microwell (cross-section).

to be achieved, as the mechanisms of regulation of the cell proliferation are not fully understood yet [25]. Lab-on-a-chip devices are prospective tools for investigations in this field. Microfluidics was already used for research on bacterial quorum sensing phenomenon [27], which is the ability of bacterial colony to restrain cell divisions in microniches by sending and receiving signals between individual microbes. A similar mechanism may have an effect on homeostasis of mammalian cells, but this hypothesis still needs to be investigated.

The biggest challenge concerning development of novel cellular model for drug screening purposes is the evaluation of an analytical method for cell viability monitoring [8]. In our work, we decided to develop an assay applicable to lab-on-a-chip system, that could be easily combined with widely available analytical systems. We have chosen electrochemical detection, as electrodes can be easily miniaturized [28].

Most of the biochemical processes in the living cell base on electron transfer between molecules with the participation of proper enzymes [25]. Therefore, cellular metabolic activity influences the red-ox potential of the extracellular environment [29]. It was expected, that changes of the cell culture medium potential could be detected by potentiometric measurements. In this work, we present our preliminary results on cell culture monitoring using platinum electrodes. HT-29 human colon cancer cells were observed under standard culture conditions and under anticancer drug treatment. Characteristic and repeatable growth phases were detected. Different media and different cell seeding densities were examined. Moreover, it was observed, that electrochemical profile changed, when cytostatic drug was added to the culture. The results indicated, that potentiometric measurements can be used for continuous monitoring of cells for drug screening purposes. In our further work, miniaturized platinum electrodes will be applied for lab-on-a-chip device for tumor spheroid culture.

Material and Methods

Cell culture

Experiments were performed on HT-29 human colon adenocarcinoma cells (ATCC). Routine cell culture was carried on RPMI medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS, Gibco), 1% L-glutamine (Sigma-Aldrich) and 0.6% antibiotics (Peniciline/Streptomicine, Sigma-Aldrich). Cell culture media used in the experiments differ according to FBS content (5%, 10%, 15% or 20%)

Cells were incubated in CO_2 incubator (HeraCell) at 37°C, 5% CO_2. Passage was performed once a week at 70–90% confluence, cell culture medium exchange — twice a week.

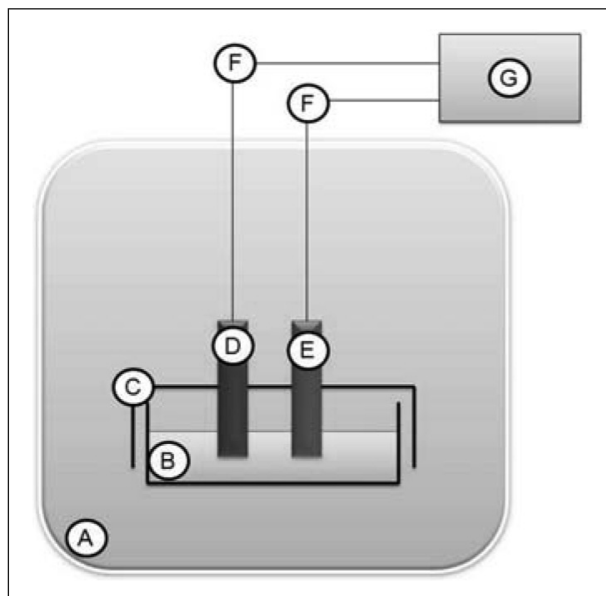

Fig. 2. Scheme of experimental setup for continuous potentiometric cell culture monitoring: (A) CO2 incubator; (B) Petri dish with cell culture medium and cells; (C) lid with holes for electrodes; (D) working electrode — platinum electrode; (E) reference electrode — Ag/AgCl electrode; (F) wires taken out of the incubator and (G) station for data collection and acquisition.

Inoculum preparation

Inocula for experiments were prepared by trypsin digestion of routine culture. First, cells were rinsed by phosphate buffered saline (PBS, Sigma-Aldrich). Next, preliminary digestion was performed using Trypsin-EDTA solution (Sigma Aldrich) for 30 sec. Same solution was used for proper digestion for 2–3 min at 37°C. Detached cells were suspended in cell culture medium. Density of inoculum was measured using Countess System (Invitrogen/Life Technologies).

Required portion of inoculum was pipetted into polystyrene petri dishes (Sarsteadt) and filled with proper amount of cell culture medium.

Endpoint potentiometric measurements

After 24 or 48 hours of incubation with cultured cells, cell culture medium was collected from the dishes and placed into sterile tubes. Probes were placed in the thermostat (37°C). Measurements were performed using combined potentiometric electrode (Pt/Ag/AgCl; EuroSENSOR, Poland).

Continuous cell culture monitoring

Continuous cell monitoring was performed using platinum working electrode and Ag/AgCl reference. Cells were seeded on 60 mm Petri dish with holes for electrodes cut in the lid. Electrodes with support were sterilized using 70% ethyl alcohol and were put in the CO_2 incubator. Wires were took out of the incubator and connected with a detection station. Scheme of the experimental set-up is presented in Fig. 2.

Results

First experiment aimed at evaluating whether changes of extracellular potential could be detected using platinum electrode. Media containing 0, 5, 10, 15 and 20% vol. FBS were used for culture of HT-29 cells. Equal inocula were added to each dish and cells were incubated with certain media for 24 hours. After this time, samples were taken and measured according to the protocol described in the “Material and Methods” section. Results are presented in Fig. 3. Differences between fresh, unreduced medium and cell-reduced medium are clearly visible. Moreover it was observed, that more intense metabolism (stronger medium reduction) was present for lower amounts of FBS in medium.

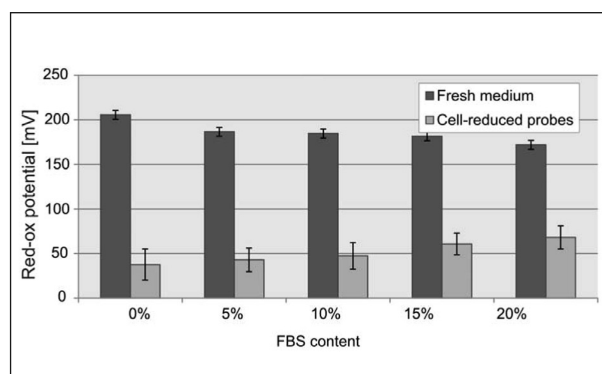

Fig. 3. Results of end-point measurements of different types of cell culture media. Error bars correspond to standard deviation.

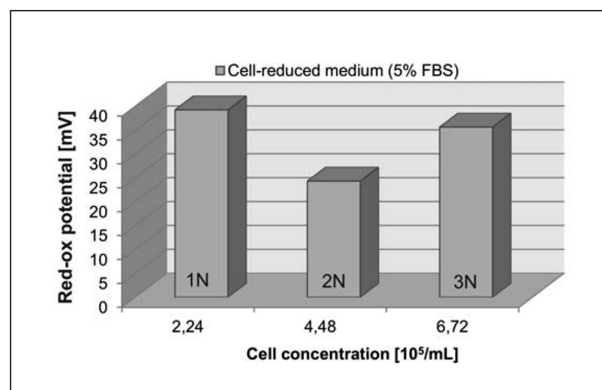

Fig. 4. Results of end-point measurements of cultures of growing cell density (for 5% FBS medium). Basic cell density was 2,24·105 cells/mL (1N) and other plates consisted of two- (2N) and three-times (3N) grater amount of cells.

Next experiment aimed at the detection of differences between different amounts of cells in the culture. The end-point experiment was performed for all types of culture media described earlier, and the biggest differences were detected for 5% FBS cell culture medium (Fig. 4). It was observed that growing number of cells indicates stronger decrease of red-ox potential (intense metabolic activity, proportional to number of cells). However, cell density close to confluence effects in inhibited metabolism (higher red-ox potential).

Described experiments show that cellular metabolic activity influences changes of red-ox potential of the cell culture medium. Next step was continuous, on-line monitoring of cellular metabolic activity using experimental setup described in the "Materials and Methods" section (Fig. 2). Culture was monitored for four days, starting from cell seeding. Repeatable profiles of changing red-ox potential of cell culture medium were detected. Example profile was presented in Fig. 5. Characteristic shape was observed. First (I) 8–10 hours were characterized by intense decrease of potential. Next, (II) there were several hours of potential increase. Next 24 hours (III) was the time of further, but gentle decrease. From the 3rd day of culture (IV) potential increase started and finally (V), a plateau was achieved. Disturbances (sudden peaks of the potential) were caused by changing condition while in-

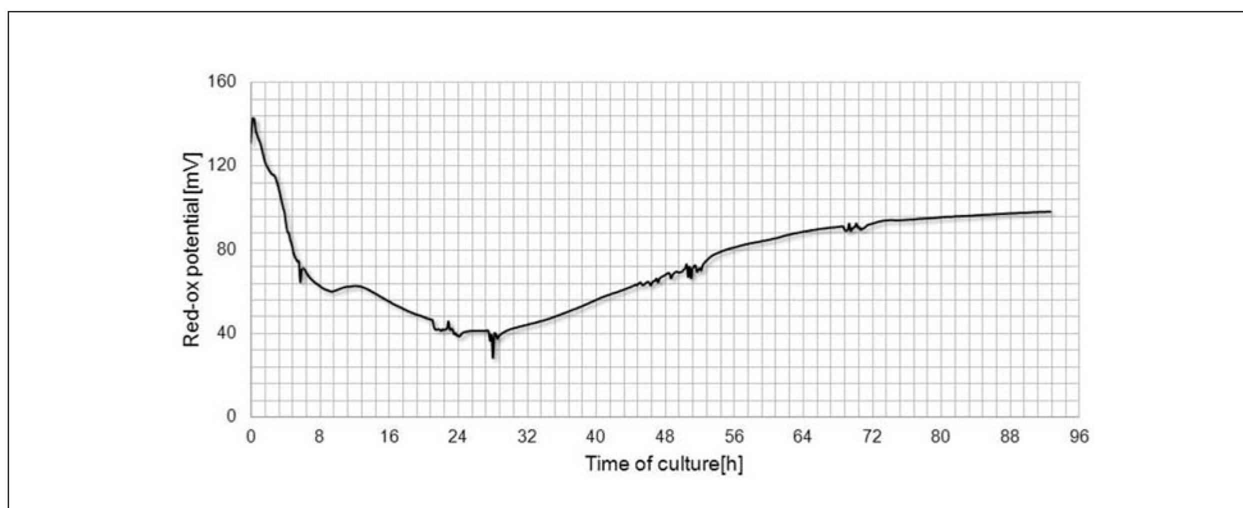

Fig. 5. Profile of changing red-ox potential during the time of cell culture.

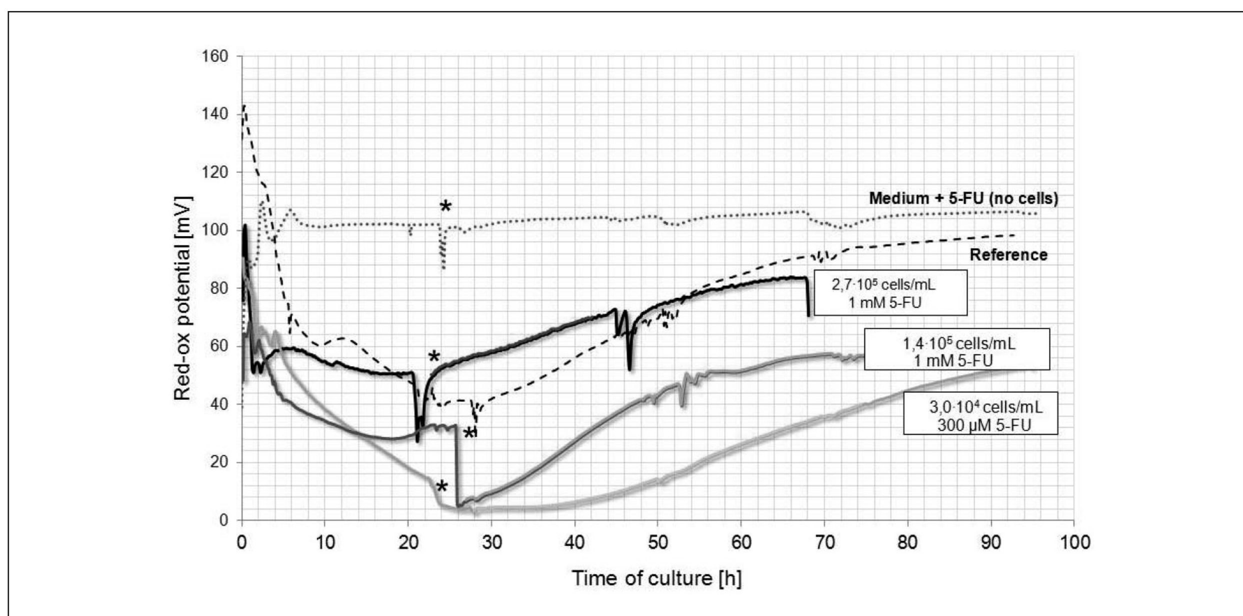

Fig. 6. Potentiometric monitoring of HT-29 cell culture exposed to 5-Fluorouracil (5-FU) cytostatic drug. Moments of addition of the drug were marked with stars (*). Dashed line refers to a drug-free culture, dotted line refers to blank probe of medium with 5-fluorouracil (without cells).

cubator was being opened (during laboratory work hours). Similar curves of potential were observed previously, using alamarBlue assay [30].

Effect of a cytostatic drug (5-Fluorouracil, 5-FU) was monitored using described experimental setup. Results are presented in Fig. 6. As was described before, times of more intense work with the incubator were visible as peaks on the graphs. The potentiometric profiles differed starting from addition of the drug (areas of the graphs marked as red). Higher concentration of the drug resulted in rapid increase of red-ox potential. On the other hand, lower concentration caused red-ox potential increase, but only after several hours of incubation. The results were compared to a drug-free culture (dashed line in Fig. 6). Gentle differences are visible, but are not conclusive. Experimental setup providing more stable and repeatable conditions is therefore required. Additionally, possible influence of 5-FU on medium red-ox potential was tested and no influence was observed (dotted line in Fig. 6).

Discussion

It was observed, that potentiometric measurements can be applied for cell culture monitoring. Significant differences were detected between different types of media and different cell densities. Media with lower amount of FBS were proved to be advantageous for HT-29 cells (Fig. 3). Higher concentration of serum could have effected in lower accessibility to carbohydrates or biochemical inhibition of cell growth.

In the experiments with different cell densities (Fig. 4) it was observed, that quantitative determination of number of cells according to medium potential is possible in some range of densities. Above the range, contact inhibition can be detected, which can be confusing. Therefore, potentiometric monitoring of cell culture should be supported with microscopic control.

The most important novelty of this paper, is the presentation of continuous potentiometric monitoring of cell culture (Fig. 5). Five characteristic features of the profiles were observed (described in the “Results” section). First phase (I) was the time of lag phase — adaptation to new conditions after passage. Majority of the cells were unattached or started to adhere and catabolic processes were the most intense. Second phase (II) was observed after adhesive protein binding — cell cycle and biosynthesis started. Therefore, anabolic processes were significant and slight potential increase was observed. The phase lasted till equilibrium between catabolism and anabolism was achieved. Then, the third phase started (III), where potential decrease was proportional to the growth of cell number. Its duration was closely related to the availability of nutrients. When they were scarce and the amount of metabolites was significant, phase of culture degeneration started (IV). It ended with a plateau (V), meaning, that no further metabolic processes occurred. The potentiometric curve was in a good correspondence with current understanding of fast-dividing cells’ cell growth curves *in vitro* [26].

Finally, the experiments with the cytostatic drug were processed. It was observed, that the shape of the profiles altered according to different concentration. In the future, it can become a base of electrochemical quantitative drug screening method. However, future work in this field should be supported by more accurate cell culture and measurement methods.

One of the biggest inconvenience of the applied analytical method was presence of signal disturbance during opening of incubator (peaks in Figs 5 and 6). According to the long-term observation, these data-points could be neglected. However, more stable conditions should be provided. It is expected that application of lab-on-a-chip device for cell culture and electrochemical detection will significantly reduce the disturbances. It will also provide higher throughput and parallel experiments under exactly the same conditions. Therefore, application of a lab-on-a-chip system can be a solution enabling more conclusive results on cellular response to applied drug.

Conclusions

In this paper, our research on electrochemical monitoring of HT-29 cells culture was presented. Significant differences between different cell culture media and cellular densities were detected. Culture was monitored continuously and characteristic, repeatable profiles were observed. Finally, the profile alteration under the exposure to anticancer drug were detected.

Based on these results we conclude that potentiometric measurements can be applied for quantitative cell viability assay in toxicology and drug screening. In our further studies, miniaturized platinum electrodes will be used for lab-on-a-chip system for three-dimensional cell culture. It will be a step toward development of a High Throughput Screening method applicable for *in vivo*-like cellular model.

Acknowledgements

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