New microfluidic device for lactate dehydrogenase (LDH) activity analysis

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In this paper, we present cytotoxicity analysis (determination of lactate dehydrogenase — LDH activity performed in a designed and fabricated microfluidic system. This method allowed for analysis of a supernatant collected from A549 (human lung cancer) and HT-29 (human colon cancer epithelial) cells, which were incubated for 24 h with selected compounds. LDH is an intracellular enzyme present in tissues, which is released into the supernatant caused by membrane damage or cell lyses. In our tests, LDH-Cytotoxicity Assay Kit (BioVision) was used. The toxic effect of drugs was measured in the developed microsystem made of PDMS (poly(dimethylsiloxane)). Analytical reaction took place in the special designed microchannel geometry. Then, the LDH activity was measured at 490 nm using spectrophotometer. In subsequent experiments, appropriate conditions for measurements using a microfluidic system were chosen. It was found that the selected reagent is sensitive to temperature changes and light exposure. Reaction time in the microsystem was determined by changes of flow rates of reagents. Afterwards, for the various reaction time, the toxic effect of 5-fluorouracil, celecoxib and 1,4-dioxane was performed. The obtained results were compared with the results carried out in 96-well plates. Based on these results, it was noted that the enzymatic reaction time in the microsystem is shorter than in 96-well plate. Moreover, the advantage of using microsystem is also the small amount of reagents.

Keywords and phrases: lactate dehydrogenase (LDH) activity, microfluidic system, PDMS, adherent cell culture, cytotoxicity analysis.

Introduction

Cancers are today the most common diseases in the world. In recent years, the number of cancer patients has been still increasing. Lifestyle of modern civilization and the relatively late diagnosis make these statistics more alarming. In medicine, various types of cancer therapy are used: chemotherapy, radiotherapy, surgery, phototherapy [1]. Very important is to test new compounds, which can be used in anticancer therapy. The development of *in vitro* cytotoxicity assays has been driven by the need to limit animal-based testing and to evaluate rapidly, effectively and reliably the toxicity of large number of compounds. A number of methods have been developed to study cell death and proliferation. They are based on different markers that indicate the number of dead cells and the number of live cells. The common endpoints being measured for cytotoxicity analysis are for example: cellular metabolic function, cellular protein content, DNA content, membrane permeability, cell number [1–3]. In order to measure those parameters, two kinds of methods are being used: direct or indirect. Direct methods are based on microscopic examination of cell cultures after incorporating an appropriate dye, i.e. trypan blue, propidium iodide. These dyes, depending on membrane integrity, either penetrate into the cell or remain on its surface, which makes it possible to determine the number of the dead and live cells. Indirect methods on the other hand are based on an indication of specific marker in cell culture supernatant, e.g. enzyme activity. Those methods have one distinctive quality, which direct methods are missing — they do not damage cells making possible to maintain a cell culture after the assay.

An example of such an assay is lactate dehydrogenase based test (LDH) [4–7]. LDH is a stable cytoplasmic enzyme present in all cells, which catalyzes the reversible oxidation of lactate to pyruvate. It is rapidly released into

cell culture supernatant upon damage of the plasma membrane. An increase in the amount of dead cells results in an increase of the LDH enzyme activity in the medium. Most often, the activity of the LDH is indirectly measured in coupled enzymatic reaction. In the first step, NAD+ is reduced to NADH/H+ by the LDH-catalyzed conversation lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to appropriate redox dye such as atetrazolium compound or resazurin. Result of LDH activity can be measured by the change of absorbance or fluorescence. Typically, cell-based assessment of compounds toxicity is evaluated in multi-well microplate systems. In recent years, we have observed a growing interest in using microfluidic devices for cell culture. It is caused by to the fact that microfluidic devices have a lot of advantages over traditional *in vitro* culture: much higher cell volume-to-extracellular fluid volume ratio, dimensions of microchannels well suited to the physical scale of cells, easy control parameters of cell microenvironment. Despite the frequent use of LDH assay in macro scale, there are only a few examples of its use in micro scale. There are research groups, who have measured LDH activity in cell culture supernatant from microchip, but they used a multi-well microtiter [8–12]. Here, we present the microfluidc system, in which the LDH activity measure is possible.

Experiments

Cells culture and LDH activity analysis

Human lung carcinoma (A549) and human colon carcinoma (HT-29) cell lines (obtained from The European Collection of Cell Cultures — ECACC) were used in experiments. First, cells were cultured in the Petri-dishes. The morphology of the A549 and HT-29 cells (Fig. 1) was analyzed using an inverted fluorescent microscope (Olympus IX-71) with an integrated CCD camera. After 24 h, growing cells attached to the bottom, were treated with compounds: 300 μM of 5-fluorouracil (5-FU); 120 μM of celecoxib and 20% v/v of 1,4-dioxane for 24h. The concentration of drugs was selected based on previous tests performed in the microfluidic system dedicated for cell culture and direct cytotoxicity assays [13, 14]. In addition, in other Petri-dishes the cells were incubated with 1% Triton-X and fresh medium. The samples — cell culture supernatant- were taken from each Petri-dishes. After that, cytotoxicity tests (with LDH-Cytotoxicity Assay Kit, BioVision) were performed. The toxic effect of drugs was measured in the fabricated microsystem made of PDMS (poly(dimethylsiloxane)). The special microchannel, performed in the microsystem, was used for an analytical reaction preparation. Then, the LDH activity was measured at 490 nm using a spectrophotometer (Specord S600, Analytic Jena). Finally, the obtained results were compared with the results carried out in 96-well plates using a plate reader (Tecan, Sunrise).

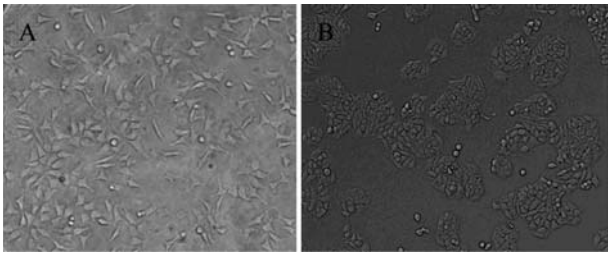

Fig. 1. (A) A549 and (B) HT-29 cells cultured in the Petri-dishes. Images were performed using an inverted fluorescent microscope (Olympus IX-71) with an integrated CCD camera. Magnification 10x.

Microfluidic devices

The aim of our research was to develop a method for cytotoxicity analysis by LDH activity analysis in the microfluidic system. The microdevice made of poly(dimethylsiloxane) — PDMS (Sylgard 184, Dow Corning) in all experiments was used. The geometry of the fabricated microdevice contains a main microchannel, in which an enzymatic reaction was carried out. The microchannel (Y-shaped) has dimensions: a length of 25 cm, a width of 300 μm and a depth of 50μm. This microstructure (designed in AutoCAD) was fabricated using soft lithography and replica molding techniques [13, 14]. First, the photosensitive material, a capillary film (Pro/Cap 50, Chromaline) was deposited on a sodium glass plate and exposed to UV light for 2.5 min through a designed photomask. Next, the exposed capillary film was developed using water and then dried — in this step the stamp was obtained. After that, the PDMS liquid was injected into the prepared stamp, and the PDMS mold was peeled off from the stamp after 1 hour at 60°C in the oven. Finally, in the PDMS plate 1.3 mm diameter holes for tubings were drilled. The PDMS plate with the obtained microchannel and access holes was bonded with the second PDMS plate (without a structure) using surface plasma activation (Plasma Preen System Inc. II 973). In Fig. 2 the fabricated microdevice is shown.

At this stage of research, the microfluidic system was connected (through the outlet) with a quartz flow cell (volume 18 μl), where absorbance was measured in the spectrophotometer (Specord S600, Analytic Jena). A microflow cell with outlet of the microchannel will be integrated ultimately. It will improve, automate and simplify examination of LDH activity.

A flow rate (enzymatic reaction time), stability of the reagent (LDH-Cytotoxicity Assay Kit), repeatability of

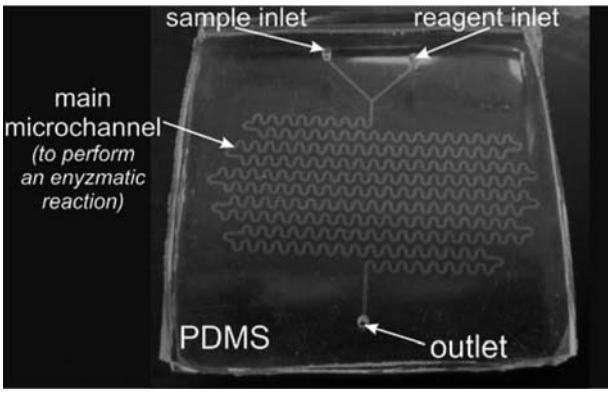

Fig. 2. The fabricated PDMS microsystem used for LDH activity analysis.

tests were examined in the fabricated microsystem before LDH activity analysis. For this purpose, a sample (supernatant with 20% of 1,4-dioxane) and a reagent in the microcystem (a flow rate 10 µl/min) were introduced. When the signal of absorbance was stable, water was introduced into the microchannel. It was one cycle of test. The LDH reagent was kept during the examination with three different conditions: (1) room temperature, light exposure (2) T = 15°C, light exposure (3) T = 15°C, without light exposure.

Results

Supernatant from the A549 and HT-29 cells incubated with 5-FU, celecoxib and 1,4-dioxane were analyzed. Moreover, the cells were cultured with Triotn-X and fresh medium. Triton-X causes complete lysis, and thus allows the determination of total LDH activity in cell culture (high control). In turn, LDH was not released in cells culture with fresh medium (low control). LDH--Cytotoxicity Assay Kit is a reagent in which reaction time is about 30 min. Therefore, cytotoxicity tests in 96-well plates after this time were performed. The test procedures were carried out according to the recommendation of LDH-Cytotoxicity Assay Kits' manufacturer. In Fig. 3 the number of the dead A549 and HT-29 cells after incubation with the tested compounds is shown. The obtained results suggested that all of the tested compounds have the toxic effect on both cell lines. Only 5-FU indicates high viability of HT-29 cells. These tests (in the macroscale) were performed as a comparison to a newly developed method in the microscale.

The next step of our research was to develop a method for determining the activity of LDH in the microsystem. The geometry (Y-shape) of the microdevice and dimensions of the main microchannel enabled to introduce two solutions and mix them in a volume ratio of 1:1. This ratio was recommended by LDH--Cytotoxicity Assay Kits' manufacturer. Finally, in the outlet of the microchip a mixture of sample and reagent was obtained. Easy control of an enzymatic reaction time and its stability were the advantages of application of the microfluidic system. In the microsystem the reaction time was only dependent on a flow rate of introduced solutions. Moreover, we observed that the reaction time was shorter in a microscale than in a macroscale. The influence of parameters such as a flow rate (enzymatic reaction time) and stability of the reagent (LDH--Cytotoxicity Assay Kit) on the viability of cells was examined. In addition, repeatability of tests was investigated. According to the description in the experimental part, we proved that reagent was not stable during the light exposure — absorbance was growing with each next cycle. Repeatability of tests and stability of reagent were the highest for cooling of a reagent and

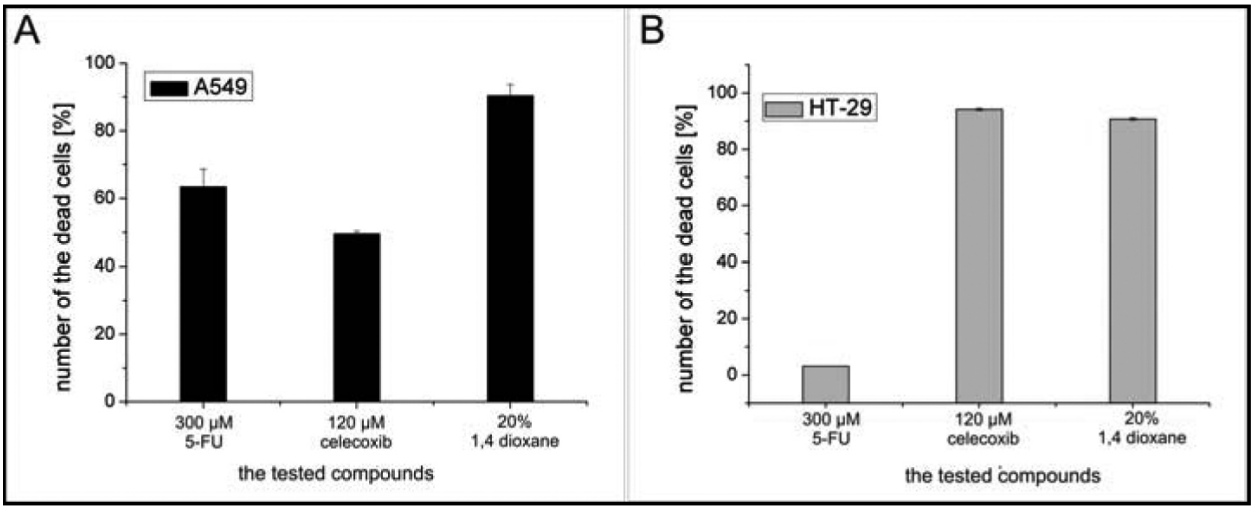

Fig. 3. The number of the dead (A) A549 cells and (B) HT-29 cells after cytotoxicity tests performed in 96-well plates — the standard method. The toxic effect of 300 μM 5-FU, 120 μM celecoxib and 20% 1,4-dioxane was examined according to the recommendation of LDH-Cytotoxicity Assay Kits' manufacturer.

lack of light exposure (Fig. 4). The compared absorbance values with a stable signal in four cycles were obtained. This means that measurements in the flow may be performed by at least 100 min.

The next step of work was focused on determining the shortest time of the enzymatic reaction in the microsystem (value of absorbance comparable with macroscale). It was expected that due to that small volume and intensive mixing of sample and reagent the reaction time will be shorter in the microdevice than on 96-well plate. First, reaction time dependence on a flow rate was examined. After that, LDH activity for 6-measurement points was analyzed (absorbance measurement). Supernatants from HT-29 and A549 cells were taken and investigated in the microsystem and 96-well plates.

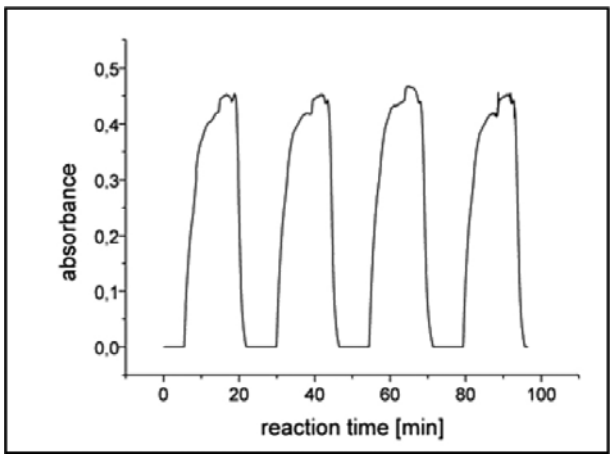

Fig. 4. Repeatability of tests and stability of reagent during a cooling of a reagent and lack of light exposure.

The cytotoxiciy results for A549 and HT-29 cells are shown in Fig. 5. The highest toxic effect was observed for HT-29 cells after incubation with 20% of 1,4-dioxane and 120 μM of celecoxib. The results for both cell lines indicate that the enzymatic reaction time in the microsystem is shorter than in 96-well plate. The number of the dead cells in a microscale over time 11.2 min (at a flow of 7 ml/min) is close to the value obtained after 30 min (time recommended by the manufacturer) in a macroscale. For example, examination of toxic effect performed during 30 min in a macroscale and 11.2 min in the microsystem gave the same or close values (50% of the dead A549 cells after incubation with 120 μM celecoxib). In turn, the number of the dead HT-29 cells in a macroscale amounted 94% (after 30 min), whereas 91% after 11.2 min in the microdevice. This dependence

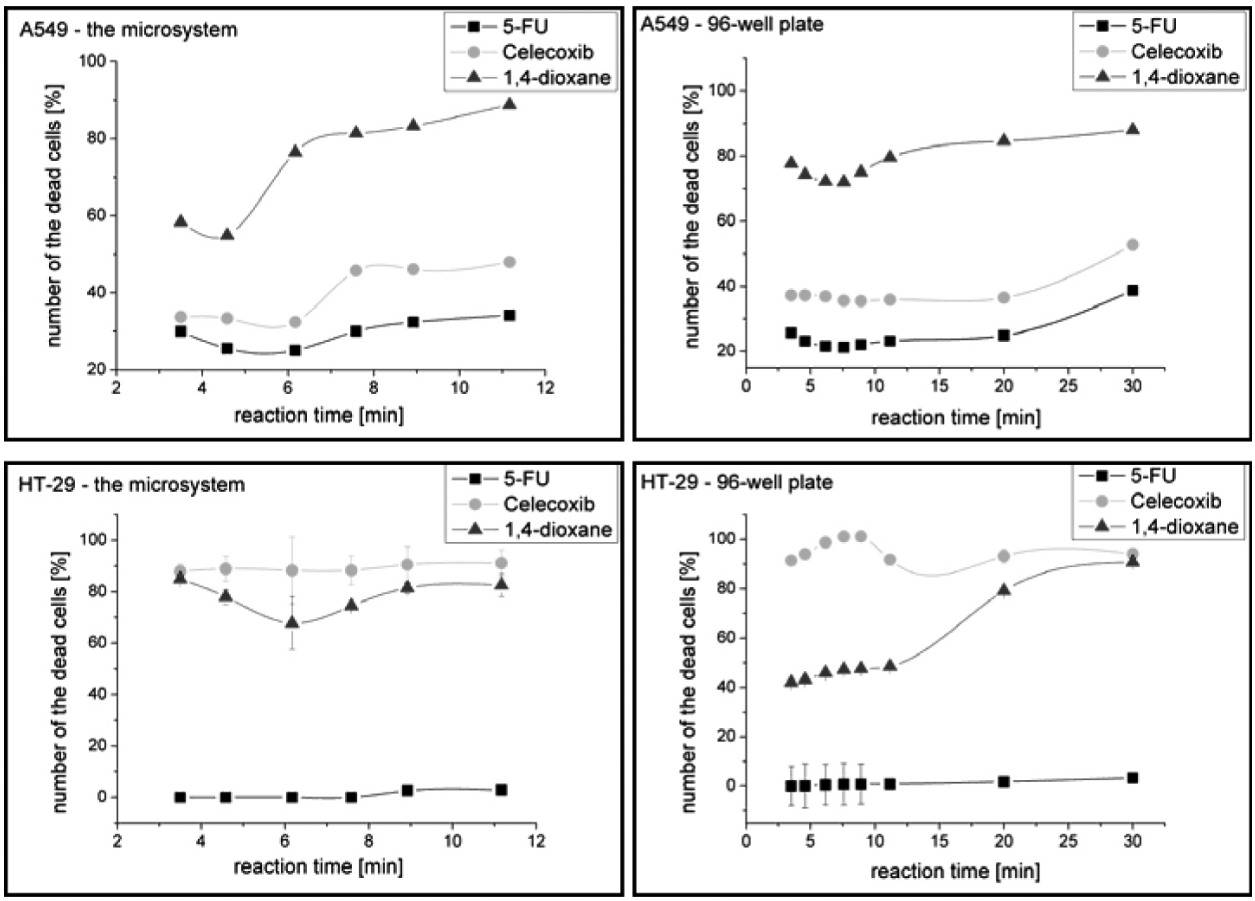

Fig. 5. The number of the dead A549 and HT-29 cells, calculated by measuring absorbance for different enzymatic reaction time. In the microsystem and 96-well plates tests were performed.

was observed for all tested compounds and the cell lines. Reduction of enzymatic reaction time may be a result of intensive mixing of sample and reagent in the microdevice. These tests confirm a possibility to analyze LDH activity in microscale. It can constitute an advantage and improvement for existing methods. In our previous works, we tested cytotoxicity of these compounds in the direct methods (using calceine-AM and propidium idoide) in a hybrid microsystem and 96-well plates [13, 14]. Here (using LDH activity tests), we also proved the toxic effect of 300 μM 5-FU, 120 μM celecoxib and 20% 1,4-dioxane on the A549 and HT-29 cells. However, here the number of the dead cells is lower than in direct tests (where the fluorescent dyes were utilized). Lim et al. [15] analyzed cytotoxicity of 5-FU and celecoxib using MTT assay and a flow cytometry. Similar results of toxic effect of tested compounds we observed. Cytotoxicity of 5-FU and celecoxib on A549 and HT-29 cells was described repeatedly [15–17]. LDH activity of 5-FU and celecoxib was examined also on various cell lines: human leukemia K562 cells [18], rat mesangial cells [19], rabbit corneal stromal fibroblasts (SIRCs) [20] and human corneal epithelial cells (HCECs) [20]. Our results, described in this paper, performed on 96-well plate and in the microfluidic system, shows that all of the tested solutions have toxic effect on the both selected cancer cells. Moreover, the usage LDH activity analysis in the tests (in particular in the microdevice) can have a significant influence on biological studies.

Conclusions

The results show the possibility to perform the cytotoxicity tests by LDH activity analysis in a microscale. It was found that the selected reagent was sensitive to temperature changes and light exposure. Moreover, we examined that reaction time in the fabricated microsystem is shorter (11.2 min) than in 96-well plate (30 min). Moreover, incubation 20% of 1,4-dioxane and 120 μM of celecoxib with HT-29 cells caused the highest toxic effect. Application of this type of microfluidic device is expected to have a significant influence on biological and engineering studies. The microsystem enables to perform fast and cheap tests with various kinds of drugs and cell lines. It can be a user-friendly device applicable in a biological laboratory. In our opinion, such tests should be helpful during optimization of real clinical photodynamic therapy parameters (*i.e.* the dose of irradiation, time of exposition, concentration of photosensitizers).

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