MICROFLUIDIC AND MICROSCALE CELL CULTURES FOR HIGH-THROUGHPUT CELL-BASED ASSAYS AND BIOPROCESS DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2009

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ABSTRACT

Cells can be cultured in different formats, such as in three-dimensional (3D) tissue constructs, in free suspension, in microcarriers, or in attached monolayer. Microscale and microfluidic cell culture devices are potentially useful in future pharmaceutical development and biomedical applications. However, culturing cells at microscale or in a microfluidic device imposes unique challenges and deserves further investigations.

For high-throughput cytotoxicity studies of anticancer drugs, increasing evidence indicates that 3D cell cultures create superior *in vitro* models to conventional monolayer cultures in multi-well plates. Due to high cell density in 3D culture, mass transport is critical. Perfusion culture via microfluidic operation provides continuous nutrient inflow and waste removal, significantly improving mass transport. To make it even more advantageous, 3D perfusion microbioreactors may help create an *in-vivo* like hydrodynamic microenvironment. In this study, a perfusion microbioreactor array was designed and fabricated for 3D culture of human colon cancer cells in modular fibrous matrix. Proliferation was monitored non-invasively with auto-fluorescence from green fluorescent protein expressed by engineered cells. Dose dependent response of the 3D cell proliferation to 5-fluorouracil was observed. The modular design allowed direct post-culture access to intact tissue constructs for various analyses. The perfusion microbioreactor array is a platform technology, which has a potential to be used as an *in-vitro* cancer model and in high throughput drug screening
One of the challenges for microfluidic cell culture in an array was parallel flow control. Flow control in multiple hydrophobic microchannels with a common inlet was thus investigated. Based on a series of designs of perfusion microbioreactor arrays, theoretical analysis and experimental validation were developed. It was found that capillary pressure played a key role in parallel flow control in the startup phase. The theory and the experiments had an excellent match. A novel method of achieving parallel flow control at significantly reduced flowrates was developed by using fibrous matrices in the microchannels. This study provided a rational approach to achieving parallel flow control by design, material selection and operations.

Another high-throughput cell culture platform developed in this study was a 24-well microbioreactor array for suspension culture. A central static mixer was fabricated to effectively break poor mixing patterns in multi-well-plate-based cell cultures, minimizing cell aggregate formation. Furthermore, evaporation was reduced with non-polar gas permeable membranes for both the bottom and the lid. Therefore, cell culture data quality was improved with the microbioreactor array. In addition, its mixing time and maximum oxygen transfer rate were characterized with optical methods. With design of experiment using the microbioreactor array, active factors for serum-free media were identified for Chinese hamster ovary cells producing monoclonal antibody (MAb).

Microcarriers are useful in culturing anchorage-dependent cells in conventional stirred tank bioreactors. However, microcarrier cell cultures are very sensitive to shear stress and microcarrier collision in a stirred tank. Furthermore, high cost of commercial microcarriers also limits large scale use of this technology. In the
perfusion microbioreactor array project, dynamic cell seeding onto small fibrous matrices was studied. Due to the similarity in operation, macroporous fibrous microcarriers were thus developed. In a comparative study, the fibrous microcarriers had comparable performances with several commercial microcarriers on cell attachment, cell growth and MAb production. Excellent protection of cells against high shear stress was achieved. In addition, the fibrous microcarriers provide over 1000-fold cost savings. These could be especially meaningful for large-scale microcarrier cell cultures, such as in viral vaccine production.
Dedicated to Yongjia and my parents
ACKNOWLEDGEMENTS

My sincere gratitude first goes to my advisor Dr. Shang-Tian Yang. For over five years in my life, it has been such a blessing to work and study under his guidance. I have learned many things from him. With care and high standards, he taught me to be a biochemical engineer having independent scientific thinking. With patience and discipline, he provides everyone in his lab the freedom and opportunities for academic growth. With energy and love, he is a model for us to lead a productive and well-balanced life. There is a Chinese proverb saying that “A teacher for one day, a father for a life time.” Dr. Yang’s mentorship to me is so invaluable and special that its constructive impact will for sure last for a life time long.

I also want to extend my appreciation to Dr. Jeffrey J. Chalmers, Dr. Jessica O. Winter, and Dr. Joanne Trgovcich for serving on my committee. I am especially grateful for their insightful advice and invaluable comments on my doctorate research.

My thanks also go to former group members in Dr. Yang’s lab, Dr. Xudong Zhang, Dr. Shubayu Basu, Dr. Anli Ouyang, Dr. Robin Ng, Dr. Xiaoguang Liu, Dr. Yunling Bai, Dr. An Zhang, Dr. Jun Luo Dr. Clayt Robinson, Dr. Liping Wang, and Ms. Shin-Chwen Wang for their support and technical advice in my research. I would like to thank Ms. Amanda Jensen and Mr. Derek Caetano-Anolles for faithfully working with me as very helpful undergrad research assistants. In addition, Ms. Ning Liu also provided helpful advice in my projects. My current lab mates, Ms. Yali Zhang, Mr.
Bingxu Song, Ms. Ching-Suei Hsu, Ms. Wei-Lun Chang, Dr. Mingrui Yu, Mr. Patrick Bennett, Ms. Baohua Zhang, Ms. Ru Zang, Ms. Congcong Lu, Mr. Kun Zhang, and Mr. Zhongqiang Wang are always friendly and willing to help, making my work and stay in Dr. Yang’s lab very easy and enjoyable.

I am also thankful to Dr. Chee Guan Koh for teaching me clean room microfabrication techniques, Mr. Paul Green and Mr. Leigh Evrard for their technical help on fabrication and facilities, and Dr. Rustin Shenkman for his care and encouragement.

I would also like to sincerely thank the NSF Center for Affordable Nanoengineering of Polymeric Biomedical Devices under the direction of Dr. L. James Lee at OSU for financial support and helpful technical discussions.

My wife Yongjia knows my dream and she is part of it. Her affections and support are endless and selfless. I feel very fortunate to have her as the best helper and friend. In addition, my parents also continuously give me selfless support and love, so my gratitude also goes to them. And this great honor belongs to my family, too.

I would like to thank God for His guidance and these many blessings around me.
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ST Yang, X Zhang, Y Wen, Microbioreactors for high-throughput cytotoxicity assays, 

Y Wen, ST Yang, The future of microfluidic assays in drug development, Expert 
Opinion on Drug Discovery, 2008, 3(10), 1237-1253.

FIELDS OF STUDY

Major field: Chemical Engineering

Minor field: Biochemical Engineering
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CHAPTER 1

INTRODUCTION

1.1 Microscale bioreactor array

Culturing cells, both prokaryotes and eukaryotes, acts as a fundamental role for today’s biotechnology, impacting both scientific discoveries in biology and medicine and also practical applications in the areas such as in vitro cell-based assays, ex vivo cell expansion, tissue engineering, and bioprocess development, etc., which are profoundly related to various aspects of the improvement for human health.

There are many different types of vessels for culturing cells, such as multiwell plates, Petri dishes, T-flasks, roller bottles, and spinner flasks [1], which are usually placed in an incubator equipped with a shaker or a spinner rack to provide temperature, CO₂ content, stirring and aeration controls. These platforms are basically for laboratory scale culturing and lack integrated monitoring and controls for pH, dissolved oxygen (DO), and optical density (OD). However, these simple culturing platforms are widely used in many applications and techniques, e.g. cloning selection, differentiation, and cytotoxicity studies, etc.

A much more complex platform than the above for culturing cells is bioreactor, which usually has monitoring and controls for pH, DO, and agitation altogether in a standalone format. Applications for bioreactors mainly focus on the production of biological or organic molecules, tissue engineering, or cell expansion. In terms of
operation, there are three modes for bioreactors: batch, fed-batch and continuous. Especially, the continuous culture mode enables perfusion through the culture, constantly providing nutrients and removing wastes with tunable hydrodynamic parameters. Therefore, a well defined culturing environment can be created in a bioreactor, which is advantageous for highly controlled cell-based studies. For example, chemostats have long been used for studying cell behaviors at steady states [2]. Currently, there is also a trend in the pharmaceutical industry that cell-based assays are increasingly used for drug target validation and drug ADMET (absorption, distribution, metabolism, elimination and toxicity) studies [3]. Especially, miniaturized culturing vessels with high controllability for microenvironment are gaining favor in these cell based assays [4]. Therefore, with enabling technologies such as microfabrication, microfluidics, biosensing and automation, high-throughput and high-controllability cell culturing platforms, such as microbioreactor or microfluidic bioreactor array, have been developed.

Altogether there are three major driving forces for developing such systems: bioprocess optimization, tissue engineering and cell-based assays. A significant increase in the number of potential new biologic therapeutics has driven bioprocess development to build microbioreactors or microbioreactor arrays for parameter characterizations in parallel, e.g. pH, DO, OD and substrate concentration, etc. [5-9]. Most of these microbioreactors allowed operations in batch mode. Only recently, has a microfluidic chemostat been developed [10], but limited to microbial cultivation.

Cell culture with bioreactors has been reported to mediate the effectiveness of tissue engineering in three-dimensional (3D) scaffolds, where perfusion was found most
beneficial to cell growth [11]. In microscale tissue engineering studies, microfluidic perfusion culture has been primarily used for *in vitro* functional liver development [12-15] and endothelial cell culture [15-17]. The tissue culturing either depends on cellular spheroid in micro-chambers or monolayer growth on microscale surface patterns. They have been successful in demonstrating functional liver cultures and endothelial cell growth. These platforms may have potential applications in cell-based assays, but the quantification of cell proliferation requires further development.

For cell-based assay purposes, several microfluidic bioreactor arrays have recently been developed, and the applications focus on toxicity studies [18-20], gene expression profiling [21,22] and quantitative cell biology [23-25]. These devices above have huge potentials in both drug discovery and fundamental biomedical studies. However, most of them rely on two-dimensional or monolayer cell cultures, which may limit their usefulness in situations where 3D cell culture is necessary to demonstrate their authentic *in vivo* physiology for certain cell types [26-30], such as tumor cells and chondrocytes [31].

Therefore, the overall objective of this work is to develop low-cost microfluidic and microscale cell culture platforms for high-throughput drug studies and bioprocess development (Figure 1.1).

1.2 Objectives

1.2.1 To design and fabricate a perfusion microbioreactor array for high-density 3D tissue cultures useful for high-throughput cytotoxicity assays. Our lab has developed a platform for fermentation and 3D cell cultures using fibrous matrix for cell immobilization, named as fibrous bed bioreactor (FBB). In addition, our lab has used
constitutively expressed enhanced green fluorescence protein (EGFP) to monitor cell proliferation. Microfabrication and microfluidic are enabling techniques for making microscale cell-based devices. Therefore, the objective was to combine microfabrication, microfluidic and fluorescence-based biosensing techniques with the concept of FBB to develop a device as a perfusion microbioreactor array applicable for proliferation and cytotoxicity assays with 3D tissue constructs.

1.2.2 To further investigate technical challenges in the development of perfusion microbioreactor array, such as parallel flow control in microchannels and dynamic cell seeding onto small fibrous matrices. For the former project, the objective was to study in both theory and experiments the fundamental forces for parallel flow control in hydrophobic microchannels. For the latter project, fibrous microcarriers were developed based on dynamic cell seeding onto small fibrous matrices, and the fibrous microcarriers were compared with commercial microcarriers for cell culture.

1.2.3 To develop a novel and low-cost 24-well microbioreactor array for high-throughput cell culture process development. Suspension cell culture is most widely used in biopharmaceutical manufacture. Scale-down models for cell cultures are useful for high-throughput medium development and bioprocess optimization. Multi-well plates are widely used for this purpose, but the data quality is generally poor due to non-uniform mixing and evaporation. Therefore, the purpose of this study was to improve data quality by building a central static mixer in each well and by using special membranes to reduce evaporation without affecting aeration. This platform should be characterized and applied in high-throughput bioprocess development, such as serum-free medium development.

1.3 Scopes of study
1.3.1 Perfusion microbioreactor array for modular 3D cell cultures and cytotoxicity assays (Chapter 3)

Perfusion microbioreactor array provides a useful tool for micro-scale tissue culture in parallel. However, high-throughput data generation has been a challenge. In this study, a 4×4 array of perfusion microbioreactors was developed for plate-reader compatible, time-series quantification of cell proliferation and cytotoxicity assays. The device was built through multi-layer soft lithography. Low-cost nonwoven polyethylene terephthalate (PET) fibrous matrices were integrated as modular tissue culture scaffolds. Human colon cancer HT-29 cells with stable expression of enhanced green fluorescent protein (EGFP) were cultured in the device with continuous perfusion and reached a cell density as high as $6 \times 10^7$ cells/mL. The microbioreactor array was used to test a chemotherapeutic drug 5-fluorouracil (5-FU) for its effect on HT-29 cells in continuous perfusion 3D culture. Compared with conventional 2D cytotoxicity assay, significant drug resistance was observed in the 3D culture.

1.3.2 Flow control and startup in parallel hydrophobic microchannels with a common inlet (Chapter 4)

Parallel flow control is a common challenge in microchannel technologies. Poly(dimethylsiloxane) (PDMS) is widely used in microfluidic devices. However, its relatively high hydrophobicity causes difficulties in pressure driven flow in parallel microchannels at low flowrates. This study presents a theoretical and experimental investigation of this phenomenon. It was found that capillary pressure was the major force for flow blockage. The threshold flowrate for uniform flow startup in parallel microchannels could be estimated by balancing the maximum capillary pressure and the hydrodynamic pressure drop in a microchannel. This hypothesis was tested in a
series of experiments using 2×4 and 4×4 perfusion microbioreactor arrays, and the results matched well with the theoretical calculations. Also, filling micro-wells with porous fibrous matrices to increase the hydrodynamic pressure drop allowed uniform flow startup at low threshold flowrates. This study established a rational method of achieving uniform flow startup in parallel hydrophobic microchannels, providing practical guidance for device design, material selection and operation in microchannel technology.

1.3.3 A novel 24-well microbioreactor array for high-throughput cell cultures with scalable performance (Chapter 5)

A novel, low-cost microplate-based 24-well microbioreactor array was developed for high-throughput cell cultures. The bottom and the lid of the array were made of a poly(dimethylsiloxane) (PDMS) membrane that reduced evaporation by 30% without affecting aeration. A central static mixer was built in each well to improve mixing patterns and reduce cell aggregates under orbital shaking conditions. Each well of the plate also had a set of observation windows at the bottom, amenable to optical measurements with a standard microplate reader. In this study, mixing time and maximum oxygen transfer rate were characterized using optical methods. Consistent cell growth and metabolism at different positions on the array were achieved for suspension culture of Chinese hamster ovary (CHO) cells. The microbioreactor array significantly improved data quality, especially reproducibility, as compared with commercial multi-well plates. The 24-well microbioreactor array was used in an experiment with 2-level fractional factorial design to screen and identify active factors in a serum-free medium (SFM) for monoclonal antibody (MAb) production by CHO cells. The SFM developed in the screening experiment was then tested in spinner flask
cultures and the results were consistent with those from the microbioreactor cultures, demonstrating the scalable performance of the microbioreactor array.

1.3.4 Low-cost fibrous microcarriers for cell attachment, growth and monoclonal antibody production: a comparative study (Chapter 6)

Small patches of poly(ethylene terephthalate) (PET) nonwoven fibrous matrices are an excellent candidate as low-cost microcarriers for cell culture. Fibrous microcarriers are a type of macroporous microcarriers, which provide large surface areas and three-dimensional capacity for high density cell growth. In this study, the fibrous microcarriers and several commercial microcarriers were used to study cell attachment kinetics, growth and monoclonal antibody production with CHO cells. The fibrous microcarriers had comparable performances with commercial ones. In addition, fibrous microcarriers provided a wider operable range for agitation rate than commercial microcarriers, effectively protecting cells from shear stress and microcarrier collisions. Moreover, the fibrous microcarriers were over 1000 times cheaper than commercial ones, which is especially meaningful for large-scale cell cultures using microcarriers.

1.4 References


Figure 1.1 Schematic diagram of research structure.
CHAPTER 2

LITERATURE REVIEW

2.1 Microfabrication and microfluidics

Microfabrication [also micromachining, micromanufacturing, or micro electromechanical systems (MEMS)] refers to the fabrication of devices with at least some of their dimensions in the micrometer range [1]. Originally, microfabrication was developed in integrated circuit (IC) industry over four decades ago, and it has been continuously driven to nanomachining, also nano electromechanical machining or NEMS, for the quest of ever smaller structures [1]. And this fast progress rate was first described in a statement that is now widely referred to as Moor’s Law, saying that the number of transistors that may be made on a unit area would double in every 18 months.

Microfabrication has found macro application opportunities in today’s world, impacting various fields such as automotive market, medical and biomedical markets, environmental monitoring, industrial/automation, IT/peripheral, and telecommunications, etc. Therefore, huge market values and potentials consist in microfabricated systems. For medical/biomedical sector alone, it had been estimated that the worldwide market shipment for microsystems would be $2.4 billion in year 2000 and grew to $7.4 billion in 2004, with a compound annual growth rate of 32.5%
Till recently, there have been over 120 companies producing and commercializing miniaturized analytic devices, ranging from miniaturized sensors, high-throughput flow cytometry, and HPLC chips to disposable electrode arrays, tissue arrays and lab CDs, etc [2].

Nowadays, MEMS techniques have been increasingly and widely extended to medical and biochemical fields for both fundamental research and device applications, altogether called BioMEMS [1,3], which is also the focus of this review on microfabrication. BioMEMS techniques provide some unique advantages: (1) small dimensions with high resolution and sensitivity, which does not only allow saving materials and energy but also makes some other advantages possible, such as (2) the ability to incorporate sensing and actuating functions in proximity or on the same substrate, (3) the ability to incorporate, probe, manipulate, and study molecules and cells, (4) minimal invasiveness, (5) portability, (6) shorter analysis time, and (7) the ability to facilitate high-throughput experimentations due to redundancy and arrays [1,3,4]. An excellent example to demonstrate these advantages is the concept and applications of lab on a chip (LOC) or micro total analysis systems (μ-TAS), which combine fluid movement with elements of processing, derivatizing, reacting, mixing, and fractioning of sample and reagents [2]. In the following sections for microfabrication, reviews on major techniques and their applications will be presented.

2.1.1 Techniques and materials for microfabrication

Based on the differences of materials, there are two categories of microfabrication, hard micromachining and soft micromachining. Hard micromachining targets “hard” materials, such as silicon, glass, GaAs and metal etc., while soft micromachining uses
polymers and gels, which are relatively “soft” materials [3]. Most of the hard micromachining techniques have their root in standard IC industry, basically including (1) thin-film deposition, (2) lithography, (3) etching, and (4) substrate bonding [3,5,6]. Soft micromachining is also referred to as soft lithography [3,7] including a plethora of techniques which may be summarized as (1) micromolding, (2) hot embossing, and (3) microcontact printing [7].

2.1.1.1 Thin-film deposition

Thin films have applications for a wide variety of areas and purposes, such as optical reflective/antireflective coatings, electrical insulation/conduction, magnetic memory discs, chemical gas/liquid sensors, mechanical tribological coatings and thermal barrier layers, etc [8]. Therefore, various materials and processes are involved to achieve a precision of thickness down to 20 Å [1]. The selections of source material, substrate material and process need to be considered for compatibility. Generally, thin-film deposition techniques mainly include oxidation, chemical vapor deposition (CVD), physical vapor deposition (PVD), and electrodeposition [3].

Oxidation is typically performed for a silicon substrate at high temperatures (between 600 and 1250°C) in a stream of water vapor at 1atm or in wet or dry oxygen/nitrogen mixtures, which results in an elevated oxide layer following the ratio that the amount of silicon consumed is 46% of the final oxide thickness [1,3]. Oxidation is usually used in semiconductor industry.

In CVD, reactive source gases, which may be from gases, evaporated liquids, or chemically gasified solids, are carried to a hot surface (typically higher than 300°C) in convection and diffusion, allowing molecular collisions and reactions to deposit a
solid film [1,3,8]. Two CVD techniques are most commonly used in microfabrication: low pressure CVD (LPCVD) and plasma enhanced CVD (PECVD), which are usually used for depositing inorganic materials, such as silicon nitride, silicon dioxide, and polycrystalline silicon. LPCVD is performed with pressures as low as 0.1 to 1 Torr and at temperatures in the range of 550-900°C, resulting in films very conformal to the substrate’s surface with excellent purity and uniformity. However, lower temperatures such as 300-400°C may often be preferable in many processes, so PECVD becomes the choice. PECVD also provides additional advantages such as fast, good adhesion, and good step coverage by using radio-frequency-driven plasma activation that provides reactive radicals, and by using ion bombardment of the substrate that provides the energy required to arrive at the desired depths. However, chemical and particulate contamination and less conformity may be the disadvantages [1].

PVD are performed with two major different principles: evaporation and sputtering. The source raw materials may be solid, liquid, or vapor. In the evaporation system, the raw material is heated and spread all over the chamber in vacuum containing the substrate, resulting in a thin film deposited on top of the substrate. During sputtering, the source raw material (usually in the form of a disc), at a high negative potential, is bombarded with positively charged argon ions created in a plasma. Neutral atoms of the source material are generated by the momentum transfer of the bombardment, which are consecutively ejected and deposited onto the substrate placed on the anode, resulting in a thin film with a wider choice of materials, better step coverage, and better adhesion to the substrate than evaporation [1,3].

Electrodeposition, also electroplating, as its name suggests, is an electrochemical
process, where the substrate, which usually has a very thinly deposited seed layer of the desired metal to make it conductive, if the substrate is dielectric, is immersed in a solution of its reducible ions at a negative potential (cathode). The ions are reduced and deposited at the substrate surface. Electrodeposition may help obtain a layer as thick as tens of microns [3], and is a middle step in a lithography technique called LIGA.

2.1.1.2 Lithography

Lithography is the technique used to transfer a master pattern onto the surface of a solid material such as a silicon wafer [1]. Currently, photolithography has been the most successful technology in microfabrication [7]. Briefly, the process is composed of the following steps: (1) A 2D pattern is first created with a computer-aided design software. (2) A photomask is then generated following the CAD design. (3) After thin-film deposition of the desired material on a silicon wafer, a thin and uniform layer of photoresist is spin coated. Then (4) the coated wafer is soft baked, removing the solvents and enhancing adhesion. (5) Subsequently, the wafer is exposed to UV light under the photomask. Then (6) the unpolymerized photoresist is dissolved off the wafer. (7) A hard bake is used to further improve the adhesion and concludes the process. The process is basically as shown in Figure 2.1. Then the patterned wafer may be later used for etching to transfer the pattern to the deposited thin film on the wafer, if necessary, which I will focus in details on the etching part of the review. Besides photolithography, there are some other types of lithography, such as e-beam lithography, extreme ultraviolet lithography, X-ray lithography [such as LIGA (German acronym for Lithographie, Galvano-formung, abformung)], charged-particle-beam lithography as the next-generation lithographies [1]. Here I
will focus on photolithography techniques in this review, as it is most widely used and plays a key role in the proposed research.

2.1.1.2.1 Masks

Masks, also photomasks, carry the designed pattern in a light field or dark field image on a flat optic medium, and transfer the pattern to the photoresist on the silicon wafer by UV exposure. In IC industry and high resolution photolithography, masks are a nearly optically flat glass or quartz plate with an absorber pattern metal (e.g., an 800 Å thick chromium layer), which is generated by e-beam lithography that has a higher resolution than photolithography, while for the photolithography experiments with a less-demanding resolution, especially rapid prototyping, a mask may even be printed with a high-resolution printer (say 4000 dpi) on a transparency [1].

2.1.1.2.2 Photoresist

The composition of a photoresist is a polymer (base resin which changes structure upon radiation), a sensitizer (controlling the photochemical reactions in the polymer), and a casting solvent (allowing thin layer formation by spin coating). There are two types of photoresist in response to UV exposure. One is called positive tone, which becomes much more soluble than the unexposed by rupture or scission of the main and side polymer chains. For example, some popular positive photoresists are poly(methylmethacrylate) (PMMA) and diazoquinone ester (DQ) and phenolic novolak resin (N), i.e., DQN. The other type acts oppositely and forms strengthened and less soluble polymer by random cross-linkage of main chains or pendant side chains. Commonly used negative tone resists are bis(aryl)azide rubber resists [1]. A very popular negative photoresist, which is also the resist used in the proposed research, is
SU-8 epoxy, invented by IBM scientists. SU-8 is composed of EPON SU-8 resin (molecular structure shown in Figure 2.2) and a photosensitizer triaryl sulfonium salt [9,10]. SU-8 resist is specifically designed for micromachining for thick films up to 500μm in a single coat and has excellent sensitivity, high resolution, low optical absorption, high aspect ratios, and good thermal, and chemical stability, with low cost. This is based on several properties of the material system: (1) photoinitiators could polymerize low-cost epoxy resins and are stable with oxygen and a large temperature range; (2) SU-8 photoepoxy, with a low molecular weight (~7000±1000) and high solubility, may have a high level of cross-linking density and form a strong polymer (with Tg more than 200ºC) in thick films. SU-8 is not only sensitive to UV light in the 365 to 436 nm range, but also to electrons and x-ray. Moreover, SU-8 has an amazingly low optical absorption in the UV range. In principle, layers up to 2mm thick could be obtained which made it an alternative method to LIGA for making high-aspect-ratio structures and is sometimes referred to as poor man’s LIGA [1,11].

2.1.1.2.2 Spin coating and soft baking

Spin coating is achieved by dispensing a viscous solution of resist onto the wafer placed on a vacuum chuck. Then the wafer is spun at a high speed, ranging from 1500 to 8000 rpm, depending on the viscosity and the desired thickness, to form a uniform film of photoresist. This is a critical step to ensure the success of pattern transfer, which should be done with extreme care to eliminate defects and ensure uniformity. After spin coating, a soft baking ensues at 75 to 100 ºC for, say, 20 min to evaporate the solvent and to remove the built-in stress, in order to enhance the adhesion of the resist to the silicon wafer.

2.1.1.2.3 Exposure and post-exposure treatment
During exposure, the photoresist-coated wafer should be aligned with the photomask. The mask may be in direct physical contact with the substrate, called hard contact, or in proximity to the substrate, known as soft contact. These two modes of contact are collectively referred to as shadow printing, which may cause quick worn-out of the mask, so they are typically just used in R&D and prototyping, while in very large scale integration (VLSI) manufacturing, projection printing is used, where the mask pattern may be reduced by 1:5 to 1:10 by projection. Most simply, an exposure system is composed of a UV lamp to deliver light in proper intensity, directionality and uniformity throughout the wafer through a photomask to transfer the desired pattern. The wavelengths used in photolithography range from extreme ultraviolet (EUV) (10 to 14nm) to deep ultraviolet (DUV) (150 to 300nm) to near ultraviolet (UV) (350 to 500 nm). For near UV, a mercury lamp is usually used for the g-line (435 nm) or i-line (365 nm). And i-line is recommended for SU-8 photoresist. The exposure time (in seconds) may be given by the division of the required incident energy (J/cm²) or dose across the photoresist surface by the incident light intensity (W/cm²) [1].

Postexposure treatment is usually necessary to stop the ongoing polymerization reaction in the photoresist. Measures that are taken to stop the reaction usually are hard baking, flood exposure with other types of radiation, treatment with reactive gas, and vacuum treatment. In photolithography with SU-8, a hard baking is usually applied as postexposure treatment [1].

2.1.1.2.4 Development

The unpolymerized photoresist needs to be washed away from the wafer, leaving the desired latent features to stand out, which may be used as a mask for further subtractive or additive steps or as a mold for pattern transfer with soft lithography.
There are two major technologies for development: wet development and dry development. The former is widely used in microfabrication in general, and the latter is beginning to substitute wet development for applications with fine line width resolution. In wet development, there are usually three exposure-induced changes of the photoresist involved for the process: variation in molecular weight of the polymers (by cross-linking or by chain scission), reactivity change, and polarity change. Wet development may also work in two ways: immersion and spray. Dry development is devised to overcome some disadvantages of wet development, such as some swelling of the resist in organic solvents and loss of adhesion. However, dry developed resists should be used [1].

2.1.1.3 Etching

Photolithography may be followed by etching to remove thin-film material according to the pattern with selectivity and directionality. Etching can also be categorized as wet and dry. In wet etching, basically, isotropic removal of material may be obtained with good selectivity with an exception of anisotropic etching of crystalline silicon. Three major anisotropic silicon etchants are commonly used: potassium hydroxide (KOH), ethylene diamine pyrochatechol (EDP), and tetramethyl ammonium hydroxide (TMAH), which can attack silicon along preferred crystallographic directions. Masking materials for wet etching is usually silicon dioxide and silicon nitride. In contrast to wet etching, dry etching may make finer structure with higher anisotropicity, but the selectivity is poor. There are three major dry etching techniques: ion milling, high-pressure plasma etching, and reactive ion etching (RIE). Ion milling is a physical process where accelerated inert ions (e.g. Ar⁺) attack the surface to remove materials, which has a very low etch rates (nm/min) and poor selectivity. In
high-pressure (0.1-5 Torr) plasma etching, chemical reactions take place between highly reactive species and the material surface, generating volatile products for continuous material removal. RIE, however, is a combination of physical and chemical processes. In deep RIE (DRIE), a covering step by coating a passivation layer of polymer and an ion bombardment step are repeated, which may achieve a deep trench with vertical walls of aspect ratios of 30:1 at silicon etching rates of 2 to 3 μm/min. Commercial DRIE etchers usually use SF₆/Ar in the etching step and a combination of Ar⁺ and a fluoropolymer (nCF₂) in the passivation [3].

2.1.1.4 Substrate bonding

In hard micromachining, substrate bonding (silicon-silicon, silicon-glass, and glass-glass) plays a key role in 3D microstructure formation, and in microsystem packaging and encapsulation. Silicon-silicon fusion and silicon-glass electrostatic (or anodic) bonding are the two most important substrate bonding techniques. In silicon fusion, the thoroughly cleaned surfaces are made hydrophilic by hydroxylation in HF or boiling nitric acid. Then the substrates are brought in contact and hydrogen bonds form between them, joining the two substrates. This can be achieved at room temperature, but a high temperature annealing is usually required (800-1200°C), which is usually necessary to strengthen the bond. Silicon-glass anodic bonding is extensively used for microsensor and device packaging, where the two substrates are brought in contact as part of an electric circuit with a voltage of around 1000V. A depletion layer of sodium ions of the glass at the silicon-glass interface is formed and the two substrates are brought together in close proximity with a strong electrostatic force. However, the precise chemistry of the process is still not clearly known [3].
Above is a review on the techniques for hard micromachining, which serves as a basic introduction of micromachining techniques with their origins. In the proposed research, however, we use soft micromachining techniques, which collectively are called soft lithography due to the use of “soft” materials with much lower cost and better biocompatible properties with a wide performance range of sizes (Figure 2.3), reviewed as follows.

2.1.1.5 Rapid prototyping and ultra-rapid prototyping

The conventional way of making a photomask with chrome patterns is time consuming and expensive, at a cost of about $200 per square inch for features larger than 5μm and $500 per square inch for features between 1 and 5 μm [7]. This constitutes a serious barrier to the application of microfabrication techniques in biomedical research. With the advent of rapid prototyping for photolithography [12], however, features that are equal to or larger than 20μm [limited by the resolution (3387 dpi) of the image-setting system] in a transparency mask could be generated with a desktop computer and high resolution printer system in a few hours at a cost of about $1 per square inch [7]. This process has substantially accelerated photolithography with a much lower cost, which helps pave the road for the wide applications of microfabrication in biomedical research. With this procedure, numerous applications have been reported. Nevertheless, the most time consuming and expensive step of this procedure is the master fabrication by photolithography. Beebe and colleagues have introduced a novel ultra rapid prototyping method of microfluidic systems by using liquid phase photopolymerization to address this issue [13]. This method bypasses the photolithography for master, directly generating the structures from design in a cartridge within several minutes. However, resolution is
even poorer for this pattern transfer process, and so far it has limited applications.

2.1.1.6 Micromolding

Soft materials, such as polymers and gels, are amenable to a series of micromolding techniques under relatively mild conditions, such as replica molding (REM), microtransfer molding (μTM), micromolding in capillaries (MIMIC), and solvent-assisted micromolding (SAMIM) (Figure 2.4). The masters for molding may be generated with hard micromachining techniques, such as photolithography, and soft lithography techniques, such as an elastic master.

Replica molding (Figure 2.4A) duplicates the shape, the morphology, and the structure from a master. It has the capability of 3D pattern transfer in one step, whereas photolithography does not have this capability. Replica molding may achieve high fidelity and resolution in complex pattern transfer with an appropriate material usually by using a prepolymer, which is determined by van der Waals interactions, wetting, and the filling of the mold. The process is reliable, fast, simple and inexpensive with a resolution as small as below 100nm for which photolithography runs to its limit due to optical diffraction [1,7]. Furthermore, structures as complex as optical surfaces may also be formed by replica molding against elastomeric masters [14].

In microtransfer molding (Figure 2.4B), a drop of prepolymer is applied on the surface of an elastic master and the excess liquid is removed, which is followed by placing it against a substrate. After the prepolymer is cured and the master is removed, a patterned microstructure is left on the surface of the substrate [15]. Both isolated and interconnected microstructures may be obtained through microtransfer molding.
with the advantage over other microfabrication techniques that microstructures may be transferred to nonplanar surfaces, which facilitates the fabrication of multiple layers of 3D structures [7].

In micromolding in capillaries (MIMIC) (Figure 2.4C), elastomeric master is placed in conformal contact with the substrate, leaving the interconnected microfeatures to form a network. Then a low-viscosity prepolymer is applied at the open ends of the network and sipped in by capillary force. After curing of the precursor and the removal of the master, an interconnected microstructure may be formed on the substrate surface. MIMIC is a convenient microfabrication technique. However, there are some limitations, especially for (1) the inability to make isolated structures and (2) the rate and distance of the prepolymer filling are small due to viscous drag in the microchannels [7].

Solvent-assisted micromolding (SAMIM) (Figure 2.4D) shares some characteristics of replica molding and embossing. In SAMIM, an elastomeric master is wetted with a solvent that well dissolves the substrate polymer but not the master, and brought into contact with the substrate. The solvent swells a thin layer of the polymer, in which the microstructures may be generated according to the master’s topology. The success of SAMIM depends on the choice of the solvent, which not only should have selectivity in dissolution but also should have high vapor pressure. The SAMIM procedure is a simple technique to form complex quasi-3D features in one step. It is different from commonly used hot embossing, in that it uses solvent to soften the polymer substrate instead of heat, and the master is elastomer instead of hard materials [7].

2.1.1.7 Hot embossing
Hot embossing is a low-cost and high-volume microfabrication technique [7]. With an embossing master, the substrate is mounted in an embossing machine which has a heating plate also containing cooling channels. A vacuum is applied and temperature is elevated just above the glass transition temperature $T_g$ of the polymer substrate. Then a force, typically of the order of 20-30 kN, is applied. With the presence of the embossing force, the system is cooled to just below the $T_g$ to further stabilize the microstructures. Then a lower temperature, say 30ºC, is reached, followed by the removal of the embossing master from the substrate, which concludes the process [16].

2.1.1.8 Microcontact printing

Microcontact printing is a remarkably simple procedure, where an elastic stamp bearing some “ink” is printed in conformability to a substrate surface, resulting in an additive self-assembled monolayer (SAM) with patterns of submicron lateral dimensions transferred from the elastomeric stamp [17]. The SAMs are only 1-3 nm thick, and the lateral dimensions may be fabricated as small as 20nm [18]. The mechanism of forming SAMs on a gold or silver substrate is that alkanethiolates $\text{CH}_3(\text{CH}_2)_n\text{S}^-$ may chemisorb spontaneously and instantaneously on gold or silver surface assumed by losing dihydrogen, resulting in the alkyl chains of up to approximately 20 atoms to form ordered structures [7]. The patterned SAMs may be used as ultrathin resist in selective wet etching [18,19] or as passivating layers in selective deposition [20-22].

2.1.1.9 Poly(dimethylsiloxane) (PDMS)
Traditional microfabrication is based on silicon and glass, which requires processes that are time-consuming and expensive. Polymer is, by contrast, inexpensive, easy to handle and with widely tunable properties and biocompatibility. Silicone elastomer PDMS (Figure 2.5) has been the most popular soft material in biomedical microfabrication applications due to very suitable physical and chemical properties (Table 2.1)[23]. These properties may be translated to the following important operation advantages: (1) PDMS has high fidelity in pattern transfer down to 10’s of nm[23]. (2) It is an elastomer which has superior conformability to other surfaces and may be actuated for making microfluidic components, such as pumps and valves [24-26]. (3) It has good sealing with itself and other surfaces, both reversibly and irreversibly, such as silicon, glass, polystyrene, polyethylene, or silicon nitride, provided both the surfaces are treated with oxygen or air plasma for about 1min [27,28] or exposed to UV/ozone [29] , for irreversible sealing. (4) PDMS may form complex 3D networks by stacking multiple layers in alignment due to its transparency and good sealing [30]. (5) PDMS is highly permeable to gases, especially nonpolar gases, such as O₂ and CO₂ [31,32], which facilitates cell-based applications. However, PDMS is hydrophobic, which may constitute some challenges in microfluidic applications due to capillary pressure. However, the surface of PDMS may be rendered hydrophilic by strong oxidants, such as oxygen plasma, UV/ozone and etc., with comparably limited degrees, but may recover to hydrophobic in a few minutes in the air. Storage in water may slow the recovery process. In addition, PDMS is prone to protein adsorption and biofouling. Therefore, surface treatments to prevent unwanted adsorption have been developed using surfactants and sol gel, etc. [33-35]. The most widely used PDMS is Sylgard 184 silicone elastomer kit from Dow Corning, composed of a base and curing agent. When forming the elastomer, the liquid
prepolymer base and the catalytic curing agent is mixed by a ratio of about 10:1 and heated to about 70°C for 2 hours. The higher the temperature for curing, the faster the polymer forms (10 min @ 150°C and 45 min @100°C) (Dow Corning website, 2006).

2.1.2 Microfluidics

The miniaturization techniques and materials reviewed above provide the enabling platforms for analytical applications in chemistry, biology and medicine. However, the control of fluid behaviors at microscale requires scientific development of microfluidic theories and engineering establishment of various functional devices.

Water’s potential in microfluidic channels, the powering of the flow, the prominent effects from large specific surface area, the parallelization of multiple flow streams, the functional component development, and system integration all are within the scope of our discussion at both fundamental and application levels.

2.1.2.1 Water in microfluidic channels described with water potential [36]

Water is transported in microfluidic channels by pressure sources (syringe pumps, micromachined pumps or peristaltic pumps), or by local body forces which can be electrical (electroosmotic flow or electrohydrodynamic flow) [37,38], magnetic (magnetohydrodynamic flow) [39,40], centrifugal [41,42], or surface-tension directed [43,44]. Water transport may also occur based on processes like evaporation [45], pervaporation, and osmosis [46]. All the water transport processes are based on different water chemical potentials with flow from high to low potentials. Factors that determine the liquid water potential may come from gravity, from water dilution by dissolving substances, from interaction with solid and gas interfaces, and from applied
pressures, for which an equation has been developed to describe the chemical potential of liquid water:

$$\Psi_i = \Psi_0 + \frac{RT}{V_w} \ln(a) + (P_{hy} - P_0) - \frac{2\gamma \cos \theta}{d} + \rho g (h - h_0)$$  \hspace{1cm} (2.1)

In the equation, $\Psi_0$ is the reference potential, usually taken as the potential of pure water at sea level and with standard temperature and pressure. The term $\frac{RT}{V_w} \ln(a)$ represents entropy of liquid water, with increased mixing decreasing the free energy. $(P_{hy} - P_0)$ indicates the applied pressure, and $-\frac{2\gamma \cos \theta}{d}$ is the term for pressure derived from surface tension for a slit orifice ($w \gg h$), with $\rho g (h - h_0)$ indicating potential due to gravity.

2.1.2.2 Pressure driven flow (PDF)

It is intuitive to use pressure to power a fluid flow due to the easiness of handling complex fluid samples and the largely available apparatus for pumping purposes, with the exceptions of challenging development of self-contained on chip pump components. It is the focus of PDF to investigate the relationship between volumetric flow rate and pressure drop, which may be expressed in the following equation [47].

$$Q = \frac{1}{\mu} \frac{\Delta P}{R_F}$$  \hspace{1cm} (2.2)

where $Q$ is volumetric flow rate, $\Delta P$ is pressure drop, $\mu$ is viscosity and $R_F$ the flow-rate resistance. For rectangular microfluidic channels, as usually seen in most microfluidic devices, the solution to $R_F$ is a geometric term with a Fourier series [47]:
where $a$ and $b$ are either width or depth, respectively, satisfying $a<b$, $L$ the length of
the microchannel, and $m(n,a)=(\pi/a)(2n-1)$. This equation may be approximated by a
linear solution:

$$R_F = \left[ \frac{ab}{12L} \left( 1 - \frac{3a}{b} \frac{64}{5\pi^5} \sum_{n=1}^{\infty} \frac{\tanh(m(n,a))b/2}{(2n-1)^5} \right) \right]^{-1} \tag{2.3}$$

where $R_F$ is the hydraulic radius, which may be expressed as:

$$R_F = \left[ \frac{1}{12} \frac{(a+b)^2}{b^2} \left( 1 - \frac{1}{8b} \frac{abR_H^2}{L} \right) \right]^{-1} \tag{2.4}$$

with $A$ being the wetted cross sectional area and $P$ the wetted perimeter of the channel.
Due to the small dimension of the microfluidic channels, the flow basically falls into
the laminar category with Reynolds number (Re) much smaller than 2000 or 2300,
which is empirically used as the transitional Re for distinguishing laminar flow and
turbulent flow. Re is defined as:

$$\text{Re} = \frac{2R_H \rho v}{\mu} \tag{2.6}$$

where $\rho$ is density, and $v$ the flow velocity. Re compares the relative magnitude of
inertial forces and viscous forces.

### 2.1.2.3 Capillary effect

A major characteristic of microfluidic devices is the large specific surface area.
Therefore, capillary pressure due to surface tension at the air/liquid meniscus walled
by solid microchannels plays a dominant role in the multiple factors affecting fluid flow. This pressure is described by Young-LaPlace equation:

$$P_c = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)$$

(2.7)

where \( \gamma \) (N.m\(^{-1}\)) is the surface tension of the liquid, and \( R_1 \) and \( R_2 \) indicate the two radii of the curvature of the water meniscus at the interface. For rectangular channels, the capillary pressure can be expressed as follows (Junker et al, 2002):

$$P_c = -\gamma \left( \frac{\cos \alpha_b + \cos \alpha_t}{d} + \frac{\cos \alpha_l + \cos \alpha_r}{w} \right)$$

(2.8)

where \( \alpha_{b,t,l,r} \) are the contact angles at the bottom, the top, the left and the right walls, respectively, with \( d \) and \( w \) representing the depth and width of the channel. For hydrophilic channel material, such as glass, the contact angle is less than 90°, while hydrophobic surface has a contact angle between 90° and 180°. The capillarity effect has been favorably used as the guiding force for microfluidic flows, since the fluid may automatically enter the microchannel without external actuation mechanisms if the wall is hydrophilic [43,44]. Also, capillary phenomena in microfluidic networks have been used for patterning proteins and for immunoassays [48-50]. And these systems are referred to as autonomous capillary systems [51,52]. For fluid flow with abruptly shrinking channels, hydrophobic walls may generate a passive valve effect [53], with which parallel multiple flows may be controlled, a very meaningful operation for high-throughput assays with microfluidic devices. And the pressure required to overcome the passive valve effect is shown in the following equation [53]:
\[ \Delta P = 2\gamma \cos(\alpha)\left[\left(\frac{1}{w_1} + \frac{1}{l_1}\right) - \left(\frac{1}{w_2} + \frac{1}{l_2}\right)\right] \]  

(2.9)

where the contact angle is the same for all walls due to the assumption of using the same hydrophobic material for the four walls, and \( w_2 \) and \( d_2 \) indicate the width and depth of the constriction channel, respectively.

2.1.2.4 Microfluidic valves and pumps

A microfluidic device may require different functional components to work together to fulfill its purpose. Valves are very common parts for a fluidic conduit. There have been various microfluidic valves developed so far. By multilayer soft lithography, Quake and colleagues developed a microfluidic system containing on-off valves and switching valves by using air-actuated control lines on top of flow lines based on elastomer [54]. Tunable hydrogels were also used to make biomimetic valves [55,56]. Another example of biomimetic valve is a passive “lymph” valve, which may allow or stop flow under different flow directions based on the elastomer and the lymph design [23]. The compression of the valve may also be obtained with magnetic forces [57]. Passive valves were also developed based on capillarity to control parallel flows [53].

Valves may be further exploited for actuation as a pump. An array of parallel control lines over the flow lines of the on-off valve above may work one by one to drive fluid flow [54]. The lymph valves with one directionality discussed above may be fabricated in a series, and when two lanes of such valves with opposite directionalities are connected with a press reservoir, liquid may be pumped through with this novel mechanism [23]. Beebe and colleagues developed a magnetically-driven oscillation bar, just as a swimming fish with its tail fin to generate vortices, pumping liquid in
one direction [58]. Surface energy, based either on hydrophobicity [59] or hydrophilicity [51] has also been utilized to make microfluidic pumps. In many examples, elastomer membrane is used as valves and diaphragm actuation is applied to make the valve work as a pump or even work as logical structures [60-62]. Effervescent chemical reaction was even used to pump fluids [63]. Furthermore, there have been novel micropumps that use cellular energy, such as the intrinsic pulsatile mechanical functions of cardiomyocytes [64,65]. There are also a number of review articles on micropumps [66-68].

2.1.2.5 Microfluidic mixers

Components such as microfluidic mixer may be very important in applications such as on-chip microreactors, drug delivery, sequencing, single cell or organism studies, and high-throughput analyses. Since laminar flow dominates in microchannels, complete mixing needs to be achieved with specific on-chip component. Passive mixing is based on a simple concept, and achieved mainly by molecular diffusion and chaotic advection, which may help to increase the contact surface and/or decrease the diffusion paths between different fluids [69], such as the T-mixer or Y-mixer [70,71], parallel lamination mixer with multiple streams either driven by pressure [72-74] or by electroosmosis [75,76], also serial lamination mixers with multiple splitting and joining stages both horizontally and vertically [77-79], and micromixers based on chaotic advection generated by special microchannel geometries or induced by an external force at high Reynolds numbers [80,81], intermediate Reynolds numbers [82-85], or at low Reynolds numbers [86-88]. There are also various active micromixers, depending on pressure field disturbance [89], electrohydrodynamic disturbance [90], dielectrophoretic disturbance, electrokinetic disturbance [90,91],
magneto hydrodynamic disturbance[92], acoustic disturbance [93,94], or thermal disturbance [95].

2.1.2.6 Microfluidic gradient generators

Chemical gradients play an essential role in developmental and physiological processes, such as axon guidance [96], immune response [97], differentiation of embryonic cells [98], chemotaxis cell migration [99,100], and drug screening. Therefore, for cell-based microfluidic devices, gradient generator is a very useful component. Gradients may be generated in a linear or a nonlinear fashion, since cells may respond differently according to the linearity of the gradient [101]. Many microfluidic gradient generators produce linear concentration gradients [102-106]. A membrane-based linear gradient generator was also developed without using streams of flow by connecting a high fluidic resistant membrane between a source and a sink for cell-signaling studies[107]. Nonlinear and complex gradients may be generated by using similar microfluidic networks in Jeon et al, 2000 [108,109]. Recently, a universal microfluidic gradient generator has been developed based on series of microdividers which are fabricated under the direction of mathematical models, especially for nonlinear spatial gradient generation [110]. Hydrogel may also be used between the source and sink reservoirs to generate concentration gradient in the underneath microchannels [111]. Multiple injectors may be pneumatically actuated to generate temporal and spatial concentration gradients of soluble molecules [112]. Similarly, arrays of horizontally-oriented mini-reservoirs may also generate different concentration gradient for cell culture [113]. Oxidative microgradients have also been generated using an array of microelectrodes [114].
gradient generators, there have been some microfluidic devices that are able to generate temperature gradient [115,116].

2.2 Microfabricated devices for cells

Microfabricated devices create unprecedented opportunities to study and utilize biological cells [117], with unique advantages of highly tailorable microenvironment that is of cellular relevant dimensions (from nm to μm) and of highly parallel experimentation that may generate abundant information. Therefore, the techniques for culturing, stimulation, handling, and analysis for cells at microscale have made tremendous progress since their combination, making microscale systematic cell-based studies possible, but still with substantial challenges in fabrication, integration and packaging (Figure 2.6). This section of the review will focus on the current progress in this field, with foci on cell culture and biosensing.

2.2.1 Microscale cell culture

Cells are fragile and sensitive, and their good maintaining and growth are usually the starting point for further experiments. Microfabricated devices for cell culturing, cell monitoring and cell-based assay has tremendous advantages with high-throughput and high-content capabilities. There are three major driving forces for developing such systems: tissue engineering, cell-based assays, and bioprocess parameter characterization.

2.2.1.1 Microtechnologies for tissue engineering

For tissue engineering, microfabrication may provide the means to create 3D extracellular matrix (ECM) structures, and microfluidics may continuously supply
nutrient and remove waste with providing mechanical cues for cell culture to mimic their *in vivo* counterpart. Microtechnologies may be applied to the studies of cell-ECM and cell-cell interactions, revealing how ECM and co-culture may affect cell attachment, proliferation, and function [118-124]. Tissue culture scaffolds may be generated with microfabrication, such as a multi-layer 3D scaffold with well defined dimensions [125], vascular tissue scaffold [126], and lay-by-lay microfluidics for biomimetic three-dimensional structures [127]. Specific tissues have been engineered with microfabrication and microfluidics techniques, such as liver cells, bone cells [128], cartilage culture [129], and neuron cell studies [130,131]. There are three good review articles on tissue engineering and cell biology in microfabricated devices [132-134].

2.2.1.2 Microtechnologies for cell-based assays

There are various noteworthy advantages of cell-based assays. Firstly, compared with animal experiments, human originated cell cultures are relevant to human physiology. Additionally, cell-based assays can leverage high throughput and integration strategies. Thirdly, specific cell types can be used to identify toxicity effects on different portals of entry and on specific distal target organs. Also, cells are sensitive and specific for showing different mechanisms of toxicity.

For cell-based assays, there has been very active research on liver cell culture facilitated with microfabrication and microfluidics due to the large need for valuable *in vitro* tools for hepatotoxicity and the challenge to maintain the phenotypes of hepatocytes *in vitro* [135-140]). In Sivaraman et al’s work, primary rat hepatocytes cultured in their system were substantially closer to native liver compared to cells cultured by other *in vitro* methods, as indicated by gene expression, protein
expression and biochemical activity assays. In Powers et al’s work, 3D hepatocyte culture was achieved with cellular aggregates without the usually necessary tissue engineering scaffold as ECM. In Leclerc et al’s work, the cell growth scaffold is monolithically created as part of the microfluidic channels, so actually it may be called a 2.5D tissue culture. Furthermore, the chambers were open to each other without segregated boundaries, which is unlikely to execute high throughput assay experiments. For other cell-based assay purposes, a perfusion microfluidic cell culture array was developed by Lee and colleagues [103]. The individual cell culture chambers were delicately-designed, and on the same chip, there was a linear gradient generator. They also reported further improvement of the continuous cell culture array on the microchannels for more effective cell filtering and uniform nutrient distribution within a chamber [141]. Recently, another improved version of the nanoliter scale microbioreactor array was developed, which achieved relatively uniform cell seeding and compared the effects of different concentrations of serum on cell proliferation [142]. Lee’s group also developed a dynamic single cell culture array, where individual single cells may be trapped at specific spot with a U-shaped hydrodynamic trapping structure in an array, and cell attachment, spreading, division and apoptosis were observed with this device [143]. For embryonic stem cell perfusion culture, a microfluidic array with logarithmical feeding concentration gradients was developed [144]. Dynamic gene profiling was also achieved with microfluidic living cell array [145]. Another excellent example is the cell culture analog (CCA) developed by Shuler and colleagues. CCA was first designed as a macroscopic prototype following physiologically based pharmacokinetic models (PBPK) [146,147]. Then this concept has evolved with microfabrication technology to a microscale CCA (μCCA) system [148], also called “animal-on-a-chip”. The system has advanced from a two-cell-type
(lung and liver), three-chamber \(\mu\)CCA system, representing “lung”-“liver”-“other tissue” with integrated dissolved oxygen sensor [60], to a two-cell-type, four-chamber \(\mu\)CCA system with “lung”-“liver”-“other tissue”-“fat” to better mimic the fluid distribution in different organs [149]. In addition, a three-cell-type, four-chamber \(\mu\)CCA system consisting of cells from liver, lung and fat have been developed to incorporate bioaccumulation effect in this toxicity evaluation device [150]. The very realistic metabolism, disposition and toxicity of a model toxicant, naphthalene, have been demonstrated with the \(\mu\)CCA system, which is impossible with other toxicity study systems alone. In essence, the objective of the \(\mu\)CCA system is to develop a surrogate of human body for chemical exposure assessment. However, \(\mu\)CCA may not be the ideal device for cell-based toxicity studies because it is based on expensive silicon microfabrication, and lacks high-throughput capability due to the design and assay techniques. Also it may overlook authentic responses due to 2D cell culture.

2.2.1.3 Microbioreactors for bioprocess development

Bioprocess development focuses on the optimization of culture conditions, where microtechnologies may play a key role in bringing up the throughput for data generation at microscale cell culture. A significant increase in the number of potential new drugs has also driven bioprocess development to build miniaturized bioreactor arrays for parameter characterizations in parallel, e.g. pH, dissolved oxygen (DO), optical density (OD) and substrate concentration, etc. [151-156]. The smallest working volume of microbioreactors has been down to 5\(\mu\)L with detection capacity of DO, OD and pH based on optical methods [154]. These microbioreactors only allowed batch operations, which is disadvantageous for microscale cell cultures. Firstly, the constantly changing culturing medium composition may camouflage the
real cause/effect relationship. Secondly, evaporation may further devastate the reliability of the culturing results obtained from microbioreactors. Not until recently, has a microfluidic chemostat been developed [157], but limited to microbial cultivation so far.

2.2.2 Analysis of living cells in microfabricated devices

This section will focus on the analysis techniques for whole cells in microfabricated devices. Basically, there are two types of readouts for living cell analysis: optical signals and electrochemical signals. The optical signals may be originated from reporter genes[158] or luminescent molecular probes (Invitrogen online handbook). The electrochemical signals are based on amperometric, potentiometric or impedance biosensing techniques [159,160].

2.2.2.1 Reporter genes and molecular probes

Reporter genes and molecular probes may have little to do with microfabricated devices, but they are the marker techniques to make cells demonstrate specific signals for qualitative or quantitative analysis. A reporter gene is the genetic sequence that encodes a selectable or distinguishable marker which is conjugated to the gene of interest and inserted into a cell or organism for expression. Luminescent reporter proteins are usually involved as the markers, such as green fluorescent protein (GFP) [161] and its variants enhanced green fluorescence protein (EGFP) [162], yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) reporter vectors [163], also red fluorescent protein dsRed reporter vector isolated from another organism [164], chloramphenical acetyltranserase (CAT) reporter vector [165,166], beta-galactosidase reporter vector [167], alkaline phosphatase reporter vector [168],
and luciferase reporter vector [169], etc. In order to express the reporter vectors, a
certain promoter sequence, which endows the specific responsiveness of the reporter
genes, needs to be conjugated, and so far thousands of proteins have been
co-expressed with reporter proteins in cells and even organisms [163]. The occurrence
of luminescence may require different conditions, such as in the presence of substrates,
an energy source or photoexcitation. EGFP-based reporter vectors are of particular
interest to the proposed research due to its unique advantages. Firstly, GFP contains
only 238 amino acids [170], which makes it a convenient tag to fused proteins for
targeting a subcellular structure. Secondly, GFP is autofluorescent, which means there
is no need to add substrates for its optical signal and thus noninvasive. Thirdly, GFP
may be readily excited with blue light or UV. The use of blue light may help reduce
phototoxicity to cells. Fourthly, wild type GFP is relatively stable with a half life of
around 26h, but it may be attenuated to around 5 hours, benefiting dynamic studies for
living cells [171]. Finally, the fluorescence intensity may be readily quantified by
means of microscope and fluorometer. Therefore, it is a simple, fast and reliable way
of probing living cells. Table 2.2 summarizes the some properties of different
fluorescent proteins. An excellent review article on GFP may be found in Tsien, 1998.

In order to monitor living cells that may convey dynamic and mechanistic information,
we need to choose a non-invasive and physiologically relevant transducer.
Fluorescence-based assays are ideal for these purposes. There have been numerous
applications so far making use the physiologically relevant optical signals. Due to the
availability of fluorescent proteins with different colors, multiple fluorescence assays
may be enabled due to simultaneous signal generations and independent
measurements if more than one reporter genes are introduced [172]. This is highly
desirable when an assay requires both cell growth assessment (an internal correction reference) and the detection of specific molecular responses [173] or when two toxicity effects are under investigation[174]. For assessment of cell growth in a dynamic mode, Hunt et al used stable green fluorescence protein (GFP)-expressing CHO cells to demonstrate the feasibility of this approach, where fluorescence intensity is linearly proportional to cell number for both static and dynamic quantifications with high sensitivity, low error rate and minimal sample preparation [175]. In addition, enhanced GFP (EGFP) and β-lactamase CCF2/AM assays have also been used in the evaluation of cytotoxicity of platinum complexes as potential antitumor drugs, where induced EGFP assay yielded a good correlation between LC50 and IC50 values for a series of known platinum complexes, and β-lactamase CCF2/AM assays were said to be a good high-throughput alternative [176]. Also, Steff et al reported that cells expressing EGFP exhibited a decrease in fluorescence when treated by various agents, such as chemotherapeutic drugs, UV irradiation, or caspase-independent cell death inducers. Kinetics and sensitivity of their EGFP-based assay were comparable to those of traditional apoptosis markers such as annexin-V binding, propidium iodide incorporation, or reactive oxygen species production. And analysis of EGFP protein content in cells undergoing cell death demonstrated that the decrease in fluorescence does not arise from degradation of the protein [177].

Besides fluorescent proteins, fluorescent dye assays have also developed into an extremely useful tool for detection of physiologically relevant events at cellular and subcellular levels. A good example is the development of 3-D collagen hydrogels with neural cells for functional cell-based biosensing [178]. Cells were stained with different dyes for proliferation assay, resting membrane potential determination and
calcium imaging. In addition, molecular probes may be used to trace cell morphology, endocytosis, receptors, ion channel signal transduction and so on (Invitrogen online handbook).

2.2.2.2 Electrochemical sensing of cells

The progress of microfabrication, such as with thin film deposition techniques, has enabled the creation of micrometer scale electrodes to probe individual living cells for determining molecular and ionic fluxes, monitoring their growth, physiological state and responses to xenobiotics [179]. In terms of the mechanisms used for electrochemical analysis of living cells, there are basically two ways to measure them, by impedance or by extracellular potential [180,181]. Cells may be considered as impedance, which is equivalent to a combination of resistors and capacitors in a circuit [182] Luong, 2003). Interdigitated electrode arrays may be used to measure cellular impedance from the adherently growing cells on the electrodes. In another scenario, certain types of cells, such as myocytes and neuron cells, are electrically excitable, which may be used to record external biopotential changes upon stimuli [183-185].

Optical and electrochemical cell-based biosensing techniques each have their own advantages and limits. For optical signals, the visualization of cellular and subcellular events may provide high content screening (HCS) information for a wide variety of cell behaviors with spatial and temporal precision [186]. However, expensive and bulky detection systems are usually necessary for optical signals. In addition, cells usually need to be first treated with fluorescent dyes or transfected with reporter genes before optical signals may be generated. In contrast, electrochemical biosensing basically does not require such expensive treatments and the peripherals for detection
could be minimal in comparison to optical equipment. However, the majority of the
electrochemical biosensors are limited to excitable cells for detecting bioelectrical
phenomena, membrane integrity, molecular or ionic fluxes, so the information is not
as rich as optical biosensors. Nevertheless, interestingly, there are some publications
taking advantages of both ways [187,188]. This combinatorial approach may provide
an even high content screening capability, especially beneficial to drug discovery in
pharmaceutical industry.

2.3 Three-dimensional (3D) cell culture

Cells may be cultivated in suspension or in an anchorage-dependent fashion. For
cell-based studies, most cell cultures, just as their in vivo situations (except cells in
hematic and lymphatic circulation systems), are maintained with a surface substrate,
either as a monolayer or in a three-dimensional matrix. 3D cell culture has been
widely used as one of the enabling technologies for tissue engineering[189], and is
also becoming more and more important in fundamental cell biology studies [190]. In
this section, we will review 3D cell culture the technology itself as tissue engineering
and compare it with its 2D counterpart in terms of how close they mimic the in vivo
cellular behaviors.

2.3.1 The “quartet” of tissue engineering

Generally, it is considered that there is a triad of key factors of tissue engineering
[191], which are cells, tissue-inducing substances and scaffolds. Following this line,
another critical factor, bioreactors, is counted in to make this group a quartet. We will
discuss them one by one.

2.3.1.1 Cells
For cell-based tissue engineering, isolated cells are the raw material. So far virtually every mammalian tissue has been attempted to be tissue engineered [189]. The sources of cells may include autologous cells from the patient, allogeneic cells from a human donor, and xenogeneic cells from a different species. Each category may also be divided as adult cells or embryonic stem (ES) cells. However, the cell sources impose the number one challenge for tissue engineering due to the lack of proper cells [192], though there have already been skin substitutes developed for successful healing surgeries [193]. There have been high hopes for ES cells, but the source for ES cells itself is a controversial problem and possible immunological barriers are not fully explored [192]. However, adult stem cells, such as bone-marrow stem cells and haematopoietic stem cells, or applied together with ex vivo gene therapy techniques, have shown huge potentials for various tissue regenerations [194].

2.3.1.2 Tissue-inducing substances

Another important factor of tissue engineering is tissue-inducing substances, which include adhesion factors and growth factors. An adhesion domain discovered from fibronectin and other ECM proteins contains the amino-acid sequence of Arg-Gly-Asp (RGD), which may be engineered into the scaffold biomaterials to promote adhesion and control cell migration speed [195]. Growth factors, such as epidermal growth factors (EGF) and angiogenic factors VEGF and FGF, may be added directly to the culture medium to promote cell growth, and they can also be incorporated into the scaffold biomaterials either tethered or with controlled release as well to better improve tissue regeneration [196-199]. In addition, these factors may also be provided through co-culture systems.

2.3.1.3 Scaffolds
Cells are placed on or within porous matrices, which could be naturally originated, such as collagen and fibrin, or synthetic, such as polymers. For synthetic polymer scaffold materials, it has been found that the bulk material, 3D morphology, structure, porosity, surface chemistry and mechanical properties of scaffolds need to be specifically designed and processed to facilitate cell proliferation, migration and function [200,201]. The methods for fabricating porous scaffolds include particulate leaching, freeze-drying, gas infusion, and phase separation, etc, which generally create isotropic microstructures [201,202]. There have also been novel techniques to make scaffolds with designed microstructures, such as solid-free-form fabrication methods (e.g. three-dimensional printing process and stereolithography) [192,203] and multi-layer scaffolds with well defined dimensions using microembossing and carbon dioxide bonding [125]. For the proposed research, well-tested polyethylene terephthalate (PET) nonwoven fibrous matrix [204-211] would be adopted as the tissue engineering scaffold. Thermal compression and boiling base treatments have been utilized to alter the porosity and surface chemistry of the PET scaffolds.

2.3.1.4 Bioreactors

Bi-dimensional tissue culture, such as for skin, is widely performed in both academia and industry. In contrast, high density 3D tissue cultures may be only achieved by well designed and operated bioreactors which have continuous control for a number of parameters, such as temperature, pH, biochemical gradients and mechanical stresses, since the environment, both biochemical and mechanical, needs to be well monitored and controlled to produce large quantities of functional tissues ([212]. There are mainly two types of bioreactors, rotating wall and fixed wall. It has been reviewed that rotating-wall bioreactors are mainly suitable for fragile tissue culture,
while fixed-wall bioreactors may provide better mechanical cues for tissues with complex geometry [212]. In terms of operation, it has also been found that perfusion culture is most beneficial to 3D cell culture [213]. If bioreactors are considered from the perspective of how tissue engineering scaffolds are incorporated, they may be categorized as hollow fiber bioreactors (HFB) and packed bed bioreactors (PBB). In HFB, porous membrane form parallel capillaries and cells grow in the extra-capillary space and medium is transported within the capillaries. High cell density may be achieved \(2\times10^8 \text{cells/mL}\), but membrane fouling and significant pressure drop may be problematic for HFB [214,215]. In PBB, a wide variety of scaffold materials may be selected depending on the requirement of tissue regeneration. A fibrous bed bioreactor (FBB) has been developed for high density 3D mammalian cell culture with the capability of ES cell expansion [216], and recombinant protein production [209,217]. In the proposed research, it is essentially the development of a miniaturized FBB array with parallel perfusion operation.

2.3.2 Make a difference: 3D vs. 2D in cell culture

Besides prosthetic purposes, 3D tissue culture also has huge potential in establishing \textit{in vitro} models for cell biology, disease models and drug testing [192,218]. In the following review, 3D and 2D cell cultures will be compared to demonstrate the differences in using the two.

For adherent cell cultures, the first difference between 3D and 2D cultures comes from attachment of cells to the ECM. An elegant report on cell-matrix adhesion studies revealed some insightful findings on this common and important cellular behavior, demonstrating that 3D-matrix adhesions differ from classically well-accepted focal and fibrillar adhesions characterized on 2D substrates in their
content of integrins, paxillin, other cytoskeletal components, and tyrosine phosphorylation of focal adhesion kinase. For the 3D matrix, three-dimensionality, fibronectin, other matrix component(s), and pliability together with functional integrin receptor $\alpha_\text{v}\beta_1$ in cells are all necessary for \textit{in-vivo} like cell-matrix adhesions, morphology generation, movement and proliferation for fibroblasts. It is also noteworthy that widely used 3D collagen gel alone may not promote these cellular behaviors [219,220]. Other in-depth investigations including gene expression and protein expression also indicate that 3D cultures are superior to the 2D in cancer cell models [221-223], liver culture models [136], and stem cell differentiation studies [224-226] \textit{in vitro}. These findings, together with many others, really have an impact on how people may choose cell culture models in either fundamental research, such as studies on cell-cell interaction, signaling transduction, and apoptosis [227,228], neural development models [229], hydrodynamic [230] and electric stimuli for cells [231], or in applicative studies such as stem cell maintenance [216,232,233], toxicity models [136,234] and disease models [235] for drug development. However, a model that may closely resemble and control the complex \textit{in vivo} microenvironment with proper dimensionality, active ECM elements, intracellular interactions and necessary hydrodynamic and electric stimuli is still the Holy Grail of 3D cell culture, which may require substantial development in both biological science and engineering.

2.4 Culture Medium

2.4.1 General components

Medium provides nutrition to cell culture in the form of an aqueous solution of sugar, amino acids, vitamins, lipids, inorganic salts, trace elements, growth factors and other components. Medium should also possess appropriate osmolality and buffered pH.
There are a number of commercial formulations in the market for various cell culture processes. In scientific community, a basal medium is usually supplemented with serum, sufficient for most cell maintenance purposes. However, for industrial processes, one should understand that serum-free medium is favorable, and the composition and feeding strategy of media has a profound impact on cell growth and biologic production. Cells exhibit deregulated metabolism in culture, which is highly dependent on the amount of key nutrients, such as glucose and glutamine, and affected by the amount of the often deleterious metabolic by-products, such as lactate and ammonia. In addition, the production rate of lactate or ammonia is cell-line and medium dependent. Therefore, given a cell line in serum-free medium culture, extensive experiments on screening and optimizing medium components and their feeding strategy are critical for a successful industrial cell culture process.

2.4.2 Serum and serum-free medium

Serum is produced from animal plasma, which has a highly complex composition, including various growth and attachment factors, lipids, protective elements, hormones, signaling proteins, enzymes, micronutrients, carrier proteins for micronutrients, and even immune molecules, etc. Its complex nature enables it to be a potent supplement for cell culture. However, the same nature poses significant disadvantages for industrial processes. The composition of serum is undefined and variable depending on production source and batch. It may even contain pathogenic agents. Therefore, strict regulations are imposed on the clinical applications of cell culture products using serum. Furthermore, serum is expensive and also increases the difficulty and cost in downstream purification of protein products. As a result, in the industry, either for pharmaceutical production or cell-based therapies, the elimination
of serum has been one major effort for medium and/or cell line development. Serum-free medium requires substantial screening and optimization for development [236], and the understanding of effective serum components can facilitate this process. Usually, growth factors, carrier proteins, and additional lipids and amino acids, among others, are necessary to supplement a basal medium with cell-line specificity. These nutrient substitutes can be categorized as undefined and chemically-defined. Undefined components can be derived from, for instance, plant hydrolysates. However, chemically-defined substitutes are more favorable for consistent performance. In addition, not only serum-free, the substitutes are also usually required to be animal-component-free for the purpose of eliminating potential pathogenic hazards.

2.5 Microcarriers

Currently, microcarriers are used in industrial processes requiring anchorage-dependent cell cultures, mostly focused on viral vaccine production [237,238]. In addition, there is an increasing trend of using microcarriers for tissue engineering or cell-based regenerative medicine [239-242]. Recently, a promising application of microcarrier cell cultures is stem cell expansion with preservation of their pluripotency. For example, Cytodex 3 was used for mESCs expansion [243], and a trimethyl ammonium-coated polystyrene was used for both hESCs and mESCs [244].

There are two types of microcarriers based on structural differences [245]: solid microcarriers such as Cytodex 1, 3 and HyqSphere, and macroporous microcarriers such as Cytopores 1, 2 and fibrous microcarriers used in this study. The materials for making microcarriers are categorized into synthetic and natural biomaterials [246].
The synthetic materials used for microcarriers can be dextran matrix substituted with positively charged DEAE (Cytodex 1), cellulose with DEAE (Cytopore 1, 2), trimethyl ammonium-coated polystyrene [244], biodegradable poly(lactic-co-glycolic acid) microcarriers [239,247], chitosan modified poly(L-lactide) microspheres [248], or polyurethane foam [249]. Natural materials used for making microcarriers can be collagen [250], gelatin (CultiSpher-G), calcium alginate [251] and chitosan [252]. There were also lactose-coated chitosan microcarriers for hepatocyte culture [253], chitosan/gelatin composite microcarriers developed for hepatocyte culture [254], and collagen-coated polylactide microspheres as chondrocyte microcarriers [255]. There was even a type of liquid micorcarriers developed based on fluorocarbon emulsion stabilized with polylysine in aqueous medium [256]. Table 2.3 summarizes a selection of commercial microcarriers.

2.6 References


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Table 2.1 Physical and Chemical Properties of PDMS (Adapted from [23])

<table>
<thead>
<tr>
<th>Property</th>
<th>Characteristic</th>
<th>Consequence</th>
</tr>
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<tbody>
<tr>
<td>Optical</td>
<td>Transparent; UV cutoff, 240nm</td>
<td>Optical detection from 240 to 1100 nm</td>
</tr>
<tr>
<td>Electrical</td>
<td>Insulating: breakdown voltage, 2e7V/m</td>
<td>Allows embedded circuits; intentional breakdown to open connections</td>
</tr>
<tr>
<td>Mechanical</td>
<td>Elastomeric; tunable Young’s modulus, typical value of ~750kPa</td>
<td>Conforms to surfaces; allows actuation by reversible deformation; facilitates release from molds</td>
</tr>
<tr>
<td>Thermal</td>
<td>Insulating; thermal conductivity 0.2W/(m.K); coefficient of thermal expansion, 310μm/(m.ºC)</td>
<td>Can be used to insulate heated solutions; does not allow dissipation of resistive heating from electrophoretic separation</td>
</tr>
<tr>
<td>Interfacial</td>
<td>Low surface free energy ~20 erg/cm² [28]; contact angle of Sylgard-184: ~110º [34]</td>
<td>Replicas release easily from molds; can be reversibly sealed to materials</td>
</tr>
<tr>
<td>Permeability</td>
<td>Impermeable to liquid water; contains aqueous solutions in permeable to gases and nonpolar solvents; oxygen diffuses through the bulk material; coefficient 3.55×10⁻⁵ cm²/s</td>
<td>Incompatible with many organic solvents</td>
</tr>
<tr>
<td>Reactivity</td>
<td>Inert; can be oxidized by exposure to a plasma; Bu4N+F-((TBA)F)</td>
<td>Unreactive toward most reagents; surface can be etched; can be modified to be hydrophilic and also reactive toward silanes; etching with (TBA)F can alter topography of surfaces</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Nontoxic</td>
<td>Can be implanted in vivo; supports mammalian cell growth</td>
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Table 2.2 Properties of fluorescent proteins (adapted from [236])

<table>
<thead>
<tr>
<th></th>
<th>Quantum yield (%)</th>
<th>Excitation peak (nm)</th>
<th>Emission peak (nm)</th>
<th>Bleaching time (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP40</td>
<td>40</td>
<td>434</td>
<td>477</td>
<td>85</td>
</tr>
<tr>
<td>EGFP60</td>
<td>60</td>
<td>489</td>
<td>508</td>
<td>100</td>
</tr>
<tr>
<td>EYFP61</td>
<td>61</td>
<td>514</td>
<td>527</td>
<td>35</td>
</tr>
<tr>
<td>dsRed68</td>
<td>68</td>
<td>558</td>
<td>583</td>
<td>145</td>
</tr>
</tbody>
</table>
Table 2.3 Selection of commercially available microcarriers and their physico-chemical parameters. Entirely adapted from [240].

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Size (µm)</th>
<th>Surface area (cm² g⁻¹)</th>
<th>Beads (g⁻¹)</th>
<th>Density (g ml⁻¹)</th>
<th>Porous</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>Amersham Biosciences, Sweden</td>
<td>147-248</td>
<td>4400</td>
<td>4.3 x 10⁶</td>
<td>1.03</td>
<td>–</td>
<td>Dextran matrix with substituted N,N-diethylaminoethyl groups</td>
</tr>
<tr>
<td>Cytodex 1</td>
<td>Amersham Biosciences, Sweden</td>
<td>135-230</td>
<td>3300</td>
<td>4.1 x 10⁶</td>
<td>1.04</td>
<td>–</td>
<td>Dextran matrix with a surface layer of N,N,N-trimethyl-2-hydroxyaminopropyl groups</td>
</tr>
<tr>
<td>Cytodex 2</td>
<td>SoloHill, USA</td>
<td>150-210</td>
<td>350</td>
<td>5.0 x 10⁶</td>
<td>1.19</td>
<td>–</td>
<td>Dextran matrix with treated surface</td>
</tr>
<tr>
<td>Plastic</td>
<td>Plastic coated</td>
<td>150-210</td>
<td>380</td>
<td>4.5 x 10⁶</td>
<td>1.02-1.04</td>
<td>–</td>
<td>Plastic coated with denatured collagen</td>
</tr>
<tr>
<td>PlasticPlus coated</td>
<td>SoloHill, USA</td>
<td>160-210</td>
<td>380</td>
<td>4.5 x 10⁶</td>
<td>1.02-1.04</td>
<td>–</td>
<td>Plastic coated with denatured collagen, promoting fast attachment</td>
</tr>
<tr>
<td>Biscilin²</td>
<td>Nunc, Denmark</td>
<td>160-300</td>
<td>255</td>
<td>5.1 x 10⁴</td>
<td>1.05</td>
<td>–</td>
<td>Poly(vinylpyrrolidone)</td>
</tr>
<tr>
<td>Cytoline 2</td>
<td>Amersham Biosciences, Sweden</td>
<td>400-2500</td>
<td>&gt;1000</td>
<td>n/a</td>
<td>1.03</td>
<td>+</td>
<td>Polytetrafluoroethylene and silica</td>
</tr>
<tr>
<td>Glass</td>
<td>Glass-coated</td>
<td>150-210</td>
<td>380</td>
<td>4.5 x 10⁶</td>
<td>1.02-1.04</td>
<td>–</td>
<td>Plastic with glass coating</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Cytopenone 1</td>
<td>200-280</td>
<td>~1200</td>
<td>n/a</td>
<td>1.03</td>
<td>+</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Cultispher G</td>
<td>133-380</td>
<td>n/a</td>
<td>1.0 x 10⁶</td>
<td>1.04</td>
<td>+</td>
<td>Cross-linked porcine gelatin</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Cultispher S</td>
<td>133-380</td>
<td>n/a</td>
<td>8.0 x 10⁶</td>
<td>1.04</td>
<td>+</td>
<td>Cross-linked porcine gelatin</td>
</tr>
<tr>
<td>Collagen</td>
<td>Cytodex 3</td>
<td>141-211</td>
<td>2700</td>
<td>3.0 x 10⁶</td>
<td>1.04</td>
<td>–</td>
<td>Dextran matrix with thin layer of denatured pig skin-derived collagen</td>
</tr>
<tr>
<td>Collagen</td>
<td>MP Biomedicals, USA</td>
<td>100-400</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>Highly cross-linked bovine collagen type I</td>
</tr>
</tbody>
</table>
Figure 2.1 Schematic process of photolithography [237].
Figure 2.2 Molecular structure of SU-8.
Figure 2.3. Schematic illustration of the size ranges that are accessible by the techniques of soft lithography that are being applied to biological problems. The upper limits of some of the techniques are not indicated because they have not yet been tested [238].
Figure 2.4 Schematic illustration of micromolding techniques. A) replica molding, B) microtransfer molding, C) micromolding in capillaries, and D) solvent-assisted micromolding [7]
Figure 2.5 Molecular structure of PDMS [239].
Figure 2.6 Tissue organization, culture and analysis in microsystems. Advanced tissue organization and culture can be performed in microsystems by integrating homogeneous and heterogeneous cell ensembles, 3D scaffolds to guide cell growth, and microfluidic systems for transport of nutrients and other soluble factors. Soluble factors — for example, cytokines for cell stimulation — can be presented to the cells in precisely defined spatial and temporal patterns using integrated microfluidic systems. Microsystems technology can also fractionate heterogeneous cell populations into homogeneous populations, including single-cell selection, so different cell types can be analysed separately. Microsystems can incorporate numerous techniques for the analysis of the biochemical reactions in cells, including image-based analysis and techniques for gene and protein analysis of cell lysates. This makes microtechnology an excellent tool in cell-based applications and in the fundamental study of cell biology. As indicated by the yellow arrows, the different microfluidic components can be connected with each other to form an integrated system, realizing multiple functionalities on a single chip. However, this integration is challenging with respect to fluidic and sample matching between the different components, not least because of the difficulty in simultaneously packaging fluidic, optical, electronic and biological components into a single system (entirely adapted from [117]).
CHAPTER 3

PERFUSION MICROBIOREACTOR ARRAY FOR MODULAR 3D CELL CULTURES AND CYTOTOXICITY ASSAYS

3.1 Abstract

One important application of tissue engineering is to provide novel in vitro models for cell-based assays. Perfusion microbioreactor array provides a useful tool for micro-scale tissue culture in parallel. However, high-throughput data generation has been a challenge. In this study, a 4×4 array of perfusion microbioreactors was developed for plate-reader compatible, time-series quantification of cell proliferation and cytotoxicity assays. The device was built through multi-layer soft lithography. Low-cost nonwoven polyethylene terephthalate (PET) fibrous matrices were integrated as modular tissue culture scaffolds. Human colon cancer HT-29 cells with stable expression of enhanced green fluorescent protein (EGFP) were cultured in the device with continuous perfusion and reached a cell density as high as $6 \times 10^7$ cells/mL. The microbioreactor array was used to test a chemotherapeutic drug 5-fluorouracil (5-FU) for its effect on HT-29 cells in continuous perfusion 3D culture. Compared with conventional 2D cytotoxicity assay, significant drug resistance was observed in the 3D culture.

3.2 Introduction
In the pharmaceutical industry, early discovery of undesirable properties for drug candidates can greatly reduce the risk of a lengthy and expensive drug development process. Recently, cell-based assays have become an attractive approach to high-throughput screening for cytotoxicity studies. The combination of cell culture, microfabrication, microfluidics, molecular biology, and sensitive monitoring techniques offers tremendous potentials for exploiting the advantages of cell-based assays [1,2]. A prominent example is the microscale cell culture analog (μCCA) based-on pharmacokinetic models developed by Shuler and colleagues [3-5]. For high-throughput quantitative cell biology studies, Lee et al developed a nanoliter microbioreactor array [6-8]. A living cell array has also been developed to demonstrate the activation and real-time monitoring of gene expressions under molecular stimuli [9,10]. A high-density microfluidic array was also developed for cytotoxicity studies [11].

Many cell types, including tumor cells, chondrocytes, and embryonic stem cells, require a three-dimensional (3D) microenvironment to properly demonstrate their in vivo physiology [12-19]. Previous work also showed that 3D culture environments affected tissue development, cell cycle, and antibody production [20-22]. For 3D cultures, perfusion can improve nutrients supply and is thus beneficial to tissue development [23,24]. Therefore, microfluidic 3D culture has become a natural resort to tissue culturing at micro-scale. For example, in a microfabricated array of bioreactors, primary rat hepatocytes were maintained in silicon scaffold channels [25], and functional cultures were achieved using preaggregated cells [26]. In another example, chondrocytes entrapped in agarose gel were cultured in a perfused microfluidic device [27]. Cells were also
maintained in 3D structures with micropillars under perfusion [28]. In these studies, however, cell proliferation could not be easily quantified on chip. Proliferation assays enable time-series studies of cell number change in response to external stimuli, revealing the trend and rate of cell growth or death under certain conditions, which can be valuable information in assessing time-dependent cellular responses to drug treatments. In addition, the end point of proliferation assays can be taken as cytotoxicity assays. Therefore, a perfusion 3D cell culture system with high-throughput quantification for cell proliferation and cytotoxicity assays is highly desirable.

The quantification of cell proliferation in 3D cultures has long been a challenge [29]. Conventional methods, such as trypan blue exclusion assay, DNA content measurement, MTT assay, Alamar Blue assay and calcein-AM assay, are invasive, so cell culture has to be disrupted. Moreover, the proliferation of 3D cell culture at microscale is even more difficult to quantify by using these methods than at macroscale due to limited accessibility to cells. However, autofluorescence-based methods can provide non-invasive quantification of engineered cell lines, thus allowing time series measurements using the same sample. Hunt et al. used stable green fluorescence protein (GFP)-expressing Chinese Hamster Ovary (CHO) cells to demonstrate the feasibility of dynamic assessment of cell growth in suspension [30]. In another study, stable red-fluorescent tumor cell lines were used to study the enhancement of breast cancer cell growth by co-culturing with human bone marrow stromal cells in 3D Matrigel [31].

In this work, a microbioreactor array for continuous perfusion 3D cell culture with high-throughput proliferation quantification was developed. The polymeric device
had a 4×4 microbioreactor array, each well containing a treated polyethylene terephthalate (PET) fibrous matrix as discrete tissue scaffold modules. Reversible packaging of the device was achieved using a frame-assisted assembly (FAA) method. This method allows direct post-culture access to the cells for further analyses. Furthermore, instead of using an specifically designed optical detection system, cell proliferation was fluorometrically quantified in a commercially available plate reader, allowing high-throughput and time-series data acquisition. Also, tissues were optically accessible for monitoring using fluorescence microscopy during the entire culture process. The microbioreactor array was used to demonstrate the cytotoxicity effect of 5-fluorouracil (5-FU) on human colon cancer cells in 3D perfusion cultures and showed advantages over conventional 2D cytotoxicity assay.

3.3 Experimental

3.3.1 Device fabrication

Figure 3.1 shows the design and construction of the microbioreactor array, which had two layers made of poly(dimethylsiloxane) (PDMS, Sylgard-184) with features facing each other. The device consisted of four independent feeding lines each containing four culture wells and three relay wells connected with channels 500 μm wide. Fluid flow path was designed to enter each cell culture well from the bottom layer and to exit from top. Inside each cell culture well, a non-woven PET fibrous matrix was filled in as a modular tissue scaffold (see Fig. 3.1.C). The PDMS pieces were fabricated using photolithography and replica molding. The process started with spin coating a layer of around 500 μm thick photoresist SU-8 100 (Microchem) on a 3 inch silicon wafer. After baking, the photoresist was exposed to UV under transparency
film masks and then developed to be photolithographically patterned with designed features. The masters for top and bottom layers were both made using this procedure. PDMS prepolymer and curing agent were mixed at a ratio of 10:1, and poured onto the masters before polymerization on a 70 °C hotplate for 2 hours. Then, the PDMS pieces were cut and peeled off. The top layer had features of 420 \( \mu \text{m} \) thick, and bottom 500 \( \mu \text{m} \). Each cell culture well had a diameter of 3 mm and a working volume of about 6.5 \( \mu \text{L} \).

3.3.2 Fluidic ports

A set of bottom and top frames made of polycarbonate were fabricated with computerized numerical control milling with open windows for the positions of cell culture wells and fluidic ports (see Fig. 3.1.D). The PDMS top layer was aligned and fitted into the top frame to form the top part of the device. Fluidic ports were drawn from the top part using blunted and bent stainless steel needles of gauge 16. PDMS was used for sealing of each fluidic port with a column-shaped mold upon heating at 97°C for 15 min, meanwhile bonding together the top PDMS layer and the top frame. The fluidic port needles were connected to a flexible connector. The flexible connector was designed as a buffer to protect the PDMS inlets and outlet from strains of external tubing. The flexible connector is made of a 2 cm long Tygon S-50-HL tubing plugged with a 2 cm long blunted stainless steel needle of gauge 16. The Tygon tubing end of the flexible connector was connected with the stainless steel needle drawn from the PDMS inlets and outlet on the chip, while the other end was connected with external tubing for syringes or waste collector (see Fig. 3.1.F). During measurement of cellular fluorescence intensity with plate reader, the pump tubing was disconnected from the flexible connectors of the chip. The flexible connectors were
capped with a short piece of Tygon tubing with one end hot-press sealed to prevent contamination (see Fig. 3.1.E), making the cell-based device portable. This provided great convenience for in situ fluorescence intensity measurement and microscopy imaging.

3.3.3 Frame assisted assembly

Before packaging, each piece of the device was sterilized by autoclaving. During assembly, the bottom PDMS layer was first placed on the bottom frame in alignment. After placing PET matrices into cell culture wells on the bottom PDMS layer, the top part was then aligned with and placed on top of the bottom part for packaging (see Fig. 3.1.E). The device was fixed in place with screws, allowing a tight sealing of multiple layers for perfusion experiments (see Fig. 3.1.F).

3.3.4 EGFP cell line development and medium

Human colon cancer HT-29 cells (ATCC: HTB-38) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, high glucose 4.5 mg/mL, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) in an incubator at 37 °C with 5% CO₂. The process for developing the clone with stable enhanced green fluorescent protein (EGFP) expression is briefly discussed as follows. The HT-29 cells were transfected with PEGFP-N3 (Clontech) using Lipofectamine 2000 (Invitrogen), with which EGFP was expressed under the control of a strong constitutive cytomegalovirus (CMV) promoter. After initial selection with geneticin (G-418, Gibco), cells were diluted and seeded in the wells of a 96-well plate at around 5 cells per well. One green fluorescent colony was isolated and
expanded in the absence of geneticin. The stability of the transfected cell line was verified by flow cytometry (>97%) using FACSCalibur (B-D Biosciences).

3.3.5 Modular PET scaffolds

The treatment and characterization of PET matrices followed the method described by Li et al (2001) [32]. Briefly, PET matrices were compressed under 30 kPa at 120 °C for 60 min. Then, the PET fibers were soaked in a solution containing 1% (w/v) Na₂CO₃ and 1% (v/v) Tween-20 and heated at 60 °C for 30–60 min. After rinsing with distilled water, PET matrices were boiled in 1% (w/v) NaOH solution for 30 min to increase surface hydrophilicity. The treated matrices were about 1 mm thick with porosity of about 0.85 and average pore size of about 45 μm [20,21,32]. Treated nonwoven polyethylene terephthalate (PET) fibrous matrices (fiber diameter: 20 μm; density: 1.35 g/cm³) were cut into square patches with a side length of about 3 mm as modular tissue scaffolds. The patches with fluorescence intensity difference within ±10% of the mean of all samples were selected for experiments. The matrices were thoroughly rinsed and then stored in phosphate buffer saline (PBS) solution.

3.3.6 Cell seeding

For perfusion cell culture experiments, selected PET matrix patches were autoclaved at 121 °C for 15 min. Each patch was then put in one well of a 384 well plate. The PET matrix was immersed in 20 μL fetal bovine serum (FBS) and kept in a cell culture incubator overnight. Cells were trypsinized from a T-flask and suspended in the maintenance medium. Each PET scaffold was seeded with 6 μL of cell suspension. Then, the 384-well plate was put into an incubator, allowing
cells to attach to the fibrous matrix. Approximately 4 hours later, 30 μL media were added to each seeded scaffold and the plate was kept in the incubator overnight to allow cellular adaptation to the 3D environment. Then, seeded scaffolds were transferred gently with a sterile tweezer to the cell culture wells of the microbioreactor array for packaging. Unless otherwise noted, the first well in each line on the chip was filled with an unseeded PET scaffold as control.

3.3.7 Perfusion culture

Four 10-mL syringes were autoclaved, aseptically filled with medium and installed on a syringe pump (PHD 2000, Harvard Apparatus). PET fibrous matrices were seeded following the process described in the section of cell seeding with different cell densities: 5.9×10⁶, 1.1×10⁷ and 2.5×10⁷ cells/mL matrix (Fig. 3.3). Aseptically, the fluidic ports of packaged microdevice were connected through tubing to syringes on the pump, and an effluent collector was connected to the other end of the device. The bubbles within the microchip were driven out by infusion at a flow rate of 100 μL/min for around 2 min. The whole system was then placed in a CO₂ incubator with perfusion at 1.2 μL/min. Perfusion cultures were maintained for over 5 days until the medium in the syringes was depleted. Cell number in each well was monitored by measuring the cellular fluorescence intensity with the microplate reader. The fluorescence intensity of control wells was subtracted from the measurement of each well containing seeded matrix.

3.3.8 5-fluorouracil (5-FU) cytotoxicity studies

The cytotoxicity effect of 5-FU on HT-29 cells in 3D perfusion culture was tested by continuously feeding the cultures with media containing the drug at different
dosages (0, 1, 10 and 100 μg/mL) for 5 days. Before running the perfusion culture experiments, 5-FU was found to have no wall adsorption in the microdevice and no fluorescence emission at the wavelengths for EGFP measurement. PET scaffolds were seeded with around 6×10^6 cells/mL. The device was fed with 5-FU containing medium at different dosages at a flowrate of 0.7 μL/min out of the consideration that this flowrate was sufficient for cell growth and drug exposure. Culture fluorescence was measured to monitor cell responses to 5-FU. In order to compare differential cytotoxicity effects of 5-FU on 2D and perfused 3D HT-29 cell cultures, a series of concentrations of 5-FU at 0.2, 0.5, 1 and 5 μg/mL were used to treat HT-29 monolayer cultures. Briefly, cells were seeded at the same density around 5.5×10^3 cells/cm^2 in a 1.2 mL suspension to each well on a 12-well plate, with 3 replicates for each drug concentration and control. After 5 days of drug exposure, cells were trypsinized, stained with trypan blue, and counted with a hemacytometer under a microscope.

3.3.9 Cell number quantification

Unless otherwise noted, cell proliferation was quantitatively monitored in a high-throughput manner using a microplate reader (Tecan GENios Pro). A lid for a multi-well plate was modified as a holder of the microchip for loading into the plate reader (Fig. 3.2). Plate definition scanning at an excitation wavelength of 485 nm and emission 535 nm was carried out to generate a map based on the difference in the fluorescence intensity between the empty non-fluorescent PDMS cell culture wells and the fluorescent polycarbonate non-well area in the bottom frame. Based on the map, a reading scheme for the 4×4 array was then defined (see Fig. 3.2 inset) by the software provided with the plate reader. To test the accuracy of plate
definition, 3 μL fluorescein (Sigma) solutions dissolved in deionized (DI) water with a series of concentrations were dispensed in the 4×4 array, the device was packaged, and the fluorescence intensity was then measured using the specific plate definition in the microplate reader, with DI water as background and subtracted from all the readings. The linear correlation between fluorescence intensity and the concentration of fluorescein [electronic supplementary information (ESI) 1] indicated that the plate definition supported data acquisition with precision and in high throughput. Similarly, a standard curve between cell number and fluorescence intensity was generated by seeding each cell culture well containing one treated PET fibrous matrix with 6 μL of cell suspension at a series of cell densities. The device was then packaged and loaded into the plate reader for measurement. The wells with only matrix and medium but without cells were used as background in the measurement. As shown in Figure 3.3, a linear correlation was established between the seeded cell number in PET matrices and the measured fluorescence intensity.

3.3.10 Hematoxylin & Eosin Staining

At the end of the perfusion culture experiment, the microdevice was dismantled, and the PET scaffolds with cells were removed and fixed with 10% formalin, embedded in paraffin, and then horizontally cut into 5 μm thick sections at a series of depths. The thin-section samples from the surface and various depths (5 μm, 60 μm, and 170 μm beneath the surface) were then deparaffinized, stained with hematoxylin and eosin (H&E) on microscope slides, and images were taken under a light microscope (Olympus BX60).

3.3.11 Scanning Electron Microscopy
Fresh tissue construct samples were fixed with 2.5% glutaraldehyde overnight at 4°C, and then progressively dehydrated in a series of ethanol solutions from 10% to 100% with 10% increment for 30 min at each concentration, followed by soaking in a series of mixtures of hexamethyldisilazane (HMDS) (Sigma) and ethanol with increasing HMDS content from 1:3 to 1:1 and finally 3:1. After sputter-coating the dried samples with gold-palladium, scanning electron microscopy (SEM) (Hitachi S-4300 FE-SEM) was used to study 3D cell morphology and distribution in the PET matrix.

3.3.12 Cell Cycle Analysis

Cell cycle analysis was conducted by flow cytometry for cells harvested from 3D perfusion cultures under 5-FU treatment at 0.2 μg/mL, 0.5 μg/mL, and 5 μg/mL. Cells were seeded at the same high density around 3.2×10⁷ cells/mL. After a 5-day drug treatment in perfusion, tissue constructs were obtained and washed twice with PBS. They were then treated in trypsin at 37°C for 10 minutes, followed by vigorous washing with culture medium twice for cell harvest. All wash fractions were collected and rinsed with PBS twice. The cells were then suspended and fixed with cold 70% ethanol, washed with PBS and resuspended in propidium iodide (PI, Sigma) staining solution containing RNase A. Cell fluorescence was measured by flow cytometry (FACS Calibur, BD Biosciences) at 488 nm excitation and 623 nm emission. The DNA content histograms were analyzed using ModFit (Verity Software House), and cell cycle distribution and the fraction of cell population in apoptosis or debris were determined accordingly.

3.4 Results and discussion
3.4.1 Perfusion cultures

Fig. 3.4 shows the growth kinetics of the perfusion cultures in the microbioreactors. The experiment was stopped when the medium in the syringes was depleted. In general, cell growth sustained over the entire culturing period (Fig. 3.4.A). Perfusion could be easily continued with a set of newly filled syringes, and culturing could be maintained for over two weeks (data not shown). The cell growth in static culture was also studied and is plotted on the same figure to illustrate the advantages of perfusion culture over static culture. The static culture, even though with a much lower initial cell density, reached its growth limit in just one day, while all perfusion cultures were continuously expanded without encountering growth limit, with the highest cell density reaching $6 \times 10^7$ cells/mL matrix. The growth rate can be calculated from the curves based on a simple equation $\frac{dX}{dt} = \mu X$ where $X$ is biomass measured by fluorescence intensity, $t$ is time and $\mu$ is the specific growth rate. The specific growth rate decreased with increasing the seeding density, with a doubling time of 67 h for $5.9 \times 10^6$ cells/mL, 100 h for $1.1 \times 10^7$ cells/mL and 173 h for $2.5 \times 10^7$ cells/mL. This could be explained by the prevalent observation that cell growth rates are lower in higher cell density perfusion cultures [33], especially for cancer cultures [17,34,35]. The results shown in Fig. 3.4.A also demonstrate good reproducibility of the data as indicated by the error bars for the growth curve with the initial seeding density of $1.1 \times 10^7$ cells/mL.

*In situ* fluorescence microscopic images were captured at the end of the culture for live cells in 3D matrices. As can be seen in Fig. 3.4.B, cells formed aggregates
along fibers and spread over the gaps between fibers, exhibiting a high density tissue-like structure. The in-growth of high-density cells in the modular tissue scaffold was confirmed with H&E stained slides (Fig. 3.5). Sections were cut at increasing depths, as illustrated in Fig. 3.5.A for surface, Fig. 3.5.B for 5 μm, Fig. 3.5.C for 60 μm, and Fig. 3.5.D for 170 μm of the tissue construct. The sections at 5 and 60 μm showed a relatively uniform distribution of high-density cells throughout the cross section. It was also identified that extracellular matrix (ECM) proteins secreted by the cultured cancerous cells spread between gaps of the fibrous matrix, as indicated in pink eosinophilic structures. Further studies on the compositions of these ECM proteins and their formation by the cancer cells may provide valuable information for cancer research [36]. For the section with a deeper cut at 170 μm, the microscopic image exhibits that cell densities were higher around the edges than in the central area. One possibility is that medium was perfused more smoothly around the edges than into the center, if the scaffold was not perfectly fitted into the cell culture well. This should be improved by producing scaffolds with the same shape and dimension as the microwell. However, gradually increased cell density during culture might also be the cause of altered medium flow distribution. For achieving uniform perfusion throughout the matrix, the microdevice may be improved with novel designs recently developed for tissue engineering bioreactors, such as a T-cup [37] or an array of microjets for bottom-up forced interstitial perfusion [38].

The high density and relatively uniform cell distribution within the fibrous matrix were also confirmed with SEM images shown in Figure 3.6. Fig. 3.6.A illustrates an overview of the uniform cell distribution in the fibrous matrix. After a vertical cut
through the centreline of the tissue construct, an image was taken right against the cut, revealing a densely occupied 3D structure by cells throughout the whole depth (see Fig. 3.6.B). In addition, individual cancerous cells exhibited a round shape (see Fig. 3.6.C), closely resembling the \textit{in vivo} morphology of cancer cells [39]. ECM proteins, which can help control bulk and local mechanics and signalling microenvironment [40], were also visible in a highly magnified SEM image (see Fig. 3.6.D), consistent with the H&E images. It should be noted that cells grown in 2D cultures showed a more spreading and flattened morphology that are different from the one found in the 3D cultures and \textit{in vivo} tissue. It is clear that the 3D perfusion microbioreactor can provide a suitable microenvironment for the colon cancer cells to grow to a high density and to exhibit similar \textit{in vivo} morphology, offering a better platform for cytotoxicity studies.

3.4.2 Cytotoxicity studies for 5-FU

5-FU is a commonly used chemotherapy drug and was thus chosen in this study. Figure 3.7 shows the growth kinetics of perfusion cultures subjected to different 5-FU concentrations, including one without 5-FU as control. Before 60 hours, the growth rate of cells under 1 $\mu$g/mL 5-FU treatment was only slightly lower than that of the control. At 10 and 100 $\mu$g/mL 5-FU, both cultures continued to grow but with much reduced rates. After 60 hours, the control continued to grow, while all the cultures with 5-FU started to decline at different rates, indicating a dose-dependent response. With the highest dosage, 100 $\mu$g/mL, the cultured tumor shrunk below its initial size at the end point. It is noted that 5-FU is an S-phase specific drug, which does not immediately kill cells, but first causes cell cycle arrest and then leads to certain cell death mechanisms, such as apoptosis [41-43].
In a separate experiment, cells after culturing in the perfusion microbioreactor under the influence of various 5-FU dosages for 5 days were analyzed for cell cycle distribution. As shown in Table 3.1, the populations at G0/G1 phases increased, while the populations at S/G2/M phases decreased with increasing the drug concentration. Furthermore, the fraction of apoptotic cells and cell debris also increased in a dose-dependent manner.

Taking the end points of the perfused 3D cultures (Fig. 3.7) and compared to the control (no drug), the cell density was reduced to 58% at 1 μg/mL, 46% at 10 μg/mL and 9% at 100 μg/mL, suggesting an IC₅₀ between 1 to 10 μg/mL of 5-FU for HT-29 3D perfusion culture. The reported IC₅₀ of 5-FU on human colon cancer HT-29 cell lines in 2D cultures ranged from 0.086 μg/mL (or 0.66 μM) [44] to 0.21 μg/mL (or 1.64 μM) [45]. Our 2D cytotoxicity studies also showed an IC₅₀ of around 0.2 μg/mL (Fig. 3.8). It is thus clear that colon cancer cells in 3D cultures were much more resistant to 5-FU than in 2D cultures. Drug resistance, which has been a well-known problem in chemotherapy, can be attributed to various mechanisms and may involve increased molecular resistance, increased quiescent cell population, changes in morphology and phenotype, and presence of cancer stem cells [46-49]. In this study, cells cultured in the perfusion microbioreactor exhibited a 3D organization that was similar to in vivo morphology of cancer cells and showed the drug effect on cell cycle, which can partially explain the increased drug resistance in the 3D perfusion culture.

It is noted that in Fig. 3.7 the control with a seeded cell density around 6×10⁶ cells/mL exhibited a slower growth rate than the culture with a similar seeded cell density in the perfusion experiment discussed before (see Fig. 3.4.A). This was
probably caused by a lower perfusion rate of 0.7 μL/min in the drug test compared with 1.2 μL/min in the earlier perfusion experiment, suggesting that flowrate could be an important parameter affecting cell growth rate as well as drug response.

3.4.3 Microbioreactor array for 3D cell-based assays

With high impacts on today’s cell biology studies and drug efficacy or safety evaluations, high-throughput perfusion microscale 3D cell culture has been a frequent focus in recent research. In most of the previous studies, three dimensionality of cell culture was attained by entrapping cells in gels, such as poly(ethylene glycol) [50], agarose [27,51-53], alginate [54], hyaluronic acid [55], peptides, gelatin, collagen and Matrigel [56-62]. Inside the gelled tissue constructs, mass transport depends on diffusion, and the cell density and cell-cell contact are relatively low. In contrast, the highly porous fibrous matrix used in this study allows both convective and diffusive mass transports within tissue constructs with mechanical cues for cells to autonomously develop 3D structures, which is an improved in-vivo like feature [63]. Furthermore, in this study, each tissue construct was integrated into the microdevice with reversible packaging as convenient modules, which offer many advantages [64], including (1) convenient choice of scaffolds for the microdevice, (2) versatile combination of different cell types in different modules, (3) wide and convenient options for post-culture analyses of the tissue constructs. This is also a feature that may benefit tissue engineering applications [64].

Moreover, the noninvasive and time-series quantification of cell proliferation and cytotoxicity in the perfusion microbioreactor array with a commercially available microplate reader enables high-throughput data acquisition. Microdevices are considered a disruptive technology, so their integration into the currently available lab
equipment for detection and control can play a positive role for removing a critical roadblock for wide applications in both academia and industry [65]. Furthermore, culturing the stable EGFP expressing cells in the microdevice affords an enabling technique for non-invasive assays of cell proliferation and cytotoxicity. This in situ monitoring technique is not only useful for the perfusion microbioreactor, but also applicable in microplate format [66]. To our knowledge, so far this has not been reported in other perfusion 3D cell culture microsystems.

In our study, with simple sequential flow for each line, the proliferation and cytotoxicity assays were performed with replicates due to relatively fast perfusion rates and a small array size. It should be noted that this approach may encounter difficulty in large arrays of microbioreactors. Recently, there have been improved designs reported for independent and parallel cell-based assays in a microfluidic device [55,67], which would benefit future designs in this field. In addition, for future work, it is also helpful to add serial drug dilution channels to the device for automatic high-throughput cytotoxicity testing. In addition, there was another concern that the debubbling step after each fluorescence intensity measurement might be detrimental to cells. While we did observe slightly slowed cell growth in some first cell culture wells in a line (data not shown), there was no significant change in the fluorescence intensity after repeated debubbling (see ESI 2). The effort of designing devices that can circumvent direct flushing to cells is in process, and future studies will ensue on this matter.

3.5 Conclusions

The microbioreactor array developed in this work has the capability of perfusion high-density 3D cell culture in modular and low-cost PET fibrous scaffolds.
Non-invasive and time-series cell proliferation and cytotoxicity assays could be achieved using a plate reader. EGFP-expressing HT-29 cells could be maintained for an extended period, reaching a cell density as high as $6 \times 10^7$ cells/mL matrix. The frame-assisted device packaging and modular design allowed direct post-culture access to cells for various further analyses. Cell proliferation and proof-of-concept cytotoxicity assays were conducted by measuring cellular EGFP fluorescence intensity. 3D colon cancer cell cultures had a time- and dose-dependent response to an S-phase specific drug 5-FU. Significant drug resistance was also observed in the perfusion 3D culture compared with conventional 2D cytotoxicity assay. Versatile applications could be envisioned using this platform, such as high-throughput cell-based drug testing, fundamental and clinical cancer research, tissue engineering studies, cell interaction assays, and scale-down models for perfusion cell culture process development.

3.6 References


47. Luqmani YA: Mechanisms of drug resistance in cancer chemotherapy. *Med Prin...


Table 3.1. Effects of 5-FU treatment on cell cycle and apoptosis for human colon cancer cells cultured in perfusion 3D microbioreactors after 5 days.

<table>
<thead>
<tr>
<th>5-FU (µg/mL)</th>
<th>G0/G1 Phase</th>
<th>S Phase</th>
<th>G2/M Phase</th>
<th>Apoptosis/Debris</th>
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<tr>
<td>0.2</td>
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<td>33.2%</td>
<td>16.6%</td>
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<td>0.5</td>
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<td>19.9%</td>
<td>6.2%</td>
<td>30.5%</td>
</tr>
<tr>
<td>5.0</td>
<td>43.8%</td>
<td>8.6%</td>
<td>0.8%</td>
<td>46.8%</td>
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</tbody>
</table>

Percentage of gated cells in various cell cycle stages and apoptosis analyzed by flow cytometry.
Figure 3.1. Design, fabrication and packaging of the 4×4 perfusion microbioreactor array. A. Schematic drawing of the device composed of top and bottom layers by AutoCAD; B. Perspective view of the assembled device to form microscale channels and wells; C. Design of individual cell culture well and relay well formed by two layers, with cell culture well that can be filled with any modular tissue engineering scaffold, e.g. in this study, PET fibrous matrix; D. Perspective drawing of the top and bottom frames by Solid Edge for frame assisted assembly; E. Photograph of device in assembly with each inlet connecting to a flexible connector capped to prevent contamination (highlighter window); F. Photograph of assembled device at work with each inlet connected to an external tubing through a flexible connector (highlighter window). Food dyes were used to indicate proper fluid segregation for each line of flow.
Figure 3.2 High throughput fluorescence intensity measurement for the microbioreactor array loaded into a microplate reader, TECAN GENiosPro™. The inset is a fluorescence intensity map generated by plate definition fluorescence scan for the 4×4 microbioreactor array at an excitation wavelength of 485 nm and emission 535 nm. The black dot array represents non-fluorescent empty PDMS cell culture wells. The greenish area is for the polycarbonate bottom frame, which is more fluorescent than PDMS upon the scanning. The red grids are generated with the microplate reader software to define relative positions of the wells for establishing a custom reading scheme.
Figure 3.3. Linear correlation of fluorescence intensity with cell number in PET fibrous matrix in the microbioreactor array, n = 2. The linear fitting equation can be used to quantify the cell density from the measured fluorescence intensity.
Figure 3.4. Growth kinetics of HT-29 cells in perfusion and static cultures on the microbioreactor array. A. Cell growth curves measured by fluorescence intensity from EGFP expressed by proliferating cells; B. A fluorescence microscopic image of live HT-29 cells in the 3D PET fibrous matrix (100X magnification; scale bar indicates approximately 60 μm)
Figure 3.5. Microscopic images of H&E stained tissue culture sections of 5 μm thickness after culturing for over 125 hours. A. Section at surface; B. Section at 5 μm depth from surface; C. Section at 60 μm depth from surface; D. Section at 170 μm depth from surface (100X magnification; scale bars indicate approximately 50 μm).
Figure 3.6. SEM images of HT-29 cells within the PET fibrous matrix cultured in the perfusion microbioreactor. A. An overview of the fibrous matrix showing high cell density and uniform cell distribution in the matrix (image at 120X magnification); B. A cross cut in the middle of the matrix showing cells densely filling the entire fibrous matrix (image at 450X magnification); C. A close-up image showing round-shaped cell morphology (1,200X magnification); D. A close-up image showing extracellular matrix proteins (indicated by the arrow) filling gap spaces around cells in the microenvironment (3000X magnification).
Figure 3.7. Kinetics of 3D perfusion cultures of HT-29 cells exposed to various 5-FU concentrations. Before 60 hours, 1 μg/mL 5-FU caused a slightly slower proliferation rate than the control, while cultures with 10 μg/mL and 100 μg/mL drug had a similarly inhibited growth. After around 60 hours, control continued to grow, but the cultures with 5-FU started to decline. The culture with 100 μg/mL 5-FU shrank below its initial size in ~ 90 hours.
Figure 3.8. Cytotoxicity tests of 5-FU against HT-29 2D culture with an IC50 approximately at 0.2 μg/mL.
CHAPTER 4

FLOW CONTROL AND STARTUP IN PARALLEL HYDROPHOBIC
MICROCHANNELS WITH A COMMON INLET

4.1 Abstract

Poly(dimethylsiloxane) (PDMS) is widely used in microfluidic devices. However, its relatively high hydrophobicity causes difficulties in pressure driven flow in parallel microchannels at low flowrates. Uneven flow startup among parallel microchannels led to uneven flow rates and permanent idleness of incompletely filled channels. This study presents a theoretical and experimental investigation of this phenomenon. It was found that capillary pressure arising from bubbles spanning the cross section of a microchannel was the major force for flow blockage. Therefore, the threshold flowrate for uniform flow startup in parallel microchannels could be estimated by balancing the maximum capillary pressure and the hydrodynamic pressure drop in a microchannel. This hypothesis was tested in a series of experiments using 2×4 and 4×4 perfusion microbioreactor arrays, and the results matched well with the theoretical calculations. Also, filling micro-wells with porous fibrous matrices to increase the hydrodynamic pressure drop allowed uniform flow startup at low threshold flowrates in parallel-flow microchannels. This study established a rational method of achieving uniform flow startup in parallel hydrophobic microchannels,
providing practical guidance for device design, material selection and operation in microchannel technology.

4.2 Introduction

Microfluidic technologies focus on processing and manipulating small amounts of fluids in channels with dimensions of tens to hundreds of micrometers [1]. The flow control in microchannels is achievable through different methods, which can be systematically integrated in their design, material selection and operation conditions [2]. For example, the design of converging-diverging microchannels can be used to generate chemical gradients distributed into multiple parallel channels made of poly(dimethylsiloxane) (PDMS) and glass [3]. For material selection, responsive hydrogels [4,5] or colloids [6] can be integrated into microchannels as novel valving and/or pumping mechanisms for flow control. In addition, surface properties, such as wettability [7-9] and surface charge [10,11], can also be employed in microfluidic flow control.

PDMS has been extensively used in making whole microfluidic devices and various functional components, ranging from cell culture, microreactors, mixers, pneumatically actuated switches and valves, to functional membranes and optical components [12,13]. PDMS is biocompatible and has attractive physiochemical properties, such as optical transparency, elasticity, flexible surface chemistry, low permeability to water, high permeability to non-polar gases, and low electric conductivity [14]. However, PDMS is hydrophobic with a contact angle of about 110° at room temperature. In hydrophobic microchannels, surface tension results in a positive pressure to hold aqueous fluid back, showing a “reluctance” to fill. Therefore, uncertainty arises when multiple hydrophobic microchannels are to be filled from one
common inlet under pressure driven flow (PDF) because bubbles in incompletely filled microchannels could block the flow. This is problematic because the incompletely filled microchannels become useless in permanent idleness, which is a nuisance often encountered in microchannel technologies. Intuitively, increasing flowrates will eliminate the blocking bubbles. However, in applications such as for cell cultures, high flowrates are prohibitive to use in order to avoid shear damage to cells. Therefore, a good understanding of the key forces in this phenomenon is desirable, which can facilitate the design and operation of such microfluidic devices.

In this study, for 3D perfusion tissue cultures, we designed and fabricated a series of microbioreactor arrays using multi-layer soft lithography. Theoretical estimations of the conditions for parallel flow startup for these devices were developed based on the balance between the maximum capillary pressure and hydrodynamic pressure drop of a fully filled microchannel. In experiments, these devices were used to verify the estimated pressure conditions and prove the theory. Based on the theoretical guidance, a novel method of achieving uniform flow startup at low flowrates in parallel-flow microchannels without changing the geometry of the devices was created. Filling cell culture wells with porous fibrous matrices increased hydrodynamic pressure drop and provided a better balance with the capillary force exerted at the juncture of a microchannel and a microwell even at a significantly reduced flowrate, thus facilitating parallel flow startup at low flowrates in the microbioreactor array. These results provide theoretical and practical guidance on device design, material selection and operation.

4.3 Materials and Methods

4.3.1 Device fabrication
Figure 4.1 shows the designs for 2×4 and 4×4 microbioreactor arrays composing of connecting microchannels, cell culture wells and/or relay wells. These microbioreactor arrays were fabricated using photolithography and replica molding [15]. Briefly, a silicon wafer was spin-coated with a negative photoresist SU-8 100 (MicroChem) at a thickness of 350 μm for the 2×4 array and 370 μm for the 4×4 array. Then the wafer was exposed to UV light under a transparency mask with desired patterns. The patterned structures on the wafer were developed, forming a mold for replica molding. PDMS (Sylgard-184, Dow Corning) prepolymer was mixed with curing agent by a ratio of 10:1 and poured over the patterns on the wafer to a thickness of about 2 mm. Heated on a hot plate at 80°C for 2 hours, PDMS pieces were polymerized, peeled off and cut according to desired boundaries. For the 2×4 array and the 4×4 array with a channel height of 740 μm, the device had two layers made of PDMS with features facing each other to form the flow path. For the 4×4 array with a channel height of 370 μm, only one patterned layer was sealed against a plain PDMS layer to form the flow path. The channel width was 500 μm, the cell culture wells had a diameter of 3 mm for all three designs, and the relay well on the 2×4 array was 1.5 mm in diameter.

4.3.2 Device packaging

The device packaging was reversible through a frame-assisted assembly, which consisted of a set of top and bottom frames (Figure 4.2), made of poly(carbonate) sheets and fabricated via computer numerical control milling. Open windows were made on the frames for fluidic ports and cell culture wells. Before packaging, the fluidic ports were built by fixing needles into the top PDMS layer at the positions for inlet and outlet and through the open windows of the top frame with alignment.
PDMS prepolymer was used to seal the connections and to bond the top PDMS and top frame together by curing at 97°C for 20 min. During packaging, the bottom PDMS layer was placed in alignment on the bottom frame, and the bonded top layers were placed on top in alignment. The four pieces were held together by screws. Good sealing was achieved due to the elasticity of PDMS. For some experiments, the cell culture and/or relay wells were filled with porous fibrous poly(ethylene terephthalate) (PET) matrices, which were cut according to the size of the well and placed into the desired positions before device packaging.

4.3.3 PET fibrous matrix

Nonwoven poly(ethylene terephthalate (PET) fibrous matrices (fiber diameter: 20 μm, porosity: 0.95, thickness: 2 mm) were compressed under 30 kPa at 120 °C for 60 min to reduce matrix porosity [16]. The treated matrices were about 1 mm thick with a porosity of about 0.85 and average pore size of about 45 μm [16]. They were cut into square patches with a side length of about 3 mm to fit into the cell culture wells or with a side length of about 1.5 mm to fit into relay wells. The height of the cell culture well on the 2×4 array was 700 μm. Assuming the cross sectional area of the fibrous matrix does not change, the porosity could be calculated to be 0.79 due to compression in the assembled device. Similarly, for the 4×4 array with cell culture well of a height of 740 μm, the porosity became about 0.80. The changed porosities were used in pressure drop calculations.

4.3.4 Parallel flow startup testing

For the 2×4 array, parallel flow startup experiments were conducted at a flowrate of 0.5 mL/min under three different conditions: with empty wells, with cell culture wells
filled with PET matrices, and with both cell culture and relay wells filled with PET matrices. Theoretical analysis was applied in each case. In order to see whether the theory holds true for a more complex design, a 4×4 microbioreactor array was also used in parallel flow startup experiments. A threshold flowrate was calculated based on the theory presented in the following section, and experiments were run for verification. In all experiments, food dye solutions were used to trace the flow. The fluid was driven by a syringe pump (PHD 2000, Harvard Apparatus). The flow was recorded with a CCD camera right above the device. Frames of pictures were captured from the videos for illustration.

4.4 Theory

The pressure drop and volumetric flow rate can be correlated with the following equation [17]:

\[ Q = \frac{1}{\mu} \frac{\Delta P_c}{R_F} \]  

(1)

where \( Q \) is the volumetric flow rate, \( \Delta P_c \) is the hydrodynamic pressure drop in the rectangular channel, \( \mu \) is the viscosity, and \( R_F \) is the flow resistance. For rectangular microfluidic channels, \( R_F \) can be estimated by a linear solution of a Fourier series [17]:

\[ R_F = \left[ \frac{1}{12} \frac{(a + b)^2}{b^2} \left(1 - \frac{1}{8} \frac{a}{b} \right) \frac{abR_{H}^2}{L} \right]^{-1} \]  

(2)

where \( a \) or \( b \) is either channel width or depth, respectively, satisfying \( a < b \), \( L \) is the length of the microchannel, and \( R_{H} \) is the hydraulic radius, which can be expressed as [17]:
\[ R_H = \frac{2A}{P} = \frac{ab}{a+b} \]  

(3)

Within cell culture well and relay well, the flow could be approximated with an axial flow through a cylinder [18]:

\[ \Delta P_w = \frac{8\mu l u_s}{R^2} \]  

(4)

where \( \Delta P_w \) is the pressure drop through a cell culture well or a relay well, \( l \) is the axial length of the well, \( R \) is the radius of the well, and \( u_s \) is the superficial velocity of the flow. Young-Laplace equation is used to analyze the capillary pressure, \( P_c \), in the microchannels [17].

\[ P_c = -\gamma \left( \frac{\cos \alpha_b + \cos \alpha_t + \cos \alpha_l + \cos \alpha_r}{d} + \frac{\cos \alpha_b + \cos \alpha_t}{w} \right) \]  

(5)

where \( \alpha_b, \alpha_t, \alpha_l, \) and \( \alpha_r \) are the contact angles at bottom, top, left and right walls, respectively, with \( d \) and \( w \) representing the depth and width of the channel, and \( \gamma \) the surface tension of water. In this study, \( \alpha_{b,t,l,r} \) are all the same as the contact angle of PDMS, which is 113°. The surface tension of water at 25°C is 0.072 N/m.

For Newtonian fluids at low flowrates, the flow through a porous fibrous matrix can be described by Darcy’s Law, as follows [19]:

\[ u_{\beta} = \frac{k}{\mu} \frac{\Delta P_f}{l} \]  

(6)

where \( k \) is the hydrodynamic permeability, \( \Delta P_f \) represents the pressure drop through the fibrous matrix of a length \( l \), and \( u_{\beta} \) is the superficial velocity of the flow in the
microchannel, which can be estimated using the following equation:

\[ u_{fs} = \frac{Q}{(1-\phi)\pi R^2} \]  

(7)

where \( \phi \) is the solid fraction of the fibrous matrix and \( R \) is the radius of the well.

The permeability \( k \) in our system can be estimated by Happel model, which describes flow perpendicular to an array of rods as follows [20]:

\[ \frac{k}{r^2} = \frac{1}{8\phi}(-\ln \phi + \frac{\phi^2 - 1}{\phi^2 + 1}) \]  

(8)

where \( r \) is the radius of the fiber.

4.5 Results

4.5.1 Non-uniform startup in 2×4 array

Two lines are the simplest case for testing parallel flow startup. The flows in the 2×4 microbioreactor array without PET fibrous matrices did not start up in parallel at 0.5 mL/min (Figure 4.3). As seen in Figure 4.3.B and C, the pause of the flow in Line 2 occurred at the spot that had the smallest dimension in the whole channel. Therefore, the capillary pressure was maximum. By Equation (5), the capillary pressure, \( P_c \), at the inlet of a cell culture well was as large as 273.3 Pa with a direction from air to liquid due to the hydrophobicity of PDMS. Furthermore, after the microchannel is filled with liquid, pressure drop through one connecting channel was only 16.4 Pa as estimated from Equations (1)-(3). Based on Equation (4), the cell culture well and relay well had a low pressure drop of 0.0037 Pa and 0.059 Pa, respectively. It is clear that capillary pressure was so dominant in the startup phase, that it was even larger...
than the summation of all the flow resistance generated in a whole line containing 8 connecting channels at a flowrate of 0.5 mL/min (273.3 Pa > 130.9 Pa). In the force analysis, the driving force at the common inlet for PDF using a syringe pump was dynamically determined by the downstream hydrodynamic pressure drop. When one line is passed, the driving force at the common inlet would be only 130.9 Pa, insufficient to surpass the capillary pressure in the non-flowing channel. This was hypothesized to be the reason why flows could not be started uniformly in parallel microchannels.

Further experiment was conducted for parallel startup in the 2×4 array with PET porous fibrous matrix filling each cell culture well. Again, the flow startup was not uniform under PDF at a flowrate of 0.5 mL/min. The same theoretical analysis may apply as above. By Equations (6)-(8), the pressure drop was 25.4 Pa through the cell culture well filled with PET matrix. The capillary pressure was still larger than all of the flow resistances in one passed line containing 8 connecting channels and 4 PET-fibrous-matrix filled cell culture wells (273.3 Pa > 232.5 Pa). Therefore, the hydrodynamic pressure drop at 0.5 mL/min was not large enough to overcome the capillary pressure.

It was a concern that manufacture defects might cause the non-uniform startup. In addition, the real situation for perfusion cell culture is that the flow will only start when the cell culture wells are filled with cell seeded PET fibrous matrices. Therefore, the following experiment was conducted. A 2×4 microbioreactor array with red dye solution pre-wetted fibrous matrices in cell culture wells was perfused with a green dye solution (Figure 4.4). The flow sequence was designed in a way that the green solution first filled the device (Figure 4.4.B), followed by air purging (Figure 4.4.C),
and then green solution filling again (Figure 4.4.D). It was found that the flow might randomly pick either Line 1 or Line 2, indicating that the manufacture imperfection was not the cause for non-uniform flows.

4.5.2 Parallel startup in 2×4 array

Previous experiments provided a hypothesis that by increasing hydrodynamic pressure drop to a value that surpasses the capillary pressure, the parallel startup can be achieved. The pressure drop in a relay well filled with PET fibrous matrix was as high as 101.7 Pa by Equations (6)-(8). Therefore, for a passed line containing 8 connecting channels, 4 fiber-filled cell culture wells, and 3 fiber-filled relay wells, the hydrodynamic pressure drop added up to 537.6 Pa, which was larger than the maximum capillary pressure 273.3 Pa. Therefore, another flow experiment was conducted at 0.5 mL/min with all the cell culture wells and relay wells filled with PET fibrous matrices. As shown in Figure 4.5, parallel flow startup was observed just as the theory predicted.

4.5.3 Parallel startup in 4×4 array

The theory for estimating the threshold flowrates to achieve parallel flow startup in a series of 4×4 microbioreactor arrays is illustrated in Figure 4.6.A. Device designs with a channel height of 740 μm had a maximum capillary pressure of 188.6 Pa. By theory, the threshold flowrate resided around 9 mL/min for the device with empty cell culture wells. The threshold flowrate can be reduced to around 2 mL/min for the device with cell culture wells filled with PET fibrous matrices. Similarly, for the device with a channel height of 370 μm, the threshold flowrate is estimated to be around 4 mL/min to match the maximum capillary pressure of 264.6 Pa. In
experiments, parallel startup was achieved with a success rate of 100% at the predicted threshold flowrate for the device with 740 μm channels (Figure 4.6.B), with a 100% success rate for the device with PET fibrous matrices filled cell culture wells (Figure 4.6.C), and a 75% success rate for the device with 370 μm channels (Figure 4.6.D). As an example, this process was also illustrated with time series photos showing how the parallel startups were achieved at the threshold flowrate for the device with 740 μm channels (Figure 4.7). The parallel startups were confirmed with a low flowrate of a distinct color solution immediately following the threshold flowrate, demonstrating that capillary pressure had no effect after filling the multiple microchannels.

4.6 Discussion

Fluids behave differently at micro- and macro-scales due to different major forces coming into play. This is not simply determined by dimensions. Material selection, design and process may altogether affect fluidic behaviors. Capillary pressure is a major force in microchannel at the flow startup phase. For hydrophilic microchannels made of, e.g., glass, aqueous solutions can autonomously fill the space occupied by air, which has already been taken advantage of in many biomedical microfluidic devices [21,22]. On the contrary, in hydrophobic microchannels, capillary pressure generates an opposing force against filling from a common inlet. This was actually employed to make passive pumping where liquid could be pumped to a higher gravitational potential due to the wettability difference between inlet and outlet of a microchannel [23]. Microchannel hydrophobicity was also used to create passive valving with which parallel dispensing of small liquid plugs was achieved for diagnostic applications [24]. In another example, hydrophobic surface patterns were
incorporated in hydrophilic microchannels to control flowrate for increasing incubation times in biochemical assays [25]. Furthermore, strong hydrophobicity can also be established by special design of microchannels. For example, abruptly changing geometry of a flow path can be used as a superhydrophobic fishbone valve to prevent hydrophobic microchannels from changing to hydrophilic after nonspecific protein binding [26] or as a component to delay liquids in hydrophilic microchannels [27].

In this study, we have shown that the capillary pressure may hinder uniform flow in parallel microchannels made of PDMS at the startup phase. However, there exists a threshold flowrate at which parallel flow startup can be achieved. The threshold flowrate can be estimated, in theory, by a balance between the maximum capillary pressure and the total hydrodynamic pressure drop in a microchannel. When the hydrodynamic pressure drop surpasses the maximum capillary pressure, parallel flow startup can be rendered. Capillary pressure is determined by the dimensions and building materials of the microchannel, while hydrodynamic pressure drop is determined by microchannel dimensions and liquid flowrate. Therefore, with a certain material, such as PDMS, and a defined flowrate, the capillary pressure and hydrodynamic pressure would change along with the dimensions of the microchannel. If we assume a square cross section and a defined length for the microchannel, two curves can be plotted: capillary pressure vs. channel width and hydrodynamic pressure drop vs. channel width, respectively (see Figure 4.8). Therefore, as an interesting extrapolation, the threshold microchannel dimension for achieving parallel flow startup can be estimated at the intersection of the two curves. For example, for a 4×4 array with a channel length of 25 mm, the threshold channel width is around 25
μm for the flowrate of 0.001 mL/min, 45 μm for 0.01 mL/min, and 120 μm for 0.1 mL/min. This kind of knowledge should be helpful for providing guidance on correlating device design and operations before it is fabricated. Furthermore, as seen in Figure 4.8, there is a sharp increase in hydrodynamic pressure drop as channel dimension decreases, which progresses much faster than the increase of capillary pressure. Therefore, for microchannels below 100 μm, parallel startup can be achieved at a relatively small flowrate, while for multiple hydrophobic microchannels larger than 500 μm, the parallel flow startup requires a much higher flowrate. Meanwhile, larger microchannels and micro-wells provide an adequate dimension where fibrous matrix can be used to significantly reduce the threshold flowrate. Therefore, the niche dimension for using the porous media is larger than 500 μm and up to 3 mm, as shown in this study. In addition, it is also noteworthy that when all the channels are fully filled, the capillary pressure no longer exists, allowing uniform parallel flow startup at any flowrates.

4.7 Conclusion

Microfluidic systems provide an enabling technology for conducting miniaturized and high-throughput experimentations. However, at microscale, fluid behaviors are affected by different factors than at macroscale. Capillary pressure plays a key role in causing non-uniform filling in multiple hydrophobic microchannels. This phenomenon was confirmed in this study through both theoretical analysis and experimental observation that parallel flow startup required balancing the maximum capillary pressure with the hydrodynamic pressure drop at the threshold flowrate. When hydrodynamic pressure drop surpasses the maximum capillary pressure, uniform flow startup in parallel hydrophobic microchannels can be ensured. When
low startup flowrates are necessary, such as for cell culture applications, PET fibrous matrices can be employed to increase the flow resistance and to balance the two flow controlling forces, capillary pressure and hydrodynamic pressure drop. Significantly reduced threshold flowrate can be achieved without changing the original device geometries. In addition, using the same theory, it is found that when microchannels are smaller, lower flowrates are sufficient for achieving the parallel flow startup. The findings in this study can help researchers to better understand the major forces at microscale, so it would benefit the design and operation of microfluidic devices with hydrophobic materials for high-throughput applications.
4.8 Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$\alpha$</td>
<td>Contact angle of PDMS</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Surface tension of water</td>
</tr>
<tr>
<td>$\theta$</td>
<td>Angle between flow front line and radius line</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Viscosity of water</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Solid fraction of fibrous matrix</td>
</tr>
<tr>
<td>$A$</td>
<td>Cross section area</td>
</tr>
<tr>
<td>$a$</td>
<td>Channel width or height, satisfying $a &lt; b$</td>
</tr>
<tr>
<td>$b$</td>
<td>Channel height or width, satisfying $a &lt; b$</td>
</tr>
<tr>
<td>$c$</td>
<td>Half length of flow front line in a well</td>
</tr>
<tr>
<td>$d$</td>
<td>Channel depth</td>
</tr>
<tr>
<td>$d_e$</td>
<td>Half length of channel width at a well entrance</td>
</tr>
<tr>
<td>$k$</td>
<td>Hydrodynamic permeability</td>
</tr>
<tr>
<td>$L$</td>
<td>Distance between cell culture well and relay well</td>
</tr>
<tr>
<td>$l$</td>
<td>Length of fibrous matrix</td>
</tr>
<tr>
<td>$P$</td>
<td>Wetted perimeter</td>
</tr>
<tr>
<td>$P_c$</td>
<td>Capillary pressure</td>
</tr>
<tr>
<td>$\Delta P_c$</td>
<td>Pressure drop in connecting channel</td>
</tr>
<tr>
<td>$\Delta P_f$</td>
<td>Pressure drop through fibrous matrix in a well</td>
</tr>
<tr>
<td>$\Delta P_w$</td>
<td>Pressure drop through a well w/o fibrous matrix</td>
</tr>
<tr>
<td>$Q$</td>
<td>Volumetric flow rate</td>
</tr>
<tr>
<td>$R_c$</td>
<td>Radius of cell culture well</td>
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<td>$R_F$</td>
<td>Flow resistance</td>
</tr>
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<td>Hydraulic radius</td>
</tr>
<tr>
<td>$R_r$</td>
<td>Radius of relay well</td>
</tr>
<tr>
<td>$r$</td>
<td>Radius of fiber</td>
</tr>
<tr>
<td>$u_{sc}$</td>
<td>Superficial velocity in cell culture well</td>
</tr>
<tr>
<td>$u_{sr}$</td>
<td>Superficial velocity in relay well</td>
</tr>
<tr>
<td>$w$</td>
<td>Channel width</td>
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4.8 References


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Figure 4.1. Schematics of 2×4 and 4×4 perfusion microbioreactor arrays. A. Top view of the 2×4 array. B. Side view of the flow path through a relay well, a cell culture well and connecting channel formed in double-layer soft lithography in the 2×4 array. C. Top view of the 4×4 array.
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5.1 Abstract

A novel, low-cost microplate-based 24-well microbioreactor array was developed for high-throughput cell cultures. The bottom and the lid of the array were made of a poly(dimethylsiloxane) (PDMS) membrane that reduced evaporation by 30% without affecting aeration. A central static mixer was built in each well to improve mixing patterns and reduce cell aggregates under orbital shaking conditions. Each well of the plate also had a set of observation windows at the bottom, amenable to optical measurements with a standard microplate reader. In this study, mixing time and maximum oxygen transfer rate were characterized using optical methods. Consistent cell growth and metabolism at different positions on the array were achieved for suspension culture of Chinese hamster ovary (CHO) cells. The microbioreactor array significantly improved the data quality, especially reproducibility, as compared with commercial multi-well plates. The 24-well microbioreactor array was used in an experiment with 2-level fractional factorial design to screen and identify active factors in a serum-free medium (SFM) for monoclonal antibody (MAb) production by CHO cells. The SFM developed in the screening experiment was then tested in spinner flask cultures and the results were consistent with
those from the microbioreactor cultures, demonstrating the scalable performance of the microbioreactor array.

5.2 Introduction

Therapeutic biologics are becoming more and more important as medicines. For example, over 130 protein therapeutics are approved by FDA in clinical use and many more are being developed [1]. However, the manufacturing of biologics is highly complex, and defined process parameters and optimized media should be developed in early clinical trials. Furthermore, it is practically infeasible to conduct all the screening and optimization experiments with commercial scale bioreactors [2]. Therefore, the biopharmaceutical industry has developed a substantial interest in scale-down and high-throughput cell culture platforms that can facilitate scalable process development with significant cost and time savings [3].

One important application of such systems is for medium development, which requires high-throughput experimentation to define the design space for screening and optimization [4]. An increasingly used scale-down model for medium development is multi-well plates due to its low cost and easy operation for both microbial fermentations [5] and animal cell cultures [6]. However, there are several challenges in using these systems, such as evaporation and poor mixing patterns. Evaporation plagues micro-scale cell cultures by medium loss and excessive variations among different wells. In addition, due to suboptimal mixing patterns, mammalian cells tend to form aggregates and accumulate at the center in the well, which causes difficulty in sampling and cell number quantification.

We have developed a 24-well microbioreactor array featured with an air-permeable and
low evaporation lid for the plate and a central static mixer in each well. With these features, data quality for cell culture was significantly improved as compared with commercial multi-well plates. In this study, mixing and oxygen transfer rate as affected by the static mixer in the microbioreactor were characterized. Consistent culture performance throughout the whole plate was demonstrated with Chinese hamster ovary (CHO) cell cultures. The microbioreactor array was successfully used in an experiment with 2-level fractional factorial design to screen and identify active factors for formulating a serum-free medium for CHO cell cultures and monoclonal antibody production. The scalability of the microbioreactor cell culture performance was demonstrated by consistent results from the microbioreactor array and spinner flasks.

5.3 Materials and Methods

5.3.1 Microbioreactor array

The microbioreactor array had the same dimensions of a commercial multi-well plate. In one plate, there were 24 corner-rounded square wells as microbioreactors in a 6×4 array. Four adjacent openings were fabricated as observation windows at the bottom of each individual microbioreactor (Figure 5.1.A). Each microbioreactor had a central static mixer. For testing purposes, there were two different sizes of static mixers: large and small (Figure 5.1.B). The working volume of the microbioreactor ranged from 0.6 to 1.5 mL. The microbioreactor array was fabricated using polycarbonate sheet via computer numerical control milling. The bottom of the microbioreactor array was cushioned with a poly(dimethylsiloxane) (PDMS) (Sylgard-184, Dow Corning) membrane of a thickness of ~500 μm. PDMS prepolymer was mixed with curing agent at 10:1 ratio, and the mixture was applied to seal the plate bottom with the PDMS membrane on a hotplate at 95°C for 15 min. Due to the high permeability of non-polar gases through PDMS, the four
observation windows could serve to enhance aeration fluxes for each microbioreactor. A flexible lid for the plate was also made from a PDMS membrane with features conforming to the top of each microbioreactor, rendering reversible sealing against evaporation without affecting aeration (Figure 5.1.C). The sterilization of the microbioreactor array was via autoclaving. The microbioreactor array was operated with agitation on an orbital shaker in an incubator, providing sufficient mixing and surface aeration (Figure 5.1.D).

5.3.2 Mixing time characterization
The mixing time of individual microbioreactor was measured by the time required to observe a color change based on an acid-base reaction [7]. Briefly, a fresh solution containing 0.1 N NaOH and a pH indicator, 1% (v/v) phenolphthalein, was diluted 1000 fold with distilled water. The base solution exhibited a pink color. 1.2 mL pre-mixed base-solution was added to a microbioreactor. At time zero when plate was under agitation at a predetermined agitation rate, 12 μL 0.1 N HCl solution was quickly dispensed into the pre-loaded base solution using a pipette, and meanwhile time recording was initiated. At the point of color disappearance, the stopwatch was terminated to record the mixing time. The mixing time of a commercial 24-well plate (Falcon) was also characterized using the same method. The tested agitation rates were 95, 115, 145, 180 and 215 rpm, respectively. There were four replicates for each mixer at each agitation rate, and Student’s t test was applied for data analysis with α = 0.05.

5.3.3 Oxygen transfer rate characterization
Due to small working volume and constant shaking, conventional electrochemical dissolved oxygen probe was impractical for use in the microbioreactor. Therefore, a non-invasive optical method based-on sulfite oxidation, shown in Equation (1), was used
to determine the oxygen transfer rate (OTR) in the microbioreactor [8]. In this method, the rate of sulfite oxidation is equal to the rate of oxygen transfer into the liquid phase, as shown in Equation (2). Because sulfite oxidation is fast and all oxygen transferred into the liquid phase would be consumed instantaneously, the dissolved oxygen concentration is close to zero, and the OTR estimated with this method represents the maximum rate, OTR$_{\text{max}}$.

\[
SO_3^{2-} + 0.5O_2 \xrightarrow{\text{Co}^{2+}} SO_4^{2-}
\]  

\[
OTR_{\text{max}} = 0.5[Na_2SO_3]/t
\]  

where [Na$_2$SO$_3$] is the initial molar concentration of sodium sulfite and $t$ is the time for its complete oxidation to sulfate. OTR$_{\text{max}}$ in the microbioreactor was tested at three agitation rates (95, 145 and 215 rpm) in an orbital shaker. Briefly, a stock solution of 0.012 M phosphate buffer (Na$_2$HPO$_4$/NaH$_2$PO$_4$, Sigma-Aldrich) at pH 8 containing $2.4\times10^{-5}$ M bromothymol blue (Sigma-Aldrich) and $10^{-7}$ M cobalt sulfate (Sigma-Aldrich) as catalyst was prepared. Fresh 0.5 M sodium sulfite (Sigma-Aldrich) solution prepared with the aforementioned phosphate buffer was adjusted to pH 8 using 2 M sulfuric acid and then purged with nitrogen gas to remove dissolved oxygen. 1.2 mL buffered sulfite solution was added to each microbioreactor. The whole plate was then covered with the elastic PDMS lid and placed on top of the orbital shaker for agitation. A modified microplate lid was used as a support for the microbioreactor array to allow bottom of the plate to be exposed to air for aeration. The completion of the sulfite oxidation was indicated by a color change from blue to yellow. The duration of the reaction was recorded. Six replicates at different positions of the plate were tested for each agitation rate and each mixer size. A Student’s t test was applied for data analysis, with $\alpha = 0.05$. 

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5.3.4 Evaporation characterization

To evaluate the water evaporation rate, 1 mL deionized (DI) water was precisely added to each well of the microbioreactor array through a pipette and the plate was covered with the PDMS lid. The microbioreactor array was immediately placed in a 5% CO\textsubscript{2} incubator at 37°C. For comparison, a commercial 24-well plate was also tested in the same way at the same time. The weight of 1 mL DI water dispensed using the same pipette was measured with 4 replicates, which was used as the initial value for the amount of water. After 7 days, the water in each well was transferred with precision to measure its weight for both the microbioreactor array and the commercial 24-well plate. Based on the weight differences of water before and after incubation, the water evaporation rates at different wells on the microbioreactor array and the commercial 24-well plate were estimated and analyzed with Student’s t test.

5.3.5 Cell line and medium

CHO cell line 6E6 (ATCC CRL-11398) producing 23F2G, a humanized anti-CD18 monoclonal antibody (MAb), was cultured and maintained in T-flasks containing the medium DMEM/F12 (Gibco) (1:1) with 5% fetal bovine serum (FBS) (Gibco), incubated in an incubator with 5% CO\textsubscript{2} at 37°C. The T-flask culture was subcultured every 5 days using trypsin (Gibco) to detach cells.

5.3.6 Suspension culture

Suspension cultures of CHO cells in DMEM/F12 with 5% FBS were carried out in the 24-well microbioreactor array to investigate the performance consistency of different wells on the array and their performance sensitivity to subtle operational variations. The array was symmetrically divided into 4 sections with each section having 6 wells. To evaluate the reproducibility of well performance, the same seeding density was used for
all 6 wells within each section and four different seeding densities were used for the four sections: 1.75±0.63×10^5 cells/mL, 0.56±0.2×10^5 cells/mL, 0.27±0.09×10^5 cells/mL, and 0.14±0.07×10^5 cells/mL. Profiles for cell proliferation, viability and metabolism were investigated. In order to compare the quality of data trending, commercial non-tissue culture treated 6, 12 and 24-well plates were also used for CHO suspension cultures with measurements for proliferation and viability profiles. Cell number and viability were quantified using trypan blue (Sigma-Aldrich) staining with hemocytometer counting under a microscope.

5.3.7 Screening for active factors in serum-free media

The microbioreactor array was used in an experiment with 2-level fractional factorial design for screening and identifying active factors in serum-free media (SFM) for MAb production in CHO suspension cultures. Cells were harvested from T-flasks with trypsin, rinsed with phosphate buffer saline (PBS) (Sigma-Aldrich) 3 times, and then cultured in serum-free media for 4 days. The basal medium used in this experiment was DMEM/F12 supplemented with 300 mg/L pluronic F68 (Sigma-Aldrich). Thirteen factors (see Table 5.1) were selected for screening in a 2-level fractional factorial Plackett-Burman design containing 20 experiments plus 4 central replicates generated using JMP (SAS) (see Table 5.2). The unit of the chemicals was in mg/L except for the solutions of MEM amino acids (Gibco, 50X), MEM non-essential amino acids (Gibco, 10 mM, 100X) and L-glutamine (Gibco, 200 mM, 100X). The factors included recombinant human insulin with zinc (Gibco, 4 mg/mL solution), aurintricarboxylic acid (ATA), sodium selenite, sodium pyruvate, L-ascorbic acid, ethanolamine, dextran sulfate, putrescine-2HCl, ferrous sulfate, and manganese sulfate (Sigma-Aldrich). Two responses, viable cell density increase ratio between the final and initial cell counts and MAb titer normalized to the highest concentration, were investigated to identify active factors in the media.
5.3.8 Enzyme linked immuno-sorbent assay (ELISA)

ELISA was used to analyze the MAb titer. Costar 96-well EIA/RIA Easy Wash plates (Corning) were coated for 1 h under mild agitation in a 37°C incubator with the first antibody, a goat anti-human IgG (Jacksonimmuno, West Grove, PA), followed by blocking with BSA under the same conditions. Diluted human IgG standard (Sigma-Aldrich) and samples were added into plates and incubated for another 1 h at 37°C. The second antibody, a goat anti-human IgG conjugated with horseradish peroxidase (HRP) was added to each well, and the plate was incubated for 1 h at 37°C. Then, the enzyme substrate solution containing 3-(p-hydroxyphenyl) propionic acid and hydrogen peroxide was added to generate fluorescence, which was monitored with a fluorescence plate reader (GENios Pro, Tecan) at excitation 320 nm and emission 405 nm. IgG standard at various concentrations were used to correlate with the reaction rate, as calculated from the fluorescence intensity increase with time, which was used as the standard curve to quantify the MAb titer in samples.

5.3.9 Scalability test in spinner flasks

After identifying the active factors in the SFM, the central points of these factors were chosen and used in spinner flask cultures for a scalability test. Four 100-mL spinner flasks each with a working volume of 40 mL were run in parallel using the SFM containing the active factors for CHO suspension cultures. The seeding density for the spinner flask cultures was $2.16 \pm 0.24 \times 10^5$ cells/mL. The profiles for cell proliferation, viability, metabolism and MAb titer were characterized. The scalability between the microbioreactor array and spinner flasks was determined based on specific growth rates and MAb titers obtained in these different-scale suspension cultures.
5.4 Results

5.4.1 Mixing time

Figure 5.2 shows the effects of agitation rate and mixer size on mixing time. In general, with increasing agitation, mixing time was decreased. Furthermore, the presence of mixer and its size also affected mixing time in a way that was dependent on the agitation rate. Counter-intuitively, the presence of the static mixer appeared to limit mixing at low agitation rates. The mixing time with the large mixer was significantly longer than that with the small mixer (p = 0.0004) and that without mixer (p < 0.0001) at 95 rpm. The mixing time with the small mixer was also significantly longer than that without mixer (p = 0.006). However, with increased agitation, there was no significant difference in mixing times between the small mixer and no mixer at 115 rpm, 145 rpm and 180 rpm, respectively, while the large mixer still had significantly longer mixing times. It is noteworthy that at 215 rpm, the beneficial effect of static mixer on mixing time became prominent, and both large and small mixers generated significantly shorter mixing times than that without a static mixer. In general, the scale of the mixing time with the microbioreactor array could well satisfy the kinetics requirements of typical cell culture processes in terms of nutrient consumption rate and cell growth [7].

5.4.2 Oxygen transfer rate

The effect of agitation rate on OTR_{max} in the microbioreactor is shown in Figure 5.3. As expected, OTR_{max} increased with increased agitation rate. In addition, the mixer size also showed a significant effect on OTR_{max} at lower agitation rates. The small mixer had a larger OTR_{max} at 95 rpm (p = 0.01) and 145 rpm (p = 0.03) than large mixer, but there was no significant difference at 215 rpm (p = 0.59). For mammalian cell culture, the oxygen uptake rate (OUR) is between $3 \times 10^{-10}$ and $2 \times 10^{-8}$ mg/cell·h [9]. When cell density is at
10^7 cells/mL, the OUR can range from 0.094 mmol/L·h to 6.3 mmol/L·h, which could be well satisfied by the OTR (9.5 mmol/L·h at 215 rpm) provided in the microbioreactor. It is thus clear that good aeration can be achieved with moderate agitation when the gas permeable PDMS membrane was used in the bottom and lid of the microbioreactor array.

5.4.3 Evaporation rate
The microbioreactor array with the elastic PDMS lid had significantly reduced water evaporation rate as compared with the commercial 24-well plate (Figure 5.4). Comparisons were made between the two platforms based on different positions on the plate, with 4 replicates for each platform. Except for the four corners, where no significant difference (p = 0.28) were found between the two platforms, the evaporation rate was reduced by 30% along the side (p = 0.002), by 36% in the center (p = 0.0002), and by 31% overall (p < 0.0001) in the microbioreactor array. The reduced evaporation rate helped improve data quality and allowed a longer culturing period in the microbioreactor array.

5.4.4 Cell culture characterization
For comparison, CHO cells were cultured in suspension in different commercial multi-well plates under agitation. It was found that, for example in a 24-well plate, there were significant cell aggregates formed in various sizes around the center of a well and a layer of cells floating on the medium surface (Figure 5.5). None of these phenomena was observed with the microbioreactor cultures. The non-uniform cell distribution made it very difficult to get reliable sampling for cell number quantification and resulted in poor data trending quality for proliferation and viability assays. Consequently, the culture data from the conventional 24-well plate had much larger errors than those in the microbioreactor cultures (see Table 5.3).
Figure 5.6 compares the cell culture kinetics from the four different sections with different seeding densities on the microbioreactor array. In general, the proliferation and viability profiles exhibited typical batch kinetics and they were replicable within each section with moderate errors. In this experiment, different seeding densities were used in different sections to show the performance sensitivity of the device to subtle variations in operation conditions. As shown in Figure 5.6.B, the specific growth rates, which were estimated from the growth curves shown in Figure 5.6.A, was higher in sections of lower seeding densities. For example, the specific growth rates in Sections III and IV were significantly higher than that in Section I, with $p = 0.0053$ and $0.0142$, respectively. This result is consistent with the general observation that a low seeding density usually results in a high growth rate at the initial phase [10]. Similarly, the metabolic profiles were reproducible within each section but showed distinct metabolic rates in different sections due to different seeding densities (Figure 5.6.C).

It is noted that the cell culture kinetics was not significantly affected by the well position on the array. With the same seeding density, data from microbioreactors on the corner, along the side and in the center of the array plate all showed the same specific growth rate, with $p = 0.66$ for corner vs. side, $p = 0.61$ for center vs. side, and $p = 0.90$ for corner vs. center. In addition, there was no significant difference in the specific growth rate between microbioreactors with large and small mixers ($p = 0.79$).

5.4.5 Screening for active factors in serum-free media

Table 5.2 shows the Plackett-Burman design for the screening of 13 factors in serum-free media using the microbioreactor array. Two responses, viable cell density (VCD) increase ratio and normalized MAb titer, were used to evaluate the effects of these medium factors. Plackett-Burman design is a 2-level fractional factorial design, and is an economic
method for screening a large number of factors. Due to its low resolution and lack of replicates, only main effects can be identified and interaction effects are confounded. Normal probability charts, however, can be used to identify the effects of significant magnitude [11]. This method has been used to accelerate identification of CHO growth factor requirements [12] and to screen active factors for antibody production by CHO cells [13]. Figure 5.7 presents the normal probability charts based on VCD increase ratio (A) and MAb titer (B). Three active factors, insulin, ferrous sulfate, and additional putrescine-2HCl, were identified using the VCD response, which could supplement the basal medium to support over 4 times of cell number amplification in a 4-day batch culture. In the normal probability chart with MAb titer, two components were consistent with the findings based on VCD, but insulin showed a negative effect on the MAb titer, which could be due to that the high level of insulin chosen in this 2-level fractional factorial design was inhibitory to MAb production. Since insulin was necessary for cell survival and growth, it was included as one of the 3 active factors in the serum-free medium for CHO suspension culture.

5.4.5 Scalability test

The three active factors, insulin, ferrous sulphate and putrescine-2HCl identified in the Plackett-Burman screening, were used at their center-point concentrations to supplement the basal medium to support batch cultures in 4 parallel spinner flasks in the scalability test. Figure 5.8 shows the cell culture kinetics in spinner flasks, including profiles for proliferation, viability, metabolic activity and MAb titer. In general, replicable performance of the medium was confirmed. In addition, the same medium was also used in 4 replicate cultures with the microbioreactor array. The scalability of the microbioreactor array cultures and the serum-free medium developed therein were confirmed by comparing the data on the specific growth rate and final MAb titer from
both microbioreactors and spinner flask cultures (Table 5.4). It is clear that good scalability could be established between the microbioreactor array and spinner flasks.

5.5 Discussion

With increasing numbers of novel therapeutic proteins, one important challenge in biologic drug development and manufacturing is bioprocess development. Bioprocess development focuses on increasing titer and maintaining product quality through optimization of culture conditions using high-throughput scale-down models. Various microscale bioreactors are being developed as promising scale-down models. The trend of microbioreactor development is miniaturization and automatic instrumentation. The volumes of microbioreactors have been decreased from milliliter scale [14, 15] to microliter scale [16-21]. Non-invasive optical sensors for pH and DO also played a key role in these microbioreactors. Several commercial scale-down microbioreactors are available on the market, including multi-well plates with sensing capabilities (PreSens GmbH, Regensburg, Germany) [22], and instrumented controllable microbioreactors such as SimCell™ (BioProcessors Corporation, Woburn, MA), Micro 24 Bioreactor (manufactured by MicroReactor Technologies, Inc., Mountain View, CA and marketed by Applikon Inc., Foster City, CA) [23,24], and Cellstation™ (Fluorometrix Corp., Stow, MA). However, these systems are expensive, requiring complex robots, and/or peripherals to operate. On the other end, multi-well plates and shake-flasks as scale-down models are commonly used in industry due to their low cost. However, they lack sensing and control with problematic mixing patterns. Especially for multi-well plates, evaporation is an issue. Therefore, the purpose of this study was to improve data quality with multi-well plates.

One major novelty of the microbioreactor array developed in this work was the central static mixer, which contributed to the improved mixing patterns in the microbioreactor.
With commercial multi-well plates, it was found that under orbital agitation in a single cell culture well the largest shear stress occurred near the edge of the well and it decreased towards the center [25]. It can be seen as a driving force to move the cells towards the center, where they accumulate, and due to poor mixing it is easy for cells to form aggregates (Figure 5.5.A). Therefore, it was difficult to get a reliable data trending. In contrast, with a central static mixer, the cells were evenly distributed throughout the whole microbioreactor with no aggregates formed (Figure 5.5.B, C). Therefore, data quality for cell number quantification could be significantly improved (Table 5.3).

However, at low agitation rates, the static mixer resulted in longer mixing times than a well without a mixer. In addition, large mixer had longer mixing times than small mixer. Similarly, at low agitation rates, large mixer had smaller OTRs than small mixer. This could be explained by the fact that at microscale, surface tension comes into play, limiting fluid motion at the liquid-gas-solid interfaces [5, 26]. With the large mixer in the microbioreactor, the interfacial area is the smallest compared with the small mixer and a bare well, resulting in the largest surface effects to retard liquid motion. Therefore, the mixing time was limited with the static mixer at low agitation rates. However, at high agitation rates such as 180 rpm, the small mixer had a shorter mixing time than that without a mixer. At 215 rpm, both the large mixer and small mixer generated a shorter mixing time than a bare well. Furthermore, the mixing times with the static mixers were generally below 140 s, which would well satisfy typical kinetics requirements of cell cultures with no limitations on mass transport. Furthermore, with cell culture media containing salts and surfactants, surface tensions would be significantly lowered due to the presence of these complex solutes [27]. Therefore, the liquid motion inhibition by the central static mixers at low agitation rates should have less effect under cell culture
conditions than under the tested conditions. It is noted that the static mixers in this study were above the liquid level, so there was the surface tension effect arising from the liquid-air-solid interfaces, which could be minimized or eliminated if they were submerged under the liquid level. Furthermore, it was found that shaking amplitude has a greater impact on mixing than frequency [28], which could also be applied to further improve the performance of a static mixer.

In addition, reduced evaporation also added to the performance consistency in the microbioreactor array with the PDMS membrane, which provided excellent sealing as a lid. Meanwhile, sufficient oxygen transfer was obtained with the same design and material. PDMS has been actively applied in the membrane aeration for microbioreactors due to its high permeability to non-polar gases, such as oxygen and CO₂, and also due to its low water vapor permeability [17, 20, 29]. In addition, there are other choices for the same purposes in microbioreactor applications, such as polymethylpentene (PMP) [7], polypropyl methylsiloxane (PPMS), polytrifluoropropylmethylsiloxane (PTFPMS), and polyphenylmethylsiloxane (PPHMS).

The microbioreactor array was applied in identifying active factors in serum-free media for CHO suspension culture. Plackett-Burman design was used for design of experiment. It is an economical method to screen a large number of factors with no replicates. Four center points were added to test the error. Insulin, ferrous sulfate, and putrescine-2HCl were identified as the active factors for CHO suspension culture. Insulin is a potent growth factor for CHO [12]. Similar growth enhancement was also reported with insulin-like growth factor 1 [30]. Ferrous sulfate has been identified as an economic replacement to transferrin for iron metabolism [31]. In this study, it indeed played a positive role to supplement the serum-free medium. One caution for using ferrous sulfate
is that it must be freshly prepared to avoid oxidation. The DMEM/F12 basal medium contained 0.081 mg/L putrescine. In this study, additional putrescine was found beneficial to both cell growth and MAb production. It has been found that polyamines and their precursor putrescine play a critical role in cell cycle and cell division [32], and polyamine starvation could cause cell cycle arrest [33]. Therefore, this could explain the benefits from additional putrescine in the serum-free medium. With this preliminary screening, however, no interaction effects were identified. It is desirable to use steepest ascent method to explore the design space and to approach the region of optimum, which is usually followed by response surface methodology for optimization [13]. Therefore, similar high-throughput experiments could be conducted with the microbioreactor array for the medium optimization. For this purpose, specific growth rate and titer could be used as responses due to the sensitivity and improved data quality of the microbioreactor array to subtle variations in the operation condition.

5.6 Conclusion

A novel and low-cost 24-well microbioreactor array featuring a central static mixer and gas-permeable membranes for good aeration and reduced evaporation was developed. Data quality was improved compared with commercial multi-well plates in CHO cell suspension culture. Due to the static mixer, poor mixing patterns of the well plates upon orbital shaking was effectively broken. A uniform cell distribution within the microbioreactor was achieved with no induction of cell aggregates. Reliable data trending could be obtained with performance consistency and sensitivity to subtle operation variations. The microbioreactor array was applied in the screening and identification of active factors in serum-free media. Both the microbioreactor array and the developed medium had scalable performances for CHO suspension culture compared with spinner
flasks based on specific growth rate and MAb titer. In addition, the standard design and
the four observation windows per microbioreactor are amenable for incorporation of
optical sensors for pH and DO. With the novelty and improvement established with this
prototype microbioreactor array, future studies could include computational fluid
dynamics evaluation of the optimal designs for static mixers, integration with optical
sensors, and high-throughput applications of the device for MAb titer improvement and
other cell culture bioprocess development.

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Table 5.1. Chemicals used in active factor screening for serum-free media. They are coded in letters. The levels of chemicals were listed with a unit of mg/L except for AA solution, NEAA solution and glutamine.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Code</th>
<th>High level (+)</th>
<th>Low level (-)</th>
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</thead>
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<td>ATA</td>
<td>A</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
</tr>
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<td>Sodium selenite</td>
<td>B</td>
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<td>0</td>
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Table 5.2 Active factor screening with a Plackett-Burman design and 4 central replicates.

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Table 5.3. Specific grow rates for CHO suspension cultures with different commercial multi-well plates and the microbioreactor array with different seeding densities.

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<th>12-Well Plate</th>
<th>24-Well Plate</th>
<th>Microbioreactor array</th>
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<tr>
<td>Seeding density (10^5 cells/mL)</td>
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<td>1.5</td>
<td>1.6</td>
<td>1.75 0.56 0.27 0.14</td>
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<td>Average Specific Growth Rate (h⁻¹)</td>
<td>0.015</td>
<td>0.007</td>
<td>0.007</td>
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<tr>
<td>Standard Deviation (h⁻¹)</td>
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<td>0.005</td>
<td>0.007</td>
<td>0.004 0.001 0.002 0.004</td>
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Table 5.4. Scalability test between cell cultures in microbioreactor array and spinner flasks

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<th>Microbioreactor Array</th>
<th>Spinner Flasks</th>
<th>p-value</th>
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<td>Specific Growth Rate (h⁻¹)</td>
<td>0.015±0.003</td>
<td>0.014±0.002</td>
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<tr>
<td>Final titer (mg/L)</td>
<td>6.09±0.67</td>
<td>6.07±0.77</td>
<td>0.9775</td>
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Figure 5.1. A. Design of the 24-well microbioreactor array. B. Each microbioreactor is a square well with corner rounding and a cluster of 4 openings as observation windows. There is a static mixer in the center of each microbioreactor. Two sizes of the central static mixers are designed for testing. C. A flexible PDMS membrane was used to reversibly seal the top of the plate. D. The microbioreactor array is conveniently agitated on an orbital shaker to provide sufficient mixing and aeration.
Figure 5.2. Effects of agitation rate and static mixer on mixing time in microbioreactors and round wells with no mixer on a commercial 24-well plate.
Figure 5.3. Effects of agitation rate and mixer size on the maximum oxygen transfer rate (OTRmax) in the microbioreactors.
Figure 5.4. Comparison of the evaporation rates from wells at different positions on the microbioreactors array and commercial multi-well plate.
Figure 5.5. A. Cell distributions with a suspension culture in a commercial 24-well plate. B. Uniform cell distribution in the microbioreactor with large central static mixer. C. Uniform cell distribution in a microbioreactor with small mixer.
Figure 5.6. Consistent cell growth and metabolic profiles in 24-well microbioreactor array. A. Cell proliferation and viability profiles in different sections with different seeding densities. B. Initial specific growth rates for different sections. C. Metabolic profiles for different sections.
Figure 5.7. Normal probability charts based on responses of viable cell density increase ratio (A) and MAb titer (B) in a Plackett-Burman design for 13 factors.
Figure 5.8. Scalability test using the serum-free medium in CHO suspension cultures with 4 parallel spinner flasks, showing profiles for proliferation and viability (A), metabolic activity (B), and MAb titer (C).
CHAPTER 6

A COMPARATIVE STUDY OF MICROFIBROUS DISKS AND MICROPARTICLES AS CARRIERS FOR SUSPENSION CELL CULTURE AND MONOCLONAL ANTIBODY PRODUCTION

6.1 Abstract

Small patches of poly(ethylene terephthalate) (PET) nonwoven fibrous matrices are an excellent candidate as low-cost carriers for cells in suspension cultures. Fibrous carriers are a type of macroporous microfibrous carriers, which provide large surface areas and a three-dimensional environment for high density cell growth. In this study, the microfibrous carriers, referred to as fibrous microcarriers, and several commercial microparticle carriers were used to study cell attachment kinetics, growth and monoclonal antibody production with Chinese hamster ovary cells. The fibrous microcarriers had comparable performances with commercial ones. In addition, fibrous microcarriers provided a wider operable range for agitation rate than commercial microcarriers, effectively protecting cells from shear stress and microcarrier collisions. In addition, the fibrous microcarriers were over 1000 times less expensive than commercial ones, which is especially meaningful for large-scale cell cultures using microcarriers.

6.2 Introduction
Currently, microcarriers are used in industrial processes requiring anchorage-dependent cell cultures, such as viral vaccine production [1,2]. In addition, there is an increasing trend of using microcarriers for tissue engineering or cell-based regenerative medicine [3-6]. Recently, microcarriers have been applied in stem cell expansion with preservation of their pluripotency. For example, Cytodex 3 was used for murine embryonic stem cell (mESC) expansion [7], and a trimethyl ammonium-coated polystyrene was used for both human embryonic stem cells (hESCs) and mESCs [8].

There are two types of microcarriers based on structural differences [9]: solid microcarriers such as Cytodex 1, 3 and HyqSphere, and macroporous microcarriers such as Cytopores 1, 2 and fibrous microcarriers used in this study. The materials for making microcarriers are categorized into synthetic and natural biomaterials [10]. The synthetic materials used for microcarriers can be dextran matrix substituted with positively charged diethylaminoethyl (DEAE) (Cytodex 1), cellulose with DEAE (Cytopore 1, 2), trimethyl ammonium-coated polystyrene [8], biodegradable poly(lactic-co-glycolic acid) microcarriers [3,11], chitosan modified poly(L-lactide) microspheres [12], or polyurethane foam [13]. The fibrous microcarrier used in this study was poly(ethylene terephthalate) (PET) nonwoven fibrous matrix, which is also a synthetic material. Natural materials used for making microcarriers can be collagen [14], gelatin (CultiSpher-G), calcium alginate [15] and chitosan [16]. Lactose-coated chitosan microcarriers and chitosan/gelatin composite microcarriers developed for hepatocyte cultures [17,18], and collagen-coated polylactide microspheres as chondrocyte microcarriers [19] have also been demonstrated. There was even a type of liquid microcarriers developed based on fluorocarbon emulsion stabilized with polylysine in aqueous medium [20].

PET fibrous matrices provide many advantageous properties for 3D cell cultures, such as
biocompatibility, large surface area and 3D porous structure [21]. They were utilized as 3D scaffolds in tissue engineering studies for human trophoblast tissue development [22], adipose tissue model construction [23], and astrocyte culturing for neurotrophic factor secretion [24], etc. PET fibrous matrices were also applied in stem cell and progenitor cell amplification and differentiation [25-27]. Furthermore, PET fibrous matrices could support long-term hybridoma cultures for monoclonal antibody (MAb) production in fibrous-bed bioreactors with continuous perfusion [28]. Similarly, packed-bed bioreactors with commercial polyester fibrous disks (Fibra-cell, New Brunswick Scientific) were also used in perfusion culture of hybridoma cells to produce monoclonal antibody [29]. However, no prior work has been done on using small fibrous disks as carriers to support cell culture and recombinant protein production in suspension under agitation.

In this study, PET fibrous microcarriers were compared with Cytodex 1, 3, Cytopore 1, 2 and HyqSphere on cell attachment kinetics, growth capacity and MAb production using Chinese hamster ovary (CHO) cells under agitation. The effect of agitation rate was investigated both in multi-well plates and spinner flasks. Comparable performances of the fibrous microcarriers were achieved. There is also a discussion on the protection effect of macroporous microcarriers on cells against high agitation rates.

6.3 Materials and Methods

6.3.1 Microcarriers

Nonwoven poly(ethylene terephthalate) (PET) fibrous matrices were characterized with the following properties: 20 μm fiber diameter, 0.93 porosity, 75 μm average pore size, and 2 mm thickness [30]. They were cut into square patches with a side length of about 3 mm as the fibrous microcarriers. Commercial microcarriers tested in this study included
Cytodex 1 (CX1), Cytodex 3 (CX3), Cytopore 1 (CP1), Cytopore 2 (CP2) (GE Healthcare) and HyqSphere CGEN102-L (HS) (HyClone). Table 6.1 summarizes the properties of these microcarriers, including their materials, density, size, surface area, suitable bioreactor, porosity, average pore size and market price, which were obtained from technical specifications of these commercial microcarriers.

6.3.2 Preparation of microcarriers

The loading of microcarriers was determined by the same total surface area provided from each type of microcarriers. The provided surface area was 12 cm²/mL, based on which the loading of CX1 was 3.26 g/L, CX3 was 5.32 g/L, CP1 was 1.31 g/L, CP2 was 1.31 g/L, HS was 39.9 g/L, and PET was 10.53 g/L. For CX1, 3 and CP1, 2, there was a hydration step in preparation for sterilization. Briefly, desired amounts of microcarriers were first swollen in Ca²⁺, Mg²⁺ free phosphate buffered saline (PBS) in small glass vials for around 3 h in a 37°C incubator. The glass vials were first siliconized with Sigmacote (SL2, Sigma-Aldrich) to prevent wall adsorption of the microcarriers. Supernatant of the swelling solution was discarded and fresh PBS was used to rinse the microcarriers once with gentle agitation. The supernatant was decanted and replaced with fresh PBS, followed by autoclaving at 121°C for 30 min. There was no hydration process for HS microcarriers. The HS microcarriers were directly immersed in Ca²⁺, Mg²⁺ free PBS and autoclaved at 121°C for 30 min. The fibrous microcarriers were autoclaved in PBS as well. Prior to use, the PBS supernatant for all microcarriers was decanted and fresh cell culture medium was added to equilibrate the microcarriers for 60 min in a 37°C incubator. Before inoculum, the medium supernatant was aspirated and fresh warm medium was added.

6.3.3 Cells and medium
A CHO cell line 6E6 (ATCC CRL-11398) producing 23F2G, a humanized anti-CD18 monoclonal antibody (MAb) was used in this study. For the convenience of visualization, CHO 6E6 cells were transfected with pEGFP-N3 plasmid using Lipofectamine 2000 (Invitrogen). Enhanced green fluorescent protein (EGFP) was expressed under the control of a cytomegalovirus (CMV) promoter. EGFP positive cells were sorted with FACS Aria (BD Biosciences). The culture consisted of over 90% fluorescent positive cells after two months of consecutive subculturing. The maintenance medium was DMEM/F12 (Gibco) (1:1) supplemented with 5% fetal bovine serum (FBS) (Gibco) for culturing in an incubator with 5% CO₂ at 37°C. Subculture was performed every 5 days using trypsin (Gibco) to detach cells.

6.3.4 Plate treatment

Non-tissue culture treated 12-well plates (Falcon) were used for cell-microcarrier attachment measurement on an agitated orbital shaker (Stovall Belly Button). In order to minimize wall adsorption of cells, Sigmacote and bovine serum albumin (BSA) (Gibco) treatments on the well surface were tested, respectively. Sigmacote was sterilized through a syringe filter (0.22 μm, PVDF, sterile, Fisherbrand). BSA was sterile as purchased in solution. Sigmacote treatment was instantaneous, and BSA treatment was overnight on an orbital shaker in a 37°C incubator. After treatment, all the liquids were aspirated and the plate was left to dry. Four wells were used for each treatment. CHO cells were seeded in each well of the 12-well plate at a density of 1.3±0.2×10⁵ cells/mL in 1.2 mL culture medium. After culturing for one day in a CO₂ incubator at 37°C, the free cell number ratio between the one-day culture and initial seeding was compared for different treatments using a Student’s t test with α=0.05.

6.3.5 Cell-microcarrier attachment
Sigmacote treated 12-well plates were used to measure cell-microcarrier attachment on an agitated orbital shaker. Two agitation rates were tested: 60 rpm and 100 rpm. For 60 rpm, CHO cells were seeded at a density of $5.0 \pm 0.7 \times 10^5$ cells/mL in 1.2 mL culture medium to each well containing the appropriate amount of microcarriers. Similarly, the test for 100 rpm had a seeding density of $2.84 \pm 0.51 \times 10^5$ cells/mL in 1.2 mL culture medium with microcarriers. The loading of each type of microcarriers was according to the same total surface area $12 \text{ cm}^2/\text{mL}$ provided by microcarriers. The attachment experiments lasted around 24 h. Liquid samples, 20 μL each, were taken from each well at different time points to quantify unattached cells using trypan blue staining and hemocytometer counting under a microscope. A first order model was used to approximate the cell-microcarrier attachment kinetics.

$$\frac{dx}{dt} = -k_{app} x \quad (1)$$

where $x$ is free cell density, $t$ is time and $k_{app}$ is the apparent cell attachment constant. Due to the experiment time scale, there could be some cell growth and some inevitable cell wall adsorption, so the kinetics was approximated with an apparent constant. The $k_{app}$ for different microcarriers and for different agitation rates were compared using Student’s t tests with $\alpha = 0.05$. There were 2 replicates for each type of microcarriers. In addition to plate-based experiments, 100 mL spinner flasks were also used in cell-microcarrier attachment tests for CX3, CP1 and fibrous microcarriers at a seeding density of $2.2 \pm 0.3 \times 10^5$ cells/mL with 40 mL working volume at 60 rpm. The loading was also based on the same total surface area of $12 \text{ cm}^2/\text{mL}$ provided by microcarriers.

6.3.6 Cell growth

Microcarrier cell cultures were conducted in Sigmacote treated 12-well plates on an
orbital shaker in a CO₂ incubator at 37°C. 1.2 mL culture medium was added to each well with microcarriers to provide the same surface area of 12 cm²/mL. Two agitation rates were tested: 60 rpm and 100 rpm. The metabolic activities were measured every day using a YSI Biochemistry Analyzer (2700 Select). Repeated batch cultures were conducted with medium replenishment. MAb expression was also measured throughout the whole process. In addition, batch cultures were conducted in spinner flasks for CX3, CP1 and fibrous microcarriers at 60 rpm.

6.3.7 Enzyme linked immuno sorbent assay (ELISA)

ELISA was used to analyze MAb titer. 96-well EIA/RIA Easy Wash plates (Corning Lowell, MA) were coated for 1 hour under mild agitation in a 37°C incubator with the first antibody, a goat anti-human IgG (Jacksonimmuno, West Grove, PA), followed by blocking with BSA under the same conditions. Diluted human IgG standard (Sigma) and samples were added into plates and incubated for another 1 hour at 37°C. The second antibody, a goat anti-human IgG conjugated with horseradish peroxidase (HRP) was added to each well, and the plate was incubated for 1 hour at 37°C. A reactant solution of 3-(p-hydroxyphenyl) propionic acid and hydrogen peroxide was added, generating fluorescence. The reaction kinetics were measured with a fluorescence plate reader (GENios Pro, Tecan) at excitation 320 nm and emission 405 nm. IgG standards at various concentrations were used to correlate with the reaction rate, as calculated from the fluorescence intensity increase with time, which was used as the standard curve to quantify the MAb titer in samples.

6.3.8 Hematoxylin & eosin (H&E) staining

In order to show the morphology of cells growing within the fibrous microcarrier, one
fibrous microcarrier with CHO cells cultured for 7 days under 60 rpm agitation was prepared for cross sectional cutting and H&E staining. The fibrous microcarrier was fixed with 10% formalin, embedded in paraffin, and a 5 μm thick section at the depth of around 500 μm from the surface was sliced, deparaffinized, and stained with hematoxylin and eosin on a microscope slide. Microscopic (Olympus IX71) images were taken for this stained section.

6.4 Results

6.4.1 Cell-microcarrier attachment

Figure 6.1 summarizes the results for cell-microcarrier attachment kinetics measurements. At 60 rpm in a 12-well plate, CP1 and CP2 had the fastest cell attachment rates followed by fibrous microcarriers, CX3 and CX1, with HS being the slowest. Similarly, at 100 rpm in a 12-well plate, CP1 and CP2 had the highest $k_{app}$ values, followed by fibrous microcarriers, CX3 and CX1, with HS having the smallest attachment rate. In spinner flasks with 60 rpm agitation, CP1 also had the highest attachment rate, while fibrous microcarriers and CX3 were comparable for CHO cell attachment rates ($p=0.5862$). When the two agitation rates were compared, 100 rpm appeared to induce faster cell-microcarrier attachment than 60 rpm in agitated 12-well plate ($p=0.0193$ with microcarrier types blocked). In addition, there was no significant difference between 12-well plate and spinner flasks agitated at 60 rpm on the cell-microcarrier attachment rates ($p=0.1134$ with microcarrier types blocked), suggesting that Sigmacote treated 12-well plates could be a convenient platform for measuring cell-microcarrier attachment kinetics under agitation. Figure 6.2 shows the fluorescence microscopic images of the microcarriers with cells after the 24-hour seeding.
6.4.2 Cell growth

In the 12-well plate under 60 rpm agitation, cells exhibited extensive growth with all microcarriers. However, the solid microcarriers could not support high-density and long-term cell cultures due to their limited spatial capacity (Figure 6.3). At 120 hours, the surfaces of solid microcarriers were fully covered with cells. At 210 hours, excessive cells got detached from the microcarriers and grew in free suspension, which is not desirable in microcarrier cultures. In contrast, the macroporous microcarriers all supported high-density cell growth (Figure 6.3), which was attributed to the three dimensional (3D) spatial capacity provided by these microcarriers in addition to the surfaces. Figure 6.4 illustrates the morphology of cells growing within the fibrous microcarriers at a depth of around 500 μm. The space between fibers provided sufficient growth potential for cells. In addition, under adequate agitation with the porous structure, mass transport is a combination of convection and diffusion, which is advantageous for high-density cell growth. Figure 6.5 demonstrates the metabolic activities of the CHO cell cultures with different microcarriers with medium replenishment. All microcarriers were comparable in terms of glucose consumption and lactate production. Table 6.2 summarizes lactate/glucose yields of these cultures. The decreased yields from around 1.8 on the first day to about 1.2 on the ninth day with repeated batches suggested increased energy utilization efficiency with the long-term microcarrier cultures. However, for solid microcarriers, the culture was a mixture of cells on microcarriers and cells in free suspension.

Unlike cell growth at 60 rpm, 100 rpm generated more shear stress and microcarrier collisions, resulting in cell death with solid microcarriers and limited cell growth with CP1 and CP2 (Figure 6.6). In contrast, extensive cell growth was observed with fibrous
microcarriers. The metabolic activities of cell cultures with different microcarriers also indicated the same difference between fibrous microcarriers and other microcarriers (Figure 6.7). With repeated batches, glucose consumption and lactate production could be continued in cycles for fibrous microcarriers, but CP1 and CP2 had very slow metabolic rates, and all the solid microcarriers did not support viable metabolism in this process. Table 6.2 summarizes the lactate/glucose yields throughout the culturing process. Commercial microcarriers generally had a high molar yield above 2 on the first day for seeding, suggesting that other nutrients might be converted to lactate as well as glucose under intensive agitation, while fibrous microcarriers had a yield of about 1.8. Along with time, the yield for fibrous microcarrier cultures decreased to 1.62 on the seventh day and 1.53 on the eleventh day, which is similar to cultures with 60 rpm agitation, suggesting more and more efficient energy utilization.

In microcarrier cell cultures with spinner flasks in a batch mode, CP1, CX3 and fibrous microcarriers were tested. With 60 rpm agitation, the macroporous microcarriers could support cell growth (Figure 6.8A,C), but the culture using solid microcarriers failed to sustain in this 5-day process (Figure 6.8B). The metabolic activities of CHO cells with these microcarriers were illustrated in Figure 6.9, indicating normal profiles for cultures with CP1 and fibrous microcarriers with minimal glucose consumption and lactate production with CX3. These results suggested that spinner flasks generated higher agitation intensity than multi-well plates on a shaker at the same agitation rate, for which macroporous microcarriers could provide sufficient protection to cells against the shear stress.

6.4.3 Monoclonal antibody (MAb) production

MAb production was compared for different microcarrier cultures under different
conditions (Figure 6.10). For cultures in a 12-well plate on an orbital shaker at 60 rpm, the MAb production profiles were comparable among different microcarriers (Figure 6.10A). However, for solid microcarrier cultures at the late stage, the MAb production was from a mixture of cell populations on microcarriers and in free suspension. At 100 rpm, excessive shear stress caused cell death with solid microcarriers, so the MAb titers were low. CP1 and CP2 produced MAb at a normal level, but there were few viable cells left under this condition (Figure 6.10.B). In contrast, fibrous microcarriers supported continuous MAb production with medium replenishment due to high-density viable cells growing within the microcarriers (Figure 6.10.C). The MAb production profiles were also studied using spinner flask batch cultures with microcarriers at 60 rpm. The macroporous microcarriers supported continuous MAb production, but solid microcarriers CX3 produced low levels of MAb due to the premature cell death under excessive agitation (Figure 6.10.D).

6.5 Discussion

Microcarrier cell cultures involve cell attachment and growth on the microcarriers, where many operation parameters may come into play and affect the performance. The attachment, spreading and growth of VERO cells on Cytodex 1 were studied as a function of presence or absence of serum and at room temperature or at 37°C for large scale cultures [31]. It was also found that there was a cell line dependency of the influence of hydrodynamic shear stress and surfactant protection efficiency on microcarrier cell growth [32]. VERO cell attachment, growth and viral production kinetics on Cytodex 1 were studied in 100 mL spinner flasks at 60 rpm and in a 15 L bioreactor at 35 rpm [33]. Culture modes and microcarrier loading also had an effect on cell expansion using microcarriers. It was reported that sustained cell expansion was achieved with Cytodex 1
in semi-batch or perfusion culture with a high microcarrier loading [34]. However, a comparative study on cell attachment kinetics, growth and MAb production in long term cultures with different microcarriers under different agitation conditions was scarce. Therefore, this study provided some insight into using different microcarriers under different agitation conditions.

In addition, in large scale bioreactor microcarrier cell cultures, there is a trade-off between the high agitation rates required for good mixing and the low agitation rates necessary to avoid cell damage due to shear stress and microcarrier collisions [35]. Therefore, the fibrous microcarriers which provide adequate protection to attached cells under high agitation could be a valuable option for large-scale microcarrier cell cultures. Furthermore, the 3D space within the fibrous microcarriers also supported high-density cell growth in long term cultures. In addition to the sufficient spatial capacity, the 3D microenvironment provided by microcarriers could also affect cell proliferation and cell cycle distribution, which may have an impact on recombinant protein productivity. For example, 3D collagen microspheres were used as a controlled proliferation technology of HEK293 cells to enhance recombinant protein productivity as compared with monolayer culture [36].

There was a concern that directly using commercial non-tissue culture treated multi-well plates might cause cell loss to wall adsorption under agitation for microcarrier cell cultures. Therefore, Sigmacote and BSA treatments were compared for the prevention of cell wall adsorption. Figure 6.11 summarizes the results for this test, indicating that Sigmacote was effective to minimize cell wall adsorption in the plastic wells, whereas BSA was totally ineffective and could not prevent cell attachment to the wall.

In this study, the cell-microcarrier attachment kinetics studies were performed with
continuous agitation. However, it was reported that intermittent stirring was beneficial to cell attachment and growth on both solid microcarriers Cytodex 1 and macroporous microcarriers Cultispher-G (Percell Biolytics AB). In addition, the pH, serum and inoculum size also had significant effects on cell attachment [37]. Therefore, with the introduction of the fibrous microcarriers in this study, future work may focus on optimization of the cell attachment conditions. In addition, the use of serum-free medium is becoming the norm for biopharmaceutical production, so serum-free medium can be tested for cell cultures using the fibrous microcarriers. It is anticipated that with the open macroporous structures, cells or cell aggregates could be easily entrapped within the fibrous microcarriers, thus facilitating the use of serum-free medium for cell-microcarrier attachment.

Cell growth was qualitatively investigated with fluorescence microscopy in this study. The quantification of cell number change during the culturing process is crucial but also challenging for microcarrier cultures, which usually requires enzymatic detachment of cells from microcarriers. This could be especially difficult for macroporous microcarriers due to the 3D internal structures, which could physically entrap the cells, causing inaccuracy in the quantification. Using GFP positive cells, we have developed a non-invasive cell number quantification method for tissue culture with fibrous matrices [38]. Similarly for the fibrous microcarriers, cell number quantification could also be achieved based on fluorescence intensity measurement (Figure 6.12).

6.6 Conclusion

Microcarriers can be used in various cell culture bioprocesses, but for large scale processes, agitation rates required for mixing may cause sensitive cell damage due to shear stress and microcarrier collisions. In this study, we demonstrated that macroporous
fibrous microcarriers could be used to support CHO cell culture with good performance in cell attachment, growth and MAb production as compared to a number of commercial microcarriers. In addition, the fibrous microcarriers could protect cells from high shear stress and their 3D internal porous structures provided large spatial capacity for high-density long-term cell growth. Also importantly, the fibrous microcarriers are over 1000 fold less expensive than commercial microcarriers. Therefore, the fibrous microcarriers are an excellent alternative to commercial microparticle carriers widely used in large-scale cell cultures, such as in vaccine production. However, there is still a challenge for the fibrous microcarriers in cell expansion applications due to the difficulty of cell harvesting. Future research may be furthered for its improvement.

6.7 References


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Table 6.1. Properties of the microcarriers tested in this study.

<table>
<thead>
<tr>
<th>Product</th>
<th>Cytodex 1 (CX1)</th>
<th>Cytodex 3 (CX3)</th>
<th>Cytopore 1 (CP1)</th>
<th>Cytopore 2 (CP2)</th>
<th>HyQSphere, CGEN102-L (HS)</th>
<th>Fibrous matrix (PET)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Company</td>
<td>GE Healthcare</td>
<td>GE Healthcare</td>
<td>GE Healthcare</td>
<td>GE Healthcare</td>
<td>Hyclone</td>
<td>nonwoven fibrous poly(ethylene terephthalate) (PET)</td>
</tr>
<tr>
<td>Material</td>
<td>cross-linked dextran matrix substituted with positively charged DEAE</td>
<td>cross-linked dextran matrix with covalently bound collagen</td>
<td>cross-linked cotton cellulose with hydrophilic DEAE exchanger, positive charge at 1.0 meq/g</td>
<td>cross-linked cotton cellulose with hydrophilic DEAE exchanger, positive charge at 1.8 meq/g</td>
<td>cross-linked polystyrene coated with collagen</td>
<td></td>
</tr>
<tr>
<td>Density (g/mL)</td>
<td>1.03</td>
<td>1.04</td>
<td>1.03</td>
<td>1.03</td>
<td>1.02</td>
<td>1.35</td>
</tr>
<tr>
<td>Size (Diameter, μm)</td>
<td>180</td>
<td>175</td>
<td>235</td>
<td>235</td>
<td>170</td>
<td>1.8 mm thick, 3mm side length</td>
</tr>
<tr>
<td>Surface area (cm²/g)</td>
<td>4,400</td>
<td>2,700</td>
<td>11,000</td>
<td>11,000</td>
<td>360</td>
<td>1500</td>
</tr>
<tr>
<td>Suitable bioreactor</td>
<td>Stirred tank</td>
<td>Stirred tank</td>
<td>Stirred tank</td>
<td>Stirred tank</td>
<td>Stirred tank</td>
<td>stirred tank, packed bed perfusion</td>
</tr>
<tr>
<td>Porosity</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>N/A</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Average pore size (μm)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>30</td>
<td>30</td>
<td>N/A</td>
<td>50</td>
</tr>
<tr>
<td>Market Price ($/g)</td>
<td>9.16 (based on 25g pack)</td>
<td>12.6 (based on 10g pack)</td>
<td>26.35 (based on 20g pack)</td>
<td>26.35 (based on 20g pack)</td>
<td>10.6 (based on 10g pack)</td>
<td>0.01 (retail)</td>
</tr>
</tbody>
</table>
Table 6.2. Lactate/glucose yield for microcarrier cultures in a 12-well plate at 60 rpm and 100 rpm agitations with medium replenishment.

<table>
<thead>
<tr>
<th>Microcarriers</th>
<th>CP1</th>
<th>CP2</th>
<th>CX1</th>
<th>CX3</th>
<th>HS</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mol/mol)</td>
<td>60 rpm</td>
<td>1 day</td>
<td>1.76±0.06</td>
<td>1.77±0.02</td>
<td>2.07±0.26</td>
<td>1.77±0.06</td>
</tr>
<tr>
<td>9 days</td>
<td>1.17±0.08</td>
<td>1.16±0.04</td>
<td>1.24±0.1</td>
<td>1.21±0.04</td>
<td>1.21±0.04</td>
<td>1.18±0.08</td>
</tr>
<tr>
<td>100 rpm</td>
<td>60 rpm</td>
<td>1 day</td>
<td>2.61±0.22</td>
<td>2.53±0.18</td>
<td>2.89±0.8</td>
<td>1.7±0.29</td>
</tr>
<tr>
<td>11 days</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1.62±0.03</td>
</tr>
<tr>
<td>11 days</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1.53±0.01</td>
</tr>
</tbody>
</table>
Figure 6.1. Kinetics of cell-microcarrier attachment in 12-well plates and spinner flasks. A. Fraction of cells in suspension in a 12-well plate at 60 rpm agitation; B. Fraction of cells in suspension in a 12-well plate at 100 rpm agitation; C. Fraction of cells in suspension in spinner flasks at 60 rpm agitation. D. Apparent attachment constants for different microcarriers under different agitation conditions.
Figure 6.2. Fluorescence microscopic images of microcarriers after a 24-hour seeding in multiwell plates at different agitation rates. A-F: at 60 rpm agitation; G-L: at 100 rpm agitation.
Figure 6.3. Microscopic images show the growth of cells on microcarriers in a 12-well plate under 60 rpm agitation with medium replenishment. At 120 hours, the solid microcarriers were almost fully covered with cells as shown in A, B and C. At 210 hours, some cells were detached from solid microcarriers and grew in suspension as shown in D, E and F. In contrast, all the macroporous microcarriers had sufficient spatial capacity for high-density and long-term cell growth on the microcarriers as shown in G-L. One unseeded fibrous microcarrier was also imaged in L to show the difference from the fibrous microcarrier with GFP positive cells grown on it.
Figure 6.4. Microscopic image of H&E staining for a section horizontally cut at around 500 μm depth of a fibrous microcarrier cultured with CHO cells in a 12-well plate agitated at 60 rpm for 7 days. This image contains the cross section cut of fibers and several long fibers, which all have cells grown around them. In addition to surface growth, cells also grow into the 3D space between fibers.
Figure 6.5. Metabolic activities of CHO cells cultured with different microcarriers in a 12-well plate at 60 rpm agitation with medium replenishment.
Figure 6.6. CHO cells cultured with microcarriers in a 12-well plate at 100 rpm agitation. Solid microcarriers did not support cell growth as shown in B, C and E, and CP1 and CP2 supported limited cell growth as shown in A and D. In contrast, fibrous microcarriers supported extensive cell growth under high agitation as shown in F.
Figure 6.7. Metabolic activities of CHO cells cultured with different microcarriers in a 12-well plate at 100 rpm agitation with medium replenishment.
Figure 6.8. Fluorescence microscopic images of CHO cells with microcarriers in spinner flasks at 60 rpm agitation in a bath culture after 5 days.
Figure 6.9. Metabolic activities of CHO cell cultures with different microcarriers in spinner flasks at 60 rpm agitation.
Figure 6.10. MAb production profiles for microcarrier CHO cell cultures. A. MAb production with different microcarriers in a 12-well plate under 60 rpm agitation with medium replenishment. B. MAb titer after a 7-day culture with commercial microcarriers in a 12-well plate under 100 rpm agitation. C. MAb production with fibrous microcarriers in a 12-well plate under 100 rpm agitation. D. MAb production with different microcarriers in spinner flasks with 60 rpm agitation.
Figure 6.11. Treatments of cell culture wells in a multi-well plate to prevent cell wall adsorption.
Figure 6.12. Cell number quantification for fibrous microcarrier cultures. A. Standard curve correlating fluorescence intensity and cell density in fibrous microcarriers. B. Cell growth profiles quantified with fluorescence intensity measurements for fibrous microcarrier cultures.
CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

7.1.1 Perfusion microbioreactor array

The perfusion microbioreactor array developed in this work could produce high-density 3D tissue constructs in modular and low-cost PET fibrous scaffolds. EGFP-expressing human colon cancer HT-29 cells could be maintained for an extended period, reaching a cell density as high as $6 \times 10^7$ cells/mL matrix. Non-invasive and time-series cell proliferation and cytotoxicity assays could be conducted in high-throughput with a microplate reader. Frame-assisted device packaging and modular design allowed direct access to cells for further microscopic and histological analyses, which showed the formation of in vivo like 3D tissue organizations. In cytotoxicity assays, 3D colon cancer cell cultures had a time- and dose-dependent response to anticancer drug 5-FU. Drug resistance was observed in the perfusion 3D culture compared with conventional 2D cytotoxicity assay.

7.1.2 Parallel flow control in multiple hydrophobic microchannels

Parallel flow control and startup is a common challenge in microchannel technologies. Capillary pressure could cause non-uniform filling in multiple hydrophobic microchannels. This phenomenon was investigated in this study through both theoretical
analysis and experimental observation. It was found that when hydrodynamic pressure drop surpasses the maximum capillary pressure, uniform flow startup in parallel hydrophobic microchannels can be ensured. When low startup flowrates are necessary, such as for cell culture applications, PET fibrous matrices can be employed to balance with capillary pressure. Significantly reduced threshold flowrate could be achieved without changing the original device geometries. In addition, using the same theory, it was found that when microchannels are smaller, lower flowrates are sufficient for achieving the parallel flow startup. The findings in this study can help researchers to better understand the major forces at microscale, so it would benefit the design and operation of microfluidic devices with hydrophobic materials for high-throughput applications.

7.1.3 24-well microbioreactor array for high-throughput bioprocess development

A novel and low-cost 24-well microbioreactor array was developed, which featured a central static mixer and gas-permeable membranes for good aeration and reduced evaporation. Data quality was improved compared with commercial multi-well plates in CHO cell suspension culture. Due to the static mixer, poor mixing patterns of commercial well plates upon orbital shaking was effectively broken. Uniform cell distribution within the microbioreactor was achieved with no induction of cell aggregates. Reliable data trending could be obtained with performance consistency and sensitivity to subtle operation variations. The microbioreactor array was applied in the identification of active factors for serum-free media. Both the microbioreactor array and the developed medium had scalable performances for CHO suspension culture compared with spinner flasks based on specific growth rate and MAb titer.

7.1.4 Fibrous microcarriers
Microcarriers can be used in various bioprocess development for cell cultures, but for large scale processes, agitation rates required for mixing may cause sensitive cell damage due to shear stress. In this study, we developed fibrous microcarriers which were macroporous and had comparable performances as a number of commercial microcarriers on cell attachment, growth and MAb production using CHO cells. In addition, the fibrous microcarriers could protect cells from high shear stress. The 3D internal porous structures also provided large spatial capacity for high-density long-term cell growth. Also importantly, the fibrous microcarriers provided over 1000-fold cost savings. Therefore, the fibrous microcarriers could be an excellent candidate for large-scale microcarrier cell cultures, such as viral vaccine production.

7.2 Recommendations

Microfluidic and microscale cell cultures have been an important focus for research and development both in the academia and in the industry. Therefore, fast progress has been made recently.

In our study of the perfusion microbioreactor array, with simple sequential flow for each line, the proliferation and cytotoxicity assays displayed good data reproducibility, probably due to relatively fast perfusion rates and a small array size. However, it should be noted that this approach may encounter difficulty in large arrays of microbioreactors. Improved designs should be developed for independent and parallel tissue construct arrays in a microfluidic device. In addition, for future work, it is also helpful to add serial drug dilution channels to the device for automatic high-throughput cytotoxicity testing. For these proposed improvements, the understanding of parallel flow control in multiple hydrophobic microchannels should play a key role for design, material selection and operation. The theory and experiments described in Chapter 4 could be a stepstone to the
successful development for the recommended improvements. Furthermore, optical sensors for pH and DO may also be integrated into the perfusion microbioreactor array.

The 24-well microbioreactor array developed in this study is a prototype for high-throughput cell cultures. In addition to the improved data quality and scalability for CHO suspension culture, there were many desirable features that were embodied in the device design but not yet fully explored in practice. For example, the four observation windows could provide convenient incorporation of optical sensors for pH and DO for each microbioreactor, which should extend its functionalities for cell cultures. In addition, the standard design of the plate should allow high-throughput data acquisition using microplate reader. In addition, computational fluid dynamics can be employed to study the optimal designs for the central static mixers and/or baffles. There were some designs discussed in Figure 5.9, which should provide a stepstone for further investigation. Another recommendation is on the application of the device, which should be applicable for the development of high titer serum-free media using design of experiment.

The fibrous microcarriers developed in this study also deserve further investigations from many aspects. Firstly, intermittent agitation can be tested for cell-microcarrier attachment, which can be compared with continuous agitation. Secondly, it is desirable to find out to what extent the fibrous microcarriers can protect cells from intense agitation. And this could be correlated with large-scale bioreactor hydrodynamic conditions to see whether the fibrous microcarriers are potent to support high-density cell cultures in a high-shear environment. Thirdly, for the fibrous microcarriers to be suitable for cell expansion applications, such as for stem cell expansion, a mild and economic method of detaching cells from the fibrous microcarriers also deserve further development.


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Figure A.1. Efficacy of plate definition confirmed by linear correlation between fluorescence intensity and the concentration of fluorescein in the microbioreactor array.
APPENDIX B. EFFECT OF DEBUBBLING ON CELL NUMBER

There is a concern that debubbling using a high flow rate might cause loss of cells every time when the device was reconnected to the perfusion system after fluorescence intensity (FI) measurement. In order to reveal any effect of debubbling on cell number, at the end of a 5-day perfusion culture, the microdevice was disconnected and loaded into a plate reader for FI measurement. Then the microdevice was reconnected to the perfusion tubing, followed by debubbling at 100 μL/min for about 2 minutes when all bubbles were visibly driven out. The device was then immediately disconnected for FI measurement again. This process was consecutively and immediately repeated twice. The effect on cell number change was evaluated from two aspects. One was to examine the FI change of the first cell culture wells of 4 independent lines after each debubbling, under the assumption that there should be no FI change, if there was no cell washout. For analysis, the FI change in each well was expressed as a percentage with regard to its previous FI measurement. The other aspect is based on a similar assumption that the total FI of all cell culture wells in one line of the microdevice should be stable, if there was no cell washout. Statistical analyses were conducted using JMP. A t test was applied for comparing means of FI change for all the 4 first wells and all the four lines to zero to see whether there was any significant change after debubbling. In addition, a Student’s t test was applied for testing whether the two immediately consecutive debubbling had any significant
difference for the effect on cell number change both on the first cell culture wells and all 4 lines. The result is illustrated in the following figure. Statistical analyses indicated that there were no significant differences of the FI changes in either the 4 first wells (p=0.28) or in the 4 lines (p=0.53) as compared to zero. Furthermore, the two consecutive debubbling also did not have significant differences in terms of changing the FI in the 4 first wells (p=0.40) or in the 4 lines (p=0.17).

![Bar graph showing the effect of debubbling on fluorescence intensity change.](image)

Figure B.1. Effect of debubbling on fluorescence intensity change.
APPENDIX C. STATIC 3D CULTURES OF HT-29 CELLS IN MICROBIOREACTOR ARRAY

Figure C.1. Static 3D cultures of HT-29 cells in microbioreactor array
APPENDIX D. COMPUTATIONAL FLUID DYNAMICS SIMULATION OF HYDRODYNAMIC MICROENVIRONMENT IN PERFUSION MICROBIOREACTOR ARRAY

A 3D model was constructed to simulate the hydrodynamic microenvironment of cell culture well filled with fibrous matrix. A finite element mesh was built through Gambit with geometric parameters representative of actual PET matrix. In each layer of the matrix, cylinders with a diameter of 20 μm were placed in parallel and 45 μm away from each other. Two adjacent layers were normal to each other. The whole matrix was formed with multiple sets of such two layers 45 μm away from one set to another. Water was used for the fluid dynamics computation governed by Navier-Stokes equation at an inflow velocity of $1 \times 10^{-5}$ m/s with FLUENT 6.2. Velocity, Reynolds number and strain rate inside the cell culture well were studied. Peclet number, defined by $\text{Pe} = \frac{UL}{D}$ (where $U$ is the velocity in the cell culture well, $L$ is the characteristic length taken as the pore size of 45 μm, and $D$ is the diffusivity of small molecules in tissue, estimated to be $1 \times 10^{-10}$ m$^2$/s), and shear stress, defined by $\tau = \varepsilon \times \mu$ (where $\varepsilon$ is shear rate, and $\mu$ is water viscosity 1 mPa•s), were evaluated for the in vitro microenvironment and compared to those of interstitial tissues.
Figure D.1. CFD simulation of a cell culture well in perfusion. A. The mesh of 3D model for a cell culture well drawn in Gambit with geometric parameters representative of the actual device parameters. B. Static pressure contours demonstrating flow entering a cell culture well from a bottom layer channel and exiting from a top layer channel at $1 \times 10^5$ m/s by finite element computation with FLUENT6.2.
Figure D.2. CFD simulation on velocity. Uniform flow distribution within the cell culture well. A. Contours of velocity magnitude for cross sectional views at different X positions (X = -0.0004, 0 and 0.0004 m) of a well. B. Histogram for velocities throughout the well showing that velocities were mainly below $7.5 \times 10^{-6}$ m/s.
Figure D.3. CFD simulation on strain rate. Uniform strain rate distribution within the cell culture well. A. Contours of strain rate for a cross section view at different X positions. B. Histogram for strain rate throughout the well showing that strain rates were mainly below 2 s\(^{-1}\).
Table D.1. CFD simulation suggested an *in vivo* like hydrodynamic microenvironment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Microfluidic well</th>
<th>Interstitial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic size (μm)</td>
<td>45</td>
<td>40~100</td>
</tr>
<tr>
<td>Fluid velocity (μm/s)</td>
<td>0.1~7.5</td>
<td>0.1~700</td>
</tr>
<tr>
<td>Reynolds number (10⁻⁵)</td>
<td>0.45~33.8</td>
<td>1~200</td>
</tr>
<tr>
<td>Shear stress (10⁻⁵ Pa)</td>
<td>1~20</td>
<td>1~4000</td>
</tr>
<tr>
<td>Peclet number</td>
<td>0.05~3.38</td>
<td>10⁻¹ ~ 10³</td>
</tr>
</tbody>
</table>
APPENDIX E. DESIGN OF 5×6 PERFUSION MICROBIOREACTOR ARRAY

Figure E.1 Design of 5×6 perfusion microbioreactor array.
APPENDIX F. CELL CYCLE ANALYSIS OF HT-29 CELLS IN PERFUSION 3D CULTURE UNDER 5-FLUOROURACIL TREATMENT

5-FU was known to cause cell cycle arrest. Therefore, cell cycle analysis for perfused 3D cultures with this drug treatment may help provide some insight into its cytotoxicity effects. The following figure demonstrates the result of cell cycle analysis for 5-FU treated perfused 3D human colon cancer cells. With increasing dosages, G0/G1 phase and apoptosis/debris populations increased in general, while S, G2 and M phase populations decreased.

Figure F.1. Cell cycle analysis for 3D perfused cultures of HT-29 with drug treatment. The 5-FU concentrations are A. 0.2 μg/mL, B. 0.5 μg/mL, C. 1 μg/mL, and D. 5 μg/mL.
Table F.1. Cell cycle analysis for perfused 3D human colon cancer cells under 5-FU treatment at different concentrations.

<table>
<thead>
<tr>
<th>5-FU Conc. (μg/mL)</th>
<th>Gated % G0,G1</th>
<th>Gated % S</th>
<th>Gated %G2, M</th>
<th>Gated % Apoptosis/Debris</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>23.82</td>
<td>26.36</td>
<td>21.23</td>
<td>16.64</td>
</tr>
<tr>
<td>0.5</td>
<td>43.69</td>
<td>20.11</td>
<td>5.19</td>
<td>30.73</td>
</tr>
<tr>
<td>1</td>
<td>30.12</td>
<td>25.3</td>
<td>6.02</td>
<td>33.73</td>
</tr>
<tr>
<td>5</td>
<td>44.27</td>
<td>8.7</td>
<td>0.79</td>
<td>47.43</td>
</tr>
</tbody>
</table>
Figure G.1. Cell specific fluorescence under drug treatment. A. Cell specific fluorescence change in 3D perfusion cultures with drug treatment for 5 days. B. Cell specific fluorescence change in 2D static cultures with drug treatment for 5 days.
APPENDIX H. PERFUSION SEEDING IN 5×6 MICROBIOREACTOR ARRAY

4 mL cell suspension at a viable cell density of 2.7±0.5×10^5 cells/mL was used in each syringe under a perfusion rate of 0.2 mL/min for all six lines. It was hypothesized that gravity would cause the majority of cells to precipitate in the syringes before they were pushed into the microbioreactor array. Therefore, the experiment was conducted with 3 syringes pushed by the pump alone until the pumping was stalled due to reaching its mechanical limit but still with some residual cell suspension left in the syringes. And the other 3 syringes were further manually pushed to drive all the residual cell suspension passing the microbioreactor array. Fluorescence microscopy (Olympus IX71) also confirmed the measurements.

Figure H.1. Perfusion seeding in microbioreactor array.
Table H.1. Statistical analysis for perfusion seeding without cell residuals left in syringes.

<table>
<thead>
<tr>
<th>Level - Level Difference</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8710.000</td>
<td>5898.56</td>
<td>11521.44</td>
</tr>
<tr>
<td>1</td>
<td>7964.667</td>
<td>5173.22</td>
<td>10796.11</td>
</tr>
<tr>
<td>1</td>
<td>7182.667</td>
<td>4371.22</td>
<td>9994.11</td>
</tr>
<tr>
<td>1</td>
<td>6945.667</td>
<td>3724.22</td>
<td>9387.11</td>
</tr>
<tr>
<td>2</td>
<td>2164.333</td>
<td>-647.11</td>
<td>4975.78</td>
</tr>
<tr>
<td>3</td>
<td>1527.333</td>
<td>-1284.11</td>
<td>4338.78</td>
</tr>
<tr>
<td>4</td>
<td>1439.000</td>
<td>-1372.44</td>
<td>4250.44</td>
</tr>
<tr>
<td>3</td>
<td>802.000</td>
<td>-2909.44</td>
<td>3613.44</td>
</tr>
<tr>
<td>4</td>
<td>725.333</td>
<td>-2966.11</td>
<td>3536.76</td>
</tr>
<tr>
<td>2</td>
<td>637.000</td>
<td>-2174.44</td>
<td>3445.44</td>
</tr>
</tbody>
</table>

Table H.2. Statistical analysis for perfusion seeding with cell residuals left in syringes.

<table>
<thead>
<tr>
<th>Level - Level Difference</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>621.000</td>
<td>1200.64</td>
<td>1991.357</td>
</tr>
<tr>
<td>1</td>
<td>1545.667</td>
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<td>1357.000</td>
<td>786.64</td>
<td>1527.357</td>
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<tr>
<td>2</td>
<td>464.000</td>
<td>93.54</td>
<td>604.357</td>
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<tr>
<td>2</td>
<td>388.667</td>
<td>16.31</td>
<td>729.024</td>
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<tr>
<td>3</td>
<td>280.333</td>
<td>-90.02</td>
<td>650.691</td>
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<tr>
<td>3</td>
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<tr>
<td>2</td>
<td>182.667</td>
<td>-186.69</td>
<td>554.024</td>
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<tr>
<td>4</td>
<td>70.333</td>
<td>-295.02</td>
<td>445.691</td>
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</table>
Figure H.2. Fluorescence microscopic images for perfusion seeding without residual cells left in syringes. A, B, C, D, E indicates well 1, 2, 3, 4, and 5, respectively.
Figure H.3. Fluorescence microscopic images for perfusion seeding with residual cells left in syringes. A, B, C, D, E indicates well 1, 2, 3, 4, and 5, respectively.
APPENDIX I. CELL CULTURE CHARACTERIZATION WITH 24- WELL MICROBIOREACTOR ARRAY

A.

Figure I.1. Cell culture characterization with the microbioreactor array. The array was divided symmetrically into four sections with each section having a different seeding density. A. $1.75 \pm 0.63 \times 10^5$ cells/mL for Section I. B. $0.56 \pm 0.2 \times 10^5$ cells/mL for Section II. C. $0.27 \pm 0.09 \times 10^5$ cells/mL for Section III. D. $0.14 \pm 0.07 \times 10^5$ cells/mL for Section IV.
Figure J.1. Designs of microbioreactors with central static mixers and baffles in different sizes and shapes in order to eliminate surface effects at microscale with optimal mixing patterns.
APPENDIX K. DYNAMIC SEEDING OF MAMMALIAN CELLS ON SMALL FIBROUS MATRICES

Figure K.1. Standard curves for three different cell lines for the correlation between fluorescence intensity and cell density in 3D PET fibrous matrices.

Figure K.2. Advantages of dynamic seeding over static seeding. A. High cell seeding density of dynamic seeding. B. High uniformity of dynamic seeding.
Figure K.3. Cell number increase on small fibrous matrices in dynamic seeding. A. HT-29-GFP. B. CHO-GFP. C. mES-GFP.
Table K.1. Dynamic seeding efficacy.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Final Cell Density (1e6 cells/mL matrix)</th>
<th>Concentrating Factor</th>
<th>Seeding Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>HT-29</td>
<td>16.09</td>
<td>5.41</td>
<td>1.47</td>
</tr>
<tr>
<td>CHO</td>
<td>7.69</td>
<td>4.15</td>
<td>1.08</td>
</tr>
<tr>
<td>ES</td>
<td>14.65</td>
<td>7.77</td>
<td>3.64</td>
</tr>
</tbody>
</table>

Figure K.4. Robustness and stability of tissue constructs generated by dynamic seeding.
Figure K.5. Free cell concentration kinetics for dynamic seeding. A. HT-29. B. CHO. C. mES.