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Fabrication and characterization of microfluidic liver-on-a-chip using microsomal enzymes

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ABSTRACT

Biotransformation in the liver plays an important role in determining the pharmacokinetic profile of drugs and food components. Current *in vitro* platforms for testing the liver metabolism suffers from the lack of resemblance to the human liver metabolism, mainly due to the lost metabolic activity of cultured hepatocytes and the absence of transport phenomena that occurs in the liver tissue. Here we report a microfluidic device with liver microsome encapsulated in 3-D hydrogel matrix, which can mimic the metabolism reaction and the transport phenomena in the liver. Photopolymerization of poly(ethylene glycol) diacrylate (PEG-DA) allows controlling the mass transfer with matrix sizes, and a gravity-induced passive flow can reproduce the blood flow through the liver. We measured the reaction kinetics of P450 enzymes in the device, and simulated the convection-diffusion-reaction characteristics inside the device with a mathematical model. Combination of mathematical analytical tool and the experimental tool allowed us to analyze and optimize the reaction kinetics inside the microfluidic chip. This novel *in vitro* platform can serve as a tool for screening the liver metabolism of various compounds.

1. Introduction

Recent advances in chemistry and biology have resulted in an increase in the number of candidates entering the drug development process. However, drug development process is still a highly inefficient process, and with a low overall success rate, many drugs fail due to unforeseen toxicity or lack of efficacy [1]. Automated screening tools have enabled high-throughput screening of drug candidates for specific properties, such as molecular binding or cell toxicity, but often the overall efficacy and toxicity observed *in vivo* differs from that observed *in vitro*.

There are several reasons behind the observed differences of *in vitro* and *in vivo* response, and one of the main reasons is that the drugs administered often go through metabolic reaction in the liver before it goes into the systemic circulation. To account for the biotransformation in the liver, a variety of *in vitro* tools exists, including primary hepatocytes, liver cell lines, recombinant hepatic enzymes, liver tissue slices, and extracted liver fraction such as microsomes [2]. All of these systems have advantages and disadvantages. While primary hepatocytes and the liver tissue slices generally reproduce the liver metabolism closely, they quickly lose activity after separation and it is difficult to handle them. Recombinant enzymes, liver cell lines and microsomes may give more consistent results and they are easier to handle, but might not reflect the true the metabolic profiles of the human liver.

All of the aforementioned *in vitro* systems are static systems, and they do not take into account the transport process occurring in the liver. In the human body, the hepatic portal vein and the hepatic artery supply the blood into the liver and the blood flows through the liver sinusoids, ultimately leaving the liver *via* the hepatic veins. Oxygen and nutrients, along with xenobiotic compounds diffuse from the sinusoids into the hepatocytes where the metabolism reactions take place. These complex transport and reaction processes affect the overall kinetics of the liver metabolism, but *conventional in vitro* systems are not able to reproduce this aspect of the liver metabolism.

During the last decade, microfabrication technology has been adapted into microfluidics and so-called 'organ-on-a-chip' technology [3–5]. The main advantage of the 'organ-on-a-chip' technology is the ability to manipulate the spatiotemporal environment in microscale to mimic *in vivo* microenvironment more closely than the conventional *in vitro* systems. The concept of organ-on-a-chip technology has been applied to various organs including the blood vessel [6], kidney [7], gastrointestinal tract [8,9], and the lung [10]. Among these novel systems, due to the importance of the liver metabolism, several 'liver-on-a-chip' devices with varying complexities have been reported [11–13]. However, characterization

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of transport phenomena and reaction kinetics inside such devices has been lacking. Quantitative characterization and optimization process is vital to correctly reflect the metabolism kinetics inside the human body, as illustrated in the allometric scaling approach to reproduce kinetics of organ interaction in the human body [14].

In this study, we have fabricated a microfluidic liver-on-a-chip device by incorporating liver microsome into 3-D hydrogel scaffold embedded inside a microfluidic channel. The metabolic reaction coupled with convection and diffusion inside the microfluidic channels have been characterized with both experimental data and mathematical simulation. This study provides an analytical background for developing more authentic liver-on-a-chip devices, reproducing the liver metabolism more closely.

2. Materials and methods

2.1. Materials

A rat liver microsome, Vivid® EOMCC Substrate and Vivid® Blue Fluorescent Standard were purchased from Invitrogen (Grand Island, NY, USA). β-Nicotinamide adenine dinucleotide phosphate (NADPH), Phosphate Buffered Saline (PBS), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Poly(ethylene glycol) diacrylate (PEGDA, 700 Da), Dimethyl sulfoxide (DMSO), and Acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). SU-8 photoresist and SU-8 developer were purchased from MicroChem (Newton, MA, USA). Sylgard 184 kit for making Polydimethylsiloxane (PDMS) was purchased from Dow Corning (Midland, MI, USA).

2.2. Microsome reaction in solution

Fluorescence standard solution was made by serial dilution. DMSO 1 ml was added to a vial containing blue fluorescence standard 1 μmol to make 100 μM blue fluorescence standard stock solutions. 195 μL of PBS and fluorescent standard 100 μM were added to 1st well of well plate and 100 μL of PBS was added to each of the remaining wells from 2nd to 7th. 100 μL of 1st well was transferred to 2nd well containing 100 μL of PBS and mixed by pipetting. This dilution steps were repeated until 6th well and threw away 100 μL of the diluted solution in the 6th. 100 μL of PBS was added to 1st to 7th wells and mixed well. The resulting fluorescent standard concentrations were 2.5 μM, 1.25 μM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, and 0 nM. Fluorescence intensity was measured at 415/460 nm wave length using a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies).

The rat liver microsome (20 mg/mL) 5 μL was added to 150 μL PBS. 205 μL acetonitrile was added to a vial with 1 mg EOMCC to make 2 mM EOMCC, which was added to PBS to make total volume of 190 μL with desired concentration. Tube was incubated at 37 °C for 10 min. 190 μL solutions was transferred to a well plate and 10 μL NADPH 20 mM in PBS was added. Fluorescence intensity was measured at 415/460 nm wave length using a fluorescence spectrophotometer every 5 min until 15 min.

2.3. Microsome-PEG reaction in solution

The rat liver microsome (20 mg/mL) 5 μL, PEGDA 3 μL, and AAPH 20% solution diluted with PBS 2 μL was added to 10 μL PBS and mixed by pipetting. 20 μL PEGDA-microsome solution was transferred 4 mm diameter PDMS well and was polymerized using UV spot curing system (OmniCure S1000, EXFO, Canada) within 10 s. Polymerized PEGDA-microsome gel was washed by PBS three times to wash non-polymerized microsome in dish. In a micro tube, 10 μL of NADPH 20 mM, EOMCC 2 mM were added to make total volume of 180 μL in PBS. Substrate solution was incubated at 37 °C for 10 min. The substrate solution and PEGDA-microsome gel were transferred to a well plate and fluorescent intensity was measured at 415/460 nm wave length every 10 min until 30 min.

2.4. Chip fabrication

Photomask designed by AutoCAD was made by HanolTech (Siheung-si, Korea). Wafer master was made by using SU-8 2050 negative photoresist. SU-8 was poured on the wafer and conducted spin-coating as 1700 rpm, 30 s. After spin-coating, wafer was baked at 95 °C for 30 min then SU-8 was polymerized by using UV contact aligner. Polymerized SU-8 wafer was baked at 95 °C for 20 min and developed. Sylgard 184 A and B were mixed as 10:1 weight ratio. After degasing, PDMS prepolymer was poured on the SU-8 master in petri dish. It was cured in the 80 °C oven for 3 h. Cured PDMS channel was punched by Biopsy punch to make inlet and outlet reservoirs. PDMS channel and slide glass were treated by plasma by using vacuum plasma system (CUTE, FEMTO science, Korea) for 3 min to irreversibly seal the channel to the slide glass and the whole device was baked 30 min at 80 °C. The rat liver microsome (20 mg/mL) 15 μL, PEGDA 9 μL, and AAPH 20% solution diluted with PBS 6 μL were added to 30 μL PBS and mixed by pipetting. 60 μL PEGDA-microsome solutions were flowed into the channel. Photomask was overlayed on the glass side of reversed chip and PEGDA-microsome was polymerized using UV spot curing system for 17 s. Polymerized PEGDA-microsome gel was washed by PBS for 30 min in 37 °C incubator.

2.5. PEG-chip microsome reaction

The length of the channel was 200 mm, width was 2 mm, and height was 100 μm. Channel volume was 40 μL and total volume of polymerized PEGDA-microsome pillars was about 14 μL. The diameter of pillar was 200 μm and the ratio of area occupied by the pillars to the total area of channels was 11.1 pillars/channel mm^2 then approximately 4400 pillars were polymerized in the channel. 10 μL of NADPH 20 mM, suitable volume assayed concentration of EOMCC 2 mM, and PBS were mixed to be 180 μL total volume and incubated at 37 °C during 10 min. Substrate solution was added to inlet reservoir and the chip was tilted to achieve flow rate of substrate solution of 5 μL/min. Fluorescence intensity of 100 μL reacted solution in outlet reservoir was measured at 415/460 nm wavelength using a fluorescence spectrophotometer every 30 min for 1 h.

2.6. Comsol simulation

The exact geometry of the fabricated chip was drawn in 2-D, and the geometry of the reaction micro well plate was drawn in 3-D using COMSOL multiphysics software. Developed model was solved to obtain time-dependent transient solution. Concentration data for each time point was extracted from the solved model and plotted. The parameters used in the model are summarized in the table below.

3. Results and discussion

3.1. Fabrication of PEG-hydrogel matrix inside chip

Microfluidic channels with width of 2 mm and height of 100 um were fabricated in PDMS with conventional soft lithography technique. PEG pillars containing microsomes were formed by UV irradiation with a photomask (Fig. 1(a)). Fig. 1(b) shows the picture of the microfluidic device. Various sizes of PEG pillars were fabricated, and it was possible to fabricate pillars with the width of 100 um and 100 um spacing in reliable manner. Reducing the size further resulted in pillars with irregular sizes due to UV diffraction. We used 200 μm pillars with 100 um spacing for further experiment (Fig. 1(c)–(e)). Using this geometry resulted in PEG-microsome pillars filling roughly 34.9% of the fluidic channel space. Time-dependent fluidic simulation of the areas around the pillars showed that the concentration profile of the reaction substrate inside the matrix was homogeneous (Fig. 2(a)). However, the transport of product molecules inside the matrix was limited by diffusion as shown in Fig. 2(b). The concentration of the substrate maintained relatively homogeneous profile inside the circular matrix, while the concentration of the product showed a gradient along the radius of the matrix. The diffusion limitation is mainly caused by the diffusion of product molecules out of the hydrogel matrix. The simulation result indicates that the effect of convection is greater than the diffusion rate, as verified by the Péclet number calculated to be greater than 1000. In order to increase the apparent reaction rate inside the microfluidic device, the diameter of the microsome-immobilized hydrogel pillars need to be reduced. Alternatively, increasing the apparent diffusivity inside the hydrogel matrix would increase the observed reaction rate.

3.2. P450 reaction in solution

P450 reaction with microsome in solution phase was tested with seven different substrate concentrations ranging from 2 μM to 80 μM. As shown in Fig. 3(a), increasing substrate concentration resulted in higher final production concentration, as well as increased rate of product formation (Fig. 3(a)). Plotting the initial reaction velocity *versus* initial substrate concentration revealed Michaelis–Menten kinetics, with maximum velocity of 0.260 μM/min and K_m value of 39.8 μM (Fig. 3(b)). The enzyme