Drug Assays Using a Cell Trapping Method in a Microfluidic Device

Department of Bio and Brain Engineering

KAIST

2010
Drug Assays Using a Cell Trapping Method in a Microfluidic Device
Drug Assays Using a Cell Trapping Method in a Microfluidic Device

Advisor: Professor Je-Kyun Park

by

Ju Hun Yeon

Department of Bio and Brain Engineering

KAIST

A thesis submitted to the faculty of the KAIST in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Bio and Brain Engineering

Daejeon, Korea
2009. 10. 22.

Approved by

________________________
Professor Je-Kyun Park
미세유체소자 내 세포 포획 방법을 이용한 약물 분석 시스템

언주현

위 논문은 한국과학기술원 박사학위논문으로 학위논문심사위원회에서 심사 통과하였음.

2009년 10월 22일

심사위원장 박제균 (인)
심사위원 조영호 (인)
심사위원 최철희 (인)
심사위원 남윤기 (인)
심사위원 민달희 (인)
Abstract

In this thesis, an efficient and accurate drug assay system in a microfluidic device was developed, considering the *in vivo* delivery path of drugs in humans. Among the various drug assays, drug permeability assays in the intestine and brain, and hepatotoxicity assay in the liver are very important for drug screening and development process. The microfluidic assay system for drug permeability and hepatotoxicity assays using a cell trapping method reduces the assay time as no cell culture in the microfluidic device is required and no complex structure, such as cellular membrane, is needed. The microhole array for cell trapping was fabricated using the poly(dimethylsiloxane) (PDMS) molding technique for mimicking the intestinal epithelial cell membrane. Based on mathematical simulations, the configuration of the microfluidic device, including a microhole array and a mixing channel, and the flow rate were optimized to trap cells firmly in each microhole without cell damage. The permeability of ten drugs was measured and
compared with the reported values of permeability in the human and rat intestine. On the other hand, drug transportation from blood to brain is restricted by the specialized membrane of brain capillary endothelial walls, namely blood brain barrier (BBB). With the same device, a permeability assay system was also performed using human umbilical vein endothelial cells (HUVECs) trapped in microholes, with or without astrocyte conditioned medium (ACM) in a microfluidic device. From the permeability assays of five widely used drugs for measuring BBB permeability, the measured permeability values were highly correlated with the permeability value of in vitro BBB model and brain uptake index (BUI). Hepatocytes have been used for in vitro hepatotoxicity assays because of their ability to sustain intact liver-specific function. In order to investigate drug effects on hepatic function using primary human hepatocytes, hepatotoxicity assays of several drugs were performed in the microfluidic device. LC50 values of the drugs assayed using the device were similar to those from the in vitro toxicity assays with human hepatocytes obtained from the literature. This novel assay system enabled us to assay in vivo-like drug assays with a short assay time, by exploiting microstructures mimicking the microenvironment of the intestine and liver.
# TABLE OF CONTENTS

Abstract: ................................................................. i

Table of Contents: ......................................................... iii

Nomenclature: ............................................................ vii

List of Tables: ............................................................. x

List of Figures: ............................................................ xi

I. Introduction: .............................................................. 1

  1.1 Background and research objectives: ................................. 2

  1.2 Chapter outlines: ...................................................... 8

References: ............................................................... 10

II. Drug Permeability Assay Using Microhole-Trapped Cells: ............... 11

  2.1 Introduction: .......................................................... 12

  2.2 Basic principle: ....................................................... 17

  2.3 Experimental: ......................................................... 19

    2.3.1 Design of a microfluidic device: ............................. 19

    2.3.2 Microfabrication process: .................................... 22
2.3.3 Cell culture ............................................................................................................. 24
2.3.4 Experimental setup ............................................................................................... 24
2.3.5 Experimental process ............................................................................................. 25
2.3.6 Computational fluid dynamics simulation ............................................................... 27
2.3.7 HPLC analysis ......................................................................................................... 27
2.3.8 Data analysis ........................................................................................................... 28
2.4 Results and discussion .............................................................................................. 30
  2.4.1 Simulation for optimizing the device structure ...................................................... 30
  2.4.2 Simulation for optimizing the flow rate ................................................................. 35
  2.4.3 Permeability assay with FITC-dextran ................................................................. 37
  2.4.4 Morphology of trapped cells and the viability test .............................................. 39
  2.4.5 Drug permeability assay ....................................................................................... 42
  2.4.6 Correlation with $F_a$ and $P_{eff}$ ............................................................................. 49
2.5 Conclusions ................................................................................................................. 51

References ....................................................................................................................... 53

III. Permeability Assay Based on Blood Brain Barrier Model ............................................. 57
  3.1 Introduction ................................................................................................................. 58
  3.2. Materials and methods ............................................................................................. 63
3.2.1. Design of a microfluidic device based on the BBB model .......... 63
3.2.2. Cell culture ............................................................................. 67
3.2.3. Experimental setup .............................................................. 68
3.2.4. Immunofluorescent staining of cell junction protein .......... 68
3.3. Results and discussion .............................................................. 70
3.3.1. Device configuration and trapped cells............................... 70
3.3.2. The expression of tight junction protein in HUVEC with/without ACM ................................................................. 73
3.3.4. Drug permeability assay of HUVECs with/without ACM .......... 80
3.3.5. Comparison of the effective permeability on the device with that of in vitro BBB model ......................................................... 85
3.3.6. Correlation with brain uptake index (BUI) and Pe on the device .. 88
3.4 Conclusion ................................................................................ 90
References ..................................................................................... 91

IV. Hepatotoxicity Assay using Suspended Human Hepatocytes Trapped in Microholes ................................................................. 95
4.1 Introduction ................................................................................ 96
4.2 Experimental ................................................................. 99
  4.2.1 Design of the microfluidic device and principle of the hepatotoxicity assay .......................................................... 99
  4.2.2 Device fabrication ....................................................... 102
  4.2.3 Cultivation of human hepatocytes .................................. 105
  4.2.4 Hepatotoxicity assay .................................................. 105
4.3 Results and Discussion .................................................. 107
  4.3.1 Primary human hepatocytes trapped in the microholes of a microfluidic device .......................................................... 107
  4.3.2 Drug sensitivity of human hepatocytes and HepG2 .......... 111
  4.3.3 Hepatotoxicity assay in a microfluidic device ................ 114
4.4 Conclusion ..................................................................... 121
References .......................................................................... 122

V. Overall Conclusions ......................................................... 126
  6.1 Summary ....................................................................... 127
  6.2 Outlook, challenges, and future works ............................ 129
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A: Fabrication process flow</td>
<td>133</td>
</tr>
<tr>
<td>Appendix B: Equation for calculating drug permeability</td>
<td>135</td>
</tr>
<tr>
<td>Summary in Korean</td>
<td>136</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>142</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>144</td>
</tr>
<tr>
<td>Publications</td>
<td>145</td>
</tr>
</tbody>
</table>
NOMENCLATURE

Alphabetic letters

$A$  The surface area

$F_d$  The dose absorbed in the human intestine

$P_{eff}$  The effective permeability coefficient

$P_e$  The effective permeability

$Q$  The number of absorbed molecules

$\nu$  The flow rate

Abbreviations

ADME/Tox  Absorption, distribution, metabolism, excretion, and toxicity

ACM  Astrocyte Conditioned Medium

AMG  Algebraic Multigrid

BBB  Blood Brain Barrier

bFGF  Basic Fibroblast Growth Factor

BSA  Bovine Serum Albumin

BUI  Brain Uptake Index
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcein AM</td>
<td>Calcein Acetoxy-Methyl Ester</td>
</tr>
<tr>
<td>CGS</td>
<td>Conjugates Gradient Squared</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IAM</td>
<td>Immobilized Artificial Membrane</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagle’s Medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PAMPA</td>
<td>Parallel Artificial Membrane Permeability Assay</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>Pgp</td>
<td>P glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>PR</td>
<td>Photoresist</td>
</tr>
<tr>
<td>RT</td>
<td>Reaction Time</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>SPIP</td>
<td>Single-Pass Intestinal Perfusion</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumor Growth Factor Beta</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonular Ocludens</td>
</tr>
</tbody>
</table>
List of Tables

Table 1  HPLC condition for measuring drug concentration.

Table 2  The effective permeability ($P_{eff}$) on the microfluidic device, human $P_{eff}$, rat $P_{eff}$, and Fa values of tested drugs.

Table 3  Comparison among $P_{eff}$ on the microfluidic device, $P_{eff}$ of in vitro BBB, brain uptake index (BUI), and $P_{eff}$ of Caco-2 cells.

Table 4  Comparison of the LC50 of drugs measured with the device versus those obtained by the MTT assay.
List of Figures

Fig. 1.1 The importance of drug discovery and development.

Fig. 1.2 The process of drug transport in \textit{in vivo}.

Fig. 2.1 The conventional assays for drug permeability test.

Fig. 2.2 Principle of cell trapping and drug absorption.

Fig. 2.3 Schematic diagrams and photograph of the microfluidic device.

Fig. 2.4 Fabrication process for manufacturing the microholes.

Fig. 2.5 Experimental process for drug permeability assay.

Fig. 2.6 Simulation results to determine the optimal slanted angle and flow rates.

Fig. 2.7 The simulated and experimented fluid flow changes before and after cell trapping in the 45° slanted microhole array structures.

Fig. 2.8 Fluorescence intensity of the absorbed FITC-dextran.

Fig. 2.9 Trapped cell morphology and cell viability over time during the drug
absorption assay.

Fig. 2.10  Comparison of drug permeability measured on the microfluidic device vs. the human and rat intestine.

Fig. 2.11  Comparison of the effective permeability ($P_{\text{eff}}$) on the device and in vivo human.

Fig. 2.12  Plot of the effective permeability ($P_{\text{eff}}$) on the device vs. the fraction of dose absorbed in the human intestine ($F_a$).

Fig. 3.1  Conventional brain permeability assays.

Fig. 3.2  Schematic illustration of a BBB model and a cross section view of BBB with an enlarged view of the transport routes across BBB.

Fig. 3.3  Design of microfluidic device mimicking the BBB model and diverse drug transport routes across HUVECs trapped in microholes.

Fig. 3.4  Schematics of the microfluidic device and the morphology of HUVECs trapped in the microholes with time.

Fig. 3.5  The expression of ZO-1 protein.
Fig. 3.6  The expression of ZO-1 of cells trapped on the microhole array in a microfluidic device.

Fig. 3.7  The absorbed concentration of Evans-blue dye.

Fig. 3.8  The absorbed concentration of FITC-dextran with a various molecular weights.

Fig. 3.9  Comparison of permeability value between only HUVECs and HUVECs supplemented ACM.

Fig. 3.10  Comparison of the effective permeability ($P_e$) on the device using HUVECs with/without ACM.

Fig. 3.11  Comparison of drug permeability measured on the microfluidic device vs. in vitro BBB model.

Fig. 3.12  Comparison of drug permeability measured on the device vs. BUI.

Fig. 4.1  Schematics of liver structure and microfluidic sinusoidal blood flow.

Fig. 4.2  Microfabricated device for hepatotoxicity assays.

Fig. 4.3  Viability of primary human hepatocytes trapped in microholes with
time after treatment with 5 mM acetaminophen.

Fig. 4.4 Comparison of live cell fraction between primary human hepatocytes and HepG2 cells at various concentrations of benzopyrene.

Fig. 4.5 Hepatotoxicity assay with various drug concentrations over time.

Fig. 4.6 Live cell fraction under various concentrations of drugs for measuring the LC50 of suspended human hepatocytes.

Fig. 4.7 Comparison of LC50 measured on the microfluidic device vs. MTT assay and in vivo human.
CHAPTER I: Introduction

1.1 Background and research objective .................................. 2
1.2 Chapter outline .................................................................. 8
References .............................................................................. 10
1.1 Background and research objective

Drug discovery and development is an expensive and time consuming process. Despite continuing effort to improve the productivity of the drug development process, only one out of ten drug candidates entering the clinical trial reaches the final approval stage (Fig. 1.1). Improved target identification and validation, manufacturing greater numbers of candidate drug compounds and being able to predict or determine absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) profiles earlier during discovery and development promises to benefit both patients and the pharmaceutical industry. As shown in Fig. 1.1, the number of new molecular entities that are approved by FDA has been declining over the last decade. The main reasons for such a low success rate are primarily unforeseen lack of efficacy or unexpectedly high toxicity is revealed in later stages of clinical trials, it increases the total cost, since the cost of clinical testing is considerably higher than the cost of preclinical testing, increasing with successive phases. Improving the ability of \textit{in vitro} assay systems to predict the efficacy and toxicity of drug candidates earlier in the drug discovery process will greatly enhance productivity. Consequently, there has been a significant amount of effort geared towards
improvement of in vitro systems for assessing drug efficacy and toxicity. As shown in Fig. 1.2, over the fifty percent of the total new drug attrition in the developmental pipeline is attributed to bioavailability including permeability and metabolism in intestine and the thirty percent of those is caused by toxicity profiles and side effects.
Fig. 1.1. The importance of drug discovery and development (a) The process of drug discovery and development. (b) The importance of permeability and toxicity assay in drug discovery.
The most of administered drugs are absorbed from intestine into the blood circulation and the absorbed drugs are metabolized in liver. Sometimes, metabolized drugs have the unexpected toxicity in liver or the side effect on the other organs. Therefore, the selection of drug candidates with a high oral absorption potentiality without any toxicity is one of the important steps in drug discovery (Fig. 1.1). Especially, in case of drugs targeting brain, transport of drugs from blood vessel to brain tissue is limited by the brain capillary endothelial wall, named blood brain barrier (BBB) [1] which is composed of specialized microvascular endothelium and glial cell elements in physical proximity with a basement membrane. Therefore, the fast and accurate assays for measuring intestinal permeability, Hepatotoxicity, and permeability of drug candidates across the BBB is therefore an important step in pharmaceutical industry [2-6].

In the present study, we developed a drug assay system using cell trapping method in a microfluidic device inspired by the in vivo drug absorption mechanisms of the intestine and in vivo liver sinusoidal structure. The device structure and experimental conditions were optimized through mathematical simulations and verified experimentally. The permeabilities of well-known drugs and LC50 of liver specific drugs were then measured using our device.
and the results were compared with their in vivo data. This system will be useful in predicting the actual human permeability and LC50 values of drugs. In addition, our drug assay system reduces the assay time as no cell culture is required and no complex structure is needed. Using this microfluidic device, it is possible to continuously monitoring the change of cellular mechanism and can be used as a valuable tool in drug discovery.
Fig. 1.2. The process of drug transport in *in vivo*. (a) Drugs are absorbed from intestine and the absorbed drugs are metabolized in liver. (b) In case of drugs targeting brain, transport of drugs is limited by the brain capillary endothelial wall, named blood brain barrier (BBB).
1.2 Chapter Outlines

This thesis is articulated into the following sections:

Chapter II describes the permeability assay using cell trapping method in a microfluidic device inspired by the in vivo drug absorption mechanisms in the intestine. Optimized device structure and experimental conditions through mathematical simulations verified experimentally. The permeabilities of ten well-known drugs were measured using this device and the results were compared with their in vivo permeabilities.

Chapter III describes a brain permeability assay system using human umbilical vein endothelial cells (HUVECs) trapped in microholes, with or without astrocyte conditioned medium (ACM) in a microfluidic device. Using trapped HUVECs and ACM, the brain permeability assay performed in in vivo-like microenvironment and the effects on shear stress by fluid flow and the interaction between HUVECs and ACM was investigated. Using five widely known drugs, it was analyzed for measuring the BBB permeability and compared with in vitro BBB permeabilities.
Chapter IV describes a hepatotoxicity assay using primary human hepatocytes trapped in microholes of a microfluidic device, which is capable of assaying with low number of hepatocytes in a short period without cell culture. The results of LC50 from the microfluidic device were compared with those of in vitro MTT assay using human hepatocytes.

Chapter V summarizes the overall results of this thesis proposal and discusses what remains to be determined by future researches.
References


CHAPTER II: *Drug Permeability Assay Using Caco-2 cells*

2.1 Introduction ................................................................. 12
2.2 Basic principle ............................................................. 17
2.3 Experimental ............................................................... 19
2.4 Results and discussion .................................................... 30
2.5 Conclusion ................................................................. 51
References .................................................................. 53

- 11 -
2.1 Introduction

As orally administered drugs must be absorbed from the intestine into the blood circulation, the selection of drug candidates with high oral absorption potential based on a permeability assay is one of the most important steps in drug discovery. Consequently, a rapid and accurate method for measuring drug permeability in the human intestine is a priority for pharmaceutical industry [1-5]. As studying and measuring the intestinal permeability in the human body or replacing animal testing is difficult, many alternative in vitro or in situ assay methods have been developed to investigate the intestinal absorptive potential and absorption mechanisms of drugs.

For an in vitro permeability assay, a method based on monolayers of the human colon adenocarcinoma endothelial cell line Caco-2 has been widely used due to the remarkable morphological and biochemical similarities of the Caco-2 cell membrane to that of small intestinal epithelium cells [6,7]. Despite the similarities, however, the limitations of this method include the long culture period of Caco-2 cells and the occasional inconsistency with in vivo permeability. To overcome the former problem, 3- to 10-day Caco-2 procedures or the fast growing Madin–Darby canine kidney (MDCK) cell line have been developed, but the 3-day culture method requires expensive media for fast
differentiation. As 80–95% of commercial drugs are absorbed primarily by passive diffusion, the parallel artificial membrane permeability assay (PAMPA) [2,8,9] and immobilized artificial membrane (IAM) [3,10] were developed for rapid and inexpensive assays dealing solely with passive diffusion. PAMPA uses lipid oils mimicking the lipid bilayer of cellular membranes and measures drugs passively diffusing through the lipid layer. The lipid layer of PAMPA replaces the cultured cells and therefore makes it possible to measure the drug permeability instantly. However, due to the lack of transport proteins of intestinal cells in the lipid layer of PAMPA, it could be implemented only for hydrophobic drugs absorbed passively. IAM, the surface of which contains one-half of the membrane bilayer, is another approach for measuring permeability because it has a good correlation with transport of drugs across Caco-2 cells. However, it is not predictive across classes of compounds because its membrane based on lipophilicity and electrostatic interaction may only be valid for compounds with similar structures.

As an in situ permeability assay, the single-pass intestinal perfusion (SPIP) approach is frequently used to provide a preserved microenvironment above the intestinal membrane because drugs are absorbed across the real intestinal cell barriers and encounter the blood circulation, resulting in a good correlation with
the *in vivo* behavior in humans [1,3,11-14]. Despite accurate measurement, however, this technique requires skilled operators due to the involvement of a surgical method in rats, and significant investment in terms of resources to set up, validate, and run. In addition, the use of anesthetics may have an influence on the membrane permeability, cellular metabolism, and lack of certain nutrients or other endogenous compounds.

Recently, microfluidic technology has been introduced in the fields of cellular assay and drug discovery because it can provide an *in vivo*-like microenvironment, continuous perfusion, and high-throughput screening [15-16]. For example, the microfluidic system for long-term perfusion culture was applied to Caco-2 cells for 2 weeks and then the transport of rhodamine 123 was monitored [16]. However, performing permeability assays using real drugs is necessary. Genes *et al* [17] presented a vascular endothelium array with blood components using microfluidic channels, and Suzuki *et al* [18] developed a planar lipid bilayer in a microfluidic device for studying membrane proteins. These studies indicated that high-throughput drug discovery would be possible using a multi-array system and lipid-bilayer structure, but it still requires complex fabrication processes and has not been used to continuously measure the permeability of real drugs.
In the present study, we developed a permeability assay system using a microfluidic device inspired by the *in vivo* drug absorption mechanisms in the intestine. The device structure and experimental conditions were optimized through mathematical simulations and verified experimentally. The permeabilities of ten well-known drugs were then measured using our device and the results were compared with their *in vivo* permeabilities.
Fig. 2.1. The conventional assays for drug permeability test. (a) Caco-2 permeability assay (b) Parallel artificial membrane permeability assay (PAMPA) (c) single pass intestinal perfusion assay (SPIP)
2.2 Basic principle

As shown in Fig. 2.2a, Caco-2 cells are injected at a high flow rate and drugs are injected at a low flow rate to the channel. Cells are automatically trapped in the microhole array in sequence due to the pressure difference in the holes. To hold the cells firmly in the holes and to supply nutrients, a medium solution containing Caco-2 cells is injected at a constant rate.

After cell trapping, a medium solution containing Caco-2 cells does not flow to the microhole array blocked by cells. The drug is then absorbed by the trapped cells and transported to the other side of the holes (Fig. 2.2b).
Fig. 2.2. Principle of cell trapping and drug absorption
2.3 Experimental

2.3.1 Design of a microfluidic device

We designed a new microfluidic permeability assay system based on absorption mechanisms in the intestine. Conventional permeability assay systems require embedded membrane structures for culturing epithelial cells or mimicking the cell membrane. Instead of a membrane structure, we used microholes for trapping Caco-2 cells and measured the permeabilities of drugs absorbed through the trapped Caco-2 cells without long-term cell culture. The microfluidic device includes two inlet parts, two outlet parts, a microhole array for cell trapping, and a mixing channel. As shown in Fig. 2.3a and b, Caco-2 cells from inlet #1 are supplied to outlet #1 at a flow rate of 20 µL/h while fresh buffer from inlet #2 are supplied to outlet #2 at a flow rate of 20 µL/h. Due to the difference of pressure in the holes, the injected cells are automatically trapped in the microhole array. After the cell trapping, fresh medium without any cells from inlet #1 is constantly supplied to outlet #1 and a fluorescent dye or drug solution to be assayed is injected into inlet #2. The dye or drug is then absorbed by the trapped cells and transported to the region (1) from the region (2) of the channel and then to the mixing channel connected to outlet #1. We
introduce the mixing channel to uniformly distribute the absorbed dye or drug because the transported chemicals are not uniformly diffused in the microchannel due to laminar flow. The concentrations of drugs are monitored in outlet #1.

A schematic side view of cross section A–A’ in Figure 1b shows the size of microhole in the microhole array (Fig. 2.3c). Because the diameter of Caco-2 cells is about 10 µm, we determined the width of microhole to 3 µm and the height of microhole to 5 µm for trapping cells firmly in the microholes. The distance between microholes was determined by cell size of 10 µm for constituting tightly compacted cell monolayer in a microhole array. The length of microhole is 30 µm for minimizing the cell lysis by fluid flow. The microchannel height is 25 µm for freely moving cells in a microfluidic device. Therefore, most animal cells with the diameter of 5–15 µm could be trapped on the microholes of this device. The overall size of the microfluidic device is 19 mm × 8 mm as shown in Fig. 2.3b.
Fig. 2.3. Schematic diagrams and photograph of the microfluidic device. (a) The drug absorption and transport process on the microfluidic device. Caco-2 cells resuspended in culture media are injected into inlet #1 (red flow) and trapped in the microhole array. Then, drugs are injected into inlet #2 (blue flow) and absorbed by the trapped cells through the holes (violet flow). Exactly, the drug is absorbed by trapped cells from the region (2) to the region (1) (indicated by small white arrows). (b) A photograph of the fabricated device for drug permeability assay. (c) A schematic side view of the A–A’ cross section showing the microhole array for cell trapping in detail.
2.3.2 Microfabrication process

The microfluidic device for drug permeability assays was fabricated using a multilayer lithography method. First, the design pattern for a microfluidic channel was printed onto a Cr mask. The mold master for devices was fabricated using a negative photoresist (PR) (SU-8 2005; Microlithography Chemical Co., Newton, MA) to manufacture a microhole array with conventional lithography. The second negative PR (SU-8 2025; Microlithography Chemical Co.) was coated and exposed after aligning with the align mark of the first layer. After the second development, the prepolymer of poly(dimethylsiloxane) (PDMS) (Sylgard 184; Dow Corning, Midland, MI) was mixed with a curing agent at a 10:1 mass ratio and poured over the mold masters. Then, the PDMS structure was cured at 65°C for 1 h and peeled from the mold (Fig. 2.4).
Fig. 2.4. Fabrication process for manufacturing the microholes.
2.3.3 Cell culture

The human colon adenocarcinoma endothelial cell line Caco-2 (ATCC HTB37) was selected for the microfluidic drug permeability assay. Cell layers cultured on Petri dishes were rinsed briefly with phosphate-buffered saline (PBS) with a pH of 7.4 (Gibco, Grand Island, NY). Then, trypsin-EDTA solution (0.25% trypsin and 1 mM EDTA·4Na; Gibco) was used to detach the cells and Modified Eagle’s medium (MEM; Gibco-BRL, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS; Gibco) was added to the dispersed cell layer. The cell cultures were maintained at 37°C under 5% CO₂ in a humidified water-jacketed incubator [19]. Prior to the permeability assay using the microfluidic device, appropriate aliquots of the cell suspension were refreshed and diluted to a suitable concentration of $2.5 \times 10^6$ cells/mL. Drug treated cells and reagents after the experiments were completely autoclaved before discharging to ensure safety according to the KAIST experimental protocols for biosafety.

2.3.4 Experimental setup

The microfluidic devices were sterilized and the bubbles within the channel were eliminated with 70% ethanol for 10 min, followed by rinsing with cell
culture media. Caco-2 cells were incubated in the culture dishes, and then 100 µL of $1 \times 10^7$ cells/mL was injected into inlet #1 of the microfluidic device and 100 µL of drugs or FITC-dextran (Sigma–Aldrich, Seoul, Korea) was also injected into inlet #2. The concentration of fluorescent dye was estimated by measuring the fluorescence intensity of reference samples in the mixing channel.

### 2.3.5 Experimental process

As shown in Fig. 2.5a, Caco-2 cells are injected into inlet #1 and then automatically trapped in the microhole array due to the difference of pressure in the holes. To hold the cells firmly in the holes and to supply nutrients, a medium solution containing Caco-2 cells is injected into inlet #1 at a constant rate. Caco-2 cells are trapped in the microhole array in sequence. After cell trapping, a fluorescent dye or drug solution to be assayed is injected into inlet #2. The dye or drug is then absorbed by the trapped cells and transported to the other side of the holes and then to the mixing channel connected to outlet #1 (Fig. 2.5b).
Fig. 2.5. Experimental process for drug permeability assay (a) The drug absorption and transport process on the microfluidic device. Caco-2 cells resuspended in culture media are injected into inlet #1 (red flow) and then trapped in the microhole array (indicated by small white arrows). (b) Then, drugs are injected into inlet #2 (blue flow) and absorbed by the trapped cells through the holes (violet flow)
2.3.6 Computational fluid dynamics simulation

Simulations of the fluid flow in the slanted microhole array structures were performed with commercial software (CFD-ACE+; ESI, Huntsville, AL). The microhole for simulation is 3 µm wide, 5 µm height, and 30 µm length (Fig. 2.1c). The number of microholes is the same as the real microfluidic device. Upwind scheme was used in the conjugates gradient squared (CGS) and preconditioning (Pre) solvers for velocity field, while algebraic multigrid (AMG) solver was used for pressure correction. The inlet fluid velocity was varied and the boundary conditions at the outlet were set at a fixed pressure. Water was used as a test sample.

2.3.7 HPLC analysis

All drugs, including propranolol, naproxen, furosemide, antipyrine, verapamil, atenolol, piroxicam, hydrochlorothiazide, cimetidine, and carbamazepine, were purchased from Sigma–Aldrich. Other chemicals or reagents were purchased from Merck (Seoul, Korea). For drugs, a solution obtained for 2 h from outlet #1 was analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Agilent 1100 series; Agilent Technologies, Santa Clara, CA) with an Eclipse Plus C18 (5 µm, 4.6 mm × 250 mm) column (Agilent Technologies).
For HPLC analysis, 5-μL aliquots of the samples were applied and buffers were passed through the column at a rate of 1 mL/min under isocratic conditions. The mobile phases were filtered through 0.46-μm sintered glass filters (Millipore, Bedford, MA) and degassed in a sonicator (Branson, Danbury, CT) before use. Each drug concentration was estimated from the drug reference samples.

2.3.8 Data analysis
The effective permeability coefficient ($P_{\text{eff}}$) was derived from eq 1, conventional equation of the steady-state condition for adapting a microfluidic perfusion system [7,20],

$$P_{\text{eff}} = \frac{1}{A \cdot C_0} \frac{dQ}{dt}, \quad (1)$$

where $P_{\text{eff}}$ is the permeability (cm/s), $A$ is the surface area (cm$^2$), $C_0$ is the initial concentration (mM), and $Q$ is the number of absorbed molecules (mol). In our device, the surface area ($A$) is determined by multiplying the hole number in the microhole array (76 trapping holes) by each hole area ($1.5 \times 10^{-7}$ cm$^2$). Here, eq 2 is an integrated form of eq 1 for time $\Delta t$,

$$Q(t) = P_{\text{eff}} \cdot A \cdot C_0 \cdot \Delta t. \quad (2)$$
As the flow rate of inlet #1 is \( v \) (cm\(^3\)/s), the volume flowing in channel #1 for \( \Delta t \) is \( v\Delta t \). The concentration of molecules transported from channel #2 to channel #1 for time \( \Delta t \) is expressed by eq 3:

\[
C = \frac{Q(t)}{v \cdot \Delta t} = \frac{P_{\text{eff}} \cdot A \cdot C_0}{v}.
\]  

(3)

From eq 3, we derived the effective permeability equation (eq 4) where \( v = 10 \) cm\(^3\)/s and \( A = 1.2 \times 10^{-5} \) cm\(^2\). The initial concentration \( C_0 \) for each drug was determined from the literature,

\[
P_{\text{eff}} = v \cdot \frac{1}{C_0} \cdot \frac{1}{A}.
\]  

(4)
2.4 Results and discussion

2.4.1 Simulation for optimizing the device structure

We performed simulations to optimize the device structure for trapping cells, including the angle of the microhole array and the flow rates in both channels. The microhole array was tilted by 90°, 60°, 45°, 30°, or 0° along the horizontal axis. For the 0° slanted microhole array, the fluid flowed from the channel of high flow rate to that of low flow rate and the flow rate crossing holes decreased gradually in the rearward holes (Fig. 2.6a and 2.6b). Due to this decreased flow rate, the cells would not be trapped in these holes. For angles of 30° and 60°, the flow rate through the holes decreased slightly in the rearward holes. For the 45° slanted microhole array, the flow rate was constant over all holes (Fig. 2.6c and 2.6d). The 90° slanted microhole array showed similar phenomena as the 45° slanted structure (Fig. 2.6e and 2.6f). The simulation results indicated that the array structure slanted at an angle of 45° produced the best conditions for cell trapping. The simulation results were also validated experimentally (data not shown). The experimental results for 30° and 60° slanted structures showed that the cells in the rearward holes were not trapped as predicted. Increasing the flow rate to trap cells in these holes resulted in lysis.
of the trapped cells. For the 90° slanted structure, cells were trapped over all holes, but cells piled up with an irregular thickness, which would result in different absorption rates in each hole. Based on the simulation and experimental results, the cell trapping structure slanted by 45° was selected for stable cell trapping to form a single layer of cell.

In addition, we optimized the flow rates in both channels to trap cells firmly, varying flow rates of 5, 10, and 20 µL/h in each inlet of a microchannel with the 45° slanted microhole array. The flow was monitored using FITC-dextran before and after the cell trapping. As the flow rate ratio of inlet #1 to inlet #2 increased, the velocities moving through holes gradually increased. The flow rate ratio of 1:1 resulted in the best results to produce similar flow velocities in each hole. The flow rates of 5 and 10 µL/h were too slow to trap cells and cell debris was caught in the holes because of the faster movement of debris compared to that of cells. From this simulation and experimental results, we determined the flow rate as 20 µL/h in each inlet for stable cell trapping. After the cell trapping, to minimize the cell damage due to pressure at a high flow rate of 20 µL/h in the two inlets, we adjusted the flow rate to 10 µL/h in both channels, maintaining the flow rate constant over all trapping holes. The simulation results also show that the pressure difference between two channels
was too small at a flow rate of 10 µL/h (data not shown). Therefore, we expect that the damage of trapped cells due to pressure would be minimized.
(a) 0° slanted Microhole Array

(b) 45° slanted Microhole Array

(c) 90° slanted Microhole Array
**Fig. 2.6.** Simulation results to determine the optimal slanted angle and flow rates. The left figures stand for the calculated velocity profiles in each microchannel, while the right figures denote the velocity distribution according to the hole number in a microhole array. (a, b) For the 0° slanted microhole array, the flow rate crossing holes gradually decreased in the rearward holes. (c, d) For the 45° slanted microhole array, the flow rate crossing holes was constant over all holes. (e, f) The 90° slanted microhole array showed the similar results of flow rate in the 45° slanted structure.
2.4.2 Simulation for optimizing the flow rate

To optimize flow rates to trap cells firmly and to minimize cell damage, simulations were carried out with flow rates of 5, 10, and 20 µL/h in inlet #1 and inlet #2 with the 45° slanted structure. As the flow rate ratio of inlet #1 to inlet #2 increased, the velocities moving through holes gradually increased. The flow rate ratio of 1:1 resulted in the best results to produce similar flow velocities in each hole. The flow rates of 5 and 10 µL/h were too slow to trap cells and cell debris was caught in the holes because of the faster movement of debris compared to that of cells. From this simulation and experimental results, we determined the flow rate as 20 µL/h in each inlet for stable cell trapping (Fig. 2.7a). In addition, to optimize the flow rate to absorb drugs passively after cell trapping, simulations on pressure differences between the two channels were performed without the microhole array for cell trapping but with the same parameters as used for cell trapping. At an inlet #1 flow rate of 20 µL/h, the difference in pressure between the two channels was too large. Although the pressure difference was negligible for a flow rate of 5 µL/h, we selected 10 µL/h because it was constant over all trapping holes and to obtain sufficient amounts of samples for HPLC analysis over 2 h (Fig. 2.7b). The flow was monitored using FITC-dextran before (a) and after (b) cell trapping in Fig. 2.7.
Fig. 2.7. The simulated and experimented fluid flow changes before and after cell trapping in the 45° slanted microhole array structures. (a) Before cell trapping, due to the slanted structure, the solution injected from the region A flows to the FITC-dextran injected region B, which therefore prevented the leakage of FITC-dextran to the region A. (b) After trapping, the FITC-dextran contacts evenly all over the holes and therefore each cell could absorb drugs under the similar drug concentration environment.
2.4.3 Permeability assay with FITC-dextran

To demonstrate the working principle of the microfluidic device using trapped cells in a microhole array, FITC-dextran was used to verify the absorption of drugs through the trapped cells. FITC-dextran is a widely used tracer in microcirculatory systems and \textit{in vitro} or \textit{in vivo} permeability studies because it is absorbed through epithelial cells and can be readily visualized under high-resolution fluorescence microscopy [21,22]. Caco-2 cells and FITC-dextran were injected into inlet #1 and inlet #2, respectively. FITC-dextran was absorbed and transported by the trapped Caco-2 cells, and the fluorescence intensity of FITC-dextran in the pressure-driven mixing channel was measured. The intensity of the absorbed FITC-dextran increased gradually over time and was saturated after 2 h as shown in Fig. 2.3. If exceptional leakage occurred on the microhole, medium fluid flowed from the region (1) to region (2) and the concentration of absorbed FITC-dextran in the mixing channel decreased. Therefore, we could notice whether microholes have leakage or not. Whenever we perform experiments, we controlled the flow rate for diminishing the leakage by the cell lysis.
Fig. 2.8. Fluorescence intensity of the absorbed FITC-dextran ($n = 3$). The intensity of absorbed FITC-dextran by the trapped Caco-2 cells increased gradually over time and became saturated after 2 h. The intensity of FITC-dextran in the mixing channel was measured under a fluorescence microscope.
2.4.4 Morphology of trapped cells and the viability test

During the assay, the lysis of trapped cells on the microhole array would reduce drug transport and also open holes causing drug leakage, consequently affecting the drug absorption rate. The key factors for controlling cell trapping are the flow rate and cell density in a microchannel. Therefore, it is possible to form single-layered cells or multi-layered cells by controlling these factors. When cells with a density of $1 \times 10^7$ cells/mL were supplied at a flow rate of 20 μL/h, cells were trapped on the microhole array with a single layer. However, a higher cell density or slower flow rate frequently resulted in cell stacking on the microholes. In this study, we precisely controlled the flow rate and cell density to form a single layer throughout our experiments. At an optimal flow rates, cells were trapped in the microhole array and observed as shown in Figure 4a. From the magnified photograph of the trapping hole, it seems that Caco-2 cells closed the holes in a single layer. To ensure cells were viable during the assay, we observed the cell viability for 3 h using Hoechst 33342 and propidium iodide (PI), which stain the nuclei of viable and dead cells blue and red, respectively. As shown in Figure 4b, most cells were viable for 2 h and the effect of dead cells would be negligible, but the number of lysed cells increased after 3 h under the influence of fluid flow. As drug absorption was saturated within 2 h (Fig. 2.9), we measured drug permeability within 2 h to exclude
measurement errors caused by the dead cells. This device can also be used for forming multilayered structure of different cells in a microfluidic device if at least two types of cells are supplied for trapping purpose. However, more experimental studies including device modification are required for forming the multiple layers of different cells on the microhole array.
Fig. 2.9. Trapped cell morphology and cell viability over time during the drug absorption assay. (a) The trapped cells on the microhole array in a single layer without lysis. (b) The Caco-2 cells trapped in the holes were stained with Hoechst 33342 and propidium iodide (PI). Viable cells were stained blue by Hoechst 33342 (upper row) and dead cells were stained red by PI (lower row).
2.4.5 Drug permeability assay

We analyzed ten well-known drugs using our microfluidic device and compared the data to the *in vivo* findings in humans and rats reported in the literature. The selected drugs are commonly used as, for example, anti-hypolipidemic agents, antiulcer agents, or diuretics. A more reliable prediction of drug permeability would be obtained using a larger number of model drugs with a broad range of physicochemical properties, *i.e.*, both high-permeability drugs, such as propranolol, naproxen, and antipyrine, and low-permeability drugs, such as cimetidine, atenolol, and furosemide. Ten drugs absorbed on the microfluidic device were analyzed by reverse-phase HPLC according to the reported experimental conditions [1,10,13,23-27]. The effective permeability, \( P_{\text{eff}} \) (cm/s), of drugs was calculated by eq 4 and shown in Table 1 [1,28-31]. The *in vivo* permeabilities in the human and rat intestine showed correlations with those measured by the microfluidic device (\( R^2 = 0.9013 \) and \( R^2 = 0.8765 \), respectively). As shown in Figure 5, the permeability of each drug was plotted with a 95% prediction interval. In particular, the \( P_{\text{eff}} \) values measured on the microfluidic device were similar with those measured in the human intestine with difference of 0.96 fold but, it was different from those measured in the rat intestine with 8 fold. The difference in the *in vivo* permeability values between human and rat is caused by the difference of effective absorptive area of the
perfused segment, permeation pathway of species, and lipid content in cellular membrane. Additionally, the diffusion coefficient and diffusion distance are also the causes for the permeability difference [32,33]. Some outlying values in the permeability correlation may be attributable to the differential effects of efflux or absorptive mechanisms among human, rat, and trapped Caco-2 cells [23]. In the case of high-permeability drugs with human $P_{\text{eff}}$ greater than $8 \times 10^{-4}$ cm/s, such as naproxen, and/or low-permeability drugs with human $P_{\text{eff}}$ less than $4 \times 10^{-4}$ cm/s, such as furosemide, the correlations were higher than for intermediate-permeability drugs with human $P_{\text{eff}}$ between $5 \times 10^{-4}$ and $7 \times 10^{-4}$ cm/s, such as verapamil, antipyrine, and carbamazepine. As the intermediate-permeability drugs are affected by solubility, the permeability of drugs can vary with absorptive conditions, such as the solvent, reaction process, and fluid flow [34]. Note that $P_{\text{eff}}$ on the microfluidic device showed a stronger correlation with that in the human intestine compared to that in the rat intestine.

In addition, we carried out $t$-test to check whether the measured permeability on our device is statistically meaningful or not, compared to that of $\textit{in vivo}$ data. As shown in Figure 6, most drugs, including naproxen, propranolol and furosemide, had a high $p$ value ($p > 0.4$) and only a few drug such as antipyrine had a low $p$ value less than 0.05. Their measured permeabilities are not the
same as those in the *in vivo* human intestine, but as shown in Figure 5, they are still highly correlated with *in vivo* permeabilities. Therefore, our device is able to produce a significant value compared to the permeability values of *in vivo* data, and to measure *in vivo* permeability.
Table 1. HPLC condition for measuring drug concentration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mobile phase</th>
<th>Wave length (nm)</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>55%(v/v) methanol, 45%(v/v) KH$_2$PO$_4$ 0.05 M (adjusted to pH 6) and 0.2%(v/v) triethylamine</td>
<td>227</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>19.9%(v/v) methanol, 27.9%(v/v) acetonitrile,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naproxen</td>
<td>51.8%(v/v) water and 0.4%(v/v) triethylamine (adjusted to pH 3.2)</td>
<td>280</td>
<td>23.3</td>
</tr>
<tr>
<td>Furosemide</td>
<td>42%(v/v) acetonitrile, 58%(v/v) water, 0.9%(v/v)</td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>glacial acetic acid and 0.1% triethylamine (adjusted to pH 5.6)</td>
<td>270</td>
<td>3.0</td>
</tr>
<tr>
<td>Verapamil</td>
<td>40% acetate buffer 10 mM (pH 5.0 with glacial acetic acid), 60% methanol</td>
<td>230</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>10%(v/v) acetonitrile, 90%(v/v) phosphate buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>0.67 M (pH=7.4) and 0.2%(v/v) triethylamine (adjusted to pH 3)</td>
<td>225</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>39%(v/v) acetonitrile, 61%(v/v) sodium acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piroxicam</td>
<td>0.1 M and 0.05%(v/v) triethylamine (adjusted to pH 8)</td>
<td>330</td>
<td>5.1</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>42%(v/v) acetonitrile, 58%(v/v) water, 0.9%(v/v) glacial acetic acid and 0.1%(v/v) triethylamine</td>
<td>270</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>(adjusted to pH 5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>78%(v/v) KH$_2$PO$_4$ 0.05 M, 22%(v/v) acetonitrile and 0.05%(v/v) triethylamine (adjusted to pH 8)</td>
<td>229</td>
<td>9.3</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>67%(v/v) methanol, 33%(v/v) water and 1% glacial acetic acid</td>
<td>230</td>
<td>8.0</td>
</tr>
</tbody>
</table>
Table 2. The effective permeability ($P_{eff}$) on the microfluidic device, human $P_{eff}$, rat $P_{eff}$, and $F_a$ values of tested drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Device $P_{eff}$ (10^{-4}) (cm/s)</th>
<th>Human $P_{eff}$ (10^{-4}) (cm/s)</th>
<th>Rat $P_{eff}$ (10^{-5}) (cm/s)</th>
<th>$F_a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>3.9 ± 1.7</td>
<td>2.9 ± 1.3 [23,35]</td>
<td>5.6 ± 2.0 [1]</td>
<td>100 [23]</td>
</tr>
<tr>
<td>Naproxen</td>
<td>10.9 ± 4.1</td>
<td>10.0 ± 4.7 [36]</td>
<td>11.9 ± 1.2 [23]</td>
<td>100 [23]</td>
</tr>
<tr>
<td>Furosemide</td>
<td>1.1 ± 0.3</td>
<td>0.3 ± 0.31</td>
<td>3.3 ± 2.0 [1]</td>
<td>61 [23]</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>9.5</td>
<td>5.6 ± 1.6 [36]</td>
<td>5.9 ± 0.2 [1]</td>
<td>100 [23]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>5.2 ± 0.1</td>
<td>6.70 ± 2.90 [23]</td>
<td>6.5 ± 0.5 [23]</td>
<td>100 [23]</td>
</tr>
<tr>
<td>Atenolol</td>
<td>0.6 ± 0.1</td>
<td>0.12 ± 0.20 [36]</td>
<td>0.60 ± 0.60 [23]</td>
<td>50 [23]</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>8.0</td>
<td>6.71</td>
<td>7.9 ± 4.0 [1]</td>
<td>100 [23]</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>2.7 ± 1.6</td>
<td>0.04 ± 0.05 [23]</td>
<td>2.0 ± 1.0 [1]</td>
<td>67 [23]</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>0.2</td>
<td>0.30 ± 0.05 [23]</td>
<td>4.8 ± 0.1 [1]</td>
<td>60 [23]</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.5</td>
<td>4.3 ± 2.7 [23]</td>
<td>6.2 ± 0.6</td>
<td>97 [1]</td>
</tr>
</tbody>
</table>
Fig. 2.10. Comparison of drug permeability measured on the microfluidic device vs. the human and rat intestine. (a) Comparison of drug permeability assayed on the microfluidic device vs. the human intestine. (b) Comparison of permeability measured on the microfluidic device vs. the rat intestine.
Fig. 2.11. Comparison of the effective permeability ($P_{\text{eff}}$) on the device and \textit{in vivo} human. *$p$ values from $t$-test are larger than 0.05 ($n \geq 3$).
2.4.6 Correlation with $F_a$ and $P_{eff}$

We compared the fraction of the dose absorbed in the human intestine ($F_a$) with the $P_{eff}$ measured on the microfluidic device because $F_a$ represents the actual drug absorption in the human intestine. As shown in Figure 7, the permeability measured on the microfluidic device and $F_a$ were significantly correlated [1]:

$$F_a \text{ (human intestine)} = 1 - e^{-1.0634P_{eff} \text{ (device)}}.$$ (5)

The obtained $P_{eff}$ on the microfluidic device showed a high correlation ($R^2 = 0.9641$) with human $F_a$ [1,30]. In the case of low-permeability drugs with $F_a$ below 70%, the $P_{eff}$ on the microfluidic device was inconsistent with $F_a$ because of differences in the effective absorptive area and mechanism of absorption. In highly permeable drugs of more than 90% $F_a$, the $P_{eff}$ values were closely correlated with $F_a$ because such drugs can be rapidly absorbed and saturated to a steady-state over the differences in the absorption mechanism between the human intestine and trapped cells on the microfluidic device. Similar to the strong correlation between in vivo human $P_{eff}$ and $P_{eff}$ on the microfluidic device, the strong correlation between $F_a$ and $P_{eff}$ on the microfluidic device indicated that this device can be used to replace conventional permeability assays by predicting human permeability more precisely.
Fig. 2.12. Plot of the effective permeability ($P_{\text{eff}}$) on the device vs. the fraction of dose absorbed in the human intestine ($F_a$).
2.5 Conclusion

We developed an efficient microfluidic device for performing permeability assays. The microfluidic structure and its experimental conditions were optimized by mathematical simulations and the results were experimentally validated. As trapped cells were viable without cell lysis on the microhole array, FITC-dextran was used to verify the absorption of drugs through trapped cells; the intensity of FITC-dextran was saturated after 2 h. Based on these experiments, we performed the permeability assay with ten drugs. The trapped cells were viable after drug absorption and the permeability coefficient between human in vivo and $P_{eff}$ on the microfluidic device was related significantly. These results indicated that our drug permeability assay device will be useful in predicting the actual human permeability values of drugs. In addition, our permeability assay system reduces the assay time as no cell culture on the microfluidic device is required and no complex structure, such as cellular membrane, is needed. Using trapped cells mimicking the intestinal epithelial cells, the integrated system including toxicity assay can be used as a valuable tool in drug discovery and its applicability will be extended to include ADME/Tox drug properties [37-40]. However, since single cells are trapped in each microhole for a short period, no tight cell junction is formed even if Caco-
2 cells mimic *in vivo* environments. Consequently, the condition on our device is not exactly the same as that of *in vivo*, and our device could not measure the drug absorption through the cell junctions. However, a total of 95% drugs are transported passively or actively in aid of transport proteins and only 5% drugs are transported by paracellular pathways through the cell junctions. In this sense, our strategy can be applied to the drugs absorbed passively or actively, which covers 95% of drugs available.
References


CHAPTER III: Drug Permeability Assay Based on

Blood Brain Barrier Model

3.1 Introduction ............................................................... 58
3.2 Experimental ............................................................ 63
3.3 Results and discussion ............................................... 70
3.4 Conclusion ............................................................... 90
References ................................................................. 91
3.1 Introduction

Transport of drugs from blood vessel to brain tissue is limited by the brain capillary endothelial wall, named blood brain barrier (BBB) [1]. The BBB is composed of specialized microvascular endothelium and glial cell elements, including astrocytes and microglia which are in physical proximity to the endothelium and a basement membrane. The physicochemical properties of the barrier are essential for determining the membrane permeability of the brain and for maintaining a precisely regulated microenvironment for reliable neuronal signaling [2-4]. Accurate assays for measuring permeability of drug candidates across the BBB is therefore an important step in pharmaceutical industry [5]. In vivo models, which have been developed in previous, provide reliable approaches for determination of the BBB permeability of drugs [5, 6]. However, such the approaches require frequent human intervention, skilled operation, and high cost.

To overcome those limitations of in vivo models, in vitro techniques for determining the BBB permeability or intestinal permeability of potential drug candidates have been developed [5]. The in vitro approaches provide several advantages over the in vivo models; they require a small amount of compounds and a few living animal for evaluation, and enable to build high-throughput
methodologies due to its relatively short assay time and easy experimental handling [7].

In the in vitro techniques, when endothelial cells are isolated from capillaries, they lose BBB characteristics quickly when cultured alone as the interactions between brain endothelium and astrocytes within neurovascular units can influence BBB under both physiological and pathological conditions. Among the various neurovascular cell types, astrocytes are essential for proper neuronal functioning and for the close proximity of neuronal cell bodies to brain capillaries. Also, astrocytes can up-regulate many BBB features leading to dense tight junctions (physical barrier), increased expression of transporters including permeability glycoprotein (P-glycoprotein, abbreviated as Pgp) and GLUT1 (transport barrier), and enhanced enzyme system (metabolic barrier) [3]. For these reasons, a great number of conventional in vitro BBB models are composed of co-cultured brain endothelial cells and astrocytes (Fig. 3.1). Claudia et al. showed that co-culture of endothelial cells and astrocytes resulted in a significant increase in endothelium occluding levels and junctional localization. Also, γ-glutamyl-transferase activity of endothelial cells was elevated by direct contact with astrocytes or by astrocyte conditioned medium (ACM) [8]. Stefan et al. compared two co-cultured in vitro models: brain
capillary endothelial cells and astrocytes mimicking BBB, and a human colon carcinoma cell line (Caco-2) behaving intestinal epithelium. The in vitro model of BBB had a strong correlation with in vivo BBB, while Caco-2 did not [5].

Venkatraman et al. suggested that human brain endothelial cells can interact with astrocytes and are also affected by the shear stress caused by blood flow. They constructed the flow-based in vitro BBB model using astrocytes and ACM, and showed an increased expression of zonular occludens (ZO-1) by co-cultivation with ACM or by the shear stress [4]. These membrane based assays are commonly used for in vitro BBB models so far, but those are lacking certain attributes that would permit a high degree of direct contact between astrocytes and endothelial cells cultured on opposing sides. Shuler et al. presented the co-culture model of endothelial cells and astrocytes on the silicon nitride membrane, and showed that the highly porous membrane allowed an increased direct interaction between endothelial cells and astrocytes and resulted in better assay results [9].

In this study, we developed a permeability assay system using human umbilical vein endothelial cells (HUVECs) trapped in microholes, with or without ACM in a microfluidic device. Using trapped HUVECs and ACM, we performed the brain permeability assay at an in vivo-like microenvironment,
and investigated the effects on shear stress by fluid flow and the interaction between HUVECs and ACM. Five widely used drugs, such as propranolol, antipyrine, carbamazepin, verapamil, and atenolol, were analyzed for measuring the BBB permeability.
Fig. 3.1. Conventional brain permeability assays.
3.2 Experimental

3.2.1 Design of a microfluidic device based on the BBB model

The BBB is a structural and functional barrier that regulates the passage of molecules into and out of the brain to maintain the neural microenvironment [4]. The physiological properties of BBB are important factors to design brain-targeted drugs. As shown in Fig. 3.2, the BBB is formed by the endothelial cells composing the cerebral capillaries together with perivascular elements such as closely associated astrocytic end-feet, perivascular neurons and pericytes. There are two major routes permeating the endothelial cells in BBB; transcellular and paracellular pathways. Transcellular pathway includes conventional passive, active, and facilitated transports while paracellular pathway is used to transport through tight junction between cells.

To perform the brain permeability assay under an *in vivo*-like microenvironment, we exploited a microchannel design for inducing a shear stress by fluid flow as well as providing an interaction between HUVECs and ACM. Fig. 3.3 shows the microfluidic device mimicking the BBB model for more accurate brain permeability assays. Under this design, cell trapping and drug permeability assay can be carried out in a rapid and easy way as
previously reported [10]. Briefly, HUVECs are trapped in the microholes due to the pressure difference between each side of microhole array, which is resulted from the different flow rate in the microfluidic channels. After cell trapping, ACM is constantly supplied to the cells through the microfluidic channel to realize an \textit{in vivo}-like environment and to verify the effect of ACM on the permeability of HUVECs. The ACM affects HUVECs to induce rapid tight junction formation [11,12], high expression of Pgp [13,14], and alteration of expression of the membrane receptors [3]. In addition, the trapped HUVECs are exposed to the shear stress due to drug solution and media flowing in each microfluidic channel, as \textit{in vivo} environment, where blood flow influence to the HUVECs. As a consequence, drugs can be transported through various transport routes, including passive transcellular pathway, active transcellular pathway by Pgp, and paracellular transport pathway by tight junction between cells.
Fig. 3.2. Schematic illustration of a BBB model and a cross section view of BBB with an enlarged view of the transport routes across BBB.
**Fig. 3.3.** Design of microfluidic device mimicking the BBB model and diverse drug transport routes across HUVECs trapped in microholes.
3.2.2 Cell culture

Primary HUVECs (ScienCell, Gaithersburg, MD) and primary human astrocytes (ScienCell) were selected for the BBB permeability assay because those cells are widely used for cell lines sustaining the properties of endothelial cells. Cell layers cultured on Petri dishes were rinsed with phosphate-buffered saline (PBS) at pH 7.4 (Gibco, Grand Island, NY). Then, trypsin-EDTA solution (0.25% trypsin and 1 mM EDTA·4Na; Gibco) was used to detach the cells. EBM-2 (Lonza, Wakersville, MD) and Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) was added to the dispersed cell layer of HUVECs and astrocytes, respectively. The cell cultures were maintained at 37°C under 5% CO\(_2\) in a humidified water-jacketed incubator [10]. We obtained ACM by cultivating astrocytes for five days. Prior to the permeability assay appropriate aliquots of the HUVECs were refreshed and diluted to a suitable concentration of 2.5 × 10\(^6\) cells/mL. Drug-treated cells and reagents after the experiments were completely autoclaved before discharging to ensure safety according to the KAIST experimental protocols for biosafety.
3.2.3 Experimental setup

The microfluidic devices were sterilized and bubbles within channels were eliminated with 70% ethanol for 10 min, followed by rinsing with cell culture media. HUVECs and astrocytes were incubated in the culture dishes, and then 100 µL of $1 \times 10^7$ cells/mL of HUVECS was injected into inlet #1 of the microfluidic device. After trapping the HUVECs on microholes, 100 µL of ACM from astrocytes cultured for five days was also injected into inlet #1. The drugs were injected into inlet #2 (Fig. 3.4).

3.2.4 Immunofluorescent staining of cell junction protein

Primary HUVECs grown on cover slips were fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were washed three times with PBS for 5 min each. Cells were then permeabilized with 0.15% Triton X-100 for 15 min. Then, the cells were blocked for 1 h in a PBS solution containing 3% BSA (Sigma–Aldrich). After then, the cells were stained with Alexa fluor® 594 tagged ZO-1 which was conjugated mouse monoclonal antibody (Molecular Probes, Eugene, OR) diluted 1:50 and incubated for 2 h at room temperature [4, 12]. The cover slips with the cells were again washed three times in PBS before
being mounted onto the stage of a confocal microscope (Axiovert25, Carl Zeiss, Germany)
3.3 Results and discussion

3.3.1 Device configuration and trapped cells

In this study, we designed a new microfluidic permeability assay system mimicking the in vivo BBB model. Conventional permeability assay systems require embedded membrane structures for culturing epithelial cells or mimicking cell membrane. Instead, we used microhole structure for trapping HUVECs and measured the permeabilities of drugs absorbed through the trapped HUVECs, with or without ACM. As shown in Fig. 3.4, the microfluidic device includes two inlet parts, two outlet parts, and a microhole array for cell trapping. HUVECs from inlet #1 are supplied to outlet #1 at a flow rate of 20 µL/h while fresh buffer from inlet #2 are supplied to outlet #2 at a flow rate of 20 µL/h. Due to the difference of pressure in the microholes, the injected cells are automatically trapped in the microholes. After the cell trapping, fresh medium without any cells or ACM from inlet #1 is constantly supplied to outlet #1 and drugs to be assayed are injected into inlet #2. The drugs are then absorbed by the trapped HUVECs and transported to the region (1) from the region (2) of the channel and the concentrations of absorbed drugs are monitored at outlet #1.
It was demonstrated that glial cell-derived soluble factors restrict the passage of albumin through endothelial cell layers after 2 h and there was the difference of BSA diffusion with or without glial cell [17,18]. L-glucose permeability and potassium permeability were also lower when endothelial cells and astrocytes were co-cultured than those cells were mono-cultured after 2 h [18,19]. For these reasons, two hours are sufficient to form tight junction between HUVECs affected by ACM. Moreover, because the HUVECs begin to migrate to region (2) after 2 h as shown in Fig. 2b, we measure the permeability value after 2 h.
Fig. 3.4 Schematics of the microfluidic device and the morphology of HUVECs trapped in the microholes with time. (a) Suspended HUVECs in culture media are injected into inlet #1 (red flow) and trapped in the microhole array. Then, ACM is injected into inlet #1 and drugs are injected into inlet #2 (blue flow), and drugs are absorbed by the trapped cells through the holes. (b) HUVECs trapped in the microholes are tightly contact with each other, but after 2 h, trapped HUVECs show the migration.
3.3.2 The expression of tight junction protein in HUVEC with/without ACM

Tight junction is a specialized cell-cell interaction that is found in almost all types of epithelial cells [12]. Previous studies suggest that permeability of endothelial and epithelial cells is regulated by components of tight and adherent junction proteins, which include ZO-1, occludin, and claudin [4]. In particular, ZO-1 is a protein participating in the specialized cell-cell interaction characterized by membrane junction between epithelial cells. However, the chemical nature of the glial cell-produced signals in induction and maintenance of the barrier properties of HUVECs is unclear. Several candidate molecules have been identified, such as tumor growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF), which can upregulate the barrier properties by increasing the resistance and decreasing the paracellular permeability in brain endothelial cells [4]. Thus, we examined the change of ZO-1 protein in HUVECs and HUVECs supplemented ACM. We found that ZO-1 expression of HUVECs was increased in the presence of ACM overall plasma membrane of HUVECs and particularly the region of cultured together with cells represent the highly expressed ZO-1 protein, as the arrows indicated (Fig. 3.5). As shown in Fig. 3.6, we confirmed the formation of tight junction protein between
HUVECs trapped on the microhole array after injecting ACM for 2 h. ZO-1 protein was more strongly expressed in the junction between HUVECs than in the surface of HUVECs.
Fig. 3.5. The expression of ZO-1 in (a) HUVECs and (b) HUVECs supplemented ACM.
Fig. 3.6. The expression of ZO-1 of cells trapped on the microhole array in a microfluidic device (a) stained tight junction of HUVECs trapped on the microhole array and (b) the magnified image of stained tight junction between HUVECs. Arrows indicate the stained region.
3.3.3 The formation of blood brain barrier in a microfluidic device

For verify the blood brain barrier in a microfluidic device, the concentration of Evans-blue dye absorbed by HUVECs was observed in HUVEC with or without ACM. Evans blue is used to assess the permeability of the blood-brain barrier to molecules. As shown in Fig. 3.7, the absorbed concentration of Evans-blue dye was decreased in HUVECs with ACM more than HUVECs without ACM because ACM increased the formation of the blood brain barrier and the BBB model is accomplished in microfluidic device.

As shown in Fig. 3.8, we used the FITC-dextran with a various molecular weights for verifying the blood brain barrier. As the results about Evans-blue dye, the absorbed concentration of FITC-dextran was also decreased in HUVECs with ACM more than HUVECs without ACM. Moreover, in the molecular weight of less than 40 kDa which is absorbed by blood brain barrier, the concentration of absorbed FITC-dextran was high, but in the molecular weight of 70 kDa, almost of FITC-dextran was not absorbed by HUVECs, both case of with and without ACM. It shows that the blood brain barrier was formed in a microfluidic device successfully.
Fig. 3.7. The absorbed concentration of Evans-blue dye. (a) HUVECs and (b) HUVECs supplemented ACM.
Fig. 3.8. The absorbed concentration of FITC-dextran with a various molecular weights.
3.3.4 Drug permeability assay of HUVECs with/without ACM

A recent study showed that contact co-cultivation of astrocytes helps endothelial cells maintain the BBB properties by upregulating Pgp on the endothelial cells. In contrast, non-contact co-cultivation of astrocytes has been shown to enhance the transendothelial resistance and decrease the transendothelial permeability in the *in vitro* model of BBB [4,7,8]. Astrocytes do not make a direct contact with endothelial cells *in vivo*, but rather, are separated from endothelial cells by an extracellular matrix. These suggest that the influence of astrocytes may be mediated by a secreted factor. The conditioned medium derived from astrocytes has been shown to be capable of maintaining the BBB characteristics, suggesting that an astrocyte-derived soluble factor is responsible for the endothelial cells to develop a BBB property [4]. Therefore, we observe the difference of the permeability value between only trapped HUVECs and when supplied ACM.

We analyzed five well-known drugs using our microfluidic device and compared their permeability values on HUVECs with/without ACM. The selected drugs are commonly used as, for example, anti-hypolipidemic agents, anti-ulcer agents, or diuretics. A more reliable prediction of drug permeability would be obtained using a larger number of model drugs with a broad range of
physicochemical properties, *i.e.*, both high-permeability drugs, such as propranolol and antipyrine, and low-permeability drugs, such as carbamazepin and atenolol. Five drugs absorbed on the microfluidic device were analyzed by reverse-phase HPLC according to the previously reported experimental conditions [20].

As shown in Fig. 3.9, the permeability value was decreased when ACM was supplemented to the microfluidic channel. In most drugs such as carbamazepin, verapamil, and atenolol, the presence of ACM decreased the permeability by 30% when compared to HUVECs alone. However, in case of propranolol, the difference of permeability is not clear. This is derived that propranolol is a highly permeable drug and the permeability value is also high despite ACM supplied to the trapped HUVECs. Antipyrine shows the higher variation than the other drugs and shows some difference of the permeability value between HUVECs and HUVECS supplied ACM. The decreased permeability value when cultured HUVECs with ACM shows that that astrocyte-derived soluble factor of ACM is responsible for the endothelial cells to develop a BBB property and it is certified that this microfluidic device is able to measure the permeability value in an *in vivo*-like environment when HUVECs cultured with ACM.
In addition, we carried out t-test to check whether the measured permeability on HUVECs is different or not, compared to that supplied ACM. As shown in Fig. 3.10, most drugs, including propranolol, carbamazepin, verapamil, and atenolol, had a low $p$ value ($p < 0.02$) and only antipyrine had a high $p$ value more than 0.05. In case of antipyrine, their measured permeabilities on HUVECs are not different as those on HUVECs supplied ACM. However, the average of HUVEC and HUVECs supplied ACM is certainly different.
Fig. 3.9. Comparison of permeability value between only HUVECs and HUVECs supplemented ACM ($n = 3$).
Fig. 3.10. Comparison of the effective permeability ($P_e$) on the device using HUVECs with/without ACM. *$p$ values from $t$-test are smaller than 0.05 ($n \geq 3$).
3.3.5 Comparison of the effective permeability on the device with that of *in vitro* BBB model

The effective permeability, $P_e$ (cm/s), of drugs was calculated by eq 4. As shown in Table 1 [1-4], the $P_e$ values measured on the microfluidic device were similar with those measured in the *in vitro* BBB. In the case of propranolol which is a high-permeability drug and atenolol which is a low-permeability drug, the permeability values on the device are different with 2 fold of those of *in vitro* BBB. In previous work, we performed the permeability assay in the microfluidic device using Caco-2 cells and obtained the permeability value which is highly correlated with *in vivo* epithelial cell permeability. Therefore, we compared the permeability on this device with those of Caco-2 cells [20]. The $P_e$ on the device is considerably different from the $P_{eff}$ of Caco-2 cells, especially in propranolol, verapamil, and atenolol with difference of ten folds. We correlate the $P_e$ on the device with $P_e$ of *in vitro* BBB and brain uptake index (BUI) for verifying the relationship between two cases, and confirming the usefulness of this device for predicting the *in vivo*-like brain permeability using the BBB model. As shown in Figure 3.11, the $P_e$ on the device are compared with the $P_e$ of *in vitro* BBB model and shows a strong correlation with those two of $P_e$ ($R^2 = 0.9982$).
Table 3. Comparison among $P_e$ on the microfluidic device, $P_e$ of \textit{in vitro} BBB, brain uptake index (BUI), and $P_{eff}$ of Caco-2 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$P_e$ on the device (cm/s)</th>
<th>$P_e$ of \textit{in vitro} BBB (cm/s)</th>
<th>BUI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HUVEC+ACM (cm/s)</td>
<td>HUVEC (cm/s)</td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>4.21e-3 ± 0.16e-3</td>
<td>4.77e-3 ± 0.23e-3</td>
<td>1.9872e-3 [7]</td>
</tr>
<tr>
<td>Carbamazepin</td>
<td>1.69e-4 ± 0.19e-4</td>
<td>5.67e-4 ± 0.31e-4</td>
<td>1.9853e-4 [7]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>2.66e-5 ± 0.02e-5</td>
<td>6.73e-5 ± 0.08e-5</td>
<td>2.3510e-5 [7]</td>
</tr>
<tr>
<td>Atenolol</td>
<td>2.48e-6 ± 0.22e-6</td>
<td>5.35e-6 ± 0.07e-6</td>
<td>2.4900e-6 [7]</td>
</tr>
</tbody>
</table>
Fig. 3.11. Comparison of drug permeability measured on the microfluidic device vs. in vitro BBB model.
3.3.6 Correlation with brain uptake index (BUI) and \( P_e \) on the device

We compared the BUI with the \( P_e \) measured on the microfluidic device because BUI represents the actual drug absorption in the BBB model as follows:

\[
BUI = \frac{brain^{14}C/brain^{3}H \times 100}{Injected^{14}C/Injected^{3}H}
\]

(5)

As shown in Fig. 3.12 the permeability measured on the microfluidic device and BUI were significantly correlated[5]. The obtained \( P_e \) on the device showed a correlation of \( R^2 = 0.8981 \) with BUI. Similar to the strong correlation between \( P_e \) of \textit{in vitro} BBB and \( P_e \) on the device, the correlation between BUI and \( P_e \) on the device indicated that this device can be used to replace conventional permeability assays by predicting the \textit{in vivo} brain permeability more precisely.
Fig. 3.12. Comparison of drug permeability measured on the device vs. BUI.
3.4 Conclusions

We developed a microfluidic device for the permeability assays based on the BBB model. The permeability assays using HUVECs with and without ACM were performed for different five drugs to determine their BBB permeability and the effect of ACM on the permeability of HUVECs. The permeability values, which were measured in our microfluidic device, showed good agreement with those in the *in vitro* BBB experiments, in which HUVECs and astrocytes were co-cultured. In addition, the measured values of the drug permeability were highly correlated with BUI and the ZO-1 expression of HUVECs, which decreased in the presence of ACM. These results indicated that our device for drug permeability assays is applicable to predict the permeability of drugs in a brain. In addition, our permeability assay system reduces the assay time as no cell culture on the microfluidic device is required and no complex structure, such as cellular membrane, is needed. Using trapped HUVECs supplied by ACM mimicking the BBB model, the integrated system including toxicity assay and metabolism assay can be used as a valuable tool in drug discovery and its applicability will be extended to include ADME/Tox drug properties [22-25].
References


CHAPTER IV: Hepatotoxicity Assay using

Hepatocytes Trapped in Microholes

4.1 Introduction .................................................................................. 96
4.2 Experimental.................................................................................. 99
4.3 Results and discussion ................................................................. 107
4.4 Conclusion .................................................................................... 121
References ......................................................................................... 122
4.1 Introduction

The liver is one of the most important organs, particularly in the detoxification and removal of many toxic chemicals. Because the liver is the major site of the metabolism of foreign compounds, it is often also the target organ of various toxins or drugs [1-4]. Understanding biochemical mechanisms and functions of the liver is key in drug discovery. Primary human hepatocytes are the major cell type, comprising 80% of the liver, and are widely used in investigating drug metabolism, the induction of drug-metabolizing enzymes, and cytotoxicity studies of drug candidates [1,3-5]. Consequently, cytotoxicity assays using primary human hepatocytes have accelerated drug development [6].

In particular, hepatotoxicity assays performed to measure drug-induced liver damage can be used in assessing cytotoxicity in drug discovery and development. For the sake of convenience, several hepatoma cell lines, such as HepG2/C3A and HepG2, retaining many biochemical synthesis pathways, are commonly used instead of hepatocytes [7]. Shuler et al. developed a microscale cell culture system using HepG2/C3A cells and demonstrated a toxicity assay for naphthalene [8]. Although HepG2 and HepG2/C3A have been widely used in toxicity assays, these cell lines cannot be used to assay
the hepatotoxicity mediated by some reactive metabolites, because they are
deficient in certain metabolic functions, including cytochrome P450 enzyme
activity. Although some cell lines do retain such enzyme activity, it fluctuates and diminishes over time after several passages [7,9,10].

Thus, there is a continuing need for an efficient and convenient
hepatotoxicity assay system using primary human hepatocytes, retaining the
original properties of *in vivo* hepatic cells. Bhatia *et al* [4] reported a
miniaturized and multi-well culture system for rat hepatocytes with an
optimized microscale architecture that maintained phenotypic function for
several weeks. However, the assay system still required a cell culture
process, lasting from one day to several weeks.

As an alternative, suspended and cryopreserved hepatocytes retaining
most liver-specific functions have been used to assay cytotoxic effects of a
variety of chemicals and pharmaceuticals. Suspended cryopreserved
hepatocytes are a convenient cell source for toxicity studies, because they
have no lag-time between isolation from organs and starting experiments, in
comparison with monolayer cultures that need an attachment period of at
least several hours [1,3,5,11-13]. However, suspended hepatocytes can only
be used for short-term metabolism or cytotoxicity studies, because such
hepatocytes in suspension have a limited life-span, and lose their morphology and liver-specific functions after a short culture period [11,14].

To overcome the short life-span of suspended hepatocytes and to realize an in vivo-like environment with a perfusion system, many research groups have developed microfluidic methods for culturing cells over a long period in microfluidic devices. A multi-layer device was developed for the characterization of drug metabolites and a simultaneous cytotoxicity assay using HepG2 cells [15]. Kane et al.[16] developed an array of primary hepatocytes for use in high-throughput liver toxicity studies, and Lee et al [17]. reported an artificial liver sinusoid structure in a microfluidic device for hepatocyte culture for up to 7 days, although these methods need a long period for the hepatotoxicity assay, over several days. Additionally, Toh et al. developed a technique for the in situ three-dimensional immobilization of primary rat hepatocytes within a localized matrix in a microfluidic channel [18,19]. However, embedding cells in hydrogels introduces operational complexity and potentially hinders mass transfer of hepatic cells; thus, there is a continuing need to develop a gel- or matrix-free cellular assay [20]. We recently developed an efficient microfluidic device for performing permeability assays based on the absorption of drugs through trapped Caco-
2 cells, mimicking intestinal epithelial cells [21].

In this article, we describe a different approach for a hepatotoxicity assay using primary human hepatocytes trapped in microholes of a microfluidic device, which is capable of assaying with low number of hepatocytes in a short period without cell culture. Hepatotoxicity assay results from the microfluidic device were compared with those of *in vitro* MTT assay using human hepatocytes. Details of the experimental results using the microfluidic device are reported.

### 4.2 Experimental

#### 4.2.1 Design of the microfluidic device and principle of the hepatotoxicity assay

Because the liver has a central role in drug metabolism and toxicity, drug-induced liver toxicity is the leading cause of acute liver failure and of post-market drug withdrawals [4]. The liver is composed of various cells, such as Kuppfer cells, epithelial cells, stellate cells, and hepatocytes, and absorbed drugs flowing continuously in blood affect hepatocytes according to the sinusoidal blood flow (Fig. 4.1a). Primary human hepatocytes are
likely to be exposed to shear stress through sinusoidal blood flow in vivo. It has been reported that shear stress by the sinusoidal blood flow has a significant influence on the hepatocyte cultivation in a microfluidic environment [22]. In contrast, static cultivation in a Petri dish may not mimic the in vivo situation for hepatocytes. To mimic the in vivo conditions of blood flow in the liver, we designed a microfluidic device for hepatotoxicity assays using suspended human hepatocytes. The assay procedures and our device are illustrated in Fig. 4.1b. Injected cells are automatically trapped in the microholes due to the difference in pressure caused by different flow rates on the two sides of the microholes. After cell trapping, fresh medium is constantly supplied to the cell side of the microchannel and fluorescent dye or drug solutions to be assayed are injected to the other side. The dye and drug then diffuse through the trapped hepatocytes and induce hepatotoxic effects in the cells. Consequently, the number of primary human hepatocytes trapped in the microholes decreases with time and dead cells become stained by the red fluorescent dye.
Fig. 4.1. Schematics of liver structure and microfluidic sinusoidal blood flow. (a) Schematic of liver and sinusoidal structure. (b) Hepatotoxicity assay process in the microfluidic device.
4.2.2 Device fabrication

The microfluidic device consists of two inlet ports, two outlet ports, and microholes for cell trapping and drug diffusion. Primary human hepatocytes are supplied from inlet #1 to outlet #1 at a flow rate of 20 µL/h, while fresh buffer is supplied from inlet #2 to outlet #2 at a flow rate of 20 µL/h (Fig.4.2a). The microfluidic device was fabricated using a multilayer lithography method. First, the designed patterns for microfluidic channels were printed onto a Cr mask. The mold master for the device was fabricated using a negative photoresist (PR; SU-8 2005; Microlithography Chemical Co., Newton, MA) to manufacture microholes in the device using conventional lithography. The second negative PR (SU-8 2025; Microlithography Chemical Co.) was coated and exposed after aligning with the align mark of the first layer. After the second development, the prepolymer of poly(dimethylsiloxane) (PDMS; Sylgard 184; Dow Corning, Midland, MI) was mixed with a curing agent at a 10:1 mass ratio and poured over the mold masters. Then, the PDMS structure was cured at 65°C for 1 h and peeled from the mold. Consequently, the microholes could be manufactured in a microfluidic device with a 45° angle, as described previously [21].
Because the diameter of hepatocytes is about 10 µm, we set the width of the microhole to 3 µm and the height of the microhole to 5 µm so as to trap cells firmly in the microholes without causing damage (Fig.4.2b). The distance between each microhole was determined by the cell size, 10 µm, to compact cells in a monolayer in the microholes. The length of the microhole connecting channels on both sides was 30 µm so as to minimize cell lysis by fluid flow. The height of the microchannels was 25 µm for the free movement of cells in the microfluidic channels. Because of the height, most animal cells with diameters in the range 5–15 µm could be trapped in the microholes of our device. As shown in Fig. 4.2c, suspended human hepatocytes were trapped in the microholes of our microfluidic device. The trapped human hepatocytes were in tight contact with other cells.
Fig. 4.2. Microfabricated device for hepatotoxicity assays. (a) Schematic illustration of the microfluidic device. (b) Configuration of the microholes. Scanning electron microscope (SEM) image of the cross-section A-A’ shows the dimensions of the microhole.
4.2.3 Cultivation of human hepatocytes

Cryopreserved human hepatocytes (HH5200, ScienCell, Gaithersburg, MD) were selected for the hepatotoxicity assay in the microfluidic device. Cryopreserved human hepatocytes cultured in Petri dishes were rinsed with phosphate-buffered saline (PBS; pH 7.4; Gibco, Grand Island, NY). Then, trypsin-EDTA solution (0.25% trypsin and 1 mM EDTA·4Na; Gibco) was used to detach the cells and hepatocyte medium (HM; Sciencell) supplemented with 25 mL fetal bovine serum (FBS; Sciencell) was added to the dispersed cell layer. The cell cultures were maintained at 37°C under 5% CO$_2$ in a humidified water-jacketed incubator.$^{23}$ Prior to the hepatotoxicity assay using the microfluidic device, appropriate aliquots of the cell suspension were refreshed and diluted to a suitable concentration, 2.5×10$^6$ cells/mL. Drug-treated cells and reagents after the experiments were autoclaved before discharging according to the KAIST experimental protocols for biosafety.

4.2.4 Hepatotoxicity assay

The microfluidic devices were sterilized and bubbles within a channel were eliminated with 70% ethanol for 10 min, followed by rinsing with cell culture media. Human hepatocytes were incubated in the culture dishes, and
then 100 μL of $1 \times 10^7$ cells/mL was injected into inlet #1 of the microfluidic device and 100 μL of drugs (Sigma-Aldrich, Seoul, Korea) was also injected into inlet #2 (Fig. 4.2a). The live or dead cells after drug treatment were counted over time and the live cell fraction was calculated from counting the dead cells stained by propidium iodide (PI) among total cells trapped in the microholes of the microfluidic device.
4.3 Results and Discussion

4.3.1 Primary human hepatocytes trapped in the microholes of a microfluidic device

This hepatotoxicity assay using suspended hepatocytes has the advantage of reducing the time required for sample preparation, including cell plating, cell scraping, and incubation, which can be performed in only 4–6 h in suspension, versus 20 h in primary culture [24]. For this reason, suspended hepatocytes are widely used as a useful cellular model during the development of new drugs and in the investigation of metabolic or toxic effects of drugs [25]. However, because suspended hepatocytes do not sustain their properties for a long time, it is difficult to obtain a large quantity of them. Thus, it is valuable to be able to perform a hepatotoxicity assay within a short period of time, without cell culture, and with a small number of human hepatocytes in a microfluidic device, which provides an in vivo-like microenvironment.

For an accurate hepatotoxicity assay using a small quantity of suspended hepatocytes in the microfluidic device, first, cells should be trapped in the microholes firmly during the assay because cells flowing away interfere with
accurate counting of dead or viable cells. Second, cells should be trapped in the microholes in a monolayer; otherwise, it is difficult to conclude whether viable cells were resistant to a toxic material or simply that chemicals did not reach the cells. Third, drugs should not diffuse through open holes, which result from lysis of the cells trapped in the holes; otherwise, it is likely that the cells need to be treated with higher concentration of drugs.

The key factors in controlling cell trapping are the flow rate and cell density in the microchannels. Thus, it is possible to form single-layered cells or multi-layered cells by controlling these factors. When cells at a density of $1 \times 10^7$ cells/mL were supplied at a flow rate of 20 μL/h, cells were trapped on the microholes in a single layer. However, a higher cell density or slower flow rate frequently resulted in cell stacking on the microholes. In this study, we precisely controlled the flow rate and cell density to form a single layer throughout our experiments; a more detailed explanation about the optimal conditions for cell trapping was provided in a previous paper [21]. To determine whether cells were viable during the assay, we observed cell viability initially using calcein AM and beyond 3 h using PI, which stain the cytosol of viable cells and the nucleus of dead cells with green and red fluorescence, respectively. As shown in Fig. 4.3, dead hepatocytes increased
over time after treatment with 5 mM acetaminophen. The cells resembled an *in vivo*-like cell layer because the suspended hepatocytes were in tight contact with each other on the microholes, which is not seen in conventional hepatotoxicity assays using single cells.
Fig. 4.3. Viability of primary human hepatocytes trapped in microholes with time after treatment with 5 mM acetaminophen.
4.3.2 Drug sensitivity of human hepatocytes and HepG2

Primary human hepatocytes have physiologically relevant properties for the evaluation of the liver-related human drug properties, such as in vivo hepatotoxicity and metabolic activity. However, primary human hepatocytes are difficult to culture and lose their metabolic properties within a short time. For this reason, hepatoma cell line, including HepG2 and HepG2/C3A, are generally used in drug toxicity studies, because HepG2 cells are relatively easy to handle and do not require expensive growth factors in their medium. However, the hepatoma cell lines have lost various drug-metabolizing enzyme activities and genotype stability decreases over time [3]. Compared with primary human hepatocytes, moreover, the hepatoma cell lines have different properties with regard to the expression of CYP450 and cytotoxic effect on cells. For example, HepG2 cells are less sensitive to diclofenac than primary cultures of hepatocytes [7]. To verify differences in toxic effects between primary human hepatocytes and HepG2 cells at various drug concentrations, our hepatotoxicity assay was performed using primary human hepatocytes and HepG2 cells trapped in a microfluidic device. Fig. 4.4 shows the differences in drug hepatotoxic effects between primary human hepatocytes and HepG2 cells at various concentrations of benzopyrene, which is one of the most sensitive drugs for hepatocytes and
HepG2 cells, because it causes acute cytotoxic effects by drug metabolism. The live cell fraction, viable cells/(viable cells+dead cells), of primary human hepatocytes and HepG2 cells gradually decreased as the concentrations of benzopyrene increased. The differences in live cell fraction between primary human hepatocytes and HepG2 cells were maximal at concentration of 15–25 μM. Primary human hepatocytes showed higher sensitivity towards treatment with benzopyrene than did HepG2 cells. These results show that primary cells were more sensitive to the drugs tested than the hepatoma cell line; this difference was due to differences in the cells’ abilities to metabolize drugs [9]. The LC50 of primary human hepatocytes was closer to the human in vivo LC50 than the LC50 of HepG2 cells (data not shown). From this result, primary human hepatocytes better reflect the properties of in vivo humans. It has also been reported that rat and human hepatocytes metabolize diclofenac at similar rates, whereas cell lines were unable to metabolize it to a measurable degree [7].
Fig. 4.4. Comparison of live cell fraction between primary human hepatocytes and HepG2 cells at various concentrations of benzopyrene.
4.3.3 Hepatotoxicity assay in a microfluidic device

Hepatotoxicity assays were performed in the microfluidic device with acetaminophen, diclofenac, verapamil, and benzopyrene using suspended human hepatocytes. The numbers of viable cells, stained by calcein AM (green), and that of dead cells, stained by PI (red), were counted. The live cell fraction, viable cells/(viable cells+dead cells), is plotted with respect to post-assay time in Fig. 4.5. The live cell fraction gradually decreased with time and increasing concentrations of drugs. The drug concentrations used were taken from a conventional hepatotoxicity assay [2,3]. In the control experiment, the live cell fraction of suspended hepatocytes decreased, because the cells were affected by microfluidic shear stress at the start of the assay, but this was constant after 2 h.

Acetaminophen is one of the most widely used antipyretic and analgesic drugs, but can cause severe hepatic failure upon overdose [26,27]. Hepatotoxicity assays with acetaminophen need relatively high concentrations, 5–20 mM (Fig. 4.5a). Verapamil, a calcium channel blocker, is widely used to treat hypertension, angina pectoris, and cluster headaches. Commonly reported adverse effects of verapamil include hepatic injury and hepatotoxicity [28]. Cells were more sensitive to verapamil than
acetaminophen; the live cell fraction rapidly decreased, even with 50 μM verapamil (Fig. 4.5b). Benzopyrene is a chemical carcinogen and is metabolized by CYP450, causing cytotoxic damage in the liver. Suspended hepatocytes were very sensitive to benzopyrene, despite the low concentration, and the live cell fraction rapidly decreased (Fig. 4.5c). Diclofenac is a frequently prescribed non-steroidal anti-inflammatory drug and undergoes hepatic metabolism in both rat and human hepatocytes [7, 29]. Diclofenac causes an important and fast depletion of ATP in hepatocytes, and ATP dropped to 10% of initial levels after 2 h of exposure to the drug. As shown in Fig. 4.5d, at 2 h in the hepatotoxicity assay, the gap in live cell fraction at various concentrations was maximal. Thus, the LC50 was measured at 2 h in the hepatotoxicity assay because this was enough time for the hepatocytes to metabolize the drug and exhibit hepatotoxic effects.
Fig. 4.5. Hepatotoxicity assay with various drug concentrations over time. (a) 5, 10, 20 mM of acetaminophen, (b) 12.5, 25, 50 μM of benzopyrene, (c) 50, 100, 200 μM of verapamil, and (d) 100, 300, 600 μM of diclofenac.
The LC50 values, which indicate the concentration of drug required to kill 50% of the analyzed cells, were calculated using the data obtained from the live cell fraction versus drug concentration, as shown in Fig. 4.6. The LC50 values measured with the device were also compared with those obtained by a conventional MTT assay using human hepatocytes. As shown in Table 3, benzopyrene had the lowest LC50 of the drugs tested, indicating that hepatocytes were most sensitive to benzopyrene. The LC50s of the other drugs measured in the microfluidic device were similar to those measured by the MTT assay using human hepatocytes. However, the LC50 from the microfluidic device was slightly different from those measured in the \textit{in vitro} MTT assay because of differences in the \textit{in vitro} conditions in the conventional MTT assay versus the more \textit{in vivo}-like environment formed in our device. On the basis of these results, this hepatotoxicity assay system can be used as a suitable alternative to conventional hepatotoxicity assays, which require culturing cells for a long time and consume expensive media and primary cells.
Fig. 4.6. Live cell fraction under various concentrations of drugs for measuring the LC50 of suspended human hepatocytes: (a) acetaminophen, (b) verapamil, (c) benzopyrene, and (d) diclofenac
**Table 4.** Comparison of the LC50 of drugs measured with the device versus those obtained by the MTT assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Major function</th>
<th>LC50 on the device (µM)</th>
<th>LC50 by MTT assay (µM)</th>
<th>LC50 of human (µM)</th>
</tr>
</thead>
</table>
Fig. 4.7. Comparison of LC50 measured on the microfluidic device vs. MTT assay and *in vivo* human. (a) Comparison of LC50 assayed on the microfluidic device vs. MTT assay. (b) Comparison of LC50 measured on the microfluidic device vs. *in vivo* human.
4.4 Conclusion

We developed an efficient microfluidic device for performing hepatotoxicity assay by counting dead cells trapped in the microholes over time. The structure of the microfluidic device was designed with optimal conditions for cell trapping and the hepatotoxicity assay. Our hepatotoxicity assay system made it possible to analyze drug hepatotoxicity using suspended human hepatocytes directly extracted from living organisms within a short time, without cell culture. The device required only a small number of primary hepatocytes; about 100 cells were sufficient for a cytotoxicity assay. Primary human hepatocytes mimicked \textit{in vivo} hepatotoxic effects. It was also shown that the LC50 values measured in the device were similar to those measured by the MTT assay using human hepatocytes. On the basis of these hepatotoxicity assay results, this hepatotoxicity assay system may be useful in drug discovery.
References


CHAPTER V: Overall Conclusions

6.1 Summary ................................................................................. 127
6.2 Outlook, challenges, and future works ................................. 129
6.1 Summary

We developed an efficient and accurate permeability and hepatotoxicity assay system in a microfluidic device. The microfluidic structure and its experimental conditions were optimized by mathematical simulations and the results were experimentally validated. As trapped cells were viable without cell lysis on the microhole array, we performed the permeability assay with ten drugs. The trapped cells were viable after drug absorption and the permeability coefficient between human in vivo and \( P_{\text{eff}} \) on the microfluidic device was related significantly. These results indicated that our drug permeability assay device will be useful in predicting the actual human permeability values of drugs.

Also, we developed a microfluidic device for the permeability assays based on the BBB model. The permeability assays using HUVECs with and without ACM were performed for different five drugs to determine their BBB permeability and the effect of ACM on the permeability of HUVECs. The permeability values, which were measured in our microfluidic device, showed good agreement with those in the in vitro BBB experiments, in which HUVECs and astrocytes were co-cultured. In addition, the measured values of the drug permeability were highly correlated with BUI and the ZO-1 expression of
HUVECs, which decreased in the presence of ACM. These results indicated that our device for drug permeability assays is applicable to predict the permeability of drugs in a brain.

An efficient microfluidic device for performing hepatotoxicity assay was performed by counting dead cells trapped in the microholes over time. The structure of the microfluidic device was designed with optimal conditions for cell trapping and the hepatotoxicity assay. Our hepatotoxicity assay system made it possible to analyze drug hepatotoxicity using suspended human hepatocytes directly extracted from living organisms within a short time, without cell culture. The device required only a small number of primary hepatocytes; about 100 cells were sufficient for a cytotoxicity assay. Primary human hepatocytes mimicked in vivo hepatotoxic effects. It was also shown that the LC50 values measured in the device were similar to those measured by the MTT assay using human hepatocytes.
6.2 Outlook, Challenges, and Future Works

We developed an efficient and accurate drug assay system in a microfluidic device, considering the *in vivo* delivery path of drugs in humans. Among the various drug assays, drug permeability assays in the intestine and brain, and hepatotoxicity assay in the liver are very important for drug screening and development process. The microfluidic assay system for drug permeability and hepatotoxicity assays using a cell trapping method reduces the assay time as no cell culture in the microfluidic device is required and no complex structure, such as cellular membrane, is needed.

The assay results of intestinal permeability were similar to those assayed *in vivo*, while the results of brain permeability and hepatotoxicity were closer to those measured *in vitro*. As the mechanisms of brain permeation and liver metabolism are more complex than those of intestinal permeation, some factors affecting the permeation in brain or liver toxicity would be missing.
Incorporation of the factors and co-trapping of various cells in the device could produce *in vivo*-close results. Also, the integrated system including permeability and toxicity assay can be used as a valuable tool in ADME/Tox drug properties.

Our drug assay device will be useful in predicting the actual human permeability and LC50 values of drugs. In this microfluidic device, it is possible to continuously monitoring the change of cellular mechanism. Using trapped cells mimicking *in vivo* environment, the various drug assay can be used as a valuable tool in drug discovery.
Appendix A: Fabrication process flow

Starting material: 4 inch-diameter silicon wafers.

The process flow listed below is for two-step lithography.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Machine</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SiO₂ removal</td>
<td>acid-hood</td>
<td>Dipping in BOE more than five times</td>
</tr>
<tr>
<td>2</td>
<td>Dehydration</td>
<td>oven</td>
<td>200°C, 15 min</td>
</tr>
<tr>
<td>3</td>
<td>SU8 spin</td>
<td>spin coater</td>
<td>SU8 2005 (5 μm thickness) Ramp to 500 rpm and hold for 10 s, and then ramp to 3000 rpm and hold for 30 s</td>
</tr>
<tr>
<td>4</td>
<td>Soft bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 2 min</td>
</tr>
<tr>
<td>5</td>
<td>UV expose</td>
<td>UV aligner</td>
<td>5 s at 20.3 mW/cm²</td>
</tr>
<tr>
<td>6</td>
<td>Post-expose bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 1 min</td>
</tr>
<tr>
<td>7</td>
<td>Develop</td>
<td>hood</td>
<td>1 min soak in SU8 developer</td>
</tr>
</tbody>
</table>
and then wash with IPA and water

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Equipment/Tool</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>SU8 spin</td>
<td>spin coater</td>
<td>SU8 3025 (25 μm thickness) Ramp to 500 rpm and hold for 10 s, and then ramp to 3000 rpm and hold for 30 s</td>
</tr>
<tr>
<td>9</td>
<td>Soft bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 10 min</td>
</tr>
<tr>
<td>10</td>
<td>UV expose</td>
<td>UV aligner</td>
<td>10 s at 20.3 mW/cm²</td>
</tr>
<tr>
<td>11</td>
<td>Post-expose bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 3 min</td>
</tr>
<tr>
<td>12</td>
<td>Develop</td>
<td>hood</td>
<td>5 min soak in SU8 developer and then wash with IPA and water</td>
</tr>
<tr>
<td>13</td>
<td>Silanize</td>
<td>hood</td>
<td>Put 10 μL of HMDS into a cup in a vacuum jar. Place the wafer in the jar for 1 h.</td>
</tr>
</tbody>
</table>
Appendix B: Equation for calculating drug permeability

\[ P = \frac{1}{A \cdot C_0} \frac{dQ}{dt} \]

where \( A = n \cdot a \)

\( A \) : surface area, \( a \) : area of each hole, \( n \) : number of holes

\[ \therefore \frac{dQ}{dt} = P \cdot A \cdot C_0 \]

\[ Q(t) = P \cdot A \cdot C_0 \cdot t \quad \therefore Q(0) = 0 \]

\( Q(t) \) denotes transferred molecules (mole)
Since the flow rate of the opposite channel is \( v \),
the passed volume in the channel for \( t \) hours is \( v \cdot t \).

The concentration of diffused molecules in the opposite channel is \( \frac{Q(t)}{v \cdot t} \)

\[ \therefore c = \frac{Q(t)}{v \cdot t} = \frac{P \cdot A \cdot C_0 \cdot t}{v} = \frac{P \cdot A \cdot C_0}{v} \]

\( c \) denote the concentration of diffused molecules in the opposite channel (\( mM \)).

\( P \) denotes the permeability (\( cm / s \))

\( A \) denotes the surface area of all holes (\( cm^2 \))

\( C_0 \) denotes the initial molecule concentration (\( mM \))

\( v \) denotes the flow rate in the opposite channel (\( cm^3 / s \))

\( \mu l / hr \) can be converted to \( cm^3 / sec \):

\[ 1 \frac{\mu l}{hr} = \frac{10^{-3} \ cm^3}{3600 \ sec} = 2.8 \times 10^{-7} \ \frac{cm^3}{sec} \]
대부분의 약물은 장내 상피세포에 의해 흡수되며 흡수된 약물은 간으로 이동하여 대사가 이루어지게 된다. 특히 뇌를 타깃(target)으로 하는 약물의 경우 뇌의 상피세포를 통해 다시 한번 흡수된다. 뇌의 상피세포는 장내의 상피세포와는 달리 세포 사이의 강한 결합을 가지고 있으며 이를 혈액-뇌 관문(blood brain barrier, BBB)이라고 한다. 뇌로 가는 약물 중 이러한 BBB를 통과하여 흡수되는지의 여부를 아는 것은 뇌를 타깃으로 하는 약물 개발에 있어 매우 중요하다. 또한 약물 개발 단계에서 실패하는 원인의 50% 이상이 약물의 흡수도와 관련되어 있고 30% 정도가 독성과 부작용 때문임을 감안할 때 약물의 흡수도와 독성을 정확하고 빠르게 예측하는 것은 약물 개발 과정에 있어 매우 중요한 요인으로 작용한다.
본 논문에서는 미세유체소자를 이용하여 약물의 흡수와 독성을 측정할 수 있는 시스템을 구축하였으며 미세유체소자 내에 형성된 마이크로홀에 세포를 포획하는 방법을 이용하여 세포 배양을 하지 않고도 빠른 시간 내에 약물의 흡수와 독성을 측정할 수 있도록 하였다. 또한 본 미세유체 시스템을 이용하여 미세소자 내에서 유체가 지속적으로 세포에 영향을 미치도록 하는 환경은 실제 생체 내의 환경과 유사하며, 실제 인간의 생체 내에서 일어나는 약물의 흡수 및 독성의 결과까지도 예측 가능하게 한다.

본 연구에서 개발된 미세유체소자는 내부에 마이크로홀 어레이 (microhole array)를 가지고 있으며 이곳에 세포를 단일막 (monolayer)으로 고정하기 위한 최적의 유속과 마이크로홀 어레이의 각도를 시뮬레이션 및 실험을 통하여 정하였다. 또한 약물을 이용한 흡수도 평가 실험이 가능한지 검증하기 위하여 형광물질을 이용하여 약물이 세포에 의해 흡수되는 지를 확인하였다. 또한 실제로 기존의 약물의 흡수도 측정에 널리 이용되는 10가지 약물을 선정하고
세포에 의해 흡수되어 나온 약물을 미세유체 소자의 배출부에서 얻어내어 HPLC를 이용하여 측정하였고, 측정된 약물의 농도는 본 시스템에 흡수도 분석 계산식을 이용하여 흡수율을 계산하였다. 미세유체 소자를 이용한 약물의 흡수도 측정 결과를 실제 human in vivo 와 rat in vivo 환경에서의 흡수도 분석결과, 미세유체 소자를 이용한 약물의 흡수도는 쥐의 생체내 약물 흡수도 보다 인간의 생체내 약물 흡수도와 더욱 유사하다는 결과를 얻을 수 있었다. 이는 미세유체 소자를 이용한 약물의 흡수도 측정법이 실제 인간의 약물 흡수도를 예측하는 대체 방법이 될 것임을 예상할 수 있게 한다.

이러한 결과를 바탕으로 본 미세유체 소자를 뇌의 BBB를 통과한 약물의 흡수도 측정에도 적용하였다. 뇌의 혈관과 유사한 특징을 보이는 HUVECs (Human Umbilical vain endothelial cells)와 이와 상호작용을 통하여 더 강한 BBB를 형성하는 것으로 알려진 성상세포 (astrocyte)를 이용하였다. 성상세포를 5일간 배양한 배지 (astrocyte conditioned medium, ACM)를 이용하여 생체
미세유체소자 내에서 BBB를 모사하고 실제로 약물의 흡수도를 측정한 결과 ACM이 있을 때 BBB가 더 강하게 형성되어 약물의 흡수도가 낮아지는 것을 확인할 수 있었고 사진을 통하여 세포 사이의 강한 결합 (tight junction)이 형성되어 있는 것도 확인할 수 있었다. 또한 실제로 뇌와 관련된 5 가지 약물의 흡수도를 측정한 결과, 기존의 모델과 유사한 결과를 나타내었다. 따라서 본 시스템은 뇌를 타깃으로 하는 약물이 BBB를 얼마나 통과하는 지를 실시간으로 측정할 수 쓰일 수 있으며, 뇌 관련 질환과 연관된 다양한 약물의 스크리닝 (screening)을 효율적이고 간편하게 할 수 있는 새로운 대체 방법이 될 것이다.

약물의 독성 측정을 위해서는 미세유체 소자 내에 마이크로 홀 구조물에 인간의 간세포를 고정하고 약물을 흘린 다음 시간에 따라 세포가 사멸하는 비율을 계산하였다. 약물의 흡수도 측정을 위한 미세유체 소자와 유사한 구조를 가지나 형광물질을 혼합하기 위한 혼합 채널을 제외하여 세포에 미치는 유체의 압력을 최소화 하고자
하였다. 미세유체 소자 내에 존재하는 마이크로 홀 구조물에 세포가 고정된 다음, 반대편으로 흐르는 약물을 흡수하게 되고 약물의 독성 정도에 따라 세포가 사멸하는 수가 달라지게 된다. 미세유체 소자 내에서 약물의 독성 측정 결과, 시간에 따라 세포의 사멸수가 점차 증가하였고 반대로 살아있는 세포의 수가 점차 감소하는 것을 확인하였다. 또한 약물의 농도가 높아질수록 세포가 사멸하는 시간이 점차 빨라지는 것을 알 수 있었으며 약물의 농도에 따라 LC50 값을 측정할 수 있었다. 그 결과 benzopyrene 의 경우가 약물에 가장 민감하다는 것을 알 수 있었고 이는 세포가 가지는 약물에 대한 민감도를 미세유체 소자로 통해 알 수 있다는 것을 증명해준다. 또한 미세유체 소자 내에서 측정된 약물의 독성은 기존의 인간의 간세포를 이용하여 MTT assay 방법 및 실제 인간의 생체 내에서의 독성 정도와도 거의 유사하였다. 이를 통하여 미세유체 소자를 이용한 약물 독성 측정 방법이 기존의 방법을 대체하여 빠르고 효율적인 독성 예측이 가능하도록 하려는 것을 알 수 있다.
본 미세유체 소자를 이용한 약물 분석 시스템은 약물의 흡수도와 독성을 측정함으로써 기존의 시스템을 대체하여 세포 배양 없이도 빠르고 효율적인 분석이 가능하다는 것을 보여주며, 앞으로 신약 개발 및 연구에 있어서 값비싼 동물 실험을 대체함으로써 약물 개발에 소요되는 시간과 비용을 획기적으로 절약할 수 있음을 시사한다. 또한 약물의 흡수도와 독성을 동시에 수행할 수 있는 소자의 기반이 되는 본 미세유체 소자는 HTS (high-throughput screening)의 가능성을 내재하고 있다. 이와 같이 미세유체 소자를 이용한 약물 분석 시스템은 국내의 신약 개발 및 약물 분석 스크리닝에 큰 도움이 될 수 있을 것이다.