DEVELOPMENT OF AN IN VITRO THREE-DIMENSIONAL MODEL FOR COLON CANCER STUDY AND DRUG EFFICACY ANALYSIS

DISSERTATION

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ABSTRACT

Colon cancer is the second most deadly cancer with 56,000 deaths estimated in the United States for 2005. The staggering average cost of developing drug therapies to the market exceeds $400,000,000, and a majority of drug candidates still fail in clinical trials. The main objective of this research was to develop an in vitro three-dimensional (3-D) model of colon cancer, utilizing tissue engineering principles, with subsequent analyses of the cancer growth within culture environments and of the application of this model for improved screening of candidate drug efficacy.

The preparation of the cancer model included scaffold treatment, cell counting method analyses, and cell seeding optimization. The fibrous scaffold, poly(ethylene terephthalate) (PET), was compressed, producing scaffold porosities ranging from 0.94 to 0.84, and treated to reduce surface hydrophobicity. The direct counting method of enzymatic detachment of cells was useful for relative counts, but the developed fluorescent model was optimal, providing accurate and noninvasive counts in the 3-D environment. Dynamic and filter seeding provided superior cell distributions and seeding rates compared to static seeding. High porosity (0.94) and treated PET had the highest attachment rate.
The colon cancer cell line, HT-29, was grown in plates on PET of four combinations of two porosities (0.94 and 0.88) and pretreatment status. Whereas cell growth was fastest in high porosity and treated PET, the low porosity and treated PET culture showed significant population growth and developed a 3-D morphology due to smaller interfiber distances. Growth within mixed spinner flask and perfusion bioreactor environments improved the aerobic conditions within the PET and sustained higher cell densities.

Two anti-cancer drugs were utilized to determine the effectiveness of the 3-D colon cancer model in predicting drug efficacy. The 3-D, low porosity PET model was more and less sensitive to 5-fluorouracil and gemcitabine, respectively, than 3-D high porosity and currently-utilized 2-D cultures were, accurately predicting their actual colon cancer efficacies. A 3-D model with transfected green fluorescent protein correlated fluorescence signals with cytotoxic effects, providing a convenient and fast method for screening drug cytotoxicity. Application of the 3-D model can be expanded to other candidate drug therapies.
Dedicated to my brother, Brett, and to Moogi
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CHAPTER 1

INTRODUCTION

1.1 Colon Cancer

Colon cancer takes the lives of more people than any other type of cancer except lung cancer. The cause and prevention mechanisms of colon cancer are still not entirely clear though, whereas there is an obvious way to largely avoid lung cancer with the omission of smoking. In 1997, colon cancer caused the deaths of 56,000 people in the United States alone (Ries et al., 2000). The third most common cancer in the U.S., colon cancer diagnoses are expected to reach approximately 105,000 in 2005. To improve the survival rate of colon cancer, several strategies are employed including chemotherapy, radiotherapy, and immunotherapy. Due to the effectiveness of these treatments, the 55% 5-year survival statistic for colon cancer is improving slowly each year.

The road from drug discovery to market availability is a long and costly journey with an approximate cost of $403,000,000 (DiMasi, 2003). Thousands of drug candidates are predicted using techniques such as molecular modeling and preliminarily screened using high-throughput techniques in the early stages of drug discovery. Promising drugs are then used in tests utilizing mostly animal and in vitro plate cell culture models to obtain
preliminary results while compiling data for an application to enter clinical trials. Clinical trials involve four stages in which the efficacy and toxicity of the drug are analyzed in human patients followed by long-term effect studies. Drugs that are approved by the FDA may reach the market following Phase III, but there is a 78% failure rate for all drugs (DiMasi, 2003). If a drug fails while in clinical trials, the financial impact can be devastating to the developing company. With 22.5% of the drugs failing due to efficacy problems, improved predictions in the preclinical trial stages would reduce failed clinical trial occurrences and be much less costly.

In the past decade, research has focused on the molecular genetics of colon cancer to determine the predisposition and specific mutations that occur in the development of a colon carcinoma. In fact, colon cancer is the most characterized cancer in regards to the etiological molecular genetics of colon cancer (Tomlinson et al., 1997). The elucidation of the oncogenes, tumor suppressor genes, and other components that take part in proposed mechanisms of carcinogenesis leads to predictions for possible tumor drug treatments and environmental carcinogens.

1.2 Tissue Engineering

Tissue engineering combines knowledge obtained from biological fields and applies it using engineering skills to repair, regenerate, or enhance tissues and organs both in vivo and in vitro (Griffith et al., 2002). To mimic the native tissue, the key concept utilized in studies is that there is a strong relationship between tissue structure and function.
Therefore, in order to achieve the desired functional attributes in a tissue-engineered construct, the culture environment must represent the native counterpart and present the inherent advantages. An important component in the tissue-engineered construct that allows for in vivo-like culture is the three-dimensional scaffold that allows cell population support, organization, and function. Many types of scaffolds are utilized for tissue regeneration and are selected based on characteristics beneficial for the tissue application.

The following are two examples of tissue engineering applications. When tendons and ligaments are regenerated for use in transplantation to repair damaged or deteriorated ones, fibroblasts are cultured in a collagen scaffold (Goulet et al., 2000). The collagen scaffold produces a construct that allows for proper organization of fibroblasts to develop with sufficient mechanical strength to withstand the stress endured by tendons and ligaments. The tubular structure of cultivated blood vessels can be directed using a tubular scaffold seeded with vascular smooth muscle cells and endothelial cells (Niklason et al., 1999). In addition, the culture system has pulsatile action to simulate the heart beating. As a result, the developed blood vessel properties and function closely mimic native blood vessels.

The development of tissues as in vitro models is important for studying tissue function, development, and disease etiology. In fact, in vitro models will be the segment of tissue engineering to have the greatest effect in the next decade (Griffith et al., 2002). In vitro models that incorporate three-dimensional culture can also be applied to cancer for developing in vivo-like tumors. Cancer models will allow for studies of etiology,
development, angiogenesis, metastasis, drug therapies, and other topics in which progress of knowledge accumulation is badly needed. This is especially true for drug therapies, where little success in developing new effective drugs for colon cancer has occurred.

1.3 Hypothesis and Objectives

Based on tissue engineering concepts, an *in vitro* three-dimensional model of colon cancer would mimic the *in vivo* cancer counterpart in function and morphology. Such a model would also present a similar susceptibility to drugs as tumor cells *in vivo*. Therefore, a three-dimensional model of colon cancer could be used to accurately and efficiently predict drug efficacy before entering into clinical trials.

The objectives and scope of this research are as follow:

1.3.1 Development of an *In Vitro* Three-Dimensional Colon Cancer Model Through Scaffold Manipulation, Cell Seeding Analyses, and Cell Counting Methods (Chapter 3)

In order to study the growth of colon cancer *in vitro*, the scaffold variations must first be prepared, and an understanding of the methods used to seed the scaffold with cells and subsequently quantify the culture growth must be attained. The scaffold undergoes compression and chemical treatment to generate fibrous matrices of varying porosities and hydrophilic status. Static, dynamic, and filter seeding methods are utilized to seed the
cells onto the various scaffold types to determine attachment rates and optimal conditions. Finally, direct and indirect cell counting methods are utilized to display effectiveness in quantifying the cell number within the three-dimensional environment.

1.3.2 Analysis of Colon Cancer Growth and Morphology in an *In Vitro* Three-Dimensional Model of Varying Scaffold Properties and Culture Environments (Chapter 4)

The proliferation and development of the colon cancer population are studied in three culture environments and four variations of scaffolding. The three environments, in increasing scale, are multiwell plate, spinner flask, and perfusion bioreactor. The scaffold types are combinations of two porosities and two pretreatment statuses. To analyze the growth, the metabolism and population growth are monitored, as well as the cell cycle populations and morphologies via scanning electron microscopy.

1.3.3 Analysis of the Utilization of a Three-Dimensional *In Vitro* Colon Cancer Model for Drug Efficacy Screening (Chapter 5)

Utilizing the developed cancer model, two drugs, 5-fluorouracil and gemcitabine, are tested on the developed model to analyze the effects under various conditions and to demonstrate the ability of the three-dimensional model to assess drug efficacy better than currently-used two-dimensional model. Cytotoxicity is determined by alteration of cell metabolism and release of lactate dehydrogenase. Additionally, the utility of fluorescence
as a signal for cell growth inhibition is investigated when colon cancer cells transfected with enhanced green fluorescent protein are exposed to an anti-cancer agent.
1.4 References


CHAPTER 2

LITERATURE REVIEW

2.1 Colon Cancer

2.1.1 Biology

The colon, also identified as the large intestine, is a component of the digestive system. The functions of the colon are to absorb the remaining nutrients from the digested food and to store the feces before release. Preceding the colon is the small intestine in which nutrients and water content are absorbed. Following the colon are the rectum and anus for completion of the digestive process. The colon is a 4.5 feet long tubular tissue with ascending, transverse, descending, and sigmoid regions based on their spatial positioning. See Figure 2.1 for a visual description of the biology.

The cross-section of the tubular colonic tissue in Figure 2.2 depicts the multilayered, multiple cell type wall composition. The vascularized outer layers consist of smooth muscle cells, fibroblasts, and endothelial cells. Within this layer is a tightly joined sheet of epithelial cells that perform the absorption function of the colon. The epithelial cells have characteristic microvilli on the exposed surface that allow for a large amount of
surface area for nutrient absorption. The inner surface of the colon also houses a symbiotic flora of microorganisms, including *E. coli*, to assist in digestion.

Cancer growth progression in the colon is described by the American Joint Committee on Cancer (AJCC) staging system (Colon and Rectal Cancer: Treatment Guidelines for Patients, 2000). The AJCC system categorizes the progression into five stages based on the level of tumor infiltration through the colonic tissue. In Stage 0, the cancer is in the earliest stage and is confined only to the mucosa after developing from epithelial cells. In Stage I, growth has entered the submucosa and muscularis propria layers. At Stage II, the tumor has extended growth into the subserosa. Stage III is characterized by the movement of cells into local lymph nodes and the presence of angiogenesis. In Stage IV, the tumor has metastasized and spread to other tissues.

2.1.2 Etiology

Colon cancer can originate from environmental induction, genetic predisposition, or a combination of both (Colon and Rectal Cancer: Treatment Guidelines for Patients, 2000). Approximately 90% of colon cancer patients are over the age of 50. A diet high in fats and physical inactivity play roles in colon cancer development. The presence of polyps in the lining of the colon is a common risk factor for colon cancer. In addition, the ailments ulcerative colitis and Crohn’s disease have the risk of developing into colon cancer. There are two main types of hereditary colon cancers: Familial adenomatous polyposis
(FAP) and Hereditary nonpolyposis colon cancer (HNPCC). All other colon cancers are termed sporadic though hereditary factors do play a part in sporadic tumors as well.

Normal, non-cancerous cells have a controlled growth process and a finite life span. Progression through the cell cycle is guarded at checkpoints by regulating molecules, and passage is allowed only when correct signals are processed. An important checkpoint is between the G1 growth phase and the DNA replication S-phase because passage into the S-phase begins the progression of mitosis. Cancer is characterized by the uncontrolled and unlimited growth of a transformed population of cells. Transformation of the cells occurs in the DNA from which all control mechanisms originate. Genes that are directly or indirectly responsible for cell growth and growth control signaling are named oncogenes and tumor suppressor genes, respectively, and mutations of these genes commonly result in cancer. Mutations in genes that have roles of DNA repair or apoptosis, the process by which a cell self-destructs due to functional errors, can also induce the transformation into cancerous cells.

2.1.3 Genetics

Colon cancer or colorectal cancer, a cancer of the colon and rectum, is characterized by a chain of specific mutation events that accumulate until the uncontrolled growth of cancerous cells form an adenocarcinoma (Tomlinson et al., 1997). Colon cancer typically originates in the epithelial cells lining the inside of the colon. Mutations at the loci of \textit{p53} and APC tumor suppressor genes cause a loss of cell cycle regulation. Mutated
oncogenes, including ras, myc, and myb, accelerate cell growth in an uncontrolled manner. The ras oncogene is critical in growth factor signal transduction and is frequently mutated into a constantly activated state in colon cancer.

2.1.4 Angiogenesis

As tumor mass increases, nutrient supply diminishes within the tumor structure usually leading to a necrotic core within the tumor. Nutrients are diverted to the tumor mass by the initiation and development of vascular structures in the cancerous structures. This process, called angiogenesis, is a natural process for vascularization of new mass during growth and during wound repair. Cancer cells take advantage of angiogenesis to supply deficient cells with nutrients obtained from blood to strengthen the cancerous progression.

Angiogenesis initiation consists of the release of angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and angiopoietin-2 (Ang2) from cells that have limited nutrient supply (Fox et al., 1998; Teichert-Kuliszewska et al., 2001). The angiogenic factors promote the migration of endothelial cells from established blood vessels through interaction with receptor tyrosine kinases. Development of the new vasculature proceeds with the endothelial cells linking and forming a sealed tubular morphology followed by the formation of the exterior cellular support structure.
2.1.5 Metastasis

When cancerous cells spread from localized tissue sites to other locations in the body, the cancer has metastasized. Metastasis occurs when cancer cells detach from the local support structure, penetrate into the blood supply, and migrate via the lymph nodes into other tissue regions. Metastatic sites are most commonly the liver and the lungs, and due to metastasis being a late-stage event, 5-year survival rates can drop down to 40% (Wirth et al., 2002). Carcinoembryonic antigen (CEA) is an adhesion molecule that plays a role in tumor structure and is an important tumor marker for metastasis.

2.1.6 Preventative Techniques

Current emphasis on improving colon cancer screening technology as a means of diagnosing the disease in early, curable stages has met the practical limitations of the negative public perceptions of utilized methods and infrequent check-ups. For these reasons, colon cancer is allowed to fully develop in many cases before identification of the disease (Bradley et al., 1997; Ries et al., 2000). Multiple screening tests are employed for early diagnosis of cancer: fecal occult blood test, digital rectal examination, flexible sigmoidoscopy, colonoscopy, and barium enema. A new method may eliminate the need for the other invasive procedures by diagnosing colon cancer risk through a blood test (Cui et al., 2003). By linking an alteration of the insulin-like growth factor II gene ($IGF2$)
with colon cancer risk and detecting it in blood samples, this blood test should improve the number of diagnoses caught in the early stages of development, improving the chance of recovery.

2.1.7 Treatments

The large inventory of techniques to combat colon cancer provides a variety of strategies to handle the estimated 130,000 new colon cancer diagnoses in the United States in 1997 (Pawlik et al., 2000). Despite the advances in treatments of many types of cancers, the most effective treatment available for colon cancer remains to be revisions of the classic radiotherapy, chemotherapy, and immunotherapy techniques (Tomlinson et al., 1997). The establishment of new surgical protocols is improving the effectiveness of surgery for the initial and late stages of tumor development. However, surgery has the disadvantages of being invasive and traumatic to the body and not being able to extract all remnants of cancer, especially from metastatic sites and tumors incorporated into vital organs. Many forms of radiotherapy, chemotherapy, and immunotherapy have systemic activity and lack specificity of action.

2.1.8 Drug Therapy

Anti-cancer drugs have a wide array of attack mechanisms. Some mechanisms and corresponding cancer drugs are DNA alkylation (nitrosoureas), antimetabolites (methotrexate), enzyme inhibition (novobiocin), free radical formation (mitomycin C),
hormone receptor inhibitors (tamoxifen), and cell cycle specific drugs (cisplatin). Angiogenic and metastatic factors are popular targets in research for cancer control treatments. Resveratrol and quercetin are compounds from grapes and wine that show inhibitive properties against endothelial cell migration during angiogenesis (Igura et al., 2001).

Specifically for colon cancer, strategies for the development of new drugs are novel and continually emerging. 5-Fluorouracil is the longest available colon cancer drug, but effective new usages of the drug have been developed within combinations with other drugs such as leucovorin and Camptosar (Pfizer) and as derivatives for improved delivery routes such as the oral drug Xeloda (Roche). 5-Fluorouracil and Camptosar are examples of DNA analogs and topoisomerase inhibitors, respectively, utilized to kill cells by disruption of DNA synthesis. 5-Fluorouracil targets both DNA and RNA of colon cancer cells for increased effectiveness (Van Laar et al., 1996). Oxaliplatin (Sanofi-Synthelabo), known for its expedient Food and Drug Administration (FDA) approval for fighting colon cancer, is an organoplatinum complex that crosslinks DNA, effectively halting essential cellular processes. Novel strategies are targeted drugs based on known molecular genetics of colon cancer and anti-angiogenic drugs that inhibit the development of neovasculature within the tumor. Erbitux (ImClone), approved in February 2004, is an antibody that competitively binds to epithelial growth factor receptors that are over-expressed in colon cancers and inhibits cell growth. Avastin (Genentech), also approved by the FDA in February 2004, is an anti-angiogenic drug that inhibits the signaling molecules utilized by cancer cells to initiate blood vessel
development. Though recent progress in development of colon cancer drugs is promising, inherited cellular resistance to these drugs is very possible, and many combinatorial regimens and novel drugs must be continually processed through the development pipeline.

Due to the lack of quantitative data for drug therapy techniques, research has turned to animals to provide data that can be extrapolated to apply to humans (Meijers et al., 1997). Discussion is ongoing about whether animals are good models for this use. Opponents describe anatomical and physiological differences between species causing data inconsistencies in addition to moral issues (Meijers et al., 1997). Proponents of the use of animals as predictive tools cite the enormous contribution the research has provided already in the advancement of drug testing (Schiffer, 1997).

Research regarding the colon cancer cellular response to various drug treatments is mainly performed in two-dimensional (2-D) culture environments due to simplicity and established protocols. However, there is extensive documentation regarding the failures of 2-D cancer cell culture systems to represent \emph{in vivo} characteristics because of a lack of allowance for the three-dimensional (3-D) orientation necessary for proper tissue function. Discrepancies in predicted drug treatment effectiveness in 2-D and 3-D cultures describe the advantage of using 3-D culture matrices (Furukawa et al., 1992; Smitskamp-Wilms et al., 1998).
Using colon cancer cells cultured in 2-D and 3-D cultures, the 3-D cultures showed up to a 180-fold increase in drug resistance to several drugs compared to the monolayer cultures. The drug resistance continued to increase as the cancer cells developed three-dimensionally (Furukawa et al., 1992). Smitskamp-Wilms et al. (1998) cultured colon and ovarian cancer cells in 2-D micro-titer plates and 3-D multilayers and found a 1000-fold decrease in cytotoxicity to gemcitabine in the multilayer cultures after the 2-D cultures erroneously predicted gemcitabine to be an effective proliferation inhibitor. The resulting dose effect of gemcitabine on cells cultured in multilayers was not significant as dosage increased which showed the ineffectiveness of using gemcitabine as a colon cancer remedy. The reason for the varying effect of gemcitabine is due to decreased DNA synthesis time in cells cultured in 3-D. Gemcitabine, effective only during DNA synthesis, has less time to act in 3-D cultures and thus is less cytotoxic. Therefore, the study of gemcitabine is useful in depicting the effect of the 3-D culture even though the drug itself is not plausible for use in treatment.

Additional research depicts the alteration of cellular performance seen between 2-D and 3-D cancer cell cultures including changes in morphology, growth kinetics, growth factor expression, and other functional properties (Haramaki, 1993; Zvibel et al., 1998; Sittinger et al., 1996; O’Conner et al., 1997; O’Conner, 1999). It is evident that the 3-D culture conditions are important for in vivo-like differentiation, proliferation, and metastatic potential of the tumor cells (Haramaki, 1993; Zvibel et al., 1998). The 3-D culture also allows for the development of the heterogeneity present in the in vivo tumor (Heppner et al., 1998). Depending on location in the tumor structure, certain cells mutate to obtain a
competitive advantage for growth (Tomlinson et al., 1997). The result is a tumor with characteristic subpopulations that determine future tumor progression. Providing the three-dimensional environment for cancer cells to propagate and interact allows for \textit{in vivo}-like representation.

2.1.9 Clinical Trials

Following drug discovery and preliminary tests to promote promising drug candidates, application to enter clinical trials is the next step. Clinical trials are composed of four stages that generally increase in sample size and duration. In Phase I, 20-80 healthy human patients are used to perform toxicity and dosage effect studies for 6-12 months. Cancer patients are used in Phase II to determine the efficacy of the drug at prescribed dosages. The sample size increases to 100-300 patients, and the second phase generally lasts 1-2 years. The progression to Phase III is a major step since the number of patients used in the studies increases by a factor of 10 and has a duration of 1-3 years. In this stage, statistical analysis is performed for drug effectiveness and side effects become known. The developing company may apply for drug availability to the market following Phase III. Phase IV is used to study long-term performance of the drug while in commercial use and to determine alternative applications of the drug. The approximate cost to bring a candidate drug through clinical trials is $403,000,000 (DiMasi, 2003). Altogether, the length of time to complete clinical trials can be between 2.5 years and 6 years showing that not only is it a monetarily costly venture, but also a time-costing procedure. Failure of drugs in clinical trials is devastating for both of these reasons.
2.2 Tissue Engineering

2.2.1 Objectives

Tissue engineering is the combination of biology and engineering for producing biological substitute structures that can reconstitute cellular or tissue function that is lost, declining, or insufficient. Theorized for ages, the field has evolved greatly within recent decades to meet the challenge of disease and a limited organ donor supply. Tissue engineering research covers a wide range of applications including many tissue substitutes, cell therapy, and diagnostic in vitro modeling. Tissue engineering techniques can be used to establish 3-D culture models for use in cancer research (Sittinger et al., 1996; Griffith et al., 2002).

Tissue engineering, as a field, is defined by two main objectives. The first objective is to apply methods and principles of engineering and life sciences to understand the biological tissue construct (Ma, 1999). In addition to deciphering the vast catalog of cellular functions, the interconnections of a cellular population, regulated by signaling pathways, and the interactions of a cellular composite with the non-cellular, surrounding environment must be understood. The three-dimensional structure and organization of tissue components are integral in defining proper function of the tissue, therefore this environmental arrangement must be mimicked to produce a tissue substitute or model.
that functions appropriately. A fundamental understanding of a tissue environment structure and how it enables or affects the tissue function is essential.

The second objective of tissue engineering is to apply established knowledge of the tissue construct towards developing biological substitutes to restore, maintain, or improve natural function to tissues or organs that are structurally or physiologically altered (Ma, 1999). As \textit{in vitro} models of tissues, including cancer, are developed that function according to the \textit{in vivo} counterparts, applied developmental and diagnostic analyses will add to the established knowledge from which medical advancements progress.

2.2.2 History

Concepts and applications of tissue engineering were present in literature, art, and cinema well before the field was named in 1987. The collections of stories that involve tissue engineering concepts show the promise and spectacular possibilities that the future could bring. However, rarely do those stories fully conceptualize or even mention the challenges involved in performing these acts in reality. In the past century, early reports of tissue engineering experimentation demonstrated important concepts that would be utilized in future applications such as immune response mechanisms and drug delivery (Langer, 2000).

The first tissue to be reconstituted in the laboratory setting was skin. During the late 1970’s and 1980’s, artificial skin was created using skin cells distributed within natural
collagen or collagen-glycosaminoglycan composite support structures (Bell et al., 1981; Burke et al., 1981). Skin became the first tissue to gain the interest of researchers due to the relatively simple two-dimensional structure compared to the complex three-dimensional structures of other tissues.

The growth of tissue engineering in the 1990’s and early 2000’s was due to interdisciplinary advancements in the fields of engineering, genomics, proteomics, cell biology, and material science (Flanagan, 2003). As the understanding of the important relationship between tissue structure and function became fully realized, three-dimensional synthetic polymers were utilized to mimic the bodily, *in vivo* environment as a support for cells to attach and grow on. The liver was the first tissue to be cultivated using these three-dimensional constructs due to the relative simplicity of the liver composition (Vacanti et al., 1988). Utilizing these newly developed tools, a multitude of tissues are being studied and mimicked with tissue-engineered products for possible future usage as a transplant.

2.2.3 Engineered Tissue Construct

The components of a tissue engineering process are determined by the specific product application. The components that must be customized based on the application include cell source, scaffold parameters, and cell culture procedures. There are five general applications of tissue-engineered constructs. Figure 2.3 summarizes the components and general applications. One application is to support the growth of human tissues *in vitro*
for future implantation into the body to replace lost tissue function. A second is utilizing a scaffold to promote tissue regeneration *in vivo* by transplanting a cell-free scaffold or a scaffold containing cells that aid in regeneration into a deficient site in the body. A third application is using scaffolds to support proper cell function within a device that supplements reduced tissue function. The seeded matrix, when located internally in the body, is encapsulated within a semi-permeable membrane to allow for provision of a therapeutic molecule to the site while protecting the cells from the host immune system. An externally-positioned, seeded matrix device provides deficient tissue function compounds through a tube directed to the body site while avoiding cellular contact with the immune system. Another application of a scaffold is to provide the environment for expanding a cell population that is later extracted from the scaffold for implementation within the body. The fifth application is to use the scaffold to promote *in vivo*-like function of a population of cells *in vitro*, and then utilize the cells and scaffold as a model for studying tissue development, pathology, pharmacology, and toxicology projects instead of using animal models.

2.2.4 Cell Sources

The cells utilized for producing a tissue substitute can come from multiple sources. When a specific cell type is needed to culture a certain tissue type, the availability of the cell type, the means of obtaining the cells, procedures for maintaining and multiplying the cell population in culture, and immune rejection upon transplantation must all be considered. Avoiding rejection is a large challenge in the development of a tissue
substitute, thus autologous cells obtained from the same person to whom the transplant will be given is ideal. The host body does not reject autologous cells. Supply of autologous cells is frequently the problem however. In many situations, a large enough population of healthy cells is not available due to the extent of disease, for example (Heath, 2000).

When autologous cells are not appropriate or available, the cells can come from either another donor of the same or different species. The transplantation of cells between similar or dissimilar species is called allotransplantation or xenotransplantation, respectively. Though the supply of these types of grafts is plentiful, immune rejection is very common, so some additional strategy must be utilized to avoid rejection. Cells provided by family members for allogeneic cells tend to be the most compatible source to avoid rejection due to similar gene sets, including, specifically, the histocompatibility complex genes. Xenografts provide an additional challenge since xenogeneic cells may contain components that are infectious when introduced into a human. That fact, in addition to ethical and moral issues regarding utilizing non-human parts in a human, has led to a general unpopularity of this procedure (Heath, 2000).

A fourth cell source is stem cells. Stem cells are characterized by the ability to proliferate indefinitely and develop into different cell types, or pluripotent, depending on the stem cell origin and given the appropriate signals. Embryonic stem cells are present as an embryo first begins to develop and differentiate to form all components of the human body. These stem cells allow for generation of any tissue cell type, yet ethics and
regulations limit their usage. An adult retains a limited supply of adult stem cells in the bone marrow and in tissues throughout the body. Most of these progenitor cells are partially differentiated into a lineage of cell types but remain multipotent to develop into a more limited range of cell types. Examples are neural stem cells, hematopoietic stem cells, and mesenchymal stem cells.

2.2.5 Scaffolds

The extracellular matrix (ECM) that supports the tissue structure and cells in vivo is a complex network of proteins and glycosaminoglycans to which cells adhere and interact. ECM molecules include collagen, laminin, and fibronectin. The three-dimensional ECM has a two-way interaction with the cells. The ECM surface properties and molecules provide cell-surface receptor-mediated signals to influence cellular spatial organization, migration, growth, differentiation, and death (Martins-Green, 2000). Important cell receptors that interact with the ECM include integrin and cadherin adhesion receptors (Griffith, 2002). The cells influence the ECM by remodeling the structure and secreting new ECM components (Hubbell, 2000).

Studies performed in vitro with cells growing on a two-dimensional surface can observe isolated cell function performance, such as proliferation, glycolysis, respiration, and gene expression, by optimizing the medium nutrient, hormone, and growth factor compositions. However, the proper regulation and control of these functions are dependant on cellular interactions only present within a three-dimensional structure (Ma,
Therefore, scaffolds, natural or artificial, are essential for creating tissue substitutes that mimic in vivo function. Advancements in three-dimensional polymer processing make customization possible for polymer composition, mechanical strength, cell-surface attachment interactions, degradation rates, and allowing a high cell density. The scaffold should enhance the cell growth while maintaining cell integrity and genotypic and phenotypic expression over extended periods of time. The cell-scaffold interactions that determine proper tissue development and function take place on multiple levels: macro-, micro-, and nanoscales. Table 2.1 summarizes the important scaffold characteristics and the effects they have on tissue development. These issues include seeding, cell adhesion, morphogenesis, cell proliferation, differentiation, apoptosis, gene expression, and tissue function.

2.2.5.1 Macroscale Interactions

At the macroscale, the scaffold properties can affect tissue shape and function, cell population proliferation and differentiation, and construct mechanical strength. Scaffolds are designed to be in a shape that guides shape-specific tissue growth to reconstitute a functional tissue. Scaffolds have been made in the shape of a bladder, and, with urinary epithelial cells and smooth muscle cells seeded on the scaffold, a morphologically and biologically similar artificial bladder was developed (Griffith and Naughton, 2002). Blood vessels have been harvested in tubular scaffolds (Niklason et al., 1999), and heart valves were developed from valve shaped support material (Langer, 2000). The thickness of a scaffold affects nutrient transport within the scaffold. As thickness increases,
diffusion of nutrients to the depths of the scaffold is minimal for sufficient nutrient supply. In a study of chondrocytes, cell proliferation decreased as matrix thickness increased (Freed et al., 1994a). Differentiation status of cultured cells can be influenced by the degree of order in the scaffold. Organization of fibrin in ordered fibrils was shown to promote differentiated angiogenic function of HUVECs (Hall et al., 2001). The mechanical strength of the scaffold determines the extent to which compression of the construct occurs. This is important in the event of osteocyte adhesion because of the subsequent constriction forces enacted on the scaffold. A scaffold with insufficient strength would compact to the extent that nutrient transport would be limited at an accelerated rate. Studies of PLLA and PLGA blends resulted in different anti-compressive properties with PLLA and PLGA (85:15) supporting forces for several weeks (Eiselt et al., 1998).

2.2.5.2 Microscale Interactions

At the microscale, the scaffold properties affect seeding adhesion, morphogenesis, proliferation, differentiation, and subsequent tissue function. In fibrous scaffolds, the fiber diameter and affiliated surface curvature affect the spreading ability of attached cells. Spreading allows cells to increase proliferation, and this is regulated by fiber dimensions. Additionally, the diameter affects the degree of cell-cell interactions allowable around the fiber which are necessary for proper tissue function. The porosity of a scaffold has many cumulative effects on the cell population. First, the porosity influences the success of cell seeding. Optimal porosities allow for penetration of cell
seeding suspensions throughout the scaffold resulting in uniform distributions. The successful mixing and distribution of cells within a scaffold results in chondrocytes functioning properly by producing ECM molecules at high cellularity for enhanced cartilage strength characteristics of the tissue (Vunjak-Novakovic et al., 1996). Porosity, closely related to pore size and distances between fibers, is key in dynamic seeding methods in which the cell attachment is aided by interception of cells within the fibrous net to bring cells into contact with the surface for adhesion. Lower porosity scaffolds have interfiber distances reduced allowing increased entrapment of cells within the scaffold. The morphogenesis of the developing tissue is influenced by the allowable migration of cells. The pore size distribution relates to the migration ability because it determines the amount of space available. The migration of endothelial cells was inhibited when pore diameter was reduced to 9 µm (Matsuda and Nakayama, 1996). Optimal pore sizes were found for enhanced bone ingrowth to establish homogeneity within the developed tissue (Chang et al., 2000). Additionally, the pore configuration played a role in the bone culture with cylindrical pores leading to bone development with higher compressive strength than with sponge pores. The available space in the scaffold determined by porosity is important for allowing cell-cell contacts and interaction. Porosity has been shown to improve the degree of cell-cell contacts by the increased expression of E-cadherin, a cell-cell adhesion protein, in hepatocytes (Moghe et al., 1996). To demonstrate how porosity results in a cascade of effects in cultured tissue function, the culture of trophoblast cells in PET will be summarized. Altering the pore size of PET, the trophoblast cells established either a spreading morphology along fibers or an aggregative morphology. Spreading resulted in increased proliferation which,
subsequently, resulted in higher gene expression of cyclin B1, utilized in cell cycle progression (Ma et al., 1999). The aggregative morphology at larger pore sizes resulted in a differentiated state for the cells. As a result, the gene expression of p27kip and the cell function of β-estradiol secretion were increased. Customizing the surface with microgrooves can affect the cell orientation to control cell-cell contact networks such as in the establishment of a neural network.

2.2.5.3 Nanoscale Interactions

At the nanoscale, the molecular interactions between the cell and the scaffold influence many key cell functions such as adhesion, morphogenesis, proliferation, differentiation, gene expression, apoptosis, and the resulting tissue function. The amino acid sequence of Arg-Gly-Asp (RGD) has been identified on fibronectin and other ECM glycoproteins as a key adhesion domain, and the design of synthetic scaffolds incorporating the peptide is successful in improving adhesion (Griffith and Naughton, 2002). The organization of RGD peptide on the scaffold surface affects adhesion as well with a clustered arrangement being optimal rather than when randomly positioned. Patterning of the surface and using microcontact printing techniques design surfaces to have sites of different sizes and functionalities to control cell size and spreading. Larger areas promoted cell spreading and increased proliferation while smaller areas induced differentiation and apoptosis in hepatocytes and endothelial cells, respectively (Chen et al., 1998; Singhvi et al., 1994). To improve cell adhesion by increasing synthetic polymer surface hydrophilicity, scaffolds are pretreated by hydrolyzing with sodium hydroxide to
create carboxyl and hydroxyl groups on the surface of PET (Phaneuf et al., 1997). Migration is a dynamic process involving adhering and detaching mechanisms working in unison. The migration on fibronectin is performed by cells adhering to integrin adherence domains such as RGD at the advancing front of the cell and detaching from heparin domains via proteoglycan receptors at the rear (Martins-Green, 2001). Bone morphogenesis and ingrowth are induced by bone morphogenic proteins bound to ECM components such as heparin and collagen (Reddi, 2001).

The surface properties of the scaffold can host adsorbed molecules that influence cell function. Scaffolds with highly-adsorbed ECM density for integrin binding cause the proliferative morphology of spreading, and a low density results in a round morphology characteristic of differentiation. Scaffolds can be designed to release growth factors efficiently for enhanced proliferation and differentiation. VEGF and fibroblast growth factor were adsorbed on an ECM to improve nerve regeneration and smooth muscle cell growth (Griffith and Naughton, 2002). Domains on the scaffold can influence proliferation directly. The laminin A chain has repeating components that are analogous to endothelial growth factor for proliferation stimulation. Conversely, heparin and heparin-like molecules on the surface of a scaffold inhibit proliferation (Martins-Green, 2001). Hepatocytes express specific differentiation genes only when in contact with the ECM. Specifically, the transcription factor eG-TF/HNF-3 is expressed when the cell is adhered to the scaffold (Martins-Green, 2001). As an alternative to adsorbing proteins to the scaffold surface since some proteins are too fragile, naked plasmid DNA is attached to the scaffold instead. Collagen scaffolds were used to release a gene for a parathyroid
hormone that was subsequently transfected into the cells to aid in bone growth regeneration (Griffith and Naughton, 2002). The scaffold can initiate programmed cell death as well. ECM degradation causes mammary epithelial cells to upgrade caspase-1 expression which initiates the apoptotic cascade (Martins-Green, 2001).

2.2.5.4 Scaffold Types

Biomaterials used as scaffolds can be natural or synthetic. Natural biomaterials, such as collagen, inherently contain the appropriate domains for cell interaction but have the disadvantages of limited adaptability and customizable processing as well as relatively scarce availability compared to synthetic biomaterials (Langer, 1999). Synthetic scaffolds have the advantage of better adaptability and customization than natural scaffolds, but the basic compositions of synthetic polymers are not sufficiently bioactive to fully interact with the cells. Therefore, synthetic scaffolds have altered surface chemistry to improve interactions. The scaffold material morphologies utilized in tissue engineering applications are usually foam-like or fibrous materials. Examples of each scaffold type are shown in Figure 2.4. Table 2.2 summarizes common scaffold materials and corresponding pore sizes and applications. Foam-like materials include foams, sponges, and gels. Natural and synthetic materials are utilized in foam-like matrices. Commonly utilized examples include PGA, PLA, PGLA, PLGA, collagen, and gelatin. Hybrids can also be developed that utilize the advantages of each component (Chen et al., 2000). Foam-like scaffolds have the advantage of being customizable in fabrication with direct control of microstructure. For this reason, foams are utilized for tissue engineering
applications in which control at the microscale is necessary like for drug delivery, for example. Furthermore, foam scaffolds can be processed with 3-D macrostructure defined for directed tissue form, such as hollow bone structures. Foams have defined pores and structure, but the pore network in not well connected. Gels suffer from limited mechanical strength.

Fibrous matrices are generally made of synthetic polymers and are arranged as woven, non-woven, or knitted. Common materials for fibrous matrices are PGA, PLA, PET, polypropylene, and polyethylene. Due to the fibrous composition of these scaffolds, pores, as classically defined, are not present, but rather, effective pore structure characteristics are used when analyzing mass transfer and fluid flow in fibrous matrices. Customized fabrication of fibrous scaffolds is difficult and is a disadvantage. Fibrous scaffolds have very high porosity with availability of nutrients to cells throughout the scaffold and a greater specific surface area for growth allowing for high cell densities (Ma et al., 1999; Li et al., 2001).

If a synthetic scaffold is transplanted, the rate of biodegradability is important to ensure that the scaffold remains to support a transplant until a natural extracellular matrix replaces it. The biodegradation or resorption rate is a function of the scaffold composition, structure, and the mechanical load present at the site of transplantation (Griffith, 2000). The necessary rate at which the scaffold is degraded varies for the tissue
type. For example, slow degradation is allowable in bone tissue whereas in other tissues, chronic inflammation may occur if the rate is too low (Griffith, 2000). It is important that the degradation by-products are non-toxic to the body.

2.2.6 Cell Culture

To develop a tissue in culture for use as a tissue substitute, the tissue cell density must be high (sometimes $10^9$ cells/mL) and uniform within the scaffold. In neomorphogenesis, cells are brought into contact with a porous scaffold and together they form a structure. To achieve uniformity within the scaffold, the cell seeding process must be optimized. Cells are injected into a matrix and incubated to allow for adhesion. Cells attach to the scaffold surfaces when introduced if the surface chemistry of the scaffold is complementary. Scaffolds can be pre-treated to alter the surface chemistry to allow for better compatibility with the cells.

The media utilized to provide complete nutrition for the growth of different cell types are based off of a standard minimum essential nutrient composition. Most media consist of a sugar source, minerals, vitamins, and amino acids. Serum commonly supplements the media to enhance cell growth and minimize contamination. Growth factors and cytokines are utilized to accelerate cell growth through interaction with specific cell receptors. Differentiation inducers are added to direct the differentiation pathway of stem cells. Important medium parameters to control include the temperature, pH, and dissolved oxygen of the medium.
There are several types of bioreactor designs utilized for cultivating new tissue growth. Bioreactor models for tissue engineering evolved from bioreactor designs utilized for mammalian cell culture to maximize productivity on large scales using microcarrier and spinner flask systems, for example (Freed et al., 1997; Ma, 1999). Figure 2.5 depicts various cell culture vessels. The simplest design is the plate or Petri dish. The spinner flask is larger in scale and has an internal agitation mechanism to provide a uniform nutrient concentration within the medium and the enclosed cell-scaffold construct. The development of microgravity systems, such as the High-Aspect Ratio Vessel (HARV), were used to reduce the shear stress encountered by cells as the culture was sustained in suspension (Akins et al., 1997). The environment within may be static or mixed using internally designed or externally applied mechanisms. Static environments rely on diffusion as the mass transfer mechanism for nutrient supply. Mixing within a culture vessel provides convective flow of oxygen and nutrients to the cells while removing waste from the surroundings. Mixing-induced shear stress may cause cell death if mixing is too high though. Perfused bioreactors are culture environments in which medium is circulated within a closed system past the immobilized cell-scaffold components. The continuous flow allows for uniform nutrient supply with enhanced mass transfer. Long-term stability of the culture is attained using continuous perfusion reactors (Freed et al., 2000).

As a novel bioreactor design, rotating-wall bioreactors spin on an axis, and the enclosed cell-scaffold constructs tumble within the rotating microgravity environment. Tissue
culture in microgravity has been shown to improve cellular aggregation and produce highly differentiated tissue products (Unsworth et al., 2000). Rotating-wall bioreactors avoid the high shear stress found within bioreactors that have agitation devices. As a result, altered gene expression favors improved aggregation resulting in aggregates up to ten times larger in diameter than those attained in conventional bioreactors. Additionally, necrosis of cells within the center of the aggregates due to mass transfer limitations is not seen. The stability of tissue constructs after removal from the microgravity environment remains an issue (Unsworth et al., 2000).

The fibrous bed bioreactor (FBB), as schematically shown in Figure 2.6, is utilized for the cultivation of cell populations for bioproduction of molecules or in vitro modeling. Utilizing the 3-D culture advantages previously described, the FBB is a continuous culture design that allows for consistent media supply with control over important culture conditions. The system can be designed to mimic in vivo environmental conditions for optimized culture. The fibrous bed bioreactor with continuous culture allows for more efficient culture and possible scale-up for industrial design (Ma et al., 2000). Long-term stability of the culture is attained using continuous perfusion reactors (Freed et al., 2000). A continuous bioreactor will ensure constant nutrient supply, stable pH, and reduced contamination (Sittinger et al., 1996).

Other stimuli may be incorporated into the culture environment to cultivate proper tissue function. Mechanical or electrical stimulation, provided at frequencies simulating in vivo conditions, has been shown to improve the resulting properties of the cultivated tissue
(Freed et al., 2000). For example, pulsatile conditions that simulate a beating heart are utilized in the culture of blood vessels resulting in improved strength and function relative to cultures lacking this stimuli (Langer, 2000).

Many tissues consist of more than one cell type. The fact that most major organs in the human body consist of more than one tissue and cell type adds to the complexity in recreating a functional organ replacement. The proper physiological function of these tissues depend on the interactions between these multiple cell types, so developing a tissue substitute with the ability to restore function should be a heterogeneous culture consisting of multiple cell types organized appropriately. Co-culture of multiple cell types within one environment to accomplish this is very difficult. Currently, two different cell types are cultured together by growing each cell type in layers with membranes. The membranes allow for signal passage without direct cell-cell contact. The concept of “tissue printing” may allow for the generation of heterogeneous, three-dimensional tissue constructs using a “printing” device that deposits multiple cell types, biomaterials, and other tissue components layer by layer to form an organized structure.

2.2.7 Mass and Fluid Transfer

Static bioreactor environments rely on diffusion as the mass transfer mechanism for nutrient supply. Mixing within a culture vessel provides convective flow of oxygen and nutrients to the cells while removing waste from the surroundings. Mixing-induced shear stress may cause cell death if mixing is too high though. The availability of the cells to
nutrients and oxygen over time is an important aspect considering that it becomes more limited as the population grows and fills more of the porous environment. Oxygen is considered the limiting nutrient in mass transfer optimization. Flow through the porous scaffold can be modeled in several ways with assumptions made. Three models attempt to simulate cell growth in fibrous environments.

In the first model, fluid flow through a fibrous scaffold was investigated to determine an expression for velocity distribution in the scaffold. The velocity distribution was then to be utilized for determining the effective diffusivity coefficient of a limiting nutrient species (Ma, 1999). In the hydrodynamic analysis to obtain the velocity distribution, one goal was to determine the permeability in the scaffold. The analysis began with Darcy’s Law for flow in a porous media:

$$\Delta p = -k \mu U \Delta L$$  \hspace{1cm} (2.1)

where \( \Delta p \) is pressure drop, \( k \) is permeability, \( \mu \) is viscosity, \( U \) is superficial velocity, and \( L \) is scaffold thickness. The flow in the scaffold was analyzed using unit cells consisting of a rod in a polygon (Happel and Brenner, 1959). The method was limited to simplified configurations such as Stokes flow perpendicular or parallel to an array of parallel rods. The configuration of a nonwoven fibrous scaffold is randomly oriented though, making this model too limited in applicability. Spielman and Goren (1968) presented a fluid flow model for randomly oriented fibrous media in the porosity range of 0.99-0.8. However, the porosity of tissue culture constructs can fall to 0.5 which renders the model applicability too limited. Therefore, using the Happel and Brenner model, the boundary
conditions were set as zero velocity at the rod surface and zero velocity gradient at the edge of the polygonal cell. With the velocity profile relation, the mass transfer analysis was performed to develop an effective diffusivity of a limiting species from the macroscopic Fick’s law and a mass conservation equation (Koch and Brady, 1986). The mass transfer analysis included flow contribution to the effective diffusivity from flow perpendicular to the scaffold. Therefore, the effective diffusivity had two components: hydrodynamic dispersion and molecular diffusion. The Peclet number was introduced to relate the relative contribution of each component. The analysis proceeded for small Peclet numbers since low flow rates are utilized in these applications to avoid shear stress damage to cells. The resulting effective diffusivity was a function of molecular diffusion coefficient in the fluid, the fiber volume fraction, Brinkman screening length, and Peclet number.

Further analysis utilizing the effective diffusivity and nutrient distribution in a scaffold with cells was not performed. However, the results were expected to over-predict the experimental results due to key factors not considered in the model. In the determination of effective diffusivity and permeability in the scaffold, the interaction of cell growth was not considered. As the culture progresses, the total solid fraction of the scaffold will increase, reducing the porosity and permeability of the scaffold. This will limit the effective diffusivity below the predicted values from this model. A cell growth model should be used to predict solid fraction dynamics. In addition, the nutrient consumption by cells was not considered. The nutrient concentration profile will decrease more
quickly through the scaffold as consumption occurs. This model would not be able to predict nutrient limiting effects on the maximum cell density maintainable in culture.

In the model development outlined by Chen (2000), the consumption rate of the limiting nutrient was considered in the nutrient profile determination. The flow situation modeled was flow parallel to the outer edges of a fibrous scaffold. In this situation, the convective transport contribution was negligible, and the diffusive mechanism was used to supply the scaffold with nutrients. The limiting nutrient was chosen to be oxygen due to its low solubility in water (0.2 mmol/L) and high consumption rate (1~5x10^{-10} mmol/cell/h). This is a common assumption in mass transfer analyses for cell culture models. It was assumed that the oxygen consumption was a constant specific rate as long as the oxygen concentration stayed above the critical dissolved oxygen level (7% saturation). The system was assumed to be homogenous, and the effective diffusion coefficient was calculated similarly to the previous model for use in the oxygen profile equation.

An oxygen balance in a differential segment of the fiber was performed accounting for accumulation equal to flux into and out of the volume with consumption of oxygen using oxygen in the volume:

\[
A \cdot dz \cdot \frac{dC}{dt} = A \cdot D_{eff} \cdot \frac{dC}{dz} (@z) - A \cdot D_{eff} \cdot \frac{dC}{dz} (@z + dz) - A \cdot X \cdot q_{o_s} \cdot dz
\]  

(2.2)
where $A$ is the area, $z$ is the location within the scaffold, $C$ is the oxygen concentration, $t$ is time, $D_{eff}$ is the effective diffusivity, $X$ is the cell density, and $q_{O2}$ is the specific consumption rate of oxygen. Assuming steady state, Equation 2.2 reduced to:

$$D_{eff} \cdot \frac{d^2C}{dz^2} = X \cdot q_{O2}.$$  \hspace{1cm} (2.3)

Using boundary conditions of oxygen concentration at the scaffold edge was equal to the bulk oxygen concentration ($C_o$) and the nutrient concentration gradient at the center of the scaffold equaled zero, the differential equation was solved to result:

$$C(z) = C_o - \frac{Xq_{O2}}{2D_{eff}}(Z^2 - z^2)$$  \hspace{1cm} (2.4)

where $Z$ is the half thickness of the scaffold. The effective diffusivity was calculated as before with Peclet number set to zero leaving the effective diffusivity as a function of solid fraction in the construct only. By setting values for the constants in Equation 2.4 to experimental amounts ($C_o = 0.18 \text{ mmol/L}$; $q_{O2} = 1 \times 10^{-10} \text{ mmol/cell/h}$; $Z = 0.2 \text{ cm}$) and varying the cell density, the oxygen concentration through the scaffold was determined. The results are shown in Figure 2.7. The critical oxygen level is denoted on the graph, and from analysis, for a culture of $1 \times 10^7$ cells/mL, the oxygen concentration is below the critical value at approximately 0.1 cm into the scaffold. Therefore, a scaffold would need to be no thicker than 0.2 cm to accommodate $1 \times 10^7$ cells/mL.

Interesting information was obtained from this model including the mass transfer-limited cell density allowable in in vitro culture. However, time information is not attainable from this model since growth kinetics is not included in the analysis of solid fraction.
increases. The assumption that all cells consumed oxygen at the same rate is different than the real situation since cell metabolism varies depending on location within aggregates that form in vitro. The diffusion of oxygen into cell masses was not considered as well. The oxygen limited state is much more pronounced for cells within aggregates. As a result, oxygen concentration predictions are probably under-predicted and thus, the allowable cell density is greater than predicted. Similarly to the first model, the determination of the effective diffusivity coefficient did not incorporate changes due to cell growth, making the diffusivity coefficient higher than actual as time passed in the culture.

A more complex and mathematically intensive analysis was performed by Galban and Locke (1999). The model incorporates the metabolic consumption of glucose and cell growth kinetics to determine the concentration profile in the model. The culture environment was static, as is found in plate cultures, and diffusion and reaction rates were assumed isotropic, so a one-dimensional solution could be determined for diffusion perpendicular down into a scaffold sitting in a plate well.

The system was designed as a multi-phase system with a cell phase and a void phase. The cell phase was made of cells growing on the scaffold into colonies initiated by cells uniformly distributed via seeding. The colony dynamics were considered in the growth models utilized for heterogeneous cell phase characteristics, an improvement over the previous models discussed. The overall analysis procedure was to break down the system into smaller multiphase volumes and to use a volume averaging method to derive the
effective diffusion coefficient. Using that to determine the nutrient concentration profile in the scaffold and incorporating growth models to characterize the cell phase increases, the model would describe the \textit{in vitro} model using multiple adjustable parameters to match experimental data.

To find an expression for effective diffusivity in the scaffold, the following equations were used:

\[
\frac{\partial C_\beta}{\partial t} = \nabla \cdot D_\beta \nabla C_\beta \tag{2.5}
\]

\[
\frac{\partial C_\gamma}{\partial t} = \nabla \cdot (D_\gamma \nabla C_\gamma) - k_m C_\gamma \tag{2.6}
\]

\[-n_{\beta \gamma} \cdot D_\beta \nabla C_\beta = -n_{\beta \gamma} \cdot D_\gamma \nabla C_\gamma \tag{2.7}
\]

\[-n_{\beta \gamma} \cdot D_\gamma \nabla C_\gamma = P(C_\gamma - K_{eq} C_\beta) \tag{2.8}
\]

where $\gamma$ is the cell phase, $\beta$ is the void phase, $k_m$ is the metabolic reaction rate, $K_{eq}$ is the equilibrium constant, and $P$ is the mass transfer coefficient at the interface of the phases. Equations 2.7 and 2.8 are boundary conditions at the interfacial area between phases where the flux and mass transfer of the nutrient occur. Quasi-steady state was assumed. Using the volume averaging method, all two-phase system variables are reduced to one averaged value simplifying the system down to one dimension. For this reason, the effective diffusion coefficient ($D_{eff}$) and effective metabolic rate constant ($k_m$) could be calculated for the averaged system.
To calculate the averaged nutrient concentration, the analysis began with:

\[
\frac{d^2 \mu}{d \delta^2} = \phi^2 \mu \tag{2.9}
\]

where \( \mu \) is a dimensionless nutrient concentration over a dimensionless distance \( \delta \) and \( \phi \) is the metabolic rate-based Thiele modulus. Using the boundary conditions of no flux at the center of the scaffold and the flux at the outer edge of the scaffold is equal to the mass transfer into the two phases present at the outer area interface, the averaged nutrient concentration was determined:

\[
\langle \mu \rangle = \frac{Sh}{\phi^2 + \phi \cdot Sh \cdot \coth(\phi)} \tag{2.10}
\]

where \( Sh \) is the Sherwood number as a function of effective diffusivity and cell volume fraction. The averaged nutrient concentration was simply a function of cell volume fraction however, since cell volume fraction determined diffusivity.

To determine the cell fraction dynamics, three cell growth models were used: Contois, Moser, and an \( n \)th-order heterogeneous model. The basic equation for mass accumulation was:

\[
\frac{d\varepsilon_\gamma}{dt} = \frac{1}{\rho_\gamma V_{v_\gamma}} \int_{v_\gamma} R_\gamma dV
\]

where \( \varepsilon_\gamma \) is the cell volume fraction, \( \rho_\gamma \) is the cell density, and \( R_\gamma \) is the cell growth rate. The Contois model, a function of cell volume fraction, depicts saturation kinetics such that inhibition of cell growth is seen when cell density increases too much. The Moser equation is similar to cooperative enzyme kinetics and can be reduced to the common
Monod model. The heterogeneous model depicts biofilm growth. In a biofilm model, the outer layer of the colony is assumed to be the only proliferating population. The cells inside the biofilm have reduced growth or cell death, but these two modes balance each other out of the total cell growth. Utilizing these cell growth kinetics models with the average nutrient concentration, Equation 2.10, the change in cell volume fraction could be analyzed.

Analyses of the growth rate constants, metabolic constant, and Moser parameter were performed compared to experimental data. The heterogeneous growth model, using first order kinetics, did not correlate well with the data at early times due to the model not incorporating culture lag time. The growth constant did increase as fiber thickness decreased which makes sense due to improved nutrient availability in thinner scaffolds, but at the minimum scaffold thickness (0.088 cm), the model was especially poor. This may be due to the cell population growing similarly to the kinetics of a two-dimensional plate culture where a higher growth rate is seen and nutrient diffusion is not an issue. The Moser (Monod) and Contois models do have lag time incorporated in the models though extended model lag times caused under-predicted growth results at lower times. Similar to before, the minimum scaffold thickness experiments were most difficult to model.

Analysis of the metabolic rate constant resulted in two models being discarded from further analysis. The heterogeneous model fit data poorly due to the lack of saturation kinetics and being weakly affected by the cell volume fraction. The Contois model fit the
data the best. The other two models were faulty because they operated in an invalid parameter constraint zone in which gradients of the nutrient were significant where, in fact, gradients should be minimal due to rapid transport.

Plotting the average nutrient concentration in the scaffold as a function of time, the concentration drops quickly at the beginning as the metabolic rate constant is increased. When the metabolic rate constant is increased, the nutrients are depleted faster resulting in less cell growth in the model than if the metabolism were lower. Therefore, cells with lower metabolic rates will produce cultures with higher cell densities than those with high metabolic rates.

An interesting feature in the Contois model is the Moser parameter which accounts for the intake of nutrients, such as glucose, into the cell but using them for cell growth or storage rather than for metabolism. As the Moser parameter increases, the extra usage of the nutrient increases. A plot of the average nutrient concentration as a function of time with varying Moser parameter results in dual effect zones. At early times, when the Moser parameter increases, the nutrient level drops more quickly due to nutrients being utilized for cell metabolism and growth. However, at longer times, when the nutrient concentration is low, increasing the Moser parameter has an inverse effect where higher Moser parameters result in lower nutrient consumption. This may be because the nutrients being taken into the cell are being placed in storage instead of being used for cell growth.
This complex model allowed for culture dynamics with time to be realized. It also included a descriptive growth model, Contois, which fit experimental data better than previous models. However, there were still variations from the model predictions to the experimental data suggesting that diffusion alone does not fully explain *in vitro* model behavior.

There are several suggestions for incorporation into future models to represent the complex situation. First, regarding the third model, the storage mechanism, modeled by the Moser parameter, could be represented as a function of time and space within the cell fraction. Utilizing flow conditions in the model will provide better estimates of the limits of cell growth in the scaffold since mass transfer is optimized with hydrodynamic dispersion. Many tissue engineering models utilize perfusion bioreactors for this reason. In perfusion systems, not only are nutrients supplied in the flow, but products are carried away. Products such as lactate can inhibit cell growth when accumulated beyond a certain level limit. The incorporation of product accumulation and diffusion may explain over-predictions of cell growth when inhibition by waste is not considered. The lactate profile in a scaffold was modeled using the following equation:

\[
\frac{\partial^2 C}{\partial z^2} + Xq_{Lac} = 0
\]

(2.12)

where \( q_{Lac} \) is the specific production of lactate. Using the boundary conditions of no concentration gradient at the center of the scaffold and the bulk concentration of lactate was zero at the scaffold edge due to parallel fluid flow, Equation 2.12 was solved:
\[ C = \frac{Xq_{lac}}{2D_{eff}} (Z^2 - z^2) \]  \hspace{1cm} (2.13)

Setting the diffusion coefficient \((1 \times 10^{-6} \text{ cm}^2/\text{s})\), lactate production \((6.5 \times 10^{-14} \text{ mmol/cell/h})\), and scaffold thickness \((0.15 \text{ cm})\), the lactate concentration was plotted against the scaffold depth for different cell densities in Figure 2.8. As shown, the lactate level increases with increasing cell density, but in this system, the lactate level was not above critical \((1.5 \text{ g/L})\) at the tested cell densities. However, with no flow, the boundary condition of zero lactate in the bulk phase would be invalid as accumulation of lactate would begin to inhibit growth.

Additional factors that are present in the culture but not modeled are the effects of cell-cell and cell-scaffold signaling and extracellular matrix secretion. Signaling in the form of growth factors and scaffold surface properties would regulate cell growth and metabolism in variation to cell growth models. The secretion of matrix components would increase the rate at which the porosity of the construct decreases thus further limiting the achievable tissue densities. The \textit{in vivo} microenvironment also includes other cell types that induce a competitive situation. In the case of tumor growth, the cancerous cells must migrate through several distinct layers of the normal tissue to make room for growth. This includes cell migration and degradation of the surrounding extracellular matrix. This impeded, complex growth environment will need to be modeled both in the \textit{in vitro} model and mass transfer models.
In vivo, as cell growth is inhibited by nutrient depletion (typically, when cells are more than 200 µm from blood supply), signals are sent to initiate endothelial cell migration to the area to establish new blood vessels and a new nutrient source. This mechanism of angiogenesis is also being attempted to be modeled in in vitro models through cell coculture. Successful in vitro angiogenic models would allow for models that bypass current mass transfer limitations. If possible, a scaffold could be developed that incorporated a synthetic vascular system with “blood vessels” spaced out within the scaffold to provide sufficient nutrient support in the spaces between.

2.2.8 Transplantation

When implanting a tissue substitute, immune rejection by the host against the foreign implant is the primary concern unless a biocompatible scaffold with autologous cells is used. Several strategies exist to circumvent this challenge depending on cell type, source, and desired function. Three specific strategies are gene modification in the cells prior to tissue development, immune system therapy before and after the transplantation procedure, and tissue encapsulation (Hardin-Young et al., 2000). Traditionally, similar to organ transplants, the complete immune system is suppressed for a period of time following the transplant to increase the chance that eventually the transplant will be accepted. There is a key 5-10 week period in which the successful integration of the transplant or the graft rejection is being determined (Hardin-Young et al., 2000). However, during this period of immunosuppression, the patient is susceptible to other illnesses. A strategy developed by Geron (Menlo Park, CA) involves building a tolerance
in the host for a cell type prior to transplantation. By first performing a bone marrow transplant using donor hematopoietic cells, the host then will circulate immune system components that match the future donor. Then, when the transplant occurs, the donor tissue is tolerated (Brower, 2003).

When a tissue engineered product is not to be used for transplantation upon its creation, cryopreservation allows for the long-term storage until a future application. Cryopreservation is commonly used for preparing cells for storage, but its utility for storage of tissues remains in development. Cryopreservation is a major focus for researchers, though, because it is a key component for improving the marketability of tissue engineered products because it will allow for development of an on-demand tissue supply, preservation of tissue genetic stability, and establishment of production quality control archives (Karlsson and Toner, 2000). During the cryopreservation process, both the freezing and thawing procedures are equally important in regulating water displacement and replacement, respectively, while maintaining cellular and tissue integrity. Cryoprotectants and thermal processing protocols are utilized in this process. Commonly utilized cryoprotectants are dimethyl sulfoxide (DMSO) and glycerol, which remove intracellular water to avoid damaging ice crystal formation. Due to the larger scale of tissues compared to cells, challenges are increased, including the induction of chemical and thermal gradients within the tissue that must be resolved utilizing optimized mass and heat transfer operations, respectively. Technologies to improve these processes and to monitor tissue parameters for performance control and modeling will further help to develop applicable cryopreservation protocols.
2.2.9 Applications

Over 20 tissues have been studied for numerous purposes including bone regeneration, recreated heart valves and vessels, skin substitutes, organ transplantation, and wound healing (Langer, 2000; Lysaght et al., 2001; Tabata, 2001; Geer et al., 2002; Griffith et al., 2002). Table 2.3 lists selected tissues engineered and the applications. Additional applications that incorporate tissue engineering tools include drug delivery, directed stem cell differentiation, and gene therapy (Langer, 1999; Fukuda, 2001; Xie et al., 2001). The development of in vitro physiological models, however, may have the greatest impact in the next decade (Griffith et al., 2002).

2.2.9.1 Gene Therapy

Gene therapy is introducing DNA that codes for an essential protein into cells that are altered to not be able to produce the protein. Gene therapy is an alternative to supplying the deficient region with the protein itself or replacing the defunct tissue with a functional tissue substitute. The DNA is carried and introduced into the cells using a delivery system that is commonly viral or plasmid-based (Fradkin et al., 2000). Once the gene can be expressed within the cell, persistence of the essential protein supply is key. Along with the great potential of this process come important safety concerns regarding the
implementation of the DNA correctly within the chromosomes. Incorrect incorporation of the gene could alter important control genes or other essential genes. Avoiding an immune response is also necessary (Fradkin et al., 2000).

2.2.9.2 In Vitro Studies

Tissue engineering can also be used to develop laboratory tools using human cells to perform pathological, developmental, pharmacological, and toxicological studies. Tissue cultured as an in vitro model can be used to study tissue or organ development processes including the signaling and control mechanisms. Additionally, toxicology and carcinogen studies on tissue models provide insight without utilizing animal or 2-D models. Cancer models can be used to study tumor biology, control, and progression as well as for cancer treatment studies.

2.2.9.3 Cell-Based Technologies

Utilizing the knowledge of cells and their interaction with materials attained from tissue engineering research, additional cell-based technologies are being developed. Biosensors take advantage of the specificity of cell surface receptors for select target molecules and the high signal amplification for detecting low levels of chemical or biological agents. The brain consists of a neuron network that processes a large amount of signals. Neurons organized on a support in specific patterns could produce in vitro neural networks that pass signals similar to the electronic structure on microchips (Mrksich, 2002).
2.2.10 Challenges

Though there has been some limited success in producing tissue substitutes, especially artificial skin, the overall tissue engineering goal of creating tissue that can replace, maintain, or improve deficient in vivo tissue is not an easy task. Multiple challenges stand in the way towards having tissue products on demand. These include creating a greater supply of cells that can avoid immune rejection such as a universal donor cell supply. Advancements in biomaterial production and bioreactor design are necessary to provide customized support and environments to replicate the complex in vivo environment. When developing complex, three-dimensional tissues, challenging issues include promoting angiogenesis to overcome tissue size limitations, the heterogeneous co-culture of multiple cell types within one tissue, and the cryopreservation of the resulting tissue before transplantation.
2.3 References


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Ma, T. 1999. “Fiber-Based Bioreactor Systems In Mammalian Cell Culture And Tissue Engineering Human Trophoblast Cells.” PhD Dissertation. The Ohio State University, Columbus, OH.


<table>
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<tr>
<th><strong>Structure Scale</strong></th>
<th><strong>Scaffold Property</strong></th>
<th><strong>Target Effects</strong></th>
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<td>Shape</td>
<td>Tissue shape</td>
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<td>Thickness</td>
<td>Proliferation</td>
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<td>Morphology</td>
<td>Differentiation</td>
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<td>Mechanical strength</td>
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<td>Tissue function</td>
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<tr>
<td>Microscale</td>
<td>Fiber diameter</td>
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<td>Porosity</td>
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<td>Surface grooving</td>
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<td>Tissue function</td>
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<tr>
<td>Nanoscale</td>
<td>Biomaterial composition</td>
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<td>Tissue function</td>
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Table 2.1: Scaffold properties at three scales and influences on tissue development
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<th>Pore Size</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate (sponge)</td>
<td>30-300 µm</td>
<td>Fibroblast</td>
<td>Shapiro et al., 1997</td>
</tr>
<tr>
<td>Collagen (gel, foam)</td>
<td>20 and 80 µm</td>
<td>Cartilage</td>
<td>Nehrer et al., 1997</td>
</tr>
<tr>
<td>Gelatin (gel)</td>
<td>40-400 µm</td>
<td>Nerves</td>
<td>Kang et al., 1999</td>
</tr>
<tr>
<td>PET (nonwoven)</td>
<td>30-40 µm</td>
<td>Trophoblast</td>
<td>Ma et al., 1999</td>
</tr>
<tr>
<td>PGA (nonwoven)</td>
<td>&gt;200 µm</td>
<td>Liver</td>
<td>Freed et al., 1994b</td>
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<td>PLA (knitted)</td>
<td>-</td>
<td>Bone</td>
<td>Kellomaki et al., 2000</td>
</tr>
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<td>PLG (foam)</td>
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<td>Bone</td>
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</tr>
<tr>
<td>PLGA (foam)</td>
<td>135-633 µm</td>
<td>Smooth Muscle</td>
<td>Harris et al., 1998</td>
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Table 2.2: Common scaffold materials used for tissue engineering applications.
<table>
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<tr>
<th>Tissue</th>
<th>Why</th>
<th>Cells</th>
<th>Scaffold</th>
<th>Application</th>
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<tbody>
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<td>PET, e-PTFE</td>
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<td>Thromboresist</td>
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<td>Bone</td>
<td>Disease, radiation</td>
<td>Periosteal, osteoblast, mesenchymal stem cells</td>
<td>PGA, PLA, calcium alginate</td>
<td>Repair, replacement, regeneration</td>
<td>Restored mechanical properties</td>
<td>Vacanti et al, 2000; Bruder et al., 2000</td>
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<tr>
<td>Brain</td>
<td>Parkinson’s and Huntington’s diseases</td>
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<td>Smooth muscle cells (SMC), fibroblast, chondrocytes</td>
<td>PGA, PLA, PCL</td>
<td>Replacement</td>
<td>Flexible transplant with induced angiogenesis</td>
<td>Lee et al., 2000</td>
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<td>Heart valves</td>
<td>Valvular disease</td>
<td>Endothelial, fibroblast</td>
<td>PGA</td>
<td>Repair, replacement</td>
<td>Durability over mechanical devices</td>
<td>Love, 2000</td>
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<td></td>
<td>e-PTFE, PMMA, PVA</td>
<td>Repair, replacement</td>
<td>Elasticity, refractive properties, transparency, curvature</td>
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<td>Alginate/poly(L-lysine)</td>
<td>Repair</td>
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<td>Pancreas</td>
<td>Diabetes</td>
<td>Islets of Langerhans</td>
<td>Alginate/poly(L-lysine)</td>
<td>Encapsulate</td>
<td>Active glucose concentration control</td>
<td>Wang et al., 2000</td>
</tr>
<tr>
<td>Kidney</td>
<td>Renal failure</td>
<td>Endothelial</td>
<td>Hollow-fiber</td>
<td>Support device, replacement</td>
<td>Blood filtration, homeostasis maintenance</td>
<td>Humes, 2000</td>
</tr>
<tr>
<td>Tendon</td>
<td>Injury</td>
<td>Fibroblasts</td>
<td>Collagen</td>
<td>Repair, replacement</td>
<td>Mechanical durability</td>
<td>Goulet et al., 2000</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>Disease, shortage</td>
<td>Artificial with hemoglobin</td>
<td>Membrane</td>
<td>Micro-encapsulate</td>
<td>Avoid removal of artificial cells from circulation</td>
<td>Chang, 2000</td>
</tr>
</tbody>
</table>

Table 2.3: Compilation of selected engineered tissues with cells, scaffolds, and applications provided.
Figure 2.1: Organs of the digestive system including components of the colon anatomy (Colon and Rectal Cancer: Treatment Guidelines for Patients, 2000)
Figure 2.2: Layers of the colon wall (Colon and Rectal Cancer: Treatment Guidelines for Patients, 2000)
Figure 2.3: Components of a tissue construct and applications.
Figure 2.4: SEM images of 4 scaffold types: a) sponge, b) foam, c) gel, and d) fibrous.
Figure 2.5: Tissue engineering culture vessels: a) multiwell plate, b) spinner flask, and c) bioreactor
Figure 2.6: Schematic diagram of the fibrous bed bioreactor
Figure 2.7: Oxygen concentration profile modeling in a fibrous scaffold at different cell densities
Figure 2.8: Lactate concentration modeling in a fibrous scaffold at different cell densities
CHAPTER 3

DEVELOPMENT OF AN IN VITRO THREE-DIMENSIONAL COLON CANCER MODEL THROUGH SCAFFOLD MANIPULATION, CELL SEEDING ANALYSES, AND CELL COUNTING METHODS

3.1 Abstract

The preparation of a tissue engineered construct consists of introducing cells into a scaffold structure suitable for cell adhesion and growing the cell population in a cell culture environment that will support high cell densities and proper function. Prior to the study of the growth of colon cancer within a three-dimensional (3-D) environment, three aspects that will be utilized when establishing the colon cancer model needed to be studied. These three are preparation of the scaffold, analysis of cell seeding methods, and determination of an optimal cell counting method within the scaffold structure.

Nonwoven, fibrous poly(ethylene terephthalate) (PET) was pretreated with sodium hydroxide to increase the scaffold surface hydrophilic properties to improve cell adhesion. PET was also compressed under different pressures, temperatures, and time durations to determine porosity change and compression stability. Scaffold porosities obtained ranged from 0.94 to 0.84. At 90°C, non-pretreated scaffolds were compressed
more than pretreated scaffolds under equivalent conditions. The apparent modulus of compression was three times larger at 90°C than at 121°C. Scaffold thickness was retained after three days in culture medium demonstrating compression stability in all tested conditions.

Cell seeding of HT-29 colon cancer cells into PET was performed at different PET conditions and seeding methods to determine optimal conditions for seeding rates and distribution of cells. Four PET scaffolds in combinations of two porosities (0.94 and 0.88) and pretreated or non-pretreated were seeded in static, dynamic, and filter environments. Cell attachment rates were highest in high porosity, pretreated scaffolds in all methods due to available pore structure and advantageous surface properties. Pretreatment of the PET had significant effects on cell attachment rates. Uniform cell distribution within the scaffold was obtained in the mixed seeding methods but was more limited in static seeding.

It is a challenge to quantify cell number in cell-scaffold constructs, and three assays for determining cell number in the 3-D model were analyzed. Detachment by enzymatic action and direct counting was found to be the best method for recovering consistent quantities of intact cells from the scaffold, yet the recovery rate was low at approximately 45%. Indirect methods included relating lysed-cell DNA or protein amounts to cell number. The DNA assay was unable to quantify significant cell presence in the scaffolds,
and the protein assay was able to quantify low cell densities but with wide variability. Therefore, an accurate method for the counting of cell number in a 3-D environment remains elusive.

3.2 Introduction

Colon cancer is the second most deadly cancer, killing approximately 56,000 people per year in the United States. With candidate drugs failing in clinical trials at over a 75% rate, many times due to inaccurate predictions of drug efficacy at the preclinical stage, improvements must be made in drug screening (DiMasi, 2003). Tissue engineering combines knowledge obtained from biological fields and applies it using engineering skills to repair, regenerate, or enhance tissues and organs both in vivo and in vitro (Griffith et al., 2002). To mimic the native tissue, the key concept utilized in studies is that there is a strong relationship between tissue structure and function. Therefore, in order to achieve the desired functional attributes in a tissue-engineered construct, the culture environment must represent the native counterpart and present the inherent advantages. A colon cancer model that incorporates an in vivo-like three-dimensional (3-D) scaffold could maintain a cell population that would mimic its in vivo counterpart in growth characteristics and, possibly, in reaction to drug exposure.

The preparation of a tissue engineered construct consists of introducing cells into a scaffold structure suitable for cell adhesion and growing the cell population in a cell culture environment that will support high cell densities and morphology development.
Prior to the study of the growth of colon cancer within a 3-D environment, three aspects that will be utilized when establishing the colon cancer model need to be studied. These three are preparation of the scaffold, analysis of cell seeding methods, and determination of an optimal cell counting method within the scaffold structure.

Two scaffold properties will be changed to obtain various scaffolds for usage in subsequent studies. To improve cell adhesion by increasing synthetic polymer surface hydrophilicity, scaffolds are pretreated by hydrolyzing with sodium hydroxide to create carboxyl and hydroxyl groups on the surface of PET (Phaneuf et al., 1997). The porosity of the fibrous PET can be changed by thermal compression. The porosity of a scaffold has many cumulative effects on the cell population. First, the porosity influences the success of cell seeding. Optimal porosities allow for penetration of cell seeding suspensions throughout the scaffold resulting in uniform distributions. The successful mixing and distribution of cells within a scaffold result in chondrocytes functioning properly by producing ECM molecules at high cellularity for enhanced cartilage strength characteristics of the tissue (Vunjak-Novakovic et al., 1996). Porosity, closely related to pore size and distances between fibers, is key in dynamic seeding methods in which the cell attachment is aided by interception of cells within the fibrous net to bring cells into contact with the surface for adhesion. Lower porosity scaffolds have interfiber distances reduced allowing increased entrapment of cells within the scaffold. The effects of pressure, temperature, time duration, and pretreatment during compression will be analyzed.
Four elements are necessary for optimization of cell seeding of scaffolds: High seeded cell density, high seeding efficiency, uniform cell distribution, and high adhesion rates (Vunjak-Novakovic et al., 1998). High initial cell density improves the rate at which cells adjust to the new environment and establish proper function. High seeding efficiency utilizes the seeding cell population effectively, and uniform cell distribution allows for the development of uniform tissue while fully utilizing the available scaffold surface area. Reducing the seeding procedure time minimizes the harmful effects on cells in suspension.

Three common methods for cell seeding are static, dynamic, and filter seeding methods. In static seeding, cells are introduced into a scaffold positioned in a multiwell plate. Dynamic seeding takes place in a mixed environment, such as a spinner flask, with agitation increasing the interaction of cells throughout the scaffold. The level of agitation influences the success of the seeding process. In filter seeding, a cell suspension is passed through the scaffold, and the scaffold acts as a filter as cells deposit on the surface. Residence time within the scaffold is an important factor in filter seeding. In addition to the seeding method, the scaffold properties also play a significant role in affecting seeding rates and deposition. Determination of the effects of these seeding methods and scaffold properties on colon cancer cell seeding in the scaffold for 3-D model establishment will be performed.

Quantifying cell growth within 3-D cultures is increasingly difficult compared to monitoring cell growth in two-dimensional (2-D) plate cultures. In 2-D cultures, the cells
are readily accessible to enzymes used to detach cells from the surface for direct counting. Due to cell incorporation deep within 3-D scaffolds and increased cell density, enzymatic detachment for direct cell count is not as effective. Other methods may be applied to attempt to accurately count cell number within a 3-D environment, such as DNA or protein assays. These are indirect methods of cell counting, relating collected DNA or protein counts from lyzed cells to original cell number. All of these methods will be analyzed for their effectiveness of representing actual cell counts in the scaffold.

3.3 Materials and Methods

3.3.1 Cells and Medium

The colon cancer cell line HT-29 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and is utilized in all of the experiments. HT-29 is a human adenocarcinoma, grade I cancer cell type of colonic epithelial origin. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose concentration, L-glutamine, and sodium pyruvate (Caisson Laboratories, Rexburg, ID). The medium was supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY).
3.3.2 Scaffold

Nonwoven, fibrous poly(ethylene terephthalate) (PET) fabric, known commercially as Dacron, was used as the 3-D culture support scaffolding. PET (fiber diameter: 20 µm; fiber density: 1.35 g/mL; porosity: 0.94) is a randomly organized, isotropic matrix with advantages of being inexpensive yet providing high specific surface area for cell growth and high porosity for cell growth and nutrient accessibility.

3.3.3 Pretreatment of Scaffolds

The surface of the PET was modified to improve serum protein and cell adhesion to the scaffold by hydrolyzing the PET in boiling sodium hydroxide solution. The scaffolds were washed in a solution of 5 g sodium bicarbonate and 5 mL Tween 20 in 500 mL of distilled water at 60°C for 30 minutes, followed by a 10 minute rinse in a 60°C water bath. The scaffolds were then hydrolyzed in boiling 1% sodium hydroxide solution for 45 minutes. Finally, the scaffolds were washed again at 60°C in a water bath for 10 minutes.

3.3.4 Compression and Stability of Scaffolds

A thermal compression method (Li et al., 2001) was used to obtain PET scaffolds of varying porosities. PET was compressed under load at 121°C, 90°C, and 60°C. Pressure was applied at 38 kPa and 86 kPa. Compression duration times studied were 30 minutes,
60 minutes, and 90 minutes. Both pretreated and non-pretreated scaffolds were compressed. Compressed scaffold thickness was determined using calipers. Compression stability was verified by soaking the scaffolds in DMEM with 10% FBS for 3 days at 37°C and 5% CO₂ followed by measurement of the scaffold thickness for signs of swelling. Porosity, the void volume fraction, was determined from the difference between the total bulk scaffold volume and the solid, fiber volume. The fiber volume was obtained from the scaffold mass and the fiber density.

3.3.5 Static Seeding

Static seeding occurred in 12-well plates with PET scaffold discs with a diameter of 2.3 cm. Four scaffold types were used: high porosity (0.94), pretreated (HP/T), high porosity, untreated (HP/UT), low porosity (0.88), pretreated (LP/T), and low porosity, untreated (LP/UT). The scaffolds were presoaked in culture medium either overnight or just before seeding, depending on the trial. Before seeding the cells, the medium was removed. Cells in quantities ranging from 0.5 million to 15 million cells were seeded in a volume below 200 µL to prevent saturation of the scaffold since saturation allows cells to reach the bottom of the well without interacting with the scaffold for seeding. The cell suspension was added slowly, in drops, across the top surface of the scaffold. The plates were then incubated at 37°C and 5% CO₂ until the next sampling time. At the sampling times of 2, 3, 4, 5, and 6 hours, duplicate samples were harvested to determine cell attachment. To do this, the scaffolds were gently washed with phosphate-buffered saline (PBS) to collect all cells that had not attached within the scaffold. The PBS was collected, and Accutase
(Innovative Cell Technologies, San Diego, CA) was added to the empty wells and incubated for 15 minutes to collect cells that had passed through the scaffold. The Accutase and PBS samples were combined and the collected cells were counted using a hemacytometer to determine the cells that were not attached to the scaffold. This indirectly provided the amount of cells attached to the scaffold.

3.3.6 Dynamic Seeding

Dynamic seeding experiments were performed in 100 mL spinner flasks (Bellco Glass, Vineland, NJ). Prior to usage, the spinner flask inner surfaces were siliconized with Sigmacote (Sigma Chemical, St. Louis, MO) to provide selective advantage for cell seeding to the PET rather than to the glass. Four scaffold types were used: high porosity (0.94), pretreated (HP/T), high porosity, untreated (HP/UT), low porosity (0.88), pretreated (LP/T), and low porosity, untreated (LP/UT). The PET scaffold was cut in 2 square pieces of 1.5 cm length and sewn onto a wire mesh ring opposite each other before positioning the mesh with PET at the bottom of the flask. The PET was soaked in 75 mL of culture medium before adding 10.8 million cells to the flask. The spinner flask was mixed at the prescribed revolution rate of 40, 80, or 120 rpm by placement on a magnetic stir plate within a 37°C and 5% CO₂ incubator until the next sample time. Samples of 0.5 mL were taken from the spinner flask medium each hour for approximately 10 hours. The cells in the medium were counted by hemacytometer, determining the amount of cells not
seeded in the PET. This indirectly provided the amount of cells seeded in the scaffold. A control experiment of cells seeded in the spinner flask with no PET was performed to demonstrate cell seeding only in the scaffold.

3.3.7 Filter Seeding

A filter seeding experiment was performed by passing a cell suspension through a column (diameter: 2.7 cm) packed with PET. The total scaffold material packed in the column consisted of 10 stacked 2.7 cm discs of HP/T PET. The cell suspension passed through the vertical column from the bottom to the top at a superficial velocity of 0.16 mm/s and was recirculated by a pump back to the column. Samples were obtained from a bypass valve each hour to determine the concentration of cells remaining in the medium by hemacytometer. This indirectly provided the amount of cells seeded in the scaffold.

3.3.8 Accutase Cell Count

Cells were statically seeded into presoaked PET in 12-well plates in triplicate samples at three cell amounts: 1.5x10^5, 6.2x10^5, and 2.5x10^6 cells. 5 hours after seeding, the wells were filled with 2 mL of culture medium, and the plates were incubated for 12 hours in a 37°C and 5% CO2 incubator. After 12 hours, the medium was removed, and the scaffolds were washed 2 times with 2 mL PBS by pulsing the PBS through the scaffold with a pipette 10 times for each wash. The PBS portions were collected together from the wells and set aside while the scaffolds were submerged in Accutase (Innovative Cell
Technologies, San Diego, CA) and incubated at 37°C. The plates were mixed gently at 10 minutes. After 20 minutes, the plates were removed from the incubator, and the Accutase was pulsed 15 times through the scaffold to release and collect the cells from the scaffold. The Accutase/cell suspension was added to the PBS wash portions, and the cells were counted by hemacyctometer. The Accutase treatment was repeated multiple times, 20 minutes each, to collect more cells from the scaffold. The cumulative cell counts were analyzed for percent recovery of cells based on the initial cell seeding amount.

3.3.9 DNA Cell Count Assay

Cells were statically seeded into presoaked PET in 12-well plates in triplicate samples at three cell amounts: 1.5x10^5, 6.2x10^5, and 2.5x10^6 cells. 5 hours after seeding, the wells were filled with 2 mL of culture medium, and the plates were incubated for 12 hours in a 37°C and 5% CO₂ incubator. After 12 hours, the scaffolds were gently washed 2 times with 2 mL of PBS. Two methods were studied to lyze the cells in the scaffold and release the DNA: freezing in water and addition of lysis buffer. For freezing, 2 mL of distilled water was added to each well, and the plate was placed in a -80°C freezer for 24 hours (Rago et al., 1990). The plates were then thawed and analyzed for DNA content. The distilled water with DNA was removed from the scaffold, and 100 µL of the sample was combined with 100 µL of Hoescht 33258 dye (200 µg/mL) in a 96-well plate. After 6 minutes, the fluorescence was read at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The fluorescence data were compared to a DNA standard graph for DNA quantification. The DNA per cell ratio used was 2.11x10^-7.
mg/cell. For the buffer lysis method, a buffer consisting of 100 mM sodium chloride (NaCl), 10 mM Tris-Cl, 25 mM ethylene diamine tetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS) was prepared, and 2 mL of the buffer was added to each well. After 24 hours of exposure at 37°C, the plates were analyzed for DNA content similar to above except that 100 µL of the sample was combined with 50 µL of Hoescht 33258 dye.

3.3.10 Protein Cell Count Assay

Cells were statically seeded into presoaked PET in 12-well plates in triplicate samples at three cell amounts: 1.5x10^5, 6.2x10^5, and 2.5x10^6 cells. 5 hours after seeding, the wells were filled with 2 mL of culture medium, and the plates were incubated for 12 hours in a 37°C and 5% CO₂ incubator. After 12 hours, the scaffolds were gently washed 2 times with 2 mL of PBS. Two methods were studied to lyze the cells in the scaffold and release the cellular protein: freezing in water and addition of lysis buffer. For freezing, 2 mL of distilled water was added to each well, and the plate was placed in a -80°C freezer for 24 hours. The plates were then thawed and analyzed for protein content. The distilled water with DNA was removed from the scaffold, and 160 µL of the sample was combined with 40 µL of 1:5 diluted Bio-Rad (Hercules, CA) Dye Reagent Concentrate in a 96-well plate. After 10 minutes, the absorbance was read at wavelength of 570 nm. The absorbance data were compared to a protein standard graph for protein quantification. The total protein per cell ratio used was 3.27x10⁻⁷ mg/cell. For the buffer lysis method, a
buffer consisting of 10 mM ethylene diamine tetraacetic acid (EDTA) was prepared, and 2 mL of the buffer was added to each well. After 24 hours of exposure at 37°C, the plates were analyzed for total protein content similar to above.

### 3.3.11 Scanning Electron Microscopy

Scaffolds containing cells were washed with PBS and fixed in 2.5% glutaraldehyde solution for 12 hours at 4°C. Samples were then washed with distilled water and dehydrated for 20 minutes in solutions of ethanol with concentrations increasing from 20% to 100% with increments of 10%. Following dehydration, the samples were dried in four successively increasing concentrations of hexamethyldisilazane (HMDS) with ethanol (1:3, 1:1, 3:1, and 1:0) for 15 minutes each and allowed to air dry for 6 hours. The scanning electron microscopy (SEM) samples were mounted and sputter coated with gold/palladium at 17 mA for 150 seconds using a Pelco (Redding, CA) Model 3 sputter coater. A Philips (New York, NY) XL 30 scanning electron microscope was used for analysis.

### 3.3.12 Data Analysis

Experiments were performed at least in duplicate or triplicate except where noted. Data shown are means with standard deviation. Analyses of variation between means were performed using a Student’s t-test, and significance was based on a 95% confidence level (α<0.05).
3.4 Results and Discussion

3.4.1 Compression of PET Scaffolds

Compression of PET under all conditions resulted in the reduction of porosity from 0.94 down to various values minimizing at 0.84 for the compression of a non-pretreated sample for 90 minutes at 86 kPa and 121°C. Figure 3.1 shows the compression results for the three temperatures studied. At 121°C, maximum compression is already attained at 30 minutes for each pressure and scaffold treatment, whereas at 90°C, the PET can be compressed more at 30 minutes at both pressures. Compression at 60°C was limited, especially at 38 kPa where only minimal compression was obtained. The glass transition temperature of PET is approximately 70°C, and thus at 60°C, the physical properties of the fibers are not yet changing, possibly limiting the compressibility. The stability of the compression was analyzed by measuring the scaffold thickness after being submerged in culture medium at 37°C for 3 days. Changes in scaffold thicknesses were random and negligible demonstrating compression stability at all levels analyzed.

The difference in compressibility between pretreated and non-pretreated scaffolds is negligible at 121°C. However, at 90°C, non-pretreated scaffolds are more compressible than pretreated scaffolds under equivalent conditions. This may be because of the pretreatment process which involves exposure of the scaffold to temperatures around
100°C when boiling in sodium hydroxide solution. During this time, the fiber properties may have changed resulting in reduced compressibility under more moderate conditions.

Figure 3.2 demonstrates the effect of compression pressure on porosity at different temperatures for pretreated scaffolds. It is evident that the porosities attained at 86 kPa are lower than those reached by applying 38 kPa of pressure at equivalent temperatures. The relationship between compression pressure and porosity alteration in nonwoven fibrous scaffolds can be correlated by the following equation:

\[ P = KY\rho_f^3[(1 - \varepsilon_c)^3 - (1 - \varepsilon_0)^3] \]  

(3.1)

where \( P \) is the pressure, \( K \) is a proportional constant, \( Y \) is the Young’s modulus of the fiber, \( \rho_f \) is the fiber density, and \( \varepsilon_0 \) and \( \varepsilon_c \) are the scaffold porosities before and after compression, respectively (van Wyk, 1946). The first three terms in the equation \((KY\rho_f^3)\) can be assumed constant for these temperatures, and it is defined as the apparent modulus for the scaffold. The data at 121°C and 90°C fit the model well with the results shown in Table 3.1. Due to the increased apparent modulus at 90°C, it takes approximately three times more applied pressure to obtain the same porosity change at 121°C.

The compression of the PET scaffolds altered the pore structure of the fibrous matrix. Using a liquid extrusion method (Miller and Tyomkin, 1994) to determine the pore size distribution within compressed and uncompressed PET scaffolds, the mean effective radii in the two PET scaffold porosities utilized in further experiments are approximately 42 \( \mu \)m for high porosity, uncompressed scaffolds and 32 \( \mu \)m for low porosity, compressed
scaffolds (Basu, 2004). In addition to the pore radius reduction of 24% in the low porosity PET, the distribution of pore sizes was narrower than that of the high porosity PET, which had a broader distribution. The porosity and the effective pore sizes influence the distribution of cells throughout the scaffold during seeding, the development of the tissue morphology, and the transfer of nutrients to the cells growing within the scaffold.

3.4.2 Static Seeding

In static seeding, a cell suspension is introduced into a presoaked PET scaffold from above, after which the cell suspension is absorbed into the scaffold in a sponge-like manner. The cells are allowed to diffuse within the scaffold to available surface attachment sites where cell adhesion can occur. To measure the rate at which cell adhesion occurs within PET under various conditions, cells were seeded statically into scaffolds and allowed to incubate for a time period. At each time, a scaffold was washed to remove all cells that were not attached. The collected cells were counted, and the balance of cells uncounted from the original number seeded were the cells that had become attached. The seeding efficiency was calculated as the percentage of the initial cells added that have been seeded onto the PET. The variables analyzed for effects on adhesion rate were the number of cells seeded, scaffold porosity, scaffold pretreatment, and scaffold presoaking.
3.4.2.1 Effect of Cell Seeding Number

The number of cells seeded statically to high porosity, pretreated PET was varied at the following levels to determine the effect on the adhesion rate: 0.5, 1, 5, 7, and 15 million cells. The results of the seeding are shown in Figure 3.3. For the first 4 hours, the adhesion profiles are similar at all seed number levels. After 4 hours, the adhesion of cells in the 15 million cells trial continued at a lower rate while the other cell seeding amounts continued to increase at the same rates.

The seeding of cells in PET can be represented by first-order kinetics. Mathematically, this is described by the following equation:

\[- \frac{dC}{dt} = kC \quad \text{or} \quad C = C_o e^{-kt}\]  \hspace{1cm} (3.2)

where \(C_o\) and \(C\) are the cell concentrations at the beginning and at a time, \(t\), respectively. The kinetic seeding rate constant is represented by \(k\). The kinetic rate constant can be determined from the slope of the line on a graph of \(-\ln(C/C_o)\) versus time. An example of the first order modeling and kinetic rate constant determination from seeding data is shown in Figure 3.4. The corresponding kinetic rate constants are shown in Table 3.2. The adhesion rate constant for the 15 million cells trial was significantly lower than the other 4 levels, which were statistically similar. This demonstrates that at the highest seed number, the available surface area within the scaffold becomes limiting, and a greater percentage of cells remain in the interstitial space of the scaffold and unattached. The
corresponding cell density at which the 15 million cells trial reached a plateau was $8 \times 10^5$ cells per mL of scaffold, which could represent a maximum seed density. As a result, the adhesion efficiency at 6 hours for the highest cell number seeded was only approximately 40% while the efficiencies at lower seed numbers approached 60%.

3.4.2.2 Effect of Porosity

As the cell suspension is seeded into the fibrous scaffold, the porosity affects the distribution of cells throughout the scaffold by limiting the diffusive mechanism by which the cells navigate the porous structure for attachment sites. The results of static seeding in 2 PET porosities, both pretreated and untreated, are seen in Figure 3.5. For the first 3 hours, the attachment kinetics for both porosities are similar, but variations occur over the final 3 hours. Comparing similar pretreatment pairings (HP/T vs. LP/T; HP/UT vs. LP/UT), the high porosity scaffolds resulted in higher attachment efficiencies in both cases. Figure 3.6 demonstrates an additional difference between static seeding in these two porosities. As seen in Figures 3.6a and 3.6b, cells were able to migrate throughout the high porosity scaffold, from top to bottom, utilizing more of the available surface area for attachment than in the low porosity scaffold, where a large proportion of the cell attachment was seen at the top of the scaffold (Figure 3.6c) and none was seen at the bottom (Figure 3.6d).

Low porosity scaffolds negatively influence the attachment efficiency and the uniformity of cell distribution. Due to the decreased and narrowed effective pore size distribution,
the seeded cells tended to collect near the top surface of the low porosity scaffold. Localization of the cells in this area decreased the available surface area for attachment for the seeded cells. As a fraction of the cells reached attachment sites, the remainder of cells rested near the top of the scaffold in aggregates, unable to penetrate into the scaffold. In high porosity scaffolds, the cells were able to move throughout the scaffold, effectively seeding the scaffold more uniformly and at a higher attachment rate.

3.4.2.3 Effect of Pretreatment

Pretreatment of the scaffolds increased the hydrophilic properties of the PET fiber surface by adding hydroxyl and carboxyl groups to the polymeric chains. This change should make the adhesion of cells to the surface more favorable because of the attractive forces between the hydrophilic PET surface and the hydrophilic cell wall. The effects of pretreatment of two porosities of PET in static seeding are shown in Figure 3.5. For both high and low porosity PET, pretreatment had an enhancing effect on cell adhesion. In high porosity PET, the pretreatment increased the seeding efficiency at 6 hours by 18%. As the cells migrated through the pretreated, high porosity scaffold, the encountered attachment sites were more favorable for immediate attachment, whereas, in the untreated, high porosity scaffold, attachment sites were less favorable for attachment. This resulted in cells positioned for attachment throughout the scaffold, and yet, the attachment was weaker, allowing for those cells to be washed away during the experiment and considered unattached. In the low porosity scaffolds, the effect of pretreatment was less significant than in high porosity with an enhancement of seeding
efficiency at 6 hours of only 9%. The effect of pretreatment was masked by the effect of
the reduced porosity. Since the low porosity did not allow a fraction of the seeded cells to
interact with the scaffold, limiting them to the top surface of the scaffold, the
pretreatment did not have a role in their unattached status.

3.4.2.4 Effect of Presoaking

The wetting of the scaffold fiber surfaces improves the transfer of cells from the bulk
suspension to the fiber surface during seeding. Pretreatment of the scaffolds and the
resulting reduction of the hydrophobic properties also aid in cell attachment by the
recruitment of medium serum proteins to the fiber surfaces. In addition to the hydrophilic
scaffold surface, some serum proteins help facilitate the attachment of cells to the PET.
To analyze the overall effect of presoaking, the PET scaffolds were presoaked in the
serum-containing medium for 1 minute or 6 hours prior to seeding of the cell suspension.
During the presoak incubation, the scaffold surfaces were wetted and the serum proteins
were positioned on the scaffold surfaces. The results of static seeding into high porosity,
pretreated PET are shown in Figure 3.7. The soaking of the scaffold for 6 hours prior to
cell seeding resulted in a 19% increase in attachment efficiency at 6 hours over
presoaking for only 1 minute. Presoaking the scaffolds prior to seeding clearly enhances
attachment efficiency, though the individual contributions to the enhancement by surface
wetness or serum proteins remain unknown.
3.4.3 Dynamic Seeding

In dynamic seeding, a cell suspension is mixed within a spinner flask in which a presoaked PET scaffold is attached to a stainless steel mesh ring. The cells move within the agitated environment, eventually coming into contact with the scaffold. In dynamic seeding, two mechanisms are important: interception and adhesion. Interception is a significant first step in the attachment process because in the mixed environment, the cells will usually first be immobilized within the scaffold before the attractive forces incorporated in the adhesion step can take effect. Interception is integral in increasing the residence time within the fibrous scaffold, enhancing the rate at which cells become attached. Following the initial time at which the cells are introduced to the spinner flask, samples of the cell suspension are removed to quantify all cells that were not seeded. The collected cells were counted, and the balance of cells uncounted from the original number added were the cells that had become seeded within the PET, whether by attachment or temporary interception. The variables analyzed for effects on seeding rate were the agitation rate, scaffold porosity, and scaffold pretreatment.

3.4.3.1 Effect of Agitation Rate

The agitation rate within the spinner flask during dynamic seeding influences the penetration capability of the cell suspension into the scaffolds. Three rates were analyzed for their effect on dynamic cell seeding: 40, 80, and 120 rpm. At 40 rpm, the agitation was not sufficient to keep the cells suspended within the medium. As a result, the cells
settled at the bottom of the spinner flask, disallowing the seeding of the cells onto the PET scaffold. The seeding profiles for dynamic seeding at 80 and 120 rpm in HP/T PET are shown in Figure 3.8. At 120 rpm, penetration of the cell suspension into the scaffold was attained with cells being intercepted within the scaffold structure. However, the agitation rate was too high, causing the release of many intercepted cells before attachment could occur. This resulted in a seeding profile with larger variability and lower seeding efficiency than those achieved at 80 rpm. The seeding profile at 80 rpm was approaching 100% seeding efficiency by 12 hours. Detachment of cells previously seeded to the scaffold was not suspected at 80 rpm. In the control experiment with no scaffolding and agitation at 80 rpm, the cells stayed in suspension for 13 hours, demonstrating effective mixing at 80 rpm. Dynamic seeding at 80 rpm also resulted in a uniform distribution of cells throughout the scaffold due to mixing. The results of dynamic seeding into HP/UT PET after 13 hours are seen in Figure 3.9. During dynamic seeding, the cells were seeded deep within the scaffold with only a limited amount of cell seeding at the exterior surfaces of the scaffold where interception was less likely.

3.4.3.2 Effect of Porosity

The porosity and affiliated effective pore size distribution within the fibrous scaffold are important for allowing access of the mixed cells to the interior surface area of the scaffold and for intercepting the cells to facilitate subsequent adhesion. The seeding results of high and low porosity PET scaffolds, pretreated and untreated, are shown in Figure 3.10. Both high porosity scaffold types resulted in higher seeding efficiencies after
7 hours compared to low porosity scaffolds. Intuitively, a lower porosity scaffold should provide smaller pores and more fiber clusters, thus increasing the amount of interception that occurs and the seeding efficiency. However, in this case, the low porosity (0.88) scaffold was too compact to allow bulk flow of the cell suspension through the scaffold. The cells were able to be intercepted at the outer surface of the low porosity scaffolds, but the inner surface areas for attachment were not utilized. Cells that were intercepted at the outer surface either quickly returned to the bulk suspension, unseeded, or penetrated enough into the scaffold for seeding. As a result, the seeding efficiencies of low porosity scaffolds were lower than those in high porosity scaffolds, which allowed flow, and seeding, throughout the scaffold.

3.4.3.3 Effect of Pretreatment

Similar to static seeding, pretreatment of the scaffolds increases the hydrophilic properties making the adhesion of cells to the surface more favorable because of the attractive forces between the hydrophilic PET surface and the hydrophilic cell wall. Once the cells are intercepted during dynamic seeding, the pretreatment status plays a significant role in completing the attachment of the cells to the scaffold. The effects of pretreatment of two porosities of PET in dynamic seeding are shown in Figure 3.10. For both high and low porosity PET, pretreatment had an enhancing effect on cell adhesion. In high porosity PET, the pretreatment increased the seeding efficiency at 7 hours by 33%. Pretreatment enhanced seeding efficiency to a much lesser extent in low porosity scaffolds at 10%. Pretreatment had a significant effect in the high porosity scaffolds due
to the flow present through the scaffold pore structure. In a mixed environment, the cells had a reduced residence time inside of the scaffold and at interception sites. With the forces of the fluid flow working against the adhesive mechanism, the attractive, hydrophilic surfaces of the pretreated scaffolds ensured cell adhesion whereas detachment due to fluid flow was more prevalent in untreated, high porosity scaffolds. In the low porosity scaffolds, fluid flow forces were not as significant because, as mentioned earlier, bulk flow through low porosity PET was limited compared to that in high porosity PET. Therefore, if a cell was able to penetrate the outer surfaces of the low porosity scaffold, it was shielded from the destructive flow forces and able to attach more similarly in pretreated and untreated scaffolds. The detachment phenomenon in untreated, high and low porosity scaffolds is seen in Figure 3.10 by the higher variability at each time point compared to the more compact error bars for the pretreated scaffolds. More fluctuation was observed in the high porosity scaffold than in the low porosity PET due to the allowance of fluid flow through the scaffold.

All four PET scaffold types were attached to the wire mesh and seeded in a spinner flask for 5 hours. At that time, the scaffolds were removed, and the cells within each scaffold type were counted. The results, shown in Figure 3.11, further demonstrate the selective ranking of seeding within each type of scaffold in dynamic seeding with HP/T scaffolds being the most favorable for seeding.
3.4.4 Filter Seeding

In filter seeding, the fibrous scaffold acts as a filter for the cells as the cell suspension flows directly through the scaffold in a recycling circuit. Interception and adhesion are the mechanisms for seeding just as in dynamic seeding. The advantage of filter seeding is that the cells are forced to pass through the scaffold, whereas in dynamic seeding, the cells can follow the fluid flow currents around the spinner flask for a longer time before eventually contacting the scaffold. The resulting seed profile for the one trial is shown in Figure 3.12. Filter seeding was shown to be highly effective at removing the cells from suspension since the seeding efficiency approached 100% in only 5.5 hours. However, the distribution of the cells throughout the stack of 10 PET discs was unknown.

3.4.5 Comparison of Seeding Methods

The effectiveness of a seeding method can be measured by the rate at which the seeding occurs, the uniformity of the cell distribution in the scaffold, and the maximum seed density. Table 3.3 summarizes the important parameter results from the three seeding methods. Comparing seeding rate constants of dynamic and static seeding, the high porosity rate constants are higher for dynamic seeding since the incorporated fluid flow allowed for full utilization of the available surface area and inclusion of interception as a mechanism for cell seeding whereas static seeding only utilized adherence properties for seeding. The rate constants for low porosity scaffolds decreased from static to dynamic seeding. Since flow through the low porosity scaffolds was limited, the surface area
utilized for seeding in the dynamic method was less than that used in the static method. Overall, the dynamic method seeding efficiencies were higher than the static method efficiencies due to the inclusion of intercepted cells counted as seeded in the spinner flasks. Cells had to be attached to the scaffold surface and not merely intercepted in the static seeding experiments for the cells to be considered seeded.

Pretreatment of PET had more of an effect on the attachment rate constant and the seed efficiency for high porosity scaffolds in dynamic seeding compared to static seeding. This is due to pretreatment allowing for quicker adherence in the mixed environment where counteractive forces attempt to detach the cells. The determined rate constants for untreated PET are actually effective rate constants that are a combination of attachment and detachment rates. In low porosity scaffolds, the pretreatment effect was lessened in dynamic seeding since flow through the scaffold was not achieved. Further, the effect of porosity was more substantial in dynamic seeding than in static seeding due to the importance of having penetration of cells via fluid flow for efficient usage of the interior surface area for attachment.

The distribution of cells throughout the scaffolds was optimized in high porosity scaffolds during dynamic seeding. In static seeding, cells were seeded entirely through the high porosity scaffolds, yet distribution was mainly one-dimensional, with diffusion occurring in the direction of gravity from the top seeding site down towards the bottom of the scaffold. Lateral diffusion to additional available surface area was limited. Low
High porosity scaffolds resulted in poor distribution of cells, mainly limited to the outer layers of the fibrous scaffold due to the reduced effective pore distribution and the limitations on bulk cell suspension flow in dynamic seeding.

Filter seeding resulted in the highest attachment rate constant and seeding efficiency. One drawback of this method, however, is that it is the most time-intensive method to prepare and maintaining sterility following seeding is difficult. The optimal scaffold type for all methods of seeding was the high porosity, pretreated PET scaffold. The maximum allowable seeding density varies by method as well, with dynamic and filter methods being higher than the static method. The maximum seed density for the static method is limited by the volume of cell suspension allowable for injecting into the scaffold. For these experiments, a maximum volume of 200 µL was observed since application of a higher volume would result in the cell suspension flowing directly through the scaffold and the loss of the cells to the bottom of the well. Therefore, only a limited amount of cells can be effectively suspended in this small volume of medium. The maximum seed densities of the dynamic and filter seeding methods are limited by the time that the cells must remain in suspension and the nutrients available in the medium. The fast seeding rate of the filter method makes these less of a concern and allows for a higher seeding density than dynamic seeding does.
3.4.6 Comparison of Cell Counting Methods

For two-dimensional (2-D) plate cultures, there are many methods that can be applied to count the number of cells present at any time. Enzymatic detachment of the cells from the surface using a product like trypsin or Accutase is the direct method since the collected cells can be counted on a hemacytometer. Indirect methods, such as the DNA or protein assay, involve lysis of the cells and quantification of cell number by relating total DNA or protein content collected to the actual cell number. To do this, the total DNA or protein per cell is necessary. These methods are all reliable tools for determining cell number in 2-D culture. A result of using the three methods to monitor cell growth in a plate culture over 10 days is shown in Figure 3.13. All three methods perform fairly well in representing the common growth curve expected during this time period. In 2-D cultures, the reagents utilized are able to make direct contact with all of the cells in the culture, and collection of the desired components is unabated. However, in 3-D cultures, the scaffolding introduces a challenge for accurate cell counting. Cell cultures in 3-D scaffolds develop a complex interior morphology, and removing cells from this environment for direct quantification has yet to be optimized. An accurate cell counting method is necessary, however, to be able to describe the growth in a 3-D model. Three methods were analyzed: Accutase treatment, DNA assay, and protein assay, with two methods of cell lysis (buffer lysis and freezing) utilized for the indirect methods.

The effectiveness of the methods to recover the number of cells seeded to PET 12 hours earlier is demonstrated in Figure 3.14. The DNA assay is not present in the results due to
negligible cell recovery using either cell lysis method. When DNA is released from lyzed cells, it aggregates with other DNA in solution. In this case, DNA probably clumped together forming a sticky aggregate. The PET was pretreated making it hydrophilic. The DNA was attracted to the PET surface, and this made it difficult to remove the DNA from the scaffold for quantification. Therefore, the DNA assay was ineffective at counting the cells from 3-D culture.

Results were obtained from both cell lysis methods using the protein assay. However, the standard deviations of the cell counts were quite large, demonstrating the lack of reproducibility using this method. The effectiveness of recovering the cells dropped significantly for higher cell numbers as well. Neither cell lysis method was significantly different from the other.

Cell quantification using three treatments of Accutase was the most effective method analyzed. The cell counts were relatively reproducible at all cell numbers. The recovery of cells over the range of cell seeding numbers was approximately constant at 45%. The Accutase method was able to collect the most cells at the highest cell number, which is useful for high cell density cultures encountered in 3-D models. Additional advantages of using Accutase are that the cells are collected intact, so they could be reused if desired, and that the viability of the culture can be determined as well using trypan blue exclusion.

The actual success of the Accutase method may be limited, however. In these experiments, the cells grew in the 3-D culture for only 12 hours before being counted.
After an extended culture time, the 3-D morphology will develop making it more difficult for Accutase to interact with the cell population. For example, Figure 3.15 shows the application of the Accutase method for 6 treatments on HP/UT and LP/UT PET scaffold cultures after 6 days. For both culture types, a maximum is attained during the fourth or fifth Accutase treatment, demonstrating the robustness of the 3-D morphology against enzymatic detachment. Furthermore, the actual cell number in both cultures is unknown. The Accutase method can be used to make relative comparisons of cell number between cell cultures, but no absolute determinations can be made.

3.5 Conclusion

In order to study the growth of a cell culture within a 3-D scaffold environment, it was necessary to alter the scaffold properties, investigate the seeding of cells into the scaffold, and to find a method for quantifying growth within the 3-D model. PET was modified by thermal compression and basic pretreatment to reduce porosity and effective pore sizes and to make the scaffold surface more hydrophilic, respectively. A range of porosities were obtained, from 0.94 to 0.84, by applying different temperatures and pressures over different time durations. The effect of temperature on the porosity change was quantified with an apparent modulus relation.

Four types of scaffolds were utilized in three seeding methods to determine optimal conditions for seeding the 3-D model. High porosity (0.94), pretreated PET scaffolds provided the best results in all three methods, allowing for high seeding rates and a
uniform distribution of cells throughout the scaffold. Porosity and pretreatment had significant, enhancing effects on cell seeding in the dynamic seeding method due to the fluid flow involved. Dynamic and filter seeding methods were optimal due to the mixing and flow involved, leading to improved distribution of cells and higher cell densities.

The methods used for cell counting of 3-D cultures turned out to be suboptimal in all cases. The DNA assay was unable to quantify cells, and the protein assay provided fluctuating results and low recovery at higher cell counts. The Accutase treatment worked the best, providing approximately 45% cell recovery at all cell numbers seeded. Multiple, time-intensive treatments were necessary, and applicability is limited when used for longer duration 3-D cultures. The Accutase method will be utilized for relative cell counts, however. A novel, fast, and noninvasive method for quantifying cell number in 3-D constructs by relating cell number to transfected green fluorescent protein fluorescence is discussed in Chapter 5.
3.6 References

Basu, S. 2004. “Effects of Three Dimensional Structure of Tissue Scaffolds on Animal Cell Culture.” PhD Dissertation. The Ohio State University, Columbus, OH.


Table 3.1: Effect of temperature and pressure on pretreated PET porosity and resulting apparent modulus for each temperature

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Pressure (kPa)</th>
<th>Porosity</th>
<th>Apparent Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>38</td>
<td>0.88</td>
<td>27239</td>
</tr>
<tr>
<td>121</td>
<td>86</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>38</td>
<td>0.91</td>
<td>84312</td>
</tr>
<tr>
<td>90</td>
<td>86</td>
<td>0.89</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Effect of temperature and pressure on pretreated PET porosity and resulting apparent modulus for each temperature
<table>
<thead>
<tr>
<th>Cell Seeding Number (million cells)</th>
<th>Attachment Rate Constant, $k$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.159 ± 0.001</td>
</tr>
<tr>
<td>1</td>
<td>0.150 ± 0.012</td>
</tr>
<tr>
<td>5</td>
<td>0.165 ± 0.008</td>
</tr>
<tr>
<td>7</td>
<td>0.145 ± 0.005</td>
</tr>
<tr>
<td>15</td>
<td>0.109 ± 0.002</td>
</tr>
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</table>

Table 3.2: Attachment rate constants for static seeding of various cell seed numbers into high porosity (0.94), pretreated PET. Ranges provided indicate standard deviation.
<table>
<thead>
<tr>
<th>Seeding Method</th>
<th>Scaffold Type</th>
<th>Attachment Rate Constant, $k$ (hr$^{-1}$)</th>
<th>Seed Efficiency (%)</th>
<th>Uniformity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td>HP/T</td>
<td>0.149 ± 0.006</td>
<td>56.1 ± 3.0</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>HP/UT</td>
<td>0.107 ± 0.006</td>
<td>38.7 ± 2.0</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>LP/T</td>
<td>0.107 ± 0.003</td>
<td>41.5 ± 1.1</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>LP/UT</td>
<td>0.074 ± 0.008</td>
<td>31.3 ± 3.8</td>
<td>Poor</td>
</tr>
<tr>
<td>Dynamic</td>
<td>HP/T</td>
<td>0.273 ± 0.007</td>
<td>87.8 ± 1.9</td>
<td>Great</td>
</tr>
<tr>
<td></td>
<td>HP/UT</td>
<td>0.108 ± 0.008</td>
<td>48.3 ± 0.0</td>
<td>Great</td>
</tr>
<tr>
<td></td>
<td>LP/T</td>
<td>0.079 ± 0.006</td>
<td>41.3 ± 1.8</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>LP/UT</td>
<td>0.064 ± 0.005</td>
<td>33.0 ± 0.0</td>
<td>Poor</td>
</tr>
<tr>
<td>Filter</td>
<td>HP/T</td>
<td>0.494 ± 0.000</td>
<td>95.0 ± 0.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.3: Seeding results for three seeding methods and 4 scaffold types: HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Seeding efficiency determined at 6 hours following seeding. Uniformity describes the extent of seeding throughout the scaffold. Ranges provided indicate standard deviation.
Figure 3.1: PET compression at: a) 121°C, b) 90°C, and c) 60°C
Figure 3.2: PET porosities after compression at: a) 86 kPa and b) 38 kPa
Figure 3.3: Static seeding of various cell seeding numbers in high porosity (0.94), pretreated PET. Error bars indicate standard deviation.
Figure 3.4: Static seeding of 500,000 cells in high porosity (0.94), pretreated PET. a) Cells remaining unseeded with time. b) Percentage of cells seeded with time. c) Determination of the first order seeding rate constant, $k$, by plotting the negative natural logarithm of the ratio of unseeded cell concentration ($C$) to original cell concentration ($C_0$) versus time and using a linear fit.
Figure 3.5: Cell attachment by static seeding in 4 types of PET. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error bars indicate standard deviation.
Figure 3.6: Scanning electron microscopy photographs of cells 2 days after statically seeded onto PET scaffolds of: a) high porosity (0.94), top view, b) high porosity (0.94), bottom view, c) low porosity (0.88), top view, and d) low porosity (0.88), bottom view
Figure 3.6 (continued)
Figure 3.7: Effect of presoak time on static seeding of cells in high porosity (0.94), pretreated PET scaffolds. Error bars indicate standard deviation.
Figure 3.8: Dynamic seeding in high porosity (0.94), pretreated PET at 2 agitation rates. Error bars indicate standard deviation.
Figure 3.9: Dynamic seeding in high porosity (0.94), untreated PET at 80 rpm after 13 hours shown by scanning electron microscopy. (a) 100x magnification; (b) 350x magnification
Figure 3.10: Cell seeding by dynamic seeding at 80 rpm in 4 types of PET. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error bars indicate standard deviation.
Figure 3.11: Seeding selectivity in 4 types of PET after 5 hours of dynamic seeding. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error bars indicate standard deviation.
Figure 3.12: Filter seeding in high porosity (0.94), pretreated PET. a) Cells remaining unseeded with time. b) Percentage of cells seeded with time.
Figure 3.13: Cell quantification of two-dimensional plate cultures using three methods. Error bars indicate standard deviation.
Figure 3.14: Comparison of the cell counting capabilities of three methods from three-dimensional, high porosity (0.94), and pretreated PET scaffold cultures at three cell seeding numbers. Error bars indicate standard deviation.
Figure 3.15: Application of Accutase treatments after 6 days of spinner flask culture in a) high porosity (0.94), untreated PET and b) low porosity (0.88), untreated PET
CHAPTER 4

ANALYSIS OF COLON CANCER GROWTH AND MORPHOLOGY IN AN
IN VITRO THREE-DIMENSIONAL MODEL OF VARYING SCAFFOLD
PROPERTIES AND CULTURE ENVIRONMENTS

4.1 Abstract

Tissue engineering combines knowledge obtained from biological fields and applies it using engineering skills to repair, regenerate, or enhance three-dimensional (3-D) tissues and organs both in vivo and in vitro. When cancer is studied in the lab setting, two-dimensional (2-D) models and animal models are utilized for analyzing drug therapies and for cancer developmental mechanism studies. Since 2-D cultures are missing this important structure, differences in cell morphology, growth kinetics, function, and expression have all been reported when comparing 2-D and 3-D cultures. For these reasons, an in vitro model based on 3-D culture should better represent the in vivo environment. The cancer tissue cultivated in the 3-D model would mimic the in vivo counterpart for better depiction of cancer progression and more meaningful reactions to stimuli in cancer assays.
The culture of colon cancer within three in vitro environments was performed to characterize the effects of scaffold parameters and environment design on the growth and developing morphology. The three culture environments investigated were static multiwell plate, spinner flask and perfusion bioreactor. The scaffolding utilized to house the cells, nonwoven, fibrous poly(ethylene terephthalate) (PET), was varied by porosity and surface hydrophilic status.

In multiwell culture, the porosity and pretreatment of PET had significant effects. In high porosity PET, the growth was characterized by mainly fiber spreading and aggregate development for 6 days before interfiber bridging was possible. This resulted in faster cell population expansion compared to low porosity PET, which was characterized with an increased extent of 3-D morphology due to reduced interfiber distances, resulting in slower proliferation. Pretreatment of PET scaffolds significantly affected the early growth in high porosity scaffolds since fiber spreading was the main mechanism for growth, and untreated surfaces inhibited cell growth. Low porosity, pretreated scaffolds had more significant growth than in untreated scaffolds at 6 days of growth.

Spinner flasks provided a mixed environment that enhanced the metabolic yield in high porosity scaffold cultures over low porosity cultures. Agitation at 80 rpm improved the metabolic status of high porosity culture over that attained at 40 rpm. The flow in a perfusion bioreactor provided the most aerobic conditions supporting a high cell population expansion. The optimal 3-D in vitro model based on these results would be a
low porosity, pretreated PET scaffold within the mixed environment of a spinner flask or perfusion bioreactor because of the speed at which the 3-D morphology would develop and the maintenance capability of the high cell density.

4.2 Introduction

Tissue engineering combines knowledge obtained from biological fields and applies it using engineering skills to repair, regenerate, or enhance tissues and organs both *in vivo* and *in vitro*. Over 20 tissues have been studied for numerous purposes including bone regeneration, recreated heart valves and vessels, skin substitutes, organ transplantation, and wound healing (Langer, 2000; Lysaght et al., 2001; Tabata, 2001; Geer et al., 2002; Griffith and Naughton, 2002). Additional applications that incorporate tissue engineering tools include drug delivery, directed stem cell differentiation, and gene therapy (Langer, 1999; Fukuda, 2001; Xie et al., 2001). The development of *in vitro* physiological models, however, may have the greatest impact in the next decade (Griffith and Naughton, 2002).

When cancer is studied in the lab setting, two-dimensional (2-D) models and animal models are utilized for analyzing drug therapies and for cancer developmental mechanism studies. Animal models have been used successfully to predict human cancer risks and carcinogen effects and to study cancer development, but differences in anatomy between the animal and humans and their effect on the interactions between the body and the cancer, in addition to public scrutiny regarding animal usage, leave animal models as an insufficient option (Meijers et al., 1997; Schiffer, 1997).
Cultures on Petri dishes and polymeric films are examples of 2-D cultures that are commonly employed in cancer studies. Colon cancer cells growing on a 2-D film surface and the common growth curve for a 2-D culture are shown in Figure 4.1. Growth is described by four stages. A lag phase may occur at the beginning of a culture, when the cells are adapting to new conditions, and this results in limited cell growth. The log phase occurs as the cell population expands exponentially and is characterized by healthy cell growth as the cells spread out across the available surface area. As surface confluence approaches, the stationary phase is entered in which cell-cell contact inhibition results in limited growth. At this stage, the growth rate and death rate of the cell population approach equivalence, resulting in a plateau of the growth curve. Following surface confluence, the cell population is unable to expand due to the lack of 2-D surface area, and cells begin to die. The death phase is characterized by the drop in cell population.

A key component of tissue engineering applications in developing functional in vivo-like tissue is the presence of a three-dimensional (3-D) scaffold. 3-D scaffolds can provide a larger amount of surface area for growth than the 2-D culture methods, while also allowing for growth into the interstitial spaces as the cell population expands. The scaffold also allows for proper morphological attributes of cells and population arrangement, heterogeneous tumor development, cell-cell and cell-scaffold interactions and communication, and differentiated function (Heppner et al., 1998; O’Conner, 1999; Li et al., 2001a). Since 2-D cultures are missing this important structure, differences in cell morphology, growth kinetics, function, and expression have all been reported when
comparing 2-D and 3-D cultures (Haramaki, 1993; O’Conner et al., 1997; Zvibel et al., 1998). For these reasons, an in vitro model based on 3-D culture should better represent the in vivo environment. The cancer tissue cultivated in the 3-D model would mimic the in vivo counterpart for better depicting of cancer progression and more meaningful reactions to stimuli in cancer assays.

The materials utilized as scaffolds in tissue engineering applications are usually foam-like or fibrous materials. Foam-like materials include foams, sponges, and gels. Commonly utilized examples include PGA, PLA, PGLA, PLGA, collagen, and gelatin (Nehrer et al., 1997; Harris et al. 1998; Kang et al., 1999; Ranucci et al., 1999). Hybrids can also be developed that utilize the advantages of each component (Chen et al., 2000). Fibrous matrices are usually made of PGA, PLA, PET, polypropylene, and polyethylene (Freed et al., 1994b; Ma et al., 1999). Fibrous matrices are arranged as woven, non-woven, or knitted. Several factors are used to match the appropriate scaffold material with the application: biocompatibility, biodegradability, pore characteristics, mechanical strength, availability, and cost of fabrication. Foam-like scaffolds have the advantage of being customizable in fabrication with direct control of microstructure. Fibrous scaffolds have higher porosity with availability of nutrients to cells throughout the scaffold and a greater specific surface area for growth allowing for high cell densities (Ma et al., 1999; Li et al., 2001a).

The cell-scaffold interactions that determine proper tissue development and function take place on multiple levels: macro-, micro-, and nanoscales. These issues include seeding,
cell adhesion, morphogenesis, cell proliferation, differentiation, apoptosis, gene expression, and tissue function. At the macroscale, the scaffold properties can affect tissue shape and function, cell population proliferation and differentiation, and construct mechanical strength. Scaffolds are designed to be in a shape that guides shape-specific tissue growth to reconstitute a functional tissue such as a bladder, blood vessel, or heart valve (Niklason et al., 1999; Langer, 2000; Griffith and Naughton, 2002). The thickness of a scaffold affects nutrient transport within the scaffold. As thickness increases, diffusion of nutrients to the depths of the scaffold is minimal for sufficient nutrient supply. In a study of chondrocytes, cell proliferation decreased as matrix thickness increased (Freed et al., 1994a). Differentiation status of cultured cells can be influenced by the degree of structural order in the scaffold (Hall et al., 2001). The mechanical strength of the scaffold determines the extent to which compression of the construct occurs. A scaffold with insufficient strength would compact to the extent that nutrient transport would be limited at an accelerated rate. Studies of PLLA and PLGA blends resulted in different anti-compressive properties with PLLA and PLGA (85:15) supporting forces for several weeks (Eiselt et al., 1998).

At the microscale, the scaffold properties affect seeding adhesion, morphogenesis, proliferation, differentiation, and subsequent tissue function. In fibrous scaffolds, the fiber diameter and affiliated surface curvature affect the spreading ability of attached cells. Spreading allows cells to increase proliferation, and this is regulated by fiber dimensions. Additionally, the diameter affects the degree of cell-cell interactions allowable around the fiber which are necessary for proper tissue function. The porosity,
and related pore size distribution, of a scaffold has many cumulative effects on the cell population. The morphogenesis of the developing tissue is influenced by the allowable migration of cells. The pore size distribution relates to the migration ability because it determines the amount of space available. The migration of endothelial cells was inhibited when pore diameter was reduced to 9 \( \mu \text{m} \) (Matsuda and Nakayama, 1996). Optimal pore sizes were found for enhanced bone ingrowth to establish homogeneity within the developed tissue (Chang et al., 2000). Additionally, the pore configuration played a role in the bone culture with cylindrical pores leading to bone development with higher compressive strength than with sponge pores. The available space in the scaffold determined by porosity is important for allowing cell-cell contacts and interaction. Porosity has been shown to improve the degree of cell-cell contacts by the increased expression of E-cadherin, a cell-cell adhesion protein, in hepatocytes (Moghe et al., 1996). Customizing the surface with microgrooves can affect the cell orientation to control cell-cell contact networks such as in the establishment of a neural network.

At the nanoscale, the molecular interactions between the cell and the scaffold influence many key cell functions such as adhesion, morphogenesis, proliferation, differentiation, gene expression, apoptosis, and the resulting tissue function. Natural biomaterials are advantageous for cell adhesion and interaction with cells because of inherent binding domains and functional groups used to interact with cell surface receptors. Synthetic scaffolds have altered surface chemistry to improve interactions. The amino acid sequence of Arg-Gly-Asp (RGD) has been identified on fibronectin and other \textit{in vivo} extracellular matrix glycoproteins as a key adhesion domain, and the design of synthetic
scaffolds incorporating the peptide is successful in improving adhesion (Griffith and Naughton, 2002). Patterning of the surface and using microcontact printing techniques design surfaces to have sites of different sizes and functionalities to control cell size and spreading. Larger areas promoted cell spreading and increased proliferation while smaller areas induced differentiation and apoptosis in hepatocytes and endothelial cells, respectively (Chen et al., 1998; Singhvi et al., 1994). To improve cell adhesion by increasing synthetic polymer surface hydrophilicity, scaffolds are pretreated by hydrolyzing with sodium hydroxide to create carboxyl and hydroxyl groups on the surface of PET (Phaneuf et al., 1997). Migration is a dynamic process involving adhering and detaching mechanisms working in unison. The migration on fibronectin is performed by cells adhering to integrin adherence domains such as RGD at the advancing front of the cell and detaching from heparin domains via proteoglycan receptors at the rear (Martins-Green, 2001).

There are several types of bioreactor designs utilized for cultivating new tissue growth. Bioreactor models for tissue engineering evolved from bioreactor designs utilized for mammalian cell culture to maximize productivity on large scales using microcarrier and spinner flask systems, for example (Freed et al., 1997; Ma, 1999). The simplest design is the plate or Petri dish. The spinner flask is larger in scale and has an internal agitation mechanism to provide a uniform nutrient concentration within the medium and the enclosed cell-scaffold construct. The development of microgravity systems, such as the High-Aspect Ratio Vessel (HARV), were used to reduce the shear stress encountered by cells as the culture was sustained in suspension (Akins et al., 1997). The environment
within may be static or mixed using internally designed or externally applied mechanisms. Static environments rely on diffusion as the mass transfer mechanism for nutrient supply. Mixing within a culture vessel provides convective flow of oxygen and nutrients to the cells while removing waste from the surroundings. Mixing-induced shear stress may cause cell death if mixing is too high though.

The fibrous bed bioreactor is utilized for the cultivation of cell populations for bioproduction of molecules or in vitro modeling. Perfused bioreactors are a type of fibrous bed bioreactor in which the nutrient medium is circulated within a closed system and flows through the immobilized cell-scaffold component. Utilizing the 3-D culture advantages previously described, a perfusion bioreactor is a continuous culture design that allows for consistent medium supply with control over important culture conditions, such as temperature, pH, and dissolved oxygen concentration. The system can be designed to mimic in vivo environmental conditions for optimized culture. The fibrous bed bioreactor with continuous culture allows for more efficient culture and possible scale-up for industrial design (Ma et al., 2000). Long-term stability of the culture is attained using continuous perfusion reactors (Freed et al., 2000). A continuous bioreactor will ensure constant nutrient supply, stable pH, and reduced contamination (Sittinger et al., 1996).

The development of an in vitro 3-D model for colon cancer would allow for the study of cancer development and drug therapy screening without the reliance on inferior 2-D and animal models. With cancer research increasing in prominence, such a model would
allow for developmental studies yet to be fully understood such as angiogenesis and metastasis. When applied to drug screening, the efficacy and mechanism of drug activity can be investigated with accuracy using the *in vivo*-like model. In this research, the growth and developing morphology of colon cancer within various 3-D scaffolds and culture environments are investigated.

4.3 Materials and Methods

4.3.1 Cells and Medium

The colon cancer cell line HT-29 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and is utilized in all of the experiments. HT-29 is a human adenocarcinoma, grade I cancer cell type of colonic epithelial origin. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose concentration, L-glutamine, and sodium pyruvate (Caisson Laboratories, Rexburg, ID). The medium was supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY).

4.3.2 Scaffold

Nonwoven, fibrous poly(ethylene terephthalate) (PET) fabric, known commercially as Dacron, was used as the 3-D culture support scaffolding. PET (fiber diameter: 20 µm; fiber density: 1.35 g/mL; porosity: 0.94) is a randomly organized, isotropic matrix with
advantages of being inexpensive yet providing high specific surface area for cell growth and high porosity for cell growth and nutrient accessibility. PET film was utilized in 2-D growth studies.

The surface of the PET was modified to improve serum protein and cell adhesion to the scaffold by hydrolyzing the PET in boiling sodium hydroxide solution. The scaffolds were washed in a solution of 5 g sodium bicarbonate and 5 mL Tween 20 in 500 mL of distilled water at 60°C for 30 minutes, followed by a 10 minute rinse in a 60°C water bath. The scaffolds were then hydrolyzed in boiling 1% sodium hydroxide solution for 45 minutes. Finally, the scaffolds were washed again at 60°C in a water bath for 10 minutes.

A thermal compression method (Li et al., 2001b) was used to obtain PET scaffolds with a porosity of 0.88. PET was compressed under 38 kPa of load at 121°C for 30 minutes. Both pretreated and non-pretreated scaffolds were compressed. Porosity, the void volume fraction, was determined from the difference between the total bulk scaffold volume and the solid, fiber volume. The fiber volume was obtained from the scaffold mass and the fiber density.
4.3.3 Cell Culture

4.3.3.1 Multiwell PET Film Culture

The PET film discs (diameter: 2.3 cm) were placed in 12-well plates and incubated overnight in culture medium. Before seeding the cells, the medium was removed. The cells were seeded onto the presoaked PET at a concentration of 91,200 cells/cm² of surface area (346,000 cells for 12-well plate wells). Medium was added at a ratio of 1.52×10⁵ cells/mL medium (2.3 mL for 12-well plate wells). Cell cultures were incubated within a pH-buffering 5% CO₂ environment at 37°C.

4.3.3.2 Multiwell PET Scaffold Culture

Four scaffold types were used: high porosity (0.94), pretreated (HP/T), high porosity, untreated (HP/UT), low porosity (0.88), pretreated (LP/T), and low porosity, untreated (LP/UT). The PET scaffolds (diameter: 2.3 cm) were placed in 12-well plates and incubated overnight in culture medium. Before seeding the cells, the medium was removed. The cells were seeded onto the presoaked PET at a concentration of 91,200 cells/cm² of surface area (456,000 cells for PET in 12-well plates) in a volume below 200 µL to prevent saturation of the scaffold. Following incubation at 37°C for 6 hours, medium was added at a ratio of 1.52×10⁵ cells/mL medium (3 mL for PET in 12-well plates). Cell cultures were incubated within a pH-buffering 5% CO₂ environment at 37°C.
4.3.3.3 Spinner Flask PET Scaffold Culture

Spinner flask experiments were performed in 100 mL spinner flasks (Bellco Glass, Vineland, NJ). Prior to usage, the spinner flask inner surfaces were siliconized with Sigmacote (Sigma Chemical, St. Louis, MO) to provide selective advantage for cell seeding to the PET rather than to the glass. Four scaffold types were used: high porosity (0.94), pretreated (HP/T), high porosity, untreated (HP/UT), low porosity (0.88), pretreated (LP/T), and low porosity, untreated (LP/UT). The PET scaffold was cut in 2 square pieces of 1.5 cm length and sewn onto a wire mesh ring opposite each other before positioning the mesh with PET at the bottom of the flask. The spinner flask was seeded maintaining the ratios of 91,200 cells/cm² of surface area (1.083x10^7 cells for PET in spinner flasks) and 1.52x10^5 cells/mL medium (72 mL for PET in spinner flasks). To allow for seeding, the spinner flask was mixed at 80 rpm for 12 hours by placement on a magnetic stir plate within a 37°C and 5% CO₂ incubator. For cell growth analyses, the spinner flask was mixed at the prescribed revolution rate of 40, 80, or 120 rpm.

4.3.3.4 Perfusion Bioreactor

The perfusion bioreactor was set up as shown in Figure 4.2. Included in the bioreactor column were 10 stacked high porosity (0.94), pretreated PET discs (diameter: 2.7 cm). The medium (200 mL) was circulated upwards through the perfusion bioreactor column and around to the stirred, jacketed medium holding tank by a pump. The temperature was maintained at 37°C by a water bath. The pH and dissolved oxygen were maintained by
PID control at 7.0 and 70% of air saturation, respectively, by the Bioflow 3000 controller (New Brunswick Scientific, Edison, NJ). Cells were added to the system via an injection port and recirculated at a superficial velocity of 0.16 mm/s.

4.3.4 Culture Analyses

4.3.4.1 Metabolism

Metabolism of the cell population was monitored by measuring the glucose uptake and lactate production of the culture. 200 μL samples were taken from multiwell plate cultures for glucose and lactate concentration analyses using a YSI Biochemistry Select Analyzer (Yellow Springs, OH). The sample sizes for spinner flask and perfusion bioreactor cultures were 0.5 mL and 3 mL, respectively.

4.3.4.2 Cell Counting

Cell number was quantified using Accutase (Innovative Cell Technologies, San Diego, CA) to release the cells from the PET scaffolds. The medium was removed, and the scaffolds were washed 2 times with 2 mL phosphate buffered saline (PBS) by pulsing the PBS through the scaffold with a pipette 10 times for each wash. The PBS portions were collected together from the wells and set aside while the scaffolds were submerged in Accutase and incubated at 37°C. The plates were mixed gently at 10 minutes. After 20 minutes, the plates were removed from the incubator, and the Accutase was pulsed 15
times through the scaffold to release and collect the cells from the scaffold. The Accutase/cell suspension was added to the PBS wash portions, and the cells were counted by hemacytometer.

4.3.4.3 Cell Cycle

To analyze the distribution of cells within the cell cycle stages, cells were harvested by Accutase treatment and fixed in 5 mL of 70% ethanol solution and stored at -20°C until all samples were collected. The day of analysis, cells were resuspended in PBS at a concentration of $10^6$ cells/mL and stained with 500 µL of propidium iodide / RNase solution for each $10^6$ cells. Following a 15-minute stain incubation, the cells were analyzed using a FACS Calibur analyzer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and ModFit software (Verity Software House, Topsham, ME).

4.3.4.4 Scanning Electron Microscopy

Scaffolds containing cells were washed with PBS and fixed in 2.5% glutaraldehyde solution for 12 hours at 4°C. Samples were then washed with distilled water and dehydrated for 20 minutes in solutions of ethanol with concentrations increasing from 20% to 100% with increments of 10%. Following dehydration, the samples were dried in four successively increasing concentrations of hexamethyldisilazane (HMDS) with ethanol (1:3, 1:1, 3:1, and 1:0) for 15 minutes each and allowed to air dry for 6 hours. The scanning electron microscopy (SEM) samples were mounted and sputter coated with
gold/palladium at 17 mA for 150 seconds using a Pelco (Redding, CA) Model 3 sputter coater. A Philips (New York, NY) XL 30 scanning electron microscope was used for analysis.

4.3.5 Data Analysis

Experiments were performed at least in duplicate or triplicate. Data shown are means with standard deviation. Analyses of variation between means were performed using a Student’s $t$-test, and significance was based on a 95% confidence level ($\alpha<0.05$).

4.4 Results and Discussion

4.4.1 Multiwell PET Scaffold Cultures

Colon cancer cells were grown in four types of 3-D PET scaffolds contained within static, multiwell plates. The types of PET used were combinations of two porosities (0.94 or 0.88) and pretreatment status (pretreated or untreated). Analyses of the growth, metabolism, cell cycle, and morphology of the cancer population were performed to characterize the early growth at 2 days and later growth at a week.
4.4.1.1 Effect of Porosity

High (0.94) and low (0.88) porosity PET scaffolds were used to house the growing cell population within the static, multiwell environment. The growth can be dependent on the type of morphology that develops within the scaffold. Proliferation of growth and morphology within a fibrous scaffold can be described by 3 mechanisms: fiber spreading, interfiber bridging, and aggregative expansion. Fiber spreading describes the growth of cells along a single fiber, with prolonged growth resulting in a coated fiber. Interfiber bridging occurs when fibers are in close proximity, allowing the connection of cells between fibers and morphological development in the interstitial space. Aggregation is characterized by a spherical expansion of cells which can commonly occur at a junction of fibers or along a fiber at which cells have proliferated into the interstitial space instead of undergoing fiber spreading. Figures 4.3 and 4.4 provide insight into the morphological development in high and low porosity PET, respectively, for a growth period of 8 days.

Early growth in high porosity PET, seen in Figures 4.3a and 4.3b, was characterized as similar to growth seen in 2-D cultures. Due to the large distance between fibers, growth progressed via fiber spreading along the surfaces of the scaffold. With most cells growing by this mechanism and the presence of excess available surface area, the population expanded at a rate similar to a highly proliferating 2-D culture at the early stage during the log phase. Within 6 days, the population had coated fibers through fiber spreading (Figure 4.3c) and aggregate morphology had developed along single fibers (Figure 4.3d).
After a full week, the cell-coated fibers were thick enough to begin bridging interfiber distances (Figure 4.3e) and aggregative morphology had expanded further (Figure 4.3f).

Growth in low porosity PET was characterized as a faster development of 3-D morphology compared to that in high porosity PET, with interfiber bridging more prevalent in the early growth. For 2 days (Figure 4.4a), fiber spreading was the dominant growth mechanism, but within 4 days, the reduced interfiber distance in low porosity PET allowed for bridging to occur (Figures 4.4b and 4.4c), developing the 3-D morphology in the interstitial spaces. By the end of the week, fiber spreading and interfiber bridging had expanded the cancer population to a localized, high cell density (Figures 4.4d, 4.4e, and 4.4f). Aggregative morphology was not detected as it was in high porosity PET culture.

In Figure 4.5, the cell population growth in each type of scaffold is shown at two time checkpoints: 2 days and 6 days. Comparing the HP/T and LP/T scaffolds, the population expansions in high porosity scaffolds were significantly higher than in the low porosity scaffolds at both times. Using the growth mechanisms prevalent in both scaffold types, the differences in population expansion were due to fiber spreading in high porosity PET culture and early 3-D morphology development by interfiber bridging in low porosity PET culture. With a majority of the cells proliferating in high porosity PET for the duration of the time studied, expansion was similar to rapid 2-D growth. Less of the population in low porosity PET was expanding by fiber spreading, but rather more cells were being incorporated in slower growing interfiber bridging.
The rate of glucose consumption is indicative of cell growth. Figure 4.6 shows the glucose consumption in high and low porosity PET cultures over 2 days and 6 days. The rate of glucose consumption in HP/T PET was significantly higher than in the LP/T PET culture during the early stage of 2 days, coinciding with the increased cell growth. During this time, the low porosity PET culture was starting to develop a 3-D morphology that proliferates more slowly. For the remainder of the week, the glucose consumption rates were similar in both porosities as the high porosity culture also began to develop three-dimensionally. The metabolic yield is the ratio of the lactate production rate and the glucose consumption rate. The yields in each type of PET in multiwell plate cultures at 2 days and 6 days are listed in Table 4.1. There was no significant difference in yields at each time point and when comparing time points to each other, indicating that population expansion was not enough to limit nutrient availability within the PET and to alter the metabolic status of the population.

Cell cycle analysis provided the population of cells that were in the growth phases of the cell cycle, representing proliferative activity. In Figure 4.7, the growth populations in 2-D, high porosity PET, and low porosity PET cultures are compared over 8 days. The growth populations decline significantly between 2 and 4 days for all three culture types due to contact inhibition in the 2-D culture and adjustment to the new environment in the 3-D cultures. At Day 6, the low porosity culture had the lowest growing population due to the developed 3-D morphology that included senescent cells within the developed structure that stopped growing due to lack of nutrients. The high porosity PET culture
remained more highly proliferative, comparable to 2-D culture, with continued fiber spreading. By Day 8, the high porosity PET growing population had declined due to expanded 3-D morphology.

4.4.1.2 Effect of Pretreatment

Pretreatment had a significant role in the success of cell seeding into PET scaffolds (see Chapter 3), but the role of increased surface hydrophilic properties on cell growth was analyzed here. Figure 4.5 demonstrates the role that pretreatment had on cell growth within two porosities of PET. At Day 2, HP/T PET had grown significantly more than HP/UT, but growth in LP/T and LP/UT were similar. By Day 6, the cell populations in pretreated high and low porosity PET cultures had expanded significantly more than their untreated counterparts. The reason for these differences can be drawn from the differing growth morphologies in each type of PET.

Since pretreatment of the scaffold made the adhesion of cells to the scaffold more favorable, the migration of cells along the fibers could be increased by this enhanced interaction as well. Therefore, a situation in which fiber spreading is the important growth mechanism will benefit more from scaffold pretreatment. High porosity PET culture growth was characterized as mainly fiber spreading until late in the culture time. Therefore, pretreatment had more of an effect on high porosity cultures with cells in HP/UT growing less due to more resistance to spreading because of less favorable surface properties and no other growth mechanism available. In low porosity PET
culture, pretreatment had less of an effect at the early growth stages because interfiber bridging was an available alternative growth mechanism in LP/UT PET. After 6 days, favorable fiber spreading in LP/T scaffolds allowed for a significant expansion of the cell population compared to the cell growth in LP/UT PET.

4.4.2 Spinner Flask Cultures

Colon cancer cells were grown in four types of 3-D PET scaffolds contained within mixed spinner flasks. The types of PET used were combinations of two porosities (0.94 or 0.88) and pretreatment status (pretreated or untreated). Analyses of the metabolism and morphology of the cancer population were performed to characterize the growth.

4.4.2.1 Effect of Porosity

High (0.94) and low (0.88) porosity PET scaffolds were used to house the growing cell population within the spinner flask environment. As discussed before, the growth can be dependent on the type of morphology that develops within the scaffold. Figure 4.8 shows the developed morphology in high and low porosity PET scaffolds after 6 days of culture. Both cultures have expanded to similar 3-D morphology with fiber spreading and interfiber bridging. The growth in the high porosity scaffold was occurring at a greater depth within the PET than it was in the low porosity scaffold. This is due to two factors:
the dynamic seeding procedure distributed cells deeper within the high porosity scaffold, and the increased effective pore sizes in high porosity PET allow for greater nutrient transport within the scaffold.

The effect of porosity can also be seen by comparing the metabolic yields of HP/UT and LP/UT PET cultures after 6 days at 80 rpm in Table 4.1. The yield in high porosity PET was significantly lower than the yield in low porosity PET signifying that the population in HP/UT was growing in more aerobic conditions. The availability of oxygen to the cells in the interior of the scaffold is increased in high porosity PET due to the allowance of fluid flow through the scaffold, delivering the necessary nutrients.

4.4.2.2 Effect of Agitation

The mixing in the spinner flask environment provides a mechanism for nutrient transfer that enables high cell density growth. The agitation rate was varied at 40, 80, and 120 rpm. The effect of agitation can be seen by comparing the metabolic yields in Table 4.1. Agitation did not have a significant impact in low porosity cultures in the range studied since cell growth was mainly located at the outer thickness of the scaffold. In high porosity PET, nutrient transfer to the cells located within the scaffold plays a more significant role. Comparing the yields at 40 and 80 rpm, the higher agitation rate effectively reduced the yield from 0.714 to 0.658 g/g, indicating that 80 rpm was better at
maintaining aerobic growth within the scaffold. At 120 rpm, the yield was higher than at 80 rpm, possibly due to an inhibitory effect on growth due to a fluid flow stress at such a high agitation rate.

4.4.3 Perfusion Bioreactor

The perfusion bioreactor experiment was only performed one time, resulting in limited insight into the operational benefits the design promises. However, based on the metabolic yield data at 6 days and 10 days, provided in Table 4.1, possible advantages of the perfusion design were suggested. At 6 days, the metabolic yield was very low, demonstrating the effectiveness of the perfused medium to deliver nutrients to the cells and to provide healthy aerobic growth. At 10 days, the metabolic yield had increased almost three-fold. This may be due to the design benefitting the development of a high cell density 3-D culture which had expanded to the point that nutrient transfer had become more limited.

4.4.4 Comparison of Culture Methods

The major difference between the three methods utilized to grow colon cancer *in vitro* was the transport of nutrients. In static multiwell plates, nutrient transfer was performed by diffusion, whereas, the in spinner flask and perfusion bioreactor, diffusion was supplemented with convective transport by bulk medium flow. Figure 4.9 compares the average metabolic yields obtained from all experiments within the culture environments.
at different times. For multiwell plate and spinner flask cultures, the metabolic yields within each environment do not significantly change between 2 and 6 days. This is probably due to the cell density increase not being large enough to start limiting the aerobic conditions within the PET scaffolds. The metabolic yield in the perfusion bioreactor does increase between 6 and 10 days, possibly because of significant cell population growth. Among culture methods, the two that incorporated convective nutrient transfer resulted in lower metabolic yields than in the static multiwell plate culture. With the mixing and flow involved in spinner flask and perfusion bioreactor cultures, a higher maximum cell density is expected since the nutrient transport would be able to support the development.

4.5 Conclusion

The culture of colon cancer within three in vitro environments was performed to characterize the effects of scaffold parameters and environment design on the growth and developing morphology. A model that provides in vivo-like growth characteristics, morphology, and cell density could be utilized to further cancer development studies and the analysis of candidate drug efficacy in drug screening applications. The three culture environments investigated were static multiwell plate, spinner flask and perfusion bioreactor. The scaffolding utilized to house the cells was varied by porosity and surface hydrophilic status.
In multiwell culture, the porosity and pretreatment of PET had significant effects. In high porosity PET, the growth was characterized by mainly fiber spreading and aggregate development for 6 days before interfiber bridging was possible. This resulted in faster cell population expansion compared to low porosity PET, which was characterized with an increased extent of 3-D morphology due to reduced interfiber distances, resulting in slower proliferation. Pretreatment of PET scaffolds significantly affected the early growth in high porosity scaffolds since fiber spreading was the main mechanism for growth, and untreated surfaces inhibited cell growth. Low porosity, pretreated scaffolds had more significant growth than in untreated scaffolds at 6 days of growth.

Spinner flasks provided a mixed environment that enhanced the metabolic yield in high porosity scaffold cultures over low porosity cultures. Agitation at 80 rpm improved the metabolic status of high porosity culture over that attained at 40 rpm. The flow in a perfusion bioreactor provided the most aerobic conditions supporting a high cell population expansion. The optimal 3-D in vitro model based on these results would be a low porosity, pretreated PET scaffold within the mixed environment of a spinner flask or perfusion bioreactor because of the speed at which the 3-D morphology would develop and the maintenance capability of the high cell density. Uniform seeding of the low porosity scaffold would be the major obstacle in developing this model.
4.6 References


Ma, T. 1999. “Fiber-Based Bioreactor Systems In Mammalian Cell Culture And Tissue Engineering Human Trophoblast Cells.” PhD Dissertation. The Ohio State University, Columbus, OH.


Table 4.1: Metabolic yields (ratio of lactate production rate to glucose consumption rate) of 3-D PET cultures in three culture environments, different agitation rates, and four PET scaffold types at different times. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error ranges indicate standard deviation.

<table>
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<tr>
<th>Culture Environment</th>
<th>Scaffold Type</th>
<th>Agitation (rpm)</th>
<th>2 days</th>
<th>6 days</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiwell Plate</td>
<td>HP/T</td>
<td>N/A</td>
<td>0.824 ± 0.013</td>
<td>0.870 ± 0.025</td>
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<tr>
<td></td>
<td>HP/UT</td>
<td>N/A</td>
<td>0.817 ± 0.026</td>
<td>0.898 ± 0.034</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP/T</td>
<td>N/A</td>
<td>0.898 ± 0.022</td>
<td>0.836 ± 0.026</td>
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</tr>
<tr>
<td></td>
<td>LP/UT</td>
<td>N/A</td>
<td>0.855 ± 0.034</td>
<td>0.879 ± 0.018</td>
<td>-</td>
</tr>
<tr>
<td>Spinner Flask</td>
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<td>0.767 ± 0.025</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HP/UT</td>
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<td>0.736 ± 0.018</td>
<td>0.714 ± 0.015</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HP/UT</td>
<td>80</td>
<td>0.754 ± 0.026</td>
<td>0.638 ± 0.023</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP/T</td>
<td>80</td>
<td>0.709 ± 0.030</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP/UT</td>
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<td>0.708 ± 0.030</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP/UT</td>
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<td>0.799 ± 0.033</td>
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<td>Perfusion Bioreactor</td>
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<td>-</td>
<td>0.308 ± 0.000</td>
<td>0.832 ± 0.000</td>
</tr>
</tbody>
</table>
Figure 4.1: a) Colon cancer growth on a two-dimensional PET film in a multiwell plate (20x magnification). b) Growth curve demonstrating two characteristic growth phases.
Figure 4.2: a) Perfusion bioreactor set-up with PET scaffolding. b) Highlight of the perfusion bioreactor, with stacked PET discs, and the medium holding tank.
Figure 4.3: SEM photographs of colon cancer cells growing on high porosity (0.94) PET in multiwell plates for various times: a) 2 days at 49x; b) 2 days at 650x; c) 6 days at 100x; d) 6 days at 650x; e) 8 days at 49x; f) 8 days at 650x
Figure 4.4: SEM photographs of colon cancer cells growing on low porosity (0.88) PET in multiwell plates for various times: a) 2 days at 49x; b) 4 days at 101x; c) 4 days at 800x; d) 6 days at 50x; e) 6 days at 350x; f) 8 days at 48x
Figure 4.5: Cell growth in multiwell plate cultures in 4 types of PET. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error bars indicate standard deviation
Figure 4.6: Glucose consumption in multiwell plate cultures in 4 types of PET for a) 2 days and b) 6 days. HP/T: high porosity (0.94) and pretreated; HP/UT: high porosity (0.94) and untreated; LP/T: low porosity (0.88) and pretreated; LP/UT: low porosity (0.88) and untreated. Error bars indicate standard deviation.
Figure 4.7: Populations of colon cancer cells in the growth phases of the cell cycle for three culture environments: two-dimensional plate culture, high porosity (0.94) PET culture, and low porosity (0.88) PET culture
Figure 4.8: SEM photographs of colon cancer cells on PET scaffolds after 6 days of spinner flask growth. a) High porosity PET at 99x; b) High porosity PET at 200x; c) Low porosity PET at 150x
Figure 4.9: Comparison of the metabolic yields in three culture environments at three times. Error bars indicate standard deviation.
5.1 Abstract

Colon cancer is the second deadliest type of cancer, killing about 56,000 people per year in the United States. Strategies for the development of new drugs against colon cancer are novel and continually emerging, but the road from drug discovery to market availability is a long and costly journey with an approximate cost of $403,000,000. There is a 78% failure rate for all drugs. If a drug fails while in clinical trials, the financial impact can be devastating to the developing company. With 22.5% of the drugs failing due to efficacy problems, improved predictions in the preclinical trial stages would reduce failed clinical trial occurrences and be much less costly. Three-dimensional (3-D) culture allows for an in vivo-like environment that is used in tissue engineering to reconstitute tissue that is morphologically and functionally equivalent to the native tissue. This suggests that a 3-D culture could be a better predictive tool for candidate drug efficacy analysis.

Analyses with two anti-cancer drugs were performed using the developed 3-D in vitro colon cancer model for drug efficacy prediction compared to the commonly used...
two-dimensional (2-D) model. Two 3-D models were studied with high and low porosity PET. In separate experiments, the cultures were exposed to 5-fluorouracil and gemcitabine after 3 days of growth. Based on changes in glucose uptake and lactate dehydrogenase concentration, the low porosity PET culture was the most susceptible to 5-fluorouracil. High porosity PET and 2-D culture models performed similarly, predicting less efficacy for 5-fluorouracil. Since low porosity culture represented a 3-D morphology after 3 culture days and 5-fluorouracil is one of the most effective drugs against *in vivo* colon cancer, the 3-D model more accurately predicted the efficacy of this drug. During gemcitabine exposure, the low porosity PET culture was not affected, whereas the 2-D culture and, to a lesser extent, the high porosity PET culture were susceptible to the drug. Gemcitabine does not have *in vivo* efficacy for colon cancer, and the low porosity model successfully demonstrated this.

Correlating cell growth or death with green fluorescent protein (GFP) fluorescence in 3-D was also investigated. Linear relationships between cell number and fluorescence in 2-D and 3-D were obtained, though the applicability of the 3-D correlation was not evident as the 3-D morphology developed. Upon exposure to 5-fluorouracil, a low porosity 3-D model with GFP-transfected cells after 1 day and 10 days of growth had fluorescence responses as expected with increasing dosages. The GFP model demonstrated sensitivity at low drug doses with fluorescence changes after one day of culture time. However, after 10 days of culture time, the 3-D morphology of the culture influenced the fluorescence sensitivity resulting in less fluorescence variation even when exposed to high drug dosages. Overall, the low porosity PET model demonstrated more accuracy in efficacy
screening than did the 2-D culture, and incorporation of fluorescence as a cytotoxicity reporter would provide for a fast and improved tool for preclinical *in vitro* drug screening.

5.2 Introduction

Colon cancer takes the lives of more people than any other type of cancer except lung cancer. The cause and prevention mechanisms of colon cancer are still not entirely clear though, whereas there is an obvious way to largely avoid lung cancer with the omission of smoking. In 1997, colon cancer caused the deaths of 56,000 people in the United States alone (Ries et al., 2000). The third most common cancer in the U.S., colon cancer diagnoses are expected to reach approximately 105,000 in 2005. Death from colon cancer can be avoided if diagnosed early with detection screenings such as colonoscopies, but progressed colon cancer must be handled through surgical extraction, chemotherapy, and immunotherapy. Due to the effectiveness of these treatments, the 55% 5-year survival statistic for colon cancer is improving slowly each year.

Strategies for the development of new drugs against colon cancer are novel and continually emerging. 5-Fluorouracil is the longest available colon cancer drug but effective new usages of the drug have been developed within combinations with other drugs such as leucovorin and Camptosar (Pfizer) and as derivatives for improved delivery routes such as the oral drug Xeloda (Roche). 5-Fluorouracil and Camptosar are examples of DNA analogs and topoisomerase inhibitors, respectively, utilized to kill cells by
disruption of DNA synthesis. 5-Fluorouracil targets both DNA and RNA of colon cancer cells for increased effectiveness (Van Laar et al., 1996). Oxaliplatin (Sanofi-Synthelabo), known for its expedient Food and Drug Administration (FDA) approval for fighting colon cancer, is an organoplatinum complex that crosslinks DNA, effectively halting essential cellular processes. Novel strategies are targeted drugs based on known molecular genetics of colon cancer and anti-angiogenic drugs that inhibit the development of neovasculature within the tumor. Erbitux (ImClone), approved in February 2004, is an antibody that competitively binds to epithelial growth factor receptors that are over-expressed in colon cancers and inhibits cell growth. Avastin (Genentech), also approved by the FDA in February 2004, is an anti-angiogenic drug that inhibits the signaling molecules utilized by cancer cells to initiate blood vessel development. Though recent progress in development of colon cancer drugs is promising, inherited cellular resistance to these drugs is very possible, and many combinatorial regimens and novel drugs must be continually processed through the development pipeline.

The road from drug discovery to market availability is a long and costly journey with an approximate cost of $403,000,000 (DiMasi, 2003). Thousands of drug candidates are predicted using techniques such as molecular modeling and preliminarily screened using high-throughput techniques in the early stages of drug discovery. Promising drugs are then used in tests utilizing mostly animal and in vitro plate cell culture models to obtain preliminary results while compiling data for an application to enter clinical trials. Clinical trials involve four stages in which the efficacy and toxicity of the drug are analyzed in
human patients followed by long-term effect studies. Drugs that are approved by the FDA may reach the market following Phase III, but there is a 78% failure rate for all drugs (DiMasi, 2003). If a drug fails while in clinical trials, the financial impact can be devastating to the developing company. With 22.5% of the drugs failing due to efficacy problems, improved predictions in the preclinical trial stages would reduce failed clinical trial occurrences and be much less costly (Walker, 2002).

Tissue engineering combines knowledge obtained from biological fields and applies it using engineering skills to repair, regenerate, or enhance three-dimensional (3-D) tissues and organs both in vivo and in vitro. When cancer is studied in the lab setting, two-dimensional (2-D) models are utilized for analyzing drug therapies. In vitro models that utilize 2-D cultures for efficacy analyses are inherently prone to error because of the lack of a 3-D scaffold to support cell growth. The multitude of differences between cultures in 2-D and 3-D environments have been detailed. Changes in cancer cell morphology (Haramaki, 1993), growth characteristics (O’Conner et al., 1997), and gene expression (Zvibel et al., 1998) between the two culture conditions depict the effect of having a 3-D scaffold available to the cell population. 3-D culture allows for an in vivo-like environment that is used in tissue engineering to reconstitute tissue that is morphologically and functionally equivalent to the native tissue. As a result, the scaffold allows for proper morphological attributes of cells and population arrangement, heterogeneous tumor development, cell-cell and cell-scaffold interactions and communication, and differentiated function (Heppner et al., 1998; O’Conner, 1999; Li et al., 2001).
Variations of drug efficacy between cultures in 2-D and 3-D have also been reported further suggesting the importance of using *in vitro* 3-D models for drug testing (Furukawa et al., 1992; Smitskamp-Wilms et al., 1998). In these studies, cells grown in a 3-D environment tend to be more drug-resistant than 2-D culture results. When colon and ovarian cancer cell lines were exposed to the drug gemcitabine (Eli Lilly), cells grown in 3-D multilayers showed over a 1000 times decrease in sensitivity to gemcitabine than the 2-D cultures, which coincides with the lack of *in vivo* efficacy for colon cancer (Smitskamp-Wilms et al., 1998). This suggests that a 3-D culture is a better predictive tool for chemosensitivity analyses.

Methods utilized to monitor the cytotoxicity of drugs applied to *in vitro* models are either direct or indirect methods. Conventional direct methods include harvesting cells and using a trypan blue exclusion method and a hemacytometer to analyze cell viability and to quantify the cell population, respectively. Indirect methods include monitoring of substrate consumption or metabolite production, relating released lactate dehydrogenase (LDH) to cell death, and loss of productivity. Analysis of cell growth using fluorescence is gaining in popularity due to its noninvasiveness (Randers-Eichhorn et al., 1997; Hunt et al., 1999; Shimada et al., 1999; Girard et al., 2001; Xie et al., 2001). Green fluorescent protein (GFP) is a marker that can be transfected into the cell. Fluorescence of GFP within the transfected cells is growth associated, and thus, the effect of a cytotoxic agent will be registered by a drop in cell fluorescence measured by a fluorometer while the cells remain in culture (Basu, 2004).
The objective of this study is to utilize the three-dimensional \textit{in vitro} model of colon cancer previously developed and analyzed (Chapters 3 and 4, respectively) for testing the efficacy of two anti-cancer drugs. The goal is to demonstrate that the 3-D model can make proper predictions of drug efficacy and should be used in further screening studies. Additionally, the effectiveness of using a GFP-transfected cell line for analyzing drug cytotoxicity will be investigated.

5.3 Materials and Methods

5.3.1 Cells and Medium

The colon cancer cell line HT-29 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and is utilized in all of the experiments. HT-29 is a human adenocarcinoma, grade I cancer cell type of colonic epithelial origin. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose concentration, L-glutamine, and sodium pyruvate (Caisson Laboratories, Rexburg, ID). The medium was supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY).
5.3.2 GFP Transfection

The cells were transfected with an enhanced green fluorescent protein plasmid, pEGFP-N3 (Clontech, Palo Alto, CA) (see Figure 5.1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The enhanced GFP (EGFP) has been modified for brighter fluorescence and higher expression in mammalian cells. EGFP expression is controlled by the CMV promoter, which constitutively controls expression independent of environmental effects. Transfected cells were selected by neomycin-resistance with G418 (Gibco, Grand Island, NY), and amplified by subculture over 20 passages. Our data showed that the state and the growth rate of the transfected cells were not affected by EGFP expression. Transfection stability was verified by flow cytometry (>97%) compared to a negative control.

5.3.3 Scaffold

Nonwoven, fibrous poly(ethylene terephthalate) (PET) fabric, known commercially as Dacron, was used as the 3-D culture support scaffolding. PET (fiber diameter: 20 µm; fiber density: 1.35 g/mL; porosity: 0.94) is a randomly organized, isotropic matrix with advantages of being inexpensive yet providing high specific surface area for cell growth and high porosity for cell growth and nutrient accessibility. PET film was utilized in 2-D growth studies.
The surface of the PET was modified to improve serum protein and cell adhesion to the scaffold by hydrolyzing the PET in boiling sodium hydroxide solution. The scaffolds were washed in a solution of 5 g sodium bicarbonate and 5 mL Tween 20 in 500 mL of distilled water at 60°C for 30 minutes, followed by a 10 minute rinse in a 60°C water bath. The scaffolds were then hydrolyzed in boiling 1% sodium hydroxide solution for 45 minutes. Finally, the scaffolds were washed again at 60°C in a water bath for 10 minutes.

A thermal compression method (Li et al., 2001) was used to obtain PET scaffolds with a porosity of 0.88. PET was compressed under 38 kPa of load at 121°C for 30 minutes. Both pretreated and non-pretreated scaffolds were compressed. Porosity, the void volume fraction, was determined from the difference between the total bulk scaffold volume and the solid, fiber volume. The fiber volume was obtained from the scaffold mass and the fiber density.

5.3.4 Drugs

5-Fluorouracil (Sigma, St. Louis, MO), shown in Figure 5.2a, is a DNA analog utilized to kill cells by disruption of DNA synthesis. It is an effective drug against colon cancer when introduced alone or in a combination therapy. The cytotoxicity of gemcitabine (Eli Lilly, Indianapolis, IN), shown in Figure 5.2b, is enacted through inhibition of
deoxynucleotides and interaction with DNA during the synthesis phase of growth. Gemcitabine is effective against ovarian cancer but has not been prescribed with efficacy against colon cancer.

5.3.5 Cell Culture

5.3.5.1 Two-Dimensional Plate Culture

Cells were added to 96-well plates at a rate of 5000 cells per well. After 30 minutes, 1 mL of culture medium was added to each well. Cell cultures were incubated within a pH-buffering 5% CO₂ environment at 37°C. The medium was replaced when the lactate concentration approached 1.5 g/L.

5.3.5.2 Multiwell PET Scaffold Culture

Two scaffold types were used: high porosity (0.94), pretreated PET and low porosity (0.88), pretreated PET. For 12-well plates, the PET scaffolds (diameter: 2.3 cm) were placed in each well and incubated overnight in culture medium. Before seeding the cells, the medium was removed. The cells were seeded onto the presoaked PET at a concentration of 91,200 cells/cm² of surface area (456,000 cells for PET in 12-well plates) in a volume below 200 µL to prevent saturation of the scaffold. Following incubation at 37°C for 6 hours, medium was added at a ratio of 1.52x10⁵ cells/mL medium (3 mL for PET in 12-well plates). Cell cultures were incubated within a
pH-buffering 5% CO₂ environment at 37°C. For 96-well plate cultures, a modified plate was prepared allowing for larger medium volume (see Figure 5.3). Seeding proceeded as above except that the scaffolds were 0.8 cm in diameter, 25,000 cells were seeded, and 3.5 mL of medium were added after 6 hours.

5.3.6 Drug Cytotoxicity Analyses

5.3.6.1 Metabolism

Metabolism of the cell population was monitored by measuring the glucose uptake and lactate production of the culture. 150 µL samples were taken from multiwell plate cultures for glucose and lactate concentration analyses using a YSI Biochemistry Select Analyzer (Yellow Springs, OH).

5.3.6.2 Lactate Dehydrogenase Assay

The lactate dehydrogenase concentration in the culture medium was assayed using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI). Prior to culture exposure to a drug, the culture medium was replaced with DMEM containing 5% FBS to reduce background LDH. Samples of 100 µL were taken from the metabolism samples and added to a 96-well plate. An equivalent volume of CytoTox-ONE reagent was added to the well, followed by 30 seconds of mixing. After a 10 minute incubation at room temperature, the wells were shaken for 10 seconds and then analyzed.
in a Cytofluor 4000 (Applied Biosystems, Foster City, CA) fluorescence multiwell plate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Relative fluorescence units were compared and directly proportional to LDH concentrations and cell death.

5.3.6.3 EGFP Expression Assay

Enhanced green fluorescence protein expression in 2-D or 3-D cultures was analyzed directly from the multiwell culture plate in a Cytofluor 4000 (Applied Biosystems, Foster City, CA) fluorescence multiwell plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Relative fluorescence units were compared and were directly proportional to the proliferating cell number within the 2-D or 3-D cultures.

5.3.7 Data Analysis

Experiments were performed at least in duplicate or triplicate. Data shown are means with standard deviation. Analyses of variation between means were performed using a Student’s $t$-test, and significance was based on a 95% confidence level ($\alpha<0.05$).
5.4 Results and Discussion

5.4.1 3-D *In Vitro* Model Screening Performance

Cells were grown in 2-D plate cultures and 3-D PET cultures in high and low porosity scaffolds located within multiwell plates. The growth period was 3 days to allow for development of 3-D morphology in the PET cultures. For studies with 5-fluorouracil, cultures were exposed to 130 µg/mL ($10^{-3}$ M) of 5-fluorouracil, and glucose, lactate, and LDH concentrations were monitored for 3 days. Figures 5.4a, 5.4b, and 5.4c show the resulting effect that 5-fluorouracil had on glucose uptake relative to the control for each culture environment. At 2 days, the glucose uptake by the low porosity cell population had decreased significantly compared to the low porosity control. The decreases in glucose utilization in 3-D high porosity and 2-D cultures were similar over the 3-day period. Figure 5.4d shows the reduction in glucose metabolism rate in each culture condition as a percentage of the metabolic rate of the corresponding control. The low porosity culture was significantly more affected by the drug than were the high porosity PET and 2-D cultures with a metabolic rate of only 23%.

In Figures 5.5a, 5.5b, and 5.5c, the fluorescence data due to LDH concentration in the three culture types over the 3-day drug exposure period are shown. Similar to the glucose uptake variations, the low porosity culture was the most affected culture type by exposure to 5-fluorouracil. Figure 5.5d depicts the percentage increase in LDH concentration relative to each control. Increasing fluorescence is proportional to increasing LDH
concentration, indicating cell death. The cell death in low porosity culture was significantly higher than that in the high porosity and 2-D cultures. Based on LDH concentration, high porosity was more susceptible to 5-fluorouracil than was 2-D culture, however, monitoring cytotoxicity in 3-D cultures using LDH does not appear to be reliable. LDH data had more fluctuation and variability than the metabolic data did, evident by the size of the error bars in Figure 5.5 compared to Figure 5.4. Therefore, other cytotoxicity reporters with more reliability than LDH are desired.

From previous studies of colon cancer growth in a 3-D PET scaffold environment (Chapter 4), low porosity PET developed a 3-D morphology faster than in high porosity PET, and by Day 3, cell population growth by fiber spreading and interfiber bridging has developed a notable 3-D morphology. High porosity culture was characterized by growth similar to 2-D culture by which cells expanded quickly along surfaces by fiber spreading. In 3 days, no prominent 3-D morphology had developed in the high porosity PET scaffold. Because of these observations, it makes sense that the 2-D and high porosity 3-D cultures were similarly affected by exposure to 5-fluorouracil after 3 days of growth.

5-Fluorouracil is an effective agent against colon cancer in vivo. Therefore, an in vitro drug screening model should predict good efficacy for this drug. In this case, the low porosity PET scaffold culture was the most susceptible to 5-fluorouracil, whereas in high porosity and 2-D cultures, 5-fluorouracil was less efficacious. Since low porosity culture was a developed 3-D morphology, this suggests that the 3-D model provided a more accurate prediction for 5-fluorouracil efficacy.
Gemcitabine could be utilized as a negative control for the 3-D model since the drug is not effective against colon cancer *in vivo*. Gemcitabine was applied to the two 3-D models and the 2-D plate culture similarly to the 5-fluorouracil studies. Two dosage levels of gemcitabine were studied (10^{-8} and 10^{-6} M) and compared to controls of each culture condition with no drug. The results are shown in Figure 5.6. According to the glucose uptake data, gemcitabine had minimal effect in low porosity PET culture at low and high doses with metabolic rates remaining at 94% and 84%, respectively. The drug had a more significant effect in 2-D culture, reducing the metabolic rate compared to the control samples. This 2-D culture data would erroneously suggest efficacy for gemcitabine against colon cancer. The high porosity PET culture was less affected by the drug than the 2-D cultures were in this case. The LDH data (not shown) in these studies had poor sensitivity and reliability, and again, the method was deemed suboptimal.

As mentioned above, the low porosity PET could be characterized with a 3-D morphology with interfiber bridging when exposed to gemcitabine. With the low porosity PET culture demonstrating the lack of efficacy for gemcitabine by being the least sensitive to the drug, the 3-D *in vitro* model demonstrated the ability to selectively predict non-efficacy better than the 2-D plate culture screening method as well. Combining these results with those obtained in the 5-fluorouracil studies, the 3-D *in vitro* model was more accurately sensitive to the two model drugs, matching their *in vivo*
effectiveness, than was the currently-utilized 2-D model. Further studies with additional drugs should be performed to completely validate the applicability of the 3-D model for preclinical drug screening.

5.4.2 GFP Fluorescence-Based Cytotoxicity Analysis

The usage of GFP fluorescence as an indicator of drug cytotoxicity would be advantageous because of the speed and simplicity of data collection. In order to utilize this method, GFP fluorescence has to be directly related to cell number, and it must be demonstrated that fluorescence readings can accurately represent culture growth or inhibition within the 3-D scaffold environment. The relationships between cell number and fluorescence on 2-D plate surfaces and in 3-D PET scaffolds are shown in Figures 5.7a and 5.7b, respectively. In each case, the fluorescence was measured 6 hours after the cells were seeded. Linearity was obtained for fluorescence data in both culture conditions. However, the slopes of the lines differ with 3-D culture having more fluorescence per cell than in 2-D culture. The reason for the enhanced fluorescence within the 3-D scaffold is unknown. The 3-D data in Figure 5.7b suggests that the cell number during growth within PET can be quantified (see Chapter 3 for analysis of other 3-D cell counting methods). Linearity between fluorescence and cell number within the scaffold would allow for determination of growth rates which is not possible with current cell counting methods. This method is also advantageous for providing 3-D cell counts in a rapid and noninvasive manner. Figure 5.8 demonstrates the qualitative increase of fluorescence as the colon cancer cells grew on PET over a span of 8 days. Future work
includes demonstration that linearity of fluorescence to cell number can be maintained once growth develops a 3-D morphology of interfiber bridging and aggregates.

The results of drug exposure at various dosages on low porosity culture after 1 day of growth are shown in Figure 5.9. The fluorescence variations were able to demonstrate the inhibitory effect of 5-fluorouracil on the 3-D culture at increasing doses with expected trends. The GFP model was able to quantify drug effects via fluorescence changes at low drug dosages, demonstrating good model sensitivity.

Transfected colon cancer cells were grown in low porosity PET scaffolds for 10 days, allowing for development of 3-D morphology, before exposing the cultures to 5-fluorouracil. The resulting effects on fluorescence are shown in Figure 5.10. The fluorescence was decreased at higher dosages, however, compared to the dosages in Figure 5.9, it took a much higher dosage to affect the fluorescence after 10 days of culture. It is not known if the decrease in fluorescence is linearly related to cell death at this stage. It is expected that at such high 5-fluorouracil dosages that the cell death would be greater than the fluorescence drop suggested.

5.5 Conclusion

Analyses were performed using the developed 3-D in vitro colon cancer model for drug efficacy prediction compared to the commonly used 2-D model. Two 3-D models were studied with high and low porosity PET. The cultures were exposed to 5-fluorouracil
after 3 days for growth. Based on changes in glucose uptake and LDH concentration, the low porosity culture was the most susceptible to the drug. High porosity and 2-D culture models performed similarly, predicting less efficacy for 5-fluorouracil. Since low porosity represented a 3-D morphology and 5-fluorouracil is one of the most effective drugs against *in vivo* colon cancer, the 3-D model more accurately predicted the efficacy of this drug. During gemcitabine exposure, the low porosity PET culture was not affected, whereas the 2-D culture and, to a lesser extent, the high porosity PET culture were susceptible to the drug. Gemcitabine does not have *in vivo* efficacy for colon cancer, and the low porosity model successfully demonstrated this.

Correlating cell growth or death with GFP fluorescence in 3-D was also investigated. Linear relationships between cell number and fluorescence in 2-D and 3-D were obtained, though the applicability of the 3-D correlation was not evident as the 3-D morphology developed. Upon exposure to 5-fluorouracil, a low porosity 3-D model with EGFP-transfected cells after 1 day and 10 days of growth had fluorescence responses as expected with increasing dosages. However, the correlation between cell number and fluorescence was less sensitive in cultures with expanded 3-D morphology. Overall, the low porosity PET model demonstrated more accuracy in efficacy screening than did the 2-D culture, and incorporation of fluorescence as a cytotoxicity reporter would provide for a fast and improved tool for preclinical *in vitro* drug screening.
5.6 References

Basu, S. 2004. “Effects of Three Dimensional Structure of Tissue Scaffolds on Animal Cell Culture.” PhD Dissertation. The Ohio State University, Columbus, OH.


Figure 5.1: Enhanced green fluorescence protein plasmid, pEGFP-N3 (Clontech, Palo Alto, CA), with CMV promoter and kanamycin and neomycin resistance
Figure 5.2: Anti-cancer drugs utilized in this study: a) 5-fluorouracil and b) gemcitabine
Figure 5.3: Modified 96-well plate with 6 scaffold sites surrounded by 8 linked medium-holding wells
Figure 5.4: Comparison of glucose metabolisms in three culture conditions after exposure to 0 (control) and $10^{-3}$ M 5-fluorouracil. Cells were grown in a) 2-D plate culture, b) high porosity (0.94) PET, and c) low porosity (0.88) PET for 3 days prior to drug exposure at $t = 0$. d) Comparison of the reduction of glucose metabolism as a percent of the corresponding control sample metabolism rate for each drug dosage in the three culture conditions. Error bars indicate standard deviation.
Figure 5.5: Comparison of lactate dehydrogenase (LDH) release, quantified by fluorescence assay, in three culture conditions after exposure to 0 (control) and $10^{-3}$ M 5-fluorouracil. Cells were grown in a) 2-D plate culture, b) high porosity (0.94) PET, and c) low porosity (0.88) PET for 3 days prior to drug exposure at $t = 0$. d) Comparison of the increase of LDH release, related to cell death, as a percent of the corresponding control sample LDH release rate for each drug dosage in the three culture conditions. Error bars indicate standard deviation.
Figure 5.6: Comparison of glucose metabolisms in three culture conditions after exposure to 0 (control), $10^{-8}$, and $10^{-6}$ M gemcitabine. Cells were grown in a) 2-D plate culture, b) high porosity (0.94) PET, and c) low porosity (0.88) PET for 3 days prior to drug exposure at $t = 0$. d) Comparison of the reduction of glucose metabolism as a percent of the corresponding control sample metabolism rate for each drug dosage in the three culture conditions. Error bars indicate standard deviation.
Figure 5.7: Relationship between cell number and GFP fluorescence 6 hours after seeding in: a) 2-D plate cultures and b) 3-D PET cultures. Error bars indicate standard deviation.
Figure 5.8: GFP-transfected HT-29 colon cancer cells growing on PET after a) 1 day, b) 5 days, and c) 8 days
Figure 5.9: Fluorescence growth curves in low porosity PET following exposure to various dosages of 5-fluorouracil at \( t = 0 \). Cell were cultured for 1 day prior to drug exposure.
Figure 5.10: Fluorescence growth curves in low porosity PET following exposure to various dosages of 5-fluorouracil. Cell were cultured for 7 days prior to drug exposure.
6.1 Conclusions

The goal of this research was to develop an *in vitro* three-dimensional (3-D) model of colon cancer that could be used in cancer developmental studies and for drug efficacy screening at the preclinical trial stage of drug development. Before the model could be studied for either application, three aspects needed to be analyzed: modifying the PET scaffold, seeding cells into the 3-D cultures, and quantifying the cell number during 3-D culture growth. The PET scaffold was thermally compressed at different pressures, temperatures, and durations resulting in PET with porosities ranging from 0.94 to 0.84. The stability of the compression was maintained after soaking in culture medium, so porosity would not increase during the 3-D cultures. The hydrophilic status of the PET surface was increased by pretreating the scaffold in boiling sodium hydroxide. The compression and pretreatment of PET scaffolds would have significant effects in the subsequent studies of colon cancer growth and drug susceptibility.

Three cell seeding methods were analyzed: static, dynamic, and filter seeding. The mixed environments provided the desired uniformity of cell distribution within the scaffolds.
Dynamic and filter seeding also would allow for higher cell density seeding than would be allowable for static seeding. It was determined that 80 rpm was optimal for dynamic seeding and that filter seeding provided superior seeding kinetics. The effects of PET porosity and pretreatment were also analyzed. The high porosity scaffold allowed better penetration of seeded cells into the interior of the scaffold, and pretreatment increased the rate of seeding and attachment in dynamic and static cultures, respectively.

None of the analyzed cell counting methods for 3-D cultures was deemed optimal due to lack of reproducibility or poor cell recovery from the scaffold. Enzymatic detachment of the cells from the scaffold by Accutase performed the best with reproducible cell counts and a modest cell recovery, and this method was chosen for usage in determining relative cell counts in upcoming studies of cell population growth in 3-D models. However, the utilization of fluorescence as a marker for cell number provided a rapid and noninvasive method for 3-D model quantification.

The studies of colon cancer growth within the 3-D in vitro model were performed within four types of PET scaffold and three culture conditions. The PET scaffold types were combinations of two porosities and 2 treatment statuses. The three culture environments were in static multiwell plates, in mixed spinner flasks, and in a perfusion bioreactor. High porosity, pretreated scaffolds were advantageous of promoting the fastest cell population expansion characterized by growth by fiber spreading. Spherical aggregates were unique to high porosity scaffold growth. Interfiber bridging did not occur until a week of culture time. Low porosity scaffolds housed the fastest developing 3-D
morphology with interfiber bridging prevalent by the fourth day of culture due to reduced interfiber distances. Due to 3-D growth into interstitial spaces, the growth was slower than that found in high porosity PET cultures. The pretreatment of scaffolds had the most significant effect on high porosity growth since fiber spreading was the prevalent growth mechanism and the one most susceptible to altered fiber surface conditions. The spinner flask and perfusion bioreactor environments provided mixing that would sustain higher cell density cultures due to convective nutrient transfer. Aerobic growth conditions were the most sustained in the perfusion bioreactor, yet the metabolic yield increased quickly possibly due to the largest cell population expansion of all three culture methods. Growth in spinner flasks were influenced by the porosity of the scaffold since flow into high porosity scaffolds to deliver nutrients was possible, whereas in low porosity scaffolds, flow was blocked by the reduced pore structure.

The application of the developed colon cancer model for drug screening applications was analyzed using two drugs with known efficacies. Cells cultured in 2-D, low porosity PET, and high porosity PET environments were grown for 3 days prior to exposure to 5-fluorouracil or gemcitabine. Based on changes in glucose uptake and lactate dehydrogenase concentrations relative to controls, the low porosity culture provided the most favorable prediction of 5-fluorouracil efficacy. Since the low porosity culture best represented 3-D morphology at 3 days, the 3-D model more accurately predicted the efficacy of this drug than did the 2-D model. In fact, the 2-D model and the high porosity culture performed similarly since cell growth in high porosity scaffolds mimicked 2-D growth at early stages with the population growing by the fiber spreading mechanism.
During gemcitabine exposure, the low porosity PET culture was not affected, whereas the 2-D culture and, to a lesser extent, the high porosity PET culture were susceptible to the drug. Gemcitabine does not have in vivo efficacy for colon cancer, and the low porosity model successfully demonstrated this.

The colon cancer cells were transfected with enhanced green fluorescent protein to analyze the effectiveness of using fluorescence to report the cytotoxic effects of drugs applied to the 3-D model. Linear relationships of cell number to fluorescence were demonstrated for cells placed into 2-D and 3-D environments, allowing for the monitoring of changes in cell number due to drug effects. The reliability of fluorescence accurately representing the viable cell count in high cell density cultures remains to be demonstrated. Various drug dosages were applied to low porosity PET cultures after 1 day and 10 days of growth, and expected trends for fluorescence decline with increasing 5-fluorouracil concentration were seen. However, at high cell density, the fluorescence seemed to less responsive to high drug dosages when pronounced cell death was expected. Overall, the low porosity PET model demonstrated more accuracy in efficacy screening than did the 2-D culture, and incorporation of fluorescence as a cytotoxicity reporter would provide for a fast and improved tool for preclinical in vitro drug screening.
6.2 Recommendations

Further studies are suggested for fully characterizing the growth of colon cancer within the 3-D model. This research suggested the proliferative capabilities involved with the perfusion bioreactor culture design. Experiments at different flow rates will provide insight into the effects on cell growth. Analysis by scanning electron microscopy will verify the extent to which the filter seeding distributed the cells and how expansive the 3-D morphology develops in 10 days.

The metabolism, morphology, cell growth, and cell cycle were utilized to characterize the colon cancer growth in 3-D. Additional analyses would provide even more interesting consequences of the developing 3-D morphology. For example, the expression of colon cancer biomarkers could be monitored throughout the expansion to study the cancer development. Epithelial growth factor receptor is overexpressed in many colon cancer tumor cells resulting in the characteristically high proliferation when the ligand binds. Changing the culture conditions in which the cells are grown may have a positive or negative effect on the receptor expression. Conditions that favor the receptor expression may represent the \textit{in vivo} condition more accurately. Also, vascular endothelial growth factor (VEGF) is secreted when cancer cells are in need of nutrients. This begins the process called angiogenesis \textit{in vivo}, as VEGF recruits the development of blood capillaries inward towards the tumor where the size of the tumor has resulted in a reduced nutrient supply. The correlation of VEGF secretion with developing 3-D morphology would define the \textit{in vivo} morphology at which angiogenesis must occur. In the broad
scope of genomic expression variations, a genetic expression profile in different culture environments would provide a global characterization of population activity that could be compared with 2-D cultures, nude mice models, and *in vivo* colon cancer tumors. Identifying the conditions that provide *in vivo*-like genetic expression would ultimately optimize the 3-D model for growth studies and drug screening analyses.

In this research, the drug efficacy was predicted using static multiwell plate 3-D cultures. The drug analyses can be expanded to include the spinner flask and perfusion bioreactor models that were studied for cancer growth. The 3-D cancer model could also be applied to additional model drugs to further validate the accuracy at which the model predicts efficacy. Analyzing drug cytotoxicity using the green fluorescent protein-transfected cell line is promising and advantageous since the speed of analysis benefits the move towards high throughput screening. Further work should be performed to correlate fluorescence changes with cell growth inhibition at higher cell densities.

The basis of applying a three-dimensional environment to the culture of cancer tissue for the applications of *in vitro* investigations of development and accurate screening of drugs in the preclinical stage is not inherently specific for colon cancer. Many types of cancer tumors inhibit 3-D environments and develop three-dimensionally, and therefore, the 3-D *in vitro* model should be applied to other cancer types with the goal of positively affecting the many fronts in the fight against cancer.
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