Development of 3-D Microbioreactor Systems for Cell-Based High Throughput Screening

Dissertation

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By

Ru Zang, M.S.

Biochemical Engineering

The Ohio State University

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Dissertation Committee:

Dr. Shang-Tian Yang, Advisor

Dr. Jeffery J. Chalmers

Dr. Andre F. Palmer
Abstract

Drug screening is a long and costly process confronted with low productivity and challenges in using animals. 3-D cell-based high-throughput platforms have been developed to improve drug-screening efficiency and minimize animal testing. However, online monitoring of cell proliferation, pH, and dissolved oxygen (DO) has been a challenge in 3-D cell-based assays. In this work, a 40 micro-well plate bioreactor (40-MBR) system was developed as a high-throughput platform for 3-D cell cultures. This 40-MBR has similar dimensions of a 384-well plate (384-MWP) can provide real-time and noninvasive monitoring of cell proliferation, pH, and DO in 3-D microenvironments. A colon cancer cell line expressing enhanced green fluorescent protein (EGFP) under the control of a constitutive CMV promoter was tested with two potential cancer drugs using the 40-MBR and 384-MWP. Compared to the 384-MWP, the 40-MBR gave more reliable and highly reproducible growth kinetic data with reduced experimental errors. This study demonstrated the potential application of the 40-MBR as a high-throughput platform for screening potential cancer drugs and evaluating their cytotoxic effects in the early-stage drug discovery.

Cytotoxicity and embryotoxicity of chemicals were also investigated in the 40-MBR using EGFP-expressing embryonic stem cells (ESCs). Embryonic stem cell test (EST) has been used as an in vitro model for assessing embryotoxicity. However, the
current EST can only provide end-point data and cannot predict embryotoxicity of chemicals affecting organs such as bone. In this study, a novel high-throughput embryotoxicity assay was developed using EGFP-expressing ESCs under the control of a survivin promoter. Survivin expression is closely associated with embryo development and cell differentiation. For control, ESCs expressing EGFP under the control of a CMV promoter were used to monitor cytotoxicity of chemicals. Using survivin as a diagnostic marker for predicting embryotoxicity was first tested and validated with various chemicals with known developmental toxicity. Then, the potential embryotoxicity of three Chinese herbal medicines, *Ginkgo biloba* (GB), *Ganoderma lucidum* spore (GLS), and *Epimedium brevicornum* (EB), was tested. The results showed that the survivin-based assay has great potential in screening compounds with different degrees of embryotoxicity.

Compared to conventional 2-D cultures, cells cultured in 3-D scaffolds generally can better recapitulate *in vivo* cellular responses to drugs. However, the next-generation 3-D scaffolds should also provide tunable ‘surface’ properties similar to those found in native microenvironments. Recently, carbon nanotubes (CNTs) have been used in several tissue-engineering studies for their unique physical properties at the nanoscale. The potential of CNTs in promoting *in vitro* differentiation of ESCs into neuronal cells was investigated in both 2-D and 3-D cultures. Using polyethylene terephthalate (PET) membranes as the base substrate, coating the PET surface with CNTs promoted neuronal differentiation, exhibiting a high degree of neurites extension and outgrowth while maintaining good cell viability. Similarly, ESCs cultured in 3-D nonwoven fibrous PET
matrices coated with CNTs showed enhanced cellular functions, including proliferation, morphology and differentiation, compared to cells cultured in the uncoated matrices. With increased biological relevance, cells cultured in 3-D PET matrices coated with CNTs can provide more representative cellular responses to drugs.
Dedication

Dedicated to my family and whom I love
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Vita

June 2005………………………………………B. S. Applied Chemistry,

Dalian University of Technology, China

June 2007……………………………………...M. S. Biochemical Engineering,

Zhejiang University, China

July 2007 – July 2008.........................Graduate Research Associate,

Nanyang Technological University,

Singapore

2008 – Present…………………………………Graduate Research Associate,

Chemical and Biomolecular Engineering,

The Ohio State University
Publications


Field of Study

Major Field: Chemical Engineering
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Chapter 1: Introduction

1.1 Cell-based high throughput screening in drug discovery

High throughput screening (HTS) plays a fundamental role in drug discovery. Conventionally, animals act as proxies for human beings in drug selection, identification, and validation and clinical trials. With the development of new drugs and the rising awareness of safety, thousands of drugs are required to be tested toxicologically, which means that more than 10 million animals will be used in the future. However, animal tests are labor intensive, expensive and present moral issues. Therefore, alternative approaches to enhance testing efficiency and reduce the number of animal tests are needed. As one of the alternative approaches, cell-based high throughput screening is extensively used in studying molecular genetics, cellular biology, pharmacology and toxicology, and in the detection of pathogens and environmental pollutants. Today, cell-based assays are used in more than half of all high throughput drug screenings for target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity). Specifically, 2-D cell-based high throughput screening in multi-well plates is widely used as they can be easily miniaturized to increase the number of wells per plate for high throughput rates [1]. However, 2-D cell cultures cannot mimic the *in vivo* microenvironment: cells form a monolayer on the surface of the culture plates, which cannot represent *in vivo* tissues and thus leads to errors in predicting tissue specific responses. Thus, methods to enhance the
biological relevance of cell cultures have received increased attention. Compared to 2-D cell cultures, 3-D cell cultures can provide another dimension for cell-cell interactions, cell migration, and cell morphogenesis, and thus better physiological microenvironments. Furthermore, cells cultured in 3-D scaffolds with interconnected porous structures, which provide templates for cell attachment and conduits for nutrients transports to support high-density cell growth and tissue development, can better mimic native tissues in vivo and give more relevant drug responses.

1.2 Survivin as a marker for screening compounds associated with cytotoxicity and embryotoxicity

As an alternative to animal tests, in vitro alternative methods including micromass test, the whole rat embryo test and embryonic stem cell test (EST) have been extensively used for embryotoxicity screening [2]. Among them, EST is the only one implemented without sacrificing animals [3]. To date, ES cell-based models have been well developed and validated as a reliable tool for in vitro developmental toxicology studies [4-6]. However, current EST approaches can only give the end-point results based on either morphological analysis of beating cardiomyocytes or molecular markers specific to neural or cardiac differentiation [3]. End-point morphological analysis requires extensive experimental experience and can lead to misjudgments [7]. In addition, the duration for cell differentiation is long, usually 10 days. Although new molecular approaches based on analysis of certain specific marker proteins during ES cell differentiation into cardiac and neural cells can reduce the duration [7], chemicals that pose potential risks on bone development may not be correctly predicted.
In this study, we propose survivin as a new molecular marker for early diagnosis of embryotoxicity of chemicals. Survivin, a 16.5-kDa protein discovered in 1997 is a member of the inhibitor of apoptosis proteins (IAPs) and has gained intense interest from disparate segments of basic and disease-related research [8]. Survivin can suppress apoptosis and thus plays a role in the transformation of benign tumors to malignant ones, in which it is over expressed. Its cancer-specific expression makes survivin a diagnostic marker for early cancer development and a special target for cancer treatment. In addition, survivin is highly expressed in undifferentiated embryonic stem cells and their derived tumors [9, 10]. Survivin expression is higher in pregnant women than in nonpregnant women. In addition, survivin is biologically important for oocyte development and maturation [11]. Survivin also plays a critical role in normal embryo development. The genetic disruption of survivin expression could be lethal in early embryo development. Therefore, survivin expression in embryonic stem cells (mESCs) may be used as an in vitro model for identifying drugs with embryotoxicity.

1.3 Carbon nanotubes-based scaffolds for tissue engineering

Although 3-D scaffolds can provide a better link between single cells and tissues than 2-D cell cultures in predicting in vivo responses, there is increased awareness that 3-D scaffolds lack nanostructures, which broadly exist in vivo and play an important role in maintaining and regulating cellular behaviors and functions. In physiological microenvironments, cells are surrounded with extracellular matrix (ECM) that is characterized by a natural web of hierarchically organized nanofibers [12]. Cells interact with ECM spanning from nanoscale to miliscale, and these interactions are capable of
controlling cellular functions, including cell alignment, migration, proliferation and differentiation. Great efforts have been dedicated to developing 3-D scaffolds with nanostructures to mimic the *in vivo* microenvironments and thus can facilitate cellular functions [13-15]. Since the discovery of carbon nanotubes (CNTs) in 1991 by Iijima [16], CNTs have attracted extensive investigations in the field of biomedical materials, biosensors, drug delivery and tissue engineering because of their high chemical stability, electrical property and biochemical property. In particular, carbon nanotubes are employed as promising scaffolds in tissue engineering. The dimension, diameter and length of carbon nanotubes are analogous to major components in the natural ECM proteins including collagens and laminins [17]. Additionally, the mechanical compliance of CNTs is similar to structural proteins in natural ECM [17]. Advances in CNT-based scaffolds have revolutionized the regenerative medicine [18-23]. However, few studies on CNT-based scaffolds have been focused on the differentiation of ES cells [24-25]. It is thus desirable to investigate the feasibility of incorporating CNTs to nonwoven fibrous matrices that have been widely used as 3-D scaffolds in cell cultures and tissue engineering.

### 1.4 Objectives

The goal of this research was to develop a microbioreactor system with 3-D scaffolds for cell cultures and cell-based high-throughput screening of cytotoxicity and embryotoxicity that is critical to early-stage drug discovery. To accomplish this goal, the following specific objectives were pursued in this research.

1. To develop a novel microbioreactor platform compatible with commercial
multiwall plate readers and capable of online monitoring of pH, DO and cell proliferation for 3-D cell cultures. Such a bioreactor platform with highly reproducible culture performance should have wide applications in cell-based high throughput screening (HTS) for drug discovery and bioprocess development.

2. To develop and evaluate the feasibility of an ESC model with survivin as the reporting marker for embryotoxicity. Such an ESC model can be used for high-throughput screening of chemicals and drugs that may cause reproductive or developmental toxicity, and thus to replace currently widely used EST that can only provide end-point data. The new ESC model capable of online monitoring without any sampling disruption can provide dynamic data on cytotoxicity and embryotoxicity.

3. To study the feasibility of using CNTs to modify scaffold surface and its effects on growth and neuronal differentiation of mES cells. Although ES cells are already successfully used in repairing spinal cord injury in small animal models, the ability to promote neuronal differentiation has always been a challenge. The study thus aimed to demonstrate CNT-based scaffolds for promoting neuronal differentiation while maintaining excellent cell viability.

4. To develop 3-D fibrous scaffolds with nanoengineered surface using CNTs for enhanced cellular functions including cell adhesion, proliferation and differentiation. Compared to 2-D cell cultures, 3-D cell cultures can better mimic *in vivo* microenvironments and provide more representative responses to drugs. However, the next generation of scaffolds for cell culture should incorporate
nanoscale features within the 3-D architecture in order to direct cell-matrix and cell-cell interactions, and thus to provide structural support for nutrients transport and tissue development. This study focused on potential beneficial effects of the nanoengineered fibrous polyethylene terephthalate (PET) scaffolds coated with multiwall carbon nanotubes (MWCNTs) on cell adhesion, morphology and proliferation of CHO and ES cells. The MWCNTs modified PET scaffolds have great potential applications in 3-D cell cultures, tissue engineering, and HTS for drug discovery.

1.5 Scopes of study

Figure 1.1 provides an overview of the major tasks performed and technologies developed in this study, including a 3-D cell culture platform for high throughput screening, an ESC-based model for embryotoxicity and cytotoxicity assays, and CNTs-based nanoengineered PET scaffolds for tissue engineering. This thesis also provides an overview on the uses of different types of cells and cell culture systems, including 2-D, 3-D and perfusion cell cultures, in cell-based HTS for drug discovery (see Chapter 2). It also discusses optical and electrochemical methods for online, non-invasive detection and quantification of cells or cellular activities and recent progresses and applications of 3-D cultures and microfluidic systems for cell-based HTS. In addition, Chapter 3 reviews the influences of 3-D microstructures and nanotopographies on cellular functions, including protein expression, cell adhesion, proliferation, migration, morphology, and differentiation. Recent progress on the development of fibrous scaffolds with 3-D microstructures and nanotextures is also discussed in Chapter 3. The major findings from
this research are summarized below with details given in Chapters 4-7. Finally, Chapter 8 provides conclusions and recommendations for future studies.

1.5.1 Microwell bioreactor system for cell-based high throughput proliferation and cytotoxicity assays (Chapter 4)

A microbioreactor platform capable of online monitoring of pH, DO and cell proliferation was developed and demonstrated with reduced variations and improved reproducibility. The cytotoxicity studies of two newly developed anticancer drugs on HT-29 colon cancer cells demonstrated the potential of the platform in determining dosage effects of drugs in the early-stage drug discovery.

1.5.2 Embryonic stem cell models for high throughput screening of cytotoxic and embryotoxic compounds (Chapter 5)

A high throughput 3-D ESC-based model was developed for real-time monitoring cytotoxicity and embryotoxicity in 3-D cell cultures. Seven compounds with known embryotoxicity were used to demonstrate and validate the model with predictive responses. The model was then used to assess cytotoxicity and embryotoxicity of three Chinese herbal medicines, demonstrating its application as a robust high-throughput screening model for evaluating embryotoxicity and cytotoxicity of chemicals and drugs.
1.5.3 Enhanced neuronal differentiation of mouse embryonic stem cells cultured on PET membranes decorated with multi-wall carbon nanotubes (Chapter 6)

CNT-based membranes were developed as scaffolds for tissue engineering. CNTs-PET membranes offered nanoscale features, which were demonstrated to promote cell adhesion and differentiation. Furthermore, CNTs played a positive role in maintaining cell viability. The increased length of neurites and improved degree of neurites branching compared to controls demonstrate that this scaffold holds great potential in nerve regeneration.

1.5.4 Multiwall carbon nanotubes (MWCNTs)-decorated polyethylene terephthalate (PET) fibrous matrices for enhanced 3-D cell cultures and functions (Chapter 7)

The next-generation tissue scaffolds should incorporate 3-D structures and nanofeatures. Carbon nanotubes can provide similar nanoscale structures to those present in natural extracellular matrices in vivo. In this work, nonwoven PET fibrous matrices decorated with multiwall carbon nanotubes (MWCNTs) were developed and used to enhance cell proliferation and differentiation. Different cell morphologies of CHO and mES cells were also observed in nanoengineered 3-D PET scaffolds. These findings indicate that the nanoengineered PET scaffolds can serve as functional biological scaffolds for tissue engineering.
1.6 References


Figure 1.1. Overview of research objectives and the scope of the study
Chapter 2: Literature Review on Cell-Based Assays in High-Throughput Screening for Drug Discovery

2.1. Introduction

The journey from drug discovery to its commercialization is complex, lengthy and expensive. It usually takes 10-12 years and well over $775 million for the research and development of a new drug. On average, approximately 250 lead compounds screened from a million compounds enter pre-clinical testing, 10 of them proceed to clinical trials, and only one will be approved by Food and Drug Administration (FDA) [1]. Historically, drug screening extensively relies on animal models as proxies for human beings in drug target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity). Although these animal models have the capacity to provide a wealth of useful information for drug screening, they are relatively expensive, low throughput and present moral issues. With the development of combinatorial chemistry and biology, the number and diversity of compounds that need to be tested for activity against targets has rapidly expanded in recent years. In addition, with the enormous amount of new data generated from the Human Genome Project, the number of proteins that can be targeted for drug screening has also significantly increased with better understanding of the mechanisms underlying human diseases [2]. These trends have spurred the development of increasingly rapid, selective and reliable high-throughput screening (HTS) assays for the
early-phase drug discovery.

HTS assays can be divided into two categories, namely biochemical assays and cell-based assays [3]. Target-based biochemical assays, mainly enzyme inhibition and receptor-ligand binding assays, have been the mainstay of HTS campaigns in the pharmaceutical industry. In a biochemical assay, the specific binding or affinity of tested compounds to the target of interest is carried out in homogeneous reactions that allow the miniaturization with low variations. However, the applications of biochemical assays are limited because not all targets can be purified or prepared in a manner suitable for biochemical measurement. Additionally, drug responses tested in biochemical assays cannot precisely represent tissue-specific responses since the activity of a small molecule in a biochemical assay is different from the activity in a cellular context [3]. Therefore, the toxicity testing market is gradually moving towards in vitro cell-based assays, as they provide an early indication of the toxicity characteristics of the drug candidates.

Cell-based assays for HTS include mainly three types: second messenger assays, reporter gene assays, and cell proliferation assays (see Table 2.1) [4]. They are extensively applied in the majority of compound screening programs performed by the biopharmaceutical industry. The global market for cell-based assays in drug discovery was valued at $6.2 billion in 2010, and is expected to increase at an annual growth rate of 11.6% to reach nearly $10.8 billion in 2015 [11]. Cell-based assays can distinguish between agonists and antagonists, identify allosteric modulators, and provide direct information on compounds with regards to cell permeability and stability inside cells, and acute cytotoxicity associated with the compounds [12]. Furthermore, cell-based assays
can be performed in a more biological relevant microenvironment and thus represent a good compromise between whole organisms and in vitro biochemical systems. These assays can provide representative tissue specific responses, and have been used throughout early drug discovery, from target identification and validation to primary screening, lead identification and optimization, and safety and toxicology screening. They have been successfully used for early drug discovery in identifying high-quality leads.

The main components of a cell-based HTS assay include cells, device for culturing the cells, and detection system for quantification of cells or cellular activities. This review provides an overview on different types of cells, cell culture systems, and in situ detection methods currently used or amenable to use in cell-based HTS assays. We highlight reporter gene techniques, which involve the expression of the reporter gene product such as an enzyme or green fluorescent protein that is used as part of a colorimetric, luminescent, or fluorescent read-out of gene activation in the cell or cellular responses to a stimulus, and recent developments of 3-D cell-based fluorescent assays and microfluidic systems for HTS. Examples and perspectives of using cell-based HTS assays in early-stage drug discovery are also provided in this chapter.

2.2. Cell sources and types

Cells used in cell-based assays should be amenable to the assays, faithfully represent the system and express the necessary factors and signaling intermediates. Various types and sources of cells have been used in cell-based assays. Table 2.2 presents a summary of
different cell types and sources with their most advantageous and disadvantageous features.

**Immortalized cell lines** are widely used for drug screening assays because they are cheap, easy to grow, reliable and reproducible. For example, HEK293 cells, derived from human kidney, are an easy, amenable experimental system [20, 21]. However, immortalized cell lines, derived from a well-known tissue type, have undergone significant mutations and their biological characteristics might be altered in the immortalization process and different from those of the native or normal cells [22]. **Primary cells** may provide representative responses; however, they have a limited life span in culture and are difficult to grow and transfect [22]. Human cancer cell lines represent the cancer of origin and are widely used for anticancer drug screening in pharmaceutical research. However, they can contain mutations that might affect the experimental results. For instance, the breast cancer cell line MCF-7 lacks a functional caspase-3 gene product [23]. Furthermore, cell-based assays employing primary cells or immortalized and cancer cell lines have been insufficient in developing effective therapeutics for cancers. Although the initial therapy and recovery is successful, the rate to a relapse of the disease is very high [24].

Recent advances in stem cell research have revolutionized the drug discovery process. **Cancer stem cells**, which might be genetically arising from oncogenic transformation of either stem cells or progenitor cells, can be isolated from tumors and used as an effective platform for cancer drug screening. Cancer stem cells can self-renew, differentiate and regenerate a phenocopy of the original tumor [25]. Therefore, they are
promising cell models for specific human cancers. However, they are rare and difficult to find or isolate from tumors. *Mesenchymal stem cells* (MSCs) isolated from bone marrow or umbilical cord blood can be differentiated into different somatic cells, such as osteoblasts, for testing drugs. However, MSCs have limited potency and are slow-growing and difficult to expand *in vitro*. *Embryonic stem cells* (ESCs) and *induced pluripotent stem cells* (iPSCs) can provide an unlimited source of normal human cells that can be expanded for drug screening and toxicological studies. ESCs are isolated from embryo and have unlimited capacity to self-renew and can be differentiated into any cell type *in vivo*. These cells can serve as better cell models for both drug efficacy and toxicity screening than primary cells or immortalized cell lines. However, ESCs present some moral issues since the embryos are destroyed during the procurement of the cells. More recently, the development of iPSCs has revolutionized the stem cell field. iPSCs are pluripotent cells artificially derived from somatic cells (e.g., fibroblasts and other adult cell types) by inducing a small set of powerful pluripotency genes. These cells lose their previous somatic cell properties and are similar to human ESCs in terms of morphology, growth properties, gene-expression profiles and differentiation potential. As iPSCs can be derived from patients with specific diseases, they have been considered as a new tool in drug discovery.

2.3. *Cell culture systems*

The majority of cell-based HTS assays are carried out in multi-well plates as they can be easily miniaturized to increase the number of wells per plate for high throughput rates, on the order of 10,000 compounds per assay per day, and handled with a robotic system for
automation [4]. More recently, there are increasing interests in developing microfluidic devices for perfusion cultures that allow for the evaluation of long-term drug effects as well as studying interactions among different cell types in a biological system like the whole animal [26]. There is also increased awareness of that conventional 2-D cell cultures in multiwell plates cannot represent in vivo tissues that are present in 3-D environments [27]. The different culture systems used in cell-based assays are discussed and compared for their advantages and disadvantages or limitations below.

2.3.1 3-D vs. 2-D cultures

In general, cell culture modes include single cells, monolayer cells on a two-dimensional (2-D) surface, multilayer cells or aggregate clusters in a 3-D scaffold [28]. 2-D cell-based assays in multiwell plates together with automated operation are widely used in drug screening because of their low costs and easy operation. However, 2-D cell cultures can result in errors in predicting tissue-specific responses due to the loss of native morphology and limited cell-cell and cell-matrix interactions. As can be seen in Figure 2.1, cells cultured in 3-D scaffolds generally show similar in vivo morphology with intimate cell-cell and cell-ECM (extracellular matrix) interactions, which are absent in 2-D cultures. It is known that cells grown on 2-D flat surfaces behave differently from those in 3-D scaffolds or in vivo tissues in their morphology, cell-matrix interactions and differentiation [29]. The third dimension in the 3-D scaffold provides another direction for cell-cell interactions, cell migration, and cell morphogenesis, which are critical in regulating cell cycle and tissue functions. Differences in spatial organization and distribution contribute to the difference in cell growth. In addition, 3-D cell cultures
provide not only the templates for cells to adhere and grow, but also the interconnectivity within the 3-D constructs to allow nutrients and metabolites to be transported in and out of the engineered tissues. Consequently, 3-D cell cultures can support a higher cell density than 2-D cell cultures [29]. The high specific surface areas offered by 3-D also allow for a long-term cell culture in vitro [30]. The cellular performances between 2-D and 3-D cell cultures are also different in many studies. For example, the malignant phenotype of human breast epithelial cells cultured in 3-D Matrigel based scaffolds can be reverted to normal morphology via the inhibition of β1-integrin and epidermal growth factor receptor (EGFR), but not in 2-D cell cultures [31]. Furthermore, cow luteal cells (primary cells) cultured in a 3-D bioreactor environment could better maintain its normal function, progesterone secretion in response to luteinizing hormone (LH) stimulation, for a longer period as compared to cells cultured in the 2-D T-flasks (see Figure 2.2) [32].

The ability for cells to maintain their normal function and response to environmental stimuli is critical in the development of cell-based HTS for drug discovery.

Numerous studies have shown that cell responses to drugs in 3-D cultures are distinct from those in 2-D cultures, which highlights the advantages of using 3-D-based models. For example, colon cancer cells in 3-D cell cultures showed an increase in drug resistance of up to 180-fold as compared to 2-D cell cultures [33]. Colon and ovarian cancer cells in 3-D cell cultures exhibited a 1000-fold decrease in cytotoxicity responses to gemcitabine, while 2-D cultures erroneously predicted gemcitabine to be an effective proliferation inhibitor [33]. Tumor cells cultured under 2-D conditions are more sensitive to PI3-kinase inhibitor than those within engineered 3-D poly (lactide-co-glycolide) tumor
microenvironments [34]. These results support the important effects of physiological microenvironments within tumors on the effectiveness of chemotherapy and suggest that 3-D engineered tumor models can offer more useful information in anticancer drug screening. Furthermore, coculture of endothelial, stromal, and/or epithelial cells within 3-D systems allows the study of the side effects of a drug on neighboring stromal cells [35]. Moreover, human skin cells (keratinocytes, dermal fibroblasts and endothelial cells) in 3-D cocultures can stand more oxidative stresses (hydrogen peroxide) and a potentially toxic heavy metal (silver) than in 2-D cultures [36]. These results suggest that 3-D culture systems outperform their counterpart 2-D culture systems in studying physiological responses to xenobiotic materials.

In summary, 3-D cultures can better recapitulate in vivo cellular responses to drug treatment and has potential to be a superior platform for drug development. Therefore, there is a great need for in vitro 3-D cell culture assays, which would bridge the 2-D monolayer cell culture systems and the animal models. However, 3-D cultures create a non-homogeneous environment that is difficult to monitor for changes in cells or cellular activities using conventional detection methods, which will be discussed later.

2.3.2 Perfusion vs. static cultures

Widely used HTS platforms (e.g. 96-, 384-, 1536-well plates) offer static microenvironments, with the medium supplied in a batch-wise manner. Although automation using robots allows the static cultures to be used as a feasible HTS platform for drug screening, static cultures cannot support a long-term cell culture due to the risk of contamination caused by repeated interventions. In addition, the intermittent medium
replacement process would result in large fluctuations in the culture microenvironments [37, 38]. Although further miniaturization of these systems holds great potentials to increase throughputs, the relatively high surface to volume ratio in microscale wells results in uncontrolled liquid evaporation and leads to undesirable culture conditions [39]. Modified multiwell plates have thus been proposed with the integration of microfluidic systems, which has been reported with high throughput for drug screening [40] and cytotoxicity evaluation of anticancer drugs [41]. Such systems, where a perfusion cell culture is achieved to compensate liquid evaporation, can maintain a cell culture for an extended period for testing long-term effects of drugs.

In addition to continuously providing nutrients and waste removal and thus keeping the cell culture system stable, perfusion can also be used to generate gradients of drug concentrations, creating a specific physical microenvironment (e.g. shear stress or interstitial fluid flow) and constructing a circulatory system to better mimic the in vivo conditions [39]. Compared to the static cell culture, perfusion increased cell content and matrix synthesis in a 3-D chondrocyte culture [42]. Furthermore, cellular responses to the perfusion and static conditions were quite different for a human hepatocarcinoma cell line [43]. Although there was a time period, wherein the cell physiology was comparable, outside this period, the cultured cells behaved differently in the two culture systems. The different culture behaviors might be attributed to the uncontrollable difference in some unknown biochemical or biophysical factors in these two culture systems.
2.4. Methods for quantification of cells and cellular activities

Cell-based assays are well established and widely used to analyze the effects of compounds on cellular activities, including nuclear size, mitochondrial membrane potential, intracellular calcium levels, membrane permeability, and cell number. The failure of early identification of toxic side effects of a compound has resulted in about 30% of the attrition of new drug candidates [44]. Therefore, cytotoxicity testing, which generally relies on the quantification of cell number and viability, has become one of the most critical steps in early-phase drug discovery. Conventional methods for cell number counting use hemacytometer, Coulter counter or flow cytometry are labor-intensive and time consuming, while Trypan blue exclusion and neutral red uptake methods for determining cell viability require the use of invasive chemicals. Furthermore, these methods have a relatively low throughput, and thus are not good choices for HTS. As offline sampling during the cell culture process is limited by the small amount of medium used in HTS assays, from several µL to several mL [45], online detection is required. Detection methods used in cell-based HTS assays can be divided mainly into two groups: electrochemical and optical techniques. In general, optical sensing is easier for miniaturization than electrochemical sensing [46]. These detection methods are discussed below.

2.4.1 Electrochemical methods

Various electrochemical biosensors, which integrate biological recognition elements and electrochemical transduction units, based on (a) cellular activity and function; (b) cellular barrier behavior; and (c) recording/stimulation of electric potential of electrogenic cells
have been developed [47]. They can be used to achieve noninvasive online monitoring of drug toxicity.

2.4.1.1 Electrochemical method based on cellular activity and function

A living cell can be considered as an electrochemical system [48]. Electron generation and charge transfer caused by redox reactions and the changes of ionic composition and concentration in living cells can be used to characterize cell viability in a homogenous solution [49]. For example, when the tumor cells are attached to a gold nanoparticle-modified carbon paste electrode, with platinum wire as auxiliary and saturated calomel electrode as reference electrodes, the cells exhibit an irreversible voltammetric response, which is related to the oxidation of guanine. The oxidation peak can be used to investigate the exogenous effect, which provides an electrochemical approach for studying antitumor drug sensitivity [50].

In addition, metabolism in cells leads to changes in metabolic products (e.g., lactic acid and carbon dioxide) or substrates (e.g., glucose and dissolved oxygen [DO]). A variety of electrochemical biosensors based on metabolic changes have been fabricated [51-54]. Electrochemical methods based on cellular activities include potentiometry and amperometry.

Conventional potentiometry cell-based sensors include an ion-selective electrode (ISE) or gas-sensing electrode (GSE) coated with a layer of cells. An ISE has been developed for screening of toxins by integrating cells with a K⁺ selective film. In such systems, a potential change caused by the ion accumulation or depletion on the electrode
surface can be used to monitor metabolic products during cell growth [54]. However, this method requires a very stable reference electrode, which limits the application of potentiometry sensors.

Amperometric electrochemical methods using a specific enzyme electrode are widely used for the determination of pH, DO or glucose. The acidification rate in the vicinity of cells can be quantified using a microphysiometer [55]. Cellular biochemical responses resulting from the accumulation of lactic acid and carbon dioxide can be approximately monitored using the pH value in pH-sensing chambers. Furthermore, heterogeneous pO$_2$ distributions around tissues could be detected using a miniaturized system [56]. However, metabolic activities can be affected by many uncontrollable environmental factors, which limit the applications of this approach in high-throughput cell-based assays.

2.4.1.2 Electrochemical method based on barrier behavior

The local ionic environment at the electrode/solution interface changes in the presence of cells. In general, cells with insulating properties would significantly increase the electrode impedance [57, 58]. Thus, biological status of cells, including cellular viability, morphology, cell number, and cell apoptosis, and cell adhesion can be monitored using electrochemical impedance spectroscopic techniques. For example, a novel electrical impedance sensor array integrated into the bottom of a microtiter plate has been developed for the quantitative detection of living cells. Real-time assessment of cytotoxicity and acute toxicity can be achieved using this device [58].
2.4.1.3 Electrochemical method based on the recording/stimulating of cellular electrical potential

Electrogenic cells and tissues, such as heart muscle, pancreas beta and nerve cells, are able to generate bioelectrical signals resulting from the orchestrated activities of ion channels embedded within cell membrane [47]. These bioelectrical signals can be used to test drugs against critical diseases such as cardiac arrhythmia, hypertension, Parkinson’s disease, diabetes, depression, and neuropathic pain [59]. A nanoelectronic biosensor was developed based on single-wall carbon nanotubes (SWCNTs). Nerve cells were grown on a SWCNT field-effect transistor, and changes in the membrane potential influenced the measurable capacity between the microelectrode and axon [60]. This method can be used to non-invasively detect cellular activities for electrogenic cells with high throughput, high sensitivity, easy use, and the capacity of long-term cell culture.

Although, electrochemical methods of detection are noninvasive and offer an appropriate temporal resolution, they cannot provide information on specific cellular activities that are directly related to certain cell functions, biomarkers or signaling pathways, which are important to better understanding of cytotoxicity effects and mechanisms of drugs. Moreover, electrochemical methods are not amenable to 3-D cell cultures as cell direct contact with the electrode is required. This limits the application of electrochemical methods to 2-D cultures, which suffer from many limitations for cell-based assays as discussed earlier.
2.4.2 Optical methods

Optical detection in cell-based HTS assays usually is carried out with colorimetric, luminescent, or fluorescent methods, which are discussed below.

2.4.2.1 Colorimetric method

Colorimetric methods are based on color change of the growth medium after cell metabolites react with chemical agents. Colorimetric assays using ruthenium dye [61] and Alamar Blue [62] have been developed. However, the low sensitivity of ruthenium dye and the poor reliability of Alamar Blue for the measurement of kinetics limit their applications in the development of HTS platforms. A spectrum of assays using tetrazolium salts such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [63], MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) [64, 65], and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [66] are commercially available. These methods are based on the reduction of a tetrazolium salt by actively growing cells to a colored formazan product that can be quantified with a spectrophotometer.

However, these colorimetric methods require multiple additions of chemicals at prescheduled time points, which interfere or can disrupt the targeted cells. In addition to the invasiveness, these methods are time consuming and laborious. Furthermore, they usually can only provide end-point data, an intrinsic drawback for in vitro cytotoxicity tests. Dynamic data, which can provide more details about the effects of drugs on cells than the end-point data, is preferred. In addition, in order to realize automation for HTS,
conventional end-point assays require the aids of expensive robotic arms. Also, miniaturization, which is an important factor to achieve HTS, is not compatible with colorimetric methods because of their relatively low sensitivity.

2.4.2.2 Luminescent methods

Many organisms, including fireflies and some marine organisms, regulate their light production using luciferase in a variety of light-emitting reactions with emission color ranging from yellow-green to red. In luminescent assays, the oxidation of luciferin catalyzed by luciferase produces light that can be detected by a light sensitive apparatus such as an illuminometer or optical microscope, allowing observation of biological processes [67]. Some luminescent reactions are mediated by ATP or calcium ions. Luciferase has been widely used as a reporter in cells expressing a luciferase gene under the control of a promoter of interest to assess its transcriptional activity [68]. Many commercial cell-based kinase activity assays use luciferase as the reporter. Luciferase can also be used to detect the level of cellular ATP in cell viability assays. In addition, some enzymes, such as caspase and cytochrome P450, can convert proluminescent molecules to luciferin, and their activities can thus be detected in a coupled or two-step luciferase assay [69].

Although firefly luciferase is widely used in cell-based assays, its application in HTS is usually limited to end-point assays because the requirements of cell lysis and addition of luciferase substrates. Unlike firefly luciferase, the luciferase (MetLuc) derived from the marine copepod *Metridia longa* is naturally secreted, which allows the development of live cell assays and multiple assays on the same cells using a no-lysis
However, in addition to the amount of luciferase, the bioluminescence intensity can also be affected by many other factors such as luciferin absorption, availability of co-factors, pH, and transparency of culture media or buffer, causing discrepancies between detected bioluminescence signals and actual changes in cellular activity [71].

2.4.2.3 Fluorescent methods

Compared to luminescent methods, fluorescent methods have higher sensitivity and are easier to be miniaturized for large-scale or high throughput measurements of cell activities, pathway activation, toxicity, and phenotypic cellular responses of exogenous stimuli [72]. Fluorescent methods for cell-based assays were initially developed using small, highly-fluorescent, organic molecules, monitoring ion concentrations, membrane potential and as intracellular substrates for reporter genes. However, conventional fluorescent molecules usually have narrow excitation spectrum and broad emission spectrum, and are difficult to use for simultaneous excitation and quantification of multi-targets with different emission peaks. In addition, they are photo-unstable and can be quenched by continuous excitation. More recently, nanoparticles such as quantum dots (QDs) have been widely used as labels in cell-based assays. These semiconductor nanocrystals are photo-chemically stable, can provide a narrow and adjustable emission, and can be excited by light of any wavelength shorter than that of the emission peak. Thus, various emission colors can be simultaneously obtained using nanoparticles of different sizes excited with a single-wavelength light [73]. Although QDs can be readily internalized into cells and used as fluorescent labels in cell-based assays [74], they can be
toxic to cells. Also, like the luminescence-based and colorimetric methods, they can only be used to quantify cells or cellular activities at the end-point, which cannot provide dynamic cell proliferation or death kinetics.

The development of reporter gene techniques using green fluorescent protein (GFP) has enabled online, non-invasive detection and quantification of cell proliferation and specific cellular functions. GFP, which was first discovered in the jellyfish, and its mutants have been developed with emission light colors ranging from blue to yellow [28]. GFP is species-independent and generally non-toxic to cells. Its detection can be performed in living samples. Therefore, cell-based assays using GFPs are amenable to real-time, automated, and non-invasive assessment of both chronic and acute cellular events [28]. In addition, GFP can be coupled with Disco soma species red fluorescent protein (dsRed) for two-color or multiplex assays [75].

Cell-based assays using cDNA encoding a fluorescent protein provide an HT platform for non-invasive analysis of cell proliferation and death kinetics. Table 2.3 shows some cell-specific promoters and reporter genes that can be used in drug screening. Since a specific cellular event or function can be monitored based on the regulatory DNA sequence or promoter used in controlling the expression of the reporter gene, the assay is responsive to targeted effects, such as activation of signal transduction pathways, and is suitable for use in disease-relevant assays. In addition, two fluorescent proteins fused with a peptide linker comprising a caspase-3 cleavage site can be used to study the activation of caspase-3 or apoptosis in live cells [105, 106] based on changes in emission wavelength due to energy transfer between two close fluorophores, a
phenomenon called fluorescence resonance energy transfer (FRET).

In general, whole-cell autofluorescence-based systems are non-invasive, fast, and simple for HTS applications. It can provide dynamic data and be used as high-content assays as well. Current commercial HTS systems [107-109] use laser scanning imaging systems with fluorescence microscopy and quantitative image analysis to perform live-cell kinetic assays with high spatial and temporal resolution. They can be used to examine the context of living cells, quantify intracellular proteins, and monitor the trafficking of proteins fused with fluorescent reporters and some subcellular structures [110]. However, their high costs and relatively low capacity limit their uses to the late-phase compound characterization [111]. Moreover, these imaging systems are limited to read planar images of cells cultured on 2-D surfaces and are not suitable for 3-D cell cultures.

Although advanced high-throughput flow cytometry has also been developed for cell sorting and detection [112], it is not amenable for use in cell-based HTS assays because cell sampling from culture wells is intrusive and difficult to implement on a large scale. On the other hand, a fluorescent plate reader or spectrofluorometer commonly used in fluorescence-based assays may not have the sensitivity high enough for use in cell-based HTS assays because of the high background noise from media and relatively weak fluorescent signals from cells cultured in conventional multiwell plates [113]. To overcome this problem, a novel 3-D culture platform with cells cultured in fibrous polyethylene terephthalate (PET) matrices in modified multiwells was developed, which is discussed in the following section.
2.5. Development of 3-D cell-based HTS assays for drug discovery

2.5.1 3-D cell-based fluorescence assays

Real-time analysis of cell proliferation based on fluorescence read-outs from a fluorometer was first demonstrated with Chinese Hamster Ovary (CHO) cells expressing GFP under the control of a human cytomegalovirus (CMV) promoter [114]. However, the weak fluorescence signals generated from cells cultured in conventional 96-well plates are hardly accurate by using a fluorometer for quantifying cells because of changes in the culture environment, including pH and other auto-fluorescent components present in the culture medium, including GFP released from dead cells [115]. High, fluctuating background signals from these non-specific effects could effectively mask live-cell GFP signal and render it unreliable or useless for assessing cytotoxicity or cell proliferation.

The aforementioned limitations can be overcome by culturing GFP-expressing cells in a PET scaffold in a modified well (see Figure 2.3), which significantly increases the total cell number per unit area and reduces the background noises [113]. Such a 3-D culture can provide a 20-fold higher cellular fluorescence and significantly improve signal to noise ratio because cells are concentrated in the scaffold at the center of the well and the background fluorescence can be measured separately and subtracted to give the true live cell signal. This new 3-D fluorescent culture platform can give highly reproducible growth kinetic data, which can be more reliably used to assess drug effects on cell proliferation. It has been successfully used to study cytotoxicity effects of chemicals, cancer drugs (see Figure 2.4) and Chinese herbal medicines, demonstrating its potential application in early-stage drug discovery.
2.5.2 Microfluidic cell-based assays

Microfluidics has emerged as a promising technology with widespread applications in engineering, biology and medicine. It has the potential to revolutionize the way we approach cell biology research. Microfluidics refers to the science and technology that allows one to manipulate tiny amounts (10^{-9} to 10^{-6} liter) of fluids using microstructures with characteristic dimensions on the order of tens to hundreds of micrometers. The controllable processing of microfluidic devices at dimensions close to cells and biomolecules enable their biological applications at the cellular level. In addition, the scale of microchannels corresponds well with the native cellular microenvironments, in which the ratio of cell volume to extracellular fluid volume can be greater than one [39]. This paves the way to create a more *in vivo*-like cellular microenvironment *in vitro*. Furthermore, microfluidic systems have the advantages of minute consumption of reagents, short diffusion path for quick reaction and fast analysis, highly paralleled operation, and versatile and precise controls for fluid transport, mixing and concentration manipulations [26]. Equipped with external physical control and online detection mechanisms, microfluidic systems can be fully automated for HTS assays with improved data quality and reduced assay time and cost.

Microfabricated cell culture devices have previously been demonstrated on silicon and polydimethylsiloxane (PDMS) substrates with hepatocytes [116], lung [117], and insect cells [118], but never for the purpose of realizing an integrated assay system. Hung et al. for the first time developed a microfluidic system integrated with concentration gradient generator capable of long-term cellular monitoring [119]. The integrated device
consists of a concentration generator and a 10×10 array of microchambers (see Figure 2.5), which enables performing 100 experiments in parallel with a unique condition in each chamber. The array can be applied to characterize the effect of culture medium components, pH, cell density, and perfusion rate on protein expression.

Most of microfluidic platforms are fabricated using PDMS, which is optically transparent, gas permeable and biocompatible [26, 39]. However, it can undesirably absorb hydrophobic drug molecules due to its hydrophobic property. Su et al. (2011) tested cytotoxicity using HEK cells using microchannels made from 2 different plastics, polystyrene (PS) and cyclo-olefin polymer (COP), and silicone elastomer. Their results showed that PS and COP might be more appropriate than PDMS devices when used in hydrophobic drugs selection [120].

The conventional format of microfluidics is not an ideal match for complete cell culture, because all of the reagents and cells are positioned in an interconnected network of enclosed microchannels, making it difficult to establish fresh, sterile sites for seeding new generations of cells. Recently, digital microfluidics (DMF) has emerged as an alternative to the conventional format of enclosed microchannels. DMF is a technique, in which nanoliter-sized droplets are manipulated on an open surface of an array of electrodes. For example, Barbulovic-Nad et al. introduced the first lab-on-a-chip platform capable of implementing all of the steps required for mammalian cell culture: cell seeding, growth, detachment, and re-seeding on a fresh surface for complete mammalian cell culture [121].

In addition, conventional mammalian cell culture protocols usually stipulate
elevated carbon dioxide and relative humidity. These parameters for microfluidic systems are maintained by placing the whole microfluidic system inside an environmental chamber and using gas permeable materials such as PDMS. Forry et al. developed a microfluidic system that allowed on-chip control of the carbon dioxide partial pressure by flowing pre-equilibrated aqueous solution through control channel across the device. The system enabled long-term microfluidic culture of mammalian cells without requiring a cell culture incubator or CO₂-independent media [122]. These pioneering research projects are excellent examples that highlight the capacity of microfluidic techniques for improving HTS via well-controlled fluid handling and without the need for complex robotics.

Most of the previous studied microfluidic systems, however, rely on 2-D cell cultures, which may limit their applications in situations where 3-D cell culture is necessary to demonstrate authentic in vivo physiology for certain cell types, such as tumor cells, hepatocytes, chondrocytes, neural cells, and ESCs. Drug testing models based on conventional cell culture technique continue to give misleading and non-predictive data for in vivo responses. This failure is mainly a result of 2-D culture’s lack of capacity to mimic in vivo microenvironments and unable to preserve their phenotypic characteristics. As discussed earlier, 3-D cultures exhibit profound differences from conventional 2-D cultures in cellular functions, morphology, and proliferation and drug responses.

2.5.3 3-D microfluidic cell cultures

3-D microfluidic cell culture systems offer a biologically relevant model to conduct
micro-scale cell-based research and applications in drug screening. Various natural and synthetic hydrogels have been incorporated into microfluidic cell culture systems to support cells in 3-D. A variety of 3-D microfluidic cell culture models have been developed [123-125]. More recent advances in modeling of the cell microenvironment have focused on the generation of in vivo-like ECM constructs for supporting 3-D cell growth and examining cell migration. For example, Vickerman et al. developed a microfluidic platform capable of mimicking the in vivo microenvironments by integrating fluidic microenvironments and 3-D microenvironments using microinjection of gel solution containing cells [123]. An open lumen-like structure was formed when human adult dermal micro-vascular endothelial cells were cultured in this microfluidic platform for up to 7 days. In another example, Toh and his group showed that cells could be maintained in 3-D structures in a microfluidic channel perfusion microbioreactor with an array of micropillars [124]. Parallel microfluidic channels can be used for multicellular 3-D cultures to study cell-cell and cell-ECM interactions in spatially well-defined geometries. Huang et al. studied the behaviors of metastatic breast cancer cells and tumor-derived macrophages cultured in adjacent ECM gels via real-time imaging [125].

However, real-time quantification of cell proliferation, which can be valuable information in assessing time-dependent cellular responses to drug treatments, could not be easily quantified in the aforementioned systems. Wen et al. developed a microbioreactor array (see Figure 2.6) that has the capability of perfusion high-density 3D cell culture in modular and low-cost PET fibrous scaffolds [126]. Non-invasive and time-series cell proliferation and cytotoxicity assays can be achieved using a plate reader.
to monitor the fluorescence emitted from the EGFP-expressing HT-29 cells cultured in the device. With continuous perfusion, cells could be maintained for an extended period, reaching a cell density as high as $6 \times 10^7$ cells/mL matrix. Such a perfusion culture system would be useful in assessing long-term drug effects on cells in a 3-D environment.

2.6. Cell-based HTS in commercial drug development

The rapid progress in combinatorial chemistry, genomics, proteomics, and bioinformatics has led to a significant increase in the number of potential therapeutics, which has also spurred the development of HTS for lead identification and optimization. Over the past two decades, HTS has emerged and matured as a platform in the early stage of drug discovery in the pharmaceutical industry. There has been a growing trend in drug discovery to perform lead identification and optimization using cell-based assays because they can provide more relevant physiological information than biochemical assays. Cell-based assays are emerging as the preferred tools for screening potential drug compounds. Pharmaceutical and biotechnology companies are gradually replacing other in vitro and biochemical assays with cell-based assays in drug discovery. Today, cell-based HTS assays represent approximately more than half of all high throughput screens currently performed, and their applications have been an integral component of drug discovery.

One successful example of cell-based HTS in drug discovery is the commercialization of Eltrombopag (Promacta/Revolade; GlaxoSmithKline), which is a thrombopoietin (TPO) receptor agonist approved by the FDA in 2008 [127]. This compound was selected out of about 260,000 compounds using a cell-based luciferase
reporter system. This screening system was based on a stable TPO-responsive cell line, BAF-3/TPO-Rluc, which was obtained by transfecting murine hematopoietic progenitor cells (BAF-3) with a human TPO receptor (hTPOr) cDNA and a luciferase reporter gene [128]. Luciferase expression was controlled under a synthetic STAT-responsive promoter, which can be regulated by TPO. Therefore, this luciferase system can be used for high throughput screening of TPO modulators. SB-497115 (Eltrombopag) was selected using this system as a candidate for clinical studies and then was commercialized in 2008 for its maximal efficacy of TPO in the proliferation of BAF-3/TPO-R cells and the increase of the amount of CD41+ (marker of megakaryocyte differentiation) cells [129-132].

Another successful example is BMS-790052 hepatitis C virus (HCV) NS5A (Bristol-Myers Squibb), a clinical candidate for the inhibition of hepatitis C virus replication [127]. O’Boyle II et al. developed the original cell-based HTS system in 2005 [133]. This system utilized a mixture of HCV and bovine viral diarrhea virus (BVDV) cell lines, isolated from human hepatocarcinoma Huh-7. In such a system, parameters including cytotoxicity, the replication of HCV replicons and the amount of active luciferase expressed from BVDV replicon cells were used to evaluate the potency and specificity of the investigated HCV replicon inhibitor [133]. Cytotoxicity was determined using Alamar blue dye, while the replication of HCV replicons was estimated using the amount of NS3 protease activity. Lemm et al. employed this cell-based replicon screening method and targeted a potential candidate named BMS-824 among a group of HCV inhibitors [134]. Finally, BMS-790052 was developed and selected as a clinical
candidate for the inhibition of hepatitis C virus replication [135].

Cell-based HTS has also made contributions to cancer chemotherapy. One example is the development of Bortezomib (Velcade®, formerly known as PS-341), which was approved by FDA in 2003 for the treatment of myeloma. It took only 8 years from initial screening to FDA approval, making Bortezomib the most rapidly developed new anticancer drug in recent history. Bortezomib along with other related peptide boronic acids were screened using the NCI60 cell line. Parameters such as GI\textsubscript{50} (50% growth inhibition, relative to no compound), TGI (total growth inhibition), and LC\textsubscript{50} (50% lethality) were used as cellular responses to drugs for the evaluation of drug efficacy [136].

2.7. Cell-based assays for screening phytochemicals

Cell-based HTS can also be applied to evaluate cellular responses to the stimuli from toxic or growth promoting phytochemicals. Like a gift from Father Nature, numerous natural products from plant food and traditional medicine have been found to be health promoting. Phytochemicals, as nonnutrient plant chemicals that contain protective, disease-preventing compounds, have become an important focus in plant research. It is necessary, however, to understand how phytochemicals can be involved in various biological activities before the full beneficial potential can be reached with any well-characterized recipe for either food supplements or clinical treatments. In general, plant chemicals can be categorized into several major types: terpenoids, phenolics, alkaloids, fiber and other nitrogen-containing plant constituents [137, 138]. Phytochemicals can provide many health benefits as: (1) substrates for biochemical reactions; (2) cofactors of
enzyme reactions; (3) inhibitors of enzymatic reactions; (4) absorbents/sequestrants that bind to and eliminate undesirable constituents in the intestine; (5) ligands that agonize or antagonize cell surface or intracellular receptors; (6) scavengers of reactive or toxic chemicals; (7) compounds that enhance the absorption and or stability of essential nutrients; (8) selective growth factors for beneficial gastrointestinal bacteria; (9) fermentation substrates for beneficial oral, gastric or intestinal bacteria; and (10) selective inhibitors of deleterious intestinal bacteria [138]. Specifically, ample research evidence indicates that the health benefits of phytochemicals can be shown in their roles against various major health-threatening diseases, including cancers, coronary heart disease, diabetes, hypertension, inflammation, microbial, viral and parasitic infections, psychotic diseases, spasmodic conditions, and ulcers, etc.

Take cancer prevention for example, Steinmetz and Potter [139] reviewed 206 human epidemiological studies and 22 animal studies to show the positive relation between vegetable and fruit consumption and risk reduction of cancer. The benefits have already been evidenced for cancers of the stomach, esophagus, breast, lung, oral cavity and pharynx, endometrium, prostate, pancreas and colon. Different mechanisms may be involved in the efficacy of different phytochemicals in preventing cancers. Although many of them are still largely a black box, we may glean some knowledge of the representative mechanisms unveiled in the literature. The known mechanisms related to inhibition of mutagenesis include antioxidant activity, alteration of biotransformation enzyme activity, and antibacterial and antiviral effects [140]. Mechanisms that affect the cancer proliferation and progression include alteration of immune function, reduction of
inflammation, modulation of steroid hormone concentrations and hormone metabolism, arrest in cell cycle progression, and stimulation of apoptosis. For instance, cell cycle arrest is an effective and important way of inhibiting cancer proliferation. In one study, terpenoids γ-tocotrienol, mixed isoprenoid, and β-ionone, suppressed the growth of both human and murine tumor cell lines via initiating apoptosis and arrest of cells in the G1 phase in the cell cycle [141]. Soy isoflavones, e.g. genistein, genistin, daidzein and biochanin A, in another study, induced G1/M phase cell cycle arrest in murine (MB49 and MBT2) and human (HT1376, UMUC3, RT4, J82 and TCCSUP) bladder cancer cell lines, evaluated by flow cytometry [142]. Triterpenoid saponins extracted from soybeans, at physiologically relevant doses, could suppress HCT-15 colon cancer cell proliferation through S-phase cell-cycle delay [143]. In another study, starting from the findings that antitumor activities of various wheat cultivars were significantly different, even when the wheat fiber content was equal [144], Qu et al. [145] found that lignans, a group of diphenolic compounds present in the outer layers of grains, were capable of arresting colon cancer SW480 cells at S phase. Resveratrol, a triphenolic stilbene present in grapes and other plants, was used to treat six human cancer cell lines (MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60), and most of the cells were arrested in the S phase of the cell cycle [146]. Another phytochemical of considerable interest is butyric acid [138], which is a short chain fatty acid from the digestion of fibers. Butyric acid was found to induce cell cycle arrest, differentiation and apoptosis of colonic epithelial cells and tumor cells in vitro.
However, there are some traditional tonic and herbal medicine plants, e.g. ginseng, found to exhibit ambiguous effects on cancer patients. Much testing has been done in humans to explore ginseng’s purported antifatigue properties, but this area remains controversial [147]. One \textit{in vitro} study [148], where cell-based reporter gene system was established, indicated that ginseng alone promoted the expression of CYP1A1, one P450 enzyme, which may profoundly influence drug-drug interactions, carcinogen activation and drug detoxification, and the high expression of this enzyme was also observed when ginseng was applied in the presence of TCDD, one tumor promoting dioxin, in sharp contrast to kava, which was a potent antagonist to the gene expression induced by the dioxin. In addition, there are \textasciitilde 13,000 medicinal substances used in China and Japan, and over 100,000 medicinal recipes recorded in the ancient literature [149, 150]. The compositions and concentrations of chemical compounds present in traditional Chinese medicines (TCM) vary with plant species, geographic area, harvest time, and storage [151], making the screening of TCM a very complex and laborious task using conventional drug screening methods.

In summary, the rapidly expanding discovery of phytochemicals with protective and disease-preventing and therapeutic effects has increasingly important impacts on human health and food and pharmaceutical industries. However, important mechanistic questions of biological activities need to be answered before the full beneficial potential can be reached with any well-characterized recipe for either food supplements or clinical treatments. Phytochemicals have been extensively reported to have an effect on at least four of the leading causes of death in the United States: cancer, diabetes, cardiovascular
disease, and hypertension [152]. Toxicity, one of the most important properties of any chemical that is used by humans, also needs to be examined for phytochemicals. This is not only true for toxicity in general; it may also be the working feature for certain phytochemicals to be effective in treating cancers. However, on one hand, *in vivo* toxicity examinations rely on animal experiments, which are expensive, ethically provoking, devoid of interaction mechanism, and most importantly, difficult to extrapolate to human responses. On the other hand, *in vitro* experiments, especially cell culture systems, have been extensively used for cytotoxicity studies. Nevertheless, there are some major problems that a static multiwell plate culture can hardly overcome. As discussed earlier in this review article, cells cultured on 2-D surfaces may be a cause large enough to camouflage the authentic responses *in vivo*, where cells exhibit morphology and interact with each other in a 3-D milieu. In addition, a relatively long-term response to phytochemicals can rarely be measured in a consistent medium due to its metabolic-waste accumulation in the static culture. Another disadvantage is that the growth inhibition is usually judged at an arbitrary point of time, lacking a dynamic long-term monitoring. Although this may be explored with multiple parallel wells for the same culture, variations between individual wells and waste accumulation may interfere with the results. In this context, 3-D fluorescent cell-based assays using microfluidics can provide a better HTS platform for evaluating and discovering phytochemicals with scientific data to support their health claims.
2.8. Conclusions and perspectives

Conventional animal tests for studying the biological effects of drugs and phytochemicals including toxins and herbal compounds are expensive and may be obscured with the actual effects by various other factors. The animal experiment is also time consuming and may impose a barrier for social acceptance. A fast, sensitive and reliable method that can be used to quickly and reliably screen potential biological effects of the vast amount and variety of drug candidates and phytochemicals is thus needed in the development of new health-promoting compounds and cancer-fighting drugs. Conventional 2-D static cell culture systems widely used in current drug discovery campaign have many inherent limitations for proliferation and cytotoxicity studies. The newly developed fluorescent cell-based 3-D culture systems are fast, sensitive, and physiologically relevant, and can be used more effectively to bridge the gap between biochemical assays and animal tests. It can save time and cost in the drug screening process. Furthermore, microfluidic microbioreactor array system, incorporating recent advances in tissue engineering, microfabrication and microfluidics, can be used to culture stem cells and carcinoma cells in 3-D fibrous or microfabricated scaffolds. Such microfluidic systems operated with continuous perfusion can be used for long-term study of drugs in an in vivo like 3-D environment and flow fields. The use of fluorescent cells in the assay also allows for real-time, non-invasive monitoring of cellular responses to drugs and dynamically changing environments. A microfluidic system can also provide on-chip serial dilutions to generate various concentrations and combinations of multiple drugs to be tested simultaneously on a single chip. In addition, it is also possible to culture multiple cell types in different but
interconnected chambers or channels to evaluate cell-cell and cell-environment interactions on a microfluidic chip, providing the biosystem-level drug responses that can only obtained in animal tests so far. Microfluidic 3-D cell-based HTS assays thus can enable the development of *in vitro* models for studying specific diseases. Such *in vitro* models may replace animal models and be used more effectively in the exploration of new drugs including phytochemicals for their therapeutic and health benefits. This could revolutionize the drug discovery process in the near future.

**2.9 Acknowledgements**

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<table>
<thead>
<tr>
<th>Assay type</th>
<th>Mechanism or method</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second messenger</td>
<td>Monitor signal transduction following activation of cell-surface receptors</td>
<td>Using fluorescent molecules that respond to changes in intracellular Ca(^{2+}) concentration, membrane potential, pH, etc. to assay receptor stimulation and ion channel activation [5, 6]</td>
</tr>
<tr>
<td>Reporter gene</td>
<td>Monitor cellular responses at the transcription/translation level</td>
<td>Coexpression of luciferase to catalyze the light-emitting luciferin reaction for detection of protein kinase C inhibitors [7, 8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quantification of G-protein coupled receptor (GPCR) internalization using a GPCR-green fluorescent protein hybrid [9]</td>
</tr>
<tr>
<td>Cell proliferation/cytotoxicity</td>
<td>Monitor the overall cell growth or death in response to external stimuli or stress</td>
<td>Virus-induced cytopathic effects on cell proliferation monitored by following the reduction of tetrazolium salt to formazan quantified by absorbance at 410 nm [10]</td>
</tr>
</tbody>
</table>

Table 2.1. Major types of cell-based assays used in HTS for drug screening.
<table>
<thead>
<tr>
<th>Cell types</th>
<th>Examples</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immortalized cell lines</td>
<td>-Myocardial cell lines for screening adrenergic drugs [13]</td>
<td>-Low cost in growth and maintenance -Homogenous cell population</td>
</tr>
<tr>
<td></td>
<td>-Skeletal muscle lines for testing cholinergic compounds [13]</td>
<td>-Lack in important aspects of native cellular function</td>
</tr>
<tr>
<td></td>
<td>-Nerve cell lines for screening neurotransmitter agonists [13]</td>
<td>-Not representative of normal cells</td>
</tr>
<tr>
<td>Primary cells</td>
<td>Human T cell for screening HIV-1 inhibitors [14]</td>
<td>-Fully differentiated cell types -Close approximation of native function</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Physiological response -Not easily accessible or available for all cell types -Need fresh preparation -Questionable reproducibility</td>
</tr>
<tr>
<td>Human cancer cell lines</td>
<td>NCI60, a panel of 60 human tumor cell lines (NCI60) representing 9 tissue types, for screening potential new anticancer agents [15]</td>
<td>-Easy to use -Cancer specific -Containing mutations that might affect the experimental outcome</td>
</tr>
<tr>
<td>Cancer stem cells</td>
<td>Phase II screening of new drugs in ovarian cancer and malignant melanoma [16]</td>
<td>-Self renewal -Multi-potency -Difficulty in maintaining as pure population in culture</td>
</tr>
<tr>
<td>Mesenchymal stem cells (MSCs)</td>
<td>Human mesenchymal stem cells derived osteoblasts for testing purmorphamine [17]</td>
<td>-Easy to obtain and in high quantity -Safe maintenance and propagation -No ethical issues -Multi-differentiation -Limited differentiation capacity</td>
</tr>
</tbody>
</table>

Table 2.2. Sources and types of cells available for drug screening.
| Embryonic stem cells (ESCs) | Human embryonic stem cell derived cardiomyocytes for electrophysiological drug testing [18] | -High quantity  
-Readily available source of all cell types  
-Capacity to fully differentiated into all cell types  
-Close approximation of native function  
-Growth and maintenance with high cost  
-Difficult to obtain fully differentiated cell types  
-Difficult to achieve purified populations  
-Moral issues |
| Induced pluripotent stem cells (iPSCs) | Mouse ES cell derived neurons for screening glutamate receptor agonists [19] | -Same advantages as ESCs  
-Can be derived from specific diseases  
-Growth and maintenance with high cost  
-Difficult to obtain fully differentiated cell types  
-Difficult to achieve purified populations  
-Difficult to generate iPSCs  
-Low efficiency of generating clones |

Note: NCI 60: The US National Cancer Institute (NCI) 60
<table>
<thead>
<tr>
<th>Specific lineage</th>
<th>Gene cassette (promoter and related reporter gene)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>α-cardiac MHC-G418</td>
<td>[76, 77]</td>
</tr>
<tr>
<td></td>
<td>α-cardiac MHC-LacZ</td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td>α-cardiac MHC-EGFP</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td>MHC-SEAP</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>MLC-2v-EGFP</td>
<td>[81-84]</td>
</tr>
<tr>
<td></td>
<td>MLC-2v-β-galactosidase</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>Cardiac α-actin-GFP, G418</td>
<td>[86, 87]</td>
</tr>
<tr>
<td></td>
<td>Cardiac α-actin-GFP</td>
<td>[88]</td>
</tr>
<tr>
<td></td>
<td>HSVtk/GCV</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>GFP-IRES-PAC</td>
<td>[89]</td>
</tr>
<tr>
<td>Chamber myocardium</td>
<td>Cardiac-specific distant upstream part of the Na+/Ca2+ exchanger-EGFP</td>
<td>[90]</td>
</tr>
<tr>
<td>Neural</td>
<td>Neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A necdin promoter-PAC</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>Neuroepithelial cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sox-1-GFP</td>
<td>[92, 93]</td>
</tr>
<tr>
<td>Neural precursor</td>
<td>Thymidine kinase promoter/nestin second intron-EGFP</td>
<td>[94]</td>
</tr>
<tr>
<td>Dopaminergic neuron</td>
<td>TH-EGFP</td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>TH-GFP</td>
<td>[96]</td>
</tr>
<tr>
<td>Others</td>
<td>Glioma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p53-Luc</td>
<td>[97]</td>
</tr>
<tr>
<td></td>
<td>p63-Luc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p73-Luc</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>Tie-1-EGFP</td>
<td>[98]</td>
</tr>
<tr>
<td>Endothelium</td>
<td>Flk-1-GFP</td>
<td>[99]</td>
</tr>
<tr>
<td>Epithelial</td>
<td>CYP7A1-GFP</td>
<td>[100]</td>
</tr>
<tr>
<td>Melanoblast</td>
<td>Dct-LacZ</td>
<td>[101]</td>
</tr>
<tr>
<td>Renal progenitor</td>
<td>LacZ/T/GFP</td>
<td>[102]</td>
</tr>
<tr>
<td>ESCs (undifferentiated)</td>
<td>OCT4-EGFP</td>
<td>[93, 103]</td>
</tr>
<tr>
<td></td>
<td>Survivin-EGFP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rex-1-GFP</td>
<td>[104]</td>
</tr>
</tbody>
</table>

Note: EGFP enhanced green fluorescent protein, ESC embryonic stem cell, GCV ganciclovir, GFP green fluorescent protein, HSVtk herpes simplex virus thymidine kinase, IRES internal ribosome entry site, Luc luciferase, MHC myosin heavy chain, MLC myosin light chain, PAC puromycin N-acetyltransferase, SEAP secreted alkaline phosphatase, TH tyrosine hydroxylase.

Table 2.3. Some cell-specific promoters and reporter genes used in drug screening.
Figure 2.1. SEM photos of some human cells grown in nonwoven fibrous PET matrices showing 3-D morphologies similar to those found in in vivo tissues. (Reproduced with permission from [28])
Figure 2.2. Decay of luteal cell’s ability in response to LH stimulation in 2-D T-flask and 3-D fibrous bed bioreactor (FBB) cultures. The 2-D T-flask culture lost almost all its response in 19 days, while 3-D FBB cultures still maintained more than 60% of the initial activity. (Reproduced with permission from [32])
Figure 2.3. Schematic illustration of culture fluorescence from cells grown on 2-D surface and in 3-D scaffold in conventional and modified 96-well plates, respectively. Cells in the 3-D culture (well dimension: 3 cm × 3 cm; area: 9 cm²) are concentrated in the scaffold (diameter: 6.2 mm; area: 0.3 cm²) at the center of the well, whereas in the 2-D culture, cells spread uniformly on the bottom surface of the well. Consequently, the noise level in the 3-D culture is reduced by a factor of 30 (9/0.3), and thus greatly increasing the signal to noise ratio. The microscopic picture shows GFP-cells in the PET matrix. (Reproduced with permission from [113])
Figure 2.4. Cytotoxicity effect of drugs assayed with GFP fluorescence in 3-D cell cultures. (A) Fluorescence kinetics of ES-GFP cells cultured with different doses of dexamethasone added one day after inoculation. (B) Effects of 5-fluorouracil (5-FU) and gemcitabine (gem) on HT-29-GFP cells. (Reproduced with permission from [113])
Figure 2.5. A perfusion microfluidic cell culture array for high-throughput cell-based assays. (A) Photograph of the microfluidic cell culture array with concentration gradient generator and $10 \times 10$ microchambers on a $2 \text{ cm} \times 2 \text{ cm}$ device. (B) Concentration gradient across 10 columns generated from the concentration gradient generator at the top of the device. All chambers were initially filled with a red dye. Blue and yellow dyes were then loaded from two separate ports at the top of the gradient generator. This array can be used to test drugs at various dosages. (Reproduced with permission from [119])
Figure 2.6. A microfluidic system for perfusion 3-D cell culture. (A) Schematic drawing of the device composed of top and bottom layers; (B) Design of individual cell culture well and relay well formed by two layers, with cell culture well filled with fibrous PET scaffold; (C) photograph of assembled device at work with each inlet connected to an external tubing through a flexible connector. Food dyes were used to indicate proper fluid segregation for each line of flow; (D) fluorescence microscopic image of live HT-29 cells in the PET fibrous matrix (scale bar indicates 60 μm); (E) Kinetics of 3-D perfusion cultures of HT-29 cells exposed to various 5-FU concentrations. Fluorescence intensity was normalized against the initial reading of each curve, respectively. (Reproduced with permission from [126])
Chapter 3: Literature Review on Three-dimensional Fibrous Scaffolds with Microstructures and Nanotextures for Tissue Engineering

3.1. Introduction

Tissue engineering has emerged as a multidisciplinary field encompassing chemical engineering, material science, chemistry and biology; it empowers the improvement of the health and quality of life by restoring, maintaining, and enhancing tissue and organ functions [1, 2]. The goal of tissue engineering is to construct functional substitutes for tissues and organs that may afford a permanent solution to damaged organs or tissues without relying on supplementary therapies. Cells and scaffolds are the two major components of tissue engineering. Cells isolated during a biopsy typically grow in a biomimetic scaffold under controlled conditions to self-regenerate, remodel, and adapt [3]. Scaffolds, which are used to facilitate cell adhesion, proliferation, extracellular matrix (ECM) production, morphogenesis, and differentiation must mimic in vivo microenvironments and offer chemical and physical cues to regulate cellular functions. In general, 3-D scaffolds can provide a better link between single cells and organs than conventional 2-D cultures because they offer spatial cell organization [4, 5]. 3-D scaffolds can be divided into four major categories: amorphous foam-like scaffolds, gel-like scaffolds, fibrous scaffolds, and scaffolds with controlled geometry and structures. Each type of scaffolds offers its own advantages and disadvantages in mimicking the
organization of native tissue structures. Considerations of scaffold design include shape of the original tissue, mechanical properties, ability to direct cell-matrix and cell-cell interactions, and porous structures for efficient mass transport [3]. Fibrous scaffolds, in particular, are favorable as a replacement for natural scaffolds because of their high porosities (up to 95%), isotropic structures, and homogeneous fiber size and pore distribution [6-8].

3-D fibrous scaffolds can support cell adhesion and proliferation, but generally lack the topographical features needed to resemble native ECM. Natural ECM proteins, such as laminin and collagen, exhibit nano-scale characteristics [9]. Laminin and collagen molecules are structurally bundled together to form fibrils with diameters ranging from 260 nm to 410 nm [9]. These sub-micron fibrils with nano-scale features are important to many cellular functions. Recent research efforts have thus focused on mimicking the nanostructural features of ECM to improve scaffold functionality [3, 10-12]. Successes in directing cell towards certain cellular morphologies, functions, and differentiations have been reported using different types of nanofibers [10]. Furthermore, studies have shown that cells cultured in scaffolds with different surface properties, including surface chemistry, geometry, and topography behaved differently [9, 13-14].

Therefore, in order to simulate in vivo microenvironments, the next generation of tissue scaffolds should incorporate both 3-D microporous structures and nano-scale architectures to enhance cellular functions. This chapter highlights the current state of the art techniques in fabricating 3-D nanoengineered fibrous scaffolds and their effects on cell growth, tissue development and functions. The important effects of microscale and
nanoscale structures and surface topography on cellular behaviors are briefly discussed, followed with the recent development of 3-D scaffolds with both microscale structures and nanoscale features.

3.2. Scaffold Effects on Cellular Behaviors

3.2.1 3-D micro-structural effects

A tissue scaffold should be biocompatible and provide the mechanical and functional support necessary for cell growth and survival. Cells grown in a 3-D scaffold behave differently from those on flat surfaces [5, 15, 16]. The third dimension in the 3-D scaffold provides another direction for cell-cell interactions, cell migration, and cell morphogenesis, which are all important in regulating cell cycle and tissue functions. 3-D scaffolds can support a higher cell density than 2-D cultures [16]. In addition, 3-D scaffolds can provide not only the template for cells to adhere and grow, and the interconnectivity within the 3-D construct allows nutrients and metabolites to be transported in and out of the engineered tissue. 3-D microenvironments and interconnectivity are also important for cellular motility and re-organization, which induce proper differentiation and morphogenesis [17]. The profound effects of 3-D microstructures have been extensively studied, and are summarized in this section. More in-depth reviews on this topic can be found elsewhere [5, 12].

3.2.1.1 Cell morphology, adhesion, and spatial organization

The majority of cells inside a 3-D scaffold attach and spread on the available surface inside the scaffold. However, spatial organization and migration patterns inside the
scaffold are also unique when cells are cultured in 3-D scaffolds. In a fibrous scaffold, cells not only attach to the surface of the scaffold, but also form bridges and aggregates between fibers [15, 16]. Previous studies have revealed that scaffold architecture, including pore structure and size, affects cell attachment, spreading, and spatial organization within the scaffold [18, 19]. When the space or distance between adjacent surfaces is large, cells only attach and stretch on the 2-D surfaces of the 3-D scaffold. However, when adjacent surfaces are nearby, cell bridging between surfaces occurs. For example, MCF-7 breast cancer cells, with an average cell diameter of 6 µm, would bridge between 60 µm wide ridges, but would not bridge across 120 µm wide ridges (see Figure 3.1A) [19]. In contrast, astrocytes, with an average diameter of 30 µm, could bridge across 120 µm wide ridges (Figure 3.1B) [19]. This bridging pattern, as opposed to confined surface attachment, was also observed when hepatocytes were cultured inside a polyethylene terephthalate (PET) fibrous scaffold with large pores, within which cells were found to cluster around the fiber surfaces (Figure. 3.1C). The formation of cell bridges could be due to the mechanical stress transmitted over cell surface receptors, which physically couple the cytoskeleton to the extracellular matrix. Biochemical responses to force-dependent changes in scaffold geometry or molecular mechanics could also influence cellular responses [20].

In addition, 3-D cultures promote cell-cell interactions and agglomerations. In one study, mesenchymal stem cells were able to attach to and spread between thinner collagen fibers situated between knitted polylactic-co-glycolic acid (PLGA) fibers, filling up all the microporous spaces inside the 3-D scaffold (see Figure. 3.1D) [21]. It has also
been reported that cells cultured in 3-D scaffolds are more spherical and usually smaller in size than those cultured on flat surfaces [22]. This phenomenon can be attributed to a reduction of surface spreading and more efficient production and sharing of ECM among cells.

### 3.2.1.2 Cell proliferation, protein production, and differentiation

The distinctive advantages of 3-D cultures over 2-D cultures in cell growth, spatial organization, and migration [23] lead to differences in cell proliferation [24], cellular function [25], gene expression [26], and differentiation [26]. Compared to cells on flat surfaces, cell proliferation in 3-D fibrous scaffolds is slower because fewer cells are directly attached to the fiber surfaces [27]. Differences in spatial organization and distribution also contribute to the difference in cell growth. The high specific surface area offered by 3-D scaffolds allows for long-term cell culturing in vitro. In addition, compared to 2-D cultures, hybridoma and astrocyte cells in 3-D cultures produced more monoclonal antibody [28] and glial cell-derived neurotrophic factor (GDNF) [19], respectively. In another study, chondrocytes produced glycosaminoglycan (GAG) more rapidly when cultured in 3-D scaffolds than in 2-D scaffolds [29].

Compared to 2-D cultures, more cells are present in the G1/G0 phase in 3-D cultures. The 3-D fibrous matrix used in long-term cultures supported a higher cell density with smaller cells than the 2-D scaffold and selectively retained healthy, non-apoptotic cells [28]. Also, distinct cellular interaction patterns and intracellular signaling were observed in 3-D scaffolds [25]. These phenomena could be caused by more efficient
ECM production and sharing between neighboring cells in 3-D scaffolds, which are not feasible in 2-D scaffolds, and the 3-D cellular arrangement inside the scaffolds. The observed difference in the expression of cell adhesion related proteins, such as E-cadherin and vinculin, also demonstrated the difference in functionality of cells cultured in 3-D scaffolds.

The proliferation and differentiation patterns of embryonic stem (ES) cells could also be directed by altering the physical and biochemical properties of 3-D matrices. By culturing mouse ES cell-derived embryoid bodies in various semi-interpenetrating polymer matrices, it was found that endothelial cell differentiation and vascularization were enhanced by the introduction of fibronectin to 3-D collagen scaffolds [30].

3.2.2 Nanostructural effects

In addition to mimicking the 3-D structural microenvironments of native tissues, it is important for the scaffold to incorporate tissue-specific microenvironments in order to properly maintain and regulate cellular behavior and function. In vivo, cells are surrounded by ECM that is characterized by a natural web of hierarchically organized nanofibers. The nanofibers play a vital role in directing cellular behaviors via cell-surface interactions [31]. Cell-surface interactions can be influenced by differences in surface chemistry, mechanical properties, and topography. Cell differentiation can be directed by controlling the mechanical properties of the surface as well as surface topography. Surfaces with different topographies have been fabricated to mimic the in vivo microenvironments and shown to affect cell attachment [32]. ECM provides cells with
both biological and mechanical supports, and serves as a cell-cell interaction mediator in the cell adhesion process. Structurally, native ECM comprises various nanoscale fibers and molecules. The nanoscale nature of ECM generally provides two benefits to cells: a high specific surface area and structural/mechanical support. Nanotopographical surfaces may not necessarily be favorable for all cell types. Different cells respond differently to nanosurfaces [33], and some cells are not beneficially affected by the nanotopography. Although extensive studies have been conducted to investigate the influence of nanosurfaces on cellular behaviors and activities [13, 14, 34, 36, 37], the underlying mechanisms of how nanofibers or nanotopographical surfaces affect cells are not well understood [35]. This section provides a brief review on nanotopographical effects reported in the literature.

3.2.2.1 Cell morphology, migration, and adhesion

The most reported cellular effects of nanofibrous topography are on cellular attachment and spreading [36]. Using osteoblasts as the study model, it was found that the stress fibers formed on the peripheral of the cells changed from dot-like to fiber-like when the cells were cultured on nanotextured surfaces, confirming that the stress fibers were highly activated and more likely to immobilize the cells [38]. The formation of stress fibers is also confirmed by the concentration of vinculin on the cytoplasmic side of the cell-substrate surface, indicating the presence of focal contacts [39]. Furthermore, as the cells spread on the surface, they flatten and stretch their microfilaments and cytoskeleton [40].
Various types of nanofibers have been fabricated and used to increase cell affinity to surfaces [41]. Several findings support the proposition that surface topography affects cellular activities and morphology [42, 43]. Some unique morphologies observed in cells cultured on nanotopographical surfaces are shown in Figure 3.2. When cultured on conventional flat surfaces, cells usually are flatly and irregularly spread out on the surface. However, when cultured on a nanofibrous surface, numerous filopodia were entangled with the tips of the nanosurfaces (see Figure 3.2A) [44]. Cells cultured on nanosurfaces also exhibited a more 3-D morphology (Figure 3.2B) [45].

Cells cultured on nanotextured surfaces tend to make focal contacts with the tips of the nanotextured surface instead of local contacts, in which most of the cell cytoplasm is in contact with the surface. These focal contacts influence the mechanotransduction process inside the cytoplasm and affect cell attachment and cellular activities. Therefore, cells formed a looser and more orderly layer on nanotextured surfaces than on flat surfaces, and variations in surface channel distribution density and size significantly affected cell attachment and morphology [46]. This phenomenon was observed through the difference in the ECM spreading between cells cultured on a 2-D surface and a nanotextured surface (see Figure 3.2C) [47]. Hepatocytes formed large clusters or aggregates on the nanofibers (see Figure 3.2D) and showed increased cell-surface interactions, attachment, and albumin synthesis [48]. In addition, cells were also shown to align in the direction of the surface nanotopography [14]. The difference on cell morphology caused by surface properties may be a result of cell migration. Surface topography may modulate migration through contact guidance, as the responses of cells
to topographical discontinuities could lead to cell polarization, lamellipodial and filopodial extension, actin bundle alignment, and focal adhesion formation preferentially along these surface features [49].

Increased cell attachment and growth can be attributed to protein adsorption. Surface energy increased with an increase in the surface roughness, and proteins would be attracted first to favorably adsorb onto the surface before cells can be physically laid on top of it [41]. The high surface energy on the ridges of rough surfaces could also assist cell proliferation by improving protein adsorption [50-52]. It has been shown that surface roughness and electrostatic interactions between the bone implant and bone can influence cell adhesion and subsequent promotion of bone growth [53]. Furthermore, both the amount of ECM proteins available on the surface and the surface roughness (in terms of anchor size and interfiber distances) affected cell spreading [54].

The orientation and alignment of brain-related cells, including neurons and astrocytes, are necessary for proper cellular communication and signal transduction. As observed in our studies, astrocytes were elongated in certain direction and orientation when cultured on nanostructured surfaces [55]. Neuron-like cells cultured on nanotextured surfaces also exhibited a substantially higher degree of neurite extension [56]. In addition, neurite outgrowth, which usually requires basal proteins, was also demonstrated on top of PLLA (poly-L-lactic acid) nanostructural surfaces [57]. These studies demonstrate that nanofeatured scaffolds have profound effects on cell adhesion and morphology critical to nerve regeneration.
3.2.2.2 Cell proliferation, cell differentiation, and protein production

Changes in cellular attachment and spreading not only dictate the mobility of the cells, but also affect cellular growth, arrangement, and ECM production [58]. When cultured on surfaces with mono-directional nanofibers, human ligament fibroblast (HLF) cells were more sensitive to strain in the direction of their longitudinal axis, i.e., parallel to fiber orientation [58]. In addition, interactions between cells and nanosurfaces led to slower cell growth because of increased cytoskeleton rearrangement and cell immobilization on the surface [59-62]. The interaction of cellular cytoskeleton with the surface can influence signal transduction within the cell, which is mediated by the $\text{Ca}^{2+}$ level in the cytoplasm of the cell [63]. These changes in intracellular signal transduction can lead to changes in cell proliferation and differentiation.

Nanofibrous scaffolds have been shown to favorably support adipogenic, chondrogenic, and osteogenic differentiation in human mesenchymal stem cells [64, 65]. In addition, nanofibrous scaffolds promoted neuronal differentiation in nerve stem cells and murine neural progenitor cells [66, 67]. Also, the expression of various proteins, including ECM proteins, proteoglycans and collagen digestible proteins (CDP), in osteoblast-like cells was found to be affected by nanosurfaces [68].

3.3. Fabrication of 3-D fibrous scaffolds with nano and microstructures

In tissue engineering, it requires a 3-D scaffold with a large surface area and volume to support high cell density and long-term culture, which is also required for most biological studies to observe significant effects [69]. Fibrous scaffolds can be fabricated from
synthetic polymers to mimic the fibrous structure of collagen and gelatin present in native extracellular matrices [70]. The ideal polymeric material should be biocompatible, economical, easy to fabricate, and with appropriate mechanical and biological properties [71]. Among the commonly used polymeric materials for scaffold fabrication are PLGA [19], polylactic acid (PLA) [72], polyhydroxyalkanoates (PHA) [73], polycarpolactone (PCL) [74], polyethylene terephthalate (PET) [75], polysaccharide [76], and polyethylene glycol (PEG) [77]. The direction of tissue scaffold fabrication is leaning towards 3-D scaffolds with nano-textures (resembling extracellular matrices), structural penetration, good mechanical (i.e. strength) and physical properties (i.e. pore size and porosity). Various approaches and methods used to fabricate 3-D fibrous scaffolds and nanotopographical features are discussed in this section.

3.3.1 Methods to fabricate 3-D fibrous scaffolds

Different fabrication techniques can be used to design and optimize distinct fibrous scaffold properties, such as pore size and structure, suitable for culturing the desired types of cells. Most of the scaffolds used in tissue engineering have random pore shapes and sizes that are of the same order as the cell. These pores contain high interconnectivity in order to ensure cell infiltration during seeding, cell interaction, and nutrient/waste transport [78]. Although the amorphous shape of scaffolds could contribute to the overall mechanical performance of the scaffolds, a scaffold with a defined pore size could help thoroughly explore the effect of pore size on cellular functions in the scaffold [79].
Table 3.1 lists methods for fabricating 3-D scaffolds with different structures and microscale pores. Nonwoven fibrous matrices with micron-sized fibers possess isotropic structure with a high porosity (usually more than 90%), good mechanical strength, and good thermal and chemical stability. In particular, needle-punched nonwoven fibrous matrices have a wide range of pore size distribution. These nonwoven fibrous matrices can be made from both biodegradable (e.g., PGA, PLA, and collagen) and non-biodegradable (e.g., PET, polypropylene, and polyethylene) polymers. To fabricate fibrous scaffolds with tightly controlled pore sizes, microembossing [19] and 3-D printing [80] have been developed, but they usually have a relative small porosity of less than 0.8.

Other methods commonly used in fabricating fibrous matrices include fiber bonding and electrospinning. In fiber bonding, individual unbounded fibers are fabricated and bonded to form scaffolds comprising the network of bonded fibers. Fiber bonding can be done with a thermal treatment [81] or treatment with supercritical carbon dioxide [82]. In electrospinning, a voltage is applied to a droplet of polymer solution so that the electrostatic repulsion causes a stream of liquid to erupt from the droplet towards a collecting plate. The polymer’s solvent evaporates in flight, and the polymer forms fibers on the collecting plate. The fiber size is controlled by controlling the voltage and the distance between the needle and the collecting plate. Although the pore structure is usually amorphous, the porosity of electrospun scaffolds can be easily controlled [83]. Due to its practicality and flexibility, electrospinning has also been used to generate nanofibrous materials, which will be further discussed in the next section.
Matrices with spongeous or foam-like structures are also widely used in tissue engineering. They can be produced by phase separation [84], gas foaming [85], solid free fabrication [86], particulate leaching [87], or particulate leaching combined with overrun processes [88]. However, none of these fabrication methods is capable of producing scaffolds with desirable nanoscale features without compromising the 3-D microscale structure.

3.3.2 Methods to fabricate nanostructured scaffolds

Table 3.2 lists methods available for fabricating nanotextured surfaces [89]. Photolithography and electron beam lithography (EBL) can create surface nanotopographies with well-ordered and geometrically precise patterns. However, they require expensive equipment and a high level of expertise, and are difficult to scale up. Polymer demixing, colloidal lithography and chemical etching provide inexpensive and scalable methods for creating nanoscale topographies. Although polymer demixing and colloidal lithography can generate nanopits and nanocolumns, they cannot produce specific feature geometries. In general, all aforementioned methods can only introduce nanostructured surface on a flat, planar surface.

Nanofibers can recreate an environment that mimics the natural ECM around cells. They can be generated using various methods, including molecular self-assembly, phase separation, and electrospinning. Molecular self-assembly can produce highly ordered nanofibrous scaffolds. However, its applications are limited to molecules that can be engineered for self-assembly. Phase separation can be used to create 3-D scaffolds
with fibers in the sub-micron range. However, this method can only produce randomly distributed fibers. Electrospinning can generate fibers, from nanometer to micrometer, with tunable properties including controllable pore size, fiber size, fiber stiffness and matrix turnover [10]. It can also create aligned nanofibers on a surface, forming a simple, ordered nanotopography.

Although electrospinning has been widely used to generate nanofibrous materials with the average fiber diameter of less than 0.5 μm [90], the electrospun nanofibrous materials have only pseudo-3-D structures with a relatively low porosity and only nanosized pores. The 3-D nanofibrous structures generated by electrospinning are 3-D relative to the size of the nanofibers, and the pores in these nanofibers are on a scale similar to that of the fibers. With a typical cell size of 10 μm, these nanofibrous substrates are 2-D surfaces to cells. Even though they do have interconnected pores, cell infiltration inside nanofibrous scaffolds is limited by the small pore size and consequently, cells can only grow in a 2-D, rather than 3-D, pattern. For instance, chondrocytes cultured in nonwoven PLLA nanofibers either wrapped around and encapsulated the nanofibers or were suspended among fibers, forming globular-shaped cells [91]. A similar phenomenon was also observed using the Ultra-Web™ Synthetic Surface (Corning) [67]. Furthermore, studies on the differentiation of human mesenchymal stem cells cultured in nanofibrous scaffolds showed that nanofibers deficient in 3-D structures could not mimic in vivo microenvironments [92].
3.3.3 Introduction of nanotopographies into 3-D fibrous scaffolds

The ideal tissue scaffolds resembling in vivo conditions should have both 3-D microstructures and nanotextured surfaces. One approach for fabricating such scaffolds is to use 3-D microfibrous scaffolds as the starting material and modify the surface of the microfibers by chemical etching or deposition of nanoparticles or nanofibers to create nanofeatures.

3.3.3.1 Chemical Etching

Chemical etching can create nanotopographies on the surface of 3-D fibrous scaffolds by soaking the scaffolds in etchants such as hydrofluoric acid (HF) [63] and sodium hydroxide (NaOH) [7]. As the material is etched away, the nanoroughness of surface is enhanced with nanoscale pits and protrusions. For example, PLGA and polyether urethane (PU) membranes with nanofeatures (50~100 nm) were obtained by soaking the films in 1N NaOH for 1 h. The viability and function of cells cultured on these surfaces increased with decreased feature size, confirming that nanotopographies are beneficial to cells [93]. In addition, NaOH was used to etch a highly porous and interconnected PET scaffold [8]. While a short treatment of ~1 h with NaOH increased the hydrophilicity of the fiber surfaces, longer treatment caused hydrolysis, and the etching process generated a nanostructured surface (see Figure 3.3B). In this approach, the degree of nanoroughness present on the surface of the microfibers is dependent on the crystalinity of the microfiber and the hydrolysis time, which also affects the resulting porosity and pore size of the treated PET scaffolds. The increased nano-roughness, surface area, and space in the
treated scaffolds also increased cell growth and improved cellular functions. However, this method can only fabricate submicron-scale features and cannot achieve uniform surface modification.

### 3.3.3.2 Polymer deposition

The sol-gel process is a low cost and versatile method for preparing nanofeatured 3-D scaffolds [94]. For instance, nonwoven PET scaffolds were used as a 3-D substrate, upon which polypyrrole nanofibers were formed using a sol-gel method. This method produced a thin layer of nanofibers uniformly coated on the surface of the PET fibers without significantly changing the original scaffold pore size or porosity (see Figure 3.3C). Another method to control the rate of polymer deposition is through dilute polymerization [95]. Similarly, a thin layer of aligned polyaniline nanofibers fabricated using a dilute polymerization method was coated on PET fibers (Figure 3.3D). Cells cultured on these nanofibers-coated PET fibers showed different behaviors and morphologies from those on the pristine PET fibers [55].

### 3.3.3.3 Bacterial cellulose deposition

A simple method to introduce nanotopographies into 3-D fibrous scaffolds was developed by directly depositing bacterial cellulose nanofibrils onto natural fibers during bacterial cellulose fermentation [96]. The mechanical properties of the modified fibers with cellulose nanofibrils (see Figure 3.3E) were not affected, and may be used as scaffolds in tissue engineering. However, no tissue engineering research has been conducted with this scaffold material yet.
3.3.3.4 Nanoparticle deposition

Nanoparticles with diameters less than 100 nm and a high surface to volume ratio can be used to produce nanofeatured scaffolds. Polyelectrolyte complex fibrous scaffolds were developed using polygalacturonic acid and chitosan via freeze drying. The resulting scaffolds had highly interconnected pores of 5~20 µm in diameter, and each individual fiber had a nanogranular structure, which promoted cell adhesion and cell proliferation [97]. Freeze drying technology can be used to control porosity and pore size. However, it is limited in its capacity to yield a variety of pore structures.

Hydroxyapatite (HA), a synthetic calcium phosphate, has a similar structure to bone mineral. HA nanoparticles can reinforce composite materials for tissue engineering and can also be used as bimolecular carriers. A novel 3-D PCL/HA composite scaffold with a porosity of 73% and pore sizes of 500 µm was developed for bone regeneration using a layer-manufacturing process [98]. A paste extruding deposition process was also developed to evenly disperse HA nanoparticles with diameters ranging from 50 to 100 nm on the surface of PCL micro-fibers (see Figure. 3.3F), and the PCL/HA scaffolds were successfully used for in vivo bone regeneration [99].

Although the merits of using nano/microscale composite structures to promote cell proliferation are established, controlling the shape of composite structures cannot be achieved using the conventional method. Solid free-form (SFF) fabrication permits shape control. Of the SFE technologies, micro-stereolithography (MSTL) has the highest resolution, allowing fabricating structures with a resolution of 10 µm [100]. During
MSTL, an ultraviolet (UV) laser irradiates the free surface of a UV-curable liquid photopolymer, causing it to solidify to form a nano/microscale composite scaffold exhibiting nanotopographies on the scaffolds surface due to the incorporation of HA nanopowder. The resulting pores, with diameters ranging from 330-360 nm, are well connected and have regular shapes. Cell proliferation and cell attachment are increased compared to the control because of the increase in the nanoroughness [100]. In addition to HA nanoparticles, other nanoparticles have also been used to modify the surface of scaffolds. 3-D nanoengineered fibrous scaffolds were also developed by incorporating tripolyphosphate (TPP) into PLGA fibrous scaffolds using an emulsion followed by melt-spinning. The TPP nanoparticles, roughly 100 nm in diameter, solved the acidic degradation problems present in PGLA scaffolds [101].

Although, 3-D fibrous scaffolds incorporated with nanoparticles have great potential in tissue engineering, it is necessary to understand the secondary effects and cytotoxicity of nanoparticles once they enter the body. The high surface to volume ratio of nanoparticles makes them highly reactive with surrounding tissues in vivo and their nanosize makes them more likely to penetrate cell membranes in the lungs, skin, and intestinal tract [102].

3.3.3.5 Composites of nanofibers and microfibers

Electrospinning can be used to fabricate nanofibers and microfibers. It can also be used with pre-fabricated scaffolds to generate composites of nanofibers and microfibers. Nanoengineered 3-D fibrous scaffolds were fabricated by impregnating the starch/PCL
microfiber meshes with electrospun starch/PCL nanofibers (see Figure 3.4A) [103]. The microfibers served as the structural support, whereas the nanofibers filling the large pores served as the networking anchors for the cells. A similar construct with electrospun type I collagen nanofibers and starch/PCL microfibers in the same structure was fabricated using a two-step process. In this process, the starch/PCL fibrous matrix was first fabricated using a wet spinning method, and collagen nanofibers were then electrospun and deposited on the matrix, followed with crosslinking with saturated glutaraldehyde vapor at room temperature for 48 h. Cells cultured in this scaffold showed increased metabolic activity and growth rate [104]. In addition, a multilayer nano/microfiber scaffold was produced by depositing multiple layers of electrospun PCL nanofibers on top of microfibrous scaffolds (see Figure 3.4B). The presence of nanofibers did not affect cell seeding in the microfiber scaffold; however, a difference in cell spreading on the surface of the scaffold was observed.

The aforementioned scaffolds with uneven distribution of nanofibers and microfibers are not ideal for tissue engineering as cell migration is limited by the nanofibrous matrices with only nanoscale pores. It is thus desirable to create “micro-voids” in nanofibrous scaffolds to enhance cell migration [105]. Another way to overcome this problem is to electrospin nanofibers onto single microfibers (see Figure 3.5A). Electrospun nanofibers can be homogeneously deposited onto individual microfibers (Figure 3.5B) and then the nanofiber-coated microfibers can be maded into scaffolds with desirable shapes and tunable porosity (see Figure 3.5C). Cellular
infiltration and spreading along the nanofibers-coated microfibers were observed in the scaffolds, which also maintained their surface and structural properties [106].

3.3.4 Introduction of 3-D structures into nanofibrous scaffolds

In contrast to the previous approach of adding nanoscale properties to a 3-D scaffold, various approaches to develop 3-D structures in nanofibrous scaffolds have also been attempted and are discussed below.

3.3.4.1 Post-processing of electrospun nanofibers

Nanofibrous scaffolds produced by electrospinning exhibit similar dimensions to collagen fibrils. However, cells cannot infiltrate into the nanofibrous meshes and generally sit on top of the electrospun fibers. The pore size of these nanofibrous scaffolds can be modified and increased through further processing with UV irradiation or assembling.

3.3.4.1.1 UV irradiation

Although UV irradiation is usually ineffective for scaffold sterilization [107], it can degrade nanotextured materials and thus could be used to fabricate microporous scaffolds from electrospun nanofibers [108]. By masking the nanofibers with porous mesh and subjecting them to UV irradiation, the porosity and pore size of the scaffolds can be controlled by controlling the pore size of the porous mask. Degrading nanofibers using UV is a promising tool for producing tissue-specific microenvironments to regulate cellular behaviors. However, there are some issues about the lack of interconnectivity of
the micro-pores and impaired structural or mechanical integrity and potential cytotoxicity caused by the degradation of and sintering in the scaffold induced by UV irradiation [109].

3.3.4.1.2 Assembly of nanofibers

Electrospun nanofibrous scaffolds can provide a nanoscale niche for cell arrangement but are limited by their low volume capacity. The assembly of nanofibers into 3-D structures can be used to overcome this limitation. For instance, a 3-D construct was produced by stacking multiple layers of electrospun nanofibrous membranes seeded with human bone marrow-derived mesenchymal stem cells [110]. The multilayered construct met the need for a 3-D construct with a larger volume containing adequate cells for the \textit{in vivo} implantation. However, the 3-D nanostructured scaffolds generated from the electrospun membranes may be too soft to be used as tissue scaffolds in bone regeneration.

Nanofibers can also be assembled by rolling into 3-D tubular scaffolds [111] or using the layer-by-layer method to form 3-D structures that can support uniform cell distribution in the multilayered nanofibrous scaffolds [112]. Polymer blends are widely used in this approach. A tubular scaffold for engineering small diameter blood vessels was fabricated by electrospinning polymer solution blends of pNSR32 (recombinant spider silk protein), PCL, and gelatin [111]. Also, PCL/collagen nanofibers were used to fabricate multilayered 3-D scaffolds, which showed a layered structure with uniform distribution [112].

3.3.4.2 Direct introduction of 3-D structures into nanofibrous scaffolds
3.3.4.2.1 Electrospinning with leaching

Electrospinning with particulate leaching can be used to produce nanofibrous 3-D scaffolds [113]. When electrospinning a salt-containing polyelectrolyte, the pore size of the resulting scaffold can be tuned by controlling the particle size of the salts. The nanofibrous polymer also contributes to the roughness of the scaffolds. In this system, although the porosity of the scaffolds could ensure appropriate mass transfer for nutrient transport and waste removal, the interconnectivity of the micropores has to be controlled to ensure cell infiltration inside the scaffolds.

Co-electrospinning of two different polymers followed by leaching of a polymer can also be used to produce a nanostructured scaffold with large pores [114]. The pores of the scaffolds would, therefore, depend on the ratio between the nanofibers and the compactness of the nanofibers. Although this method can generate hybrid scaffolds with amorphous pore structures, the thickness of the scaffolds has to be properly controlled to ensure appropriate mechanical strength for structural support of the scaffold.

3.3.4.2.2 Cryogenic electrospinning

Cryogenic electrospinning can control the pore size of electrospun scaffolds, which is impossible to do with conventional electrospinning processes [115]. By lowering the temperature of the collection plate during electrospinning, the pore size of the electrospun scaffold can be increased. In this approach, the electrospun fibers were collected with the ice crystals formed within the fibrous matrix on the plate because of the higher humidity environment caused by the lower temperature. Macro-sized pores or voids could be
formed in the nanofibrous network after the drying [115]. However, the size of the ice crystal, which was critical to the control of the scaffold pore size, could not be controlled to produce scaffolds with homogeneous structures.

3.3.4.2.3 Wet electrospinning

Wet spinning or electrospinning with a liquid reservoir, such as a coagulation bath, to collect the nanofibers before being drawn into a rotating roller can be used to produce continuous and bundled yarns [116]. A 3-D nanofibrous scaffold with large pores and a high porosity was fabricated using silk fibroin (SF) via wet electrospinning (Figure 3.6A). The SF nanofibers were dropped directly into a coagulation bath containing methanol below the spinneret, and a SF nanofiber foam was obtained after freeze-drying (see Figure 3.6B), which can be molded into foams with desirable size and shape (see Figure 3.6C). Cells in the 3-D SF nanofibrous foam proliferated well [117]. However, scaffolds with desirable pore sizes are difficult to fabricate using this method.

3.3.4.2.4 Phase separation

Phase separation with gelatinization can generate microporous scaffolds with nanotextures [118]. In this system, the nanotextures were generated as the result of phase separation, while larger pores were caused by gelatinization. This approach yielded a scaffold with surface roughness that improved protein adsorption and cell attachment. Thermally induced phase separation (TIPS) has been widely used to fabricate porous composite structures. By changing the temperature, a homogeneous polymer solution can be separated into a polymer-rich phase and a polymer-lean phase. After the removal of
solvent using freeze-drying or vacuum, a 3-D porous scaffold can be formed. A variety of
3-D nanofibrous scaffolds have been fabricated using the phase separation process. For
example, a PLLA solution was thermally induced to phase separation and then self-
assembled into a 3-D interconnected fibrous network with a fiber diameter ranging from
50 to 500 nm and a high porosity up to 98.5%.

One drawback of the scaffolds generated by phase separation is the lack of
interconnected macropores. To overcome this problem, TIPS combined with sugar sphere
template leaching were developed to produce a nano-fibrous PLLA scaffold with
interconnected spherical macropores (Figure 3.7A and 3.7B) [119]. The macropore shape
and size can be tuned by controlling the sugar spheres. In addition, phase separation with
salt particle leaching was also developed to produce nanofibrous scaffolds with tunable
pore size and porosity (Figure 3.7C and 3.7D). The nanofibrous scaffolds consisting of
interconnected microscale pores could mimic the physical architecture and chemical
composition of natural ECM, which significantly promoted cell differentiation and
neurites outgrowth [120]. However, phase separation is limited to a few polymers and
cannot produce long and continuous fibers with control over fiber orientation.

3.3.5 Carbon nanotubes

Carbon nanotubes (CNTs) have attracted great attention due to their mechanical strength
and excellent thermal, magnetic and electrical properties. Their dimensions, diameter and
length, are analogous to major components in natural ECM, and their mechanical
properties are similar to those of the ECM proteins [121]. These unique characteristics
have found them numerous applications in biomedical materials, biosensors, drug delivery, and tissue engineering.

Thin-film networks of carbon nanotubes with 3-D sieve architecture consisting of aligned CNTs with cavity sizes ranging from 5 to 65 µm (see Figure 3.8A) were fabricated and used to culture human fibroblasts, which adsorbed and spread on the surface of the carpet-like carbon nanotubes [122]. Microchannel porous scaffolds composed of multiwall carbon nanotubes (89 wt %) and a small portion of chitosan (see Figure 3.8B) increased the differentiation of mouse myoblasts (C2C12) to osteoblastic cells [123]. However, the lack of interconnectivity of these nanostructured 3-D materials hindered oxygen supply, nutrient transfer, and cell migration. To overcome this limitation, a variety of nanoengineered 3-D polymer materials have been developed by attaching CNTs, using electrophoretic deposition (EPD), to porous scaffolds, such as polypropylene fumarate (PPF) [124], collagen [44, 125], and polyurethane foams [126]. In our recent work, CNTs were deposited on the surface of nonwoven PET matrices (see Figure 3.8C) to provide nano features, resulting in increased cell proliferation and differentiation. Although CNTs can be used to improve the performance of 3-D scaffolds, their applications in tissue engineering can be hampered by their potential adverse effects on human life and the environments [127, 128].

3.4. Conclusions and future perspectives

The recent advancements in scaffold fabrication methods have provided biologists and tissue engineers the scaffolds needed for better understanding of cell-scaffold
interactions. Scaffolds with nanoscale features offer great promise for enhancing the biological performance of cell and tissue cultures and will play an increasingly important role in tissue engineering [3]. Several fabrication methods have been developed to produce 3-D scaffolds with micro-porous structures and tunable ‘surface’ properties with nanostructures similar to those present in natural extracellular matrices. These fabrication methods take different approaches, including bulk processing, incorporation of nanostructures onto the surface of microstructures, and incorporation of microstructures into nanoscaffolds. Each of them has brought us one step closer to creating an ideal scaffold for tissue engineering applications but also has its own limitations. The major challenge is how to scale up the fabrication processes capable of producing macro-scale scaffolds with high precision, control and homogeneity in micro- and nano-structures suitable for various clinical applications. Also, in-depth understanding and control of cellular functioning through topographical and microstructural modifications are critical to the design and development of a functional synthetic tissue scaffold.

In addition, the next-generation scaffolds also need to be functionalized with growth factors and other biomolecules that can accelerate tissue regeneration and enhance regenerated tissue function through temporal and spatial control of the release of these biomolecules [129]. How to induce vascularization or incorporate blood-vessel like conduits for nutrients supply and metabolites transport in a large-scale scaffold remains a challenge for tissue engineering of an organ such as liver [130]. Finally, it may be necessary to integrate scaffolds in a bioreactor that can provide a dynamic environment with control of physical parameters (e.g., pH, temperature, etc.) and exert mechanical
forces such as shear stress and hydrodynamic pressure to guide cell growth, differentiation and functional assembly [131], which is important for in vitro applications, including mass production of stem cells for regenerated medicine and drug screening [132]. Overall, it will require a collaborative effort from biologists, chemists, material scientists, engineers, and clinical surgeons to design and assemble a functional scaffold that is clinically and economically feasible for the intended applications.

3.5 Acknowledgements

R.Z and R.N contributed equally to the majority of the work. S.T.Y, R.N, R.Z and N.L wrote the manuscript. K.Y contributed to part of the work.
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<th>Matrix structure</th>
<th>Porosity</th>
<th>Reference</th>
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<td>[78]</td>
</tr>
<tr>
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<td>0.81</td>
<td>[79]</td>
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<td>[82]</td>
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<td>Solid free fabrication</td>
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<tr>
<td>Particulate leaching</td>
<td>Spongeous</td>
<td>0.87</td>
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Table 3.1. Fabrication methods for various types of 3-D scaffolds.
<table>
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<th>Fabrication method</th>
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<tr>
<td>Electrospinning</td>
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</tr>
<tr>
<td>Colloidal lithography</td>
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<td>Uncontrollable geometries</td>
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<td>Micro stereolithography</td>
<td>330-360 nm</td>
<td>Controllable geometries and adequate pore size [100]</td>
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<td>E-beam lithography</td>
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<td>Precise geometry and patterns can be created</td>
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<tr>
<td>Polymer demixing</td>
<td>&gt; 13 nm</td>
<td>Limited sample features can be created</td>
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<tr>
<td>Phase separation</td>
<td>Pore sizes &gt; 1 nm</td>
<td>Uncontrollable patterns</td>
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<tr>
<td>Self assembly</td>
<td>Tailored by molecular design</td>
<td>Limited to only molecules that will undergo self-assembly</td>
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<tr>
<td>Chemical etching</td>
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<td>Uncontrollable geometries</td>
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<tr>
<td>Nanoporous membrane</td>
<td>Pore sizes &gt; 1 nm</td>
<td>Lack of mechanical and physical properties</td>
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Note: conventional photolithography can only create features at the micrometer scale (> 0.5 µm).

Table 3.2. Nanoscale fabrication methods and their feature sizes. (Reproduced from [89])
Figure 3.1. Scanning electron micrographs of cells cultured in 3-D scaffolds. (A) MCF-7 breast cancer cells covering the surface of and forming aggregates in a PLGA scaffold; (B) Astrocytes bridging in a PLGA scaffold; (C) Hepatocytes bridging and forming aggregates in a PET scaffold; (D) Mesenchymal stem cells growing on collagen fibers in a knitted PLGA-collagen scaffold. (A and B are reproduced with permission from [19], and D from [21])
Figure 3.2. Scanning electron micrographs of cells cultured on nanofibrous surfaces. (A) Osteosarcoma cells spread on the carbon nanotubes (CNTs)-coated surface with many filopodia extended and entangled with the CNTs; (B) Rat neural stem cells cultured on polyether sulfone nanofibers with a “stand-up” posture instead of spreading on the surface; (C) Keratinocytes on electrospun silk fibroin nanofibers; (D) Spheroid-like hepatocytes spread on galactose-grafted nanofibers showing that the aggregates engulfed the functional nanofibers. (Reproduced with permission from [44], [13], [47], and [48], respectively)
Figure 3.3. 3-D microfibrous scaffolds with nanofeatures on their surfaces created by various methods. (A) Prestine PET fibers with smooth surface; (B) PET fibers with rough surface after etching with NaOH; (C) PET fibers coated with polypyrrole nanofibers via sol-gel polymerization; (D) PET fibers coated with polyaniline nanofibers via sol-gel polymerization; (E) Bacterial cellulose nanofibers attached on sisal fibers after fermentation; (F) Composite PCL scaffold with HA nanoparticles obtained by extrusion of the mixture of nano-HA and PCL and stacked in layers to form the 3-D scaffold. (B, C, and D are reproduced with permission from [55], E and F are reproduced with permission from [96] and [99], respectively)
Figure 3.4. Composites of nanofibers and microfibers produced by electrospinning of nanofibers to existing microfibers. (A) Nanofibers impregnated in a microfibrous scaffold; (B) Cross-sections of layered scaffolds generated by sequential electrospinning of nanofibers on top of microfibers. (Reproduced with permission from [103] and [105], respectively)
Figure 3.5. Electrospinning of PCL nanofibers onto a single PLA microfiber. (A) Schematic diagram illustrating the process; (B) A single PLA microfiber with electrospun PCL nanofibers coated on its surface; (C) 3-D scaffold made of PLA microfibers coated with PCL nanofibers. (Reproduced with permission from [106])
Figure 3.6. Wet electrospinning of silk fibroin (SF) nanofibers. (A) Schematic diagram illustrating the process; (B) Nanofibrous scaffold from the wet spinning (×1000); (C) Various foam shapes made from the electrospun fibrous scaffolds. (Reproduced with permission from [117])
Figure 3.7. PLLA nanofibrous scaffolds produced by phase separation followed with particles bleaching. (A) and (B) are SEM images of the scaffolds from sugar spheres bleaching and (C) and (D) are from salt particles bleaching. (A and B are reproduced with permission from [119] and C and D from [120])
Figure 3.8. 3-D scaffolds made from carbon nanotubes (CNTs). (A) Vertically aligned CNTs on surface with micron-sized cavity; (B) CNTs/chitosan scaffold with a chamber-like structure; insert shows the monolithic scaffold prepared by the ice segregation induced self-assembly (ISISA) process; (C) PET fibers coated with CNTs via deposition. (A and B are reproduced with permission from [122] and [123], respectively)
Chapter 4: Microwell Bioreactor System for Cell-Based High Throughput Proliferation and Cytotoxicity Assays

Abstract

3-D cell-based high throughput proliferation and cytotoxicity assays are increasingly used in drug screening and bioprocess development. However, online monitoring of cell proliferation, pH, and dissolved oxygen (DO) has been a challenge in 3-D cell-based assays. In this work, a 40-microwell bioreactor (40-MBR) system was developed from a 384-well plate (384-MWP) for real-time, noninvasive monitoring of pH, DO, and cell proliferation in 3-D microenvironments using a fluorescence plate reader. 3-D cultures of Chinese Hamster Ovary (CHO) and MCF-07 breast cancer cells in 40-MBR confirmed that the bioreactor system was capable of simultaneously monitoring DO and cell proliferation based on culture fluorescence intensity. In addition, online monitoring of pH was also achieved by measuring the absorbance of phenol red. Furthermore, cytotoxicity studies of sodium butyrate on CHO cells demonstrated that the 3-D cultures in 40-MBR gave more reliable and highly reproducible growth kinetic data with reduced errors compared to 384-MWP. Furthermore, the dosage effects of two new anticancer drug candidates, 5,7-dihydroxy-2-(4-hydroxyphenyl)-8-[(E)-2-phenylethenyl]-3,4-dihydro-2H-1-benzopyran-4-one (DH-8P-DB) and 5, 7-dihydroxy-2-(4-hydroxyphenyl)-6-[(E)-2-
phenylethenyl]-3,4-dihydro-2H-1-benzopyran-4-one (DH-6P-DB), on HT-29 colon cancer cells were assessed in the 40-MBR, which indicated that DH-6P-DB would be a better drug candidate in treating colon cancer than DH-8P-DB. These studies demonstrated that 40-MBR could serve as a platform to evaluate potential cancer drugs in the early-stage drug discovery.

4.1. Introduction

The rapid progress of drug discovery has spurred the development of rapid, selective and reliable scale-down and high-throughput screening (HTS) cell culture platforms [1-3]. Current trends are directing towards developing high throughput cell-based platforms with high biological relevance for drug target validation and ADMET (absorption, distribution, metabolism, elimination and toxicity) [4]. Three-dimensional (3-D) cultures can better recapitulate in vivo cellular responses to drug treatment. A microbioreactor suitable for 3-D cell cultures is thus a superior platform for drug screening. Biotek has developed 3D inserts compatible with commercially available multi-well plates, allowing 3-D cell cultures and monitoring of cell growth using a light microscope. However, online quantification of cell proliferation remains a challenge for 3-D cell cultures.

Cell density and cell viability are two major parameters for evaluating cell culture performance in high throughput screening for drug discovery. Conventional methods for determining cell density include hemocytometer counting, antigen detection, and DNA synthesis measurement, while Trypan blue exclusion, neutral red inclusion, and lactate dehydrogenase are broadly used to determine cell viability [4]. However, these conventional approaches are invasive and can only provide end-point data. New
approaches to achieve online monitoring with no sampling disruption to obtain dynamic data are preferred. Recent advances in fluorescence protein technology have made non-invasive online monitoring achievable [5]. Hunt et al. demonstrated the feasibility of dynamic assessment of cell growth in suspension using stable GFP-expressing Chinese Hamster Ovary (CHO) cells [6]. Additionally, stable red-fluorescent tumor cell lines were used to study growth enhancement of breast cancer cells co-cultured with human bone marrow stromal cells in 3-D Matrigel [7]. In these previous studies, an auto fluorescence-based method was utilized to achieve on-line monitoring of cell proliferation [8-10]. Although cells expressing fluorescence proteins enabled real-time measurement of cell growth using a commercial plate reader in HTS [5-8], during cell death phase the extended GFP stability resulted in slower total fluorescence decline than the decrease of the number of viable cells [11]. In addition, Zhang et al. reported that changes in culture medium caused by cell activities and cell debris also played an important role in the discrepancy between the total fluorescence and the total number of viable cells [8, 9].

On the other hand, cell growth and viability are highly dependent on cell culture environments such as dissolved oxygen (DO) and pH. Online measurements of DO and pH during cell cultures can offer non-invasive methods for monitoring cellular activities [12, 13]. DO and pH can be optically determined with no disruption based on optical methods [14-18]. Microbioreactors for mammalian cell culture equipped with non-invasive optical sensors for pH and DO monitoring have been developed [19-22]. Furthermore, a 24-microbioreactor system integrated with pH and DO sensors developed
by Pall Corporation is commercially available. However, simultaneously online monitoring of pH, DO, and cell proliferation for 3-D cell cultures has rarely been reported. Therefore, there is a great need to develop a platform capable of online quantifying DO, pH and cell proliferation for 3-D cell cultures.

In order to achieve online monitoring of cell proliferation, pH and DO, we designed and developed a 40-microwell bioreactor plate (40-MBR) with pH and DO online detection capability. A simple method based on the platform was developed for monitoring cell proliferation, which was fluorometrically quantified in a commercial multi-well plate reader, allowing high-throughput and time-series data acquisition with significantly improved data quality. 3-D cultures of CHO cells and MCF-07 breast cancer cells in the platform were used to evaluate its potential application as a HTS platform capable of online monitoring pH, DO and cell proliferation. EGFP-expressing HT-29 colon cancer cells and CHO cells were then used to demonstrate the potential application of the new platform for 3-D cell-based assays in HTS for drug discovery.

4.2. Materials and methods

4.2.1 EGFP Cell lines and media

Human colon cancer HT-29 (ATCC: HTB-38) and breast cancer MCF-07 cells 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). CHO cells (ATCC CRL-11398) were cultured in DMEM/F12 (Gibco) (1:1) supplemented with 5% FBS. These cultures were incubated in a humidified incubator at 37 °C with 5% CO₂.
Stable cell lines expressing enhanced green fluorescence protein (EGFP) were obtained by the transfection with PEGFP-N3 plasmids (Clontech) using Lipofectamine 2000 (Invitrogen). The expression of EGFP was under the control of human cytomegalovirus (CMV) promoter, which is a strong constitutive promoter and can control the fluorescence protein expression independent of environmental effects. After an initial 10-day selection using geneticin (G-418, Gibco), fluorescence protein expressing colonies were selected using a fluorescence microscope. Then, cells were subcultured in the absence of the selective pressure every 5 days for 20 passages. The stability of the transfected cell line was verified (>97%) using FACS Calibur (B-D Biosciences).

4.2.2 Design and fabrication of 40-MBR

The 40-MBR system was fabricated by modifying a commercial 384-well plate (BD Optilux TM Black/clear bottom) using a computer numerical controlled (CNC) machine (Sherline 2010). There were 40 microbioreactors on the modified 384-well plate (Figure 4.1A). Each microbioreactor (Figure 4.1B) with a dimension of 10.5 mm×10.5 mm consisted of a center well and eight surrounding wells with their original walls removed. Each microbioreactor had a working volume (liquid) of 1 mL. It was sterilized in 70% ethanol for 2 h and dried overnight at 70 °C. For 3-D cell culture, a sterile polyethylene terephthalate (PET) (fiber diameter: 20 μm; porosity: 0.85; pore size: 45 μm) fibrous disk (diameter: 4 mm; thickness: 1 mm) pre-seeded with cells was placed in the center well of each microbioreactor (Figure 4.1B).
An oxygen sensitive film was used to achieve online monitoring of dissolved oxygen (DO). The film was prepared by dissolving 9 mg of oxygen-sensitive fluorescent dye Ru(dpp)$_3^{2+}$ dichloride (Alfa Aesar) in 100 µL of ethanol and then mixed with 11 g of polydimethylsiloxane (PDMS, Dow Corning) (final concentration: 0.82 mg Ru(dpp)$_3^{2+}$Cl$_2$/g PDMS). The solution was casted onto a glass plate to fabricate a membrane with a controlled thickness using a GARDCO adjustable micrometer film applicator (Paul N. Gardner Co., Pompano Beach, FL). After drying at 90 °C for 1 h in dark, the dried membrane was carefully peeled off from the glass plate and its thickness was measured using a Mitutoyo electronic indicator (Model 543-252B, Mitutoyo America Corp., Aurora, IL) with an accuracy of 0.5 µm. Finally, the membrane with a thickness of 60 µm was punched into round-shaped disks with a diameter of 4 mm. Each membrane disk was fitted into a side well of each microbioreactor for DO sensing by measuring the fluorescence at the excitation wavelength of 485 nm and the emission wavelength of 612 nm (Figure 4.1C). Prior to use, the films were soaked in PBS and then sterilized in an autoclave at 121 °C for 30 min. The concentration of the oxygen was inversely proportional to the phase shift, which can be described by the Stern–Volmer equation ($I_0/I = 1+K_{sv}[O_2]$) [22]. In this equation, $I_0$ is the fluorescence intensity without oxygen, while $I$ is the fluorescence intensity when the concentration of oxygen is $[O_2]$. $K_{sv}$ is a constant and can be calculated through the linear relationship between $I_0 / I$ and oxygen concentration.

The absorbance of phenol red at 560 nm was used to achieve online monitoring of pH. Phenol red, as a reversible indicator, exists in two states: HIn and In$^-$, which are yellow and red, respectively [14]. A linear correlation exists between the pH ranging
from 6.4 to 8.0 and absorbance at 560 nm. By measuring the absorbance, pH can be calculated from the linear correlation.

4.2.3 3-D cell cultures

The PET scaffolds were thoroughly rinsed with DI water, soaked in PBS and then sterilized in an autoclave at 121 °C for 30 min. Before use in cell culture, the scaffolds were soaked in growth medium at 37 °C in a CO₂-incubator overnight. Each sterilized PET disk was placed in one well of a 96-well plate. Unless otherwise stated, each PET scaffold in the 96-well plate was seeded with 10 μL of cell suspension with a cell density of 2 million cells per mL. The 96-well plate was put into a CO₂-incubator for 6 h, allowing cells to attach to the fibrous matrix. Then, 200 μL of growth medium were added to each well and the 96-well plate was incubated for another 24 h. Finally, the seeded scaffolds were transferred gently with a sterile tweezer to the center well of each microbioreactor. After adding 1 mL culture medium containing a drug of a prescribed concentration to each well, the 40-MBR was placed on an orbital shaker (Belly Button Shaker, Stovall, Greensboro, NC) at a rotating speed of 90 rpm in a CO₂-incubator.

4.2.4 Culture fluorescence for growth kinetics

Cell proliferation of EGFP-expressing cells in 40-MBR was studied using online fluorescence signals monitored in a high-throughput manner using TECAN GENiosPro™ at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence of the live cells (F\textsubscript{cells}) in the microbioreactors on 40-MBR was determined from the fluorescence signal measured directly from the center well (F\textsubscript{center})
subtracting background fluorescence signals from the scaffold \( F_{\text{scaffold}} \) and the culture medium \( F_{\text{medium}} \), which was taken from the average fluorescence signals measured from 8 surrounding wells. Both \( F_{\text{center}} \) and \( F_{\text{medium}} \) were taken throughout the culture period, whereas \( F_{\text{scaffold}} \) was measured in culture medium before cell seeding. For comparison, the fluorescence for the live cells in 40-MBR was also determined from the total fluorescence signal subtracting background fluorescence signals from the scaffold \( F_{\text{scaffold}} \) and the fresh culture medium \( F_{\text{fresh medium}} \) measured before cell seeding, which can also be used to represent the situation for commercial multiwell plates such as 384-MWP in which only the initial fresh medium can be used for background correction. The specific growth rates for cells cultured under different conditions were estimated for both cases based on the corrected culture fluorescence time-course data.

4.2.5 Cytotoxicity studies of sodium butyrate on CHO-GFP cells

In order to demonstrate the potential applications of the 3-D platform with reduced variations and improved reproducibility, the effects of sodium butyrate on CHO-GFP cells were investigated. Sodium butyrate was found to have no fluorescence at the excitation wavelength of 485 nm and emission wavelength of 535 nm. 20,000 cells were inoculated in each PET scaffold. After 24 h of inoculation, cells were exposed to different concentrations of sodium butyrate ranging from 0 to 150 mM. Each condition was conducted with 4 replicates. Culture fluorescence as stated above was measured to monitor cell responses to sodium butyrate.
4.2.6 Cytotoxicity of DH-8P-DB and DH-6P-DB on HT-29-GFP colon cancer cells

The 3-D platform was then used to study the cytotoxic effects of DH-8P-DB and DH-6P-DB on HT-29-GFP cells. The chemical structures of DH-8P-DB and DH-6P-DB are shown in Table 4.1. The culture medium was supplemented with either 10 mg/mL stock solution of DH-8P-DB or 5 mg/mL stock solution of DH-6P-DB in dimethysulfoxide (DMSO, Invitrogen). The concentration of DMSO in the culture medium was less than 0.002% (w/v), which was found to have no effect on HT-29 colon cancer cells. The dosage-dependent cytotoxic effects of both drugs on EGFP-expressing HT-29 colon cancer cells were investigated. Before the cytotoxic test, it was found that both DH-8P-DB and DH-6P-DB had no fluorescence at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. Each scaffold was seeded with about 30,000 HT-29 cells, which were exposed to DH-8P-DB or DH-6P-DB at different doses ranging from 0 μg/L to 100 μg/L. Each experiment was conducted in triplicates. Culture fluorescence as stated above was measured to monitor cell responses to DH-8P-DB and DH-6P-DB.

4.2.7 Statistical analysis

Descriptive statistics including 95% confidence interval (CI) and standard deviation were calculated from the specific growth rate data to estimate variations in 40-MBR and 384-MWP. The coefficients of variations (CV), which were calculated as the ratio of the standard deviation and the mean, were also used to assess the variations [23, 24]. Student’s t test analysis of data was performed using JMP version 7.0, with \( p < 0.05 \) as the threshold for significant difference.
4.3. Results and Discussion

4.3.1 Background and total fluorescence

Previous studies in our lab demonstrated that there was a good linear correlation between the measured fluorescence intensity and the cell number when cells were cultured in the fibrous PET scaffolds [9]. It should be noted that EGFP cells exhibited the same specific growth rate and doubling time as compared to their parental cells, non-transformed (wild type) cells. Therefore, EGFP-cells could be used to study cell proliferation by monitoring the fluorescence signals. Figures 4.2A and 4.2B show total fluorescence and normalized total fluorescence kinetics, respectively, when CHO cells were treated with various concentrations of sodium butyrate. The specific growth rate based on the total fluorescence was 0.015 h\(^{-1}\) and doubling time was 46 h, which was different from previously reported value (doubling time = 20 h) [26]. Therefore, the total fluorescence cannot represent the true growth kinetics and it is clear that the background fluorescence needs to be eliminated from the total fluorescence. In 3-D cell culture, the total fluorescence (\(F_{\text{center}}\)) consisted of the fluorescence from cells (\(F_{\text{cells}}\)), scaffolds (\(F_{\text{scaffold}}\)), and culture medium (\(F_{\text{medium}}\)). There was no obvious difference between the fluorescence for fresh culture medium and the fluorescence for the medium and scaffolds. Therefore, \(F_{\text{scaffold}}\) could be ignored when fluorescence was used to quantify cell growth. In addition, the fluorescent compounds in the fresh medium elicited a background signal of approximately 500 RFU, which was considered as background fluorescence for 384-MWP. However, Figures 4.2C and 4.3D indicate that the background fluorescence and normalized background fluorescence from the culture medium varied with time and the
concentration of sodium butyrate. Changes in cell culture medium components, cell debris, and EGFP released from dead cells contributed to the variations. Figure 4.2E shows the corrected fluorescence kinetics by subtracting background fluorescence from the total fluorescence in 40-MBR. The calculated doubling time was 20 h, which was in good agreement with previously reported value [26]. It is thus clear that the culture fluorescence must be corrected before it can be reliably used to represent cell growth kinetics. Therefore, fluorescence for live cells in 384-MWP might not represent the true growth kinetics and might not be suitable for in situ quantification of live cells because of its changing background fluorescence cannot be measured and corrected accordingly. Detailed comparison of 40-MBR and 384-MWP are given in cytotoxicity studies discussed later.

4.3.2 Real-time quantification of DO, pH and cell proliferation

Oxygen is an important factor involved in regulating cellular behavior. Continuous measurements of oxygen levels are thus crucial to maintain proper cell behavior. An oxygen sensitive film was used for monitoring DO in this work. The response of non-invasive oxygen sensor for in vitro cell culture to oxygen generally follows the linear Stern-Volmer equation, $I_0/I = 1 + K_{sv} [O_2]$. In this study, $K_{sv}$ was found to be 0.054 L/mg, and a saturated concentration of oxygen in water yielded a 1.45-fold increase in the intensity as compared to 0% oxygen (Figure 4.3A). Figure 4.3B shows that with pH increase, there was an increase in the absorbance at 560 nm, with a linear correlation for the pH ranging from 6.4 to 8.0 as follows: $\text{pH} = 6.424 + 3.697A_{560}$. 

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CHO cells and breast cancer cells were cultured in 40-MBR to demonstrate its capability of online monitoring DO, pH and cell growth simultaneously, and the results are shown in Figure 4.4. For pH, there was a decrease during the cell culture because of the production of lactate. The oxygen level decreased as a result of oxygen consumption by the cells. The per-cell oxygen utilization rate (OUR), defined as the ratio between total oxygen consumption rate and total cell number, was determined by following the method stated by Guarino et al [20]. The fold increase in cell number was similar to that in fluorescence intensity and thus cell number could be estimated from the fold increase in fluorescence intensity and initial seeding density. The per-cell oxygen OUR obtained from the kinetics was calculated as 2.2 fmol·min\(^{-1}\)·cell\(^{-1}\) for breast cancer cells and 0.8 fmol·min\(^{-1}\)·cell\(^{-1}\) for CHO cells, which were similar to those reported in the literature [20, 26]. Besides, the calculated doubling time of 20 h for CHO cells and 43 h for breast cancer cells are in good agreement with literature values [25, 27]. These results demonstrated that the 40-MBR capable of online monitoring of pH, DO and cell proliferation could serve as a platform for controlling and studying cell growth kinetics relevant to bioprocess development and early-stage drug discovery.

4.3.3 Cytotoxicity studies of sodium butyrate on CHO-GFP cells

To demonstrate the 40-MBR as a platform for cytotoxicity studies, the effects of sodium butyrate on CHO cells were investigated. Figures 4.5A and 4.5B show the growth kinetics for the CHO cells exposed to different concentrations of sodium butyrate in 40-MBR and 384-MWP, respectively. The specific growth rates obtained from the 4 replicates under each concentration were used for statistical analysis. In general, the
higher drug concentration would lead to an earlier termination of cell growth as indicated by the time reaching a maximum cell number, which was consistent with the previous study [28]. Sodium butyrate concentrations larger than 3 mM would decrease the specific growth rate due to cell apoptosis caused by sodium butyrate. However, when cells were exposed to 1 mM sodium butyrate, the average specific growth rate based on the fluorescence signal was slightly higher than that for 0 mM, which was probably because sodium butyrate, a widely used enhancer for protein production, at a low concentration could enhance the production of green fluorescence protein. Nevertheless, a statistical analysis using student’s t test showed that sodium butyrate at 1 mM did not affect cell growth significantly. When the butyrate concentration was increased to 3 mM, it significantly inhibited cell growth in 40-MBR due to increased apoptosis, which is in agreement with the reported butyrate concentration that significantly reduced cell viability measured using PE-Annexin V affinity assay [29]. There was no statistical difference in the growth rate for cells treated with sodium butyrate at concentrations ranging from 5 to 30 mM.

The growth kinetics in 384-MWP (Figure 4.5B) also showed that sodium butyrate would inhibit cell growth, which was generally consistent with the observation from 40-MBR (Figure 4.5A). However, there was one inconsistent butyrate effect on cell growth observed in 384-MWP, which showed that cells grew faster when exposed to 10 mM than 5 mM. Figures 4.5C and 4.5D compare the mean specific growth rates obtained from the 40-MBR and the 384-MWP, with Y error bars representing standard deviations and 95% confidence interval (95% CI), respectively. Compared to 384-MWP, 40-MBR
gave smaller standard deviation as well as 95% CI, indicating the new platform can provide more reliable and reproducible growth kinetic data with reduced experimental errors. The smaller coefficients of variations (see Figure 4.5E) further confirmed that the 40-MBR had reduced data variations and improved reproducibility. The improved data quality, as measured by the smaller variations, for the 40-MBR over 384-MWP can be attributed to the timely correction of background fluorescence. Consequently, the 40-MBR was more sensitive and reliable than commercial 384-MWP in detecting drug responses of cells in a HTS manner.

4.3.4 DH-8P-DB and DH-6P-DB cytotoxicity studies on HT-29-GFP colon cancer cells

The potential application of the 40-MBR in drug discovery was further evaluated by determining the dosage-dependent cytotoxicity effects of two potential anticancer drugs, namely DH-8P-DB and DH-6P-DB, on HT-29 colon cancer cells. Figures 4.6A and 4.6B show the growth kinetics of HT-29 colon cancer cells exposed to different concentrations of DH-8P-DB in 40-MBR and 384-MWP, respectively. Although 3-D cell culture enabled epithelial cells to regain polarity [30], which was essential for the maintenance of tissue function, the proliferation rate of HT-29 colon cancer cells was suppressed in the presence of DH-8P-DB. Figures 4.6C and 4.6D compare the specific growth rates from 40-MBR and 384-MWP, with Y error bars representing standard deviations and 95% CI, respectively. In general, the growth data from both platforms showed that DH-8P-DB inhibited HT-29 colon cancer cells with reduced growth rate or increased death rate at a higher drug concentration. However, compared to 384-MWP, the 40-MBR showed
significantly reduced standard deviation, 95% CI, and coefficients of variations (see Figure 4.6 E), a clear indication of improved reproducibility. When statistical difference in specific growth rates occurred in 40-MBR and 384-MWP, the concentration of DH-8P-DB was 100 µg/L.

Figures 4.7 shows the dose-dependent cytotoxicity effects of DH-6P-DB on colon cancer cells cultured in 40-MBR and 384-MWP. Again, the 40-MBR gave better specific growth rate data with reduced variations (Figures 4.7C-E). The concentration of DH-6P-DB to statistically inhibit colon cancer cell growth was found to be 10 µg/L in 40-MBR and 100 µg/L in 384-MWP. Based on the data from the 40-MBR, DH-6P-DB is more effective than DH-8P-DB in inhibiting colon cancer cell growth and thus could be considered as a potential candidate in future drug discovery. However, the data from 384-MWP suggested that both DH-8P-DB and DH-6P-DB required the same concentration of 100 µg/L in order to significantly inhibit colon cancer cell growth. In this case, DH-6P-DB might be missed as a more potent anticancer drug candidate.

The drug concentration causing 50% decrease in cell number (IC₅₀) is usually used to evaluate the cytotoxicity of the drug, and is thus estimated for DH-8P-DB and DH-6P-DB. However, the IC₅₀ value could vary greatly with the time points chosen. In this study, IC₅₀ is expressed as the drug concentration inhibiting the specific growth rate by 50% of the control level (IC₁₅₀), which is independent of time points chosen [8]. In 40-MBR, IC₁₅₀ values for DH-8P-DB and DH-6P-DB were 10 µg/L and 2 µg/L, respectively (see Figures 4.8A and 4.8B), suggesting DH-6P-DB was more effective in inhibiting HT-29 colon cancer cell growth than DH-8P-DB. IC₁₅₀ values from 384-MWP
data also suggested that DH-6P-DB might be a more effective drug candidate in treating colon cancer. However, the estimated IC_{50} values were lower from 40-MBR than from 384-MWP, indicating that the 40-MBR was more sensitive than 384-MWP in detecting drug cytotoxicity due to reduced data variations.

**4.4. Conclusions**

A reliable, high throughput 40-MBR capable of real-time monitoring of cell proliferation, pH, and DO in 3-D microenvironments was developed from a commercial 384-well plate. Based on the culture fluorescence measurement, the 40-MBR showed increased reproducibility and reduced variations in assessing the dosage-dependent cytotoxicity effects of potential drugs such as DH-8P-DB and DH-6P-DB. The integrated, high throughput continuous monitoring of pH, DO and cell proliferation in 3-D cell cultures provides a reliable platform for high throughput screening in the early-stage drug discovery and bioprocess development with increased biological relevance.
4.5 References


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<td>DH-6P-DB</td>
<td><img src="image" alt="Chemical structure of DH-6P-DB" /></td>
<td>C_{23}H_{18}O_{5}</td>
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<td>5,7-dihydroxy-2-(4-hydroxyphenyl)-6-[(E)-2-phenylethenyl]-3,4-dihydro-2H-1-benzopyran-4-one</td>
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Table 4.1. Compound information
Figure 4.1. A 40-MBR for 3-D cell cultures on a modified 384-well plate. (A) Picture of the 40-MBR. (B) Picture of a microbioreactor with a PET scaffold in the center well of the microbioreactor. (C) Schematic illustration of detection of pH, DO, and culture fluorescence from cells grown in 3-D scaffold and medium (middle) with fluorescent images of CHO-EGFP cells in the PET fibrous scaffold (left) and oxygen sensitive film (right) with no oxygen and saturated oxygen.
Figure 4.2. Kinetic fluorescence signals for CHO cells cultured in the presence of sodium butyrate at various concentrations (0 mM to 70 mM). (A) Total culture fluorescence, (B) Normalized total fluorescence, (C) Background fluorescence from the culture medium, (D) Normalized background fluorescence, (E) Normalized culture fluorescence after subtracting background fluorescence.
Figure 4.3. Linear correlations for online monitoring of pH and dissolved oxygen (DO). (A) Correlation between DO and normalized fluorescence intensity ($I_o/I$). (B) Correlation between pH and absorbance at 560 nm.
Figure 4.4. Culture kinetics showing cell growth, pH and DO profiles in MBR. (A) MCF-07 breast cancer cell; (B) CHO cell.
Figure 4.5. Effects of sodium butyrate on CHO-GFP cells. (A) Cytotoxicity of sodium butyrate on CHO cells in 40-MBR; (B) Cytotoxicity of sodium butyrate on CHO cells in 384-MWP; Comparison of specific growth rates for 40-MBR and 384-MWP with Y error bars representing (C) standard deviations and (D) 95% confidence interval (CI); (E) Comparison of coefficients of variations for 40-MBR and 384-MWP.
Figure 4.6. Cytotoxic effects of DH-8P-DB on HT-29 colon cancer cells. (A) Cytotoxicity of DH-8P-DB on HT-29 colon cancer cells in 40-MBR; (B) Cytotoxicity of DH-8P-DB on HT-29 colon cancer cells in 384-MWP; Comparison of specific growth rates for 40-MBR and 384-MWP with Y error bars representing (C) standard deviations and (D) 95% confidence interval (CI); (E) Comparison of coefficients of variations for 40-MBR and 384-MWP.
Figure 4.7. Cytotoxic effects of DH-6P-DB on HT-29 colon cancer cells. (A) Cytotoxicity of DH-6P-DB on HT-29 colon cancer cells in 40-MBR; (B) Cytotoxicity of DH-6P-DB on HT-29 colon cancer cells in 384-MWP; Comparison of specific growth rates for 40-MBR and 384-MWP with Y error bars representing (C) standard deviations and (D) 95% confidence interval (CI); (E) Comparison of coefficients of variations for 40-MBR and 384-MWP.
Figure 4.8. Effects of doses of DH-8P-DB (A) and DH-6P-DB (B) on the specific growth rates in 40-MBR and 384-MWP. Note: $SGR_{\text{max}}$ indicates maximum specific growth rate.
Chapter 5: Embryonic Stem Cell Models for High Throughput Screening of Cytotoxic and Embryotoxic Compounds

Abstract

The embryonic stem cell test (EST) has been validated and used as an in vitro model for assessing embryotoxicity. However, the current EST is based on morphological analysis of beating cardiomyocytes and the quantification of marker protein expression specific to myocardiac or neural differentiation, which can only provide end-point data and cannot predict embryotoxicity of chemicals affecting organs other than hearts and nerve systems. Survivin is overexpressed in undifferentiated embryonic stem cells (ESC) and is involved in oocyte development and maturation as well as normal embryo development. Furthermore, the expression of survivin in ESC is significantly reduced or down-regulated upon differentiation, suggesting that survivin can be used as a diagnostic marker for predicting embryotoxicity. In this study, a non-invasive, high-throughput embryotoxicity assay was developed based on differential expression of enhanced green fluorescent protein (EGFP) driven by a survivin promoter in ESC. ES cells expressing EGFP driven by a constitutive CMV promoter were used to monitor cell proliferation and for cytotoxicity assay. The specific survivin expression in ESC as determined by the ratio of EGFP fluorescence from survivin and from CMV promoters showed down-regulation in the presence of chemicals with known embryotoxicity. In contrast, there was no
change in the survivin expression for ESC cultured in the presence of non-embryotoxic chemicals. These results suggested that the ESC models can be used to evaluate cytotoxicity and embryotoxicity of drugs. Three Chinese herbal medicines, *Ginkgo biloba* (GB), *Epimedium brevicornum* (EB), and *Ganoderma lucidum* spore (GLS), were then tested, and the results showed that GB and EB were embryotoxic while GLS was not, all at the non-cytotoxic level. The ESC models with high predictability offer a promising HTS platform for assessing cytotoxicity and embryotoxicity.

5.1 Introduction

Many chemicals are embryotoxic and some pharmaceuticals administered to pregnant women may interfere with embryo development and induce abnormal embryogenesis and malformations [1]. Therefore, prior to the marketing of a drug, its possible harmful effects on pregnant women must be assessed. A large number of experimental animals are widely used as proxies for human beings in regulatory reproductive and developmental toxicology [2]. Animal-based approach is time-consuming, costly, labor-intensive, and presents moral issues. Alternative *in vitro* methods including the embryonic stem cell test (EST), the rat whole-embryo culture test, and the micro-mass test for prediction of toxicity without relying on animals have thus been developed to assess embryotoxicity and cytotoxicity [3]. Among the alternative methods, the EST, first introduced by Spielmann *et al* in 1997 using embryonic stem cells (ESCs) and mouse 3T3 cells, is the only one implemented without sacrificing pregnant animals [4]. ESCs derived from the inner cell mass in early embryonic development stages are highly pluripotent and capable of differentiating into all three germ layers and various cell
lineages. With the unlimited proliferation capability, ESCs are sensitive to certain drugs or environmental changes caused by the drugs. To date, several ESC-based models have been developed and validated as a reliable tool for in vitro developmental toxicology studies [5-7]. However, the current EST relies on morphological endpoint analysis [4], which requires extensive experience and can lead to misjudgments [7]. It is also limited to the detection of mesodermal differentiation (e.g., cardiomyocytes) and neural differentiation during prenatal development [7]. Chemicals affecting organs other than hearts and nerve systems may not be correctly predicted. In addition, the duration for cell differentiation is long, usually 10 days. Recently, new molecular approaches based on quantitative gene expression or proteomics analysis of certain marker proteins specific to ES cell differentiation into myocardiac and neural cells have also been developed [8-13]. Like the EST, the molecular methods can only provide endpoint data. To reduce the test duration and explore new endpoints of the EST for early detecting differentiation toxicity are in urgent need.

In this work, survivin as a new molecular marker for early diagnosis of embryotoxicity was studied. Survivin was discovered in 1997 as a member of the inhibitor of apoptosis proteins (IAPs) [15], and since then, it has gained intense interest from disparate segments of basic and disease-related research. Fujino et al (2008) reported that survivin expression in pregnant women was higher than nonpregnant women and survivin is biologically important for oocyte development and maturation [14]. Survivin is highly expressed in undifferentiated embryonic stem cells and their derived tumors [16, 17]. In addition, survivin plays a critical role in normal embryo development, and its genetic disruption is lethal in the early stage of embryo. Forced
survivin expression or inhibition caused by drug administered during fetal development can lead to fetus anomalies. Therefore, monitoring survivin expression in embryonic stem cells may provide a method for identifying drugs inhibiting survivin expression and thus causing developmental toxicity.

In this study, a novel high-throughput embryotoxicity assay was developed using EGFP-expressing ESCs under the control of a survivin promoter. Survivin expression is closely associated with embryo development and cell differentiation. For control, ESCs expressing EGFP under the control of a CMV promoter were used to monitor cytotoxicity of chemicals. Using survivin as a diagnostic marker for predicting embryotoxicity was first tested and validated with various chemicals with known developmental toxicity. Then, the potential embryotoxicity of three Chinese herbal medicines, *Ginkgo biloba* (GB), *Ganoderma lucidum* spore (GLS), and *Epimedium brevicornum* (EB), was tested. The results showed that the survivin-based assay has great potential in screening compounds with different degrees of embryotoxicity.

### 5.2 Materials and methods

#### 5.2.1 Chemicals

Seven chemicals used in the European Center for Validation of Alternative Methods (ECVAM) international validation study were dissolved in appropriate solvents, namely, dimethylsulfoxide (DMSO), distilled water (DI) or ethanol as indicated in Table 5.1.
5.2.2 Preparation of Chinese herbal medicines

Chinese herbal medicines including *Ganoderma lucidum spore* (GLS) powder, *Ginkgo biloba* (GB) and *Epimedium brevicornum* (EB) were purchased from Cai Zhi Lin (Guangzhou, China). GLS powder was directly dissolved in DMEM knockout medium, which was filter sterilized prior to use. GB and EB extracts were prepared as follows: 1 g of the dried leaves was added to distilled water (100 mL) in a 500 mL round-bottom flask equipped with a condenser and refluxed for 30 minutes. After centrifuging the obtained suspension at 4,000 rpm for 10 minutes, the supernatant was freeze-dried at -40°C for 24 h. Both the extract powders were then dissolved in knockout DMEM and filter-sterilized before use.

5.2.3 Cell line and culture media

Mouse embryonic stem D3 cells (ATCC CRL-1934) were maintained on gelatin pre-coated T-flasks in the growth medium containing knockout Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 µM β-mercaptoethanol (Sigma-Aldrich), 50 U/mL penicillin (Invitrogen), 50 µg/mL streptomycin (Invitrogen), and 100 µM leukemia inhibitory factor (LIF) (Millipore). The EB formation medium was the same as the growth medium but without LIF, and the neuronal differentiation medium was the same as the EB formation medium but supplemented with 10⁻⁷ mM of retinoic acid (Sigma-Aldrich).
5.2.4 Construction of plasmids

The Psurvivin-EGFP plasmid was generated by inserting a 303-bp survivin promoter fragment from PGL3-survivin into the Ase I site of the PEGFP-N3 (Clontech). Briefly, PEGFP-N3 was digested using Ase I and Bgl II to remove the CMV promoter. The 303-bp survivin promoter fragment was amplified from PGL3-survivin using PCR with the forward primer (5’-ATTAATGGATTCAGGCGTGAGCCACTG-3’) and reverse primer (5’-AGATCTGCCGCGCGCCACC-3’). During the PCR process, Ase I site was introduced to the fragment. Then the PCR products were digested with Ase I and Bgl II and cloned into the (Ase I/ Bgl II) sites of linear pEGFP-N3 with removed CMV promoter to generate Psurvivin-EGFP. The constructed plasmids were confirmed by DNA sequencing (Applied Biosystems, Foster City, CA).

5.2.5 Establishment of stable transgenic mouse ES cell lines expressing EGFP

ES cells were transfected with PEGFP-N3 and Psurvivin-EGFP using Lipofectamine 2000 (Invitrogen), respectively. Stable transgenic ES cells were established after 10-day selection using 500 µg/mL of geneticin (G-418, Invitrogen). The stability of the transfected cell line was verified (>97%) using FACS Calibur (B-D Biosciences).

5.2.6 Characterization of stable transgenic ES cells

To confirm that survivin expression can be monitored using GFP, regulation of GFP expression during neuronal differentiation and embryoid bodies (EB) formation was investigated. Two million ES cells were inoculated onto a non-adherent 9-cm Petri dish containing 10 ml EB medium and incubated at 30 rpm for 4 days to form EBs or cell
aggregates. The formed EBs or cell aggregates were exposed to the neural differentiation medium and incubated for another 4 days. Medium was changed every other day. Next, 10 EBs were inoculated into a gelatin-coated well on the 24-well plate and incubated at 37 °C in a humidified CO₂-incubator (Thermo Electron) for 10 d. After 10-day culture, immunocytochemistry was performed on the EBs to identify neuronal differentiation of ES cells. Cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) overnight at 4 °C. Then, the cells were permeabilized with 0.1 % Triton-100 (Sigma-Aldrich) in PBS for 30 min, followed by blocking the cells using 3% fetal bovine serum (FBS) in PBS for 30 min. Then, primary antibody against β-III tubulin (DHSB) (1 µg per million cells) were added and incubated overnight at 4°C. The cell samples were then washed three times with PBS to remove unbounded primary antibody. Subsequently, goat anti-mouse R-phycoerythrin conjugated secondary antibody (Invitrogen) was applied for 1 h. Cell images were taken using an inverted fluorescence microscope (Fluorescence Inverted Microscope IX71, Nikon).

5.2.7 Reverse Transcriptase PCR

ES cells after 10-day differentiation were harvested. Then, total RNA was isolated from the harvested cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. Yield and purity of RNA were determined with a NanoDrop (NanoDrop Technologies, Wilmington, DE). One µg of RNA was used as template to synthesize single-stranded complementary DNA (cDNA) using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Then the cDNA was amplified by Taq DNA polymerase (Promega) with 10 µM of each of the forward and reverse primers,
which were listed in Table 5.2. The annealing temperatures were 56 °C, 60 °C and 58 °C, respectively. Final RT-PCR products were separated on a 3% agarose (Fisher Scientific, Pittsburgh, PA) gel at 70 V for 60 min. Then, DNA was stained with ethidium bromide for visualization and photographed with a Gel Doc 2000 Gel Documentation System (Bio-Rad, Hercules, CA). The intensity of bands was analyzed using Image J (National Institute of Health).

5.2.8 3-D cell culture

Polyethylene terephthalate (PET) fibrous materials were used as scaffolds to support 3-D cell cultures. Prior to use, the disk-shaped PET scaffolds, with a diameter of 4 mm, were sterilized at 121°C for 30 minutes. Furthermore, a 40-MBR platform with improved reproducibility developed from a commercial 384-well plate was used for 3-D cell culture of EGFP-cells [29]. The disk-shaped PET scaffolds were immersed in the culture medium and incubated at 37 °C overnight. Each sterilized patch was placed in one well of a 96-well plate. Cells were trypsinized from a T-flask and then suspended in the growth medium. Each PET scaffold in the 96-well plate was seeded with 10 μL of cell suspension with a cell density of 1 million cells per mL. The 96-well plate was then incubated at 37 °C for 6 h, allowing cells to attach to the fibrous matrix. Finally, the seeded scaffolds were transferred gently with a sterile tweezer to the center well of each unit in the 40-MBR. One mL growth medium containing different drugs was then added to each well, and the platform was placed on an orbital shaker (Belly Button Shaker) at a rotating speed of 90 rpm in a humidified CO2-incubator.
5.2.9 Real-time monitoring

Fluorescence signal was measured using TECAN GENiosPro™ at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Cytotoxic effects of test chemicals on ES cells were determined following the previously described method [29]. The concentrations of test chemicals are listed in Table 5.1. In addition, three Chinese herbal medicines were tested at 1 g/L (based on the weight of herbal medicines before extraction). Specific survivin expression was estimated as the ratio between culture fluorescence signal driven by survivin promoter and that by CMV promoter. The fluorescence signal under a CMV promoter here was used to rule out false positive hits due to the cytotoxic effects of compounds.

5.2.10 Cell cycle analysis

ESCs harvested 48 h after drug treatments were fixed in ice-cold 70% ethanol at -20°C overnight. The fixed cells were re-suspended in PBS containing 100 µg/ml RNase A and 50 µg/ml propidium iodide (PI, Invitrogen) and incubated at 37 °C for 30 min. Finally, the stained cells were analyzed using flow cytometry (FACS Calibur, BD Biosciences).

5.2.11 Statistical analysis

Each experiment was conducted in triplicate. Statistical analysis of percentage of cells in G2/M phase and drug response curves from embryotoxicity/cytotoxicity assays were carried out using JMP version 7.0, with $p < 0.05$ as the threshold for significant difference.
5.3 Results

5.3.1 Establishment and validation of mouse embryonic stem cells expressing EGFP under the control of survivin promoter

To characterize the developed transgenic ES cells with survivin-EGFP, the correlation between survivin expression and EGFP expression was investigated. It is reported that survivin was highly expressed in undifferentiated human ESC and downregulated in mature embryoid bodies [17]. Figure 5.1A shows fluorescent images for 6-day old and 30-day old EBs, respectively, suggesting a rapid decrease in EGFP expression with the aging of EBs. This is in contrast to transfections with CMV-EGFP, in which GFP was observed also in differentiated EBs (Figure 5.1B). Furthermore, survivin is also reported to be rapidly downregulated upon the differentiation of embryonic stem cells [16]. When 8-day old EBs were plated on gelatin-coated surfaces, cells were differentiated into neuronal cells as indicated by β-III tubulin staining. EGFP expression driven by the survivin promoter was greatly reduced when neuronal differentiation occurred (see Figure 5.2A). Therefore, changes in the survivin gene expression were correlated to those in EGFP expression during both the EB maturation and neuronal differentiation processes. This correlation was further demonstrated by RT-PCR analysis. Figure 5.2B reveals that EGFP and survivin were abundant in undifferentiated ES cells and both EGFP and survivin were greatly decreased after 10-day differentiation. In addition, the relative decrease in survivin expression was similar to that in EGFP expression. These findings demonstrate that EGFP expression can be used for monitoring survivin expression.
5.3.2 Strongly embryotoxic compounds

The embryotoxicity and cytotoxicity of three strongly embryotoxic compounds categorized by ECVAM were analyzed using PCMV-ES and Psurvivin-ES cells. The results are shown in Figure 5.3. Figure 5.3A shows kinetic profiles including cell proliferation, survivin expression and specific survivin expression for the control (no drug). In general, cell growth kinetics and survivin expression kinetics were similar, and the specific survivin expression was constant throughout the cell culture process. Figure 5.3B shows kinetic profiles for cells treated with retinoic acid at 0.3 µg/L. Compared to the control, RA at 0.3 µg/L exhibited no cytotoxicity, which was in agreement with the previous study [1]. However, survivin expression was greatly downregulated as indicated by the declining specific survivin expression. Although no cytotoxicity at such a low dose was observed, the decrease in specific survivin expression may result in abnormal embryo development. This is in agreement with a previous study that RA at a concentration of 0.1 µg/L could greatly inhibit the differentiation of ES cells into cardiomyocytes [1]. Furthermore, Figure 5.3C shows kinetic profiles for cells exposed to another known strongly embryotoxic compound 5-FU at 10 µg/L. Similarly, 5-FU at a low concentration exhibited no cytotoxicity, but survivin expression was significantly decreased. Both of the two embryotoxic drugs led to a rapid decrease in survivin expression. The deficiency in survivin expression could result in lethality at the blastocyst stage. The 3-D ESC model correctly determined the two embryotoxic compounds RA and 5-FU, which was in agreement with the recently performed validation study of ECVAM [1]. However, in the case of MTX exposure, survivin
expression was increased, suggesting no embryotoxicity using the ESC model. Figure 5.3D indicates that MTX showed strong cytotoxicity even at a low concentration of 50 µg/L. MTX could cause skull and limb abnormalities as the consequence of general cytotoxicity [18].

5.3.3 Moderately embryotoxic compounds

The embryotoxic effects of compounds including tetracycline and boric acid were investigated. ES cells exposed to tetracycline at a concentration up to 0.1 mg/L showed similar growth kinetics (Figure 5.3E) to that of the control. However, survivin expression was slightly decreased. The result was consistent with the previous result that tetracycline downregulated survivin expression, indicating the negative effect on fetus bone development [19]. Furthermore, in ECVAM validation study, boric acid is a moderate embryotoxic compound. Figure 5.3F shows that boric acid at 10 mg/L exhibited no cytotoxicity. However, survivin expression was downregulated, unveiling its embryotoxic potential. Moderate embryotoxic compounds were shown with inhibition of differentiation due to embryotoxicity not relying on cell viability [20].

5.3.4 Non-embryotoxic compounds

Two well-known non-embryotoxic compounds, saccharin and acrylamide, were also investigated. As can be seen in Figure 5.3G, saccharin at a concentration of 0.18 g/L did not inhibit cell growth or downregulate survivin expression, suggesting that saccharin should not have any embryotoxicity or cytotoxicity. Acrylamide at 25 mg/L exhibited significant cytotoxicity and upregulated survivin expression (Figure 5.3H). Acrylamide
can penetrate the skin and cause reproductive and developmental toxicity in animal studies [21]. Additionally, acrylamide neurotoxicity occurs in both the central and peripheral nervous systems, likely through microtubule disruption, which has been suggested as a possible mechanism for genotoxic effects of acrylamide in mammalian systems [21]. However, Piersma et al. (2007) reported that acrylamide has no embryotoxicity [20]. Although no embryotoxicity was caused by acrylamide, it does cause developmental toxicity due to cytotoxicity.

The cytotoxic effects of acrylamide at different concentrations were investigated and the results are shown in Figure 5.4. When the concentration was below 10 mg/L, there was no obvious cytotoxicity ($p = 0.1192$). Besides, at 5 mg/L (standard concentration release in water), no significant increase in survivin expression was observed ($p = 0.8670$). However, when the concentration was increased to 25 mg/L, there was a significant increase in survivin expression ($p < 0.0001$) and cytotoxicity was obviously observed. The result was consistent with a previous *in vivo* study that the lowest observed teratogenic effect occurred at 20 mg/L [21]. Acrylamide did inhibit the differentiation of ES D3 cells, which was due to cytotoxicity rather than embryotoxicity. Furthermore, the median lethal dose (LD50) for acrylamide in rats, guinea pigs and rabbits is 150-180 mg/L [22]. In the present investigation using the *in vitro* model, the dose to kill 50% of cells after 24-h drug treatment was about 150-175 mg/L, which was close to the *in vivo* data.
5.3.5 Analysis of embryotoxicity of Chinese herbal medicines

Using the ESC model, the embryotoxicity and cytotoxicity of three Chinese herbal medicines of great interest were assessed and identified. GB is one of the top selling herbal medicines in the world and it contains some compounds that can be used to treat neurodegenerative diseases. Whether GB is safe on pregnant women remains a controversial issue. ES cells treated with GB at 1 g/L yielded similar cell growth kinetics to the control (Figures 5.5A and 5.5B), suggesting no cytotoxicity. However, survivin expression was greatly downregulated, indicating that GB might pose risks on the pregnant women. This is consistent with some in vitro and in vivo studies that high doses of GB would lead to degeneration of the oocyte development [23]. In vivo studies also showed that although high doses of GB were not toxic to mothers, GB caused intrauterine growth retardation in fetuses of Wistar rats during fetogenesis [24]. EB is often used as a supplement for the enhancement of sexual activity for both men and women. Figure 5.5C indicates that EB at 1 g/L had no cytotoxicity. However, similar to GB, EB greatly reduced survivin expression, suggesting potential embryotoxicity. This work together with some of the previous studies suggests that it may not be good to have GB and EB during pregnancy. In addition to GB and EB, the bioactivity of GLS was also investigated using the model. GLS has been widely used in China for health and longevity. It has been reported that GLS exhibited anti-cancer effects [25]. Its safety on pregnant animals has been rarely studied. The effects of GLS at 1 g/L were investigated using the model. Figure 5.5D shows that GLS at 1 g/L has no cytotoxic effects on ES cells. Besides, survivin expression was not affected as compared to the control.
Therefore, the ES model indicates that it might be safe for pregnant animals to take GLS during pregnancy. However, before the administration of GLS by pregnant women, animal studies are still needed to further analyze the possible developmental toxicity of this Chinese herbal medicine.

5.3.6 Cell cycle analysis

Survivin gene involved in cell cycle regulation has been identified with the highest expression level reported in G2/M and lower level in G1 [26]. FACS analysis was performed to determine whether over-expression of survivin was caused by cell cycle arrest at the G2/M phase. FACS analysis results (Figure 5.6) show that the percentage of G2/M population treated by acrylamide (37%) was significantly higher than the control (30%) \( (p = 0.0395) \), whereas FACS analysis clearly showed no significant difference in the percentage of G2/M population between 4-TH (27%) treated cells and the control (30%) \( (p = 0.2306) \). Compared to the control, forced expression of survivin using acrylamide did not block the accumulation of cells in G1 phase, but did increase G2/M cell populations. Besides, reduced expression of survivin caused by tetracycline did decrease G2/M cell populations in comparison with the control. In addition, it is reported that RA can accelerate cell death by enforcing G1 progression through the down-regulation of survivin [27]. Furthermore, GB and EB greatly reduced the percentage of G2/M population \( (p < 0.0001) \), whereas GLS did not affect G2/M population (31%) significantly compared with the control \( (p = 0.8762) \). The cell cycle analysis further demonstrated that survivin expression was related to EGFP expression under the survivin promoter.
5.4 Discussion

The development of the fluorescence-based ESC model coupling a commercial well plate reader allows high throughput, noninvasive and online monitoring embryotoxicity and cytotoxicity. The ESC model has several advantageous features: high throughput, noninvasive, and online monitoring, reduced duration period for assessing developmental and reproductive toxicity, and capacity of assessing chemicals associated with embryotoxic effects on organs other than hearts and nerve systems.

Current trends in drug screening are directed towards developing assays with high throughput (HT) manners. Generally, the HT capability is compromised with inferior data quality and less information suffered from off-line sampling. Real-time and online measurement of cellular performances including cell proliferation and gene expression is thus desirable. EGFP as a non-invasive reporter in mammalian cells does not interfere with cell growth and other activities and thus can be used to study dynamic properties of cultures including cell growth, cell morphology and protein expression [28]. In this work, the feasibility of using EGFP under the control of survivin promoter to estimate survivin expression was demonstrated. In the established stable transgenic ES cells with a survivin promoter upstream of enhanced green fluorescence protein (EGFP) reporter gene, changes in gene expression was related to EGFP expression during neuronal differentiation and embryoid bodies (EB) maturation, suggesting that online monitoring could be achieved by fluorometrically quantifying EGFP in a commercially available well plate reader allowing high-throughput and time-series data acquisition. For the analysis of embryotoxicity, 3-D cell cultures of undifferentiated ES cells were conducted in a previously developed 40-MBR [28]. This platform used for fluorescent assays was
demonstrated with reduced variations and increased reproducibility.

Since the first development of the EST for assessing embryotoxicity by Spielman et al. in 1997, the EST has been widely used and validated as a reliable tool to reduce the number of animal tests in drug screening [4]. Current EST methods are usually based on end-point morphological analysis of beating cardiomyocytes, which requires 10 days [7]. Reduction in the assay duration contributes to simplicity, economy, and reproducibility. Recent studies on altered gene expression specific to embryotoxic treatment during embryonic stem cell differentiation into myocardiac and neural cells have been conducted [12]. Detectable expression levels were observed at days 4-6. The quantitative analysis of marker gene expression may empower the EST with reduction in duration. Stable transgenic ES cells for detection of marker gene expression by luciferase reporter genes have been developed for high throughput screening or prediction of embryotoxicity of chemicals [13]. The expression of Hand1 and Cmya1 during mouse ES cell differentiation into cardiomyocytes was estimated using luciferase expression. This model showed reliable data at day 6. In this work, EGFP expression under survivin promoter was used as an indicator for quantitative analysis. This is the first demonstration on proposing survivin as a diagnostic marker for embryotoxicity. The method showed reliable data at day 3, shorter than 10 days required for microscopic examination of contracting cardiomyocytes in the original EST and 6 days required for marker proteins specific to cardiac differentiation in a newly developed EST.

Although the EST based on quantitative determination of marker genes involved in the embryotoxicity under cardiomyocyte and neuron differentiation processes has greatly reduced duration [12,13], the EST based on those marker proteins is limited and
cannot be used in testing chemicals that affecting other organs development such as bone
development. Table 5.3 summarizes the cytotoxic and embryotoxic effects of the
compounds and Chinese herbal medicines studied in the chapter. Tetracycline is
demonstrated to reduce survivin expression [19] and can impair development of bone in
the fetus. Besides, tetracycline exhibited embryotoxic properties evidenced by a higher
intrauterine death rate and congenital anomalies in individual fetuses. Using the current
EST based on end-point analysis of marker protein expression cannot predict
embryotoxicity caused by tetracycline because of the limitations of current EST. On the
contrary, the fluorescence-based ESC model with survivin as a diagnostic marker
identified tetracycline as an embryotoxic compound. Therefore, these results
demonstrated that this model could reliably predict the embryotoxic responses of drugs to
fetuses. In addition, developmental and reproductive toxicity can be caused by
cytotoxicity and embryotoxicity. Online monitoring of cytotoxicity has also been
developed and validated by measuring fluorescence signals under CMV promoter.
Chemicals including MTX and acrylamide were assessed to interfere with embryo
development and cause abnormal embryogenesis and development by analyzing
cytotoxicity assays. Some drugs at low doses may not exhibit cytotoxicity. But they are
indeed harmful to the early embryo development. Retinoic acid, 5-FU, boric acid, and
tetracycline led to a decrease in survivin expression, suggesting their potential in
negatively affecting embryo development. Furthermore, the ESC model successfully
identified acrylamide and saccharin as non-embryotoxic compounds. Furthermore, the
embryotoxicity and cytotoxicity of three Chinese herbal medicines with great interest was
assessed using the ESC model. Compared to in vivo studies, the assessment of GB
indicated that the developed model could provide reliable determination of embryotoxicity and cytotoxicity. So far, no studies have been focused on the embryotoxicity of EB. Therefore, this study could serve as a reference for future studies of this Chinese herbal medicine. In addition, since GB would cause growth retardation, the current EST will not correctly detect its effects on embryo development. Furthermore, the analysis of GLS suggests that GLS might be safe on pregnant women since no cytotoxicity or embryotoxicity was observed.

5.5 Conclusion

The *in vitro* three dimensional (3-D) embryonic stem (ES) cell-based model with high throughput manners proposed in the paper provides as a tool to analyze the developmental and reproductive toxicology of pharmaceuticals and xenobiotics by examining cytotoxic and/or embryotoxic effects. The novel ESTs allow online determination of cytotoxicity and embryotoxicity in a short duration in high throughput manners by EGFP detection. Before the developed ES model could possibly be used to reduce animal tests for testing a variety of chemicals, pharmaceuticals or xenobiotics, further studies using a wider selection of test compounds are necessary to assess the capabilities and limitations of the present ESTs in prediction of embryotoxicity.
5.6 References


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Table 5.1. Chemicals and their concentrations and solvents used in this study
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Table 5.2. Primes and annealing temperatures used in RT-PCR
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Table 5.3. Summary of cytotoxic and/or embryotoxic effects of chemicals and Chinese herbal medicines.

Note: GLS: *Ganoderma Lucidum* spore powder; GB: *Ginkgo Biloba* extract; EB: *Epimedium brevicornum*. 
Figure 5.1. Characterization of ES cells transfected with CMV-EGFP and Survivin-EGFP. (A) Images for 6 days and 30 days old EBs formed by stable transgenic cells with Psurvivin-EGFP. (B) Images for 6 days and 30 days old EBs formed by stable transgenic cells with PCMV-EGFP. Scale bar: 100 µm.
Figure 5.2. (A) Fluorescent images for fully differentiated ES cells transfected with Psurvivin-EGFP. Scale bar indicate 100 µm, nuclei were stained with DAPI. (B) RT-PCR analysis. (Middle) Semiquantitative analysis of the relative levels of survivin and EGFP expression in controls (Con) and ES cells after 10-day neural differentiation (DES). The intensity of each band in the gel images was measured using ImageJ program and normalized by GAPDH. (Right) The relative decrease in survivin and EGFP.
Figure 5.3. Kinetic profiles for embryotoxic and non-embryotoxic compounds. (A) Controls. (B) Retinoic acid. (C) 5-FU. (D) MTX. (E) Tetracycline. (F) Boric acid. (G) Saccharin. (H) Acrylamide.

Figure 5.3 continued
Figure 5.4. Growth kinetics (A) and specific growth rates (B) for mES cells cultured in the presence of acrylamide at various concentrations (0 to 200 µg/ml).
Figure 5.5. Kinetic profiles for Chinese herbal medicines. (A) Control. (B) Ginkgo biloba (GB). (C) Epimedium brevicornum (EB). (D) Ganoderma lucidum spore (GLS).
Figure 5.6. Analysis of control, RA, Tet, AAD, GB, EB, and GLS treated ES cells in G2/M
Chapter 6: Enhanced Neuronal Differentiation of Mouse Embryonic Stem Cells Cultured on PET Membranes Decorated with Multi-wall Carbon Nanotubes

Abstract

Embryonic stem cells (ESCs) capable of self-renewal and differentiation into various cell types are good cell sources for cellular transplantation and tissue engineering. Neurons differentiated from stem cells can serve as promising cell therapy tools in treating neurological pathologies of the central and peripheral nervous systems. Although ESCs are already successfully used in repairing spinal cord injury in small animal models, in vitro differentiation of ESCs into neuronal cells has been a challenge. In this work, surface nanotopography and cell attachment were enhanced for polyethylene terephthalate (PET) membranes decorated with multi-wall carbon nanotubes (MWCNTs), which also promoted the differentiation of mouse ESCs into neuronal cells while maintaining excellent cell viability. In addition, a high degree of neurites extension and outgrowth was observed. These findings suggest that MWCNTs-decorated PET membranes hold great promises for nerve regeneration.
6.1 Introduction

Embryonic stem cells, derived from the inner cell mass (ICM) of the blastocysts, possess the ability to self-renew and differentiate into various cell types. Much effort has been dedicated to investigating the potential of ES cells or its derivatives in treating neurological pathologies of the central and peripheral nervous systems such as spinal cord injuries, Parkinson’s disease, and glaucoma. Although embryonic stem cells have been already successfully used in repairing spinal cord injury in small animal models, the ability to promote neuronal differentiation of ES cells in vitro has been a challenge. Hence, scaffolds that can mimic extracellular matrix (ECM) for nervous system repair and regeneration have attracted great attention.

In vivo, cells are surrounded by ECM that is characterized by a natural web of hierarchically organized nanofibers. The nanofibers play a vital role in directing cellular behaviors via cell-surface interactions [1]. Cell-surface interactions can be influenced by differences in surface chemistry, mechanical properties, and topography. Cell differentiation can be directed by controlling the mechanical properties of the surface as well as surface topography [2]. With the development of nanotechnology, researches to mimic the nanostructural features of ECM to regulate cell-surface interactions and promote neural regeneration have been widely carried out [3-5]. Since the discovery of carbon nanotubes (CNTs) in 1991 by Iijima et al. [6], CNTs have been at the forefront of nanotechnology due to their unique physicochemical properties, allowing the development of a variety of miniaturized devices with remarkable performance. Recently, CNTs have also gained great attention for their potential as tissue engineering scaffolds.
Carbon nanotubes, when used in nerve tissue engineering, exhibit many stimulating effects, including reestablishing the intricate connections between neurons, and guiding and enhancing the intrinsic capacity of the brain to reorganize in a controlled fashion via regulating the interactions between scaffolds and biological cell membranes [7]. The dimension, diameter and length of CNTs are analogous to those of the natural ECM proteins, including laminin and collagen [8]. The size, high electrical conductivity and aspect ratio, and large surface area of CNTs favor their interactions with distal dendrites, which promote nerve regeneration [7]. Also, CNT-based scaffolds can maintain structural integrity as they have similar mechanical properties to structural proteins found in natural ECM [8]. Recent studies have shown that CNTs could aid nerve tissue regeneration and deliver drugs to repair damaged neurons associated with disorders such as epilepsy, Parkinson’s disease, and even paralysis [9]. Furthermore, CNTs-based scaffolds [4, 5, 10-12] for neural cell proliferation were assessed for their biocompatibility and ability to promote neurites elongation and branching. Moreover, the incorporation of CNTs in scaffolds provides electrical conductivity [13, 14], which may aid in directing cell growth. In addition, several recent studies have shown that the use of an electrical field would aid neuron outgrowth. However, few studies on CNTs-based scaffolds have been focused on the investigation of differentiation of ES cells into neuronal cells [15, 16].

In the present study, the ability of MWCNTs-decorated microporous PET membranes to promote neuronal differentiation was investigated. Furthermore, the beneficial effects of carbon nanotubes on cell adhesion, cell viability and cell morphology were studied.
6.2 Materials and methods

6.2.1 Cell lines and medium

Mouse embryonic stem D3 cells (ATCC CRL-1934) were maintained on gelatin pre-coated T-flasks in the growth medium containing knockout Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 µM β-mercaptoethanol (Sigma-Aldrich), 50 U/mL penicillin (Invitrogen), 50 U/mL streptomycin (Invitrogen), and 100 µM leukemia inhibitory factor (LIF) (Millipore). The EB formation medium was the same as the growth medium but without LIF, and the neuronal differentiation medium was the same as the EB formation medium but supplemented with $10^{-7}$ mM of retinoic acid (RA, Sigma-Aldrich).

ES cells were transfected with PEGFP-N3 (Invitrogen) using Lipofectamine 2000 (Invitrogen), respectively. After an initial 10-day selection using 500 µg/mL of geneticin (G-418, Gibco), fluorescence-expressing colonies were selected using a fluorescence microscope (Fluorescence Inverted Microscope IX71, Nikon). Then, cells were subcultured in the absence of the selective pressure every 5 days for 20 passages. Stable transgenic ES cells were established and the stability of the transfected cell line was verified (>97%) using FACS Calibur (B-D Biosciences).

6.2.2 Fabrication of MWCNT-decorated PET membranes

One microgram of multiwall carbon nanotubes (Nanostructured & Amorphous Materials, Inc., Houston, TX) was dispersed in 100 ml of 0.4% gelatin solution and sonicated for 30
min. Ten milliliters of CNTs solution were filtered through the microporous PET membrane (Whatman, pore size: 1 µm) disk with a diameter of 21 mm, which could be fitted into wells on 12-well plate. Later the CNT-decorated PET membranes were rinsed with DI water three times. The gelatin-pretreated PET membranes were used as the control. Prior to fitting into wells on 12-well plate, the membranes were sterilized at 121°C for 30 min.

6.2.3 Neuronal cell differentiation

ES cells were differentiated into neuronal cells using the 4-/4+ protocol as previously reported [17]. Two million ES cells were inoculated onto a non-adherent 9-cm Petri dish containing 10 mL EB medium and incubated at 30 rpm for 4 days to form EBs in a humidified CO₂-incubator (Thermo Electron). Then, the formed EBs were exposed to neural differentiation medium and incubated for another 4 days. Medium was changed every other day. After the 8-day incubation, 30 EBs were placed into each gelatin-coated well on a 12-well plate inserted with either MWCNTs-decorated membranes or the control. Then ES growth medium was added and cells were incubated for another 2 weeks.

6.2.4 Immunocytochemical Analysis

Immunocytochemistry was performed on EBs after 2-week cell culture to identify neuronal differentiation of ES cells. Cells cultured on membranes were fixed using 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) at 4°C overnight. Cells were then permeabilized using 0.1 % Triton-100 (Sigma-Aldrich) in PBS for 30
After blocking the cell samples using 3% fetal bovine serum (FBS) in PBS for 30 min, primary antibody against β-III tubulin (TUJ1, early neuronal marker, DHSB) and nestin (Rat 401, DHSB) was added respectively and incubated at 4°C overnight. The cell samples were then washed three times with PBS to remove unbounded primary antibody. Then, goat anti-mouse R-phycoerythrin conjugated secondary antibody (Invitrogen) was applied for 1 h. The images were taken using an inverted fluorescence microscope (Fluorescence Inverted Microscope IX71, Nikon).

6.2.5 Flow cytometry analysis

To quantitatively identify neuronal differentiation, cells stained against nestin and β-III tubulin were analyzed using flow cytometry, respectively. ES cells were harvested from the membranes and dissociated into individual cells in 0.25% trypsin (Invitrogen) prior to the flow cytometry analysis. Samples were then fixed with 4% paraformaldehyde at room temperature for 20 min and later cell membranes were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min for intracellular marker assays. Next, samples were blocked using 3% FBS in PBS for 1 h. After blocking the unspecific binding sites, primary antibody against nestin and β-III tubulin were added and incubated at room temperature for 1 h. The cell samples were then washed three times with PBS to remove unbounded primary antibody. Subsequently, diluted goat anti-mouse Alexa 647 conjugated secondary antibody (Invitrogen) was applied to the cells for 1 h at room temperature. Finally, β-III tubulin and nestin positive cells were detected and quantified using BD FACS LSR II and Cell Quest software (Becton Drive).
6.2.6 Reverse Transcriptase (RT)-PCR

Total RNA was isolated from the harvested cells using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. Yield and purity of RNA were determined with a NanoDrop (NanoDrop Technologies). One µg of RNA was used as template to synthesize single-stranded complementary DNA (cDNA) using SuperScript™ III First-Strand Synthesis System (Invitrogen). Then the cDNA was amplified using Taq DNA polymerase (Promega) with 10 µM of each of the forward and reverse primers listed in Table 6.1. RT-PCR products were separated on a 3% agarose (Fisher Scientific) gel at 70 V for 60 min. Then DNA was stained with ethidium bromide (Sigma-Aldrich) for visualization and photographed with a Gel Doc 2000 Gel Documentation System (Bio-Rad).

6.2.7 Statistical analysis

Student’s t test analysis of the percentage of β III tubulin and nestin positive cells was performed using JMP version 7.0, with $p < 0.05$ as the threshold for significant difference.

6.2.8 Analytical Methods

Trypan blue exclusion method with the aid of a hemocytometer (Superior, Marienfeld, Germany) was used to determine cell viability.
6.2.9 Scanning electron microscopy

Samples were rinsed with PBS three times, fixed in 2.5% glutaraldehyde (Sigma-Aldrich) at 4°C overnight and then progressively dehydrated in 10%, 30%, 50%, 70%, 90% and 100% ethanol for 10 min, respectively. After dehydration, samples were soaked in hexamethyldisilazane (HMDS) (Sigma-Aldrich) for 1 min, and then placed in a desiccator for a week. Finally, the chemical-dried samples were sputter-coated with gold and scanning electron microscopy (SEM, FEI) was used to study cell morphologies on the membranes.

6.3 Results

6.3.1 Scaffold characterization

Figure 6.1A shows that PET (white) and MWCNTs-decorated PET (black) membranes. SEM images were used to study the surface structures of both scaffolds (Figures 6.1B and 6.1C). Figure 6.1B shows that the surface of PET membrane was full of homogenous pores with a diameter of 1 µm. On the other hand, Figure 6.1C displays the PET membrane with MWCNTs deposited, which increased the nanoroughness.

6.3.2 Cell adhesion

Figure 6.2 shows that cell adhesion in MWCNTs-PET membranes was significantly enhanced compared to the control (p = 0.0013). After one-hour incubation in CO₂-incubator, about 23% EBs were attached to the PET membrane, whereas 74% EBs were attached to the MWCNTs-PET membrane. Carbon nanotubes can offer nanofibril
surfaces, mimicking the characteristics of ECM, which enhances growth factor adsorption and thus increase cell adhesion.

**6.3.3 Cell viability**

In order to investigate the biocompatibility of the scaffold, cell viability needed to be examined. Figure 6.3 indicates that after 2-week cell culture cell viability in the MWCNTs-PET membrane was enhanced as compared to that in PET membrane. This result suggests that this scaffold has beneficial effect on cell growth, providing a microenvironment similar to *in vivo* microenvironments for cells to survive and differentiate.

**6.3.4 Cell morphology**

Figure 6.4 include a set of SEM images displaying differentiated cell morphologies in the MWCNTs-PET and PET membranes. EBs were attached to the membranes and the peripheral cells displayed neural differentiation first. In addition, numerous cells migrated out of the EBs to the peripheral area and the cells were differentiated into neuronal cells. Figures 6.4A-C display that cells were flatly spread out on the non-decorated membranes and few neurites were observed. However, on the MWCNTs-decorated membranes, more neurons were observed and the neurons grew across the surfaces of carbon nanotubes and neural network was formed (see Figure 6.4D). In addition, neurites bridges were formed between adjacent neuronal cells (see Figure 6.4E). Also, MWCNTs-PET scaffolds improved neurites outgrowth and branching (see Figure 6.4F).
6.3.5 Neuronal differentiation of ES cells

Immunocytochemical analysis was used to characterize neuronal differentiation of ES cells. After EB attachment and being cultured for another 10 days, the differentiated cells were immunostained with primary antibodies against β-III tubulin. β-III tubulin contributes to microtubule stability in neuronal cell bodies and it is regarded as a marker to identify differentiated neurons. Figure 6.5 is a set of fluorescent images displaying the morphology of differentiated cells. Figure 6.5B and 6.5D were taken after staining with antibodies against β-III tubulin. Abundant expression of β-III tubulin was visible when cells were cultured on the MWCNTs-PET membranes. Also, β-III tubulin was in the radial direction of EBs, as indicated with an arrow (Figure 6.5D). Figures 6.5A and 6.5D are the corresponding GFP images for Figures 6.5B and 6.5E, respectively. Figures 6.5C and 6.5F demonstrate that more abundant β-III tubulin positive cells were present in MWCNTs-PET membranes.

FACS analyses of β-III tubulin and nestin were performed to further demonstrate enhanced neuronal differentiation on the MWCNT-decorated PET membranes. After 2-week cell culture, cells were stained and analyzed for β-III tubulin and nestin. The quantitative results from FACS analysis of protein expressions are shown in Figure 6.7. 3% of cells cultured in either PET or MWCNTs-PET membrane were nestin positive, whereas around 90% of the cells were β-III tubulin positive in the MWCNTs-PET membranes and 80% positive when cultured in the control. A majority of cells expressed β III tubulin in both scaffolds. Furthermore, percentage of β III tubulin positive cells in the MWCNTs-PET membranes was significantly higher (p = 0.0016) than that in the
control, whereas the percentages of nestin positive cells was not significantly influenced (p = 0.1162). For both membranes, a small fraction of cells were nestin positive. Nestin is an intermediate filament structural protein and it is used to determine neural precursor cells. Nestin is expressed in the early stage of neuronal precursor cells and its expression is down-regulated and replaced by neuron-specific makers such as β-III tubulin upon further differentiation [18].

Differentiation of ES cells into neurons was further confirmed using RT-PCR analyses of specific gene expressions (β-III tubulin, Nestin, and Nurr1) and ES-specific marker (Oct4) (see Figure 6.7). The expression of Oct4 was strong in undifferentiated ES cells, and was down regulated, as indicated by the weak expressions in both scaffolds when they had undergone differentiation. Abundant expression of β-III tubulin was observed in both scaffolds. In addition, the expression of nestin was detected in both scaffolds and the level of expression in MWCNTs-PET scaffolds was slightly higher than that in the control, which was consistent with FACS analysis. Furthermore, Nurr1, a marker of mesencephalic dopaminergic neuron, was detected. Its expression can promote the further differentiation of neural precursor cells into neuronal cells, which may explain the low percentage of nestin positive cells and high percentage of β-III tubulin positive cells. These results indicate that MWCNTs-PET materials can offer desirable chemical and physical cues for the neuronal differentiation of ES cells.
6.4 Discussion

Nerve tissue engineering has emerged as a promising strategy for rebuilding the lesioned circuits of the central and peripheral nervous systems. Current trends in nerve regeneration are directing towards developing scaffolds with nanotopography to regulate cell-surface interactions and thus promote neuronal regeneration. This study presents the development of MWCNTs-decorated PET scaffolds. Figure 6.1C shows that the membrane was covered with MWCNTs, exhibiting enhanced surface nanoroughness. Compared to the control with only gelatin coating, MWCNTs increased cell adhesion efficiency. Surfaces with different topographies have been fabricated to mimic the in vivo microenvironments and shown to affect cell attachment [19]. Structurally, native ECM comprises various nanoscale fibers and molecules. Carbon nanotubes, resembling ECM, provide mechanical support, and serve as cell-cell interaction mediators in the cell adhesion process. The most reported cellular effects of nanofibrous topography are on cellular attachment and spreading [20]. The increase in the surface roughness is effective for the initial cell adhesion. Increased cell attachment and growth can be attributed to protein adsorption. Surface energy increased with an increase in the surface roughness, and proteins would be attracted first to favorably adsorb onto the surface before cells can be physically laid on top of it [21]. The high surface energy on the ridges of rough surfaces could also assist cell proliferation by improving protein adsorption [22-24]. Therefore, carbon nanotubes have positive effects on cell growth.

Furthermore, the effect of MWCNTs on cell viability was investigated. Recent researchers reported that carbon nanotubes in suspension are detrimental to cells [25, 26].
However, Hu et al. reported good neural cell viability on MWCNTs deposited on the polyethylenimine-coated cover glass [27]. In this study, gelatin-modified carbon nanotubes were deposited on the surface of the PET-membrane. No adverse effect on cell growth was observed. Cell viability on the MWCNT-decorated PET scaffolds was higher than gelatin-coated PET surface, suggesting the beneficial effect of CNTs on cell survival. This study indicates that the MWCNTs-PET membranes may have positive influence on cell survival and can serve as promising candidates in neural cell differentiation.

As shown in Figure 6.4, the differentiated neurons on MWCNTs-PET surface exhibited a substantially high degree of neurites extension and outgrowth. Nerve fibers can be entangled with carbon nanotubes, since the dimension of carbon nanotubes is similar to that for nerve fibers [12]. In addition, cells on the modified surface exhibited a nerve network morphology. It is reported that neuron-like cells cultured on nanotextured surfaces exhibited a substantially higher degree of neurite extension [28]. In addition, neurite outgrowth, which usually requires basal proteins, was also demonstrated on top of PLLA (poly-L-lactic acid) nanostructural surfaces [29]. Besides, mature neurons were generated on PAA-g-CNT (poly acrylic acid grafted CNT) thin film [16]. These studies demonstrated that CNTs-based scaffolds have great effects on cell adhesion and morphology critical to nerve regeneration. In addition, the MWCNTs-PET membrane exhibit significant enhancement in neuronal differentiation from FACS analysis. The capability of CNTs to promote neuronal differentiation for mES cells might be a result of the increase in protein adsorption and cell adhesion [16]. The enhanced differentiation
suggests that the introduction of CNTs into scaffolds may be an effective strategy in promoting neuronal differentiation.

6.5 Conclusions

The present investigation demonstrated that carbon nanotubes can enhance cell adhesion and neuronal differentiation of mES cells. Besides, cell viability in the MWCNTs-decorated PET membranes was enhanced as compared to that in the control, demonstrating the beneficial effects of carbon nanotubes on cell growth. Furthermore, the incorporation of carbon nanotubes in PET membranes enhanced neurites outgrowth and branching. The MWCNTs-PET membranes can offer desirable nanocues on cell adhesion and neuronal differentiation. Therefore, the CNTs-based scaffolds coupling its electrical property hold great promises for enhancing the restoration of lost nerve functions.
6.6 References


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Table 6.1. Primes and annealing temperatures used in RT-PCR
Figure 6.1. Images of the scaffolds. (A). Photos of MWCNTs-PET and PET membranes compared to one dime coin; SEM images showing the surface of PET scaffolds (B) and MWCNTs-PET scaffolds (C).
Figure 6.2. Comparison of cell adhesion efficiency (relative to PET membranes) in MWCNTs-PET and PET membranes. Note: * indicates significant difference.
Figure 6.3. Comparison of cell viability in MWCNTs-PET and PET membranes. Note: * indicates significant difference.
Figure 6.4. SEM images showing differentiated cell morphologies in PET (A-C) and MWCNTs-PET (D-F) membranes.
Figure 6.5. Immunohistochemistry performed on EBs after 10-day neuronal differentiation of ES cells in MWCNT-decorated PET membranes. (A) and (D) are corresponding respectively GFP images for (B) and (E); β III tubulin stained cells cultured in PET (B) and MWCNTs-PET (E) membranes; (C) is merged image of (A) and (B), while (F) is merged image of (D) and (E). Scale bar indicate 200 µm.
Figure 6.6. RT-PCR analysis of gene expressions after neuronal differentiation of ES cells in MWCNTs-PET and PET membranes.
Figure 6.7. Comparison of β-III tubulin and nestin expression in neural differentiation of mES cells in MWCNTs-PET and PET membranes. Note: * indicates significant difference.
Chapter 7: Multiwall Carbon Nanotubes (MWCNTs)-Decorated Polyethylene Terephthalate (PET) Fibrous Matrices for Enhanced 3-D Cell Cultures and Functions

Abstract

Much effort has been dedicated to developing three-dimensional (3-D) scaffolds with nanofeatures for cell cultures in tissue engineering and drug screening. In this study, nonwoven polyethylene terephthalate (PET) fibrous matrices were decorated with multiwall carbon nanotubes (MWCNTs). Cellular functions of Chinese Hamster Ovary (CHO) and mouse Embryonic Stem (mES) cells were investigated, respectively. Statistical analysis of specific growth rates indicates that cell proliferation was significantly enhanced, suggesting that MWCNTs had beneficial effects on cell growth. In addition, different cell morphologies were observed. Generally, cells cultured in MWCNTs-decorated PET scaffolds were stretched and well spread out, whereas cells in non-decorated PET scaffolds were sporadically distributed. Furthermore, the interactions between scaffolds and cells were enhanced because of the presence of filopodia in MWCNTs-decorated PET matrices. Besides, neuronal differentiation of mES cells in the 3-D nanoengineered scaffolds was improved. Also, neuronal cells differentiated from mES cells in the scaffolds with nanotopography formed nerve network around the fiber.
and neurites bridges. These findings suggest that the MWCNT-decorated PET fibrous matrices can serve as functional biological scaffolds for tissue engineering.

### 7.1 Introduction

Since the discovery of carbon nanotubes (CNTs) in 1991 by Iijima et al. [1], CNTs have attracted extensive attention in the field of biomedical materials, biosensors, drug delivery and tissue engineering because of their high chemical, electrical and biochemical properties. In particular, CNTs are employed as promising scaffolds in tissue engineering [2-19]. Carbon nanotubes exhibit similar dimension to major components in the natural extracellular matrix (ECM), including collagens and laminins, which form fibrils with diameters ranging from 260 nm to 410 nm [20]. These fibrils are important for cellular functions and the mimics of their structure can enable the scaffolds to better resemble in vivo microenvironments. Additionally, the structural integrity of cells can be maintained, as the mechanical compliance of CNTs is similar to structural proteins in natural ECM [2].

Furthermore, the incorporation of CNTs in scaffolds provides electrical conductivity [3-4], which aided in promoting cell growth. Several studies revealed that the use of an electric field would aid bone regeneration, neural regeneration and wound healing [5-7]. Polymer grafted carbon nanotubes films [2], CNTs-coated surfaces [8-9], CNTs sheets [10-11] and patterned carbon nanotubes on polymer surfaces [12-13] were developed to investigate protein absorption and cell responses including cell morphology, proliferation and differentiation. However, these CNT-based scaffolds are two-
dimensional (2-D) materials that will lead to a loss of cell morphology and result in errors in predicting tissue-specific responses.

3-D cell culture technologies empower drug screening and tissue engineering by providing better mimics of in vivo microenvironments than 2-D cell culture and enable the use of primary and stem cells in drug discovery and bioprocess development. Therefore, the next-generation scaffolds for cell culture should incorporate 3-D features and nano-scale architecture to offer adequate physiological behavior in vitro for directing cell-matrix and cell-cell interactions as well as pore properties for mass transport [21]. Recently, a variety of nano-engineered 3-D polymer materials have been developed by attaching CNTs to porous scaffolds, such as polypropylene fumarate (PPF) [6], collagen [14-16], and polyurethane foams [17]. Besides, Correa-Duarte et al. fabricated thin film networks of carbon nanotubes with 3-D sieve architecture to investigate the extensive cell growth, the spreading and the adhesion of the common mouse fibroblast cell line L929 [18]. Also, Abarrategi et al. developed microchannel porous scaffolds composed of a major fraction of multiwall carbon nanotubes (89 wt%) and a small portion of chitosan and reported enhanced cell differentiation of the C2C12 cell line into osteoblastic lineage, suggesting the potential application of the scaffolds for bone regeneration [19]. However, the lack of interconnectivity of those nanostructured 3-D materials hinders oxygen and nutrients transfer.

In this study, using nonwoven PET fibrous matrices as the base substrate, the PET surface was decorated with CNTs to provide nano architectures. Cellular functions (cell proliferation, morphology and differentiation) of EGFP-cells (epithelial-like CHO cells
and mouse embryonic stem cells) were investigated in both the 3-D MWCNT-based nonwoven PET fibrous matrices and PET matrices. This is the first demonstration studying cell proliferation, morphology and differentiation in MWCNTs-based fibrous matrices.

7.2 Materials and methods

7.2.1 EGFP cell lines and medium

Chinese Hamster Ovary (CHO) cell line (ATCC CRL-11398) was maintained in DMEM/F12 (Gibco) (1:1) with a supplement of 5% FBS (Gibco). In addition, mouse embryonic stem D3 cells (ATCC CRL-1934) were maintained on gelatin pre-coated T-flasks in the growth medium containing knockout Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 µM β-mercaptoethanol (Sigma), 50 U/mL penicillin (Invitrogen), 50 U/mL streptomycin (Invitrogen), and 100 µM leukemia inhibitory factor (LIF) (Chemicon). Both cell lines were transfected with PEGFP-N3 (Clontech) using Lipofectamine 2000 (Invitrogen). After an initial 10-day selection using geneticin (G-418, Gibco), fluorescence-expressing colonies were selected using a fluorescent microscope. Then, cells were subcultured in absence of the selective pressure every 5 days for 20 passages. Finally, transgenic cells with stable EGFP expression were established and the stability of the transfected cell line was verified (>97%) using FACS Calibur (B-D Biosciences).
7.2.2 Fabrication of MWCNTs-decorated PET fibrous materials

Multiwall carbon nanotubes (Nanostructured & Amorphous Materials, Inc., Houston, TX) were directly dispersed in 0.4% (v/v) gelatin solution and homogenous MWCNTs suspension at a concentration of 0.1 mg/ml was formed, which could be stable for 2 months. The disk-shaped PET materials with a diameter of 6 mm were soaked into the MWCNTs suspension, followed by sonication for 30 min. Then the treated scaffolds were rinsed with PBS several times to remove the residual MWCNTs. Prior to use for cell culture, the scaffolds were sterilized in an autoclave at 121 °C for 30 min. In addition, sterilized PET matrices soaked in 0.4% gelatin were used as controls.

7.2.3 3-D cell cultures of EGFP cells

3-D cell cultures of EGFP-cells were conducted in a previously developed platform with improved reproducibility based on a commercial 96-well plate [22]. Before seeding cells, both PET matrices and MWCNTs-decorated PET matrices were immersed in the culture medium and kept at 4 °C overnight. Each scaffold was then seeded with 25 μL of cell suspension (1 million cells per mL) and placed into the modified 96-well plate, followed by 6-h incubation in a humidified CO2-incubator (Thermo Electron), allowing cells to attach to the fibrous matrix. Finally, 3 mL growth medium was added to each well and then the platform was placed on an orbital shaker (Belly Button Shaker, Stovall, Greensboro, NC) at a rotating speed of 90 rpm in the humidified CO2-incubator.
7.2.4 Neuronal cell differentiation

ES cells were differentiated into neuronal cells using the 4/-4+ protocol as previously reported [23]. Two million ES cells were inoculated onto a non-adherent 9-cm Petri dish containing 10 ml EB medium (ES growth medium without LIF) and incubated at 30 rpm for 4 days to form embryoid bodies (EBs). Then the EBs were exposed to differentiation medium containing 10^{-7} M retinoic acid to induce neural cell differentiation for another 4 days. Medium was changed every other day. Then, 10 EBs were seeded into one MWCNTs-decorated scaffold and the control and incubated for another 2 weeks. ES growth medium was then added and was changed every other day.

7.2.5 Flow cytometry analysis

To quantitatively identify neuronal differentiation, cells stained against β-III tubulin, a neuron-specific marker, were analyzed using flow cytometry. ES cells were harvested from the 3-D scaffolds and dissociated into individual cells in 0.25% trypsin (Invitrogen) prior to the flow cytometry analysis. Samples were then fixed with 4% paraformaldehyde at room temperature for 20 min and later cell membranes were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min for intracellular marker assays. Next, samples were blocked using 3% fetal bovine serum (FBS) in PBS for 1 h. After blocking the unspecific binding sites, primary antibody (E7, DHSB) against β III tubulin were added and incubated at room temperature for 1 h. The cell samples were then washed three times with PBS to remove unbounded primary antibody. Subsequently, diluted goat anti-mouse Alexa 647 conjugated secondary antibody (Invitrogen) was applied to the cells for 1 h at room temperature. Finally, β-III tubulin positive cells were
detected and quantified using BD FACS LSR II and Cell Quest software (Becton Drive).

7.2.6 Quantification of cell proliferation

Cell proliferation was quantitatively monitored in a high-throughput manner using TECAN GENiosProTM at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Cell density increases linearly with the fluorescence density [22]. Using this method, dynamic and non-invasive assessment of cells can be achieved. Furthermore, the specific growth rate was calculated from the growth curves based on a simple equation \( \frac{dX}{dt} = \mu X \) where \( X \) is cell biomass recorded as normalized fluorescence, \( t \) is time and \( \mu \) is specific growth rate during exponential growth phase.

7.2.7 Raman spectrascopy

The Raman spectra were collected on a Renishaw Invia Raman spectrometer. The excitation source at 514.5 nm was generated using the 514.5 nm diode laser with maximum power of 500 mW.

7.2.8 Statistical analysis

Student’s t test analysis of data was performed using JMP version 7.0, with \( p < 0.05 \) as the threshold for significant difference.

7.2.9 Scanning electron microscopy

Samples were rinsed with PBS three times, fixed with 2.5% glutaraldehyde overnight at 4 °C and then progressively dehydrated in 10%, 30%, 50%, 70%, 90% and 100% ethanol
(v/v) for 10 min, respectively. After dehydration, samples were soaked in hexamethyldisilazane (HMDS) (Sigma) for 1 min. Finally, the chemical-dried samples were sputter-coated with gold and scanning electron microscopy (SEM, FEI) was used to study cell morphologies in the PET matrices and MWCNTs-decorated PET matrices, respectively.

7.3 Results

7.3.1 Scaffold characterization

The fabricated MWCNTs-decorated fibrous PET matrices were characterized using SEM. As shown in Figure 7.1A, the scaffolds fitted into 96-well plate were disk-shaped (diameter: 6 mm, thickness: 1 mm). Figure 7.1B shows the microstructure of the nonwoven PET matrices, revealing good interconnectivity and porosity. The high surface area and excellent interconnectivity within pores could enable essential nutrients and oxygen transfer, high cell density and cell migration. Figures 7.1C and 7.1D display the surface topography of the PET matrices and the MWCNTs-decorated matrices, respectively. The surface without carbon nanotubes was smooth with few blemishes, while MWCNTs were distributed on the surface of every individual fiber of the decorated matrices with increased nanoroughness. To further confirm the presence of MWCNTs, Raman spectroscopy was used. MWCNTs have distinct Raman resonances, namely D and G modes observed at 1330 and 1580 cm\(^{-1}\), respectively. Figure 7.2 shows the presence of D and G bands in MWCNTs-decorated PET scaffolds, confirming the presence of traces of MWCNTs.
7.3.2 Cell morphology

CHO and mouse ES cells were used to demonstrate the feasibility of the MWCNTs-decorated PET matrices as scaffolds in mimicking the in vivo microenvironments. Fluorescent images in Figure 7.3 indicate that both the epithelial-like cells and ES cells cultured on the non-decorated PET matrices were distributed sporadically, whereas those cultured on the MWCNT-decorated PET scaffolds were well stretched and elongated along the direction of the fiber. SEM images (Figures 7.4 and 7.5) confirm that cells were well expanded, stretched, and attached tightly on the modified surface. More cells were present on the decorated surface (see Figure 7.4C) than the controls (see Figure 7.4A). Cells were entrapped in the fibrous scaffold and adhered to the surface of the scaffold. After attaching to the surface of MWCNTs-decorated scaffold, cells grew faster compared to the control. Besides, the images at higher magnification reveal that more filopodia were present in decorated surface than nondecorated one (see Figures 7.4B and 7.4D). Cell-matrix interactions were enhanced due to the force between the filopodia and nanofeatures on the surface of the scaffolds provided by MWCNTs. Cells-matrix and cell-cell interactions also existed and cells tended to form a colony through filopodia.

7.3.3 Cell proliferation

Cell growth kinetics was evaluated as a function of normalized fluorescence. Figures 7.6A and 7.6C show the growth kinetics for CHO cells and mES cells, respectively. Both CHO and mES cells grew faster in the MWCNTs-decorated PET matrices as compared to
the control. In addition, Figures 7.6B and 7.6D indicate the comparison of the specific growth rates of CHO and mES cultured on the non-decorated scaffold and MWCNTs-decorated one, respectively. Statistical analysis indicates that the specific growth rate was statistically increased when CHO cells were cultured in the MWCNTs-decorated PET matrices ($p = 0.0194$). Similarly, mES cells cultured in the MWCNTs-decorated PET scaffold proliferated faster than those cultured in the control ($p = 0.0081$). CNTs can be designed to mimic the extracellular matrix proteins such as laminin and collagen, and therefore provide as better mimics of in vivo microenvironments in cell culture. The enhanced cell proliferation compared to non-decorated matrices indicates that the MWCNTs-decorated PET matrices may have positive influence on cell growth and could serve as promising materials in tissue engineering.

7.3.4 Cell differentiation

Besides the effects of carbon nanotubes on cell proliferation and morphology, its role in cell differentiation was also investigated. Neuronal differentiation in both scaffolds was confirmed with FACS analyses of β-III tubulin. After 2-week cell culture, cells were stained against β-III tubulin. The quantitative results from FACS analysis of protein expressions are shown in Figure 7.7. Around 71% of the cells were β-III tubulin positive cells in the MWCNT-decorated PET matrices, whereas 60% positive when cultured in the control. Therefore, the decoration of PET matrices using MWCNTs enhanced neuronal differentiation of ES cells.

Furthermore, SEM images of cells (see Figure 7.8) in MWCNTs-decorated PET matrices illustrate that neurites network was formed around the fiber (see Figure 7.8A).
Besides, neural cells were stretched and expanded between fibers and cells (see Figures 7.8B and 7.8C). Furthermore, neurites bridges were created between neuronal cells or between neuronal cells and scaffolds (see Figures 7.8C and 7.8D).

7.4 Discussion

Compared to conventional 2-D flat surfaces, 3-D cell cultures provide another dimension for cells to adhere and grow, and thus can support a long-term cell culture. In addition, a natural tissue scaffold comprises nanofibrous materials that provide a nanotextured network and 3-D physical/structural support. Consequently, 3-D fibrous scaffolds are favorable as a replacement for natural scaffolds because of their high porosities (up to 95%), isotropic structures, homogeneous fiber distribution and fiber size, and homogeneous pore distribution [24-26]. Although 3-D fibrous scaffolds can support cell adhesion and cell proliferation, they generally lack the topographical features needed to resemble native tissue ECM proteins, such as laminin and collagen, which exhibit nanoscale characteristics that are important for cellular functions [20]. In this work, CNTs were incorporated in the PET fibrous matrices to provide nanocues. The 3-D nanoengineered scaffolds provide a promising platform for cell to adhere, proliferate and differentiate.

Figure 7.6 shows that cell proliferation was significantly improved in the 3-D nanoengineered scaffolds, revealing the positive effects of CNTs on cell growth. Carbon nanotubes, resembling ECM, provide cells with mechanical support, and serve as a cell-cell interaction mediator in the cell adhesion process. The most reported cellular effects of nanofibrous topography are on cellular attachment and spreading [27]. The
introduction of CNTs to PET fibrous matrices increased the nanoroughness of the surface. The increase in the surface roughness would be effective for the initial cell adhesion. Furthermore, surface energy increased with an increase in the surface roughness, and proteins would be first attracted to favorably adsorb onto the surface before cells can be physically laid on top of it [28]. The high surface energy on the ridges of rough surfaces could assist cell proliferation by improving protein adsorption [29-31]. In addition, Figures 7.4 and 7.5 illustrate that cells were stretched and tightly attached to the surface, signifying good cell attachment and thus promoting cell proliferation. This is in agreement with a previous study that cell adhesion on the MWCNTs-decorated surface was strong because of the interactions between carbon nanotubes and pseudopods [9].

Besides cell proliferation and cell morphology, cell differentiation is another important parameter for evaluating the potential of biomaterials for tissue engineering. The capacity of mES cells to undergo neuronal differentiation in the 3-D nanoengineered scaffold was assessed. Figure 7.7 shows that more cells were differentiated into neurons than the control, suggesting CNTs could promote neuronal differentiation of ES cells. This is in agreement with a previous study that cell differentiation can be directed by controlling the mechanical properties of the surface as well as surface topography [19]. The enhanced differentiation suggests that the use of carbon nanotubes may be an effective strategy to regenerative medicines and the nanofeatures created by CNTs play an important role in tissue-specific stem cell differentiation. Furthermore, Figure 7.8 displays that differentiated cells cultured on MWCNT-decorated surfaces formed nerve
network and bridges. These findings further demonstrate that nanofeatured scaffolds have great effects on cell adhesion and morphology critical to nerve regeneration.

Although the application of CNTs in 3-D scaffolds seems to have great potential in tissue regeneration, there are still concerns about the adverse effects of carbon nanotubes on human life and the environments [32-33].

7.5 Conclusion

MWCNTs were used to decorate the surface of the non-woven PET matrices to provide nano features. Cells responded to nanofeatures on the surface. ESCs cultured in 3-D nonwoven fibrous PET matrices coated with CNTs showed enhanced cellular functions, including proliferation, morphology and differentiation, compared to cells cultured in the nondecorated matrices. In addition, neural network and bridges were formed in the MWCNTs-decorated PET matrices. With increased biological relevance, cells cultured in 3-D PET matrices coated with CNTs can provide more representative cellular responses to drugs.
7.6 References


Figure 7.1. Images of the scaffolds. (A). Photos of MWCNTs-decorated and non-decorated PET matrices compared to one dime coin; SEM images showing the microstructure of the PET fibrous scaffolds (B), the surface of non-decorated PET matrices (C) MWCNTs-decorated PET matrices (D).
Figure 7.2. Raman spectrometry for MWCNTs, MWCNTs-decorated and non-decorated PET matrices.
Figure 7.3. Fluorescent images displaying cell morphologies of EGFP-cells. CHO cells in non-decorated PET matrices (A) and MWCNT-decorated PET matrices (B); ES cells in non-decorated PET matrices (C) and decorated PET matrices (D).
Figure 7.4. SEM images showing CHO cell morphologies in non-decorated PET matrices (A) and in MWCNT-decorated PET matrices (C); (B) was the magnified image of the boxed area in (A) and (D) was the magnified image of the boxed area in (C).
Figure 7.5. SEM images showing mES cell morphologies in non-decorated PET matrices (A), in MWCNT-decorated PET matrices (C); (B) was the magnified image of the boxed area in (A) and (D) was the magnified image of the boxed area in (C).
Figure 7.6. Growth kinetics files for CHO cells (A) and mES cells (C); comparison of specific growth rates for CHO cells with $p=0.0194$ (B) and mES cells with $p=0.0081$ (D) in MWCNT-decorated and non-decorated PET matrices. Note: * indicates statistical difference.
Figure 7.7. Comparison of β III tubulin positive cells in neuronal differentiation of mES cells in MWCNTs-decorated and non-decorated PET matrices. Note: * indicates statistical difference.
Figure 7.8. SEM images showing differentiated cell morphologies in MWCNT-decorated PET-matrices.
Chapter 8: Conclusions and Recommendations

8.1 Conclusions

Compared to conventional 2-D cell cultures, 3-D cell cultures can provide more in vivo relevant responses to drug treatments and thus better bridge the gap between single cells and native tissues. In the dissertation, a 40-MBR system as a 3-D high throughput platform capable of real-time quantification of cell proliferation, pH and DO was developed. The potential applications of the platform in early-stage drug screening were demonstrated. In addition, cytotoxicity and embryotoxicity of chemicals and drugs were investigated in the 40-MBR using a fluorescent survivin-based ES model. The model was demonstrated and validated as a reliable tool for assessing chemicals and pharmaceuticals associated with developmental or reproductive toxicity. Furthermore, in order to better mimic in vivo microenvironments and provide more tissue-specific responses in drug screening, carbon nanotubes were introduced to 2-D and 3-D PET scaffolds. Cellular functions including cell adhesion, proliferation, and differentiation were enhanced in the nanoengineered scaffolds. The following sections summarize the major findings.

8.1.1 Microwell bioreactor system for cell-based high throughput proliferation and cytotoxicity assays

3-D cell culture is superior to 2-D cell culture in cell-based high throughput screening because 3-D can better mimic in vivo microenvironments and thus provides a better link
between single cells and tissues. A 40-MBR system for 3-D cell cultures was developed with the capacity of online monitoring of cell proliferation, pH and DO. Cytotoxicity studies of sodium butyrate demonstrated that this platform could provide reliable data with reduced variations and improved reproducibility as compared to 384-MWP. Furthermore, dosage effects of potential anticancer candidates were investigated in the 40-MBR, suggesting its promising application in early-stage drug screening.

8.1.2 Embryonic stem cell models for high throughput screening of cytotoxic and embryotoxic compounds

Prior to marketing of a drug, its possible harmful effects on pregnant women need to be assessed. Although cytotoxicity of chemicals could be assessed in the 40-MBR, developmental toxicity could be caused either by cytotoxicity or embryotoxicity. Therefore, embryotoxicity screening is also of great importance in determining developmental toxicity. In order to estimate embryotoxicity, stable transgenic ES cells with a survivin promoter upstream of enhanced green fluorescence protein (EGFP) reporter gene were established. Online monitoring was achieved by fluorometrically quantifying EGFP in a commercially available well plate reader, allowing high-throughput and time-series data acquisition. The integration of the model with the developed cytotoxicity model enabled the fast screening of compounds associated with developmental toxicity. Furthermore, developmental toxicity of three Chinese herbal medicines, including *Ginkgo Biloba* extract (GB), *Ganoderma Lucidum* spore powder (GLS), and *Epimedium brevicornum* (EB) was assessed using the model. These studies
suggest that the model serves as a robust high-throughput tool to evaluate embryotoxicity and cytotoxicity of chemical compounds.

8.1.3 Enhanced neuronal differentiation of ES cells in nanoengineered PET membranes

In order to provide more *in vivo* responses to drug treatment, it is necessary to introduce some features to mimic the natural tissue scaffold. A natural tissue scaffold comprises of nanofibrous materials that provide a nanotextured network. In addition to its function as a structural template, the nanofibrous scaffold increases the efficiency of cell-cell interactions and provides a template to support the interactions among proliferating cells. Carbon nanotubes, exhibiting similar dimension to natural ECM, can enhance cell adhesion and neuronal differentiation of mES cells. Besides, CNTs played a positive role in cell survival, suggesting the biocompatibility of carbon nanotubes. Furthermore, the incorporation of carbon nanotubes in PET membranes increased neurites outgrowth and the degree of neurites branching. The MWCNTs-PET membranes can offer desirable chemical and physical cues on cell survival, adhesion and neuronal differentiation. Therefore, the CNT-based scaffolds coupling its electrical property hold great promises for enhancing the restoration of lost nerve functions.

8.1.4 3-D nanoengineered fibrous scaffolds for tissue engineering

The next-generation tissue scaffolds should incorporate 3-D structures and nanofeatures to mimic the physiological microenvironments. In this study, PET fibrous scaffolds were used as 3-D structural support and MWCNTs were used to provide nanofeatures on the surface of the non-woven PET matrices. Cells responded to nanofeatures on the surface.
Compared to the scaffolds without carbon nanotubes, cells cultured on MWCNTs-decorated PET matrices were more spread out and stretched. Moreover, cell proliferation in the decorated scaffolds was significantly enhanced. Neuronal differentiation was also increased in the decorated matrices. Furthermore, neural network and bridges were formed in the MWCNTs-decorated PET matrices. This study indicates that the MWCNT-decorated nonwoven PET matrices can serve as promising scaffolds in tissue engineering.

8.2 Recommendations

The integrated fluorescence systems (survivin-EGFP and CMV-EGFP) for embryotoxicity screening might cause variations due to experimental operations. Therefore, further studies should focus on developing a dual-fluorescence system, which enables real-time quantification of cell proliferation (RFP) and survivin expression (EGFP) in the same system simultaneously. Furthermore, in order to discover the capabilities and limitations of the model, a larger pool of chemicals needs to be tested using this system. In addition, the application of survivin-EGFP could be extended in anticancer drug screening. The specific expression of survivin in malignant cancers makes survivin an ideal diagnostic marker for early cancer development and a special target for cancer treatment. The suppression of survivin production can make cancer cells more susceptible to apoptosis during chemotherapy. Its specific properties make anti-survivin therapy, which shows efficacy without overt toxicity, a promising cancer treatment strategy. Therefore, transgenic cancer cell lines with EGFP expression under a survivin promoter can be used to study cancer specific effects of drugs and provide more reliable data in predicting in vivo responses to drug treatment than conventional
cytotoxicity assays.

Furthermore, in order to better recapitulate in vivo responses to drug treatment, 3-D nanoengineered scaffold to mimic in vivo microenvironments was developed. It was demonstrated that nanofeatures in the scaffolds enhanced cell adhesion, proliferation, and differentiation, suggesting the beneficial effects of CNTs-based scaffolds. Therefore, the incorporation of 3-D nanoengineered scaffolds into cell-based assays in drug screening process is desirable. With the increased biological relevance, cells cultured in the nanoengineered scaffolds can provide highly predictive in vivo responses in drug discovery and thus minimize animal tests and accelerate drug discovery process.

Besides potential applications of the nanoengineered scaffolds in drug screening, CNTs-based scaffolds can also be used in nerve regeneration, affording a permanent solution to damaged organs or tissues without relying on supplementary therapies. As possible scaffolds in nerve tissue regeneration, they should have electric and magnetic properties because electricity and magnetic field can promote nerve regeneration. The introduction of carbon nanotubes can provide conductive property. Thus, further research will be focused on the incorporation of magnetic nanoparticles in the CNT-based scaffolds to provide magnetic properties. The scaffolds with magnetic properties can be manipulated in situ using magnetic fields according to the individual patient’s needs. These next-generation tissue scaffolds can promote nerve cell proliferation and thus hold great promises in nerve regeneration.
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Appendix A: Linear correlations between cell number and fluorescence intensity in 3-D PET scaffolds and PBS suspension
Figure A.1. Fluorescence intensity is proportional to the number of EGFP expressing cells including. (A) CHO, (B) MCF-07 breast cancer, (C) HT-29 colon cancer, (D) ES-D3 with EGFP under CMV promoter, and (E) ES-D3 with EGFP under survivin promoter either in PBS suspension or in 3-D scaffolds. Each point represents the average fluorescence intensity from triplicate samples minus average fluorescence of blanks. The blanks for suspension are the fluorescence from the same volume of PBS without cells. The blanks for 3-D consist of the fluorescence signals from the same volume medium and scaffolds without cells.
Appendix B: Survivin-EGFP plasmid construction
Figure B.1. Survivin-EGFP plasmid construction.
Appendix C: SEM images showing cell morphologies when cells treated with different drugs associated with developmental toxicity
Figure C.1. SEM images for nontreated (A, B), tetracycline-treated (C, D), and acrylamide-treated (E, F) ES cells. A, C, E: magnification 200; B, D, F: magnification 600.
Appendix D: Kinetics for GFP expression during cell differentiation process
Figure D.1. (A). Kinetics of EGFP expression driven by survivin and CMV promoters; (B). Kinetics of specific survivin expression during cell differentiation process. When the 8-day old EBs were used for differentiation in gelatin-coated PET scaffolds, EGFP driven by CMV promoter was increasing faster than EGFP driven by survivin promoter (Figure D.1A). Specific survivin expression, as the ratio between EGFP under survivin promoter and EGFP under CMV promoter, was downregulated due to the differentiation for the first 50 h, whereas survivin was kept at the same level after 50 h probably because of the cease of differentiation (see Figure D.1B).
Appendix E: Flow cytometry analysis of β III tubulin positive cells when cells were differentiated in 3-D scaffolds
Figure E.1. Flow cytometry analysis of β III tubulin positive cells. (A) Cells differentiated in 3-D PET fibrous scaffolds, 52%; (B) Cells differentiated in 3-D MWCNTs-decorated PET scaffolds, 80%.