EMBRYONIC STEM CELL CULTURE IN FIBROUS BED BIOREACTOR

DISSERTATION

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ABSTRACT

Embryonic stem (ES) cells possess unlimited self-renewal ability and the potential of differentiating to all tissue organs, making ES cells the ideal source for cell therapy and drug screening. However, present culture techniques are both expensive and labor intensive, unable to meet the projected high demand for ES cells.

Three-dimensional culture is capable of mimicking in vivo growth environment and therefore, cells show their native morphology and functions. 3-D culture is superior to 2-D culture by eliminating the necessity of gelatin coating and frequent subculture. The STO cells conditioned medium is capable of replacing expensive cytokine leukemia inhibitory factor (LIF). Dynamic culture of ES cells in spinner flasks showed better cell growth and maintenance of pluripotency. Further scale up to a two stage perfusion culture system was successful, providing higher cell expansion and Oct-4 and SSEA-1 expressions. Human ES cells expansion in perfusion bioreactor also reached the success. hES cells grows faster in FBB than on 2-D surface. The hES cells pluripotency was also maintained well. However, neural differentiation efficiency was lower in 3-D culture than in 2-D system due to the higher possibility of causing de-differentiation in 3-D system. Astrocyte conditioned medium was superior to retinoic acid in inducing neural differentiation.

Hydrodynamics and its influence on cell attachment and growth in 3-D cultures were also
studied. Increased mixing intensity enhanced both cell attachment and detachment rates. Cell detachment kinetics can be described by Bell model. The interaction between Kolmogorov eddy size and the scaffold’s pore size played a vital role in affecting shear damage to cells growing in the scaffold. When Kolmogorov eddy size is smaller than the pore size, shear damage to cells is apparent.

Mathematical modeling of the fibrous bed bioreactor (FBB) was investigated too. Axial dispersion model can represent the mass transfer in ordered disc packed FBB. However, both radial and axial dispersion plus convection contribute significantly to the mass transfer in spiral wound FBB. Packing methods showed different performance in different scales. Cell aggregate size should be controlled less than at 170 \( \mu \text{m} \) if 30% DO is present in the medium and 250 \( \mu \text{m} \) if 70% DO is present.
Dedicated to My Parents and Who I Love
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Chapter 1

Introduction

1.1 Embryonic stem cells

Embryonic stem cells were isolated from inner cell mass. They possess unlimited proliferation potential and ability to differentiate into all kinds of organs. ES cells find wide applications in cell therapy and drug screening. The mammalian ES cell lines were derived from mouse blastocyst in 1981 by Evans and Martin separately. Upon first isolation, ES cells were maintained on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells with doubling time of 14 hours (Martin et al., 1981 and Evans et al., 1981). However, undifferentiated ES cell growth requires gelatin coated surface and LIF-containing medium in feeder cell free culture. Also, frequent subculture (every two or three days) is critical to avoid spontaneous differentiation. Current expansion method is labor intensive and expensive and can not meet the projected high demand. Up to now, ES cells have been successfully differentiated into neural cells, hematopoietic cells, cardiac cells, muscle cells, pancreatic cells and epidermal keratinocytes (Guan et al., 1999). Dopamine neural cell transplantation is one promising alternative for the Parkinson disease treatment. Current neural differentiation involves the use of cocktail of cytokines or co-culture with stromal cells. Nevertheless, high cost of the cytokines and hazard of feeder cell cross contamination prevent the therapy from
ordinary patients. Moreover, both methods are carried out in T-flask or multiwell. A scalable process of ES cell differentiation at low cost is required.

1.2 Bioreactors for Cell Culture

Hitherto, there are mainly four types of bioreactors: stirred tank, airlift, hollow fiber and packed bed bioreactors. Stirred tank bioreactors are widely used in industry because of their homogeneous culture environment and easiness of automatic control. However, high shear damage to animal cells limits their applications, especially in anchorage-dependent cell culture. Airlift bioreactors possess smaller shear damage and better oxygen transfer efficiency. But bubble rupture is a potential cause of cell death. Foaming is another problem in airlift bioreactors. Hollow fiber bioreactors were applied in hepatocyte cultures (Hu et al., 1997). The major mass transfer mechanism involved in a hollow fiber bioreactor is diffusion. Moreover, fouling of the hollow fiber membrane can build additional mass transfer barriers. Therefore, a large nutrient gradient and high pressure drop in hollow fiber bioreactors are usually observed, which are notoriously for scale up. The most commonly used support materials are glass beads in packed bed bioreactors. Uneven cell distribution is observed in glass beads packed bioreactor because it works as an in depth filter during inoculation.

A novel fibrous bed bioreactor (FBB) has been developed in our group (Li et al., 2003; Ma et al., 2000; Zhu and Yang 2004; Chen et al., 2002). Nonwoven PET scaffold provides cells a three-dimensional culture environment, which mimics their in vivo growth condition. Therefore, cells show their native morphology and functions in the FBB (Li et al., 2003; Ma et al., 2000; Zhu and Yang 2004; Chen et al., 2002)
1.3 Objectives

1. To develop a scalable process for embryonic stem cell expansion. Tissue transplantation and cell therapy require large amounts of the embryonic stem cells and current in vitro ES cell cultures are carried out in gelatin pretreated multiwells with frequent subculturing. These operations are both labor intensive and expensive, making it not a satisfactory solution to the projected high demand. The objective was to develop an economical process for mass production of embryonic stem cells and their derived cell lineages for cell therapy and other biomedical applications at low costs.

2. To study the hydrodynamics and momentum and mass transport phenomena in the FBB. The FBB has found many applications in microbial fermentation, biocatalysis, and cell culture processes. However, its theoretical analysis is left behind due to the complexity of the problem. The objectives were to study the effects of hydrodynamics on cell attachment and growth, to mathematically model the transport phenomena in the FBB, and to provide theoretical support for bioreactor scale up.

1.4 Scopes of study

1.4.1 Expansion of undifferentiated embryonic stem cells in a two-stage perfusion culture system (Chapter 3)

Three dimensional cultures of ES cells in PET scaffolds were proven to be superior to the traditional two dimensional cultures. Expensive cytokine leukemia inhibitory factor (LIF) was replaced with STO conditioned media for mES cells. A two-stage perfusion fibrous bed bioreactor system was developed for mass production of murine and human embryonic stem cells.
1.4.2 Neural differentiation from ES cells (Chapter 4)

Astrocyte conditioned medium was demonstrated to be superlative to retinoic acid in inducing neural differentiation from ES cells. The differentiation efficiency was lower in 3-D than 2-D cultures due to the high potential of causing de-differentiation in the 3-D system.

1.4.3 Effects of mixing intensity on cell attachment and growth in 3-D cultures (Chapter 5)

Dynamic seeding in spinner flasks significantly improved the seeding efficiency. Mixing intensity can influence cell attachment and detachment. The interaction between mixing intensity and scaffold pore size played a crucial role in the hydrodynamic damage to the cells growing in the scaffold.

1.4.4 Modeling and scale up a fibrous bed bioreactor for mammalian cell culture (Chapter 6)

Three packing methods are employed in the FBB design. Axial dispersion model can well fit the data from the ordered disc packed FBB. However, both axial and radial dispersion and convection contribute significantly to mass transfer in the spiral wound FBB. Different reactor designs have different performance under different scales. Cell aggregate size should be controlled to avoid nutrient starvation in the center of the cell aggregate.
1.5 References


Martin GR 1981, Isolation of a pluripotential cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634_7638

Chapter 2

Literature review

2.1 Introduction

Animal cells are capable of producing properly folded, glycosylated and secreted proteins and have attracted more and more attention in pharmaceutical industry. Many recombinant proteins, such as monoclonal antibodies, interferon-α, β, λ, tissue plasminogen activator (tPA), and erythropoietin (EPO), are produced by animal cell culture technology (Arathoon and Birch, 1986; Eridani, 1990). Moreover, animal cell culture technology has found application in tissue engineering. Instead of culturing existing cell lines, primary cells, such as embryonic stem cells, epidermal keratinocytes, and hematopoietic cells, separated from tissues were cultured and employed in in vitro organ construction.

In order to scale up the animal cell culture process, many kinds of bioreactors were employed. A novel fibrous bed bioreactor (FBB) was developed in our group for both anchorage-dependent and anchorage-independent cells. Higher cell density and productivity were achieved in this FBB. It has great potential for protein production and tissue engineering.
Embryonic stem cells were separated from inner cell mass. They possess an unlimited ability to self-renew and can differentiate into all kinds of tissues. Because of these characteristics, ES cells are widely used in drug screening, tissue transplantation, and cell therapy. Their induced differentiation is carried out by various cytokines combination or co-culture after the embryoid body formation.

2.2 Stem Cells

Great efforts were made in the past century to overcome the debilitating influence of organ and tissue loss. Synthetic replacements were used to treat diseases. However, living tissue in a multicellular organism can adapt and remodel itself in response to physiological and environmental cues. Synthetic implants are not capable of perceiving local conditions and responding appropriately. This lack of adaptation to the local tissue environment can be a major factor in implant failure. Therefore, more sophisticated biological tissue or cellular implantations attract more attention. Due to the cell source limitation, stem cells are accepted as the most promising cell source for therapeutic applications (Vats et al., 2005). Different stem cell sources are discussed below.

2.2.1 Embryonic Stem Cells

Shortly after fertilization, a mammalian egg begins to divide into totipotent cells which, if isolated, have the ability to develop into a fetus. Following division, these cells form a hollow ball of cells called a blastocyst. While the outer layer of this blastocyst differentiate into the placenta and other tissue necessary for fetus growth, the inner layer, which is called the inner cell mass, will eventually mature to become a fetus (Martin, 1981). The inner cell mass is present on day 3.5-4 in mice and consists of pluripotent
embryonic stem cells with unlimited proliferation potential. Hwang and colleagues (2004) have shown lately that somatic-cell nuclear transfer (SCNT) techniques can be applied to human oocytes, resulting in successful blastocyst formation and the derivation of an embryonic stem-cell line. Martin and coworkers (2005) demonstrated that hES cells derived from mouse feeder cells resulted in the acquisition of an immunogenic, non-human sialic-acid residue. Limited hES cell lines and restrictions in some cultures due to ethnical issues preclude the use of hES cells in therapy.

2.2.2 Fetal Stem Cell

Circulating human fetal mesenchymal stem cells from fetal blood provides an alternative, less controversial source of cells for therapy. The fetal mesenchymal-stem-cell population includes adherent cells that divide in culture for 20 to 40 passages; these cells can differentiate into mesenchymal lineages including bone and cartilage and also oligodendrocytes and haemopoietic cells (Campagnoli et al., 2001). However, the pluripotency of fetal stem cells has not been proven.

2.2.3 Adult Stem Cell

The self renewal ability of some tissues such as skin, haemopoietic system, bone, and liver indicates the presence of stem or progenitor cells. The autologous or allogeneic cells from adult patients might provide a less difficult route to regenerative-cell therapies. Bone marrow transplantation has been approved for patients with blood disorders or who are undergoing chemotherapy or radiotherapy. In addition to bone marrow stem cells, adult stem cells have also been found in the blood, central nervous system, epidermis, liver, and pancreas etc. Adult stem cells sources and their applications was summarized
in Table 2.1. Adult stem cells are believed to differentiate into certain cell types, which are related to their origin organ. Their proliferation potential is also not comparable to ES cells (Campagnoli et al., 2001). Adult stem cells are rare in adult tissue and difficult to isolate and maintain \textit{in vitro}.

2.2.4 Multipotent Adult Stem Cells

Adult stem cells differentiation potential was believed to be strictly limited to the tissue of origin. However, Poulsom and co-workers (2002) ascertained the plasticity of the bone marrow stem cells. Multipotent adult stem cells also express Oct-4, which was previously observed only in pluripotent embryonic stem cells. These findings initiate the research interest in adult stem cells. Table 2.2 compares adult stem cell and embryonic stem cells are listed in table 2.2.

2.3 Embryonic Stem Cells

2.3.1 Different Embryonic Stem Cell Lines

Shortly after fertilization, a mammalian egg begins to divide into totipotent cells which, if isolated, have the ability to develop into a fetus. Following division, these cells form a hollow ball of cells called blastocyst. While the outer layer of this blastocyst differentiate into the placenta and other tissue necessary for fetus growth, the inner layer which is called the inner cell mass will eventually mature to become a fetus (Martin, 1981). Figure 2.1 demonstrated the procedures of derivation of embryonic stem cells from human embryo. The summary of different human embryonic stem cell was listed in Table 2.3 (Haffman and Carpenter, 2005)
2.3.2 The Molecular Characterization of Embryonic Stem Cell

At present, several criteria are accepted to define embryonic stem cell. Human and mouse ES cells have different surface markers. Mouse ES cells are specific stage embryonic antigen-1 (SSEA-1) positive, while human ES cells are SSEA-3 and SSEA-4 positive. Several groups have demonstrated that surface makers can not truly distinguish ES cells. Differentiated mES cells sometimes remain SSEA-1 positive. Alkaline phosphatase (ALP) activity is present in all pluripotent stem cells derived from mouse or primate. However, the functional significance of ALP activity has not yet been ascertained. Telomerase activity is another marker of self-renewable ES cells. Telomerase is known to maintain the chromosome length during cell replication. Telomerase activity of other cells diminishes during the proliferation, which leads to cell aging and the loss of proliferation potential. To date, the best characterized marker for ES cell is the expression level of transcription factor Oct-4. All pluripotent stem cells from mice and primates including human highly express Oct-4. Also, pluripotent cells cannot form without Oct-4. The combination of ALP, and surface markers with Oct-4 expression were employed in identifying undifferentiated ES cells (Laslett AL et al. 2003).

2.3.3 Embryonic Stem Cells Expansion

2.3.3.1 Mouse Embryonic Stem Cell Expansion

The first mammalian ES cell lines were derived from mice blastocyst in 1981 (Evans and Martin, 1981). Since this first isolation, ES cells have been kept on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells (Martin, 1974). Some unknown factors secreted to the medium or cell contact mediated mechanisms maintain the ES cells’
pluripotency. Murine ES cells were cultured on MEF feeder cell layers until their replacement by cytokine leukemia inhibitory factor (LIF) (Smith et al., and William et al., 1988). LIF is a member of the interleukin (IL)-6 family. Upon cytokine withdrawal, spontaneous differentiation can occur and a variety of cell lineages such as hematopoietic, cardiac, and neural cells are obtained. Zandstra et al (2000) found that LIF regulated ES cells’ differentiation, but not their growth. They also demonstrated that LIF concentration should be higher than 500 pM to prevent differentiation. LIF in the medium was consumed quickly and frequent media changes are necessary to maintain the elevated SSEA-1 expression. Autocrine LIF effect was evident at higher ES cell densities, suggesting the use of higher inoculums when pluripotency maintenance was the priority. Moreover, ES cells must be seeded on gelatin pre-coated surfaces in feeder cell free cultures to facilitate ES cell attachment and prevent spontaneous differentiation. In non-treated petri-dishes, significant amounts of embryoid body formation was observed (our own observation). There are few publications about murine undifferentiated ES cells proliferation possibly due to the rapidness of ES cell growth. Normally, ES cells need subculturing every 2-3 days; even at the split ratio of 1:10 to inhibit the differentiation. Greater than 70% cell confluence was believed to be detrimental to pluripotency. The feasibility of using microcarriers to expand murine ES cells has been tested (Fok et al., 2005). They found that ES cell can expand well in glass microcarriers without significant differentiation. Nonetheless, frequent subculturing (every 3 days) is still necessary. Albranches et al. (2003) discovered that ES cells cultured in different sized petri-dishes had different doubling times and maximum cell densities. Their result showed that 35 mm dishes were better than 90 mm dishes, and explanation is attributed to better oxygen
transfer. Three dimensional cultures of ES cells in PET fibrous matrices demonstrated better growth than 2-D cultures, and the controlled bioreactor system can enhance ES cell proliferation rate while maintaining pluripotency (Li et al., 2003).

2.3.3.2 Human Embryonic Stem Cell Expansion

Human embryonic stem cells were not isolated until 1998 and are more difficult to culture than murine ES cell. LIF counterparts have not been found in hES cell cultures. Animal component free cultures of hES cells are important from the perspective of therapeutic applications. Feeder-free growth of undifferentiated human embryonic stem cells on Matrigel or laminin in MEF conditioned medium was published by Xu et al. in 2001. hES cells can maintain their pluripotency for more than 130 population doublings, and results are replicable in four cell lines (H1, H7, H9, H14). Moreover, Xu and the coworkers succeeded in cryopreserving ES cells in ES culture medium supplemented with 10% DMSO and 30% knockout serum replacement. hES cells have shown poor growth on gelatin coated surfaces and started to differentiate after the first passage. Different source of conditioned medium have also been studied and only MEF conditioned medium can sustain satisfactory undifferentiated hES cell growth. Matrigel is a complex mixture of mouse sarcoma origin. Therefore, animal components still exist in the culture system. The use of human laminin to replace Matrigel can partly solve the problem. Amit et al. (2004) maintained hES cells on a fibronectin matrix using unconditioned medium by supplementing bFGF and TGF-β1. Nevertheless, hES cell showed lower cloning efficiencies and growth rates and a higher rate of spontaneous differentiation. Large advances in serum free hES cell cultures have been made in the
past few years. Xu et al. (2005) showed that bFGF alone or with other growth factors supports hES cells growth which is comparable to that in MEF conditioned medium. SCF, LIF, TPO, Flt3L alone or combination without bFGF is not sufficient for maintaining undifferentiated hES cell growth. Levenstein et al., (2006) developed a new recipe for serum free cultures of hES cells, using DMEM-F12 as the basal medium. High concentration of bFGF (250 ng/ml) was found to be effective in supporting undifferentiated hES cells growth, while 4, 24 or 40 ng/ml of bFGF failed to sustain hES cells growth in three passages in unconditioned medium. This was attributed to the rapid degradation of bFGF in the medium. However, this degradation is slower in conditioned medium. However, other scientist reported that 4 ng/ml bFGF in conditioned medium can maintain hES cells on Matrigel for 130 doublings (Xu et al., 2000). Beattie et al (2005) reported that 50 ng/ml of activin A is capable of sustaining hES cell pluripotency over 20 passages without the feeder layer or MEF conditioned medium. Without the presence of bFGF, Pluripotency can be maintained in unconditioned medium by supplementing TGF-β or activin, but cell growth is poor. Along with the development of serum free medium for feeder free cultures, the mechanisms of extracellular factors regulating self-renewal were also investigated. hES cells differentiated into trophoblast lineage in response to BMP4. The addition of BMP-2 and BMP-7 leads to differentiation into primitive endoderm (Pera MF et al, 2004). This work indicates that the suppression of BMP mediated signaling (i.e. inhibition of Smad1/5/8) is critical for maintaining hES cell pluripotency. SR (serum replacement) containing unconditioned medium exhibited BMP-like differentiation activity. The undifferentiated hES cells possess high levels of phosphorylated Smad 2/3, indicating a crucial role for TGF-β/ activin A/ nodal mediated
signaling (Xu et al., 2005; James D., et al., 2005; Besser D., 2004). FGF signaling is active in undifferentiated hES cells and plays a role in hES cell mitogenesis (Amit et al., 2000). High concentration of bFGF can replace noggin in suppressing BMP induced differentiation (Kim et al., 2004).

When a microcurrent of 6 µA and 1 to 10 Hz was applied to ES cells for one hour each day. They expanded and remained undifferentiated after 10 to 14 cell cycles without MEF feeder cells (Rader, W.C. and Scheller, A., 2003). Amit et al (2000) observed that clonally derived hES cell lines are capable of maintaining pluripotency and proliferation potential for a prolonged time. The parental hES cell line, H9, was cultured for 6 months and its clonal derivation continued to culture for another 8 months with high proliferation potential, high levels of telomerase and normal karyotypes. They also demonstrated that bFGF was only critical in serum-free culture by increasing the initial cloning efficiency and maintaining undifferentiated expansion. Cheng et al (2003) observed that human adult marrow cells support prolonged hES cells expansion. Although hES cells growth rate was slower on marrow stromal cells than on MEF cells in the first 6 passages, they gained better growth after adapting on marrow stromal cells. In this way, hES cells can be subcultured up to 13 passages.

2.3.3.3 Embryonic Stem Cell Expansion and Bioreactor

Embryonic stem cell expansion in bioreactors has yet not been reported. However, cultures of adult stem cells, such as blood stem cell, (Cabral JMS, 2001), neural stem cells (Sen et al., 2002) and mesenchymal stem cells (Mantalaris and Wu, 2006) in stirred tank bioreactors have been reported. ES cells develop into embryoid bodies and initiate
the differentiation process in suspension culture. Therefore, traditional stirred tank bioreactors are not capable of fulfilling the goal of undifferentiated ES cell expansion. However, bioreactors are applied for embryoid body formation and detailed review information is summarized in the following paragraphs.

2.3.4 ES Cell Differentiation

ES cells have been successfully differentiated into cardiogenic, myogenic, hematopoietic, neurogenic cell types as well as skeletal muscle cells, vascular smooth muscle cells, epithelial-like cells and pancreatic cells. Figure 2.2 illustrates ES cell differentiation. Cell-lineage selection is mainly determined by the growth factors in medium. For examples, insulin-like growth factor has been shown to be essential for the formation of functional heart (Sachinidis, 2003). Unknown factors in fetal calf serum (FCS) can induce hematopoietic differentiation (Gordon MK, 1995). Insulin can initiate differentiation into pancreatic cells (Lester 2004). Figure 2.3 illustrates neural and pancreatic differentiations of ES cells. Table 2.4 summarizes the ES cell differentiation examples.

2.3.4.1 Embryoid Body Formation

Compared with ES cell expansion, there are much more published works on ES cell differentiation. In vitro differentiation protocol includes the formation of embryo-like aggregates, so called embryoid bodies (EB) as the first step. EBs differentiated into endodermal, mesodermal and ectodermal cell lineages (Guan et al., 1999). EB formation requires a suspension environment (non-adherent dish). Wobus et al (1991) developed the “hanging drop” method. However, Dang et al (2002) observed that agglomeration of EBs
in static culture resulted in cell death and necrotic centers due to nutrient transfer limitations. This agglomeration also had negative effects on ES cell differentiation. Controlled bioreactor systems should be applied in ES cell culture systems. Unfortunately, suspension cultures of ES cells in stirred tanks resulted in significant cell agglomerations and poor cell proliferation and differentiation (Dang et al., 2004). Non-conventional bioreactors, such as rotating cell culture systems (RCCS) systems have been studied. RCCS have mild mixing intensities and very low shear stresses. Furthermore, bubble bursting, which damages cells, and foaming are precluded by diffusing oxygen into the reactor, rather than by sparging (Gerecht-Nir et al. 2004). They investigated hEB formation and differentiation in high aspect rotating vessels (HARV) and slow turning lateral vessels (STLV). Small and relatively homogeneous populations of hEB were observed in STLV, and big cell clumps in HARV. Compared with static cultures in petri dishes, EB formation efficiency improved four-fold and the EB size was seven times smaller in STLV. It was also demonstrated that the number of EBs didn’t increase after 7 days, but their size increased (Gerecht-Nir et al. 2004). Dang et al. (2004) developed a novel encapsulation culture method for EB formation and differentiation. ES cells formed aggregates first, and then these aggregates were encapsulated in size-controlled agarose hydrogel capsules and cultivated in the stirred tank for growth and differentiation. By controlling capsule size and number, a consistent cell aggregation and more uniform size were obtained. Mouse ES cell aggregates grew faster in the capsule than human ES cells did. Cell expansion in the encapsulation stirred tank system was similar to that in the non-encapsulated static control. However, ES cell almost did not grow in stirred tanks without the protection of encapsulation.
2.3.4.2 ES Cell differentiation and The Culture Environment

In addition to bioreactor optimization, the culture environment can also affect ES cell differentiation. Chen et al (2003) found that rhesus monkey ES cells differentiate differently in 3-D culture environments (collagen matrix) and 2-D monolayers. Unlike cells in 2-D slides, ES cells in 3-D collagen gel did not form epithelial cells and large spread cells. Instead, they form tubular or spherical gland-like structures. ES cell differentiation is also influenced by the physical properties of the matrix structures. ES cells penetrate into collagen gel, while they migrate and develop into aggregates in collagen sponges due to their porous structure. It has also been shown that coculturing with fibroblasts in collagen gel facilitates neural lineage differentiation, while coculturing with keratinocytes in collagen sponges facilitates endothelial lineage differentiation. Levenberg et al (2003) exploited three-dimensional polymer scaffolds to study hES cell differentiation. A 50/50 mixture of poly (lactic-co-glycolic acid) (PLGA) and poly (L-lactic acid) (PLLA) matrix was coated with matrigel or fibronectin to assist ES cell attachment. In the presence of various growth factors, hES cells formed the 3-D structure on the polymer scaffold. Some cell differentiation on two-dimensional fibronectin coated dished, but no 3D structure was observed. Matrigel itself can form a 3D environment, but it failed to support hES cell growth and 3-D organization.

2.3.4.1 ES Cell Neural Differentiation

Wouter et al (1996) applied $10^{-7}$ M of retinoic acid (RA) to induce neuron differentiation. After 4-5 days treatment, the neuron markers GAP-43 and NF-165 were detected. However, neurogenesis is a complex process; different neural cells appear at different
stages. ES cells differentiate into neural stem cells first and then further into astrocyte progenitors, oligodendrocyte progenitors and neuronal progenitors. These progenitors finally differentiate into more specific neural cell types. Reubinoff et al (2001) studied neural progenitors derived from ES cells. ES cells were cultured for 3-4 weeks in order to obtain enriched neural progenitor cells. The neuroectodermal markers nestin and PAX-6 were detected during the first week. After one week, the differentiation process sped up, and the early neuroectodermal marker N-CAM (neural cell adhesion molecule) was expressed. It was found that a combination of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) is favorable for human fetal and adult derived neuroepithelia progenitors. Midbrain dopaminergic neuron derivation is elaborated because of its application in Parkinson disease treatment. Two distinct procedures for deriving dopaminergic neurons from ES cells have been published (Lee et al. 2000 and Kawasaki et al 2000). One is based on defined culture conditions and the other is based on co-culturing with stromal feeder cells. Lee et al. (2000) generated midbrain neurons in defined culture medium. Their protocol was summarized as five steps with the whole process of 3-5 weeks: undifferentiated ES cell expansion, EBs formation, nestin-positive cell selection, nestin-positive cell expansion, and dopaminergic neuron differentiation. Tyrosine hydroxylase (TH) was selected to identify the midbrain neurons. It was found N2 medium improved TH+ cell generation, while HEPES buffer strongly inhibited the differentiation of TH+ neurons. It was also observed that sonic hedgehog (SHH), FGF8, and ascorbic acid (AA) increased the yield of ES-derived TH+ neurons. At the same year, Kawasaki H. et al (2000) developed another co-culture system for inducing midbrain dopaminergic neuron differentiation from mouse ES cells. They identified a stromal cell-
derived inducing activity (SDIA) that increased neural differentiation. ES cells were co-cultured with stromal PA6 cells in serum free medium (It is essential to remove serum since FBS will strongly inhibit neuron differentiation) without retinoic acid or embryoid body formation. They obtained 16% TH+ dopaminergic neural cells, 52% neuron, 9% GABAergic, 5% cholinergic and 1% serotoninergic neurons. PA6 cells retained high dopaminergic neuron inducing activity even after being fixed with paraformaldehyde (PFA). This phenomenon indicates that this neuron-inducing activity might be attributed to something on the surface of the cell rather than to secreted factors. Kawasaki H. et al (2000) also compared neuron differentiation in systems in direct contact with PA6 cells, separated from PA6 cells by a filter and in medium conditioned by PA6 cells. The results showed that direct contact was most efficient, with the filtered system in second place and the conditioned medium having almost no neuron inducing ability. FGF8, SHH, IL1, IL11 and GDNF in this co-culture system had no significant effect on the dopaminergic neural differentiation. Perrier et al. (2004) also applied a similar strategy using different stromal feeder cells, MS5 and S2 from bone marrow, to derive midbrain dopamine neurons from human embryonic stem cells. The coculture of hES cells on MS5 stroma resulted in efficient differentiation into neuroepithelial structures named neural rosetters. After harvesting and re-inoculating these rosetters into medium with FGF8 and SHH for an additional 7-9 days, then changing to BDNF-containing medium, midbrain dopaminergic neural cells were obtained. Their results showed that exposure to SHH and FGF8 from day 12 to 20 followed by differentiation in the presence of AA and BDNF resulted in a 3-times more TH+ cells. During the differentiation toward dopaminergic neurons, differentiating ES cells sequentially expressed key transcription factors such as
Pax2, Pax5, and engrailed-1 (En-1). Nakayama et al. (2003) discovered that astrocyte-derived factors induced the differentiation of ES cells into neurons. Their protocol involved two stages: culturing in astrocyte conditioned medium under floating conditions to form floating spheres and then transfer to adherent cultures in conditioned medium with N\textsubscript{2} supplementation. In order to stimulate the proliferation of neural stem cells, the astrocyte conditioned medium was supplemented with 20 ng/ml fibroblast growth factor-2 (FGF-2). It was observed that the spheres obtained in the first stage possessed a periphery of neural stem cells, a core of dividing ES cells and an intermediate layer of cells in a transitional stage of differentiation. The author demonstrated that prolonged culturing facilitated complete differentiation. Upon the measurement of a panel of neuron markers during the culture, it was found that Oct-4 (undifferentiating ES cell marker) expression was down-regulated in the first stage, nestin (neural stem cell marker) expression was up-regulated in the first stage, and TH (dopaminergic neural cell marker) expression was up-regulated during the late period of the second stage. When culturing neural stem cells derived from ES cells without astrocyte-conditioned medium, only small amounts of neuritis or migrated neurons were detected, proving that soluble factors from astrocyte are critical for effective neurogenesis. Buytabert-Hoefen et al. (2004) did similar work on hES cells. They generated TH\textsuperscript{+} neurons from hES cells after coculture with astrocytes or PA6 stromal cells. They observed that the use of astrocytes from embryonic striatum produced almost 10-fold more TH\textsuperscript{+} cells than the use of astrocytes from mesencephalon. In addition, coculturing hES cells and the PA6 cells exposed to embryonic striatum in hanging baskets increased differentiation efficiency by 20 times compared with growth on PA6 cells alone. Moreover, their result also showed that glial-
derived neurotrophic factor (GDNF) can double the number of TH\(^+\) cells in cocultures with PA6 cells. In this way, a high yield of TH\(^+\) cells can be obtained in 3-4 weeks. In addition, Shim et al. (2004) applied molecular genetics to enhance midbrain dopamine neuron differentiation efficiency. This work was based on the five stages differentiation process developed by Lee. The limiting step in that protocol is the third stage. extensive cell death occurred in the selection of ectodermal/neuroectoderal precursors. Bcl-XL expression might prevent cell death and therefore, increase the efficiency of the neural differentiation. Their results demonstrated that overexpressing Bcl-XL during \textit{in vitro} differentiation resulted in a higher expression of genes related to midbrain dopamine neuron development and increased the number of TH\(^+\) cells. Moreover, dopaminergic neurons derived from Bcl-XL ES cells possess higher resistance to 1-methly-4-phenylpyridium, a toxin. Further investigation showed that these Bcl-XL derived dopaminergic neurons had more extensive fiber outgrowths, resulting in a more significant reversal of behavioral symptoms than with wild type ES cell derived dopaminergic neurons during the transplantation into Parkinsonian rats.

2.3.5 ApplicationS of ES Cells

ES cells possess unlimited proliferation potential and can differentiate into all kinds of tissues. These two characteristics make ES cells a promising cell source for drug screening and tissue transplantation.

2.3.5.1 Tissue Engineering and Cell Therapy

It has been estimated that up to 128 million people suffer from chronic, degenerative, and acute diseases in the United States. Embryonic stem cells are exploited as cell therapy
tools in tissue transplantation (Gorba T, Allsopp T.E., 2003). They may be used to treat
spinal cord injuries, diabetes, Parkinson’s disease, leukemia, heart infarcts, etc. However,
ES cells develop teratoma if directly injected into the body. Therefore, they must be
induced to develop into a the certain cell lineage prior to transplantation.

Type I diabetes is an autoimmune disease characterized by the destruction of insulin
producing cells in the pancreas. The current treatment with human islet transplantation in
order to restore insulin secretion is severely restricted by the availability of donated
pancreases and the toxicity of the immunosuppressive drug treatments required to prevent
graft rejection. ES cell derived pancreatic cells can overcome the shortage of donors.
These cells may also be accepted by the patients’ immune system without attack.

Many nervous system diseases result from the loss of nerve cells. Mature nerve cells
cannot divide to replace those that are lost. Parkinson’s patients have been treated by
transplanting fetal cells into their brains with some benefits.

Presently, there are more than 70 different recognized congenital and inherited
deficiencies of the immune system, such as bubble body disease, Wiskott-Aldrich
Syndrome, and AIDS. Transplantation of ES cells with normal genes can result in the
restoration of immune function.

Table 2.5 shows the applications of ES cell application in tissue transplantation in animal

2.3.5.2 Drug Screening

There is a particular interest in the development of an ES cell based Alzheimer’s disease
screening assay. Also, ES cells can be employed in compound identification and
optimization. Established mouse lines are applied in embryonic stem cell test (EST) for investigating toxicology. Furthermore, the particular lineages differentiated from ES cells are employed for early stage evaluation of drug adsorption, metabolism, and toxicity. Embryonic stem cell technology offer the opportunity to develop much improved functional models by expanding cell populations in the pluripotent state and subsequently converting the cell populations into mature, fully differentiated cells through directed differentiation. Figure 2.4 illustrates the protocol of using mouse ES cells in drug screening and development (Pouton and Haynes, 2005)

2.4 Large Scale Cell Cultures

2.4.1 Challenges in Large Scale Culture

Animal cells have poor growth rates with an approximate doubling time of 24–48 h and an average productivity of 250 mg/L protein. These two values are significantly lower than those of *E.coli*. Despite these drawbacks, animal cell cultures have gained more and more interest because of their unique glycosylation ability. The general biopharmaceutical process chain was illustrated in Figure 2.5.

In addition to upstream genetic modification and downstream purification, scaling up the cell culture process from a 25 cm² T-flask to a 10,000 L or bigger bioreactor is a great challenge to chemical engineers. The main obstacles are oxygen supply limitation, waste metabolite accumulation, automatic process control, shear damage to cells, and growing anchorage-dependent cells (Glacken et al., 1983). Among these challenges, shear sensitivity and oxygen supply are believed to be mostly resolved. The remaining issues
are controlling the product quality, CO₂ concentration during scale up etc (Chu L. and Robinson D.K., 2001).

Animal cells can be categorized into two types: anchorage-dependent cells and suspension cells. Suspension cell cultures are preferred in industry because it provides homogenous environment and thus the process is easy to automatically control. Therefore, most cell cultures are adapted to suspension culturing prior to scale-up. However, anchorage dependent cells are also exploited in industry, especially in the cases of virus or vaccine production, such as adenovirus production by 293 cells. Anchorage-dependent cells cultures are more difficult to scale up than suspension cell cultures because of the additional requirement of economically providing enough surface area.

2.4.2 Cell Culture Bioreactors

Cell culture bioreactors can be divided into four main types; many currently used bioreactors are modifications of these four.

2.4.2.1 Stirred Tank Bioreactor:

Stirred tank bioreactors are the most widely used bioreactors in biotechnology. They can provide homogenous environments. The homogenization is crucial in order to attain a uniform temperature, nutrient concentration and cell distribution, making them easy to monitor and control. Cell culture stirred tank bioreactors are adapted from microbial fermentation. However, animal cells are much more sensitive to shear stress than microbial cells are. Baffles and other sharp protrusions that cause turbulence should be avoided in cell culture bioreactors. Stirred tank bioreactors are mainly used for the culture
of suspension cells. 3000 L vessels are used for FMD vaccine production (Radlett et al., 1985). 8000 L bioreactors are used to produce interferon (Phillips et al., 1985). They are also widely used in anchorage-dependent cells culture by microcarriers. The major problem with stirred tank bioreactors is the damage to the cells caused by mechanical agitation and gas sparging (Chalmers, 1994; Kunas and Papaoutsakis, 1990; Handa 1989). Foaming is another issue because it can not only cause contamination but also kill the cells by absorbing cells and through bubble rupturing (Tan et al., 1994 and Zhang et al., 1992).

2.4.2.2 Airlift Bioreactor

Airlift bioreactors apply the bubble column principle to simultaneously mix and aerate. Air bubbles are introduced at the bottom of the bioreactor and rise through a central draft. Aerated medium has a lower density than non-aerated medium. Upon oxygen consumption, the medium’s density increases, and thus it falls down in the region out of the draft. Airlift bioreactors possess the advantages of structural simplicity in structure, low shear stress, and good oxygen transfer rate (Birch J.R. et al., 1987). Nevertheless, they still experience problems caused by direct sparging as discussed in the previous session.

2.4.2.3 Hollow Fiber Bioreactors

In hollow fiber bioreactors, Cells growing in the extra-capillary space (ECS) and medium are re-circulated in the intra-capillary space (ICS). Nutrients diffuse into the fibers at one end, and toxic metabolites diffuse back into the lumen at the other end. Cells and products can be harvested from the ECS. Very high cell densities ($2 \times 10^9$ cells/ml) can be
reached in hollow fiber bioreactor (Hopkinson J., 1985 and Tharakan J.P., Chau P.C., 1985). However, hollow fiber bioreactor have poor stability due to fouling of the fiber membrane. Cells detached from the membrane are subject to nutrient starvation because diffusion is the main mass transfer mechanism. Moreover, the pressure drop along the reactor also prevents its scale-up potential.

2.4.2.4 Packed bed bioreactors

Cells are immobilized in packed bed bioreactors by certain immobilization carriers. Immobilization can protect cells from damage caused by shear stress and gas sparging. High cell densities \(1 \times 10^8 \text{ cells/ml}\) have been reached in the Celligen Plus bioreactor (Wang et al., 1992). Cell immobilization can increase the specific productivity, and improve product bioactivity and culture stability because of the improved microenvironment provided by the immobilization matrices (Chesmel et al., 1995; Edgington S.M., 1992; Kumar et al., 1990, Lee and Palsson 1990; Yamaguchi et al., 1997). Glass beads are the most commonly used packing materials (Burbidge 1980; Whiteside 1981; Griffiths et al., 1982). Glass beads possess the advantage of a consistent and known packing geometry, high stability, and low cost. It has been reported that large matrix spheres (2-6 mm) increase the productivity 4 to 20 fold (Nilsson et al., 1986). Although small beads (3 mm) provide larger specific surface areas, it was found that better yields were obtained with large beads (5mm). This may be attributed to better flow characteristics through the bed and thus better mass transfer thanks to the bigger channel size. However, glass beads work as a depth filter during inoculation and thus cause uneven cell distribution through the bed. This limits its scale-up potential.
2.4.3 Cell Culture Processes

**Batch cultures:** These are the most commonly used large-scale industrial production method because it is easy for FDA to validate. There is no nutrient addition or waste removal except for oxygen sparging during the process, so cell growth is inhibited after some time due to nutrient starvation or metabolic waste inhibition. Despite its simplicity, the cell densities obtained are low (1~2×10^6 cells/ml).

**Fed batch cultures:** After initial cell seeding and medium addition, necessary nutrients are continuously or intermittently fed into the system during the culture. However, the culture medium is not removed. Fed batch operation extends cell culture time by alleviating nutrient depletion. However, metabolic waste inhibition still exists.

**Perfusion cultures:** These are continuous cultures in which fresh medium is continuously fed and cell free solution continuously withdrawn. Nutrient starvation and metabolic waste inhibition are thus alleviated. Perfusion culturing increases cell density to a higher level (10^7~10^8 cells/ml) and allow a prolonged production period. Currently, the fouling of cell retention devices makes it difficult to run perfusion cultures for longer than six months or a year. Meanwhile, continuous operation introduces complexity to the process’ validation, and thus perfusion cultures are not widely used in the biopharmaceutical industry.

2.4.4 Three Dimensional Cell Cultures

Three-dimensional cell cultures resemble the *in vivo* tissue environment. Therefore cells show their native morphology and functions. 3-D culture systems sustains higher cell
densities, longer proliferation period and progressively increased differentiation activity, while cell proliferation is limited due to the limited available surface and the differentiation potential may be lost in 2-D system. Temenoff et al. (2000) showed that chondrocytes dedifferentiate in 2-D Petri dishes, but maintain their differentiated phenotype and functions in 3-D cultures. The expression of various ECM proteins and their receptors in 3-D cultures reflect the native situation much better than those 2-D cultures (Wueller, 1997). 3-D cell cultures have also demonstrated higher drug resistance than 2-D cultures because they form aggregates (Hoffman, 1994).

Recently, three-dimensional cell cultures using fibrous matrices have been developed for culturing various cell types, including CHO, hybridoma, human osteosarcoma, human cytотrophoblast ED27 and mouse embryonic stem cells (Luo, 2002; Chen et al., 2002; Ma et al., 2000; Li et al., 2003). Previous studies demonstrated the 3-D fibrous PET matrices can support high cell densities \(3 \times 10^8\) cells/ml matrix) with high volumetric productivities (Chen et al., 2002). The advantages of using fibrous matrices include their high porosity, high specific surface area, high permeability, low pressure drop, high mechanical strength, protection from shear damage, facilitation of downstream processes, and low material costs. These virtues allow fibrous PET scaffolds to be an ideal material for cell cultures.

2.5 Bioreactor modeling

Solid and microporous spheres are the most commonly used packing materials for bio-applications in enzyme biocatalysis (Dadvar and Sahimi, 2003), waste water treatment (Gu and Syu, 2004) and cell cultures. Differential equations from chromatography with
reaction term are borrowed. Specifically, axial dispersion models are widely employed, with the assumption that mixing obeys Fick’s law (Backer and Baron, 1994; Gu and Syu, 2004; Iliuta I. et al. 1999). However, the drawback of this model is the tailing (Hoogendoorn and Lips, 1965). Multistage dispersion model integrating the axial dispersion and plug flow are particularly accurate in describing reactors with separate flow regions. Axial dispersion-exchange model considered that axial dispersion and mass exchange between dynamic and static regions resulting from back mixing (Hoogendoorn and Lips, 1965). Three potential mass transfer limiting steps exist in microporous particle packed bed bioreactors: external mass transfer, diffusion through the liquid film around the particles, and intra particle diffusion. Only diffusion through the liquid film and intra particle diffusion are considered to be mass transfer limiting steps in most of the papers. Backer and Baron (1994) included the intra particle diffusion terms in the dispersion equation. They applied residence time distribution (RTD) data to validate their model. At low Reynolds numbers (<10), their predictions fit the experimental data very well, indicating its correctness. Low biomass loading has no effect on the mass transfer. However, high biomass loading (> 0.02 g cell/g beads) significantly decreases the rate of mass transfer between the extraparticle and intraparticle fluid phase significantly. Gu and Syn (2004) also established a model to describe the immobilized cell column in bioconversion and waste water treatment, in which interfacial film diffusion is considered to be the mass transfer limiting step. Moreover, reaction and cell death are included in their model, making it closer to reality. Iliuta and coworkers (1996) developed an axial dispersion-exchange model with the assumption that intraparticle diffusion leads to the long tails of residence time distribution (RTD) curves.
Spiral wound hollow fiber or membrane reactor modeling is much more difficult than conventional packed bed reactor modeling because of the complex 3-dimensional momentum and mass transport. Crowder and Gooding (1997) calculated the mass transfer efficiency by applying empirical correlations in a spiral wound membrane reactor, but their study of the mathematical modeling was limited. Madireddi and coworkers (1999) developed a transient model for mass transfer, especially for concentration polarization in spirally wound membrane reactors. Membrane thickness and the distance between membrane layers were taken into account in their work. Hollow fiber bioreactor are considered to be similar to spirally wound fibrous bed bioreactors. Channels in FBB are similar to the lumen space in hollow fiber bioreactors and the PET sheet is similar to the membrane. Therefore, some mathematical models that describe hollow fiber bioreactor are here introduced to FBB systems. Hollow fiber bioreactor can be separated into three parts: the extra cellular space (ECS), the lumen and the fiber membrane. Waterland and coworkers (1974) developed an equation to describe mass transfer in the lumen. Both radial diffusion and convection are considered in this equation. Transport in the membrane and ECS occurs only through radial diffusion. In diffusion limited situations, the following two equations are used. They are the basis for hollow fiber bioreactor modeling.

ICS: \[ 2Pe_{ax}(1-r^2) \frac{\partial C_1}{\partial z} = \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_1}{\partial r} \right) \]  

ECS: \[ \frac{\partial C_\varepsilon}{\partial t} + Pe_{ax} \left( u_i \frac{\partial C_\varepsilon}{\partial z} + v_i \frac{\partial C_\varepsilon}{\partial r} \right) = \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_\varepsilon}{\partial r} \right) + \frac{\partial^2 C_\varepsilon}{\partial r^2} - R \]
If the Monad equation is considered for the reaction term (oxygen consumption), the equation is

$$R = \phi^2 X \frac{C_e}{K_m + C_e}. \quad (3)$$

And the cell growth equation is

$$\frac{\partial X}{\partial t} = \psi \phi^2 X \frac{C_e}{K_m + C_e}. \quad (4)$$

$\phi$ is the Thiele modulus and $\psi$ is the dimensionless yield (Brotherton and Chau, 1996). One step beyond a diffusion-limited model is to consider the mass transfer resistance in the fiber membrane or the transient diffusion problem in the ECS (Webster and Shuler, 1979 and 1981). With the development of a more permeable membrane, hollow fiber bioreactors were no longer diffusion-limited. Convection enhanced transport in ECS was taken into consideration. Apelblat et al (1974) modeled the fluid dynamics in a capillary network as flow in a porous medium. Salmon et al. (1988) imposed the velocity profiles derived by Apelblat (1974) on the substrate mass balance equations and derived a model. Kelsey et al. (1990) combined the substrate mass balance and cell growth equations to simulate bioreactor performance during the initial stage before cells obstruct the ECS. In their model, the magnitude of radial convective transport is indicated by wall Peclet number. Brotherton and Chau (1990) did a comprehensive study on momentum and mass transport in spirally wound hollow fiber bioreactor for animal cell culture, but none of the aforementioned works verified their model with experimental data.

2.6 Reference


Andersen DC. and Gooche CF, 1994, The effect of cell culture conditions on the
oligosaccharides structures of secreted glycoproteins. Current Opinion in Biotechnology 5, 546-549


Buytaert-Hoefen KA, Alvarez E, Freed CR, 2004, Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF. Stem Cells. 22: 669-674


Cass B, Pham PL, Kamen A, Durocher Y, 2005, Purification of recombinant proteins from mammalian cell culture using a generic double-affinity chromatography scheme. Protein Expression and Purification 40, 77-85
Chalmers JJ, 1994, Cells and bubbles in sparged bioreactors. Cytotechnology, 15: 311


Chen CN, 2000, Animal cell culture in a fibrous-bed bioreactor: protein production, cell immobilization, and cell cycle and apoptosis. Ph..D. dissertation, Ohio State University


lead to heterogeneity in batch culture. Biochem J 272: 333-337


cell biology, 7: 862-869


Lavon N, Yanuka, O, Benvenisty N, 2004, Differentiation and isolation of hepatic-like
cells from human embryonic stem cells. Differentiation 72: 230-238


Mantalaris A, Wu JHD, 2006, Ex vivo culture of hematopoietic and mesenchymal stem cells for tissue engineering and cell-based therapies. Biotechnology and Bioprocessing 30: 723-743

Martin GR 1981, Isolation of a pluripotential cell line from early mouse embryos cultured
in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634-7638


Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T, 2001,
Neural progenitors from human embryonic stem cells. Nature Biotechnology. 19: 1134-1140


Tan WS, Dai GC, Chen YL, 1994, Quantitative investigation of cell-bubble interactions using a foam fraction technique. Cytotechnology. 15: 321


of N-linked oligosaccharides of recombinant human tissue kallikrein produced by Chinese hamster ovary cells on microcarrier beads and in serum-free suspension culture. Biotechnol. Prog. 10: 39-44


Whiteside JP, and Spier RE, 1981, The scale up from 0.1 to 100 liter of unit process system based on 3 mm diameter glass spheres for the production of four strains of FMDV from BHK monolayer cells. Biotechnol. Bioeng. 23: 551-561


Wobus AM, Wallukat G and Hescheler J, 1991, Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation. 48:173-182


Wueller-Klieser M, 1997, Three-dimensional cell cultures: from molecular mechanisms
to clinical applications. Am J Physiol Cell Physiol 273: c1109-c1123


<table>
<thead>
<tr>
<th>Stem Cells</th>
<th>Source</th>
<th>Differentiation</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchymal stem cells</td>
<td>Bone Marrow</td>
<td>non-haemopoietic lineages</td>
<td>Myocardial infarction</td>
<td>Caplan and Bruder, 2001</td>
</tr>
<tr>
<td></td>
<td>Subventricular zone and the dentate gyrus in the hippocampus</td>
<td>neurons, oligodendrocytes, and astrocytes</td>
<td>Central nervous system (CNS) repair</td>
<td>Reynolds and Weiss, 1992</td>
</tr>
<tr>
<td>Neural stem cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocyte stem cell</td>
<td>Liver</td>
<td>hepatocytes and biliary cells</td>
<td>Liver repair</td>
<td>Alison, 1998</td>
</tr>
<tr>
<td>Pancreatic stem cell</td>
<td>Pancreas</td>
<td>adult ductal cells, β cell</td>
<td>Islet formation</td>
<td>Bonner-Weir and Sharma, 2002</td>
</tr>
<tr>
<td>Hematopoietic stem cell</td>
<td>Cord Blood or activated peripheral blood</td>
<td>T-cells, B-cells and NK cells, and granulocyte/monocyte progenitor (GMP) and the megakaryocyte/erythrocyte progenitor (MEP).</td>
<td>Leukemia, and blood related disease</td>
<td>Bellantuono 2004</td>
</tr>
<tr>
<td>Adipose stem cell</td>
<td>Adipose</td>
<td>adipocytes, osteoblasts, myoblasts, and chondroblasts.</td>
<td>Cell therapy</td>
<td>Rodriguez et al., 2005,</td>
</tr>
</tbody>
</table>

Table 2.1 Adult Stem Cell Sources and Applications
## Table 2.2 Comparison Between Adult Stem Cell and Embryonic Stem Cell

<table>
<thead>
<tr>
<th></th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Stem Cell</td>
<td>Less ethical controversy,</td>
<td>Rare in adult tissue and difficult to isolate and maintain <em>in vitro</em>,</td>
</tr>
<tr>
<td></td>
<td>No nuclear transfer,</td>
<td>Not pluripotent,</td>
</tr>
<tr>
<td></td>
<td>Existing through the life span of the organism,</td>
<td>Limited life span,</td>
</tr>
<tr>
<td></td>
<td>Successful examples in clinical treatment</td>
<td>Immune rejection in heterologous transplantation</td>
</tr>
<tr>
<td></td>
<td>Unlabeled life span,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pluripotent,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remarkable resistance to genomic instability and malignant</td>
<td>Only existing at a certain stage in ICM,</td>
</tr>
<tr>
<td></td>
<td>transformation,</td>
<td>Form the teratoma by direct injection to the body,</td>
</tr>
<tr>
<td></td>
<td>Less immune rejection problem</td>
<td>Ethical controversy</td>
</tr>
<tr>
<td>Embryonic Stem Cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>Embryos numbers</td>
<td>Fresh/frozen</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>USA¹</td>
<td>36</td>
<td>Both</td>
</tr>
<tr>
<td>Australia/Singapore³</td>
<td>N/A</td>
<td>Frozen</td>
</tr>
<tr>
<td>USA³</td>
<td>N/A</td>
<td>Fresh</td>
</tr>
<tr>
<td>Israel³</td>
<td>5</td>
<td>Frozen</td>
</tr>
<tr>
<td>Singapore²</td>
<td>1</td>
<td>Frozen</td>
</tr>
<tr>
<td>Sweden⁸</td>
<td>5</td>
<td>N/A</td>
</tr>
<tr>
<td>USA¹⁰</td>
<td>19</td>
<td>Frozen</td>
</tr>
<tr>
<td>Korea⁶⁵</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Sweden⁵²</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Korea⁶⁴</td>
<td>20 pronuclei</td>
<td>Frozen</td>
</tr>
<tr>
<td>20 blastocyst</td>
<td>Frozen</td>
<td>MitoC STO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israel⁶⁵</td>
<td>(abnormally</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.3 Published Human Embryonic Stem Cell Derivation (Hoffman and Carpenter, 2005), To be continued
Continued

<table>
<thead>
<tr>
<th>Location</th>
<th>N/A</th>
<th>N/A</th>
<th>MitoC MEF</th>
<th>8–10% SR, 5% FBS, + LIF + bFGF</th>
<th>N/A</th>
<th>N/A</th>
<th>HUES1-17</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>286 cleaved embryos &amp; 58 blastocysts</td>
<td>Frozen</td>
<td>MitoC MEF</td>
<td>plasmate, 5% FBS, + LIF + bFGF</td>
<td>N/A</td>
<td>97</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Iran</td>
<td>N/A</td>
<td>N/A</td>
<td>MitoC MEF</td>
<td>20% FBS + LIF</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>Royan H1</td>
</tr>
<tr>
<td>UK</td>
<td>11 day-2 embryos</td>
<td>Fresh</td>
<td>Irrad MEF</td>
<td>FBS, then SR + bFGF</td>
<td>7</td>
<td>7</td>
<td>8 (culture in BRL-CM for d 6-8)</td>
<td>Royan H1</td>
</tr>
<tr>
<td>USA</td>
<td>117 (46 morula, 71 blastocyst)</td>
<td>MitoC MEF or BRL</td>
<td>10–20% FBS or SR + bFGF</td>
<td>39 blast</td>
<td>32 ICM</td>
<td>15, 18, 21, 24, 27, 28, 31, 33</td>
<td>Royan H1</td>
<td>17</td>
</tr>
<tr>
<td>Korea</td>
<td>8 PN-stage embryos</td>
<td>Frozen</td>
<td>MitoC human uterine endometrial cells</td>
<td>20% SR + bFGF</td>
<td>19</td>
<td>16</td>
<td>N/A</td>
<td>Miz-hES4-8, 10-13</td>
</tr>
<tr>
<td>Korea</td>
<td>N/A</td>
<td>N/A</td>
<td>MitoC MEF</td>
<td>20% SR + bFGF</td>
<td>19</td>
<td>16</td>
<td>N/A</td>
<td>Miz-hES4-8, 10-13</td>
</tr>
<tr>
<td>Korea</td>
<td>N/A</td>
<td>Frozen</td>
<td>MitoC STO</td>
<td>20% SR + bFGF</td>
<td>73</td>
<td>10</td>
<td>5–7</td>
<td>SNUhES1-3</td>
</tr>
<tr>
<td>China</td>
<td>N/A</td>
<td>Fresh</td>
<td>Irrad MEFs</td>
<td>20% FBS + LIF</td>
<td>7</td>
<td>4</td>
<td>N/A</td>
<td>CHES-1</td>
</tr>
<tr>
<td>Spain</td>
<td>40</td>
<td>Frozen</td>
<td>Placental fibroblasts</td>
<td>20% SR + bFGF</td>
<td>16</td>
<td>16</td>
<td>N/A</td>
<td>VAL-1, VAL-2</td>
</tr>
</tbody>
</table>

Table 2.3 Published Human Embryonic Stem Cell Derivation (Hoffman and Carpenter, 2005) To be continued
<table>
<thead>
<tr>
<th>USA</th>
<th>N/A</th>
<th>Frozen Lysed MEFs</th>
<th>plasmate</th>
<th>N/A</th>
<th>5</th>
<th>N/A</th>
<th>ACT-14</th>
<th>20</th>
</tr>
</thead>
</table>

*Number of cell lines divided by the number of ICMs x 100. N/A, information not available from published sources; irrad, irradiated; MitoC, Mitomycin C; MEF, mouse embryonic feeders; HEF, human embryonic feeders; STO, STO cells; FBS, fetal bovine serum; SR, serum replacement; BRL-CM, BRL conditioned medium; PN, pronucleus; ICM, inner cell mass.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Induction Reagents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth Muscle Cell</td>
<td>DMSO</td>
<td>Dinsmore et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Retinoic acid, SHH, FGF8, Ascorbic acid</td>
<td>Wouter, 1996; Lee et al., 2000</td>
</tr>
<tr>
<td>Neural Cell</td>
<td>FGF, TPO</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>Haemopoietic Cell</td>
<td>BMP, TGF</td>
<td>Passier and Mummery, 2005</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>Mitogen, FGF, B27 supplement, nicotin-amide a FGF, b FGF, BMP4, HGF</td>
<td>Lumelsk, et al., 2001</td>
</tr>
<tr>
<td>Pancreatic Cell</td>
<td></td>
<td>Lavon et al., 2004</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>BMP4, Ascorbate</td>
<td>Heng et al., 2005</td>
</tr>
</tbody>
</table>

Table 2.4 Embryonic Stem Cell Differentiation Ability
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Disease model</th>
<th>Site of transplantation</th>
<th>Survival and marker expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA induced neurons and precursors</td>
<td>Quinolinic acid injection in rats</td>
<td>Striatum</td>
<td>+</td>
</tr>
<tr>
<td>GRP expanded with bFGF and PDGF</td>
<td>Lyelin-deficient mutant rats</td>
<td>Intraventricular injection</td>
<td>+</td>
</tr>
<tr>
<td>EB differentiated 4 days, no RA</td>
<td>6-OHDA injection in rats</td>
<td>Median forebrain bundle</td>
<td>+</td>
</tr>
<tr>
<td>Shh, FGF-8 induction of Nurr1 overexpressing cells</td>
<td>6-OHDA injection in rats</td>
<td>Striatum</td>
<td>+</td>
</tr>
<tr>
<td>RA induced neurons and precursors</td>
<td>Spinal cord contusion in rats</td>
<td>Spinal cord</td>
<td>+</td>
</tr>
<tr>
<td>Lineage-selected cardiomyocytes</td>
<td>Dystrophic mdx mice</td>
<td>Ventricular myocard</td>
<td>+</td>
</tr>
<tr>
<td><strong>Lineage-selected insulin-secreting cells</strong></td>
<td>Streptozotocin-induced diabetes in mice</td>
<td>Spleen</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.5 Application of ES Cells in Cell Therapy
Figure 2.1 Schematic of derivation of hES cell lines from a human blastocyst (Hoffman and Carpenter, 2005)
Figure 2.2 ES cell differentiation diagram
Figure 2.3 ES cell differentiation into neural and pancreatic cells
Figure 2.4 Culture protocols for use of mouse embryonic stem cells (ESCs) in drug discovery and development.
Figure 2.5 The flowchart of general biopharmaceutical process
Chapter 3

Expansion of Undifferentiated Embryonic Stem Cells in Fibrous Bed Bioreactor

3.1 Abstract

Embryonic stem (ES) cells have unlimited proliferation potential and capability to differentiate into all cell and tissue types, and thus are ideal cell sources for tissue engineering and cell therapy. However, the supplies of ES cells are limited to the cell lines approved by the federal government. Current in vitro cultures of ES cells are carried out on gelatin pre-coated surface and require frequent subcultures to maintain their undifferentiated state. Feeder cell free culture of human ES cells requires Matrigel or laminin coated surface. These labor intensive and expensive culturing methods cannot meet the market demand. mES cells grown in three-dimensional (3D) polyethylene-terephthalate (PET) scaffolds were demonstrated to maintain their undifferentiated state after 30 days of culturing in a conditioned medium from STO fibroblast culture without subculturing. The 3-D mES cell culture was improved by controlling the scaffold pore size and increasing the mass transfer efficiency in the culture system. mES cells in small pore size scaffold proliferated more and had higher Oct-4 and specific stage embryonic antigen-1 (SSEA-1) expression. mES cells in spinner flasks have higher growth rates and
also keep their undifferentiated stage better than those in static cultures. Furthermore, a two-stage perfusion system that integrated the conditioned medium production and the mES cells proliferation was also developed. When the pH, DO, and perfusion rates were controlled, mES cells expanded 193 folds in 15 days and more than 94% of them maintained pluripotency. 3-D culture of human embryonic stem (hES) cells showed that Matrigel was not necessary for the feeder cell free culture. Similar bioreactor system was also applied to human embryonic stem cells. hES cells expanded 145-fold in 18 days without subculture and SSEA-4 expression dropped from 83% to 79.6%, indicating most of hES cells maintained pluripotency during the proliferation in fibrous bed bioreactor system.

3.2 Introduction

Embryonic stem (ES) cells, which are derived from the inner cell mass of blastocyst (Martin 1981), have unlimited proliferation potential and are capable of differentiating into all types of cells and tissues (Guan et al., 1999). These two characteristics make ES cells an ideal cell source for tissue engineering and cell therapy. The vast potential biomedical applications of ES cells include clinical treatments of diabetes, Parkinson’s disease, liver malfunction, heart failure, spinal cord injury, skin wounds, etc. ES cells are also invaluable tools for drug discovery and gene therapy (Gorta et al., 1999). Genetically modified ES cells can be used for high-throughput drug screening and to transmit and express specific genes in target organs. With the expected demand for ES cells, efforts have been carried out to mass produce them. However, spontaneous differentiation usually occurs when embryonic stem cells are cultured in vitro (Thomson 1998). Upon
the first isolation, it was assumed that murine embryonic fibroblasts (MEF) were necessary as the feeder layer cells in maintaining mouse ES cell pluripotency (Martin and Evan 1974). This coculture method generated a complication in their application due to the cell source contamination from the feeder layer cells when harvesting the ES cells. Further investigation later demonstrated that leukemia inhibitory factor (LIF) could be used as a substitute for the feeder cells to sustain undifferentiated mES cell growth (Smith et al., 1988). The minimum concentration of LIF necessary was later found to be 500 pM (Zandstra et al., 2000). Xu et al. (2001) first set up the feeder-free culture of hES cells on Matrigel-coated surface with MEF conditioned medium. Later research showed that bFGF and other growth factors such as TGF-β1, activin A, SCF, LIF, TPO, Flt3L were beneficial for the maintenance of hES cells pluripotency (Rao and Zandstra, 2005; Cheng et al., 2003; Xu et al., 2005; Levenstein et al., 2005; Beattie et al., 2005).

Current methods available for ES cell expansion are two-dimensional based systems, which are limited by space and very inefficient in their specific surface area. Furthermore, the surfaces need to be pre-coated with gelatin (for mES) or Matrigel (for hES), and frequent subcultures are necessary in order to maintain the undifferentiated stage of the ES cells (Zandstra et al., 2000; Xu et al., 2001). It is clear that these expensive, labor intensive, and time consuming methods are inappropriate for the projected high market demand.

An important component in the tissue-engineered construct that allows for in vivo-like cell culture is three-dimensional scaffolds supporting cell population, organization, and function mimicking those of native tissues. Although it is well recognized in the tissue
engineering community that the scaffold structure has strong effects on tissue development and function, very little has been done to study the effects of three-dimensional (3-D) scaffolds on ES cell cultures. Cells cultured in 3-D environment showed distinct characteristics in terms of their cell cycle and ability to expand and differentiate. It has been reported that ES cells could form 3-D structures in 3-D scaffolds that were not observed in 2-D cultures during induced differentiation (Chen et al., 2003; Levenberg et al., 2003). 3-D cellular structures could be critical to the ES cell’s ability to maintain its native functions and thus important for applications in cell therapy.

A novel three dimensional cell culture based fibrous bed bioreactor system was reported successfully to culture human cytotrophoblast ED27 (Ma et al., 2000), CHO cells, hybridoma cell and human osteosarcoma cell (Chen et al., 2002; Luo 2002). In this system, polyethylene-terephthalate (PET) is used as the scaffolding material. Fibrous PET matrices have the advantages of high porosity, high specific surface area, high permeability, low pressure drop, high mechanical strength, thermal and chemical stability, as well as low cost. Furthermore, the fact that non-woven PET scaffold can provide a 3-D culture that mimics the in vivo growth environment makes PET an ideal material for tissue engineering as well as cell cultures. Preliminary work by Li and coworkers also showed that 3-D PET scaffold can be used to sustain ES cell growth in short term cultures. Furthermore, they also reported the ability of these ES cells to differentiate into hematopoietic cells (Li et al., 2003). However, the possibility for long term mass production of undifferentiated ES cells was not investigated.
The large scale production of undifferentiated ES cells is rarely reported due to its anchorage dependency and high expense of growth factors. One possible scalable way using microcarriers was reported for mass production of the ES cells by Zandstra’s group (Fok and Zandstra 2005).

In this study, an economic and scalable method for the mass production of undifferentiated mES cells was developed by replacing LIF with conditioned medium from STO cell culture as well as providing a 3-D culture environment. Similar method was also tested in hES cells culture. Further optimization of the culture system was also performed and analyzed.

3.3 Materials and Methods

3.3.1 Cultures and Media

Murine ES D3 (mES) cells (CRL-1934, ATCC) were maintained on gelatin pre-coated T-flasks containing the ES growth medium, which consisted of the knock-out Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 μM β-mercaptoethanol (Sigma, St. Louis, MI) and 100 μM leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA). Both STO (CRL-1503, ATCC) and mouse embryonic fibroblast (MEF) (SCRC-1045, ATCC) cells were cultured in DMEM with 10% FBS, and they were used to prepare the conditioned media described later. Human ES (hES) cells (SCRC-2002, ATCC) were maintained on Matrigel (BD, San Jose, CA) coated T-flasks and cultured in the MEF conditioned medium described below.
Unless otherwise noted, these T-flask cultures were incubated in a CO₂ incubator at 37°C. The mES cells were passaged every three days and hES cells were subcultured every week to maintain their undifferentiated stage. All other cell culture materials were obtained from Gibco unless otherwise specified.

3.3.2 STO-Conditioned Media.

To replace LIF in the mES growth medium, STO-conditioned media were prepared in either T-flasks or spinner flasks, as follows. After the STO fibroblast cell culture reached 80% confluence in 75 cm² T-flasks, the media were refreshed and cells were cultured for another four days. Supernatant was then collected and used as the conditioned medium after supplementing with glucose, non-essential amino acid, L-glutamine, β-mercaptoethanol, and antibiotics to the same concentrations as those in the mES growth medium. The conditioned medium used in the long-term mES culture was prepared in a 250-mL spinner flask by inoculating 3~5 × 10⁶ STO cells to the flask containing PET matrix and 110 mL media. After incubation for six days, the medium was refreshed and cells were cultured for another four days before the supernatant was collected and supplemented with the additional media components in the mES growth medium.

3.3.4 MEF-conditioned Medium

The conditioned medium for hES cell culture was prepared in a 250-mL spinner flask with the PET matrix seeded with the MEF cells. One day after inoculation of the MEF cells, the medium was changed to knock-out DMEM plus 10% serum replacement (SR), which was then collected after incubation for three days. Before use, the conditioned
medium was adjusted in its composition to: 4 g/L glucose, 10% SR, 0.1 mM non-essential amino acid, 2 mM L-glutamine, 100 µM β-mercaptoethanol, 50 U/mL penicillin, 50 µg/mL streptomycin, and 4 ng/mL bFGF (Sigma)

3.3.5 Nonwoven PET Matrices

Needle-punched nonwoven polyethylene terephthalate (PET) matrices (thickness, 2 mm; porosity, 0.93; pore size: 60–130 µm; fiber diameter, ~20 µm; fiber density, 1.35 g/cm³) were used as cell culture scaffolds. The PET matrices were pretreated with NaOH to increase their surface hydrophilicity. The thickness, porosity, and pore size of the PET matrices were reduced to ~1 mm, 0.88, and 30–60 µm, respectively, by thermal compression (Li et al., 2001). In this paper, HP refers to the original PET matrices (without thermal compression) with the higher porosity, while LP refers to the matrices with the lower porosity obtained after thermal compression.

3.3.6 Static Cultures in Multiwells

Unless otherwise noted, ES cells were inoculated into either gelatin coated 24 well plate (2-D culture) or uncoated 3-D PET matrices in 24 well plate. For 3-D cultures, each well containing one sterile PET matrix and 100 µL medium was inoculated with 3–10 × 10⁴ cells. The cell suspension was carefully added from the center of the matrix using a micropipette. Following three hours incubation in a CO₂ incubator to allow for cell attachment to the matrix, the seeded matrix was washed with the culture medium to remove unattached and loosely attached cells. Then, the matrix was transferred to a new well and 1 ml of fresh medium was added to the well. The static cultures were used to
study the effects of gelatin coating, STO-conditioned media, lactic acid, and matrix pore size on mES cell proliferation and maintenance of its pluripotency.

3.3.7 Effects of Gelatin Coating.

The necessity and effects of gelatin coating of the support surface on ES cells were studied first. In this study, mES cells were cultured in multiwells each containing either a PET film disk (1.5 cm in diameter) or PET fibrous matrix (~1 cm square). For gelatin coating, the PET film or matrix was soaked in 0.3 ml of 0.5% gelatin solution for 2 h at room temperature, followed by air drying prior to use.

3.3.8 Effects STO-conditioned Medium

The feasibility of using the STO-conditioned medium to replace LIF for maintaining mES cells in the undifferentiated stage was studied. Each well was inoculated with $2 \times 10^4$ mES cells and cultured in either the growth medium (with LIF or without LIF) or the STO-conditioned medium (without LIF). Cells were harvested every 2–3 days for analyses of total cell number and SSEA-1 positive cells. The harvested cells were subcultured in the same type of medium for 6 consecutive passages; each time with the same inoculation amount of $2 \times 10^4$ cells.

3.3.9 Effects of Lactic Acid

The effects of lactic acid on ES cell proliferation and maintenance were also studied in 2-D static cultures. The growth media containing various initial amounts of lactic acid (0.162 to 3 g/L) were used to culture mES cells ($2 \times 10^4$) in gelatin coated 24-well. The
initial medium pH was adjusted by sodium bicarbonate to ~7.0. After incubation for 4 days, cells were harvested to quantify the total cell number and SSEA-1 positive cells.

3.3.10 Effects of Pore Size

About $3.5 \times 10^4$ mES cells were inoculated into each well containing a piece of the PET matrix (1×1 cm) and 100 µL of the ES growth medium. Cells were harvested every 5 days for analyses of cell number, SSEA-1 and Oct-4. Results obtained from the normal PET matrices with 60~130 µm pore size and thermal compressed PET matrices with reduced pore size of 30~60 µm were compared to evaluate the effects of matrix pore size.

3.3.11 Dynamic Cultures in Spinner Flasks

In order to avoid cell growth stagnation caused by oxygen limitation in static cultures, mES cells were cultured in a dynamic environment to increase oxygen transfer efficiency. The dynamic cultures were carried out in 25-mL spinner flasks, which were pre-siliconized with Sigmacote® (Sigma) to eliminate the possibility of cell attachment to the flask wall. The PET matrix was cut into 10 cm × 1 cm sheet and affixed onto a stainless steel mesh around the wall of the spinner flask. After autoclaving at 121°C for 30 min, 10 ml of cell suspension ($5 \times 10^4$ cells/mL) were added to the spinner flask. Unless otherwise noted, the spinner flasks were incubated in a CO$_2$ incubator at 37°C, agitated at 80 rpm, and with media change once every 2–3 days. Cell attachment kinetics in PET matrices was evaluated by counting the number of cells in the suspension during the first 2–5 h. The spinner cultures were studied for 15 days in five different media: the growth
media with and without LIF, and the conditioned media of 15%, 25%, and 50% in the growth media without LIF.

3.3.12 Perfusion Cultures in Controlled Bioreactor System

The two-stage perfusion bioreactor system set-up is illustrated in Figure 3.1. A 450-mL spinner flask with the PET matrix (1×25×0.1 cm) was used as the first-stage bioreactor with its pH and DO probes connected to a New Brunswick Bioflow 3000® control system. Approximately $1 \times 10^7$ STO cells were inoculated into this bioreactor, with 200 mL of working volume. The pH and DO were controlled at 7.0 and 70% air saturation, respectively, by continuously surface aeration of a gas mixture (air, CO$_2$, O$_2$, and N$_2$). After culturing the STO fibroblast cells for three days, ~7.2×$10^6$ mES cells in 4 mL were inoculated into the second-stage bioreactor, which was a glass column (2.5 cm diameter and 8 cm height) packed with PET matrices (2.5 cm diameter and 3 cm height). The entire bioreactor system was moved into a CO$_2$ incubator for the first 6~8 h after mES inoculation. Then, it was re-connected to the control tower and medium circulation between the two bioreactors was started at 7.5 ml/min. The continuous perfusion was started 24 h after mES cell inoculation, at a dilution rate of 1 day$^{-1}$ or 8.3 ml/h. Liquid samples were taken every two days to monitor the concentrations of glucose, lactic acid, and suspended cells.

For the perfusion culture of hES cells, they were cultured in the 450-mL spinner flask reactor described above. Six million hES cells were inoculated into the bioreactor with 200 mL working volume and pH and DO controlled at 7.0 and 70 % air saturation,
respectively. The MEF conditioned medium with 4 ng/mL bFGF was perfused into the system from 7th day at a perfusion rate of 1 day⁻¹.

At the end of the culture, cells were harvested from the PET scaffold. The culture medium was discarded and the reactor was filled with fresh PBS solution. Thereafter, the scaffold was washed by circulating PBS for 3 min. Following the washing, the PBS solution was removed from the vessel and 60 ml of Accutase® was pumped into the tank. The scaffold was immersed in the Accutase® for 20 minutes before being flushed. An additional amount of Accutase® was circulated into the fibrous bed and incubated for another 20 minutes. The solution was then flushed and the total flushed solutions were subjected to centrifugation to collect the harvested cells.

3.3.13 Embryoid Body (EB) Forming Efficiency

To evaluate the pluripotency of the ES cells produced from various culturing systems, their EB forming efficiency was determined, as follows. Approximately one thousand ES cells were inoculated into a non-adhesive petri dish. After culturing for 6 days in the ES growth medium without LIF, the resulting EBs were counted. The EB forming efficiency was defined as the number of EB divided by the number of inoculated cells (my own definition, which is similar to the CFU definition).

3.3.14 Flow Cytometric Analyses

ES cells were trypsinized from the PET matrices before flow cytometric analyses. For SSEA-1 assay, 2–5 ×10⁵ cells were washed with PBS containing 0.5% BSA and 2 mM EDTA (Sigma). Human FcR blocking reagent (Pharmigen, San Jose, CA) was added to
the single cell suspension to prevent nonspecific binding; this was followed by incubation of SSEA-1 antibody (Developmental studies hybridoma bank, Iowa city, IO) for 60 min. at 4°C. After washing, the samples were incubated with goat anti-mouse IgM-PE secondary antibody (Jackson ImmunoResearch lab, West Grove, PA) for 30 min. After fixing the cells with 2% formaldehyde, the SSEA-1 positive cell population was analyzed using a flow cytometer (BD FACS Calibur). For intracellular protein Oct-4 quantification, cells were fixed with 4% formaldehyde for 20 min. at room temperature, followed by cell membrane perforation with the washing buffer containing 0.5% saponin and 0.1% sodium azide (Sigma) for 20 min. at room temperature. The samples were later incubated with anti-Oct-4 monoclonal antibody (Chemicon, Temecula, CA) at room temperature for 30 min. and then IgG-FITC for another 30 min. The Oct-4 positive cells were then analyzed and quantified using a flow cytometer. SSEA-4 assay for hES cells was similar to SSEA-1 assay except that IgG-FITC, instead of IgM-PE, was used as the secondary antibody. Unstained cells were used for locating the population and cells only labeled with IgM-PE or IgG-FITC were used to evaluate the non-specific binding or background fluorescence reading.

3.3.15 Scanning Electron Microscopy (SEM)

Cell morphology and distribution in the PET matrices were observed using a scanning electron microscope (Philip XL 30, Philips Electronics, Eindhoven, The Netherlands). Each sample (cells in the PET matrix) was washed with PBS solution and incubated in 2.5%(v/v) glutaraldehyde overnight at 4°C. The samples were then rinsed with distilled water and progressively dehydrated in 10%(v/v) - 100%(v/v) ethanol in increments of
10% by soaking the sample for 30 min. at each concentration. The dehydrated samples were then dried by soaking in hexamethyldisilazane (HMDS) (Sigma) and ethanol mixture with ascending HMDS concentrations of 1:3, 1:1, and 3:1 for final dehydration. The dried samples were sput-coated with gold-palladium at an argon pressure of 14 Pa for 120 seconds and a current of 17 mA to convey electrical conductivity, and then observed in the SEM at 5-25 kV accelerating voltage.

3.3.16 Analytical Methods

Cell Counting. The PET matrices were placed into the standard nuclei counting solution (0.1 M citric acid, 0.1 % (w/v) crystal violet) and incubated at 37°C for 24 h. The matrix was then vigorously vortexed to release cell nuclei, which were counted under a microscope. Cells on 2-D surface of multiwells or T-flasks were counted, after trypsinization, using a hemocytometer.

Metabolic Assays. Lactic acid and glucose concentrations in the medium were measured with YSI Biochemistry Select Analyzer (Yellow Spring, OH).

3.4 Results and Discussion

3.4.1 Effects of Gelatin Coating on 2-D and 3-D Cultures

Figure 3.2 compares the results of mES cells cultured in 2-D and 3-D with or without surface coating with gelatin. In general, cells grew better on the gelatin-coated well surface than on the uncoated surface in 2-D cultures, as indicated by the higher expansion fold and fraction of SSEA-1 positive cells. This finding is consistent with common belief.
that gelatin or ECM protein coating on the support surface is indispensable for cell attachment and growth in mES cell cultures (Zandstra et al., 2001). However, the 3-D culture with PET scaffolds without gelatin pre-coating was able to support good cell growth and maintain high SSEA-1 expression (Figure 3.2). This advantage of 3-D cultures over conventional 2-D cultures can significantly reduce the cost of the ES cell expansion process by eliminating the expensive ECM coating of the support surface. It is noted that gelatin coating of the PET matrices resulted in poorer cell growth because the uneven coating blocked and deteriorated the 3-D pore structure of the matrices. In addition, ES cells harvested from the 3-D culture showed a higher EB forming efficiency of 24.7 % ± 7.2% (vs. 18.7% ±7.6% in 2-D cultures). The EB forming efficiency is an indicative of the pluripotency of ES cells or their ability to differentiate into various cell types (Zandstra et al., 2001). Therefore, ES cells grown in 3-D PET matrices can maintain their high potential for differentiation.

3.4.2 Effects of Conditioned Media and Lactic Acid

STO cells are commonly used as feeder cells to support the undifferentiated mES cell growth (Xu et al., 2001). These cells are believed to secrete several cytokines essential for mES cells to maintain their undifferentiated stage. The conditioned medium from STO cell culture was thus studied as an economic alternative to the more expensive cytokine LIF commonly used in mES cell growth media. Murine ES cells were cultured in the growth media (with and without LIF) and the conditioned medium (without LIF) for six passages. As shown in Figure 3.3, no significant difference in cell growth, as indicated by the total cell number at the end of each passage, was found among the three
media studied in 2-D cultures. However, SSEA-1 was significantly down-regulated in mES cells cultured in the growth medium without LIF, whereas the cells cultured in the conditioned medium and the LIF-containing growth medium had similar SSEA-1 expression level, which was maintained at the level of ~85%. The results suggested that the STO-conditioned medium was as good as LIF in maintaining mES cells in the undifferentiated stage.

However, it was later found that the conditioned media produced in the spinner flask could not sustain cell growth and maintain their undifferentiated stage in long-term 3-D mES cultures. SSEA-1 expression dropped from 98% to 58% in the 3-D culture after 20 days. Further investigation revealed that the lactic acid production in the 3-D STO culture was much higher than that in 2-D STO cultures, resulting in the higher lactic acid concentration of the conditioned media from the 3-D culture (2.2–2.4 g/L vs. 0.6–0.9 g/L from the 2-D culture). The higher density of STO cells in the 3-D culture resulted in limited oxygen transfer and higher lactic acid production.

Since lactic acid is a known inhibitor to most mammalian cells, it was believed to be the main factor limiting mES cells growth in the conditioned media with a high lactic acid content. The effects of lactic acid on mES cells were thus studied and the results are shown in Figure 3.4. As expected, lactic acid strongly inhibited ES cell growth at a concentration of 1.5 g/L and higher. At 3 g/L of lactic acid, it was observed that mES cells could not attach well on the flask surface and there was no significant cell growth. Moreover, increasing the lactic acid concentration also significantly decreased the cell populations expressing Oct-4 and SSEA-1, implying that mES cells had undergone
spontaneous differentiation at elevated lactic acid concentrations. It is clear that ES cells are more sensitive to lactic acid than other animal cells, which usually can tolerate lactic acid up to 3.5 g/L (Patel et al., 2000; Hassell et al., 1991). This result concurred with the finding that frequent media replacement to minimize the accumulation of metabolic wastes was necessary for long-term ES cell cultures (Viswanathan et al., 2004).

The conditioned medium was mixed with fresh mES growth medium at different ratios to reduce the amount of lactic acid in the resulting media, which were used to test their effects on the long-term mES cell culture. In the long-term culture, the media in the culture wells were refreshed at 50% rate periodically. The results are shown in Figures 3.5 and 3.6. In general, mES cells grown in these media showed similar growth kinetics. Except for the 50% conditioned medium, mES cells had expanded approximately 80–98 times and reached their maximum numbers after 15 days (Fig. 3.5A). The fraction of cells expressing Oct-4 decreased significantly faster in the culture without LIF than with LIF or the conditioned media (Fig. 3.6B). All cultures showed significant decrease in SSEA-1 positive cell population after 15 days. This dramatic drop in SSEA-1 expression occurred during the stationary phase when cell growth was limited by oxygen, which will be further discussed later in this paper. SSEA-1 is a surface marker and thus more sensitive to the culture environment than the intracellular protein Oct-4. It is noted, however, that SSEA-1 expression decreased much faster in 25% and 50% conditioned media, in which the lactic acid concentrations increased above the inhibiting level of 1.5 g/L (see Fig. 3.5C). It is thus clear that a high lactic acid concentration not only inhibited cell growth but also induced spontaneous differentiation. In general, the 10% conditioned medium was able to replace the LIF containing medium for the long-term 3-D mES
culture. ES cells growth was 16% higher and Oct-4 and SSEA-1 levels were within the range as compared to the normal ES growth medium.

3.4.3 Effects of Matrix Pore Size

Murine ES cells were cultured in PET matrices with two different pore sizes. As shown in Figure 3.7, mES cells cultured in the smaller pore scaffold (LP) grew faster with 60% more cells after 20 days as compared to the HP culture. There were also more SSEA-1 and Oct-4 positive cells in the LP culture. With smaller pores, the contact probability between the cells and the fibers is higher. Accordingly, more attachment and bridging, which are beneficial for cell proliferation, are likely to occur. This pore size effect was confirmed by SEM images showing that ES cells were more likely to form big aggregates in large-pore matrices (HP), and on the other hand attached to individual fibers when cultured in small-pore matrices (LP) (Figure 3.8). Aggregation of cells, which was mostly found in large-pore matrices, were not beneficial for cell proliferation and could induce differentiation due to the contact inhibition they caused. These findings are similar to the results previously reported by others (Ma et al., 2000). This might explain why cells grown in the larger pore matrices were more likely to differentiate.

3.4.5 Dynamic Culture in Spinner Flasks

Cell Seeding Kinetics. The kinetics of cell seeding in the spinner flask culture was studied by monitoring the number of suspended cells remaining in the culture medium after seeding. The adsorption kinetics of mES cells to the PET matrix followed the first order reaction kinetics, as illustrated in Figure 3.9. In addition, the adsorption rate was
influenced by the agitation rate, with the adsorption rate constants found to be 0.58 h\(^{-1}\), 1.76 h\(^{-1}\) and 1.58 h\(^{-1}\) at 40 rpm, 80 rpm and 120 rpm, respectively. In all three conditions, the seeding efficiencies were above 90%. Since the contact between cells and fibers was random, increasing the agitation rate increased the contact probability between cells and fibers. However, an overly high agitation rate can also result in an increase in the detachment rate. The optimal agitation rate for cell seeding in the PET matrices was 80 rpm.

3.4.6 Effects of Conditioned Media.

Experiments were carried out using conditioned medium to replace the LIF. Results shown in Table 3.1 imply that there is no significant difference in the cell growth rate among the media studied. However, Oct-4 expression was up-regulated with the increased ratio of the conditioned medium in the culture media; SSEA-1 expression was similar in the three conditioned media. This was not observed in static cultures because the lactic acid inhibition and oxygen limitation suppressed this phenomenon. As expected, mES cells differentiated significantly in the growth medium without LIF, as indicated by their low Oct-4 and SSEA-1 expressions.

In general, the dynamic cultures in spinner flasks performed better than static cultures because of agitation resulting in better oxygen transfer. As shown in Table 1, ES cell expansion was much higher in spinner flasks than in static cultures. Furthermore, Oct-4 and SSEA-1 expressions were also higher in the dynamic cultures. The increase in cell expansion and cellular markers was conjectured to be partly caused by the significant decrease in lactic acid production. Since most of lactic acid is produced anaerobically, the
production of lactic acid was suppressed by increased oxygen transfer in the dynamic cultures. This was further proven by the lower $Y_{\text{lac/glu}}$ values in the dynamic cultures. Consequently, lactic acid concentrations were kept lower than the inhibiting level for most of the time, except for the 50% conditioned media culture, as shown in Figure 10.

3.4.7 Effects of Agitation Rate.

mES cell cultures under different agitation rates were also compared. The results shown in Table 3.2 confirmed that increasing the mixing intensity improved mES cell expansion and reduced spontaneous differentiation. When mixing intensity was increased from 80 rpm to 120 rpm, both cell density and Oct-4 and SSEA-1 expressions increased, indicating that mES cell cultures in the 3-D system were mass transfer limited. Mixing provides fresh gas/liquid contact surfaces, and thus more oxygen can be delivered from the gas phase to the media. Also, the mass transfer mechanism involved diffusion as well as convection, which is more effective than diffusion that was the only mechanism in static cultures. The improved mass transfer was needed to ensure that cells in the center of the 3-D matrix did not undergo nutrient (oxygen) starvation.

3.4.8 Perfusion Culture in Controlled Bioreactor

In the previous results, we have shown that the undifferentiated mES cells could grow well in 3-D PET scaffolds when cultured in conditioned media obtained from STO cells. However, as our ultimate objective is to mass-produce ES cells, further up-scaling optimization needed to be performed. For this purpose, a two-stage perfusion culture system shown in Figure 3.1 was developed. The glucose consumption and lactic acid
production profiles in the bioreactor are shown in Figure 3.11, and the final cell number and SSEA-1 and Oct-4 positive cell fractions are given in Table 3.2. In 15 days, mES cells in this perfusion bioreactor system expanded 198-fold to 1.4 billion cells in the FBB bioreactor, which was much higher than those attained in spinner-flask and static multi-well cultures. Last but not least, the Oct-4 and SSEA-1 marker expression levels were 94.6% and 92.4%, respectively, higher than those of the mES cells cultured in spinner flasks and multiwells using LIF-containing growth medium.

By controlling pH and DO as well as proper perfusion, the cell culture environment was optimized in the bioreactor. Since both STO and mES cells were immobilized in the PET matrices and no viable cells were circulating in the culture media, there was almost no cross contamination between the two bioreactors. Furthermore, with the continuous supply of conditioned media from STO cells in the first bioreactor, the majority of the mES cells could conserve their pluripotency, as proved by the high percentage of undifferentiated mES cells. At the end of the study, ES cells in the bioreactor were harvested by rinsing with Accutase, which gave a 47.6% recovery rate.

3.4.9 Human ES Cell Cultures

hES cells culture in spinner flask was also investigated and the results are summarized in Table 3.3. A great advantage of 3-D culture of hES cells is eliminating the necessity of the Matrigel coating though hES cell growth slowed down to some extent in the 3-D spinner system. Nevertheless, this problem was solved in the bioreactor system. Matrigel is a complex mixture of mouse sarcoma origin. Therefore, animal components still exist in the culture system and this is not desirable for clinical therapy. Although human
laminin can replace the Matrigel in hES cells culture, it still imposes a high cost to the process.

Human ES cells culture in the perfusion bioreactor also demonstrated the success. hES cells expanded 145-fold and the SSEA-4 expression dropped slightly from 83.2% to 79.4% in 18 days, indicating that most of hES cells in the bioreactor maintained their pluripotency during the in vitro expansion. Furthermore, the hES cell growth rate in the bioreactor also surpassed that of the 2-D culture. In summary, no need for the ECM coating, higher growth rate, good maintenance of pluripotency, and automatic control assure the perfusion fibrous bed bioreactor system is a very promising platform for the scale up of the mass production process of undifferentiated ES cells.

hES cells were cultivated on Matrigel or laminin in MEF conditioned medium, where they could maintain their pluripotency for more than 130 population doublings (Xu et al., 2001). A microcurrent of 6 µA for 1 to 10 Hz was applied to ES cells for one hour each day. The hES cell expanded and remained undifferentiated after 10 to 14 cell cycles without MEF feeder cells (Rader et al., 2003) Amit and coworkers (2000) found that clonally derived human embryonic stem cell lines could maintain pluripotency and proliferation potential for prolonged periods of time. Amit et al. (2004) also maintained hES cells on a fibronectin matrix using unconditioned medium by supplementing bFGF and TGF-β1. Nevertheless, hES cell showed lower cloning efficiencies and growth rates and higher possibility of spontaneous differentiation. Cheng et al. (2003) observed that human adult marrow cells support prolonged hES cell expansion. Xu et al. (2005) showed that bFGF alone or with other growth factors supports hES cells growth which is
comparable to the hES cells growth in MEF conditioned medium. SCF, LIF, TPO, Flt3L alone or combination without bFGF is not sufficient to maintain undifferentiated hES cell growth. Levenstein and the coworkers (2005) in Wisconsin ascertained that high concentration of bFGF (250 ng/ml) was efficient to support undifferentiated hES cells growth. Beattie et al (2005) reported that 50 ng/ml of activin A was capable of sustaining hES cells pluripotency over 20 passages without the feeder layer or MEF conditioned medium. Without the presence of bFGF, hES cells pluripotency can be maintained in unconditioned medium by supplementing of TGF-β or activin, but the cell growth is poor.

3.4.10 Effects of Oxygen Transfer

The reason for cell growth stagnation in static culture was further investigated. We assumed that it was not because of contact inhibition, since according to the SEM pictures and the data, there was still a tremendous amount of unoccupied space in the scaffold. Nutrient limitation in the media was another factor for cell growth inhibition. In the 3-D static culture, the main mass transfer mechanism was by diffusion. Oxygen must diffuse through the liquid that occupied the pores of the scaffolds to reach the cells in the scaffold. When cell density increased, the permeability of the scaffold decreased and the oxygen consumption rates increased which resulted in poor oxygen transfer rates in the scaffold. Even without considering the decrease in permeability, our model below suggested that the oxygen concentration will be completely depleted after 387 h of culturing.
\[ \frac{\partial C}{\partial t} = D_1 \frac{\partial^2 C}{\partial z^2} \quad \text{for } z \leq 0.415 \text{ cm, oxygen diffusion in liquid} \]

\[ \frac{\partial C}{\partial t} = D_2 \frac{\partial^2 C}{\partial z^2} - QX_0 \exp(\mu t) \quad \text{for } 0.415 \text{ cm} < z \leq 0.515 \text{ cm, oxygen diffusion in scaffold} \]

B.C. \quad z = 0, C = 0.212 \text{ mM} \quad (100\% \text{ DO})

\[ z = 0.515 \text{ cm, } \frac{\partial C}{\partial z} = 0 \quad \text{(No diffusion through at the well bottom)} \]

I.C. \quad t = 72n, C = 0.212 \text{ mM}

\[ n=0,1,2,3,4,5 \quad \text{(Medium was changed every 72 h)} \]

where \( D_1 = 2.5 \times 10^{-5} \text{ cm}^2/\text{s}, D_2 = 1.8 \times 10^{-5} \text{ cm}^2/\text{s}, Q = 1 \times 10^{-10} \text{ mmol/cell/h} [18], \ X_0 = 3.75 \times 10^4 \text{ cells}, \mu = 0.012 \text{ h}^{-1}. \)

The transient partial differential equations were solved by Matlab\textsuperscript{®}. Figure 3.12 shows the oxygen concentration profile during the culture time. After 387 h, Oxygen concentration at the bottom of the well became zero. It is thus obvious that oxygen limitation is the dictating factor for long-term culture; in our case, we could not observe cell growth after 15 days of culture. The peak time for our 3-D culture to reach the maximum cell number, which appears to be 15 days, was actually 387 hours.

The effect of oxygen on stem cell growth and differentiation has been reported. Lennon et al. (2001) concluded that oxygen is an important factor that influences stem cell growth and differentiation. They found that rat mesenchymal stem cells could produce
more bone cells at 5% oxygen than 20% oxygen. Ouyang et al (2002) concluded that low oxygen tension could prolong epidermal stem cell’s life span. On the other hand, Kallos et al (2003) established that oxygen should be kept above 20% in neuron stem cell suspension culture in order to obtain more cells. Since the effect of oxygen on cell culture differ from one cell line to another, the influence of oxygen on ES cell differentiation need to be further investigated in a well controlled bioreactor system.

3.5 Conclusions

The 3-D culture system is an easy and economical way to mass produce undifferentiated ES cells. ECM coating and frequent subculture were circumvented in 3-D ES cell culture. Lactic acid above a certain threshold has a negative effect on ES cell growth and induces spontaneous differentiation. The success of two-stage perfusion fibrous bed bioreactor system demonstrated the promising future for up-scaling the process to satisfy market demands.

3.6 References


Beattie GM, Lopez AD, Bucay N, Hinton A, Firpo MT, King CC, Hayeka A, 2005
Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers, Stem Cells, 23: 489-495


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Table 3.1 mES cell culture performance after 15 days of culturing in various media.
Table 3.2 mES cell culture performance after 15 days of culturing in spinner flasks and a two-stage perfusion bioreactor.
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Table 3.3  hES cells cultures in different systems.

0.5 million hES cells were cultivated on Matrigel coated 25 cm² T-flask with MEF conditioned medium supplemented with 4 ng/ml bFGF. 0.3 million hES cells were inoculated into a 25 ml spinner flask with 10 ml working volume packed with 10 × 1 cm PET scaffold.
Figure 3.1 The two-stage perfusion ES cell culture system
Figure 3.2. ES cell culture in 2-D and 3-D culture with and without gelatin pre-coating, (A) ES cell growth, (B) SSEA-1 expression
Figure 3.3 Embryonic stem cells growing in LIF containing medium, without LIF medium, and conditioned medium (CM) in 2-D system within 6 passages, (A) cell growth, (B) SSEA-1 expression.
Figure 3.4 Effects of initial lactic acid concentration on ES cell in 2-D culture system, (A) cell number, (B) SSEA-1 and Oct-4 expressions
Figure 3.5 ES cells growth in LIF containing medium, without LIF medium, 10% conditioned medium, 25% conditioned medium, 50% conditioned medium in 3-D static culture. (A) cell number, (B) glucose concentration kinetics, (C) lactic acid concentration kinetics.
Figure 3.6 ES cells differentiation in LIF containing medium, without LIF medium, 10% conditioned medium, 25% conditioned medium, 50% conditioned medium in 3-D static culture, (A) SSEA-1 expression, (B) Oct-4 expression.
Figure 3.7 Effect of pore size on ES cell growth, (A) cell number, (B) SSEA-1 expression, (C) Oct-4 expression
Figure 3.8 SEM images of ES cell in PET scaffold, (A,B) ES cell growing in PET fibers, (C) ES cell distribution in large pore size scaffold, (D) ES cell distribution in small pore size scaffold
Figure 3.9 ES cell attachment kinetics in 3-D PET scaffold at 40 rpm, 80 rpm and 120 rpm
Figure 3.10 Glucose and lactic acid kinetics of ES cell culture in different medium in spinner flask at 80 rpm, (A) LIF containing medium, (B) without LIF medium, (C) 10% conditioned medium, (D) 25% conditioned medium, (E) 50% conditioned medium
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Chapter 4
Neural Differentiation from Embryonic Stem Cells in 2-D and 3-D Culture Systems

4.1 Abstract

The interaction between cells and their culturing environment is known to play a vital role in ES cell differentiation. In this study, the effects of tissue scaffolding and culturing methods on neural differentiation were studied. Murine embryonic stem (ES) cells were cultured on 2-D surfaces, microcarriers and in 3-D polyethylene terephthlate (PET) fibrous scaffolds. In general, sophisticated neural network prototype was observed in all three systems using media conditioned with astrocyte. The conditioned medium was found to be as efficient as retinoic acid in inducing neural differentiation and yet did not inhibit cell proliferation as did retinoic acid. Among the three culturing systems studied, 2-D system presented the highest overall neural differentiation efficiency. Partially differentiated ES cells were then used to study the potential of cell de-differentiation. It was found that Oct-4 expression increased in the cells grown in the growth medium, suggesting de-differentiation of ES cells. However, a higher degree of de-differentiation
occurred in the 3-D culture system with PET scaffolds than in the 2-D system. These results suggest that the high potential of de-differentiation in the 3-D culture system may limit its application in producing differentiated ES cells, but is favorable for expanding and maintaining undifferentiated ES cells. A scalable process for neural differentiation from ES cells in a 3-D bioreactor was shown to be feasible but would require further process optimization.

4.2 Introduction

Embryonic stem (ES) cells have unlimited proliferation potential and can differentiate into all cell and tissue types found in the whole animal (Guan et al., 1999; Wobus et al., 1991). The combination of these two characteristics makes ES cells an ideal cell source for tissue engineering and cell therapy. The commonly used in vitro ES cell differentiation protocol involves the formation of embryo-like aggregates, so called embryoid body (EB) as the first step. Then EBs are differentiated into endoderm, mesoderm and ectoderm cell lineages (Guan et al., 1999). However, differentiation into different cell lineages is mainly determined by the growth factors in the medium. For examples, insulin-like growth factor was essential for the formation of functional heart cells (Sachinidis et al., 2003); unknown factors in fetal calf serum (FCS) induced hematopoietic differentiation (Gordon 1995); and insulin in the medium initiated differentiation into pancreatic like cells (Lester et al., 2004). In order to achieve high
differentiation efficiency, cocktails of cytokines or growth factors are usually employed in ES cells differentiation (Lee et al., 2000; Cerdan et al., 2004; Parisi et al., 2003; Kawai et al., 2004; Kanno et al., 2004). However, these growth factors and cytokines are expensive to use and their high costs can greatly impede the ES cell differentiation process scale up. An alternative to using the well defined media with expensive growth factors is by co-culturing ES cells with stromal feeder cells, which can induce ES cell differentiation to a specific lineage (Kawasaki et al., 2000).

Although ES cell differentiation has been extensively studied. A scalable mass production method of differentiated ES cells remains to be developed. Recently, a controlled bioreactor system was used to grow and differentiate ES cells encapsulated in gels to hematopoietic cells and cardiomyocytes, which were augmented in their population by 3 times under low oxygen tension control in the bioreactor (Bauwens et al., 2005). This study indicated the possibility of regulating ES cell differentiation by engineering tools and the advantages of using a bioreactor with proper process control in the production of differentiated ES cells.

The interaction between cells and the culture environment plays a vital role in the embryonic stem cell differentiation in vitro. Unlike cells in 2-D slides, ES cells in 3-D collagen gel did not form epithelia cells and large spread cells. Instead, they formed tubular or spherical gland-like structures (Chen et al., 2003). Other three-dimensional
polymer scaffolds were also applied to study hES cell differentiation. A 50/50 mixture of poly (lactic-co-glycolic acid) (PLGA) and poly (L-lactic acid) (PLLA) matrix was coated with Matrigel or fibronectin to facilitate ES cell attachment. In the presence of various growth factors to induce ES cell differentiation, hES cells formed a 3-D structure on the polymer scaffold that was not observed in 2-D cultures (Levenberg et al., 2003). Another study showed that 3-D collagen gel network promoted neuron cells differentiation (Ma et al., 2005). The 3-D structure was also found to be important in other types of stem cell cultures. Porous hydroxyapatite/chitosan–gelatin (HCG) scaffolds improved osteogenic differentiation from human mesenchymal stem cells (MSC) (Zhao et al., 2006). Cartilage cell differentiation from MSCs was enhanced in a 3-D porous silk scaffold and the spatial cell arrangement and the collagen type-II distribution in the MSCs-silk scaffold constructs resembled those in native articular cartilage tissue (Wang et al., 2005).

Notwithstanding all these previous studies, the effect of culture environment on neural differentiation from embryonic stem cells is still not clear. In this work, neural differentiation of ES cells in different culturing systems, including static cultures on 2-D surfaces, in 3-D PET scaffolds, and dynamic culturing in bioreactors with microcarriers and PET matrices as the solid support for cell anchorage. The feasibility and advantages of using an astrocyte-conditioned medium to improve neural differentiation of ES cells were also studied. The effects of these different tissue scaffolding and culturing
methods on ES cell neural differentiation were studied and the results are reported in this paper.

4.3 Materials and Methods

4.3.1 Cultures and Media

ES D3 cells (CRL-1934) obtained from ATCC were maintained on gelatin pre-coated T-flasks consisting knock-out Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 µg/mL streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 µM β-mercaptoethanol (Sigma) and 100 µM leukemia inhibitory factor (LIF) (Chemicon). For neural differentiation, LIF was excluded in the above ES medium and either retinoic acid (10⁻⁷ M) or an astrocyte-conditioned medium (30% v/v) was added to induce neural differentiation.

Astrocytes (CRL-2253) from ATCC were cultured in DMEM with 10% FBS. Approximately three to five million astrocyte cells were inoculated to a PET matrix submerged in 110 mL of DMEM with 10% FBS in a 250-mL spinner flask. The medium was refreshed at day six and cells were cultured for another four days. Supernatant was then collected and used as conditioned medium to mix with fresh ES cell medium without LIF for neural differentiation.
4.3.2 Embryoid Body (EB) Formation and Re-inoculation

To produce the EB body for neural differentiation study, 2~5 × 10^5 ES cells were inoculated into the 9-cm bacterial culture grade Petri dish containing 10 ml of the differentiation medium. After 4 days of culturing, 9 ml of supernatant was discarded and EBs were harvested and suspended in 1 ml medium and used in neural differentiation study in various culturing systems described below. The total number of cells in the EB suspension was estimated by nuclei counting of cells present in 100 µl of EB suspension.

4.3.3 Neural Differentiation of ES Cells in Static Cultures

In static cultures, EBs were inoculated into either gelatin coated 24 well plate (2-D culture) or 3-D PET matrices in 24 well plate. Each well was inoculated with EBs equivalent to ~0.1 million cells (2-D culture). In 3-D inoculation, 50 µl of EBs suspension equivalent to ~0.12 million cells were slowly added to the matrix from the center by pipette. After incubation in a CO₂ incubator for 3 h to allow for cell attachment to the matrix, the seeded matrix was washed with medium to remove unattached and loosely attached cells. Then, the matrix was transferred to a new well and 1 ml of fresh medium was added.
4.3.4 Dynamic Cultures in Spinner Flasks

The EBs were inoculated and cultured in 25-ml spinner flasks with 10 ml of conditioned media and 2 mg/ml of microcarriers (Cytodex-3, Pharmacia, Uppsala, Sweden) or a piece of PET fibrous matrix (10 cm × 1 cm × 0.18 cm), representing 2-D and 3-D culturing environments, respectively. Spinner flasks were pre-siliconized with Sigmacote® (Sigma) to eliminate the possibility of cell attachment to the flask wall. The PET matrix was affixed on a stainless steel mesh around the wall of the spinner flask. After autoclaving at 121 °C for 30 min, 10 ml of EB suspension (equivalent to ~1 million cells) were added to the spinner flask. For the 2-D dynamic culture, microcarriers were pre-hydrated with PBS for 5 h, autoclaved at 121 °C for 30 min, and then soaked with the culture medium overnight prior to seeding with 10 ml of EB suspension (equivalent to ~1 million cells). Unless otherwise noted, the spinner flasks were incubated in a 5% CO₂ incubator at 37°C, agitated at 80 rpm, and with media change once every 2-3 days.

4.3.5 De-differentiation of Partially Differentiated ES cells

The partially differentiated ES cells from 2-D cultures in the astrocyte conditioned medium were harvested after 7 days and then re-inoculated into ES cell growth medium, with or without LIF, and the differentiation medium (30% v/v conditioned medium plus 10⁻⁷ M retinoic acid) in both 2-D and 3-D cultures to study the potential of de-differentiation of ES cells under various culturing conditions.
4.3.6 Flow Cytometric Analyses

To identify neural differentiation, two intracellular proteins, nestin and NCAM were analyzed. Nestin is expressed in the early stage of neural differentiation; while NCAM is expressed in the late stage. Undifferentiated ES cell marker Oct-4 was also analyzed. ES cells were trypsinized from the solid supports (2-D surface, 3-D PET matrices, and microcarriers) prior to the flow cytometric analysis. To measure the intracellular proteins Oct-4, nestin, and NCAM, cells were fixed with 4% formaldehyde for 20 min. at room temperature, and then the cell membranes were perforated with the permeabilizing buffer containing 0.5% saponin and 0.1% sodium azide (Sigma, St. Louis, MO) for another 20 min at room temperature. The samples were incubated with anti-Oct-4 (Chemicon, Temecula, CA), anti-nestin, or anti-NCAM monoclonal antibody (Developmental studies hybridoma bank, Iowa city, IO) at room temperature for 30 min. After washing with the permeabilizing buffer, cells were incubated with IgG-FITC (Jackson Immuno Lab, West Grove, PA) for another 30 min. Finally, cells were fixed with 2% formaldehyde and analyzed with a flow cytometer (BD FACS Calibur). Unstained cells were used for locating the population and cells only labeled with IgG-FITC were used to evaluate the non-specific binding or background fluorescence reading.
4.3.7 Scanning Electron Microscopy

Samples (0.5×0.5 cm²) were fixed with 2.5% glutaraldehyde overnight at 4°C. After washing with PBS several times, samples were dehydrated for 30 min in each of the ethanol solutions from 20% to 100% at 10% increment. Samples were then transferred to hexamethyldisilazane (HMDS) (Sigma) and ethanol mixtures with ascending HMDS concentrations: 1:3, 1:1, and 3:1 for final dehydration. Samples were coated with gold/palladium and examined with a Philips XL 30 scanning electron microscope (SEM) (Philips Electronics, Eindhoven, The Netherlands).

4.3.8 Analytical Methods

4.3.8.1 Cell Counting.

The PET matrices or microcarriers were placed into the standard nuclei counting solution (0.1 M citric acid, 0.1 % (w/v) crystal violet) and incubated at 37°C for 24 h. The matrix or carrier was then vigorously vortexed to release cell nuclei, which were counted under a microscope. Cells on 2-D surface were counted by a hemocytometer after trypsinization.

4.3.8.2 Metabolic Assays.

Lactic acid and glucose concentrations in the medium were measured with YSI Biochemistry Select Analyzer (Yellow Spring, OH).
4.4 Results

4.4.1 Effect of Conditioned Medium on Neural Differentiation

Wouter et al. (1996) reported that $10^{-7}$ M of retinoic acid (RA) can induce neural differentiation in ES cells. However, it was found that retinoic acid treatment strongly inhibited cell proliferation in both 2-D and 3-D cultures (Figure 4.1 A). Cells in astrocyte-conditioned medium grew ten times faster than cells in retinoic acid containing medium. Cells cultured in the conditioned medium with retinoic acid also grew faster than those in retinoic acid medium. The ability of conditioned medium to induce neural differentiation was studied and it was found that the conditioned medium was more efficient than retinoic acid in terms of neural differentiation induction (Figure 4.1 B). Nestin expression in the conditioned medium was 3 times higher than that in the medium containing retinoic acid. Although conditioned medium plus retinoic acid had the highest nestin expression, conditioned medium by itself was better for neural differentiation because of its better performance in both proliferation and differentiation. It is noted that cells in 2-D cultures generally had higher nestin expressions, suggesting that neural differentiation was more efficient in 2-D cultures. This was further studied with additional neural differentiation marker NCAM discussed below.
4.4.2 Neural Differentiation in Static Cultures

Figure 4.2 shows the kinetics of neural differentiation of ES cells grown either on 2-D surfaces or in 3-D matrices under static culture conditions. In general, ES cells cultured in these systems proliferated and gradually differentiated into neural cells as indicated by the increasing cell populations expressing nestin and NCAM and decreasing cell population expressing Oct-4 during the 15-day culturing period studied. However, both proliferation and neural differentiation were faster for ES cells grown on the 2-D surface in Petri dishes than they did in 3-D PET matrices.

Figure 4.3 shows the cell morphology in 2-D and 3-D culture systems. For 2-D cultures, cells in the peripheral of the attached embryoid body differentiated into neural type cells first (Fig. 4.3A). These peripheral cells also migrated out and communicated with other attached embryoid bodies if there was one nearby by forming neuritis or bridges (Fig. 4.3B) and eventually created a neural network (Fig. 4.3C). For 3-D cultures in PET fibrous matrices, cells can form sophisticated neural networks in high cell density regions (Fig. 4.3D). They stretched out on the fiber as they did on the 2-D surface (Fig. 4.3E). Two cells on the adjacent fibers could also communicate with each other by forming the bridge (Fig. 4.3F).
4.4.3 Neural Differentiation in Dynamic Cultures

Dynamic culturing in a bioreactor with mixing can improve mass transfer and afford a more scalable cell culture process, and was thus studied for its use in neural differentiation of ES cells. Figure 4.4 shows neural differentiation of ES cells in the 2-D microcarrier culture system for a period of 21 days. In general, the ES cells in the dynamic culture continued to grow and expanded ~14-fold (Fig. 4.4A). During this period, Oct-4 was increasingly down-regulated, while both neural markers, nestin and NCAM were up-regulated (Fig. 4.4B). The metabolic rates, as indicated by the glucose and lactic acid concentrations in the culture medium (Fig. 4.4A), remained stable, indicating a good potential for long-term culture in this system. However, large aggregates of embryoid bodies and multiple microcarriers (see Fig. 4.4C) were found in this system. The formation of large EB-microcarrier aggregates lowered the efficiency of utilizing the surface areas provided by the microcarriers and might have slowed neural differentiation as compared to the static culture in multiwells.

Neural differentiation in 3-D dynamic culture in the spinner flask with the PET fibrous matrix as the cell support is shown in Figure 4.5. Compared to the 2-D microcarrier culture system, the 3-D culture had a similar growth rate, but a lower efficiency in inducing neural differentiation as indicated by the higher Oct-4 and lower nestin and
NCAM expressions at 21 days (Fig. 4.5B). This finding was consistent with the results from the static cultures discussed before.

It should be noted that the degree of ES cell neural differentiation in these dynamic cultures was lower than those obtained in static cultures, indicating that the dynamic cultures were not optimal for neural differentiation. The reasons for this will be discussed later in this paper.

4.4.4 De-differentiation of Partially Differentiated ES Cells

The de-differentiation of partially differentiated ES cells were studied in both 2-D and 3-D static cultures and the results are shown in Table 4.1. In general, partially differentiated ES cells continued their differentiation process when they were re-cultured in the differentiation medium, as indicated by the increased expressions of nestin or NCAM after 14-day culturing. However, the differentiation process was interrupted for cells re-cultured in the two growth media, either with or without LIF. As expected, cells cultured in these media showed a much higher growth rate than that in the differentiation medium. Furthermore, significant increase in Oct-4 (from 48% to 60~70% in 2-D and 70~80% in 3-D cultures) and decreases in both nestin and NCAM expressions were observed in these cultures at 14 days. This population shift toward undifferentiated cells might be because the non-differentiated ES cells in the mixed population grew faster than the differentiated cells. However, it is also possible that “de-differentiation” occurred
when partially differentiated cells were re-cultured in the growth media. This hypothesis was made with more confidence based on the observation that the cell growth rate in 3-D cultures was usually less than that in 2-D cultures. The 3-D culture clearly is more favorable for the proliferation of undifferentiated ES cells than 2-D culture and has the higher potential to induce de-differentiation under the culturing conditions studied.

4.5 Discussion

Wouter et al. (1996) applied $10^{-7}$ M of retinoic acid to induce neuron differentiation of ES cells. After 4-5 days of treatment, they detected neuron marker GAP-43 and NF-165 expression. However, neurongenesis is a multifaceted process-- different neural cells appear at different stages. ES cells are capable of differentiating into neural stem cells, which can further differentiate into astrocyte progenitor, oligodendrocyte progenitor, and neuronal progenitor cells. These progenitor cells finally differentiate into more specific neural cell types. Reubinoff et al. (2001) studied neural progenitors derived from ES cells. In order to obtain enriched neuron progenitor cells, ES cells were cultured for 3-4 weeks, and during the first week, neuronectodermal markers nestin and PAX-6 were detected. After one week, the differentiation process speed up, and early neuronectodermal marker NCAM (neural cell adhesion molecule) was expressed. They also found that combining basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) is favorable for human fetal and adult derived neuroepithelia progenitors.
Dopamine neurons attracted particular attention due to their application in Parkinson disease treatment. Two different procedures to derive dopaminergic neurons from ES cells have been reported (Lee et al., 2000; Kawasaki et al., 2000). One is based on defined culture conditions and the other is based on co-culturing with stromal feeder cells. Lee et al. (2000) developed a method for generating midbrain neurons in a defined culture medium; the whole process took 3-5 weeks. Kawasaki et al. (2000) developed a co-culture system to induce midbrain dopaminergic neuron differentiation from mouse ES cells. They identified a stromal cell-derived inducing activity (SDIA) that increased neural differentiation. Perrier et al. also used a similar strategy to derive midbrain dopamine neurons from human embryonic stem cells by using MS5 and S2 as feeder cells (Perrier et al., 2004). Nakayama et al. found that astrocyte-derived factors induced the differentiation of ES cells into neurons (Nakayama et al., 2003). The astrocyte conditioned medium was supplemented with 20 ng/mL fibroblast growth factor-2 (FGF-2) to stimulate the proliferation of neural stem cells. Buytaert-Hoefen et al. also did a similar work on hES cells (Buytaert-Hoefen et al., 2004). In addition, Shim applied molecular genetic tools to enhance the midbrain dopamine neuron differentiation efficiency (Shim et al., 2004).

Although retinoic acid was effective in inducing neural differentiation (Wouter et al., 1996), its induction on neural differentiation was dose dependent. It has been reported that cardiac muscle cells were observed when retinoic acid was depleted in the culture
medium (Gajovic et al., 1997). Moreover, retinoic acid significantly inhibited cell proliferation. Lee and coworkers (2000) applied a cocktail of chemicals and growth factors for neural differentiation, but this method involved expensive growth factors. A more delicate but also economical neural differentiation method thus should be developed. Therefore, astrocyte conditioned medium was studied as an alternative method to induce dopamine neurons from ES cells. Our results proved that astrocyte conditioned medium can induce neural differentiation in ES cells more efficiently than retinoic acid. It also has the advantage of stimulating cell growth.

The significance of interaction between the culture system and cell growth or differentiation has long been recognized. The concept of 3-D cell culturing has been proved to be superior to 2-D culturing in many ways and is widely used in tissue engineering (Chen et al., 2003; Levenberg et al., 2003). Three-dimensional tissue scaffolds can mimic cells’ in vivo growth environment and thus, cells show their native morphology and functions in 3-D scaffolds (Wang et al., 2005). Our previous work also suggested that ES cells were able to maintain their pluripotency and proliferation potential in 3-D PET scaffolds (Ouyang 2006). However, the 3-D culture is not optimal for neural differentiation as compared to traditional 2-D culture systems. Further investigation on this issue revealed an interesting phenomenon of “de-differentiation” in 3-D culture. At least some of the differentiated cells regained their Oct-4 expression ability. Oct-4 is the marker that identifies un-differentiated ES cells. This might be one
reason why it worked better for undifferentiated ES cells expansion in 3-D PET scaffolds (Ouyang 2006). A dynamic culture system with microcarriers was also found to be better for ES cell neural differentiation than that using 3-D PET scaffolds. Microcarriers provided a 2-D surface area while PET matrices provided a 3-D culture environment. Again, the different differentiation efficiencies can be attributed to de-differentiation in 3-D culture systems.

Compared to static cultures in multiwells, dynamic culturing in bioreactors presents a more scaleable process but was found to be less favorable to ES cell neural differentiation. Mixing provided a better mass transfer rate and thus reduced the accumulation of lactic acid, which is widely considered a cytotoxic metabolic waste. It has been shown that mixing can improve undifferentiated ES cell expansion while still retaining their pluripotency (Ouyang 2006). Bioreactors were also demonstrated to be able to improve the EB forming efficiency (Gerecjt-Nir et al., 2004). However, Sean and his co-workers found that mechanical stress could inhibit human embryonic stem cell differentiation (Palecek et al., 2005). Dang and co-workers found that embryoid bodies did not grow if put directly into the agitated bioreactor, and they used encapsulation to solve this problem (Dang et al, 2004 and 2002). Bauwens (2005) exploited gel encapsulation technique to generate cardiomyocyte from ES cells in bioreactors. Therefore, shear stress induced by agitation may contribute to the poor differentiation efficiency, which presents a challenge to the differentiation process scale up in the dynamic culture.
Other culture parameters such as pH and DO can also influence stem cells differentiation (McAdams et al., 1997; Bauwens et al., 2005). McAdams (1997) found the differentiation efficiency of erythroid progenitors enhanced when increasing the pH from 6.95 to 7.4. Bauwens (2005) illustrated that 4% oxygen tension promoted more cardiomyocyte differentiation from ES cells than 20% oxygen tension. A scalable neural differentiation process from ES cells in a 3-D bioreactor was demonstrated in this study, which provided a good platform for investigating the effects of these culture parameters. It should be mentioned that ES cells can grow well in the 3-D PET scaffold without pre-coating with any extracellular matrix proteins, a potential cost advantage over 2-D cultures, which usually require coating the tissue flask surface with expensive gelatin or Metrigel (Ouyang 2006). However, more sophisticated process development, control and bioreactor design would be required to optimize ES cell differentiation in the dynamic environment inside a bioreactor.

4.6 Conclusion

The astrocyte conditioned medium can induce neural differentiation in ES cells more efficiently than retinoic acid and still stimulate cell proliferation. ES cell neural differentiation was more efficient in 2-D than in 3-D cultures due to the higher potential of de-differentiation in 3-D environment. Neural differentiation from ES cells in a 3-D bioreactor is feasible but requires further process optimization.
4.7 Acknowledgement

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4.8 References


Gajovic, S., St-Onge, L., Yokota, Y., Gruss, P. Retinoic acid mediates Pax6 expression during in vitro differentiation of embryonic stem cells. Differentiation 1997, 62, 187-192,


Levenberg, S., Huang, N.F., Lavik, E., Rogers, A., Itskovitz-Eldor, J., Langer, R.


Ouyang A. Embryonic stem cell culture in a fibrous bed bioreactor, Ph.D thesis, The Ohio State University, 2006


Sachinidis, A., Fleischmann, B.K., Kolossov, E., Wartenberg, M., Sauer, H., Heschelere, J. Cardiac specific differentiation of mouse embryonic stem cells. Cardiovascular Res. 58, 125

Wang Y, Kim U-J, Blasioli DJ, Kim, H-J, Kaplan DL, In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells, Biomaterials, 26, 7082-7094, 2005

Wobus, A.M., Wallukat, G. and Hescheler, J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca\(^{2+}\) channel blockers. Differentiation. 48,173-182, 1991

Wouter, G.I., Maikel, P.P., Maria, W.M., Neuron differentiation of embryonic stem cells. Biochemica et Biophysica Acta. 1312, 21-26, 1996

Zhao F.; Grayson WL, Ma T, Bunnell B, Lu WW, Effects of hydroxyapatite in 3-D chitosan–gelatin polymer network on human mesenchymal stem cell construct development, Biomaterials 27, 1859-1867, 2006
Table 4.1 De-differentiation of partially differentiated ES cells in various media in 2-D and 3-D static cultures.

<table>
<thead>
<tr>
<th></th>
<th>Cell density (10^4/well)</th>
<th>Oct-4 (%)</th>
<th>Nestin (%)</th>
<th>NCAM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell population</td>
<td>~10</td>
<td>48</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>After 2-D culturing in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth medium with LIF</td>
<td>70 ± 15</td>
<td>69</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Growth medium without LIF</td>
<td>73 ± 29</td>
<td>60</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Differentiation medium</td>
<td>25 ± 14</td>
<td>50</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>After 3-D culturing in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth medium with LIF</td>
<td>33 ± 3.1</td>
<td>80</td>
<td>9</td>
<td>0.1</td>
</tr>
<tr>
<td>Growth medium without LIF</td>
<td>66 ± 7.5</td>
<td>78</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Differentiation medium</td>
<td>5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The cell number was lower than the total cell number used to inoculate the PET matrix because not all cells were attached to the matrix in the initial cell seeding.
Figure 4.1 Neural differentiation of embryonic stem cells in various static culture systems. (A) cell expansion fold (B) nestin expression. (Abbreviation: 2-D: cells grown on the surface in multwells; 3-D: cells grown in the 3-D PET matrix in multiwells; DM: differentiation medium; CM: conditioned medium; RA: retinoic acid)
Figure 4.2 Kinetics of neural differentiation from ES cells in different static culture systems. (A) 2-D culture (B) 3-D culture.
Figure 4.3 Morphology of ES cells from neural differentiation in 2-D and 3-D cultures. (A) cells at the peripheral of the EB differentiated first on 2-D surface at 4th day, (B) cells at the peripheral of the EB migrated and formed bridge with other EBs on 2-D surface at 6th day, (C) neural network formation on 2-D surface at 15th day, (D) neural network formation in 3-D PET scaffold at 10th day, (E) differentiated ES cells on the PET fiber, (F) two neural cells on adjacent fibers communicate with each other by forming the neurite bridge.
Figure 4.4 Neural differentiation of ES cells in the dynamic culture with microcarriers in a spinner flask agitated at 60 rpm. (A) Kinetics of cell growth, glucose consumption, and lactate production; (B) Cell populations expressing Oct-4, nestin, and NCAM, respectively; (C) Aggregates of ES cells and microcarriers.
Figure 4.5 Neural differentiation of ES cells in the dynamic culture with 3-D PET matrix in a spinner flask agitated at 60 rpm. (A) Kinetics of cell growth, glucose consumption, and lactate production; (B) Cell populations expressing Oct-4, nestin, and NCAM, respectively.
Chapter 5

Effects of Mixing Intensity on Cell Seeding and Proliferation in Three-Dimensional Fibrous Tissue Scaffolds

5.1 Abstract

Nonwoven fibrous matrices have been widely used in cell and tissue cultures because their three-dimensional (3-D) structures with large surface areas and pore spaces can support high-density cell growth. Although cell adherence and growth on 2-D surfaces have been thoroughly investigated, very little is known for cells cultured in 3-D matrices. The effects of mixing intensity on cell seeding, adherence, and growth in fibrous matrices were thus investigated. CHO and osteosarcoma cells were inoculated into nonwoven PET matrices by dynamic and static seeding methods, the former was found to be superior in the seeding efficiency and cell distribution in the matrices. Dynamic seeding increased seeding efficiency from ~40% to more than 90%. When higher mixing intensities were applied in the dynamic seeding, both cell attachment and detachment rates increased. The cell attachment was transport limited, as indicated by the increased attachment rate with increasing the mass transfer coefficient of the cells. Meanwhile, cell detachment from the 3-D matrix can be described by the Bell model. The effects of the matrix pore size on
cell adherence and proliferation were also investigated. In general, the smaller pore size is favorable to cell attachment and proliferation. Further analysis revealed that the interaction between mixing intensity and pore size played a vital role in hydrodynamic damage to cells, which was found to be significant when the size of Kolomogorov eddy was smaller than the matrix pores. Increasing the mixing intensity also increased oxygen transfer, decreased the lactate yield from glucose, and improved cell growth.

5.2 Introduction

The growth of anchorage-dependent mammalian cells commonly used in tissue engineering and recombinant protein production usually requires a large surface area for cell attachment. Seeding or attaching cells onto the solid support is the first and often the most critical step in mammalian cell cultures, and has long been a challenge for the scale-up of anchorage-dependent cell cultures. For decades, microcarriers have been widely used as the solid support for large-scale anchorage-dependent cell cultures because of their large specific surface areas and the homogeneous culture environment afforded by them in the conventional stirred tank bioreactor (Varani, et al., 1998; Sun et al., 2000; Shiragami et al., 1997; Bouchet et al., 2000; Malda et al., 2003; Clark et al., 1980). However, microcarriers are expensive and can clog the spin filter used in a perfusion culture. Recently, a three-dimensional (3-D) cell culture method using fibrous matrices such as polyethylene terephthalate (PET) has been developed for culturing various cell types, including CHO, hybridoma, human osteosarcoma, human cytотrophoblast ED27 and mouse embryonic stem cells (Chen et al., 2002; Luo and Yang, 2004; Ma et al., 2000; Li et al., 2003; Zhu and Yang, 2004). In general, the 3-D fibrous matrices can support
high cell densities ($3 \times 10^8$ cells/mL matrix) with a high volumetric productivity of the recombinant protein or monoclonal antibody (Yang et al., 2004). The PET fibrous matrices are also advantageous for use as tissue scaffolds because of their high porosity, specific surface area, permeability, and mechanical strength. The unique 3-D structure of PET scaffolds can provide cells a biomimetic environment that closely resembles their $in vivo$ growth conditions. Cells cultured in the porous matrices are also protected from shear damage, a major concern in microcarrier suspension cultures. However, how to seed a large number of cells uniformly into fibrous matrices has not been well studied and remains a challenge, especially in large-scale cell and tissue cultures.

Seeding is one of the most important steps in 3-D culturing because seeding can greatly affect cell growth and morphogenesis in the scaffold. Conventional static seeding methods usually have a low seeding efficiency of less than 50%. Several dynamic methods for seeding 3-D tissue scaffolds have thus been studied, including filtration and perfusion seeding (Carrier et al., 1999; Kim et al., 2000; Li et al., 2001; Zhao et al., 2005). In general, dynamic seeding methods improved the seeding efficiency in fibrous scaffolds to 60% – 76% (Kim et al., 1998; Nasseri et al., 2003; Xiao et al., 1999; Zhao et al., 2005). However, an investigation of cell adherence mechanism in 3-D matrices that may lead to better understanding and further improvement of the seeding efficiency has not been carried out. Orsello et al. (2001) proposed a receptor-ligand model for describing cell adhesion mechanism on the 2-D surface. However, the cell adherence mechanism in the 3-D matrix is more complicated as cells not only attach to the fiber surface but also may be entrapped in the crevices of the porous matrix and form bridges between fibers when the distance between adjacent fibers is compatible to cell size (Basu
and Yang, 2005; Ma et al., 1999; Yang et al., 2005). In addition, mixing has profound effects on cell attachment and detachment. A high mixing intensity can increase the contact frequency between cells and the matrix, which is critical to initial cell attachment. On the other hand, a high mixing intensity also can result in cell detachment (Gooch et al., 2001; Mascari et al., 2002) and hydrodynamic damage (Nienow et al., 1996). Whether and how the mixing intensity, an important parameter in bioreactor scale up, affects cell adherence and growth in 3-D fibrous matrices remains to be answered.

In this work, two cell lines with different cell sizes and attachment strengths were chosen as model cell lines to study the seeding of 3-D PET matrices. The effects of mixing intensity on Chinese hamster ovary (CHO) and osteosarcoma (OST) cell adherence and growth in PET matrices with two different porosities and pore sizes were investigated. It was found that cell attachment to the fibrous matrix under the dynamic seeding conditions can be simulated with a reversible adsorption model. Also, the mixing intensity had strong effects on cell adhesion, growth, and damage by the hydrodynamic shear, but the effects were dependent on the matrix pore size. These results and their implications on improving seeding efficiency and cell growth in porous fibrous matrices are discussed in this paper.

5.3 Materials and Methods

5.3.1 Cultures and Media

Chinese hamster ovary cells (CHO 4 AMLP), expressing a secreted alkaline phosphatase (SEAP) under the control of the S-phase specific adenovirus major late promoter (Lee et al, 1998), were cultured in F12 (GIBCO) medium supplemented with 10% (v/v) fetal
bovine serum (GIBCO). The recombinant human osteosarcoma cells OST 143b (Chen et al., 2002) were cultured in DMEM (GIBCO) medium supplemented with 5% (v/v) fetal bovine serum. Cells were subcultured in 75 cm² T-flasks and harvested before use for seeding 3-D PET matrices.

5.3.2 PET Matrices

Needle-punched nonwoven polyethylene terephthalate (PET) matrices (fiber diameter, ~20 µm; fiber density, 1.35 g/cm³) were used as cell culture scaffolds. The PET matrices were pretreated with NaOH before inoculation in order to increase the hydrophilicity as previously reported (Ma et al., 2000). PET fibrous matrices with reduced pore sizes were prepared by the thermal compression method (Li et al., 2001). The physical characteristics of the thermally compressed (denoted as LP) and uncompressed (denoted as HP) PET matrices are listed in Table 5.1.

5.3.3 Static Seeding

Unless otherwise noted, the seeding experiments were conducted with uncompressed (HP) matrices. Each well containing one piece of the PET matrix (10 mm in diameter) was inoculated with 0.2 mL of a cell suspension containing 1–3 × 10⁶ cells/mL, which was slowly added to the center of the matrix with a micropipette. After incubation in a CO₂ incubator for 3 h to allow for cell attachment to the matrix, the seeded matrix was washed with the medium to remove unadhered and loosely attached cells. Then, the matrix was transferred to a new well.
5.3.4 Dynamic Seeding

The PET matrix was cut into a 21 cm × 1 cm sheet and affixed on a stainless steel wire mesh around the wall of a 250-mL spinner flask (Fig. 5.1). The spinner flask was presiliconized with Sigmacote® (Sigma) to eliminate the possibility of cell attachment to the flask wall. A 5-cm long paddle impeller was installed in the spinner flask with a 5 mm clearance distance from the bottom. The whole system was autoclaved at 121 °C for 30 min before use. The spinner flask was seeded with 60 mL of a cell suspension containing 1.5~2.6 × 10^5 cells/mL. Cell attachment kinetics on the PET matrices was followed by monitoring the cell number remaining in the suspension during the first 6–8 h at various agitation rates (10, 20, 40 and 80 rpm). A control experiment was done with cells in the spinner flask without the fibrous matrix to monitor if there was any reduction in the suspended cells caused by hydrodynamic shear damage instead of cell attachment to the fibrous matrix. All experiments were repeated at least three times and the average values are reported in this paper.

5.3.5 Analytical Methods

The PET matrix was placed into the standard nuclei counting solution (0.1 M citric acid, 0.1 % (w/v) crystal violet) and incubated at 37 °C for 24 h. The matrix was then vigorously vortexed to release cell nuclei, which were counted under a light microscope. Lactic acid and glucose concentrations in the medium were measured with YSI Biochemistry Select Analyzer (Yellow Spring, OH). The lactate dehydrogenase (LDH) level in the medium was measured with the CytoTox One® Assay kit (Promega, Madison, WI).
5.3.6 Cytological Assessment of Cell Distribution in PET Matrices

PET matrices with cells were fixed in a 10% formalin solution overnight and then embedded in 4 μm thick layers of paraffin. Cross-sections were cut through the center of the scaffolds. Sections were stained with hematoxylin and erosin (H&E). Cell distribution in the matrix was examined under a light microscope (Eclipse TE200, Nikon, Japan).

5.4 Results and Discussion

5.4.1 Cell Seeding in PET Matrices

The fraction of cells remained in suspension after dynamic seeding was monitored. Figures 5.2 and 5.3 show the kinetics of seeding CHO and OST cells, respectively, into high-porosity (HP) and low-porosity (LP) matrices at various agitation rates. In general, the seeding to the matrices was good for all cases studied, with most cells attached to the matrices within 6 h. Except for the one at 10 rpm agitation rate, more than 90% of the initially seeded cells were attached to the matrices. The seeding efficiency was calculated from the initial and final cell density in the suspension and the results are shown in Table 5.2. It is clear that the dynamic seeding was much more efficient than static seeding, which gave a low seeding efficiency of ~40% for both CHO and OST cells. The greater than 90% seeding efficiency obtained in this study was also significantly higher than those from other dynamic seeding methods. A previous study showed that filtration seeding in PET scaffolds gave a seeding efficiency of 60% (Li et al., 2003). A similar dynamic seeding method employed in polyethylene oxide-terephthalate (PEO)/polybutylene terephthalate (PBT) scaffolds achieved a seeding efficiency of 76%
(Xiao et al., 1999). A maximum seeding efficiency of 68% was obtained for human mesenchymal stem cells cultured in a perfusion bioreactor system (Zhao et al., 2005).

The results from this work clearly showed a greater improvement in the dynamic seeding of 3-D fibrous matrices. The lower seeding efficiency for CHO cells in the HP matrix at 10 rpm was attributed to the non-turbulent mixing environment in the spinner flask. For the spinner flask, the impeller Reynolds number, $Re = \frac{d^2 n \rho}{\mu}$, at 10 rpm was 595, whereas at and above 20 rpm the Reynolds number was greater than 1000, which would be in the turbulent region with the geometric shape and agitation blade used in this work (Ho and Wang, 1991). This suggests that turbulent mixing is important to efficient seeding of cells into the fibrous matrices. However, once the flow was in the turbulent region, further increasing the agitation rate did not have significant effect on the seeding efficiency. This finding is consistent with the study on a tissue-engineered cartilage by Gooch and coworkers (2001). They also concluded that the existence of mixing itself and not the mixing intensity was the primary determinant factor of cartilage development. In addition to the agitation rate, the cell type and matrix pore size also would affect cell adherence and the seeding efficiency, which will be discussed in the following section.

5.4.2 Kinetics of Cell Adherence

The cell attachment process usually follows the first-order reaction kinetics that can be described by the following equations (Sun et al., 2000; Fang et al., 2000; Yashiki et al., 2001):

$$\frac{dC}{dt} = -k_{app} C$$  \hspace{1cm} (1)
\[-\ln\left(\frac{C}{C_0}\right) = k_{\text{app}} t\]  

(2)

As shown in Figures 5.4 and 5.5, the attachment of CHO and OST cells to PET matrices followed the first-order adsorption kinetics. The apparent adsorption rate constant, \(k_{\text{app}}\) can be determined from the slopes of these linear plots, and are listed in Table III. However, cell adherence, especially in the dynamic 3-D environment, is actually a reversible process with simultaneous cell attachment and detachment, which can be represented as follows:

\[
\begin{align*}
    C & \xrightarrow{k_f} C_a \\
    C & \xleftarrow{k_r} C_a
\end{align*}
\]

The rate of change in the free cell number in the suspension thus can be represented by the following equation:

\[
\frac{dC}{dt} = -k_f C + k_r C_a
\]

(3)

By introducing the dimensionless cell number, \(x = C/C_0\) and using the material balance, \(C_0 = C + C_a\), eq. (3) can be reduced to:

\[
\frac{dx}{dt} = -(k_f + k_r)x + k_r
\]

(4)

Eq. (4) can be integrated to the following form:

\[
t = \frac{-\ln\left(\frac{(k_f + k_r)x - k_r}{k_f}\right)}{k_f + k_r}
\]

(5)
Based on eq. (4), the time course data shown in Figures 5.2 and 5.3 were replotted in the form of \( \frac{dx}{dt} \) vs. \( x \) to validate the reversible model and find the rate constants, \( k_f \) and \( k_r \). As shown in Figure 5.6, the data generally fit well in the linear plots, confirming that the reversible model is valid. The rate constants can be estimated from the intercept on the y-axis, which equals to \( k_r \), and the slope, which equals to \(- (k_f + k_r)\). However, to minimize the error or uncertainty stemming from derivatizing the time course data to get \( dx/dt \), the \( k_f \) and \( k_r \) values found from the linear plots were used as the initial values in a nonlinear regression fitting of eq. (5) with Polymath® 5.1. As also seen in Figures 5.2 and 5.3, the reversible model fits the experimental data well (p value <0.05 and \( R^2 > 0.9 \)). The best values of \( k_f \) and \( k_r \) found from the nonlinear regression are listed in Table 5.3. In general, both \( k_f \) and \( k_r \) values increase with increasing the agitation rate; however, \( k_f \) is one order of magnitude larger than \( k_r \).

5.4.3 Cell Adherence Mechanism in 3-D Fibrous Matrices

The cell adherence mechanism in 3-D matrices is multifaceted. Cell adherence to a solid surface usually can be described by the ligand and receptor model, and consists of two steps: the first step is cell delivery to the region where the ligand is close enough to the receptor; and the second step is the formation of a bond between the ligand and receptor (Orsello et al., 2001). The first step is related to the collision frequency, while the second step is associated with the contact time. In general, cell-fiber collision frequency increases with increasing the agitation rate and is higher in the low-porosity (LP) matrix and for the larger cells. On the other hand, the contact time should decrease as the agitation rate increases since cells would be more quickly swept from the surface, which
also would increase the detachment rate. In addition, cells are easier to be trapped in a matrix with smaller pores, which would increase the contact time. Cells could have multiple contacts with adjacent fibers when the fibers are close enough. Therefore, the $k_f$ values for seeding into the LP matrix were generally higher than those of the HP matrix at the same agitation rate. As expected, the $k_f$ values for the CHO cells, which had an average diameter of 12 $\mu$m, were smaller than those of the larger OST cells (diameter: 15 $\mu$m).

Since the probability of cell attachment to fibers increases with increasing the collision frequency and thus the transport or mass transfer rate of the cells, $k_f$ should increase with increasing the mass transfer coefficient of the cells, $k'$, which can be estimated from the following mass transfer correlation (Harnby et al., 1992; Gooch et al., 2001):

$$Sh = 2 + 0.72 \frac{Re}{Sc}^{1/2}$$

(6)

where $Sh = \frac{k'd^*}{D}$, $Sc = \frac{\nu}{D}$ and $Re = \frac{d^2n\rho}{\mu}$

In order to find the mass transfer coefficient, $k'$, the diffusivity of the cells was estimated using the following equation (Bird et al., 2001):

$$D = \frac{k_BT}{6\pi\mu R}$$

(7)

For CHO cells with $R = 6$ $\mu$m and $\mu = 0.7$ mPa·s at 37$^\circ$C, the diffusivity $D = 5.41 \times 10^{-10}$ cm$^2$/s.
As can be seen in Figure 5.7, there is a positive correlation between $k_f$ and $k'$. However, the increase in $k_f$ with $k'$ was asymptotic, suggesting that cell attachment was no longer limited by transport or mass transfer at high mass transfer or agitation rates; instead, it was limited by reaction or bond formation between the cells and the fiber surface, which required a sufficient contact time.

Cell detachment rate from the fibrous matrix also increased with increasing the agitation rate, which would affect the fluid flow field and the shear stress exerting on the attached cells. The shear stress in the fibrous matrix can be evaluated according to eq. (8) (Perry and Wang, 1989), in which the local fluid velocity through the matrix, $\nu_{matrix}$, at a given agitation rate was estimated using FLUENT® as shown in Fig 5.8.

\[
\tau = \frac{\mu d' \nu_{matrix}}{4k(1 - \varepsilon')}
\]  

(8)

Figure 5.9 shows a strong correlation between the cell detachment rate constant, $k_r$, and shear stress, $\tau$. After data regression, it was found that cell detachment from the fibrous matrix followed the Bell model (Bell, 1978), which predicts that cell detachment is proportional to the exponential of mechanical force, $F$, or shear stress, $\tau = F/A$, as follows:

\[
k_r = k_{r0} e^{\frac{\sigma F}{k_BT}} = k_{r0} e^{\sigma' \tau}
\]  

(9)

Where $k_{r0}$ is the detachment rate constant at no force circumstance, $\sigma$ is the distance range of bond energy potential minimum, and $\sigma' = \sigma A/k_BT$. Several other models have
also been proposed for describing the ligand and receptor bond dissociation (Chen and Springer, 2001), including Hookean spring model and Evans model that give different relationships between the detachment rate constant and the mechanical force (Dembo et al., 1988; Evans and Ritchie, 1997). However, Bell model fits best with the data. Table 5.4 summarizes Bell model parameters for CHO and OST cells in HP and LP matrices. As expected, $k_{\text{det}}$ was higher in the HP matrix than in the LP matrix, and higher for CHO than for OST cells. Different cell lines also have different $\sigma'$ values; however, the matrix porosity has little effect on $\sigma'$.

5.4.4 Cell Growth in PET Matrices

Following dynamic seeding, the cells in the PET matrices were allowed to grow for seven days with media change every two to three days. As can be seen in Table 5.2, dynamic culturing greatly improved cells proliferation. As expected, increasing agitation would improve mass (oxygen) transfer in the spinner flask and thus should lead to faster cell growth and higher final cell density. In general, the lactate yield from glucose metabolism, $Y_{\text{lac/glu}}$, decreased with increasing in the agitation rate, indicating that the oxygen transfer rate increased with increasing the agitation rate or mixing intensity. With improved oxygen transfer and sufficient oxygen supply, cells were undergoing aerobic metabolism and producing less lactic acid from glucose.

However, the increased shear stress at a high mixing intensity such as at 80 rpm agitation rate could severely damage cells, as indicated by the significantly increased level of lactate dehydrogenase (LDH) in the culture media (see Table 5.2). Lactate dehydrogenase (LDH) was released into the media from cells with damaged membrane caused by a high
shear stress. Because of the severe shear damage of the cells, the final cell density in the HP matrices at 80 rpm was lower than that at 20 rpm or 40 rpm. It is noted that when the agitation rate increased from 40 rpm to 80 rpm, the LDH level or shear damage increased dramatically for cells grown in the HP matrices, but not in the LP matrices. Apparently, the shear effect on cell damage was different in HP and LP matrices with different pore sizes.

Croughan and Wang (1989) suggested that the relative size of a turbulent eddy to a microcarrier should play a strong role in the hydrodynamic damage of cells on the surface of the microcarrier. They found that when the Kolmogorov eddy size was similar to or smaller than the microcarrier size, hydrodynamic damage was apparent. The Kolmogorov eddy size, $\eta$, in the spinner flask can be calculated from the following equation (Gooch et al., 2001):

$$\eta = \left(\frac{V^3}{\varepsilon}\right)^{0.25}$$  \hspace{1cm} (10)

where the energy dissipation rate, $\varepsilon = N_p n^3 d^5/V$. In most of cell culture cases, the power number, $N_p$, is ~0.5 (Gooch et al., 2001). As can be seen in Table 5.5, the Kolmogorov eddy size decreased with increasing the agitation rate. When the agitation rates were low at 40 rpm and lower, $\eta$ (150 $\mu$m and higher) was larger than the pore sizes of both HP (60–130 $\mu$m) and LP (30–60 $\mu$m) matrices. However, at 80 rpm, $\eta$ (~90 $\mu$m) was similar to or smaller than the pore size of the HP matrix but still larger than that of the LP matrix. It thus can be concluded that hydrodynamic damage is apparent when the Kolmogorov eddy size is similar to or smaller than the pore size of the 3-D fibrous matrix. This is
understandable as the smaller eddies are more likely to reach cells inside the pore areas, while eddies larger than the pores cannot reach cells inside the matrix. Also, only at 80 rpm the shear stress, $\tau = \sim 42 \text{ dyne/cm}^2$, was larger than the reported critical shear stress of 30 dyne/cm$^2$ (Sprague et al., 1987). It is noted that the fibrous matrix, which was placed about 0.5 cm from the impeller tip, was exposed to the high shear region where most of the power input was dissipated (Venkat et al., 1996). Therefore, even a modest agitation rate of 80 rpm could cause severe shear damage to cells grown in the HP matrix with relatively large pores.

5.4.5 Effects of Matrix Pore Size

It is clear that the pore size of the fibrous matrix can have significant effects on both cell adherence and proliferation, and thus should be carefully optimized for cell culture and tissue engineering applications. In this study, both CHO and OST cells have higher cell expansion folds when cultured in the smaller pore size (LP) matrix (see Table 5.2). The higher expansion folds or net cell growth can be attributed to both the higher growth rate and lower or negligible death rate due to less shear damage. It has been reported that the matrix pore size can affect cell proliferation and differentiation, and fibrous matrices with smaller pores generally give higher proliferation rates (Ma et al., 1999; 2000). In addition, the matrix pore size also can have a significant effect on cell seeding (Li et al., 2001). As observed in this study, the dynamic seeding process was faster (higher attachment rate constants) and generally has a higher seeding efficiency in the LP matrix than in the HP matrix.
It has also been reported that the seeding method and matrix pore size both can affect the cell distribution inside the matrix, which in turn can affect cell proliferation and tissue development (Li et al., 2001; Ma et al., 1999). The pore size or distance between adjacent fibers also can affect cell’s ability to migrate or spread in the matrix. Yang et al. (2005) found that cells could not bridge over to the neighboring fibers if the apparent pore size or the distance between adjacent fibers were more than ~4 times of the cell diameter.

Figure 5.10 shows cells after seeding and culturing for 7 days in the fibrous matrices. In general, cells were evenly distributed in the matrix (Fig. 5.10a and 5.10d). Most cells either attached to the fibers (Fig. 5.10b and 5.10e) or formed small aggregates or bridging between closely adjacent fibers (Fig. 5.10c and 5.10f). These observations further confirmed that the dynamic seeding and culturing in the LP matrix with pore sizes of 30–60 µm gave good cell expansion due to good seeding (both high efficiency and uniform), cell growth, and protection from high shear damage.

5.5 Conclusions

Seeding anchorage-dependent cells to support matrices is one of the most important steps in cell cultures and tissue engineering. The dynamic seeding method demonstrated with the nonwoven PET matrices in this study is capable of improving the seeding efficiency to 95%–99%. Cell adherence in the 3-D dynamic culture environment is a reversible process. In general, both cell attachment and detachment rates increased with the mixing intensity, which also influenced cell metabolism, growth, and death caused by shear damage. Increasing the mixing intensity increased oxygen transfer and decreased the lactate yield from glucose. However, the optimal agitation rate for seeding and culturing
is a compromised one that must balance the intricate interactions among cell type (size), mixing intensity, and matrix pore size. By comparing the matrices with two different pore-size ranges, it was concluded that the smaller matrix pore sizes of 30–60 µm was more favorable to cell attachment, proliferation, and protection from hydrodynamic damage for both CHO and OST cells studied.
5.6 Nomenclature

\(A\) \hspace{1cm} \text{area (m}^2\text{)}

\(C\) \hspace{1cm} \text{free cell number}

\(C_0\) \hspace{1cm} \text{initial cell number}

\(C_a\) \hspace{1cm} \text{attached cell number}

\(d\) \hspace{1cm} \text{impeller paddle diameter (cm)}

\(d'\) \hspace{1cm} \text{fiber diameter (cm)}

\(d''\) \hspace{1cm} \text{cell diameter (cm)}

\(F\) \hspace{1cm} \text{mechanical force (N)}

\(k\) \hspace{1cm} \text{permeability (m}^2\text{)}

\(k_B\) \hspace{1cm} \text{Boltzmann constant (1.3806503 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1})}

\(k_{app}\) \hspace{1cm} \text{apparent attachment rate constant (h}^{-1}\text{)}

\(k_f\) \hspace{1cm} \text{attachment rate constant (h}^{-1}\text{)}

\(k_r\) \hspace{1cm} \text{detachment rate constant (h}^{-1}\text{)}

\(k_{ro}\) \hspace{1cm} \text{detachment rate constant at no force circumstance (h}^{-1}\text{)}

\(n\) \hspace{1cm} \text{agitation rate (rpm)}

\(N_p\) \hspace{1cm} \text{power number (= 0.5 in this study)}

\(R\) \hspace{1cm} \text{radius of cell (cm)}

\(Re\) \hspace{1cm} \text{Reynolds number}

\(V\) \hspace{1cm} \text{reactor working (liquid) volume (mL)}

\(v_{\text{matrix}}\) \hspace{1cm} \text{local fluid velocity in the matrix (cm/s)}

\(\varepsilon\) \hspace{1cm} \text{energy dissipation rate (cm}^2\text{s}^{-3}\text{)}

\(\varepsilon'\) \hspace{1cm} \text{voidage or porosity}

\(\eta\) \hspace{1cm} \text{Kolmogorov eddy size (\text{µm})}

\(\mu\) \hspace{1cm} \text{viscosity (cP or mPa}\cdot\text{s)}

\(\nu\) \hspace{1cm} \text{kinematic viscosity (m}^2\text{/s)}

\(\rho\) \hspace{1cm} \text{fluid density (kg/m}^3\text{)}

\(\sigma\) \hspace{1cm} \text{the distance range of bond energy potential minimum (m)}

\(\sigma'\) \hspace{1cm} \text{= } \sigma A / k_B T \text{ (kg}^{-1} \text{ m s}^2\text{)}

\(\tau\) \hspace{1cm} \text{shear stress (dyne/cm}^2\text{)}
5.7 References


Bell GI. 1978, Models for the specific adhesion of cells to cells. Science, 200, 618-627


Chen S, Springer TA. 2001, Selectin receptor-ligand bonds: formation limited by shear rate and dissociation governed by the Bell model. Proc Natl Acad Sci USA 98, 950-955


Li Y, Ma T, Kniss DA, Lasky LC, Yang ST. 2001, Effects of filtration seeding on cell density, spatial distribution, and proliferation in nonwoven fibrous matrices. Biotechnol Prog 17, 935-944

Li Y, Ma T, Yang ST, and Kniss DA. 2001, Thermal compression and characterization of 3-D nonwoven PET matrices as tissue engineering scaffolds, J Biomaterials 22, 609-618.


Malda J, Kreijveld E, Temenoff JS, Blitterswijk CA van, Riesle J. 2003, Expansion of human nasal chondrocytes on macroporous microcarriers chances redifferentiation. Biomaterials 24, 5153-5161


Nasseri BA, Pomerantseva I., Kaazempur-Mofrard MR, Sutherland FWH, Perry T, Ochoa E, Thompson CA, Mayer JE, Oesterle SN, Vacanti JP. 2003, Dynamic rotational seeding and cell culture system for vascular tube formation. Tissue Eng 9, 291-299


<table>
<thead>
<tr>
<th>Property</th>
<th>LP</th>
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<tr>
<td>Thickness (mm)</td>
<td>1.07 ± 0.03</td>
<td>1.51 ± 0.04</td>
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<tr>
<td>Porosity</td>
<td>0.849± 0.004</td>
<td>0.926 ± 0.004</td>
</tr>
<tr>
<td>Pore size range (µm)</td>
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<td>60–130</td>
</tr>
</tbody>
</table>

LP: low porosity matrix obtained after thermal compression; HP: high porosity matrix without thermal compression

Table 5.1 Physical characteristics of nonwoven PET fibrous matrices.
Table 5.2 Effects of mixing intensity and pore size on cell seeding and growth in PET matrices.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Agitation rate (rpm)</th>
<th>HP</th>
<th>LP</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>( k_{app} ) (h(^{-1}))</td>
<td>( k_f ) (h(^{-1}))</td>
</tr>
<tr>
<td>CHO</td>
<td>10</td>
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<tr>
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<td>80</td>
<td>0.30</td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>80</td>
<td>0.47</td>
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Table 5.3 Effects of agitation rate on cell attachment and detachment rate constants
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<td>$k_{ro}$ (h^{-1})</td>
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Table 5.4 Bell model parameters for the detachment rate constant in PET matrices
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<th>Agitation rate (rpm)</th>
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<td>24.7</td>
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<tr>
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<td>42.2</td>
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<td>80</td>
</tr>
<tr>
<td>OST</td>
<td></td>
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Table 5.5  Hydrodynamic parameters under different mixing conditions
Figure 5.1 The experimental set up of the spinner flask with the fibrous matrix around the reactor wall.
Figure 5.2 Kinetics of CHO cell adherence to the fibrous matrices. (A) high porosity matrix (HP); (B) low porosity matrix (LP). Curves show simulation from the reversible model.
Figure 5.3 Kinetics of OST cell adherence to the fibrous matrices. (A) high porosity matrix (HP); (B) low porosity matrix (LP). Curves show simulation from the reversible model.
Figure 5.4 First order reaction kinetics for CHO cell adherence to the fibrous matrices. (A) high porosity matrix (HP); (B) low porosity matrix (LP).
Figure 5.5 First order reaction kinetics for OST cell adherence to the fibrous matrices. (A) high porosity matrix (HP); (B) low porosity matrix (LP).
Figure 5.6 Linear regression of $k_f$ and $k_r$ value. According to the equation 7, the intercept of the trend line is $k_r$ and slope is $-(k_f+k_r)$. 
Figure 5.7 Correlation between the mass transfer coefficient and the attachment rate constant, $k_f$. 
Figure 5.8 Fluent simulation of dynamic culture in spinner flask. (A) contour of radial velocity, (B) path lines of radial velocity
Figure 5.9 Correlation between the shear stress and the detachment rate constant, $k_r$. The line shows the simulation from the Bell model.
Figure 5.10 Cell distributions in 3-D PET LP matrices in dynamic cultures at 7 days. (A) OST (100×), (B) OST (200×), (C) OST (400×), (D) CHO (100×), (E) CHO (200×), (F) CHO (400×)
Chapter 6

Modeling and Scale Up a Fibrous Bed Bioreactor for Mammalian Cell Culture

6.1 Abstract

The fibrous bed bioreactor (FBB) has wide applications in mammalian cell culture and tissue engineering. A fibrous matrix of polyethylene-terephthalate (PET) scaffold provides a 3-D culture environment for animal cells; such an environment more closely mimics their in vivo growth situation. FBB has been proven to be superior to conventional bioreactors for both anchorage dependent and independent cells. Mathematical models were established for ordered disc packed and spiral wound bioreactors in this study. The comparison between model prediction and step response residence time distribution data shows that the models are valid. For the ordered disc packed bioreactor an axial dispersion model was used. However, both radial and axial convection and dispersion contributed significantly to the mass transfer in spiral wound bioreactors. As such, a model that considers both axial and radial convection was employed for the spiral wound bioreactors. For both reactor types, models show that embryonic stem cells can reach tissue like cell density of $10^9$ cells/ml in 15~22 days in a lab scale fibrous bed bioreactor by perfusion culture. Models predict significant oxygen
depletion in scaled versions of both types of FBB, and, therefore, an uneven cell distribution is observed. Increasing the superficial velocity alleviates nutrient limitation. Spiral wound FBB with side exit performed best in scaled up FBB. In order to avoid diffusion limitation to the cell aggregate, the cell aggregate size should be less than 250 µm for 70% DO in the medium and 170 µm for 30% DO in the medium. A hydrodynamic study of FBB shows that the pressure drop is much higher in an ordered disc packed FBB than a spiral wound FBB and that the hydrodynamic permeability decreases rapidly after cell density reaches $10^8$ cells/ml. Models predict that the superficial velocity could reach a maximum of 72 cm/min without causing shear damage to the cells in the reactor.

6.2 Introduction

The fibrous bed bioreactor has wide applications in fermentation, enzyme catalysis, and animal cell culture (Albayrak and Yang, 2002 (a,b); Luo and Yang, 2004; Wang et al., 1992). The fibrous bed bioreactor is a modification of the packed bed bioreactor by applying novel packing materials such as cotton towel, non-woven PET scaffold (Albayrak and Yang, 2002b; Chen et al., 2002). The traditional packed bed bioreactor has a large pressure drop along its axis which restricts its scale up potential (Calis, et al., 2001; Achenbach, E., 1995). On the other hand, cotton towel and PET scaffold have the merits of high porosity, high specific surface area, high permeability, low pressure drop, high mechanical strength, and low material costs. These advantages assure the fibrous bed bioreactor a promising future. However, the theoretical study of FBB has been left
behind its applications, which hinders its scale up potential. Thus, it’s necessary to model the FBB before scale up.

It was noted in traditional packed bed bioreactor that even though small beads (3 mm) provide large specific surface area, 4-20 times better yields were obtained in large bead (5mm) packed beds (Nilsson et al., 1986). Larger void space due to less efficient packing may attribute to better flow characteristics through the bed which benefits mass transfer. This unexpected result suggests that the design of the bioreactor is of great importance.

Two packing methods were applied in the fibrous bed bioreactor. One is ordered disc packing and the other is spiral wound packing. There are many papers which discussed packed bed bioreactor modeling, but they focused on the modeling of solid sphere and microporous sphere packed beds because of their bio-applications in enzyme biocatalysis, waste water treatment and cell culture (Dadvar and Sahimi, 2003; Gu and Syu, 2004). The governing differential equations for chromatography were borrowed and a reaction term was added in order to model the packed bed bioreactor. Particularly, the axial dispersion model has been widely used (Backer and Baron, 1994; Gu and Syu, 2004; Iliuta I. et al. 1999). However, the accuracy of the axial dispersion model is limited by tailing (Hoogendoorn and Lips, 1965). The multistage dispersion model integrates the axial dispersion and plug flow. It is accurate in describing a reactor with separate flow regions. In microporous particle packed bed bioreactors, there are three potential mass transfer limiting steps: external mass transfer, diffusion through the liquid film around the particles and intra particle diffusion. Backer and Baron (1994) included the intra particle diffusion terms in the dispersion equation for their yeast cell culture bioreactor. At low
Reynolds number (<10), the model prediction fit the experimental data very well indicating the validity of the model. In the presence of low biomass loading, there was no effect on the mass transfer. However, at high biomass loading (> 0.02 g cell/g beads), mass transfer rate between the extraparticle and intraparticle fluid phase dropped significantly. Gu and Syn (2004) also established a model to describe the immobilized cell column in bioconversion and waste water treatment. They considered interfacial film diffusion as the mass transfer limitation step. What’s more, reaction and cell death was included in their model, vastly increasing model accuracy. Iliuta and coworkers (1996) developed an axial dispersion-exchange model with the assumption that intra particle diffusion led to the long tails of the residence time distribution (RTD) curve.

Spiral wound hollow fiber or membrane reactor modeling is much more difficult than conventional packed bed reactor modeling because of the complex 3-dimensional momentum and mass transport. Crowder and Gooding (1997) calculated the mass transfer efficiency by applying empirical correlations in a spiral wound membrane reactor, but their study of the mathematical modeling was limited. Madireddi and coworkers (1999) develop a transient model for mass transfer, especially for concentration polarization in spiral wound membrane reactors. Membrane thickness and distance between two membrane layers were taken into account in their work. Brotherton and Chau (1990) did a comprehensive study on momentum and mass transport in spiral wound hollow fiber bioreactor for animal cell culture, but none of the aforementioned works verified their model with experimental data.
Modeling of fibrous bed bioreactor will be addressed in this study and supportive information of nutrients and cell distribution, hydrodynamic studies for future scale up will be gathered in terms of both bioreactor design and operation.

6.3 Material and Method

6.3.1 Fibrous Bed Bioreactor Set Up

Two packing methods were compared for their performance. The first packing method is ordered disc packing. A PET scaffold was cut into several round shaped pieces of similar diameter to the reactor column – The reactors dimensions are 2.5 cm diameter and 8 cm height as Figure.6.1A illustrated. Another packing method is spiral packing. One PET sheet of 8× 20 cm was spirally wound into the column. The approximate distance between layers is 1 mm. Two types of spiral wound reactors were used. One was with normal top exit (Figure 6.1B) and the other was with side exit at the top (Figure.6.1C).

6.3.2 Residence Time Distribution

The pulse input method was used to ascertain the mean residence time and variance (Fogler S. 2002). Step response data was also collected to compare with model prediction. For pulse input measurement, 0.2 ml of saturated NaCl solution was rapidly injected into the fibrous bed bioreactor from the bottom. The conductivity of the effluent was measured every 5 seconds until the reading returned to its initial value and remained stable for 30 seconds. For the step response experiment, 0.125 M NaCl solution was pumped into the fibrous bed bioreactor. The conductivity of the effluent was measured
every 5 seconds until the reading was stable for 30 seconds. Three superficial velocities of 2.2, 7.5, and 11.41 cm/min were tested. All experiments were duplicated.

6.3.3 Pressure Drop Measurement

Axial pressure drop of the three bioreactors were measured by a water manometer. Fluid superficial velocity ranged between 0 to 13.2 cm/min. The packed fibrous bed had a height of 8 cm and a diameter of 2.5 cm. Matrices with different solid fraction were prepared by thermo compression method for 0, 30 min, 60 min and 90 min (Li, et al., 2001).

6.4 Mathematical Models

Mean residence times and variance were determined by equations 1-4 below (Fogler S. 2002):

\[
E(t) = \frac{C(t)}{\int_0^\infty C(t) dt} \quad (1)
\]

\[
t_m = \int_0^\infty tE(t) dt \quad (2)
\]

\[
\sigma^2 = \int_0^\infty (t - t_m)^2 E(t) dt \quad (3)
\]

\[
\sigma^2_\theta = \frac{\sigma^2}{t_m^2} \quad (4)
\]

The Bodenstein number is recognized as the theoretical plate number in a packed column and is calculated by equation 5 (Tong et al., 2003).

\[
\sigma^2_\theta = \frac{2}{Bo} - \frac{2}{Bo^2} (1 - e^{-Bo}) \quad (5)
\]
The axial dispersion coefficient in fibrous bed bioreactor is represented by equation 6 (Tong et al., 2003).

\[ D_l = \frac{uL}{\varepsilon Bo} \]  

(6)

The axial dispersion model is represented in equations 7-10 below. In this study, the axial dispersion model is applied to the ordered disc packed fibrous bed bioreactor (Fogler S. 2002):

\[ \frac{\partial c}{\partial t} = D_l \frac{\partial^2 c}{\partial z^2} - \frac{u}{\varepsilon} \frac{\partial c}{\partial z} \]  

(7)

I.C. \( t = c(z,0) = 0 \)  

(8)

B.C. \( z = 0, c = c_0 + \frac{D_l \varepsilon}{u} \frac{\partial c}{\partial z} \)  

(9)

\[ z = L, \frac{\partial c}{\partial z} = 0 \]  

(10)

However, the axial dispersion model is incapable of fitting the data from the spiral wound FBB. In order to model the spiral wound FBB, much more sophisticated momentum and mass transfer phenomena had to be accounted for. The following equations capture the complexities of these two transport phenomena in a spiral wound FBB.

**Momentum Transfer**

Continuity equation:

\[ \frac{1}{r} \frac{\partial}{\partial r} (rv_r) + \frac{\partial u_z}{\partial z} = 0 \]  

(11)

Equations of motion:

\[ v_r \frac{\partial u_z}{\partial r} + u_z \frac{\partial u_z}{\partial z} = - \frac{1}{\rho} \frac{\partial P}{\partial z} + \nu \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial u_z}{\partial r} \right) + \frac{\partial^2 u_z}{\partial z^2} \right] \]  

(12)

\[ v_r \frac{\partial v_r}{\partial r} + u_z \frac{\partial v_r}{\partial z} = - \frac{1}{\rho} \frac{\partial P}{\partial r} + \nu \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial v_r}{\partial r} \right) + \frac{\partial^2 v_r}{\partial z^2} \right] \]  

(13)
Assuming L>>d, the inertial terms can be neglected and thus,

\[ u_z = \frac{R^2}{4\mu} \left( -\frac{dP}{dz} \right) \left[ 1 - \left( \frac{r}{R} \right)^2 \right] \]  

(14)

\[ v_r = \frac{R^3}{8\mu} \left( \frac{d^2P}{dz^2} \right) \left( \frac{r}{R} \right)^2 - \frac{1}{2} \left( \frac{r}{R} \right)^4 \]  

(15)

at r=R, \( v_w = \frac{R^3}{16\mu} \left( \frac{d^2P}{dz^2} \right) \)  

(16)

Wall velocity is pressure driven (Darcy’s law)

\[ v_w = A_m \left[ P(z) - P_m \right] \]  

(17)

Where \( P_m \) is matrix pressure and can be considered constant (Brotherton and Chau, 1990). \( A_m \) is the hydrodynamic permeability. The dimensionless pressure equation is given below:

\[ \frac{d^2P'}{dz'^2} - \alpha^2 \left( P' - P_m' \right) = 0 \]  

(18)

\( \alpha \) is the pressure modulus,

\[ \alpha = L \sqrt{\frac{16\mu A_m}{d^3}} \]  

(19)

The boundary conditions are different for the two types of spiral wound FBB. The following dimensionless equations correspond to the boundary conditions of a FBB with normal top exit.

\( z'=0, P'=1 \) (inlet pressure)  

(20)

\( z'=1, P'=0 \) (ambient gauge pressure)  

(21)

After integration, the following is obtained:

\[ P'(z) = P_m' + (1 - P_m') \cosh \alpha z' - \beta \sinh \alpha z' \]  

(22)

\[ \beta = \frac{P_m' + (1 - P_m') \cosh \alpha}{\sinh \alpha} \]  

(23)

\[ v_w' = \alpha^2 [(1 - P_m') \cosh \alpha - \beta \sinh \alpha] \]  

(24)

The boundary conditions for spiral wound FBB with side outlet are as follows:

\( z'=0, P'=1 \)  

(25)
\[ z' = 1, \frac{dP'}{dz'} = 0 \]  

(26)

After integration, new dimensionless pressure and radial wall velocity function is obtained as:

\[ P'(z) = P_m' + (1 - P_m') \cdot \left[ \cosh \alpha z' - \tanh \alpha \cdot \sinh \alpha z' \right] \]  

(27)

\[ v_w' = (1 - P_m') \cdot \alpha^2 \left( \cosh \alpha z' - \tanh \alpha \cdot \sinh \alpha z' \right) \]  

(28)

Axial velocity and radial velocity are described by equations 29 and 30.

\[ v_r = v_w \frac{r}{R} \]  

(29)

\[ u_z = u_0 \left( 1 - \left( \frac{r}{R} \right)^2 \right) \left( 1 - \frac{z}{v_w dz} \right) \]  

(30)

Radial dispersion coefficient can be evaluated by equation 31 (Pereira et al., 2005)

\[ D_r = D \left( 1 + \frac{63}{320} \sqrt{2(1 - \varepsilon)} \frac{u d_{bed}}{D} \right) \]  

(31)

Mass Transfer

Mass transfer in spiral wound FBB was further investigated. The general equation is below.

\[ \frac{\partial c}{\partial t} = - \frac{u_z}{\varepsilon} \frac{\partial c}{\partial z} + \frac{v_r}{\varepsilon} \frac{\partial c}{\partial r} + D_r \left[ \frac{1}{r} \frac{\partial c}{\partial r} + \frac{\partial^2 c}{\partial r^2} \right] + D_l \frac{\partial^2 c}{\partial z^2} \]  

(32)

Equation 32 has to be modified into its dimensionless form in order to apply previous axial and radial velocity functions to the mass transfer equation. The dimensionless Groups below depict the dimensionless method used in this study.

\[ t' = \frac{t u_0}{L}, \quad u_z' = \frac{u_z}{u_0}, \quad v_r' = \frac{v_r}{R}, \quad c' = \frac{c}{c_{fed}}, \quad z' = \frac{z}{L}, \quad \frac{r}{R} = \frac{D_{r,j}}{u_0 R^2} \]

The exit concentration profile was compared with RTD data to validate the model. For this purpose, concentration was integrated over the cross sectional area with consideration given to the axial velocity profile.
Experimental data was gathered using a sodium chloride solution – because the substrate of interest is oxygen, the limiting substrate, the dispersion coefficient calculated from equation 6 requires modification. Equations 34-36, along with equation 6, demonstrate the steps needed for such modification.

\[
D_{NaCl} = \frac{2}{\frac{1}{D_{Na^+}} + \frac{1}{D_{Cl^-}}} \tag{34}
\]

\[
P e = a\left(\frac{Re}{\nu}\right)\left(\frac{\nu}{D}\right) \tag{35}
\]

\[
Bo = Pe \tag{36}
\]

The models derived for mass transport are validated by RTD data. The ultimate goal is to use the model equations to predict the oxygen profile in the fibrous bed bioreactor throughout a long term culture. Oxygen consumption due to cell metabolism must be taken into consideration in the model. As such, an oxygen consumption term is added to the model and is represented by equations 37 and 38. The Monad equation is employed for the specific cell growth rate calculation.

\[
reaction = Q_n X_0 e^{\mu' t} \tag{37}
\]

\[
\mu' = \mu_{\max} \frac{S}{K + S} \tag{38}
\]

Where \(Q_n=10^{-10}\) mmol/cell/h, \(\mu_{\max}=0.0146\) h\(^{-1}\) and \(X_0=5\times10^5\) cell/mL matrix (from our own experimental data). Accurate prediction of an oxygen profile will allow for determination of a cell density profile in the FBB.
Hydrodynamic parameters of permeability and shear stress were also calculated. Permeability could be defined by Darcy’s law as:

\[
\frac{F}{A} = u = \frac{k \Delta P}{\mu L}
\]

(39)

Where, k is the hydrodynamic permeability, F is the volumetric flow rate through a cross-section of area A, u is the flow velocity, \(\mu\) is the fluid dynamic viscosity, and \(\Delta P\) is the pressure drop over a length L. This equation is valid for Newtonian fluids at low flow rates (Spielman et al., 1968). Permeability of porous fibrous matrix is also a function of the void fraction of the packed bed. Void fraction decreases with increasing cell density in the following manner.

\[
\varepsilon = \varepsilon_0 - \frac{4X_0 e^{(\varepsilon_0 / 3)} (r^3)}{3R^2 L}
\]

(40)

Additionally, the permeability is a function of fiber radius (a), solid fraction (\(\phi\)) and fiber arrangement. For different flow patterns, there are different empirical equations to calculate permeability. Jackson (1986) gave an excellent summary of empirical models which follow:

flow parallel to an array of rods

\[
\frac{k}{a^2} = \frac{1}{4\phi} (-\ln \phi - \frac{3}{2} + 2\phi - \frac{\phi^2}{2})
\]

(Langmuir) (41)

flow perpendicular to an array of rods

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

(Happel) (42)

flow through three-dimensional arrays
\[
\frac{k}{a^2} = \frac{3}{20\phi} (-\ln \phi - 0.931 + O(\ln \phi)^{-1}) \quad \text{(Drummond & Tahir)} \tag{43}
\]

Apparent fiber diameter, \(d_f\), also changes with the void fraction according to Equation 44
\[
d_f^2 = \frac{1 - \varepsilon}{1 - \varepsilon_0} d_{f,0}^2 \tag{44}
\]

Shear stress is calculated as follows (Perry and Wang, 1989)
\[
\tau = \frac{\mu ud_f}{4k(1 - \varepsilon)} \tag{45}
\]

Oxygen diffusion into the cell aggregate was also considered. The equations listed below describe this phenomenon.
\[
\frac{\partial c'}{\partial t} = D \left( \frac{\partial^2 c'}{\partial r^2} + \frac{2}{r} \frac{\partial c'}{\partial r} \right) - Q_0 X \tag{46}
\]
\[
X = \frac{R^3}{r_{cell}^3} \tag{47}
\]
\[
\text{I.C. } t=0, \ c' = 30\% \text{ DO} \tag{48}
\]
\[
\text{B.C. } r=0, \ \frac{\partial c'}{\partial r} = 0 \tag{49}
\]
\[
r=R, \ c' = c\big|_{r=L} \tag{50}
\]

An implicit finite difference method was used to solve all transient partial differential equations.
6.5 Results

6.5.1 Effects of Flow Rate and Packing Method on Residence Time Distribution:

Three flow rates were chosen to investigate the effect of flow rate on RTD data. Equations 1~6 were employed to evaluate the dispersion coefficient, and mean residence time. It’s obvious that as the flow rate increases, the mean residence time decreases (Figure 6.2). Moreover, different packing methods greatly influence mean residence time. At the same flow rate, the mean residence time in the normal spiral wound FBB was 100% higher than that in the ordered disc packed FBB and is also slightly larger than that in the spiral wound FBB with side exit (Table 6.1). Different packing methods also affect the residence time distribution curve. The curves weren’t normally distributed at a low flow rate of 2.2 cm/min in spiral wound FBB with side exit. Instead, several peaks were observed in the RTD curve. Additional peaks were also noticed in the higher flow rate RTD curves of 6.5 cm/min and 11.4 cm/min. Such peculiar RTD curves can be attributed to the complex mixing phenomena and flow pattern in a spiral wound FBB. Dispersion coefficients under different conditions are listed in Table 6.1. Table 6.1 elucidates that, in all cases, dispersion coefficients increase with increasing flow rate. Axial dispersion coefficients of spiral wound FBB with side exit are much higher than the other two cases.

6.5.2 Model Validation:

Figure 6.3 presents the model prediction versus experimental data. It is evident that the axial dispersion model fits the experimental data of the ordered packed disc FBB well (Figure 6.3A). However, this model is not able to fit the RTD data from spiral wound
reactor. More sophisticated models were developed in succession. First, both axial
dispersion and radial dispersion were included into the model giving improved but
insufficient results. Then, radial convection was considered and model prediction fit the
RTD data well (Figure 6.3B) proving the validity of this model. Because the mass
transfer equation for the side exit spiral wound FBB is identical to that of the top exit
spiral wound FBB, this model is also used to fit the experimental data from spiral wound
FBB with side exit. However, there is a crucial distinction – the boundary conditions
differ. After applying the different boundary conditions, it is shown that the model fits
the experimental data well. Equations 11-32 above depict transport phenomena in a
spiral wound reactor by theoretical derivation from momentum and mass transfer
fundamental equations.

6.5.3 Oxygen and Cell Density Profile in Lab Scale FBB

Dispersion coefficients change with superficial velocity. Different substances have
different dispersion coefficient. Experimental results show that the Bodenstein number
remains constant after the Reynolds number surpasses 40 (Figure 6.4). For the actual cell
culture process the Reynolds number always exceeded this requirement. Therefore,
equations 33-36 are applied to evaluate the oxygen dispersion coefficients under different
conditions.

After model validation, oxygen consumption by cells is taken into consideration. A
maximum cell density of $10^9$ cells/ml is assumed; the maximum cell density is that of
human tissue. According to previous experimental observation (Luo, 2002), another
criterion for cell growth arrest is that dissolved oxygen (DO) in the medium is less than
30%. Figure 6.5A indicates that oxygen concentration decreases to 30% DO in lab scale ordered disc packed FBB at superficial flow rate of 0.00231 m/s. After 25 days, the oxygen concentration drops to 50% DO at the exit in spiral wound FBB with top exit (Figure 6.5B). However, it dropped to almost zero in the spiral wound FBB with side exit at the same flow rate (Fig.6.5C). The cell density reaches tissue like cell density in 20 days and is evenly distributed throughout the ordered disc packed reactor (Figure 6.6A). Cells grow slower in the lab scale spiral wound FBB with top exit and took 22 days for the entire reactor to reach the maximum cell density. It is evident from the uneven cell distribution and low exit cell concentration that cell growth is not satisfactory in the lab scale spiral wound FBB with side exit. The ordered disc fibrous bed reactor is best in regards to its performance and efficiency.

6.5.4 Oxygen and Cell Density Profile in Scaled up FBB

The scaled up bioreactors’ performance was also investigated. For this purpose, the bed length was increased 10 fold and the diameter was increased 3 fold. Oxygen depletion is observed in all three cases as shown in Figure 6.7. The oxygen is consumed faster in the ordered disc packed reactor and the concentration falls to zero on the 16th day (Figure 6.7A). Oxygen concentration in the other two cases falls to zero on the 18th day (Figure 6.7B, C). However, a larger portion (~10 cm) of the packed bed suffers from nutrient limitation in the two spiral wound reactors than in the ordered disc packed reactor (~ 7 cm).

In all three reactors, cell density is not uniformly distributed as witnessed by Figure 6.8. Cells cease to grow after the DO reaches 30%. Figure 6.8 demonstrates that spiral wound
reactor with side exit has the best cell growth with a smaller non-even distribution region and maintains a higher cell density in the nutrient limiting area. This result indicates that different bioreactor designs should be considered for different scales.

6.5.5 Oxygen and Cell Density Profile in Scaled up FBB with Higher Superficial Velocity

Since nutrient depletion is observed in all three designs, a higher superficial velocity of 69.3 cm/min was considered to improve mass transfer inside of the reactor. However, higher superficial velocity also causes greater shear damage to cells. The greatest safe velocity is determined according to Equations 42, 44, and 45. It is found to be 72 cm/min.

The higher velocity alleviates oxygen depletion to ~45 %DO in ordered disk packed reactor (Figure 6.9A) and ~55 % in spiral packing with side exit (Figure 6.9C). Because oxygen concentration remained above 30 %DO, cell growth continued until the cell density reached a tissue like state (Figure 6.10A, C). To some extent, cells grow faster in the spiral wound with side exit. For the normal spiral wound reactor, the higher superficial velocity is only able to delay complete oxygen depletion (Figure 7.9B). This results in non-uniform cell distribution. Under these circumstances, the ordered disc packed reactor is best.

6.5.6 Oxygen Diffusion in Cell Aggregate:

Mammalian cells form aggregate in 3-D PET scaffold during growth. However, cell aggregate size adds to the diffusion resistance and undesirable oxygen limitation might occur in the center of a large aggregate. Oxygen diffuses through the aggregate to the
center as described by equations 46-50. It is found (Figure 6.11) that cell aggregates are capable of reaching a size of 250 µm if the medium contains 70% DO. However, the maximum aggregate size is 170 µm with 30% DO in the medium. Cell aggregate size is partially controllable by the pore size of the scaffold. Thermal compression has proven to be able to permanently reduce the pore size – to some extent, this process is able to reduce the aggregate size (Li et al., 2001).

6.5.7 Hydrodynamic Study of Fibrous Bed Bioreactor

Pressure drops in the three bioreactor designs were measured as illustrated by Figure 6.12A. The pressure drop along the axial direction is much higher in the ordered disc packed FBB than in the spiral wound reactors with the same packed bed height. This result is unsurprising because there are channels of small pressure resistance between two layers of scaffold. A smaller pressure differential is advantageous; it is well recognized that pressure drops along the axial direction make scale up work difficult.

Permeability and porosity decrease with increasing cell density. However, it’s impossible to measure these parameters without destroying the cells. Mathematical models have been developed to account for changes in permeability. Figure 6.12B demonstrates that the Happel model is better able to fit the solid fraction data. Figure 6.12C makes evident that permeability begins to decrease as cell density approaches $10^8$ cells/ml.

Animal cells are believed to be more sensitive to shear damage than bacteria, fungi and plant cells due to their lack of a cellular wall. Cells in a flowing liquid medium are
subject to shear stress resulting in hydrodynamic damage. For this reason, shear stress is one of the most important considerations in mammalian cell culture reactor design. Experimental data indicates that animal cells suffer from hydrodynamic damage when shear stress exceeds 20 dyne/cm² (our own data). Figure 6.12D indicates that, with regard to the aforementioned limitation, the maximum obtainable superficial velocity is 72 cm/min. Furthermore, Figure 6.12D shows that shear stress increases with culture time until the 22nd day and thereafter rapidly falls to zero. Equation 45 indicates that shear stress is a balance between permeability and solid fraction – these two conflicting factors account for the peak in the shear stress profile.

6.6 Discussion

Fibrous bed bioreactor was successfully used in mammalian cell culture. However, its mathematical modeling work has been left behind due to the complex momentum and mass transport phenomena in the reactor. Residence time distribution measurement provides a tool for model validation.

Pressure gradients increase along the axial direction. The results demonstrate that the ordered disc packed FBB has a much higher pressure drop along its axial direction. This may give rise to non-uniform nutrient delivery and cell growth in the bioreactor. This disadvantage is especially apparent in a scaled bioreactor. Nutrients are consumed in the lower portion of the reactor limiting their availability to the upper portion; cells in the upper part experience nutrient limitation resulting in slower growth. By increasing superficial velocity, a greater portion of the bioreactor can be alleviated of nutrient limitation. However, increasing velocity also contributes negatively to shear damage to
the cells. Therefore, other methods of nutrient depletion alleviation, such as reactor design, have to be considered. There is more void space in spiral wound reactor due to channels between PET. Nutrients are more efficiently delivered to the upper portion of the reactor in these bioreactors. However, this advantage is only observed in scaled versions of the bioreactors. Recent design and modeling efforts have focused on radial convective enhanced nutrient delivery. Based on the above analysis, the spiral wound reactor with side exit is a good alternative for the design because it’s capable of increased radial convection.

6.7 Conclusions

The axial dispersion model is able to describe the mass transfer in ordered disc packed FBB while both axial and radial convection and dispersion contribute significantly to the mass transfer in spiral wound FBB. Regarding bioreactor performance, ordered disc packed FBB is best in lab scale while spiral wound FBB with side exit is best on a larger scale. In order to avoid diffusion limitation to the cell aggregate, the cell aggregate size should be less than 250 µm for 70% DO in the medium and 170 µm for 30% DO in the medium. A hydrodynamic study of FBB shows that the pressure drop is much higher in an ordered disc packed FBB than a spiral wound FBB and that the hydrodynamic permeability decreases rapidly after cell density reaches $10^8$ cells/ml. Models predict that the superficial velocity can reach a maximum of 72 cm/min without causing shear damage to the cells in the reactor.
6.8 Nomenclature:

\( \nu \)  Kinematic viscosity (m\(^2\)/s)
\( \mu \)  Medium viscosity (cp)
\( \tau \)  Shear stress (dyne/cm\(^2\))
\( \phi \)  Solid fraction
\( \varepsilon \)  Void fraction
\( \sigma^2 \)  Variance (s\(^2\))
\( \sigma_{0}^2 \)  Dimensionless variance
\( \mu' \)  Cell specific growth rate
\( a \)  Fiber radius
\( Am \)  Permeability (m\(^3\)/s/kg)
\( Bo \)  Bodenstein Number
\( C \)  Tracer or oxygen concentration
\( D \)  Diffusivity (m\(^2\)/s)
\( d \)  Distance between two layers of scaffold (m)
\( d_f \)  Apparent fiber diameter (m)
\( D_l \)  Axial dispersion coefficient (m\(^2\)/s)
\( Dr \)  Radial dispersion coefficient (m\(^2\)/s)
\( E(t) \)  Residence-time distribution function
\( H \)  Packed bed height (m)
\( k \)  Hydrodynamic permeability (m\(^2\))
\( L \)  Packed bed length (m)
\( P \)  Pressure (Pa)
\( P' \)  Dimensionless Pressure
\( Pe \)  Peclet Number
\( Pm \)  Dimensionless pressure of the matrix
\( Q_0 \)  Oxygen consumption rate (mmol/cell/h)
\( R \)  Radius of the reactor (m)
\( r_{cell} \)  Single cell radius
\( t_m \)  Mean residence time (s)
\( u \)  Superficial velocity (m/s)
\( u_a \)  Axial velocity (m/s)
\( v_r \)  Radial velocity (m/s)
\( v_w \)  Wall velocity (m/s)
6.9 Reference:


Table 6.1 Effect of Flow Rate on Dispersion Model Parameters.

<table>
<thead>
<tr>
<th>Packing method</th>
<th>Flow rate (cm/min)</th>
<th>Mean residence time (s)</th>
<th>Variance (s²)</th>
<th>Dispersion coefficient (10^6) m²/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordered disc Packing</td>
<td>2.2</td>
<td>238.51</td>
<td>3473.77</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>72.68</td>
<td>683.88</td>
<td>6.95</td>
</tr>
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<td></td>
<td>11.4</td>
<td>46.13</td>
<td>292.41</td>
<td>16</td>
</tr>
<tr>
<td>Spiral wound</td>
<td>2.2</td>
<td>543.24</td>
<td>19037.21</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>143.16</td>
<td>1611.89</td>
<td>4.23</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>109.85</td>
<td>823.23</td>
<td>6.4</td>
</tr>
<tr>
<td>Spiral wound with side exit</td>
<td>2.2</td>
<td>505.94</td>
<td>65153.9</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>143.5</td>
<td>7390.52</td>
<td>22</td>
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<td></td>
<td>11.4</td>
<td>89.98</td>
<td>3138.235</td>
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</table>
Figure 6.1 Fibrous bed bioreactor set up (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.2 The effects of flow rate and packing method on residence time distribution: (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.3 Model prediction vs experiment data: (A) ordered disc packed FBB at flow rate 13.2 cm/min. (B) spiral wound FBB at flow rate 13.2 cm/min. Axial dispersion model, axial and radial dispersion model, two-way dispersion with one way convection, and two-way dispersion with two-way convection model were tested against experimental data. (C) spiral wound FBB with side exit at flow rate of 13.2 cm/min.
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Figure 6.7 Oxygen profile in scaled up fibrous bed bioreactor with $L=30$ cm, $D=7.5$ cm and $u=13.2$ cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.
Figure 6.8 Cell density profile in scaled up fibrous bed bioreactor with $L=30$ cm, $D=7.5$ cm and $u=13.2$ cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.
Figure 6.9 Oxygen profile in scaled up fibrous bed bioreactor with $L=30$ cm, $D=7.5$ cm and $u=66$ cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.
Figure 6.10 Cell density profile in scaled up fibrous bed bioreactor with $L=30$ cm, $D=7.5$ cm and $u=66$ cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.
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Chapter 6

Modeling and Scale Up a Fibrous Bed Bioreactor for Mammalian Cell Culture

6.1 Abstract

The fibrous bed bioreactor (FBB) has wide applications in mammalian cell culture and tissue engineering. A fibrous matrix of polyethylene-terephthalate (PET) scaffold provides a 3-D culture environment for animal cells; such an environment more closely mimics their in vivo growth situation. FBB has been proven to be superior to conventional bioreactors for both anchorage dependent and independent cells. Mathematical models were established for ordered disc packed and spiral wound bioreactors in this study. The comparison between model prediction and step response residence time distribution data shows that the models are valid. For the ordered disc packed bioreactor an axial dispersion model was used. However, both radial and axial convection and dispersion contributed significantly to the mass transfer in spiral wound bioreactors. As such, a model that considers both axial and radial convection was employed for the spiral wound bioreactors. For both reactor types, models show that embryonic stem cells can reach tissue like cell density of $10^9$ cells/ml in 15~22 days in a lab scale fibrous bed bioreactor by perfusion culture. Models predict significant oxygen
depletion in scaled versions of both types of FBB, and, therefore, an uneven cell distribution is observed. Increasing the superficial velocity alleviates nutrient limitation. Spiral wound FBB with side exit performed best in scaled up FBB. In order to avoid diffusion limitation to the cell aggregate, the cell aggregate size should be less than 250 µm for 70% DO in the medium and 170 µm for 30% DO in the medium. A hydrodynamic study of FBB shows that the pressure drop is much higher in an ordered disc packed FBB than a spiral wound FBB and that the hydrodynamic permeability decreases rapidly after cell density reaches $10^8$ cells/ml. Models predict that the superficial velocity could reach a maximum of 72 cm/min without causing shear damage to the cells in the reactor.

6.2 Introduction

The fibrous bed bioreactor has wide applications in fermentation, enzyme catalysis, and animal cell culture (Albayrak and Yang, 2002 (a,b); Luo and Yang, 2004; Wang et al., 1992). The fibrous bed bioreactor is a modification of the packed bed bioreactor by applying novel packing materials such as cotton towel, non-woven PET scaffold (Albayrak and Yang, 2002b; Chen et al., 2002). The traditional packed bed bioreactor has a large pressure drop along its axis which restricts its scale up potential (Calis, et al., 2001; Achenbach, E., 1995). On the other hand, cotton towel and PET scaffold have the merits of high porosity, high specific surface area, high permeability, low pressure drop, high mechanical strength, and low material costs. These advantages assure the fibrous bed bioreactor a promising future. However, the theoretical study of FBB has been left
behind its applications, which hinders its scale up potential. Thus, it’s necessary to model the FBB before scale up.

It was noted in traditional packed bed bioreactor that even though small beads (3 mm) provide large specific surface area, 4-20 times better yields were obtained in large bead (5mm) packed beds (Nilsson et al., 1986). Larger void space due to less efficient packing may attribute to better flow characteristics through the bed which benefits mass transfer. This unexpected result suggests that the design of the bioreactor is of great importance.

Two packing methods were applied in the fibrous bed bioreactor. One is ordered disc packing and the other is spiral wound packing. There are many papers which discussed packed bed bioreactor modeling, but they focused on the modeling of solid sphere and microporous sphere packed beds because of their bio-applications in enzyme biocatalysis, waste water treatment and cell culture (Dadvar and Sahimi, 2003; Gu and Syu, 2004). The governing differential equations for chromatography were borrowed and a reaction term was added in order to model the packed bed bioreactor. Particularly, the axial dispersion model has been widely used (Backer and Baron, 1994; Gu and Syu, 2004; Iliuta I. et al. 1999). However, the accuracy of the axial dispersion model is limited by tailing (Hoogendoorn and Lips, 1965). The multistage dispersion model integrates the axial dispersion and plug flow. It is accurate in describing a reactor with separate flow regions. In microporous particle packed bed bioreactors, there are three potential mass transfer limiting steps: external mass transfer, diffusion through the liquid film around the particles and intra particle diffusion. Backer and Baron (1994) included the intra particle diffusion terms in the dispersion equation for their yeast cell culture bioreactor. At low
Reynolds number (<10), the model prediction fit the experimental data very well indicating the validity of the model. In the presence of low biomass loading, there was no effect on the mass transfer. However, at high biomass loading (> 0.02 g cell/g beads), mass transfer rate between the extraparticle and intraparticle fluid phase dropped significantly. Gu and Syn (2004) also established a model to describe the immobilized cell column in bioconversion and waste water treatment. They considered interfacial film diffusion as the mass transfer limitation step. What’s more, reaction and cell death was included in their model, vastly increasing model accuracy. Iliuta and coworkers (1996) developed an axial dispersion-exchange model with the assumption that intra particle diffusion led to the long tails of the residence time distribution (RTD) curve.

Spiral wound hollow fiber or membrane reactor modeling is much more difficult than conventional packed bed reactor modeling because of the complex 3-dimensional momentum and mass transport. Crowder and Gooding (1997) calculated the mass transfer efficiency by applying empirical correlations in a spiral wound membrane reactor, but their study of the mathematical modeling was limited. Madireddi and coworkers (1999) develop a transient model for mass transfer, especially for concentration polarization in spiral wound membrane reactors. Membrane thickness and distance between two membrane layers were taken into account in their work. Brotherton and Chau (1990) did a comprehensive study on momentum and mass transport in spiral wound hollow fiber bioreactor for animal cell culture, but none of the aforementioned works verified their model with experimental data.
Modeling of fibrous bed bioreactor will be addressed in this study and supportive information of nutrients and cell distribution, hydrodynamic studies for future scale up will be gathered in terms of both bioreactor design and operation.

6.3 Material and Method

6.3.1 Fibrous Bed Bioreactor Set Up

Two packing methods were compared for their performance. The first packing method is ordered disc packing. A PET scaffold was cut into several round shaped pieces of similar diameter to the reactor column – The reactors dimensions are 2.5 cm diameter and 8 cm height as Figure.6.1A illustrated. Another packing method is spiral packing. One PET sheet of 8×20 cm was spirally wound into the column. The approximate distance between layers is 1 mm. Two types of spiral wound reactors were used. One was with normal top exit (Figure 6.1B) and the other was with side exit at the top (Figure.6.1C).

6.3.2 Residence Time Distribution

The pulse input method was used to ascertain the mean residence time and variance (Fogler S. 2002). Step response data was also collected to compare with model prediction. For pulse input measurement, 0.2 ml of saturated NaCl solution was rapidly injected into the fibrous bed bioreactor from the bottom. The conductivity of the effluent was measured every 5 seconds until the reading returned to its initial value and remained stable for 30 seconds. For the step response experiment, 0.125 M NaCl solution was pumped into the fibrous bed bioreactor. The conductivity of the effluent was measured
every 5 seconds until the reading was stable for 30 seconds. Three superficial velocities of 2.2, 7.5, and 11.41 cm/min were tested. All experiments were duplicated.

6.3.3 Pressure Drop Measurement

Axial pressure drop of the three bioreactors were measured by a water manometer. Fluid superficial velocity ranged between 0 to 13.2 cm/min. The packed fibrous bed had a height of 8 cm and a diameter of 2.5 cm. Matrices with different solid fraction were prepared by thermo compression method for 0, 30 min, 60 min and 90 min (Li, et al., 2001).

6.4 Mathematical Models

Mean residence times and variance were determined by equations 1-4 below (Fogler S. 2002):

\[
E(t) = \frac{C(t)}{\int_0^\infty C(t)dt} \tag{1}
\]

\[
t_m = \int_0^\infty tE(t)dt \tag{2}
\]

\[
\sigma^2 = \int_0^\infty (t - t_m)^2 E(t)dt \tag{3}
\]

\[
\sigma^2_{\theta^2} = \frac{\sigma^2}{t_m^2} \tag{4}
\]

The Bodenstein number is recognized as the theoretical plate number in a packed column and is calculated by equation 5 (Tong et al., 2003).

\[
\sigma^2_{\theta^2} = \frac{2}{Bo} - \frac{2}{Bo^2} (1 - e^{-Bo}) \tag{5}
\]
The axial dispersion coefficient in fibrous bed bioreactor is represented by equation 6 (Tong et al., 2003).

\[ D_l = \frac{uL}{\varepsilon Bo} \]  

(6)

The axial dispersion model is represented in equations 7-10 below. In this study, the axial dispersion model is applied to the ordered disc packed fibrous bed bioreactor (Fogler S. 2002):

\[ \frac{\partial c}{\partial t} = D_l \frac{\partial^2 c}{\partial z^2} - \frac{u}{\varepsilon} \frac{\partial c}{\partial z} \]  

(7)

I.C. \quad t = 0, c(z,0) = 0

(8)

B.C. \quad z = 0, c = c_0 + \frac{D_l \varepsilon}{u} \frac{\partial c}{\partial z}

(9)

\[ z = L, \frac{\partial c}{\partial z} = 0 \]

(10)

However, the axial dispersion model is incapable of fitting the data from the spiral wound FBB. In order to model the spiral wound FBB, much more sophisticated momentum and mass transfer phenomena had to be accounted for. The following equations capture the complexities of these two transport phenomena in a spiral wound FBB.

**Momentum Transfer**

Continuity equation:

\[ \frac{1}{r} \frac{\partial}{\partial r} (rv_r) + \frac{\partial u_z}{\partial z} = 0 \]  

(11)

Equations of motion:

\[ v_r \frac{\partial u_r}{\partial r} + u_z \frac{\partial u_z}{\partial z} = -\frac{1}{\rho} \frac{\partial P}{\partial z} + v \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial u_z}{\partial r} \right) + \frac{\partial^2 u_z}{\partial z^2} \right] \]  

(12)

\[ v_r \frac{\partial v_r}{\partial r} + u_z \frac{\partial v_z}{\partial z} = -\frac{1}{\rho} \frac{\partial P}{\partial r} + v \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial v_z}{\partial r} \right) + \frac{\partial^2 v_z}{\partial z^2} \right] \]  

(13)
Assuming $L >> d$, the inertial terms can be neglected and thus,

$$u_z = \frac{R^2}{4\mu} \left( -\frac{dP}{dz} \right) \left[ 1 - \left( \frac{r}{R} \right)^2 \right]$$

(14)

$$v_r = \frac{R^3}{8\mu} \left( \frac{d^2P}{dz^2} \right) \left( \frac{r}{R} \right)^2 - \frac{1}{2} \left( \frac{r}{R} \right)^4$$

(15)

at $r=R_v$, $v_w = \frac{R^3}{16\mu} \left( \frac{d^2P}{dz^2} \right)$

(16)

Wall velocity is pressure driven (Darcy’s law)

$$v_w = A_m \left[ P(z) - P_m \right]$$

(17)

Where $P_m$ is matrix pressure and can be considered constant (Brotherton and Chau, 1990). $A_m$ is the hydrodynamic permeability. The dimensionless pressure equation is given below:

$$\frac{d^2P}{dz^2} - \alpha^2 (P' - P_m') = 0$$

(18)

$\alpha$ is the pressure modulus,

$$\alpha = L \sqrt{\frac{16\mu A_m}{d^3}}$$

(19)

The boundary conditions are different for the two types of spiral wound FBB. The following dimensionless equations correspond to the boundary conditions of a FBB with normal top exit.

$$z' = 0, P' = 1 \quad \text{(inlet pressure)}$$

(20)

$$z' = 1, P' = 0 \quad \text{ (ambient gauge pressure)}$$

(21)

After integration, the following is obtained:

$$P'(z) = P_m' + (1 - P_m') \cosh \alpha z' - \beta \sinh \alpha z'$$

(22)

$$\beta = \frac{P_m' + (1 - P_m') \cosh \alpha}{\sinh \alpha}$$

(23)

$$v_w' = \alpha^2 \left[ (1 - P_m') \cdot \cosh \alpha z' - \beta \sinh \alpha z' \right]$$

(24)

The boundary conditions for spiral wound FBB with side outlet are as follows:

$$z' = 0, P' = 1$$

(25)
\[ z' = 1, \frac{dP'}{dz'} = 0 \] (26)

After integration, new dimensionless pressure and radial wall velocity function is obtained as:

\[ P'(z) = P_m' + (1 - P_m') \cdot [\cosh \alpha z' - \tanh \alpha \cdot \sinh \alpha z'] \] (27)

\[ v_w'(z) = (1 - P_m') \cdot \alpha^2 (\cosh \alpha z' - \tanh \alpha \cdot \sinh \alpha z') \] (28)

Axial velocity and radial velocity are described by equations 29 and 30.

\[ v_r = v_w \frac{r}{R} \] (29)

\[ u_z = u_0 (1 - \left( \frac{r}{R} \right)^2 ) (1 - \int_0^z v_w \, dz) \] (30)

Radial dispersion coefficient can be evaluated by equation 31 (Pereira et al., 2005)

\[ D_r = D(1 + \frac{63}{320} \sqrt{2(1 - \epsilon)} \frac{u_d \epsilon}{D}) \] (31)

**Mass Transfer**

Mass transfer in spiral wound FBB was further investigated. The general equation is below.

\[ \frac{\partial c}{\partial t} = -\frac{u_z}{\epsilon} \frac{\partial c}{\partial z} + \frac{v_r}{\epsilon} \frac{\partial c}{\partial r} + D_r \left[ \frac{1}{r} \frac{\partial c}{\partial r} + \frac{\partial^2 c}{r^2 \partial r^2} \right] + D_{r,j} \frac{\partial^2 c}{\partial z^2} \] (32)

Equation 32 has to be modified into its dimensionless form in order to apply previous axial and radial velocity functions to the mass transfer equation. The dimensionless Groups below depict the dimensionless method used in this study.

\[ t' = \frac{t u_0}{L}, \quad u_z' = \frac{u_z}{u_0}, \quad v_r' = \frac{v_r}{u_0 R}, \quad c' = \frac{c}{c_{fed}}, \quad z' = \frac{z}{L}, \quad r' = \frac{r}{R}, \quad D_{r,j}' = D_{r,j} \frac{L}{u_0 R^2} \]

The exit concentration profile was compared with RTD data to validate the model. For this purpose, concentration was integrated over the cross sectional area with consideration given to the axial velocity profile.
Experimental data was gathered using a sodium chloride solution – because the substrate of interest is oxygen, the limiting substrate, the dispersion coefficient calculated from equation 6 requires modification. Equations 34-36, along with equation 6, demonstrate the steps needed for such modification.

\[
D_{NaCl} = \frac{2}{\frac{1}{D_{Na^+}} + \frac{1}{D_{Cl^-}}} 
\]

(34)

\[
Pc = a\left(\frac{Re}{\nu}\right)\left(\frac{\nu}{D}\right) 
\]

(35)

\[
Bo = Pe 
\]

(36)

The models derived for mass transport are validated by RTD data. The ultimate goal is to use the model equations to predict the oxygen profile in the fibrous bed bioreactor throughout a long term culture. Oxygen consumption due to cell metabolism must be taken into consideration in the model. As such, an oxygen consumption term is added to the model and is represented by equations 37 and 38. The Monad equation is employed for the specific cell growth rate calculation.

\[
\text{reaction} = Q_0 X_0 e^{\mu' t} 
\]

(37)

\[
\mu' = \mu_{\text{max}} \frac{S}{K + S} 
\]

(38)

Where \( Q_0 = 10^{-10} \text{ mmol/cell/h} \), \( \mu_{\text{max}} = 0.0146 \text{ h}^{-1} \) and \( X_0 = 5 \times 10^5 \text{ cell/mL matrix} \) (from our own experimental data). Accurate prediction of an oxygen profile will allow for determination of a cell density profile in the FBB.
Hydrodynamic parameters of permeability and shear stress were also calculated.

Permeability could be defined by Darcy’s law as:

$$\frac{F}{A} = u = \frac{k \Delta P}{\mu L}$$

(39)

Where, k is the hydrodynamic permeability, F is the volumetric flow rate through a cross-section of area A, u is the flow velocity, \(\mu\) is the fluid dynamic viscosity, and \(\Delta P\) is the pressure drop over a length L. This equation is valid for Newtonian fluids at low flow rates (Spielman et al., 1968). Permeability of porous fibrous matrix is also a function of the void fraction of the packed bed. Void fraction decreases with increasing cell density in the following manner.

$$\varepsilon = \varepsilon_0 - 4X_0 \varepsilon^{1/3} r_{cell}^3$$

$$3R^2 L$$

(40)

Additionally, the permeability is a function of fiber radius (a), solid fraction (\(\phi\)) and fiber arrangement. For different flow patterns, there are different empirical equations to calculate permeability. Jackson (1986) gave an excellent summary of empirical models which follow:

flow parallel to an array of rods

$$\frac{k}{a^2} = \frac{1}{4\phi} \left( -\ln \phi - \frac{3}{2} + 2\phi - \frac{\phi^2}{2} \right)$$

(Langmuir)

(41)

flow perpendicular to an array of rods

$$\frac{k}{a^2} = \frac{1}{8\phi} \left( -\ln \phi + \frac{\phi^2 - 1}{\phi^2 + 1} \right)$$

(Happel)

(42)

flow through three-dimensional arrays
\[ \frac{k}{a^2} = \frac{3}{20\phi} (-\ln \phi - 0.931 + O(\ln \phi)^{-1}) \]  

\text{(Drummond & Tahir)} \quad (43)

Apparent fiber diameter, \(d_f\), also changes with the void fraction according to Equation 44

\[ d_f^2 = \frac{1 - \varepsilon}{1 - \varepsilon_0} d_{f,0}^2 \]  

\text{(44)}

Shear stress is calculated as follows \cite{Perry and Wang, 1989}

\[ \tau = \frac{\mu u d_f}{4k(1 - \varepsilon)} \]  

\text{(45)}

Oxygen diffusion into the cell aggregate was also considered. The equations listed below describe this phenomenon.

\[ \frac{\partial c'}{\partial t} = D\left(\frac{\partial^2 c'}{\partial r^2} + \frac{2}{r} \frac{\partial c'}{\partial r}\right) - Q_0 X \]  

\text{(46)}

\[ X = \frac{R^3}{r_{cell}^3} \]  

\text{(47)}

I.C. \( t=0, c' = 30\% \text{ DO} \) \quad (48)

B.C. \( r=0, \frac{\partial c'}{\partial r} = 0 \) \quad (49)

\( r=R, c' = c|_{z=L} \) \quad (50)

An implicit finite difference method was used to solve all transient partial differential equations.
6.5 Results

6.5.1 Effects of Flow Rate and Packing Method on Residence Time Distribution:

Three flow rates were chosen to investigate the effect of flow rate on RTD data. Equations 1~6 were employed to evaluate the dispersion coefficient, and mean residence time. It’s obvious that as the flow rate increases, the mean residence time decreases (Figure 6.2). Moreover, different packing methods greatly influence mean residence time. At the same flow rate, the mean residence time in the normal spiral wound FBB was 100% higher than that in the ordered disc packed FBB and is also slightly larger than that in the spiral wound FBB with side exit (Table 6.1). Different packing methods also affect the residence time distribution curve. The curves weren’t normally distributed at a low flow rate of 2.2 cm/min in spiral wound FBB with side exit. Instead, several peaks were observed in the RTD curve. Additional peaks were also noticed in the higher flow rate RTD curves of 6.5 cm/min and 11.4 cm/min. Such peculiar RTD curves can be attributed to the complex mixing phenomena and flow pattern in a spiral wound FBB. Dispersion coefficients under different conditions are listed in Table 6.1. Table 6.1 elucidates that, in all cases, dispersion coefficients increase with increasing flow rate. Axial dispersion coefficients of spiral wound FBB with side exit are much higher than the other two cases.

6.5.2 Model Validation:

Figure 6.3 presents the model prediction versus experimental data. It is evident that the axial dispersion model fits the experimental data of the ordered packed disc FBB well (Figure 6.3A). However, this model is not able to fit the RTD data from spiral wound
reactor. More sophisticated models were developed in succession. First, both axial dispersion and radial dispersion were included into the model giving improved but insufficient results. Then, radial convection was considered and model prediction fit the RTD data well (Figure 6.3B) proving the validity of this model. Because the mass transfer equation for the side exit spiral wound FBB is identical to that of the top exit spiral wound FBB, this model is also used to fit the experimental data from spiral wound FBB with side exit. However, there is a crucial distinction – the boundary conditions differ. After applying the different boundary conditions, it is shown that the model fits the experimental data well. Equations 11-32 above depict transport phenomena in a spiral wound reactor by theoretical derivation from momentum and mass transfer fundamental equations.

6.5.3 Oxygen and Cell Density Profile in Lab Scale FBB

Dispersion coefficients change with superficial velocity. Different substances have different dispersion coefficient. Experimental results show that the Bodenstein number remains constant after the Reynolds number surpasses 40 (Figure 6.4). For the actual cell culture process the Reynolds number always exceeded this requirement. Therefore, equations 33-36 are applied to evaluate the oxygen dispersion coefficients under different conditions.

After model validation, oxygen consumption by cells is taken into consideration. A maximum cell density of $10^9$ cells/ml is assumed; the maximum cell density is that of human tissue. According to previous experimental observation (Luo, 2002), another criterion for cell growth arrest is that dissolved oxygen (DO) in the medium is less than
30%. Figure 6.5A indicates that oxygen concentration decreases to 30% DO in lab scale ordered disc packed FBB at superficial flow rate of 0.00231 m/s. After 25 days, the oxygen concentration drops to 50% DO at the exit in spiral wound FBB with top exit (Figure 6.5B). However, it dropped to almost zero in the spiral wound FBB with side exit at the same flow rate (Fig.6.5C). The cell density reaches tissue like cell density in 20 days and is evenly distributed throughout the ordered disc packed reactor (Figure 6.6A). Cells grow slower in the lab scale spiral wound FBB with top exit and took 22 days for the entire reactor to reach the maximum cell density. It is evident from the uneven cell distribution and low exit cell concentration that cell growth is not satisfactory in the lab scale spiral wound FBB with side exit. The ordered disc fibrous bed reactor is best in regards to its performance and efficiency.

6.5.4 Oxygen and Cell Density Profile in Scaled up FBB

The scaled up bioreactors’ performance was also investigated. For this purpose, the bed length was increased 10 fold and the diameter was increased 3 fold. Oxygen depletion is observed in all three cases as shown in Figure 6.7. The oxygen is consumed faster in the ordered disc packed reactor and the concentration falls to zero on the 16th day (Figure 6.7A). Oxygen concentration in the other two cases falls to zero on the 18th day (Figure 6.7B, C). However, a larger portion (~10 cm) of the packed bed suffers from nutrient limitation in the two spiral wound reactors than in the ordered disc packed reactor (~ 7 cm).

In all three reactors, cell density is not uniformly distributed as witnessed by Figure 6.8. Cells cease to grow after the DO reaches 30%. Figure 6.8 demonstrates that spiral wound
reactor with side exit has the best cell growth with a smaller non-even distribution region and maintains a higher cell density in the nutrient limiting area. This result indicates that different bioreactor designs should be considered for different scales.

6.5.5 Oxygen and Cell Density Profile in Scaled up FBB with Higher Superficial Velocity

Since nutrient depletion is observed in all three designs, a higher superficial velocity of 69.3 cm/min was considered to improve mass transfer inside of the reactor. However, higher superficial velocity also causes greater shear damage to cells. The greatest safe velocity is determined according to Equations 42, 44, and 45. It is found to be 72 cm/min.

The higher velocity alleviates oxygen depletion to ~45 %DO in ordered disk packed reactor (Figure 6.9A) and ~55 % in spiral packing with side exit (Figure 6.9C). Because oxygen concentration remained above 30 %DO, cell growth continued until the cell density reached a tissue like state (Figure 6.10A, C). To some extent, cells grow faster in the spiral wound with side exit. For the normal spiral wound reactor, the higher superficial velocity is only able to delay complete oxygen depletion (Figure 7.9B). This results in non-uniform cell distribution. Under these circumstances, the ordered disc packed reactor is best.

6.5.6 Oxygen Diffusion in Cell Aggregate:

Mammalian cells form aggregate in 3-D PET scaffold during growth. However, cell aggregate size adds to the diffusion resistance and undesirable oxygen limitation might occur in the center of a large aggregate. Oxygen diffuses through the aggregate to the
center as described by equations 46-50. It is found (Figure 6.11) that cell aggregates are capable of reaching a size of 250 \( \mu \text{m} \) if the medium contains 70\% DO. However, the maximum aggregate size is 170 \( \mu \text{m} \) with 30\% DO in the medium. Cell aggregate size is partially controllable by the pore size of the scaffold. Thermal compression has proven to be able to permanently reduce the pore size – to some extent, this process is able to reduce the aggregate size (Li et al., 2001).

6.5.7 Hydrodynamic Study of Fibrous Bed Bioreactor

Pressure drops in the three bioreactor designs were measured as illustrated by Figure 6.12A. The pressure drop along the axial direction is much higher in the ordered disc packed FBB than in the spiral wound reactors with the same packed bed height. This result is unsurprising because there are channels of small pressure resistance between two layers of scaffold. A smaller pressure differential is advantageous; it is well recognized that pressure drops along the axial direction make scale up work difficult.

Permeability and porosity decrease with increasing cell density. However, it’s impossible to measure these parameters without destroying the cells. Mathematical models have been developed to account for changes in permeability. Figure 6.12B demonstrates that the Happel model is better able to fit the solid fraction data. Figure 6.12C makes evident that permeability begins to decrease as cell density approaches \( 10^8 \) cells/ml.

Animal cells are believed to be more sensitive to shear damage than bacteria, fungi and plant cells due to their lack of a cellular wall. Cells in a flowing liquid medium are
subject to shear stress resulting in hydrodynamic damage. For this reason, shear stress is one of the most important considerations in mammalian cell culture reactor design. Experimental data indicates that animal cells suffer from hydrodynamic damage when shear stress exceeds 20 dyne/cm² (our own data). Figure 6.12D indicates that, with regard to the aforementioned limitation, the maximum obtainable superficial velocity is 72 cm/min. Furthermore, Figure 6.12D shows that shear stress increases with culture time until the 22nd day and thereafter rapidly falls to zero. Equation 45 indicates that shear stress is a balance between permeability and solid fraction – these two conflicting factors account for the peak in the shear stress profile.

6.6 Discussion

Fibrous bed bioreactor was successfully used in mammalian cell culture. However, its mathematical modeling work has been left behind due to the complex momentum and mass transport phenomena in the reactor. Residence time distribution measurement provides a tool for model validation.

Pressure gradients increase along the axial direction. The results demonstrate that the ordered disc packed FBB has a much higher pressure drop along its axial direction. This may give rise to non-uniform nutrient delivery and cell growth in the bioreactor. This disadvantage is especially apparent in a scaled bioreactor. Nutrients are consumed in the lower portion of the reactor limiting their availability to the upper portion; cells in the upper part experience nutrient limitation resulting in slower growth. By increasing superficial velocity, a greater portion of the bioreactor can be alleviated of nutrient limitation. However, increasing velocity also contributes negatively to shear damage to
the cells. Therefore, other methods of nutrient depletion alleviation, such as reactor design, have to be considered. There is more void space in spiral wound reactor due to channels between PET. Nutrients are more efficiently delivered to the upper portion of the reactor in these bioreactors. However, this advantage is only observed in scaled versions of the bioreactors. Recent design and modeling efforts have focused on radial convective enhanced nutrient delivery. Based on the above analysis, the spiral wound reactor with side exit is a good alternative for the design because it’s capable of increased radial convection.

6.7 Conclusions

The axial dispersion model is able to describe the mass transfer in ordered disc packed FBB while both axial and radial convection and dispersion contribute significantly to the mass transfer in spiral wound FBB. Regarding bioreactor performance, ordered disc packed FBB is best in lab scale while spiral wound FBB with side exit is best on a larger scale. In order to avoid diffusion limitation to the cell aggregate, the cell aggregate size should be less than 250 µm for 70% DO in the medium and 170 µm for 30% DO in the medium. A hydrodynamic study of FBB shows that the pressure drop is much higher in an ordered disc packed FBB than a spiral wound FBB and that the hydrodynamic permeability decreases rapidly after cell density reaches 10^8 cells/ml. Models predict that the superficial velocity can reach a maximum of 72 cm/min without causing shear damage to the cells in the reactor.
6.8 Nomenclature:

- $\nu$: Kinematic viscosity (m$^2$/s)
- $\mu$: Medium viscosity (cp)
- $\tau$: Shear stress (dyne/cm$^2$)
- $\phi$: Solid fraction
- $\varepsilon$: Void fraction
- $\sigma^2$: Variance (s$^2$)
- $\sigma^2_0$: Dimensionless variance
- $\mu'$: Cell specific growth rate
- $a$: Fiber radius
- $A_m$: Permeability (m$^2$/s/kg)
- $B_0$: Bodenstein Number
- $C$: Tracer or oxygen concentration
- $D$: Diffusivity (m$^2$/s)
- $d$: Distance between two layers of scaffold (m)
- $d_f$: Apparent fiber diameter (m)
- $D_l$: Axial dispersion coefficient (m$^2$/s)
- $D_r$: Radial dispersion coefficient (m$^2$/s)
- $E(t)$: Residence-time distribution function
- $H$: Packed bed height (m)
- $k$: Hydrodynamic permeability (m$^2$)
- $L$: Packed bed length (m)
- $P$: Pressure (Pa)
- $P'$: Dimensionless Pressure
- $Pe$: Peclet Number
- $P_m$: Dimensionless pressure of the matrix
- $Q_0$: Oxygen consumption rate (mmol/cell/h)
- $R$: Radius of the reactor (m)
- $r_{cell}$: Single cell radius
- $t_m$: Mean residence time (s)
- $u$: Superficial velocity (m/s)
- $u_x$: Axial velocity (m/s)
- $v_r$: Radial velocity (m/s)
- $v_w$: Wall velocity (m/s)
6.9 Reference:


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Table 6.1 Effect of Flow Rate on Dispersion Model Parameters.

<table>
<thead>
<tr>
<th>Packing method</th>
<th>Flow rate (cm/min)</th>
<th>Mean residence time (s)</th>
<th>Variance ((s^2))</th>
<th>Dispersion coefficient ((10^6 , m^2/s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordered disc Packing</td>
<td>2.2</td>
<td>238.51</td>
<td>3473.77</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>72.68</td>
<td>683.88</td>
<td>6.95</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>46.13</td>
<td>292.41</td>
<td>16</td>
</tr>
<tr>
<td>Spiral wound</td>
<td>2.2</td>
<td>543.24</td>
<td>19037.21</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>143.16</td>
<td>1611.89</td>
<td>4.23</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>109.85</td>
<td>823.23</td>
<td>6.4</td>
</tr>
<tr>
<td>Spiral wound with side exit</td>
<td>2.2</td>
<td>505.94</td>
<td>65153.9</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>143.5</td>
<td>7390.52</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>89.98</td>
<td>3138.235</td>
<td>47</td>
</tr>
</tbody>
</table>
Figure 6.1 Fibrous bed bioreactor set up (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.2 The effects of flow rate and packing method on residence time distribution: (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.

Figure 6.3 Model prediction vs experiment data: (A) ordered disc packed FBB at flow rate 13.2 cm/min. (B) spiral wound FBB at flow rate 13.2 cm/min. Axial dispersion model, axial and radial dispersion model, two-way dispersion with one way convection, and two-way dispersion with two-way convection model were tested against experimental data. (C) spiral wound FBB with side exit at flow rate of 13.2 cm/min.
Figure 6.4 Bodenstein number under different Reynolds number
Figure 6.5 Oxygen profile in lab scale fibrous bed bioreactor with L=3 cm, D=2.5 cm and u=13.2 cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.6 Cell density profile in lab scale fibrous bed bioreactor with $L=3$ cm, $D=2.5$ cm and $u=13.2$ cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit.
Figure 6.7 Oxygen profile in scaled up fibrous bed bioreactor with L=30 cm, D=7.5 cm and u=13.2 cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.8 Cell density profile in scaled up fibrous bed bioreactor with L = 30 cm, D = 7.5 cm and u = 13.2 cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.9 Oxygen profile in scaled up fibrous bed bioreactor with L=30 cm, D=7.5 cm and u=66 cm/min (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.10 Cell density profile in scaled up fibrous bed bioreactor with L = 30 cm, D = 7.5 cm and \( u = 66 \text{ cm/min} \) (A) ordered disc packing, (B) normal spiral packing, (C) spiral packing with side exit
Figure 6.11 Oxygen concentration in the center of cell aggregate (A) 70% DO in the medium (B) 30% DO in the medium
Figure 6.12 Hydrodynamic study of fibrous bed bioreactor (A) pressure drop (B) permeability model fit (C) effect of cell density on permeability and porosity (D) shear stress in the reactor during the cell culture.
Chapter 7

Conclusions and Recommendations

7.1 Conclusions

The fibrous PET matrix provides not only a large surface area, but also the three-dimensional structure to facilitate \textit{in vivo} like cell organization. The fibrous bed bioreactor with 3-D fibrous matrices is superior to conventional bioreactors using only 2-D surface for cell attachment in tissue engineering applications. The feasibility and advantages of the fibrous bed bioreactor for mass production of undifferentiated embryonic stem cells was demonstrated. Neural differentiation patterns in 2-D and 3-D cultures were studied and compared. Effects of reactor hydrodynamics on cell attachment and growth were studied. Mathematical models for the fibrous bed bioreactor with different packing designs were developed and used to predict the mass transfer and cell growth in the reactor. The important results presented in previous chapters are summarized below.

7.1.1 3-D Culturing of Embryonic Stem Cells in Fibrous Bed Bioreactor

3-D cultures are superior to 2-D cultures by eliminating the requirement for gelatin coating and frequent subculturing. STO cells conditioned medium can replace LIF in
sustaining long term undifferentiated ES cell cultures. Lactic acid above 1.5 g/L has a negative effect on ES cell growth and induces spontaneous differentiation. A low porosity scaffold is beneficial for maintaining undifferentiated ES cell growth. The two-stage perfusion fibrous bed bioreactor system was successfully demonstrated and has promising future for up-scaling to satisfy market demands. hES cells were also successfully cultivated in a perfusion bioreactor. The hES cell growth rate in FBB surpassed at in 2-D cultures. Matrigel coating was not necessary in FBB.

7.1.2 Neural Differentiation in 2-D and 3-D Systems

The astrocyte conditioned medium can effectively induce ES cells neural differentiation in replacing retinoic acid, which inhibits cell proliferation. Neural differentiation efficiency was generally higher in 2-D than 3-D cultures because of the higher possibility of de-differentiation in 3-D cultures. Dynamic cultures in 3-D bioreactors are feasible for producing neural differentiated ES cells will require further optimization.

7.1.3 Effect of Mixing Intensity on Cell Attachment and Growth in Fibrous Scaffold

Increasing mixing intensity increased both cell attachment and detachment rates. However, the attachment rate is one magnitude higher than the detachment rate under the conditions studied. The cell detachment behavior from the 3-D matrix can be described by the Bell model. A smaller pore size is favorable to cell attachment and proliferation. Further analysis revealed that the interaction between mixing intensity and pore size played a vital role in causing hydrodynamic damage to cells, which was found to be significant when the size of Kolomogorov eddy was smaller than the matrix pores.
Mixing intensity can also influence cell metabolism; increasing the mixing intensity increases oxygen transfer and decreases the lactate yield from glucose. It is suggested that 3-D PET scaffold with a smaller pore size should be used in the future because, to some extent, the smaller pore size can stimulate cell proliferation and prevent hydrodynamic damage.

7.1.4 Modeling and Scale up of Fibrous bed Bioreactor

The axial dispersion model is able to describe the mass transfer in ordered disk packed FBB while both axial and radial convection and dispersion contribute significantly to the mass transfer in the spiral wound FBB. Regarding the bioreactor performance, ordered disk packed FBB is best in the lab scale while the spiral wound FBB with side exit is best on a larger scale. In order to avoid diffusion limitation to cell aggregates, the cell aggregate size should be less than 250 µm for 70% DO in the medium and 170 µm for 30% DO in the medium. A hydrodynamic study of FBB shows that the pressure drop is much higher in an ordered disk packed FBB than a spiral wound FBB and that the hydrodynamic permeability decreases rapidly after cell density reaches $10^8$ cells/ml. The model predicts that the superficial velocity can reach a maximum of 72 cm/min without causing shear damage to the cells in the reactor.

7.2 Recommendations

Regarding to therapeutic applications of ES cells, an animal-component free culture of ES cells is required. Though mass production of ES cells in the two-stage perfusion culture system was successful, mouse embryonic fibroblast cells are still involved in the
system and make the process validation and approved by FDA difficult. Therefore, a serum-free ES cell culture of ES cells process in bioreactor needs to be developed. Accordingly human origin cells should be used for the production of conditioned medium to replace MEF cells.

The results from neural differentiation of ES cells indicated that 2-D cultures was better than 3-D cultures and static culture was better than dynamic culture. However, this comparison is based on two different support materials (PET and gelatin coated polystyrene). Future study of ES cells differentiation should be carried out on the same PET material for both 2-D and 3-D cultures. Also, the effects of pH and DO on ES cells differentiation should be investigated in a controlled fibrous bed bioreactor. Further optimization is required to achieve satisfactory differentiation efficiency. Shear stress might contribute to the lower neural differentiation efficiency in the bioreactor and a separate study of effect of this factor in a well defined shear environment is recommended. Furthermore, how to scale up the differentiation process is an interesting question and deserves more attention. Other types of bioreactor can be taken into consideration for this purpose.

Additional biomarkers should be measured to quantify the differentiation efficiency. A panel of neural markers expressed during the differentiation. Only nestin and NCAM were detected in this study. It might be another story if full panel of neural markers were measured. Oct-4 expression delays during the differentiation process, meaning some differentiated ES cells are still Oct-4 positive. Therefore, neural markers expression should be considered as the criteria for differentiation efficiency.
Bibliography


Anastasius JK., Theodore HW., and Thomas, JH, 1971, Use of asymptotic relations to
correlate mass transfer data in packed beds. Chemical Engineering Science. 26: 1581-1589


Bell GI. 1978, Models for the specific adhesion of cells to cells. Science 200, 618-627


Buytaert-Hoefen KA, Alvarez E, Freed CR, 2004, Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF. Stem Cells. 22: 669-674


Cass B, Pham PL, Kamen A, Durocher Y, 2005, Purification of recombinant proteins from mammalian cell culture using a generic double-affinity chromatography scheme. Protein Expression and Purification 40, 77-85

Chalmers JJ, 1994, Cells and bubbles in sparged bioreactors. Cytotechnology, 15: 311


Chen CN, 2000, Animal cell culture in a fibrous-bed bioreactor: protein production, cell immobilization, and cell cycle and apoptosis. Ph.D. dissertation, Ohio State University


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Mantalaris A, Wu JHD, 2006, Ex vivo culture of hematopoietic and mesenchymal stem cells for tissue engineering and cell-based therapies. Biotechnology and Bioprocessing 30: 723-743

Martin GR 1981, Isolation of a pluripotential cell line from early mouse embryos cultured
in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634–7638


(TSH) isoforms produced by Chinese hamster ovary cells: the role of sialylation and sulfation in TSH bioactivity. Endocrinology. 133: 1490-1503

Tan WS, Dai GC, Chen YL, 1994, Quantitative investigation of cell-bubble interactions using a foam fraction technique. Cytotechnology. 15: 321


Whiteside JP, and Spier RE, 1981, The scale up from 0.1 to 100 liter of unit process system based on 3 mm diameter glass spheres for the production of four strains of FMDV from BHK monolayer cells. Biotechnol. Bioeng. 23: 551-561


Wobus AM, Wallukat G and Hescheler J, 1991, Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca$^{2+}$ channel blockers. Differentiation. 48:173-182


Xiao YL, Riesle J, Blitterswijk CA, 1999, Static seeding and dynamic fibroblast seeding and cultivation in porous PEO/PBT scaffold. J of Materials Science: Materials in Medicine 0, 773-777


Appendix A Matlab Codes for Ordered Disc Packed FBB
1.1 Model validation by residence time distribution in FBB

clear;
cle;

\[ u = \frac{0.0231}{2}; \text{ m/s} \]
\[ L = 8; \text{ cm} \]
\[ L = \frac{L}{100}; \text{ m} \]
\[ R = \frac{2.5}{2}; \text{ cm} \]
\[ R = \frac{R}{100}; \text{ m} \]
\[ \text{volreactor} = \pi R^2 L; \]
\[ D = 2.15476 \times 10^{-5}; \text{ m}^2/\text{s} \]
\[ dt = 0.1; \text{ s} \]
\[ C_{\text{fed}} = 0.7 \times 0.212; \text{ millimol/L} \]
\[ \epsilon_o = \frac{26}{41}; \]
\[ C_{\text{fed}} = \frac{C_{\text{fed}}}{C_{\text{fed}}}; \]
\[ D = D \times L/(u \times R^2); \]
\[ dt = dt \times u/L; \]
\[ u_{\text{nondim}} = \frac{u}{u}; \]
\[ z_{\text{node}} = 51; \]
\[ dz = \frac{L}{(z_{\text{node}} - 1)}; \]
\[ dz = \frac{dz}{L}; \]
\[ \text{Matrix1}(1:z_{\text{node}},1:z_{\text{node}}) = 0; \]
\[ \text{Matrix1}(1,1) = 1; \]
\[ \text{Matrix1}(z_{\text{node}},z_{\text{node}}) = 1; \]
\[ \text{Matrix2}(1:z_{\text{node}},1:z_{\text{node}}) = 0; \]
\[ \text{boundary_vector}(1:z_{\text{node}}) = 0; \]
\[ \text{boundary_vector} = \text{boundary_vector}'; \]
\[ \text{boundary_vector}(1) = C_{\text{fed}}; \]
\[ C_{\text{prof}}(2:z_{\text{node}},1) = 0; \]
\[ C_{\text{prof}}(1,1) = C_{\text{fed}}; \]
\[ \text{dummy} = 1; \]
\[ \text{tindex} = 1; \]
\[ \text{while dummy} = 1 \]
a=1/dt+R^2/L^2*D/dz^2;
aprime=1/dt-R^2/L^2*D/dz^2;
b=-R^2/L^2*D/(2*dz^2)-u_nodim/(4*epsilon_o*dz);
c=-R^2/L^2*D/(2*dz^2)+u_nodim/(4*epsilon_o*dz);

for i=2:znode-1
    coeff1=[b a c];
    coeff2=[-b aprime -c];
    Matrix1(i,i-1:i+1)=coeff1;
    Matrix2(i,i-1:i+1)=coeff2;
end

boundary_vector(end)=Cprof(end-1,tindex);

Cprof(:,tindex+1)=Matrix1^(-1)*(Matrix2*Cprof(:,tindex)+boundary_vector);

if tindex*(dt*L/u)/60>=5
    break
end

tindex=tindex+1;
end

Cexit=Cprof(end,:);

save('RTDfit','Cexit','tindex','dt','dz','znode') %all dimensionless

clear;
cle;

uMax=0.00231/2; %m/s
L=8; %cm
L=L/100; %m
R=2.5/2; %cm
R=R/100; %m

exp_data=textread('flowrate7.txt');
exp_data=exp_data/max(exp_data);
flowrate7=exp_data;
load('RTDfit')
dt=dt*L/uMax

node=5/dt;

a=1;
for i=1:node:length(Cexit)
    Cexit_cut(a)=Cexit(i);
    a=a+1;
end

Cnew=Cexit_cut(2:end);
len=60-length(flowrate7);
flowrate7(end:end+len)=flowrate7(end);

if length(Cnew)>length(flowrate7)
    tspan=[1:length(flowrate7)]*5;
    plot(tspan/60,Cnew(1:length(flowrate7)),tspan/60,flowrate7,'.')
    xlabel('Time (s)')
    ylabel('C/C_0')
    Legend('Model','Experimental',0)
else
    tspan=[1:length(Cnew)]*5;
    plot(tspan,Cnew,tspan,flowrate7(1:length(Cnew)),'.')
    xlabel('Time (s)')
    ylabel('C/C_0')
    Legend('Model','Experimental',0)
End
1.2 Oxygen and cell growth prediction in lab scale FBB

function Anlimodel_plusReaction

clear;
clc;

m=0;

len=3; %cm
len=len/100; %m
Diam=2.3; %cm
Diam=Diam/100; %m
volReactor=pi*Diam^2/4;
D=[1.17356e-5 3.06889e-5]*1.6/2.1; %m^2/s
u=[7.6 13.49]; %cm/min
u=u/100*1/60; %m/s
intc=0; %mol/L
intc=intc*1000; %mol/m^3
Cfed=.7*.213; %mol/m^3
epsilon=.643; %void fraction
Q=1e-10; %mmol/(cell*h)
Q=Q/3600*1e-3; %mol/(cell*s)
oxo=8e6; %cells
mu=.015; %hr^-1
mu=mu/3600; %s^-1
time=20; %days
time=time*24*3600; %s
time2=30; %day
time2=time2*24*3600; %s
p(1:2,1:7)=0;

xmesh=linspace(0,len,100);
tspan=linspace(0,time,100);

for i=1:2

sol=pdepe(m,@pdefun,@icfun,@bcfun,xmesh,tspan,[],Cfed,intc,D(i),u(i),epsilon,Q,xo,len,mu,volReactor,p(i,:));
    [row col depth]=size(sol);
for j=1:row
    for k=1:col
        if sol(j,k,1)<=0
            sol(j,k,1)=0;
        end
    end
end

solution1(:,:,i)=sol(:,:,1);
p(i,:)=polyfit(xmesh,solution1(end,:,i),6);
end

tspan=linspace(time,time2,100);
tinterval=tspan(2)-tspan(1);
Cfed=.5*.213;

for i=1:2
    sol=pdepe(m,@pdefun,@icfun,@bcfun,xmesh,tspan,[],Cfed,inc,D(i),u(i),epsilon,Q,xo,le
    n,mu,volReactor,p(i,:));
    [row1 col1 depth1]=size(sol);
    for j=1:row
        for k=1:col
            if sol(j,k,1)<=0
                sol(j,k,1)=0;
            end
        end
    end
    solution2(:,:,i)=sol(:,:,1);
end

index=1:99;
tgraph(1:100)=linspace(0,time,100);
tgraph(101:199)=time+index*tinterval;
for i=1:2
    totalsolution(1:row,:,i)=solution1(:,:,i);
    totalsolution((row+1):(row+1)-(row1-1)-1,:,i)=solution2(2:row1,:,i);
figure(i)
surf(xmesh/len,tgraph/(3600*24),totalsolution(:,:,i)/.212*100)
title(['Flowrate ',num2str(u(i)*6000),', (cm/min)'])
xlabel('Fraction of Bed Length')
ylabel('Time (days)')
zlabel('Concentration (%DO)')
end

function [c,f,s]=pdefun(z,t,C,Cfed,inct,D,epsilon,Q,xo,len,mu,volReactor,p)
c=1;
f=D*dCdz;
s=-u/epsilon*dCdz-(Q*xo*z/len*exp(mu*t))/volReactor;

function [Po,Qo,PL,QL]=bcfun(zo,Co,zL,CL,t,Cfed,inct,D,epsilon,Q,xo,len,mu,volReactor,p)
Po=Co-Cfed;
Qo=-epsilon/u;
PL=0;
QL=1;

function Cint=icfun(z,Cfed,inct,D,epsilon,Q,xo,len,mu,volReactor,p)
Cint=p(1)*z^6+p(2)*z^5+p(3)*z^4+p(4)*z^3+p(5)*z^2+p(6)*z+p(7);
1.3 Oxygen and cell density profiles prediction in large scale at various velocities

clear;
clc;

u=.00231/2; %m/s
L=30; %cm
L=L/100; %m
R=7.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
D=1.0037e-5*1.6/2.1; %m^2/s
dt=20; %s
Cfed=.7*.212; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
oxo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=26/41;
rcell=4.5e-6; %m

sizeMesh=75;

znode=20;
dz=L/(znode-1);

Matrix1(1:znode,1:znode)=0;
Matrix1(1,1)=1;
Matrix1(znode,znode)=1;
Matrix2(1:znode,1:znode)=0;

boundary_vector(1:znode)=0;
boundary_vector=boundary_vector';
boundary_vector(1)=Cfed;
growth_vector(1:znode)=0;
growth_vector=growth_vector';

growth(2:znode-1)=xo/L;
growth(1)=0;
growth(znode)=0;
Cprof(2:znode,1)=0;
Cprof(1,1)=Cfed;
dummy=1;
tindex=1;
totalcells(1)=xo;
while dummy==1
    for i=2:znode-1
        if Cprof(i,tindex)>=.3*.212
            growth_vector(i)=dz*(i-1)*Qo/volreactor*growth(i)*exp(muMax*Cprof(i,tindex)/(Cprof(i,tindex)+K)*dt);
            growth(i)=growth(i)*exp(muMax*Cprof(i,tindex)/(Cprof(i,tindex)+K)*dt);
        end
        if growth(i)/volreactor>10^15
            growth(i)=10^15*volreactor;
        end
        CD(i,tindex)=growth(i)/volreactor;
        epsilon(i,tindex)=epsilon_o-4/3*pi*rcell^3*CD(i,tindex);
    end
    a=1/dt+D/dz^2;
aprime=1/dt-D/dz^2;
    for i=2:znode-1
        b=-D/(2*dz^2)-u/(4*epsilon(i,tindex)*dz);
c=-D/(2*dz^2)+u/(4*epsilon(i,tindex)*dz);
coeff1=[b a c];
coeff2=[-b aprime -c];
Matrix1(i,i-1:i+1)=coeff1;
Matrix2(i,i-1:i+1)=coeff2;
end
boundary_vector(end)=Cprof(end-1,tindex);
\[
\text{Cprof(:,tindex+1) = Matrix1}^{-1} \times (\text{Matrix2} \times \text{Cprof(:,tindex)} + \text{boundary_vector} - \text{growth_vector});}
\]

\[
\text{for } i = 1: \text{length(Cprof(:,tindex+1))}
\]

\[
\text{if } \text{Cprof}(i,\text{tindex+1}) < 0
\]

\[
\text{Cprof}(i,\text{tindex+1}) = 0;
\]

\[
\text{end}
\]

\[
\text{end}
\]

\[
\text{if } \text{tindex} \times \text{dt/(3600*24)} \geq 25
\]

\[
\text{break}
\]

\[
\text{end}
\]

\[
\text{tindex} = \text{tindex+1};
\]

\[
\text{end}
\]

\[
\text{node} = \text{floor(tindex/sizeMesh)};
\]

\[
a = 1;
\]

\[
\text{for } j = 1: \text{node:tindex}
\]

\[
\text{Ccut(:,a) = Cprof(:,j)};
\]

\[
\text{CDcut(:,a) = CD(:,j)};
\]

\[
a = a + 1;
\]

\[
\text{end}
\]

\[
\text{clear};
\]

\[
\text{clc};
\]

\[
\text{u} = 5 \times 0.00231/2; \text{ % m/s}
\]

\[
\text{L} = 100; \text{ % cm}
\]

\[
\text{L} = \text{L}/100; \text{ % m}
\]

\[
\text{R} = 13/2; \text{ % cm}
\]

\[
\text{R} = \text{R}/100; \text{ % m}
\]

\[
\text{volreactor} = \pi \times \text{R} \times 2 \times \text{L};
\]

\[
\text{D} = 1.0037 \times 16/2.1; \text{ % m^2/s}
\]

\[
\text{dt} = 10; \text{ % s}
\]

\[
\text{Cfed} = 7 \times 212; \text{ % millimol/L}
\]

\[
\text{Qo} = 1 \times 10^{-10}; \text{ % millimol/(cell*hr)}
\]

\[
\text{Qo} = \text{Qo}/3600 \times 1/1000; \text{ % mol/(cell*s)}
\]
xo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=26/41;
rcell=4.5e-6; %m

sizeMesh=75;

znode=40;
dz=L/(znode-1);

Matrix1(1:znode,1:znode)=0;
Matrix1(1,1)=1;
Matrix1(znode,znode)=1;
Matrix2(1:znode,1:znode)=0;
boundary_vector(1:znode)=0;
boundary_vector=boundary_vector';
boundary_vector(1)=Cfed;
growth_vector(1:znode)=0;
growth_vector=growth_vector';
growth(2:znode-1)=xo/L;
growth(1)=0;
growth(znode)=0;
Cprof(2:znode,1)=0;
Cprof(1,1)=Cfed;

dummy=1;
tindex=1;
totalecells(1)=xo;
while dummy==1
    for i=2:znode-1
        if Cprof(i,tindex)>=.3*.212
            growth_vector(i)=dz*(i-1)*Qo/volreactor*growth(i)*exp(muMax*Cprof(i,tindex)/(Cprof(i,tindex)+K))*dt;
        end
    end

end
growth(i)=growth(i)*exp(muMax*Cprof(i,tindex)/(Cprof(i,tindex)+K)*dt);
end

if growth(i)/volreactor>10^15
    growth(i)=10^15*volreactor;
end
CD(i,tindex)=growth(i)/volreactor;
epsilon(i,tindex)=epsilon_o-4/3*pi*rcell^3*CD(i,tindex);
end

a=1/dt+D/dz^2;
aprime=1/dt-D/dz^2;

for i=2:znode-1
    b=-D/(2*dz^2)-u/(4*epsilon(i,tindex)*dz);
c=-D/(2*dz^2)+u/(4*epsilon(i,tindex)*dz);
coeff1=[b a c];
coeff2=[-b aprime -c];
Matrix1(i,i-1:i+1)=coeff1;
Matrix2(i,i-1:i+1)=coeff2;
end

boundary_vector(end)=Cprof(end-1,tindex);

Cprof(:,tindex+1)=Matrix1^-1*(Matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);

for i=1:length(Cprof(:,tindex+1))
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if tindex*dt/(3600*24)>=25
    break
end

tindex=tindex+1;
end
node=floor(tindex/sizeMesh);

a=1;
for j=1:node:tindex
    Ccut(:,a)=Cprof(:,j);
    CDcut(:,a)=CD(:,j);
    a=a+1;
end

CDcut(1,:)=CDcut(2,:);
CDcut(end+1,:)=CDcut(end,:);

save('NoRadMatrixScaledhighvelocity2','Ccut','tindex','dt','dz','znode','CDcut','node','size Mesh')

clear;
clc;

load('NoRadMatrix')

tspan=node*dt*[0:74];
xmesh=[0:znode-1]*dz;

figure(1)
title('Oxygen Concentration Profile')
surf(tspan/(3600*24),xmesh*100,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
% title('Oxygen Concentration Profile')
axis([0 25 0 3 0 70])

figure(2)
surf(tspan/(3600*24),xmesh*100,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')
load('NoRadMatrixScaled')

tspan=node*dt*[0:74];
xmesh=[0:znode-1]*dz;

figure(3)
title('Oxygen Concentration Profile')
surf(tspan/(3600*24),xmesh*100,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
axis([0 25 0 30 0 70])

figure(4)
surf(tspan/(3600*24),xmesh*100,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
title('Cell Density Profile')

load('NoRadMatrixScaledhighvelocity')

% K=.05*.212;
% muMax=.0146;
% muMax=muMax/3600;
% xo=8e6*7.5^2/2.5^2*30/3;
% volReactor=pi*(7.5/2)^2*30; %cm^3

% [row col]=size(Ccut);
% 
% tindex=1:length(Ccut(1,:));
% timeinc=25/(length(tindex)-1);
% tcalc=timeinc*3600*24;
% time=timeinc*tindex;
% 
% for i=1:row
%     CDo=xo/volReactor;
%     
% load('NoRadMatrixScaled')
for j=1:col-1
    growth=CDo*exp(muMax*Ccut(i,j)/(Ccut(i,j)+K)*tcalc);
    CellDensity(i,j+1)=growth;
    CDo=growth;
end
end

CellDensity(:,1)=xo/volReactor;
[row col]=size(CellDensity);

for i=1:row
    for j=1:col
        if CellDensity(i,j)>10^9
            CellDensity(i,j)=10^9;
        end
    end
end

figure(5)
title('Oxygen Concentration Profile')
surf(tspan/(3600*24),xmesh*100,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
figure(6)
surf(tspan/(3600*24),xmesh*100,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
load('NoRadMatrixScaledhighvelocity2')
figure(7)
title('Oxygen Concentration Profile')
surf(tspan/(3600*24),xmesh*1000/3,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')

figure(8)
surf(tspan/(3600*24),xmesh*1000/3,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')

%title('Cell Density Profile')
1.4 Oxygen diffusion in cell aggregate size

function CellAggregate

clear;
cle;

m=2;
Dab=1.8e-5; %cm^2/s
Dab=Dab/100^2; %m^2/s
mu=.35; %per day
mu=mu/(24*3600); %s^-1
Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/(1000*3600); %mol/(cell*s)
rcell=4.5e-6; %m
time=1; %min
time=time*60; %s

DOfrac=.7;
DO=.212;
Cmedium=DOfrac*DO;
Ccenter=Cmedium;
Ragg(1)=50e-6;
CellDensity=10^15;

tspan=linspace(0,time,100);

packingefficiency=1-(1-1/6*pi);
index=1;
while Ccenter>0 %3*DO
    rmesh=linspace(0,Ragg(index),100);
    sol=pdepe(m,@pdefun,@icfun,@bcfun,rmesh,tspan,[],Dab,Qo,Ragg(index),Cmedium,CellDensity);
    Ccenter=sol(end,1);
    C(:,index)=sol(:,1);
    Ragg(index+1)=Ragg(index)+1e-5;
    index=index+1;
end

[row col]=size(C)
node=ceil(col/5);

a=1;
for i=1:node:col
    Cplot(:,a)=C(:,i);
    RaggPlot(:,a)=Ragg(:,i);
    a=a+1;
end

figure(1)
plot(tspan/60,Cplot(:,1)/.212*100,tspan/60,Cplot(:,2)/.212*100,':',tspan/60,Cplot(:,3)/.212*100,'-.',tspan/60,Cplot(:,4)/.212*100,'--',tspan/60,Cplot(:,5)/.212*100,'.')
xlabel('Time (min)')
ylabel('Center Concentration (%DO)')
legend(['Aggregate Radiuius = ',num2str(RaggPlot(1)/1e-6),' micrometers'],...
      ['Aggregate Radiuius = ',num2str(RaggPlot(2)/1e-6),' micrometers'],...
      ['Aggregate Radiuius = ',num2str(RaggPlot(3)/1e-6),' micrometers'],...
      ['Aggregate Radiuius = ',num2str(RaggPlot(4)/1e-6),' micrometers'],...
      ['Aggregate Radiuius = ',num2str(RaggPlot(5)/1e-6),' micrometers'])

function [c,f,s]=pdefun(r,t,C,dCdr,Dab,Qo,Ragg,Cmedium,CellDensity)
c=1;
f=Dab*dCdr;
s=-Qo*CellDensity*(1-r^2/Ragg^2);

function [Po,Qo,PR,QR]=bcfun(ro,Co,rR,CR,t,Dab,Qo,Ragg,Cmedium,CellDensity)
Po=0;
Qo=1;
PR=CR-Cmedium;
QR=0;

function Cin=icfun(r,Dab,Qo,Ragg,Cmedium,CellDensity)
Cint=.3*.212;
1.5 Shear stress during cell culture

```matlab
clear;
clc;

Eo=.634;
oxo=8e6; %cells
mu=.0146; %hr^-1
mu=mu/3600; %s^-1
visc=7e-4;
rcell=4.5e-6; %m
R=2.5; %cm
R=R/100; %m
len=3; %cm
len=len/100; %m
dfo=20e-6; %m

i=20;
E=1;
while E>0
    t=linspace(0,i,10); %day
    t=t*24*3600; %s
    E=Eo-xo*exp(mu*t)*4/3*pi*rcell^3/(pi*R^2*len);
i=i+.5;
end

t=linspace(0,i-.5,20);
display(num2str(max(t)))
t=t*24*3600;
E=Eo-xo*exp(mu*t)*4/3*pi*rcell^3/(pi*R^2*len);

dfsq=(1-E)/(1-Eo)*dfo^2;
phi=1-E;
k=dfsq./(2*phi).*(-log(phi)+(phi.^2-1)/(phi.^2+1))

tao=0*t;
j=1;
while max(tao)<2
    u=j; %cm/min
    u=u/(100*60);
tao=(visc*u./k).*sqrt(dfsq)./(4*phi);
j=j+.1;
```

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end

umax=u

u=linspace(2.5/6000,umax,4)
for i=1:length(u)
    tao(i,:)=(visc*u(i)./k).*sqrt(dfsq)./(4*phi);
end

set(0,'DefaultAxesColorOrder',[0 0 0])
set(0,'DefaultAxesLineStyle',{'-','--',':','-.'})
plot(t/(24*3600),tao*10)
xlabel('Time (days)')
ylabel('Shear Stress (dyne/cm^2)')
axis([0 25 0 22])
legend(['u = ',num2str(u(1)*6000),', (cm/min)'],...'
     ['u = ',num2str(u(2)*6000),', (cm/min)'],...'
     ['u = ',num2str(u(3)*6000),', (cm/min)'],...'
     ['u = ',num2str(u(4)*6000),', (cm/min)'],0)
Appendix B Matlab Codes for Spiral Wounded FBB with Top Exit
2.1 Model validation by residence time distribution data

clear;
clc;

uMax=0.00231; %m/s
L=8; %cm
L=L/100; %m
R=2.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=3.218e-6; %m^2/s
DiffR=DiffR*L/(uMax*R^2); %dimensionless
DiffL=4.83623e-5; %m^2/s
DiffL=DiffL*L/(uMax*R^2); %dimensionless
dt=.01;%s
dt=dt*uMax/L; %dimensionless
Cfed=.7*.212; %millimol/L
Cfed=Cfed/Cfed; %dimensionless
epsilon=.84;

znode=11;
rnode=11;

dr=R/(rnode-1);

dr=dr/R; %dimensionless
dz=L/(znode-1);
dz=dz/L; %dimensionless

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;
index1=1;
index2=1;
for i=1:znode*rnode
  for j=1:rnode*rnode
    if i==j & ((i<=rnode)||(i>=znode*(rnode-1)))
      matrix1(i,j)=1;
    end
    if ((rem(i,rnode)==1)||(rem(i,rnode)==0))
      if i==j
        matrix1(i,j)=1;
        boundary(index1)=i;
        index1=index1+1;
      end
    elseif (i>=rnode) & (i<=znode*(rnode-1))
      pos_r=rem(i,rnode);
      pos_z=ceil(i/znode);
    end
    if pos_r==0
      pos_r=rnode;
    end
  end
end
uz=uMax*(1-(pos_r-1)^2*dr^2/R^2)*(1-Alpha*(1/2*sinh(Alpha*(pos_z-1)*dz/L)-Beta*(cosh(Alpha*(pos_z-1)*dz/L)-1)));
ur=uMax*Alpha^2*(1/2*cosh(Alpha*(pos_z-1)*dz/L)-Beta*sinh(Alpha*(pos_z-1)*dz/L))*(pos_r-1)*dr/L;
a=1/dt+DiffR/dr^2+R^2/L^2*DiffL/dz^2;
aprime=1/dt-DiffR/dr^2-R^2/L^2*DiffL/dz^2;
b=uz/(epsilon*4*dr)-R^2/L^2*DiffL/(2*dr^2);
c=-uz/(epsilon*4*dr)-R^2/L^2*DiffL/(2*dr^2);
d=ur/(epsilon*4*dr)-R*DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2);
e=R*DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2)-ur/(epsilon*4*dr);
coeff1(1)=c;
coeff1(1+rnode)=a;
coeff1(rnode)=e;
coeff1(2+rnode)=d;
coeff1(1+2*rnode)=b;
coeff2(1)=-c;
coeff2(1+rnode)=aprice;
coeff2(rnode)=-e;
coeff2(2+rnode)=-d;
coeff2(1+2*rnode)=-b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
    not_boundary(index2)=i;
    z_not_boundary(index2)=ceil(i/znode);
    r_not_boundary(index2)=rem(i,rnode);

    if r_not_boundary(index2)==0
        r_not_boundary(index2)=rnode;
    end

    index2=index2+1;
end

end

dummy=1;
while dummy==1
    for i=2:length(boundary)-1
        if rem(i,2)==1
            boundary_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
        else
            boundary_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
        end
    end

boundary_vector(1:rnode)=Cfed;
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);
boundary_save(:,tindex)=boundary_vector;
Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector);

if (tindex*(dt*L/uMax)/60>=5)
    break
end

for i=1:znode*rnode
    if Cprof(i,tindex)<0
        Cprof(i,tindex)=0;
    end
end

tindex=tindex+1;
end

for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    uz=(1-(pos_r-1)^2*dr^2)*(1-1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz)-tanh(Alpha)*(cosh
    Alpha*(pos_z-1)*dz)-1));
    Area=(j^2*dr^2-(j-1)^2*dr^2);
    Vol(j)=Area*uz;
end
VolFrac=Vol/sum(Vol);

for i=1:tindex
    for j=1:rnode-1
        Cfrac(j)=VolFrac(j)*Cprof_org(end,j,i);
    end
    Cexit(i)=sum(Cfrac);
end

save('RTDfit','dt','tindex','Cexit') %all dimensionless
clear;
clc;

uMax=0.00231;  \%m/s  
L=8;  \%cm  
L=L/100;  \%m  
R=2.5/2;  \%cm  
R=R/100;  \%m

exp_data=textread('flowrate7.txt');  
exp_data=exp_data/max(exp_data);  
flowrate7=exp_data;

% exp_data=textread('flowrate6step.txt');  
% exp_data=exp_data/max(exp_data);  
% flowrate6=exp_data;

load('RTDfit')  
dt=dt*L/uMax;

node=5/dt;

a=1;
for i=1:node:length(Cexit)
    Cexit_cut(a)=Cexit(i);
    a=a+1;
end

Cnew=Cexit_cut(2:end);

if length(Cnew)>length(flowrate7)
    tspan=[1:length(flowrate7)]*5;
    plot(tspan,Cnew(1:length(flowrate7)),tspan,flowrate7,'.
else
    tspan=[1:length(Cnew)]*5;
    plot(tspan,Cnew,tspan,flowrate7(1:length(Cnew)),'.
end
clear;
clc;

C1=csvread('Spiral_Dispersion.txt')
C2=csvread('Spiral_2Dispersion.txt')
C3=csvread('C_Profile_Spiral_axialuz.txt')
C4=csvread('C_Profile_Spiral_axialradialuz.txt')
C2=C2/max(C2);

Exp=csvread('flowrate7spiral.txt')
Exp=Exp/max(Exp);
Exp2(2:length(Exp)+1)=Exp;
Exp2(1)=0;
time2=[0:length(Exp)-1]*5;

time=5; %min
 time=time*60; %s

time=0:5:time;

plot(time2,Exp,'.',time2,C1,'.-',time,C2,':',time,C3,time,C4,'--')
xlabel('Time (s)')
ylabel('C/C_o')
legend('Experimental','Dispersion','Dispersion_{two-way}','Dispersion_{one-way convection}','Dispersion_{two-way convection}')
2.2 Oxygen and cell density profiles prediction in lab scale spiral wounded FBB with top outlet (Codes for figures are in the later section)

clear;
cle;

uMax=0.00231; %m/s
L=3; %cm
L=L/100; %m
R=2.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*.212; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
xo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=11;
rnode=11;
dr=R/(rnode-1);
dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector=growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;

%beginning of calculation
%________________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1
    index1=1;
    index2=1;
    for i=1:znode*rnode
        for j=1:rnode*znode
            if i==j & ((i<=rnode)|(i>=znode*(rnode-1)))
                matrix1(i,j)=1;
            end
            if ((rem(i,rnode)==1)|(rem(i,rnode)==0))
                if i==j
                    matrix1(i,j)=1;
                    boundary(index1)=i;
                    index1=index1+1;
                end
            elseif (i>=rnode) & (i<=znode*(rnode-1))
                pos_r=rem(i,rnode);
                pos_z=ceil(i/znode);
                if pos_r==0
                    pos_r=rnode;
                end
            end
        end
    end
    uz=uMax*(1-(pos_r-1)^2*dr^2/R^2)*(1-Alpha*(1/2*sinh(Alpha*(pos_z-1)*dz/L)-Beta*(cosh(Alpha*(pos_z-1)*dz/L)-1)));
end
\[ ur = u_{\text{Max}} \cdot \alpha^2 \cdot \left( \frac{1}{2} \cosh(\alpha \cdot (z-1) \cdot dz/L) - \beta \sinh(\alpha \cdot (z-1) \cdot dz/L) \right) \cdot (r-1) \cdot dr/L; \]

\[ a = 1/\Delta t + \text{Diff}_R/\Delta r^2 + \text{Diff}_L/\Delta z^2; \]
\[ a' = 1/\Delta t - \text{Diff}_R/\Delta r^2 - \text{Diff}_L/\Delta z^2; \]
\[ b = uz/(\varepsilon(j) \cdot 4 \cdot dz) - \text{Diff}_L/(2 \cdot dz^2); \]
\[ c = -uz/(\varepsilon(j) \cdot 4 \cdot dz) - \text{Diff}_L/(2 \cdot dz^2); \]
\[ d = ur/(4 \cdot \Delta r \cdot \varepsilon(j)) - \text{Diff}_R/(4 \cdot (r-1) \cdot \Delta r^2) - \text{Diff}_R/(2 \cdot \Delta r^2); \]
\[ e = \text{Diff}_R/(4 \cdot (r-1) \cdot \Delta r^2) - \text{Diff}_R/(2 \cdot \Delta r^2) - ur/(4 \cdot \Delta r \cdot \varepsilon(j)); \]
\[ \text{coeff1}(1) = c; \]
\[ \text{coeff1}(1+rnode) = a; \]
\[ \text{coeff1}(rnode) = e; \]
\[ \text{coeff1}(2+rnode) = d; \]
\[ \text{coeff1}(1+2*rnode) = b; \]
\[ \text{coeff2}(1) = c; \]
\[ \text{coeff2}(1+rnode) = a'; \]
\[ \text{coeff2}(rnode) = e; \]
\[ \text{coeff2}(2+rnode) = d; \]
\[ \text{coeff2}(1+2*rnode) = b; \]

\[ \text{matrix1}(i,i-rnode:i+rnode) = \text{coeff1}; \]
\[ \text{matrix2}(i,i-rnode:i+rnode) = \text{coeff2}; \]

\[ \text{if } i == j \]
\[ \text{notboundary}(\text{index2}) = i; \]
\[ \text{znotboundary}(\text{index2}) = \text{ceil}(i/\text{znod}); \]
\[ \text{rnotboundary}(\text{index2}) = \text{rem}(i,\text{rnod}); \]
\[ \text{if } \text{rnotboundary}(\text{index2}) == 0 \]
\[ \text{rnotboundary}(\text{index2}) = \text{rnod}; \]
\[ \text{end} \]
\[ \text{index2} = \text{index2} + 1; \]
\[ \text{end} \]
\[ \text{end} \]
\[ \text{end} \]

\[ \text{for } i = 1: \text{length(notboundary)} \]
\[ \text{if } \text{Cprof}(i,\text{tindex}) > 0.3*0.212 \]

\[ 260 \]
growth_vector(not\_boundary(i))=((r\_not\_boundary(i)-1)*dr)^2/R^2*(z\_not\_boundary(i)-1)*dz/L*Qo*growth(i)/volreactor*(ceil(not\_boundary(i)/znode)-1)*dz/L*exp(muMax*C prof(not\_boundary(i),tindex)/(Cprof(not\_boundary(i),tindex)+K)*dt);

growth(i)=growth(i)*exp(muMax*Cprof(not\_boundary(i),tindex)/(Cprof(not\_boundary(i) ,tindex)+K)*dt);

if growth(i)/(volreactor)>10^15
  growth(i)=10^15*volreactor;
end

CD(not\_boundary(i),tindex)=growth(i)/volreactor;

epsilon(not\_boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(not\_boundary(i),tindex);

%inside boundary positions
for i=2:length(boundary)-1
  %r=0
  if rem(i,2)==1
    boundary\_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
    CD(boundary(i),tindex)=CD(boundary(i)+1,tindex);
    epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)+1,tindex);
    %r=R
  else
    boundary\_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
    CD(boundary(i),tindex)=CD(boundary(i)-1,tindex);
    epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)-1,tindex);
  end
end

boundary\_vector(1:rnode)=Cfed;
boundary\_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

CD(1:rnode,tindex)=CD(rnode+1,tindex);
CD(end-(rnode-1):end,tindex)=CD(end-rnode,tindex);
epsilon(1:rnode)=epsilon_o-4/3*pi*rcell^3*CD(rnode+1,tindex);
epsilon(end-(rnode-1):end)=epsilon_o-4/3*pi*rcell^3*CD(end-rnode,tindex);

boundary\_save(:,tindex)=boundary\_vector;

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Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);

for i=1:znode*rnode
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if (tindex*dt/(3600*24)>=25)
    break
end

tindex=tindex+1;
end
%
________________
%end of calculation

for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CD_org(j,:,i)=CD(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a = a + 1;
end

save('SpiralEpsilonLabNew','Ccut','dz','znode','dt','node','CDcut')

2.3 Oxygen and cell density for large scale FBB

clear;
clc;

uMax=0.00231; %m/s
L=30; %cm
L=L/100; %m
R=7.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*2.12; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
oxo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*2.12; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=11;
rnode=11;
dr=R/(rnode-1);
dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector=growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;

%beginning of calculation
%_______________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1

    index1=1;
    index2=1;
    for i=1:znode*rnode
        for j=1:rnode*rnode
            if i==j & ((i<=rnode)|(i>=znode*(rnode-1)))
                matrix1(i,j)=1;
            end
            if ((rem(i,rnode)==1)|(rem(i,rnode)==0))
                if i==j
                    matrix1(i,j)=1;
                    boundary(index1)=i;
                    index1=index1+1;
                end
            end
        end
    end

end

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elseif (i>=rnode) & (i<=znodex(rnode-1))
    pos_r=rem(i,rnode);
    pos_z=ceil(i/znode);
    if pos_r==0
        pos_r=rnode;
    end

uz=uMax*(1-(pos_r-1)^2*dx^2/R^2)*(1-Alpha*(1/2*sinh(Alpha*(pos_z-1)*dz/L)-Beta*(cosh(Alpha*(pos_z-1)*dz/L)-1)));

ur=uMax*Alpha^2*(1/2*cosh(Alpha*(pos_z-1)*dz/L)-Beta*sinh(Alpha*(pos_z-1)*dz/L))*rnode*dr/L;

a=1/dt+DiffR/dx^2+DiffL/dz^2;
aprime=1/dt-DiffR/dx^2-DiffL/dz^2;
b=uz/(epsilon(j)*4*dx)-DiffL/(2*dx^2);
c=-uz/(epsilon(j)*4*dx)-DiffL/(2*dx^2);
d=ur/(4*dx*epsilon(j))-DiffR/(4*(pos_r-1)*dx^2)-DiffR/(2*dx^2);
e=DiffR/(4*(pos_r-1)*dx^2)-DiffR/(2*dx^2)-ur/(4*dx*epsilon(j));
coeff1(1)=c;
coeff1(1+rnode)=a;
coeff1(rnode)=e;
coeff1(2+rnode)=d;
coeff1(1+2*rnode)=b;
coeff2(1)=-c;
coeff2(1+rnode)=aprime;
coeff2(rnode)=-e;
coeff2(2+rnode)=-d;
coeff2(1+2*rnode)=-b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
    not_boundary(index2)=i;
z_not_boundary(index2)=ceil(i/znode);
r_not_boundary(index2)=rem(i,rnode);
    if r_not_boundary(index2)==0
        end
r_not_boundary(index2)=rnode;
end

index2=index2+1;
end
end
end

for i=1:length(not_boundary)
    if Cprof(i,tindex)>.3*.212
        growth_vector(not_boundary(i))=((r_not_boundary(i)-1)*dr)^2/R^2*(z_not_boundary(i)-1)*dz/L*Qo*growth(i)/volreactor*(ceil(not_boundary(i)/znode)-1)*dz/L*exp(muMax*Cprof(not_boundary(i),tindex))/(Cprof(not_boundary(i),tindex)+K)*dt);
    end
    growth(i)=growth(i)*exp(muMax*Cprof(not_boundary(i),tindex))/(Cprof(not_boundary(i),tindex)+K)*dt);
    end
    if growth(i)/(volreactor)>10^15
        growth(i)=10^15*volreactor;
    end
    CD(not_boundary(i),tindex)=growth(i)/volreactor;
    epsilon(not_boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(not_boundary(i),tindex);  
end

%inside boundary positions
for i=2:length(boundary)-1
    if rem(i,2)==1
        boundary_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
        CD(boundary(i),tindex)=CD(boundary(i)+1,tindex);
        epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)+1,tindex); 
    end
else
    boundary_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
    CD(boundary(i),tindex)=CD(boundary(i)-1,tindex);
    epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)-1,tindex);
end

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boundary_vector(1:rnode)=Cfed;
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

CD(1:rnode,tindex)=CD(rnode+1,tindex);
CD(end-(rnode-1):end,tindex)=CD(end-rnode,tindex);
epsilon(1:rnode)=epsilon_o-4/3*pi*rcell^3*CD(rnode+1,tindex);
epsilon(end-(rnode-1):end)=epsilon_o-4/3*pi*rcell^3*CD(end-rnode,tindex);
boundary_save(:,tindex)=boundary_vector;

Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);

for i=1:znode*rnode
  if Cprof(i,tindex+1)<0
    Cprof(i,tindex+1)=0;
  end
end

if (tindex*dt/(3600*24)>=25)
  break
end

end

%________________________
%end of calculation

for i=1:tindex
  for j=1:znode
    Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    CD_org(j,:,i)=CD(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
  end
end

for j=1:rnode-1
  AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

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for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);
a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end
save('SpiralEpsilonScaledNew','Ccut','dz','znode','dt','node','CDcut')

2.4 Oxygen and cell density in large scale FBB with high velocity

clear;
clc;

uMax=5*0.00231; %m/s
L=30; %cm
L=L/100; %m
R=7.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*.212; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
xo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=11;
rnode=11;
dr=R/(rnode-1);
dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector=growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;

%beginning of calculation
%_______________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1
index1=1;
index2=1;
for i=1:znodernode
    for j=1:rnode*rnode
        if i==j & ((i<=rnode)||(i>=znodernode(rnode-1)))
            matrix1(i,j)=1;
        end
    end
    if ((rem(i,rnode)==1)||(rem(i,rnode)==0))
        if i==j
            matrix1(i,j)=1;
            boundary(index1)=i;
            index1=index1+1;
        end
    elseif (i>=rnode) & (i<=znodernode(rnode-1))
        pos_r=rem(i,rnode);
        pos_z=ceil(i/znodernode);
        if pos_r==0
            pos_r=rnode;
        end
    end
end
uz=uMax*(1-(pos_r-1)^2*dr^2/R^2)*(1-Alpha*(1/2*sinh(Alpha*(pos_z-1)*dz/L)-Beta*(cosh(Alpha*(pos_z-1)*dz/L)-1)));
ur=uMax*Alpha^2*(1/2*cosh(Alpha*(pos_z-1)*dz/L)-Beta*sinh(Alpha*(pos_z-1)*dz/L))*((pos_r-1)*dr/L);
a=1/dt+DiffR/dr^2+DiffL/dz^2;
aprime=1/dt-DiffR/dr^2-DiffL/dz^2;
b=uz/(epsilon(j)*4*dz)-DiffL/(2*dr^2);
c=-uz/(epsilon(j)*4*dz)-DiffL/(2*dr^2);
d=ur/(4*dr*epsilon(j))-DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2);
e=DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2)-ur/(4*dr*epsilon(j));
coeff1(1)=c;
coeff1(1+rnode)=a;
coeff1(rnode)=e;
coeff1(2+rnode)=d;
coeff1(1+2*rnode)=b;
coeff2(1)=-c;
coeff2(1+rnode)=aprime;
coeff2(rnode)=-e;
coeff2(2+rnode)=-d;
coeff2(1+2*rnode)=-b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
    not_boundary(index2)=i;
z_not_boundary(index2)=ceil(i/znode);
r_not_boundary(index2)=rem(i,rnode);

    if r_not_boundary(index2)==0
        r_not_boundary(index2)=rnode;
    end

    index2=index2+1;
end
end
end

for i=1:length(not_boundary)
    if Cprof(i,tindex)>0.3*212

        growth_vector(not_boundary(i))=((r_not_boundary(i)-1)*dr)^2/R^2*(z_not_boundary(i)-1)*dz/L*Qo*growth(i)/volreactor*(ceil(not_boundary(i)/znode)-1)*dz/L*exp(muMax*Cprof(not_boundary(i),tindex))/(Cprof(not_boundary(i),tindex)+K)*dt);

        growth(i)=growth(i)*exp(muMax*Cprof(not_boundary(i),tindex))/(Cprof(not_boundary(i),tindex)+K)*dt);
    end

    if growth(i)/(volreactor)>10^15
        growth(i)=10^15*volreactor;
    end

    CD(not_boundary(i),tindex)=growth(i)/volreactor;
end

epsilon(not_boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(not_boundary(i),tindex);
%inside boundary positions
for i=2:length(boundary)-1
    %r=0
    if rem(i,2)==1
        boundary_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
        CD(boundary(i),tindex)=CD(boundary(i)+1,tindex);
        epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)+1,tindex);
    %r=R
    else
        boundary_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
        CD(boundary(i),tindex)=CD(boundary(i)-1,tindex);
        epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)-1,tindex);
    end
end

boundary_vector(1:rnode)=Cfed;
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

CD(1:rnode,tindex)=CD(rnode+1,tindex);
CD(end-(rnode-1):end,tindex)=CD(end-rnode,tindex);
epsilon(1:rnode)=epsilon_o-4/3*pi*rcell^3*CD(rnode+1,tindex);
epsilon(end-(rnode-1):end)=epsilon_o-4/3*pi*rcell^3*CD(end-rnode,tindex);

boundary_save(:,tindex)=boundary_vector;

Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);

for i=1:znode*rnode
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if (tindex*dt/(3600*24)>=25)
    break
end

tindex=tindex+1;
end
for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CD_org(j,:,i)=CD(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end
for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end
for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);
a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end
save('SpiralEpsilonScaledNewhighvelocity','Ccut','dz','znode','dt','node','CDcut')
clear; % figures for previous cases
clc;
load('SpiralEpsilonLabNew')

tspan(1:75)=node*dt*[0:74];
zmesh=[0:10]*dz;

figure(1)
%title('Lab Scale ')
surf(tspan/(3600*24),zmesh*100,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
%title('Oxygen Concentration Profile')

figure(2)
surf(tspan/(3600*24),zmesh*100,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')

load('SpiralEpsilonScaledNew')

figure(3)
%title('Lab Scale ')
surf(tspan/(3600*24),zmesh*1000,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
%title('Oxygen Concentration Profile')

figure(4)
surf(tspan/(3600*24),zmesh*1000,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')

load('SpiralEpsilonScaledNewhighvelocity')
figure(5)
%title('High velocity ')
surf(tspan/(3600*24),zmesh*1000,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
%title('Oxygen Concentration Profile')

figure(6)
surf(tspan/(3600*24),zmesh*1000,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')
Appendix C Matlab Codes for Modeling of Spiral Wounded FBB with Side Exit
3.1 Model validation by residence time distribution

clear;
clc;

uMax=0.00231; %m/s
L=8; %cm
L=L/100; %m
R=2.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=3.218e-6; %m^2/s
DiffR=DiffR*L/(uMax*R^2); %dimensionless
DiffL=4.83623e-5; %m^2/s
DiffL=DiffL*L/(uMax*R^2); %dimensionless
dt=0.25;%s
dt=dt*uMax/L; %dimensionless
Cfed=.7*.212; %millimol/L
Cfed=Cfed/Cfed; %dimensionless
epsilon=.84;

znode=31;
rnode=31;
dr=R/(rnode-1);
dr=dr/R; %dimensionless
dz=L/(znode-1);
dz=dz/L; %dimensionless

Alpha=4*L*sqrt(2e-10/(.001^3*R));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;

index1=1;
index2=1;
for i=1:znode*rnode
    for j=1:rnode*rnode
        if i==j & ((i<=rnode)&&(i>=znode*(rnode-1)))
            matrix1(i,j)=1;
        end
        if ((rem(i,rnode)==1)&&(rem(i,rnode)==0))
            if i==j
                matrix1(i,j)=1;
                boundary(index1)=i;
                index1=index1+1;
            end
        elseif (i>=rnode) & (i<=znode*(rnode-1))
            pos_r=rem(i,rnode);
            pos_z=ceil(i/znode);
            if pos_r==0
                pos_r=rnode;
            end
            uz=-1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz)-tanh(Alpha)*(cosh(Alpha*(pos_z-1)*dz)))*(1-(pos_r-1)^2*dr^2);
            %uz=(1-1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz)-tanh(Alpha)*cosh(Alpha*(pos_z-1)*dz)-1))*(1-(pos_r-1)^2*dr^2);
            ur=1/2*Alpha^2*(cosh(Alpha*(pos_z-1)*dz)-tanh(Alpha)*sinh(Alpha*(pos_z-1)*dz))*(pos_r-1)*dr;
        end
        end
    end
end

a=1/dt+DiffR/dr^2+R^2/L^2*DiffL/dz^2;
a_prime=1/dt-DiffR/dr^2-R^2/L^2*DiffL/dz^2;
b=uz/(epsilon*4*dz)-R^2/L^2*DiffL/(2*dz^2);
c=-uz/(epsilon*4*dz)-R^2/L^2*DiffL/(2*dz^2);
d=ur/(epsilon*4*dr)-R*DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2);
e=R*DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2)-ur/(epsilon*4*dr);
coeffl(1)=c;
coeffl(1+rnode)=a;
coeffl(rnode)=e;
coeff1(2*rnode)=d;
ccoeff1(1+2*rnode)=b;
ccoeff2(1)=-c;
ccoeff2(1+rnode)=aprim;
ccoeff2(rnode)=-e;
ccoeff2(2+rnode)=-d;
ccoeff2(1+2*rnode)--b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
    not_boundary(index2)=i;
z_not_boundary(index2)=ceil(i/znode);
r_not_boundary(index2)=rem(i,rnode);

    if r_not_boundary(index2)==0
        r_not_boundary(index2)=rnode;
    end

    index2=index2+1;
end
end
end
end

tindex=1;
dummy=1;
while dummy==1

    for i=2:length(boundary)-1
        if rem(i,2)==1
            boundary_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
        else
            boundary_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
        end
    end

    boundary_vector(1:rnode)=Cfed;
end
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

boundary_save(:,tindex)=boundary_vector;
Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector);

if (tindex*(dt*L/uMax)/60>=5)
    break
end

for i=1:znode*rnode
    if Cprof(i,tindex)<0
        Cprof(i,tindex)=0;
    end
end

tindex=tindex+1;
end

for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    uz=(1-(pos_r-1)^2*dr^2)*(1-1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz)-tanh(Alpha)*cosh(Alpha*(pos_z-1)*dz)-1)));
    Area=(j^2*dr^2-(j-1)^2*dr^2);
    Vol(j)=Area*uz;
end
VolFrac=Vol/sum(Vol);

for i=1:tindex
    for j=1:rnode-1
        Cfrac(j)=VolFrac(j)*Cprof_org(end,j,i);
    end
    Cexit(i)=sum(Cfrac);
end

save('RTDfit','dt','tindex','Cexit') \%all dimensionless

clear;
cle;

\textit{uMax}=0.00231; \%m/s
\textit{L}=8; \%cm
\textit{L}=L/100; \%m
\textit{R}=2.5/2; \%cm
\textit{R}=R/100; \%m

exp\_data=textread('flowrate7step.txt');
exp\_data=exp\_data/max(exp\_data);
flowrate7=exp\_data;

exp\_data=textread('flowrate6step.txt');
exp\_data=exp\_data/max(exp\_data);
flowrate6=exp\_data;

load('RTDfit')
dt=dt*L/uMax;

node=5/dt;

a=1;
for i=1:node:length(Cexit)
    Cexit\_cut(a)=Cexit(i);
a=a+1;
end

Cnew=Cexit\_cut(2:end);

if length(Cnew)>length(flowrate7)
    ts\_span=[1:length(flowrate7)]*5;
    plot(ts\_span/60,Cnew(1:length(flowrate7)),ts\_span/60,flowrate7,:)\newline
    xlabel('Time (min)')
    ylabel('C/C_0')
    Legend('Model','Experimental',0)
else
    ts\_span=[1:length(Cnew)]*5;
end
plot(tspan/60,Cnew,tspan/60,flowrate7(1:length(Cnew)),',')
xlabel('Time (min)')
ylabel('C/C_o')
Legend('Model','Experimental',0)
end
3.2 Oxygen and cell density profiles prediction in lab scale FBB

clear;
clc;

uMax=0.00231; %m/s
L=3; %cm
L=L/100; %m
R=2.5/2; %cm
R=R/100; %m
volreactor=\pi R^2 L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*.212; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
xo=8e6*R^2/0.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=11;
rnode=11;
if R/(rnode-1);
dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector = boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector = growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:rnode*znode)=0;

%beginning of calculation
%_____________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1

    index1=1;
    index2=1;
    for i=1:znode*rnode
        for j=1:rnode*rnode
            if i==j & ((i<=rnode)|(i>=znode*(rnode-1)))
                matrix1(i,j)=1;
            end
            if ((rem(i,rnode)==1)|(rem(i,rnode)==0))
                if i==j
                    matrix1(i,j)=1;
                end
                boundary(index1)=i;
                index1=index1+1;
            elseif (i>=rnode) & (i<=znode*(rnode-1))
                pos_r=rem(i,rnode);
                pos_z=ceil(i/znode);
                if pos_r==0
                    pos_r=rnode;
                end
            else
                uz=-uMax*1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*(cosh(Alpha*(pos_z-1)*dz/L)))*(1-(pos_r-1)^2*dr^2/R^2);
                ur=uMax*1/2*Alpha^2*(cosh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*sinh(Alpha*(pos_z-1)*dz/L))
\[ (*dz/L)*((pos_r-1)*dr/R; \]

\[ a = \frac{1}{dt} + \frac{\text{DiffR}}{dr^2} + \frac{\text{DiffL}}{dz^2}; \]

\[ a' = \frac{1}{dt} - \frac{\text{DiffR}}{dr^2} - \frac{\text{DiffL}}{dz^2}; \]

\[ b = \frac{uz}{(\epsilon(j)\times4\times dz)} - \frac{\text{DiffL}}{2\times dz^2}; \]

\[ c = \frac{uz}{(\epsilon(j)\times4\times dz)} - \frac{\text{DiffL}}{2\times dz^2}; \]

\[ d = \frac{ur}{4\times dr\times \epsilon(j)} - \frac{\text{DiffR}}{4\times ((pos_r-1)\times dr^2)} - \frac{\text{DiffR}}{2\times dr^2} - \frac{ur}{4\times dr\times \epsilon(j)}; \]

\[ e = \frac{\text{DiffR}}{4\times ((pos_r-1)\times dr^2)} - \frac{\text{DiffR}}{2\times dr^2} - \frac{ur}{4\times dr\times \epsilon(j)}; \]

\[ \text{coeff1}(1) = c; \]

\[ \text{coeff1}(1+r\text{node}) = a; \]

\[ \text{coeff1}(r\text{node}) = e; \]

\[ \text{coeff1}(2+r\text{node}) = d; \]

\[ \text{coeff1}(1+2*r\text{node}) = b; \]

\[ \text{coeff2}(1) = -c; \]

\[ \text{coeff2}(r\text{node}) = a'; \]

\[ \text{coeff2}(2+r\text{node}) = -e; \]

\[ \text{coeff2}(2+2*r\text{node}) = -d; \]

\[ \text{coeff2}(1+2*r\text{node}) = -b; \]

\[ \text{matrix1}(i,i-r\text{node}:i+r\text{node}) = \text{coeff1}; \]

\[ \text{matrix2}(i,i-r\text{node}:i+r\text{node}) = \text{coeff2}; \]

\[ \text{if } i == j \]

\[ \text{notboundary}(\text{index2}) = i; \]

\[ \text{z_not_boundary}(\text{index2}) = \text{ceil}(i/\text{znod}); \]

\[ \text{r_not_boundary}(\text{index2}) = \text{rem}(i,\text{rnod}); \]

\[ \text{if } \text{r_not_boundary}(\text{index2}) == 0 \]

\[ \text{r_not_boundary}(\text{index2}) = \text{rnod}; \]

\[ \text{end} \]

\[ \text{index2} = \text{index2} + 1; \]

\[ \text{end} \]

\[ \text{end} \]

\[ \text{end} \]

\[ \text{for } i = 1:\text{length(\text{not_boundary})} \]

\[ \text{if } \text{Cprof}(i,\text{tindex}) > .3* .212 \]

\[ \text{growth_vector(\text{not_boundary}(i))} = ((\text{r_not_boundary}(i)-1)\times dr^2/R^2\times (\text{z_not_boundary}(i)-285) \]
\frac{dz}{L} Q_0 \text{growth}(i) / \text{volreactor} \times (\text{ceil}(\text{not_boundary}(i) / znode) - 1) \times \frac{dz}{L} \exp(\mu_{\text{Max}} \times \text{Cprof}(\text{not_boundary}(i), t) / ((\text{Cprof}(\text{not_boundary}(i), t) + K) \times dt);

\text{growth}(i) = \text{growth}(i) \times \exp(\mu_{\text{Max}} \times \text{Cprof}(\text{not_boundary}(i), t) / ((\text{Cprof}(\text{not_boundary}(i), t) + K) \times dt);

\text{end}

\text{if growth}(i) / (\text{volreactor}) > 10^{15}
\text{growth}(i) = 10^{15} \times \text{volreactor};
\text{end}

\text{CD}(\text{not_boundary}(i), t) = \text{growth}(i) / \text{volreactor};

\text{epsilon}(\text{not_boundary}(i)) = \epsilon_o - \frac{4}{3} \times \pi \times rcell^3 \times \text{CD}(\text{not_boundary}(i), t);
\text{end}

\% \text{inside boundary positions}
\text{for } i = 2: \text{length(boundary) - 1}
\% r = 0
\text{if } \text{rem}(i, 2) = 1
\text{boundary_vector}(\text{boundary}(i)) = \text{Cprof}(\text{boundary}(i) + 1, t);
\text{CD}(\text{boundary}(i), t) = \text{CD}(\text{boundary}(i) + 1, t);
\text{epsilon}(\text{boundary}(i)) = \epsilon_o - \frac{4}{3} \times \pi \times rcell^3 \times \text{CD}(\text{boundary}(i) + 1, t);
\% r = R
\text{else}
\text{boundary_vector}(\text{boundary}(i)) = \text{Cprof}(\text{boundary}(i) - 1, t);
\text{CD}(\text{boundary}(i), t) = \text{CD}(\text{boundary}(i) - 1, t);
\text{epsilon}(\text{boundary}(i)) = \epsilon_o - \frac{4}{3} \times \pi \times rcell^3 \times \text{CD}(\text{boundary}(i) - 1, t);
\text{end}
\text{end}

\text{boundary_vector}(1: \text{rnode}) = \text{Cfed};
\text{boundary_vector}(\text{end} - (\text{rnode} - 1): \text{end}) = \text{Cprof}(\text{end} - 2 \times \text{rnode} + 1: \text{end} - \text{rnode}, t);

\text{CD}(1: \text{rnode}, t) = \text{CD}(\text{rnode} + 1, t);
\text{CD}(\text{end} - (\text{rnode} - 1): \text{end}, t) = \text{CD}(\text{end} - \text{rnode}, t);
\text{epsilon}(1: \text{rnode}) = \epsilon_o - \frac{4}{3} \times \pi \times rcell^3 \times \text{CD}(\text{rnode} + 1, t);
\text{epsilon}(\text{end} - (\text{rnode} - 1): \text{end}) = \epsilon_o - \frac{4}{3} \times \pi \times rcell^3 \times \text{CD}(\text{end} - \text{rnode}, t);

\text{boundary_save}( :, t) = \text{boundary_vector};

\text{Cprof}( :, t + 1) = \text{matrix1}^{-1} \times (\text{matrix2} \times \text{Cprof}( :, t) + \text{boundary_vector} - \text{growth_vector});
r);

for i=1:znode*rnode
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if (tindex*dt/(3600*24)>=25)
    break
end

tindex=tindex+1;
end

%________________________

%end of calculation

for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CD_org(j,:,i)=CD(j*rnode -(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end

save('SpiralNewLab','Ccut','dz','znode','dt','node','CDcut')

3.2 Oxygen and cell density profiles in large scale FBB

clear;
clc;

uMax=0.00231; %m/s
L=30; %cm
L=L/100; %m
R=7.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*.212; %millimol/L

Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
xo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=11;
rnode=11;
dr=R/(rnode-1);
dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/(.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector=growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:rnode*znode)=0;
matrix2(1:znode*rnode,1:mode*znod)=0;

%beginning of calculation
%_____________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1

index1=1;
index2=1;
for i=1:znode*rnode
    for j=1:rnode*rnode
        if i==j & ((i<=rnode)|(i>=znode*(rnode-1)))
            matrix1(i,j)=1;
        end
    end
    if ((rem(i,rnode)==1)|(rem(i,rnode)==0))
        if i==j
            matrix1(i,j)=1;
            boundary(index1)=i;
            index1=index1+1;
        end

    end
end

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elseif (i>=rnode) & (i<=znode*(rnode-1))
    pos_r=rem(i,rnode);
    pos_z=ceil(i/znode);
    if pos_r==0
        pos_r=rnode;
    end

uz=-uMax*1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*(cosh(Alpha*(pos_z-1)*dz/L))*(1-(pos_r-1)^2*dr^2/R^2);

ur=uMax*1/2*Alpha^2*(cosh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*sinh(Alpha*(pos_z-1)*dz/L))*(pos_r-1)*dr/R;

a=1/dt+DiffR/dr^2+DiffL/dz^2;
aprime=1/dt-DiffR/dr^2-DiffL/dz^2;
b=uz/(epsilon(j)*4*dz)-DiffL/(2*dz^2);
c=-uz/(epsilon(j)*4*dz)-DiffL/(2*dz^2);
d=ur/(4*dr*epsilon(j))-DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2);
e=DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2)-ur/(4*dr*epsilon(j));
coeff1(1)=c;
coeff1(1+rnode)=a;
coeff1(rnode)=e;
coeff1(2+rnode)=d;
coeff1(1+2*rnode)=b;
coeff2(1)=-c;
coeff2(1+rnode)=aprime;
coeff2(rnode)=-e;
coeff2(2+rnode)=-d;
coeff2(1+2*rnode)=-b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
    not_boundary(index2)=i;
    z_not_boundary(index2)=ceil(i/znode);
    r_not_boundary(index2)=rem(i,rnode);
    if r_not_boundary(index2)==0
        not_boundary(index2)=i;
    end

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\[ r_{\text{not\_boundary}}(\text{index}2) = r_{\text{node}}; \]

\[ \text{index}2 = \text{index}2 + 1; \]

\[ \text{end} \]

\[ \text{end} \]

\[ \text{end} \]

\[ \text{end} \]

\[ \text{for } i=1: \text{length(not\_boundary)} \]
\[ \text{if } C_{\text{prof}}(i, \text{tindex}) > 0.3 \times 0.212 \]
\[ \text{growth\_vector}(\text{not\_boundary}(i)) = ((r_{\text{not\_boundary}}(i)-1) \times dr)^2 / R^2 \times (z_{\text{not\_boundary}}(i)-1) \times dz/L \times Q_{\text{o}} \times \text{growth}(i) / \text{vol\_reactor} \times (\text{ceil}(\text{not\_boundary}(i)/z_{\text{node}})-1) \times dz/L \times \exp(\mu_{\text{Max}} \times C_{\text{prof}}(\text{not\_boundary}(i), \text{tindex}) / (C_{\text{prof}}(\text{not\_boundary}(i), \text{tindex}) + K) \times dt); \]
\[ \text{growth}(i) = \text{growth}(i) \times \exp(\mu_{\text{Max}} \times C_{\text{prof}}(\text{not\_boundary}(i), \text{tindex}) / (C_{\text{prof}}(\text{not\_boundary}(i), \text{tindex}) + K) \times dt); \]
\[ \text{end} \]

\[ \text{if } \text{growth}(i) / \text{vol\_reactor} > 10^{15} \]
\[ \text{growth}(i) = 10^{15} \times \text{vol\_reactor}; \]
\[ \text{end} \]
\[ \text{CD}(\text{not\_boundary}(i), \text{tindex}) = \text{growth}(i) / \text{vol\_reactor}; \]
\[ \text{end} \]

\[ \text{epsilon}(\text{not\_boundary}(i)) = \epsilon_{\text{o}} - 4/3 \pi r_{\text{cell}}^3 \times \text{CD}(\text{not\_boundary}(i), \text{tindex}); \]
\[ \text{end} \]

\% inside boundary positions
\[ \text{for } i=2: \text{length(boundary)}-1 \]
\[ \% r = 0 \]
\[ \text{if } \text{rem}(i, 2) == 1 \]
\[ \text{boundary\_vector}(\text{boundary}(i)) = C_{\text{prof}}(\text{boundary}(i)+1, \text{tindex}); \]
\[ \text{CD}(\text{boundary}(i), \text{tindex}) = \text{CD}(\text{boundary}(i)+1, \text{tindex}); \]
\[ \text{epsilon}(\text{boundary}(i)) = \epsilon_{\text{o}} - 4/3 \pi r_{\text{cell}}^3 \times \text{CD}(\text{boundary}(i)+1, \text{tindex}); \]
\[ \% r = R \]
\[ \text{else} \]
\[ \text{boundary\_vector}(\text{boundary}(i)) = C_{\text{prof}}(\text{boundary}(i)-1, \text{tindex}); \]
\[ \text{CD}(\text{boundary}(i), \text{tindex}) = \text{CD}(\text{boundary}(i)-1, \text{tindex}); \]
\[ \text{epsilon}(\text{boundary}(i)) = \epsilon_{\text{o}} - 4/3 \pi r_{\text{cell}}^3 \times \text{CD}(\text{boundary}(i)-1, \text{tindex}); \]
\[ \text{end} \]
boundary_vector(1:rnode)=Cfed;
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

CD(1:rnode,tindex)=CD(rnode+1,tindex);
CD(end-(rnode-1):end,tindex)=CD(end-rnode,tindex);
epsilon(1:rnode)=epsilon_o-4/3*pi*rcell^3*CD(rnode+1,tindex);
epsilon(end-(rnode-1):end)=epsilon_o-4/3*pi*rcell^3*CD(end-rnode,tindex);
boundary_save(:,tindex)=boundary_vector;

Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);

for i=1:znode*rnode
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if (tindex*dt/(3600*24)>=25)
    break
end

tindex=tindex+1;
end
%________________________________________________________________________
%end of calculation

for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CD_org(j,:,i)=CD(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
    292
for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end
save('SpiralNewScaled','Ccut','dz','znode','dt','node','CDcut')

3.3. Oxygen and cell density in large scale FBB with higher velocity

clear;
clc;

uMax=5*0.00231; %m/s
L=30; %cm
L=L/100; %m
R=7.5/2; %cm
R=R/100; %m
volreactor=pi*R^2*L;
DiffR=6.4165e-6*(2.5/1.6)^-1; %m^2/s
DiffL=8.25206e-6*(2.5/1.6)^-1; %m^2/s
dt=30; %s
Cfed=.7*.212; %millimol/L
Qo=1e-10; %millimol/(cell*hr)
Qo=Qo/3600*1/1000; %mol/(cell*s)
xo=8e6*R^2/.0125^2*L/.03; %cells
muMax=.0146; %hr^-1
muMax=muMax/3600; %s^-1
K=.05*.212; %mol/m^3
epsilon_o=.84;
rcell=4.5e-6;

sizeMesh=75;

znode=21;
rnode=21;

r=dr=R/(rnode-1);
dz=dz=L/(znode-1);
epsilon(1:znode*rnode)=epsilon_o;

Alpha=4*L*sqrt(2e-10/.001^3*R));
Beta=(1+cosh(Alpha))/(2*sinh(Alpha));

Co(1:rnode)=Cfed;
Co(rnode+1:znode*rnode)=0;
Co=Co';
Cprof(:,1)=Co;
boundary_vector(1:znode*rnode)=0;
boundary_vector=boundary_vector';
growth_vector(1:znode*rnode)=0;
growth_vector=growth_vector';
CD(1:znode*rnode,1)=0;

matrix1(1:znode*rnode,1:mode*znodemode)*znode*znodemode)=0;
matrix2(1:znode*rnode,1:mode*znodemode)=0;

%beginning of calculation
%_______________________________
tindex=1;
dummy=1;
growth(1:znode*rnode)=xo;
while dummy==1
index1=1;
index2=1;
for i=1:znode*rnode
    for j=1:rnode*rnode
        if i==j & ((i<=rnode)|(i>=znode*(rnode-1))
            matrix1(i,j)=1;
        end
        if ((rem(i,rnode)==1)|(rem(i,rnode)==0))
            if i==j
                matrix1(i,j)=1;
                boundary(index1)=i;
                index1=index1+1;
            end
            elseif (i>=rnode) & (i<=znode*(rnode-1))
                pos_r=rem(i,rnode);
                pos_z=ceil(i/znode);
                if pos_r==0
                    pos_r=rnode;
                end
    end
end
uz=-uMax*1/2*Alpha*(sinh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*(cosh(Alpha*(pos_z-1)*dz/L)))*(1-(pos_r-1)^2*dr^2/R^2);
ur=uMax*1/2*Alpha^2*(cosh(Alpha*(pos_z-1)*dz/L)-tanh(Alpha)*sinh(Alpha*(pos_z-1)*dz/L))*(pos_r-1)*dr/R;

a=1/dt+DiffR/dr^2+DiffL/dz^2;
aprime=1/dt-DiffR/dr^2-DiffL/dz^2;
b=uz/(epsilon(j)*4*dz)-DiffL/(2*dz^2);
c=-uz/(epsilon(j)*4*dz)-DiffL/(2*dz^2);
d=ur/(4*dr*epsilon(j))-DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2);
e=DiffR/(4*(pos_r-1)*dr^2)-DiffR/(2*dr^2)-ur/(4*dr*epsilon(j));
coeff1(1)=c;
coeff1(1+rnode)=a;
coeff1(rnode)=e;
coeff1(2+rnode)=d;
coeff1(1+2*rnode)=b;
coeff2(1)=-c;
coeff2(1+rnode)=aprime;
coeff2(rnode)=-e;
coeff2(2+rnode)=-d;
coeff2(1+2*rnode)=-b;

matrix1(i,i-rnode:i+rnode)=coeff1;
matrix2(i,i-rnode:i+rnode)=coeff2;

if i==j
  not_boundary(index2)=i;
  z_not_boundary(index2)=ceil(i/znode);
  r_not_boundary(index2)=rem(i,rnode);

  if r_not_boundary(index2)==0
    r_not_boundary(index2)=rnode;
  end

  index2=index2+1;
end
end
end
end

for i=1:length(not_boundary)
  if Cprof(i,tindex)>.3*.212

    growth_vector(not_boundary(i))=((r_not_boundary(i)-1)*dr)^2/R^2*(z_not_boundary(i)-1)*dz/L*Qo*growth(i)/volreactor*(ceil(not_boundary(i)/znode)-1)*dz/L*exp(muMax*Cprof(not_boundary(i),tindex)/(Cprof(not_boundary(i),tindex)+K))*dt);

    growth(i)=growth(i)*exp(muMax*Cprof(not_boundary(i),tindex)/(Cprof(not_boundary(i),tindex)+K))*dt);
  end

  if growth(i)/(volreactor)>10^15
    growth(i)=10^15*volreactor;
  end

  CD(not_boundary(i),tindex)=growth(i)/volreactor;
end
epsilon(not_boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(not_boundary(i),tindex);

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%inside boundary positions
for i=2:length(boundary)-1
    % r=0
    if rem(i,2)==1
        boundary_vector(boundary(i))=Cprof(boundary(i)+1,tindex);
        CD(boundary(i),tindex)=CD(boundary(i)+1,tindex);
        epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)+1,tindex);
    else
        boundary_vector(boundary(i))=Cprof(boundary(i)-1,tindex);
        CD(boundary(i),tindex)=CD(boundary(i)-1,tindex);
        epsilon(boundary(i))=epsilon_o-4/3*pi*rcell^3*CD(boundary(i)-1,tindex);
    end
end

boundary_vector(1:rnode)=Cfed;
boundary_vector(end-(rnode-1):end)=Cprof(end-2*rnode+1:end-rnode,tindex);

CD(1:rnode,tindex)=CD(rnode+1,tindex);
CD(end-(rnode-1):end,tindex)=CD(end-rnode,tindex);
epsilon(1:rnode)=epsilon_o-4/3*pi*rcell^3*CD(rnode+1,tindex);
epsilon(end-(rnode-1):end)=epsilon_o-4/3*pi*rcell^3*CD(end-rnode,tindex);

boundary_save(:,tindex)=boundary_vector;

Cprof(:,tindex+1)=matrix1^-1*(matrix2*Cprof(:,tindex)+boundary_vector-growth_vector);
for i=1:znode*rnode
    if Cprof(i,tindex+1)<0
        Cprof(i,tindex+1)=0;
    end
end

if (tindex*dt/(3600*24)>=25)
    break
end

tindex=tindex+1;
end
for i=1:tindex
    for j=1:znode
        Cprof_org(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CD_org(j,:,i)=CD(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Cprof_org(i,j,k);
            CDfrac(j)=AreaFrac(j)*CD_org(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end

[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end

save('SpiralNewScaledhighvelocity','Ccut','dz','znode','dt','node','CDcut')
clear;
clc;
load('SpiralNewLab')
tspan(1:75)=node*dt*[0:74];
zmesh=[0:10]*dz;
tspan(1:75)=node*dt*[0:74];
zmesh=[0:10]*dz;

figure(1)
title('Lab Scale ') 
surf(tspan/(3600*24),zmesh*100,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
%title('Oxygen Concentration Profile')

figure(2)
surf(tspan/(3600*24),zmesh*100,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')

load('SpiralNewScaled')

figure(3)
title('Lab Scale ') 
surf(tspan/(3600*24),zmesh*1000,Ccut/.212*100)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('% DO')
%title('Oxygen Concentration Profile')

figure(4)
surf(tspan/(3600*24),zmesh*1000,CDcut*10^-6)
xlabel('Time (days)')
ylabel('Length (cm)')
zlabel('Cell Density (cell/mL)')
%title('Cell Density Profile')

% load('SpiralNewScaledhighvelocity3')
Due to the long running time, the whole program was cut into 5 days work as below:

clear;
clc;

load('cut1')

R=7.5/2*1/100; %m
dr=R/(rnode-1);
z=30/100
dz=z/(znode-1)
z=30/100

sizeMesh=15;

[row col]=size(Cprof);

tindex=length(Cprof(:,1));

for i=1:tindex
    for j=1:znode
        Corg(j,:,i)=Cprof(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CDorg(j,:,i)=CD(j*rnode -(rnode-1):(j+1)*rnode-rnode,i);
    end
end
for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Corg(i,j,k);
            CDfrac(j)=AreaFrac(j)*CDorg(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end

[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end
save('cutcut','Ccut','dz','znode','dt','node','CDcut','node','sizeMesh')
clear;
clc;
load('cut2')

R=7.5/2*1/100; %m
dr=R/(rnode-1);
z=30/100
dz=z/(znode-1)
z=30/100

sizeMesh=15;

[row col]=size(Cprof2)
tindex=length(Cprof2(1,:));

for i=1:tindex
    for j=1:znode
        Corg(j,:,i)=Cprof2(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CDorg(j,:,i)=CD2(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Corg(i,j,k);
            CDfrac(j)=AreaFrac(j)*CDorg(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end

[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut2(:,a)=Cfinal(:,j);
    CDcut2(:,a)=CDfinal(:,j);
    a=a+1;
end

save('cutcut2','Ccut2','dz','znode','dt','node','CDcut2','node','sizeMesh')
clear;
cle;

load('cut3')
R = 7.5 / 2 * 1 / 100; \% m
dr = R / (rnode - 1);
z = 30 / 100
dz = z / (znode - 1)

sizeMesh = 15;
[row col] = size(Cprof3);
tindex = length(Cprof3(1,:));

for i = 1:tindex
    for j = 1:znode
        Corg(j,:,i) = Cprof3(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CDorg(j,:,i) = CD3(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j = 1:rnode-1
    AreaFrac(j) = (j^2 * dr^2 - (j-1)^2 * dr^2) / R^2;
end

for k = 1:tindex
    for i = 1:znode
        for j = 1:rnode-1
            Cfrac(j) = AreaFrac(j) * Corg(i,j,k);
            CDfrac(j) = AreaFrac(j) * CDorg(i,j,k);
        end
        Cfinal(i,k) = sum(Cfrac);
        CDfinal(i,k) = sum(CDfrac);
    end
end

[row col] = size(Cfinal);
node = round(col / sizeMesh);

a = 1;
for j = 1:node:col
    Ccut3(:,a) = Cfinal(:,j);
    CDcut3(:,a) = CDfinal(:,j);
a=a+1;
end

save('cutcut3','Ccut3','dz','znole','dt','node','CDcut3','node','sizeMesh')
clear;
clc;

load('cut4')

R=7.5/2*1/100; %m
dr=R/(rnode-1);
z=30/100
dz=z/(znole-1)

sizeMesh=15;

[row col]=size(Cprof4);

tindex=length(Cprof4(1,:));

for i=1:tindex
    for j=1:znole
        Corg(j,:,i)=Cprof4(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CDorg(j,:,i)=CD4(j*rnode -(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znole
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Corg(i,j,k);
            CDfrac(j)=AreaFrac(j)*CDorg(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
        CDfinal(i,k)=sum(CDfrac);
    end
end
[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut4(:,a)=Cfinal(:,j);
    CDcut4(:,a)=CDfinal(:,j);
    a = a + 1;
end

save('cutcut4','Ccut4','dz','znode','dt','node','CDcut4','node','sizeMesh')

clear;
cle;

load('cut5')

R=7.5/2*1/100; %m
dr=R/(rnode-1);
z=30/100
dz=z/(znode-1)

sizeMesh=15;

[row col]=size(Cprof5);
tindex=length(Cprof5(1,:));

for i=1:tindex
    for j=1:znode
        Corg(j,:,i)=Cprof5(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
        CDorg(j,:,i)=CD5(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end
for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            \[ C_{frac}(j) = Area_{Frac}(j) \times Cor(g(i,j,k)); \]
            \[ CD_{frac}(j) = Area_{Frac}(j) \times CD_{org}(i,j,k); \]
        end
        C_{final}(i,k) = \text{sum}(C_{frac});
        CD_{final}(i,k) = \text{sum}(CD_{frac});
    end
end
[row col] = size(C_{final});
node = round(col/sizeMesh);

a = 1;
for j = 1:node:col
    Ccut5(:,a) = C_{final}(:,j);
    CDcut5(:,a) = CD_{final}(:,j);
    a = a + 1;
end

save('cutcut5', 'Ccut5', 'dz', 'znode', 'dt', 'node', 'CDcut5', 'node', 'sizeMesh')
clear;
cle;

sizeMesh = 25;
load('cut1')
load('cut2')
load('cut3')
load('cut4')
load('cut5')

[row col] = size(C_{prof});

Cend = C_{prof};
Cend(:,end:end+col-1) = C_{prof2};
Cend(:,end:end+col-1) = C_{prof3};
Cend(:,end:end+col-1) = C_{prof4};
Cend(:,end:end+col-1) = C_{prof5};
pack Ccombine

```
tindex=length(Cend(1,:));

for i=1:tindex
    for j=1:znod
        Corg(j,:,i)=Cend(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znod
        for j=1:rnode-1
            Cfrac(j)=AreaFrac(j)*Corg(i,j,k);
        end
        Cfinal(i,k)=sum(Cfrac);
    end
end

[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    Ccut(:,a)=Cfinal(:,j);
    a=a+1;
end

save('FinallyDone1','Ccut','dz','znode','dt','CDcut','node','sizeMesh')
clear;
CDend=CD;
CDend(:,end:end+col-1)=CD2;
CDend(:,end:end+col-1)=CD3;
```
pack CDcombine

for i=1:tindex
    for j=1:znode
        CDorg(j,:,i)=CDend(j*rnode-(rnode-1):(j+1)*rnode-rnode,i);
    end
end

for j=1:rnode-1
    AreaFrac(j)=(j^2*dr^2-(j-1)^2*dr^2)/R^2;
end

for k=1:tindex
    for i=1:znode
        for j=1:rnode-1
            CDfrac(j)=AreaFrac(j)*CDorg(i,j,k);
        end
        CDfinal(i,k)=sum(CDfrac);
    end
end

[row col]=size(Cfinal);
node=round(col/sizeMesh);

a=1;
for j=1:node:col
    CDcut(:,a)=CDfinal(:,j);
    a=a+1;
end

save('FinallyDone2','dz','znode','dt','node','CDcut','sizeMesh')

clear;
cle;

K=.05*.212;
muMax=.0146;
muMax = muMax/3600;
xo = 8e6 * 7.5^2 / 2.5^2 * 30 / 3;
volReactor = pi * (7.5/2)^2 * 30; % cm^3

load('cutcut')
load('cutcut2')
load('cutcut3')
load('cutcut4')
load('cutcut5')

[row col] = size(Ccut);

Cend = Ccut;
Cend(:, end:end+col-1) = Ccut2;
Cend(:, end:end+col-1) = Ccut3;
Cend(:, end:end+col-1) = Ccut4;
Cend(:, end:end+col-1) = Ccut5;

tindex = 1:length(Cend(1,:));
timeinc = 25 / (length(tindex)-1);
tcalc = timeinc * 3600 * 24;
time = timeinc * tindex;

zmesh = linspace(0, 30, length(Cend(:, 1)));

mu = muMax * Cend./(Cend+K);

CDend = CDcut;
CDend(:, end:end+col-1) = CDcut2;
CDend(:, end:end+col-1) = CDcut3;
CDend(:, end:end+col-1) = CDcut4;
CDend(:, end:end+col-1) = CDcut5;

[row col] = size(Cend);

for i = 1:row
    CDo = xo / volReactor;
    for j = 1:col-1
        growth = CDo * exp(muMax * Cend(i,j) / (Cend(i,j)+K) * tcalc);
        CellDensity(i,j+1) = growth;
        CDo = growth;
    end
end
CellDensity(:,1)=xo/volReactor;

[row col]=size(CellDensity);

% for i=1:row
%     for j=1:col
%         if CellDensity(i,j)>10^9
%             CellDensity(i,j)=10^9;
%         end
%     end
% end

figure(1)
surf(time,zmesh,Cend/.212*100);
ylabel('Length (cm)')
xlabel('Time (days)')
zlabel','% DO')
axis([0 25 0 30 0 70])

figure(2)
surf(time,zmesh,CellDensity);
ylabel('length (cm)')
xlabel('time (days)')
zlabel('Cell Density (cells/mL)')

figure(3)
surf(time,zmesh,CDend/10^6);
ylabel('length (cm)')
xlabel('time (days)')
zlabel('Cell Density (cells/mL)')
Appendix D Flow Cytometric Analysis of Biomakers for ES cells
**FITC only control**

### Histogram Statistics

File: fitc.005  
Sample ID: fitc  
Acquisition Date: 01-Oct-04  
Gated Events: 10000  
X Parameter: FITC (Log)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Left, Right</th>
<th>Events</th>
<th>% Gated</th>
<th>% Total</th>
<th>Mean</th>
<th>Geo Mean</th>
<th>CV</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>1, 9910</td>
<td>10000</td>
<td>100.00</td>
<td>58.53</td>
<td>2.48</td>
<td>2.06</td>
<td>68.66</td>
<td>1.93</td>
</tr>
<tr>
<td>M1</td>
<td>1, 14</td>
<td>9993</td>
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Oct-4 of mES cells expression from two stage reactor at 15 days
PE only control
SSEA-1 expression of mES cells from two stage reactor at 15 days
FITC only control
SSEA-4 expression of hES cells from reactor at 18 days