DEVELOPMENT OF A MICROMIXING SYSTEM FOR TREATMENT OF ORL-48 MICROTISSUES IN DIFFERENT CONCENTRATIONS OF CYTOCHALASIN-B

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For my beloved mother, Maliga D/O Kanthan, and grandmother, Kamatchy, for their encouragement and blessing…

To my beloved supervisor, co-supervisor and friends for their support and caring…

-I wish I can learn more and better. I wish I have all the opportunities in my life and time flies slower…-

கற்றது கைமண் அளவு, கல்லாதது உலகளவு

"What you have learned is a mere handful; What you haven't learned is the size of the world"

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ABSTRACT

Mixing and dilution are essential procedures in pharmaceutical operation to process two or more components in a separate or thoroughly mixed condition until homogenous solution was obtained. However, conventional serial dilution method used in laboratory assessment causes high usage of reagents, higher complexity procedures and costly. Micromixing method provides a better platform that enables mixing and dilution of liquid-based reagents which is convenient solutions preparation, easy liquid handling and time-saving. In this study, a polydimethylsiloxane (PDMS) micromixer was designed, simulated and prototyped using vinyl tape method and successfully applied to mix and dilute Cytochalasin-B in culture media (CB-DMEM, 30.0 µM) with 0.05 % ethanol solutions (diluent) to produce four different concentrations of CB-DMEM (5.3, 10.6, 14.8, and 20.2 µM). The different concentrations of CB-DMEM were applied on to ORL-48 microtissues produced by using flicking technique. The morphological responses, cell viability and cell proliferation of ORL-48 monolayer cells (2D) and microtissues (3D) treated in four different CB concentrations were assessed via phase contrast microscopy, live/dead staining and Alamar Blue® staining respectively. The results show that both 2D and 3D of ORL-48 microtissues were only morphologically affected (fibroblastic spreading to round shape) while cell viability and cell proliferation show that CB treatment solely does not causes apoptosis (≈ 90 % cells are alive and able to proliferate). The micromixer employed in solution preparation of CB-DMEM (5.3, 10.6, 14.8, and 20.2 µM) provide a convenient and faster method to prepare cytochemical solution for drug screening and experiments. Besides that, application of micromixer consumes less volume of reagents and cost efficient.

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ABSTRAK

Pencampuran dan pencairan adalah prosedur penting dalam operasi farmaseutikal untuk memproses dua atau lebih komponen dalam keadaan yang berasingan atau menyeluruh sehingga larutan homogen diperolehi. Walau bagaimanapun, kaedah pencairan siri konvensional yang digunakan dalam penilaian makmal menyebabkan penggunaan reagen yang berlebihan, prosedur yang rumit dan mahal. Kaedah pencampuran-mikro menyediakan platform yang lebih baik yang membolehkan pencampuran dan pencairan reagen berasaskan cecair dengan proses yang tidak merumitkan, memudahkan pengendalian cecair dan menjimatkan masa. Dalam projek ini, pencampur-mikro berasaskan polydimethylsiloxane (PDMS) direka, disimulasikan dan diprototaipkan melalui kaedah pita vinil dan berjaya digunakan untuk mencampur dan mencairkan Cytochalasin-B dalam media kultur (CB-DMEM, 30.0 μM) dengan larutan etanol 0.05% untuk menghasilkan empat kepekatan CB-DMEM (5.3, 10.6, 14.8, dan 20.2 μM) yang berlainan. Kemudian, empat kepekatan CB-DMEM yang berbeza diperkenalkan pada mikrotisu ORL-48 yang dihasilkan dengan menggunakan teknik penggilapan melalui pengkapsulan mikro. Respons morfologi, daya maju sel dan percambahan sel sel tunggal (2D) dan mikrotisu (3D) ORL-48 yang dirawat dalam empat kepekatan CB yang berbeza telah diperhati melalui mikroskopi kontras fasa, pewarnaan hidup /mati dan pewarnaan Alamar Blue®. Keputusan menunjukkan bahawa kedua-dua sel tunggal (2D) dan mikrotisu (3D) ORL-48 hanya terjejas secara morfologi (berselerak ke bentuk bulat) manakala daya maju sel dan proliferasi sel menunjukkan bahawa rawatan CB semata-mata tidak menyebabkan apoptosis (≈ 90% sel hidup dan mampu membiak). Pencampur mikro yang digunakan dalam penyediaan cecair CB-DMEM (5.3, 10.6, 14.8, dan 20.2 μM) menyediakan kaedah yang mudah dan cepat untuk menyediakan penyelesaian sitokimia untuk pengskrinan ubat dan eksperimen. Selain itu, penggunaan pencampur-mikro kurang menggunakan reagen dan menjimatkan kos.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	-	Percentage
<<	-	Very much less than
>>	-	Very much larger than
µFCCD	-	Microfluidic cell culture device
µM	-	Micromolar
µm	-	Micrometer
2D	-	Two – dimensional
3D	-	Three – dimensional
CB	-	Cytochalasin-B
CB-DMEM	-	Cytochalasin-B in Dulbecco's Modified Eagle Medium
COC	-	Cyclic olefin copolymer
CRM	-	Cancer Research Malaysia
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribonucleic acid
ECM	-	Extracellular matrix
EGF	-	Epidermal growth factor
EthD-1	-	Ethidium homodimer-1
FADH	-	Reduced form of flavin adenine dinucleotide (FAD)
FDA	-	Flavin adenine dinucleotide
FITC	-	Fluorescein isothiocyanate
FMNH	-	Metabolic activity reducing coenzymes
HBSS	-	Hank's balanced salt solution
HeLa	-	Henrietta Lacks, cervical cancer cells
IC_{50}	-	Half maximal inhibitory concentration
KCL	-	Kirchhoff Current Law
LCD	-	Liquid crystal display

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LN	-	Lymph nodes
L.O.C	-	Lab on Chip
MEMS	-	Micro-electromechanical
ml	-	Milliliter
ml/min	-	Milliliter per minute
mm	-	Millimeter
MOEMS	-	Micro-optoelectromechanical systems
mol/m³	-	Molar per meter cube
NADH	-	Reduced nicotinamide adenine dinucleotide
NADPH	-	Reduced nicotinamide adenine dinucleotide phosphate
ORL-48	-	Asian oral cancer cell lines
OSCC	-	Oral squamous cell carcinoma
PC	-	Polycarbonate
PCR	-	Polymerase chain reaction
PDMS	-	Polydimethylsiloxane
P_e	-	Péclet number
PI	-	Polyimide
PMMA	-	poly-(methyl methacrylate)
PS	-	Polystyrene
PVC	-	Polyvinyl chloride
R^2	-	Coefficient of determination
R_e	-	Reynold number
REDOX	-	Reduction-oxidation reaction
RGD	-	Tripeptide Arg-Gly-Asp
RIE	-	Reactive ion etching
RPM	-	Rotation per minute
Si	-	Silicon
SU-8	-	Negative epoxy-based photoresist
TPZ	-	Tirapazamine
UV	-	Ultraviolet

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CHAPTER 1

INTRODUCTION

This chapter covers the research background, problem statement, objectives, and scope of research.

1.1 Introduction

Microfluidic systems have been widely applied for identification of biochemical products, diagnosis, drug testing and screening in chemical and biological fields. General information concerning micromixing techniques and microfluidic technologies employed in in biological and chemical application is briefly explain in research background. The problem statement highlights the weakness of the current method for mixing and diluting (serial conventional dilution method) in laboratory assessment. Consequently, a convenient and time saving method was introduced to enable linear mixing and dilution of cytochemical solutions.

1.2 Research background

Dilution and mixing is one of the important procedure in chemical and biological analysis to mix and dilute single or multiple reagents such as enzymes, cytochemical solutions, biological and chemical assays into desired concentration solutions to be employed in both chemical and biological analysis [1]. The conventional serial dilution method used in laboratory assessment to mix and dilute consume more chemicals and solutions, time and laboratory plastic wares. This is because the procedures repeat the

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calculations, titration and accurate pipetting which to withdraw a specific volume of stock solution and dilute them in the separate conical tube [2, 3]. In addition, most of the laboratory plastic wares and pipettes are not reusable and cleanable which contribute to many laboratory plastic wares waste that need to be managed. Application of microfluidic mixer (micromixer) to dilute and mix reagents provide a solution which consumes fewer reagents, time-saving and less laborious [4, 5].

Microfluidic is defined as a system consists of integrated microchannels which are able to be fabricated in micro or nano-scales with at least one of the dimensions is less than or equal to 1.0 mm [6]. The general idea of microfluidic mixing is to achieve thorough and rapid mixing of two or numerous samples in microscale devices [7]. Based on mixing principles, micromixers are categorised into two groups: active and passive micromixers. Active micromixers require external perturbation energy to blend the sample species and achieve optimum mixing. Types of external perturbation energy includes pressure field [8], electrokinetic [9], dielectrophoretic [10], electrowetting [11], magneto-hydrodynamic [12] and ultrasound [13]. Passive micromixers are dependent on the mass transport phenomena and are driven by molecular diffusion and chaotic advection. Generally, these devices are designed with channels geometry in order to increase the surface area between the different fluids and contact time. Passive micromixers can be categorised as T- and Y-shaped micromixers [14], parallel lamination micromixers [15], sequential lamination micromixers [16], focusing enhanced mixers [17], chaotic advection micromixers [18] and droplet micromixers [19]. However, design and fabrication of microfluidic mixer involves complex operational control, require well equipped clean room and expensive and highly toxic chemicals such SU-8 photoresists and etchants [20].

Passive micromixer utilises no energy input except the mechanism of infused liquid (flow rate) at a constant rate. In addition, the magnitude of flow rate applied dependent on the design of the micromixer to achieve micromixing. A previous study [21], shows that twisted T-shaped micromixer with 200 µm in depth and 200 µm wide requires a flow rate lower than 1.00 ml/min for acceptable mixing performance. Based on a reported study [22], parallel liquid infusion into micromixer at high flow rates may enhance mixing by induction of turbulence. It is observed that size distribution of emulsion decreases when the flow rate increases due to increase in turbulent energy. The maximum flow rates fed into the micromixer was 40 ml/min.

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In clinical medicine and biological studies, microfluidic systems have been applied for identification of biochemical products, diagnosis, and drug discovery. Implementation of micromixing technologies in the microfluidic system enables biological screening [23], enzyme assay [24], cell lysis [25] and biochemical analytical [26] to be conducted. Integration of micromixing into enzyme assays contribute several advantages such as improved cost efficiency, low sample consumption and reagents can be thoroughly mixed with enzymes [27]. High-throughput permit parallelisation of molecular sorting and a small volume of samples used enable microfluidic to be selected as a tool for biological screening [28]. By integrating micromixers with microvalves and micropumps, a micromixer is able to add two or more samples before furthered into polymerase chain reaction (PCR) which is a key process in biological engineering. Due to its high sensitivity, high throughput, less material consumption, low cost, portable and easily designed, microfluidics devices offer limitless potential and application in point-of-care diagnostics, disease management and patient care such as in cancer studies [29, 30].

Oral cancer is in eleventh order of the most common cancer worldwide as reported by World Health Organization. Annually, there are 400,000 new victims of oral cancer and approximately 300,000 died of this disease [31]. South and South East Asian countries are among the countries with 80% of these cases occurs [32]. Smoking, excessive alcohol consumption and betel chewing are the contributing factors estimated to account for about 90% of oral cancer [33]. Most of the cancer research publications [34, 35], are still working on monolayer cells for assessment of therapy drugs on cancer cells. The validity of results published for cell biology research using monolayer of cells (2D cell culture) are questionable and criticised due to simplified model for cell biology study [36]. Biosensor and Bioengineering Laboratory of Universiti Tun Hussein Onn Malaysia (UTHM) had engineered a flicking technique to culture oral squamous carcinoma cells (ORL-48) in three dimensions (3D) using calcium alginate [37]. The collaborator of the laboratory is, Cancer Research Malaysia (CRM) which has mission to establish new laboratory models to find new ways to treat oral cancer. CRM has one of the largest collections of Asian oral cancer cell lines that have been used by other researchers in many countries including the UK, USA, Thailand, and India to study the cancer therapeutic drugs. In this study, ORL-48 microtissues and Cytochalasin-B were employed to understand the resistivity of 3D cancerous cell lines on cell permeable mycotoxin as

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the 3D cancerous cell lines have higher towards apoptosis inducing drugs [38]. Besides that, morphological responses, cell viability, and cell proliferation of ORL-48 microtissues reveal the resistance towards Cytochalasin-B while employment of micromixer provides convenient method to prepare Cytochalasin-B in different concentrations. The mixing and dilution performance of the micromixer was assessed via spectrophotometry. The morphological responses, cell viability and cell proliferation of treated microtissues of ORL-48 were analysed using phase contrast microscopy, live/dead staining and Alamar Blue® staining. The backbone of this project is to be useful to prepare cytochemical solution in different concentrations via time saving and convenient method and understand the resistance of 3D cancerous cell lines toward cytochemical treatment.

1.3 Problem statement

Mixing and dilution are important processes in diagnostic and biological analyses while the conventional way of mixing and diluting different fluids consume a large volume of reagents and time to be applied and analyse [39]. By using a micromixer to generate different concentration of treatment reagents, fewer stock reagents (approximately 5.0 ml) are used and economically cost effective to analyse the reactions of tissues and cells towards a range of concentrations of treatment reagents and identifying the half maximal inhibitory concentration, (IC_{50}) of the treatment reagents. IC_{50} is a quantitative measure indicates quantity of particular drug or inhibition substance is needed to inhibit a given biological process. Micromixer provides convenient, fast and linear mixing and dilution which shorten the time and reduces the cost for solution preparations compared to conventional serial dilution method.

Infusion flow rates and parallel fluid infusion are important parameters to allow fluids to mix and dilute well in micromixer. Current commercial infusion pump which provides flow rates in nl/min to µl/min may not be able to infuse at an optimum flow rate for enhanced mixing and dilution. Customised infusion pump with optimum flow rate for the micromixer provide linear dilution and mixing of the solutions. Although monolayer of cell are commonly used for drug screening, the reliability of the results is questionable due to its simplified 2D cell model which does not reflect the real microenvironment of cancerous cell lines and tumours. Based on literature

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reviews, a micromixer could be designed via simple vinyl tape method to mix and dilute cytochemical solutions and generate four different output concentrations. Hence, the purpose of this project is to design, simulate and prototype a micromixer and customised infusion pump system to mix and dilute Cytochalasin-B in culture media (CB-DMEM, 30.0 µM) with 0.05 % ethanol solution to produce four different concentrations of CB-DMEM by linear dilution and mixing. The micromixing system used provide convenient and time saving for cytochemical solutions preparation into different concentrations. The four different concentrations of Cytochalasin-B were used to treat ORL-48 microtissues which reveals the effects of different concentrations of CB-DMEM on ORL-48 microtissues (3D) compared to monolayer of ORL-48 cells (2D).

1.4 Objectives of the research

This study embarks on the following objectives:

a. To design a PDMS micromixer for mixing and dilution of Cytochalasin-B in culture media (CB-DMEM).
b. To develop a customised infusion pump system which enable optimum flow rate for linear mixing and dilution via the PDMS micromixer.
c. To investigate the morphological responses of ORL-48 microtissues, cell viability and proliferation of ORL-48 microtissues treated in different concentrations of Cytochalasin-B

1.5 Scopes of research

The scope of the study is limited to design and development of a PDMS based microfluidic mixer (micromixer) via vinyl tape method. The micromixer was applied to dilute and mix CB-DMEM (30.0 µM) with 0.05 % ethanol solution to produce four different concentrations of CB-DMEM (5.3, 10.6, 14.8 and 20.2 µM). In order to achieve linear mixing and dilution, a customised electronic infusion pump was developed to provide flow rates of 0.5, 1.0 and 2.0 ml/min based on fluid mixing simulation via COMSOL Multiphysics version 4.2. Dilution and mixing performance of the micromixer was assessed by using spectrophotometry, photometric analysis and

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Beer's standard calibration curve of concentration against absorbance of CB-DMEM produced from serial dilution method as a reference graph. Four different concentration of CB-DMEM (5.3, 10.6, 14.8 and 20.2 µM) were collected into four separated centrifuge tubes and introduced to monolayer (2D) and microtissues (3D) of ORL-48 in petri dishes. The morphological response of ORL-48 microtissues and monolayer cells towards four different concentrations of CB were assessed via phase contrast microscopy. The study of cell viability and proliferation of treated ORL-48 microtissues and monolayer of cells were investigated by using live and dead staining and Alamar Blue® staining.

1.6 Thesis outline

This thesis is divided into five chapters. Chapter 1 provides an overview of this project and the objectives, scopes and problem statement of the research. Chapter 2 briefly explains microfluidic mixer and mixing principles, reviews of the methods to fabricate microfluidic device, review of common polymer as microfluidic materials, previous research on microfluidic device in cell culture system, oral squamous carcinoma cells, comparison of 2D and 3D cell culture, review of commercialised products in cell culture system, review of microencapsulation cells and biopolymer, Cytochalasin-B (CB), review of pharmacology studies in 2D and 3D cell model, and review of biophysical characterisation techniques. Chapter 3 outlined the experimental procedure of this research including the design and simulation of fluid mixing in micromixer, prototype and fabrication of PDMS micromixer using vinyl tape method, development of customised infusion pump system, performance assessment of the micromixer, microencapsulation of ORL-48 cells, and characterisation of biophysical properties of treated ORL-48 microtissue. Chapter 4 presented the results and discussions on the research project which includes simulation of linear fluid mixing, the prototype of the microfluidic mixer, electronic infusion pump system, micromixing and dilution of CB stock solution with 0.05 % ethanol solution, the growth of ORL-48 microtissue, and characterisation of treated ORL-48 microtissue using CB. At last but not least, Chapter 5 delivered the conclusion, thesis contribution and recommendations for future work.

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