Development of a Point-of-Care system for early diagnostics of genetic diseases

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Lysosomal storage disorders (LSDs) represent a group of more than 45 genetically inherited diseases caused by the absence or deficiency of one or more specific lysosomal enzymes. Nowadays, there is a lack of reports on fast, reliable methods for the diagnostics of LSDs. Currently applied diagnostic approaches generate many false-negative and false-positive results, which results in classification of patients to inappropriate therapeutic groups. Moreover, these methods are time-consuming (even 20 hours), and are carried out only in a few laboratories in the world.

The goal of this work was to develop a method and a tool, a Point-of-Care system, for diagnostics of LSDs. The polymeric microdevice consists of a cell lysis module, a mixing microchannel and an optical detection module. The system enables to determine the activity of α-galactosidase (deficient in Fabry disease), and to reduce the time of analysis to 10 min. Due to its easy fabrication steps and low price, it seems to be a prospective tool for a point-of-care approach.

Keywords and phrases: lysosomal storage disorders, Fabry disease, Gaucher disease, early diagnostics, intracellular enzyme activity, Point-of-Care system, lab-on-a-chip system.

Introduction

Due to the present development of science and technology, diagnostics and treatment of currently incurable disorders become feasible. The early identification of organism dysfunction is as crucial as development of new methods and diagnostic tools. Fast and accurate recognition of a disorder enables to start a proper therapy, and, in fact, it improves patient's comfort and quality of life, and often prevents from early death.

Lysosomal storage disorders (LSDs) are a group of approximately 45 rare inherited metabolic diseases that result from defects in lysosomal function, *i.e.* Fabry, Gaucher, Niemann-Pick, Pompe, Tay-Sachs diseases, or 7 types of mucopolysaccharidoses. 70% of them is caused by abnormal activity of a proper enzyme. In consequence, an accumulation of specific compounds, which are normally degraded inside a lysosome, is observed [1]. This accumulation may lead to cell degradation, tissue and organ damage, and eventually to death of a patient [2, 3]. The importance of an early and proper diagnosis has grown since first methods of treatment were found, *i.e.* enzyme replacement therapy, residual enzyme activation, chemical chaperone therapy, substrate reduction therapy, promoter activation, and protein homeostasis regulation [4].

There are many approaches to diagnose LSD patients: histological examinations, DNA-mutation analysis, the identification of a storage material in patient's tissues or in the urine, and finally the determination of enzyme activity using a natural or an artificial substrate [5]. Molecular analysis seems to be the best diagnostic method. However, there is a large number of mutations of genes coding enzymes, and in fact it is not possible to develop screening molecular-based diagnostics methods. Thus, biochemical study on enzyme activity seems to be more accessible [6].

Development of novel, effective diagnostic strategies and procedures is strongly related with applying micro total analysis systems (μTAS). Apart from low reagents consumption, short reaction time, versatility, possibility of automation, continuous monitoring and stimulation of each step of the procedure, the use of miniaturized microdevices in progressive clinical medicine (point of care systems, POCS) affects the patients' comfort [6].

The aim of this project was to design and develop an integrated microdevice for diagnostics of Fabry disease (FD). FD is a hereditary, X-linked lysosomal storage disorder caused by a partial or complete deficiency of α-galactosidase A (GLA) [7]. Although most symptoms of FD occur in early childhood, diagnosis is often delayed or missed. FD diagnosis is a relatively new issue and is hindered due to the rarity of the disorder [8].

Experimental

Microdevice design and fabrication

A schematic view of the integrated microfluidic system is presented in Fig. 1. The microsystem consists of a cell lysis module based on a *sheath flow* geometry, a mixing microchannel and an optical detection module connected with a spectrofluorimeter by optical fibers.

The *sheath flow* geometry lets to obtain a maximum efficiency of a chemical cell lysis process. Three 100 μm wide and 150 μm deep inlet microchannels (*i.e.* two side-focusing streams used for lysis buffer and the middle one for cell suspension and substrate mixture introduction) merge into a single 300 μm wide and 150 μm deep microchannel. Then, a meander-shape micromixer is formed, in which the lysis process and the analytical enzymatic reaction undergo simultaneously. Among many tested micromixers we have decided to apply a smooth and relatively long microchannel (~35 cm long). This solution causes that the time of response is short (there is no accumulation of compounds in grooves).

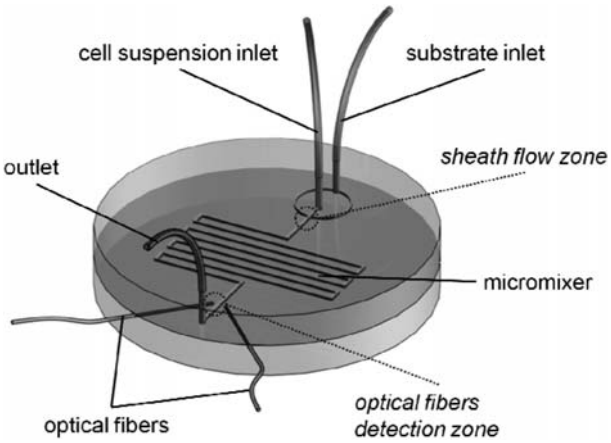

Fig. 1. A schematic view of the microsystem.

The microsystem was fabricated in poly(dimethylsiloxane) (PDMS) by the replica moulding technique using a micromilled poly(methyl methacrylate) (PMMA) master. First, geometry of the structure was designed in AutoCAD. Then, the microchannels' geometry was milled in a PMMA plate. Degassed mixture of PDMS prepolymer with curing reagent with 10:1 weight ratio was poured into the master, and was cured for 2 h at 75°C. After this time the PDMS plate with precisely formed microchannels' network was obtained. Then, the PDMS slab was frozen in liquid nitrogen, which enabled to drill inlet and outlet holes for tubings. Fabricated PDMS structure was sealed with a plain PDMS slab after oxygen plasma treatment.

Measurement method

α-galactosidase A (EC 3.2.1.22) (GLA) is an enzyme deficient in Fabry disease, and the activity of GLA mostly using isolated from blood leukocytes, or cultured fibroblasts is being determined for diagnostic purposes [9]. A principle of GLA activity determination in our investigations was fluorometric measurement of a protonated form of a product released in the enzymatic reaction (λ_{ex} = 320 nm, λ_{em} = 445 nm). We used 4-methylumbelliferyl-α-D-galactopyranoside as a substrate for the reaction catalyzed by GLA, and lysed L929 fibroblasts as a source of the enzyme. In order to eliminate presented in human tissues, interfering α-galactosidase B, an inhibitor, N-acetylgalactosamine was added to the reaction mixture. Moreover, the reaction mixture contained phenylmethylsulfonyl fluoride (PMSF) to inhibit proteases, and sodium orthovanade to inhibit phosphatases, all of them released after cell lysis. The concentration of released fluorescent product, 4-methylumbelliferone (4-MU) was determined against a 4-MU standard curve. The curve of advancement of the enzymatic reaction performed in the presented microsystem was estimated for different reaction times obtained by the change of reagents' flow rates maintained in the constant 4:1 ratio (see Table 1). This ratio enabled to obtain a width of a focusing stream with cell suspension on the order of 15 μm (matched to a diameter of mammalian cells).

Table 1.

Flow rate of substrate [μL/min]	Flow rate of cell suspension [μL/min]	Time of the enzymatic reaction in the microsystem [min]
12	3	1
6	1.5	2
4	1	3
2	0.5	6

Results and Discussion

Determination of the α-galactosidase activity in the presented microsystem was performed for 10 samples from different stock L929 fibroblast suspensions.

Investigations were performed at room temperature (RT, ~21°C) and at physiological temperature (PT, 37°C). We have decided to carry out assays at RT, because at RT the enzyme remains more stable, its activity is essentially unchanged. Carrying out investigations at RT does not require special thermostating modules, which enables to develop low cost, and portable HTS or POC systems. At both temperatures the α-galactosidase kinetics follows simple Michaelis-Menten model [10]. The enzyme saturation is observed for the 4-methylumbelliferyl-α-D-galactopyranoside concentration above 5 mM. We have checked that significant background fluorescence occurs when the substrate concentration is increased. Thus, in our investigations, the substrate concentration in a final reagents' mixture of 5 mM was used.

The calculated GLA activities at RT and PT were 2.72±0.40 μU/106 cells and 5.34±0.77 μU/106 cells, respectively. Measurements were highly repeatable (SD < 15%), what is extremely difficult to obtain when biological material is used in experiments. The activities of α-galactosidase in the microsystem were successfully determined. The method sensitivity at RT was invariable. Possibility of determination of the enzyme activity at RT without a special thermostating module is very prospective.

Moreover, we determined the activity of α-galactosidase in macro-scale. Macro-scale assays were performed in a 4ml quartz couvette. The activity of α-galactosidase at RT was 2.52±0.71 μU/106 cells, and at PT was 4.98±0.85 μU/106 cells. Values of the enzyme activity obtained in both scales are in good correlation. It confirms that elaborated micro-scale method is accurate and may be an alternative for quite expensive Fabry disease diagnosis procedures utilized only in a few clinical laboratories in Europe.

Conclusions

Future research should address the development of protocols and tools for early diagnostics of Fabry disease [11]. The presented work rises to this challenge. The presented microsystem is inexpensive, easy to fabricate, accurate, and well suited to the medical context of the developing world. The microdevice may be used not only for FD diagnostics, but also for other lysosomal storage diseases, which diagnostic procedures are based on the same principle (*e.g.* Gaucher disease).

Further works should be focused on determination of GLA activity using samples from patients. After the validation of the analytical procedure the presented microsystem will be ready for preliminary tests in clinical laboratories.

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