BIOPHYSICAL EFFECTS OF MATRICES ON CELLULAR COLONIZATION

By

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BIOPHYSICAL EFFECTS OF MATRICES ON

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CHAPTER I

INTRODUCTION

Significance of Tissue Engineering

The inability of an organ to function in tandem with other organs, thereby resulting in either its loss or repair, causes trauma to more than 20 million patients (Lysaght and Reyes 2001) worldwide. Physicians used to treat organ or tissue loss by transplanting organs from one individual to the other, performing reconstructive surgery or using mechanical devices (Langer and Vacanti 1993). For organ transplantation, it is a difficult task to recruit available and matching donors. At the end of 2006, there were 98,263 recipients on the waiting list for organ transplantation, and a total of 28,291 patients received organ transplants (OPTN / SRTR Annual Report: Transplant Data 1997-2006). Also, donor organs are not always an effective alternative, running the risk of infection and rejection by the body. This has fueled interest in a new and emerging area of organ/tissue manufacturing, where suitable biomaterials are investigated for use in tissue regeneration, providing an alternative to organ transplantation. With the advent of tissue engineering, there would be lesser dependence on donor organs. Also, engineers could manufacture regenerative tissues on a large scale with easily available materials, thereby reducing the cost and time it takes to repair a damaged organ by a significant factor.

Biopolymer Structures as Regenerative Alternatives

Regenerative tissues are fabricated using materials which possess the essential properties of biocompatibility, biodegradability and strength. Examples of structures that are formed using such materials are three dimensional (3D) porous structures, two dimensional (2D) membranes and hydrogels. Three dimensional (3D) structures can be cultivated to replace or repair missing parts, 2D membranes can be used as films for wound closure and hydrogels offer the advantage of being a minimally invasive alternative for cartilage repair. Further, hydrogels offer the advantage of being liquids at room temperature and gelling at body temperature, cementing their capability as a minimally invasive material. Apart from the structure, there is also interest in the chemical nature of the biomaterials that are used, chitosan by far being the most popular. In spite of being biodegradable, biocompatible, inexpensive and available aplenty, chitosan does not have a cell binding domain. As a result, there is interest in evaluating the effect of a material cross linked with chitosan, which would account for cell binding characteristics. Gelatin satisfies the same criteria of biocompatibility and biodegradability, along with possessing cell binding characteristics. Therefore, for this study, structures have been made with chitosan and chitosan-gelatin separately to study their effect on cellular colonization. The polymeric structures are synthesized to mimic the properties of the Extracellular Matrix (ECM), and the functionality of the ECM depends on a variety of proteins such as collagen, elastin and proteoglycans. Therefore, in this study, to analyze the potential application of the polymeric structures, their functionality with respect to the proteins secreted (in particular collagen and elastin) has been evaluated. Also, if these structures are made available, there is interest in studying

the mechanism of regeneration. To understand the outcome of this study, we will have to take a glimpse at the concept of wound repair.

Repair and Regeneration of Wounds

The long practiced method of healing a wound was to close it using surgical procedures. However, this technique had its limitations. It was restricted to easily accessible anatomical locations; whereas if the wound was deep or wide, it would be required to keep it uninfected for as long as possible, keep it painless and substitute a material that would aid in the healing process. Therefore, there was increased interest for materials that could better integrate into the surrounding tissue region when seeded with the appropriate cells, aid in the growth of new tissue, and ultimately degrade into non-toxic components that could be discarded by the body.

One of the widely studied areas of tissue engineering is wound repair, which follows a sequence of events – inflammatory cells migrate into plasma clot, followed by fibroblasts which form collagen and matrix elements, and eventually contract to close the wound (Montesano and Orci 1988). The amount of collagen formed is the net of synthesized and degraded collagen. Degradation is accomplished by enzymes, Matrix metalloproteinases (MMP) (M. S. Ågren 1998). MMPs use the ECM as a substrate and can change the functionality of the ECM. Although several processes are involved in the wound repair process, MMPs have been found to play a crucial role during the final stage of wound healing (Anne-Cécile Buisson 1996). It has been demonstrated that gelatinases (MMP-2 and MMP-9), in particular MMP-9 (gelatinase B) are expressed by migrating epithelial cells during wound repair, and the wound repair process depends on

this MMP (Anne-Cécile Buisson 1996). Hence this study has looked at the activity linked to the production of gelatinase A (MMP-2) and gelatinase B (MMP-9) enzymes.

Objectives and Hypothesis

There have been extensive studies on 2D membranes, 3D porous scaffolds and hydrogels (Burdick, Peterson et al. 2001; Cukierman, Pankov et al. 2001; Griffith and Swartz 2006). These structures vary in their mechanical properties, and their elastic moduli range from 2 Pa to 2 MPa. There has been no comprehensive single study of all the three structures, and the effects that the varying mechanical properties might have on cellular colonization on the materials. *In this study, we hypothesize that bulk mechanical properties of polymeric structures affect cellular viability and colonization. In addition, it is also hypothesized that the presence or absence of a cell binding domain has a significant effect on cellular response to these structures.* To test the hypothesis, we need to use a matrix composed of a polymer with and without cell binding domain, on cell adhesion and functionality. For this purpose, we chose the chitosan, a biodegradable and biocompatible polymer which can be processed into various forms without altering the functional groups. In addition, it has a net positive charge which can immobilize negatively charged molecules such as gelatin and glycosoaminoglycans. Further, it has no cell binding domain and is therefore a suitable material for comparison purposes for this study. Gelatin, on the other hand has a cell binding domain. It has an Arg–Gly–Asp (RGD)-like sequence that promotes cell adhesion and migration (Huang, Onyeri et al. 2005). In addition, it can be blended with chitosan without the presence of a cross linker

(Mao, Zhao et al. 2003). Therefore, the objectives of this study can be summarized as follows:

1. To study the influence of a matrix composed of a polymer without cell binding domain (chitosan) and varying mechanical properties (2Pa to 2MPa) on cellular activity.

2. To study the influence of a matrix composed of a polymer with cell binding domain (chitosan-gelatin) and varying mechanical properties (2Pa to 2MPa) on cellular activity.

CHAPTER II

REVIEW OF LITERATURE

Materials used in Tissue Engineering Applications

The underlying concept of tissue engineering is that cells can be isolated from a patient, grown to multiply in an outside environment in suitable growth conditions (mimicking biological conditions), seeded onto a template and then grafted into the patient as a replacement tissue (Yang, Leong et al. 2001). The template is a material that has to satisfy the basic criteria of biocompatibility and biodegradability. Biocompatibility involves the use of materials that do not provoke an unwanted tissue response to the implant and at the same time promote cell attachment and functional characteristics (Yang, Leong et al. 2001). Biodegradability implies that the material can degrade over time (once its use has been realized) into non-toxic products, leaving only the living tissue (Yang, Leong et al. 2001). Some materials possessing these characteristics include natural and synthetic polymers, ceramics, metals and a combination of these materials.

Metals are used as biomaterials because of their strength and toughness (SV Madihally, Introduction to BioEngineering, Classnotes). Most common are stainless steel, cobalt-chromium alloy, titanium, aluminum, zinc and their alloys (SV Madihally, Introduction to BioEngineering, Classnotes). Ceramics are used for their hardness, high wear

resistance, stiffness, high compressive strength and low coefficient of friction (SV Madihally, Introduction to BioEngineering, Classnotes). However, many of these materials possess the disadvantages of having little biodegradable characteristic and limited scope for processing into various forms. Also metals may release ions into the body over a prolonged period of time, causing undesirable reactions. These limitations roused the interest in investigating the use of polymers for biomedical applications.

Natural polymers like collagens have been used to repair nerves, skin, cartilage and bone (Yang, Leong et al. 2001). Though they may simulate the native biological environment, they pose problems of functional consistency from batch to batch (Yang, Leong et al. 2001). Poor mechanical strength is another drawback, which kindled interest in alternate polymers that can provide strength and functional reliability. Various polymers (the fundamental being chitosan) have been investigated for their potential as biocompatible, biodegradable and strong materials, that can be used as a tissue regenerative or regrowth alternative. The scope for investigating biomaterials for tissue replacement is very wide. To focus the scale on one specialized area, this study deals with tissue research on soft tissues.

For this study, chitosan was chosen as the reference biomaterial, because it satisfies various suitable criteria like biocompatibility, biodegradability, strength, and it is also available in abundant quantities at low cost. Chitosan has repeating units of β(1–4) 2-amino-2-deoxy-D-glucose and is formed through the N-deacetylation of chitin, an abundant polysaccharide produced from crustacean shells (Nettles, Elder et al. 2002). Most importantly, Chitosan can be processed into various forms without the loss of its functionality.

The structure of chitosan is represented in **Figure 1**. What chitosan lacks is a cell binding domain. The question that we now ask is why is a cell binding domain important?

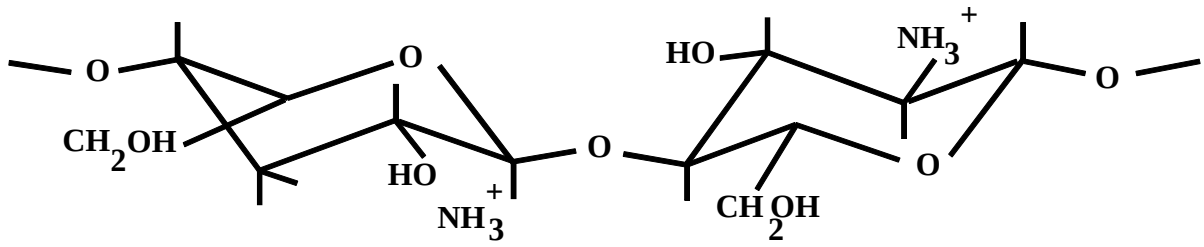

Figure 1: Chemical structure of Chitosan

The Importance of the Cell Binding/Adhesion Domain

Multicellular organisms require specific mechanisms for intercellular communication and adhesion. Also, a specific mechanism is needed for carrying signals between the cell and matrix. Gumbiner (Gumbiner 1996) has done an extensive review of cell adhesion. From his review, we can infer that the functional units of cell adhesion domains are made of three classes of proteins – the adhesion receptors, the ECM proteins and the cytoplasmic plaque/peripheral membrane proteins. He also explains that the cell adhesion receptors mediate binding interactions at the extracellular surface and include members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan superfamilies. The ECM proteins include the collagens, fibronectins, laminins, and proteoglycans. Further, cell adhesion receptors associate with cytoplasmic plaque proteins at the interface of the plasma membrane and serve as a link between the adhesion systems and the cytoskeleton.

For this study, it is more important to understand cell-matrix interactions to get a better insight into the significance of matrix architecture simulating the natural ECM. Focal adhesion points are the sites of contact between a matrix and the cell, and are associated with actin microfilaments at their cytoplasmic aspect, playing a significant role in the organization of actin, and thereby impacting cell spreading, cell morphogenesis and cell migration (Zamir and Geiger 2001).

Integrins are the most widely investigated receptors on the cell surface that communicate between the inside of the cell and the matrix. In the extracellular space the ligands that help in these adhesions are fibronectin, vitronectin and various collagens (Geiger, Bershadsky et al. 2001). Until the turn of the 20^{th} century there were extensive studies on 2D cell matrix interaction with the help of in vitro models. The shift in focus started when research focus shifted to using 3D porous structures for tissue regeneration. The study by Cukierman *et al* (Cukierman, Pankov et al. 2001) suggests that cell derived 3D matrix is more effective in binding cells than a 2D substrate, by at least a factor of six. They also suggested that 3D matrix conditions improved the functionality of the cellular environment. Hence, we need to choose a material, in addition to chitosan, to provide this essential feature, and to test our hypothesis that a polymeric structure composed of a cell binding domain can influence cellular colonization to a significant extent.

For this study, gelatin was chosen as it has a binding domain and can be cross linked with chitosan without the need for a cross linker. Gelatin is a partially denatured derivative of collagen, and collagen is present as a connector for most body tissues. The Arg–Gly–Asp (RGD)-like sequence on gelatin contributes to the cell binding property of this material. Gelatin has a coiled helical structure as shown in **Figure 2**.

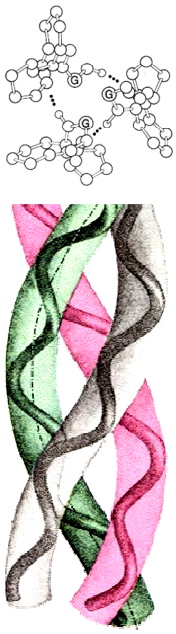

Figure 2: Structure of gelatin, taken from Zubay G, Biochemistry, 3rd edition

Also, when chitosan and gelatin are linked together, the resulting structure can affect the spatial distribution of integrin ligands and polycationic chitosan interaction with the anionic cell surface (Huang, Onyeri et al. 2005).

The Extra-Cellular Matrix (ECM) and Elements of Interest

Prior to investigating specific structures of interest, there is need for a better understanding of the Extracellular Matrix (ECM) and the matrix elements, because the materials used for tissue regrowth and regeneration would be required to mimic the properties of the ECM, to a certain extent. The ECM can be considered as the foundation that holds the cells together in a tissue to control the tissue structure and regulate the cell phenotype. The ECM architecture appears in a variety of forms in different tissues, and the diversity arises through specific molecular interactions and arrangements of

collagens, elastins, proteoglycans and adhesion proteins such as fibronectins and laminins (Stevens and George 2005). Collagens are present as a connector for most body tissues and are responsible for a large portion of the ECM structure. They are the most abundant protein in the ECM structure. Collagen fibers provide tensile strength, while hydrated gel of proteoglycans fills the extracellular space, creating a space for the tissue while allowing the diffusion of nutrients, metabolites and growth factors (Kim and Mooney 1998). As a biomaterial, it is biodegradable, biocompatible, facilitates wound repair and is used in several drug delivery devices (Friess 1998). Collagen processing occurs in fibroblasts by cell receptor clustering, followed by invagination of the cell membrane (Lee and McCulloch 1997). It is reported that there was a significant impact of the collagen matrix structure on both primary human lung fibroblast and human bone marrow-derived mesenchymal stem cell (Leah C. Abraham 2004; Mauney, Volloch et al. 2005). Another study suggested that remodeling collagen matrices and using denatured collagen could be a potential model for studying disease states (Abraham, Dice et al. 2007). Based on the studies mentioned, we chose to study the production of collagen as an extracellular matrix element.

Elastin, another connector, provides elastic characteristics to ECM. It helps to restore tissues to their original shape when they stretch. It is found as elastic fibers in the ECM and comprises an important fraction of the dry weight of the ECM. They are composed of two different portions – a amorphous component which lacks a definite structure and 10-12nm fibers, which are located along the periphery of the amorphous component (Rosenbloom, Abrams et al. 1993). Since they form such a vital portion of the ECM, elastin content has also been investigated in this study.

Molecules, such as fibronectins, mediate cell adhesion (Kim and Mooney 1998). Actin is also an important constituent of the ECM. It plays a significant role in cell-cell or cell-matrix adhesion. Integrin, an important matrix element is found in most cells at points of cell-matrix adhesion, especially beneath the actin-containing microfilament bundles (Buck and Horwitz 1987). Also cell migration involves polymerization of actin at the leading edge, to push out the membrane, and contraction of actin-myosin cables at the rear (Machesky and Hall 1997). Actin fibers generate sufficient force in muscle cells to support contraction. The ECM serves as a storage depot for growth factors and provide these factors in a controlled manner to adjacent cells. **Figure 3** (courtesy Dr.Sundar Madihally) shows the some of the cell- ECM interactions.

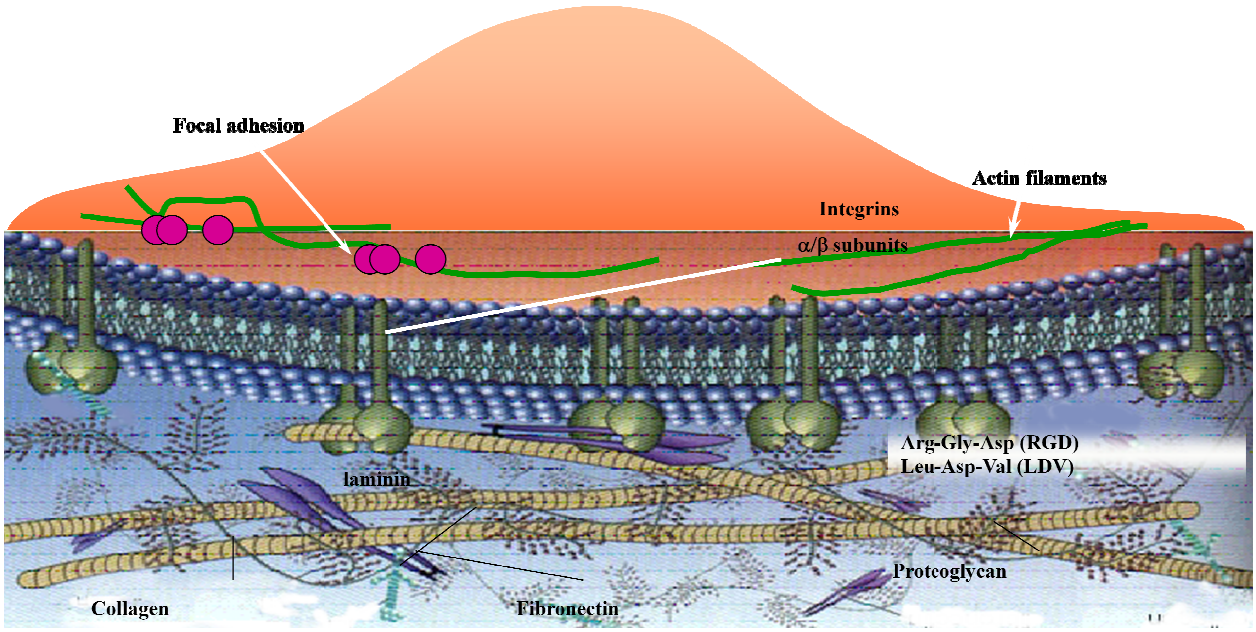

Figure 3: The Cell -Extracellular Matrix Interactions (provided by Dr.Sundar Madihally)

Matrix Synthesis and Degradation

One of the underlying features of a polymeric biomaterial used for tissue regeneration is its biodegradability. Once the function of the polymer has been realized, it should ideally degrade and be eliminated from the body as non-toxic byproducts. Another issue is the fate of the matrix elements that are synthesized by these biomaterials during the process of regeneration. It is essential that these elements be present, and utilized to lend functional stability to the diseased organ even after the degradation of the polymeric tissue alternatives. So in addition to evaluating the synthesis of matrix elements by the polymeric structures (this primarily happens due to the cells synthesized on the structures), it is necessary to investigate their degradation characteristics. Both chitosan and gelatin possess biodegradable characteristics. The loss or degradation of matrix elements is manifested in the form of an inflammatory response. There are enzymes that act to nullify the effect of the inflammation, and they belong to a family called Matrix metalloproteinases (MMPs). There are several kinds of MMP enzymes, but the most important are the gelatinase enzymes, MMP-2/MMP-9 that play a crucial role during the final stages of wound healing. In addition, it is necessary to comprehend the detailed mechanism behind wound repair to evaluate the potential use of polymeric biomaterials for tissue regeneration purposes.

Wound Healing Mechanism (Adam J Singer 1999)

Wound healing involves continuous tissue inflammation, formation and repair. Tissue injury causes an inflammation of blood vessels. Homeostasis is established by blood clots formed at the site of the wound. Migrating epithelial cells dissect the wound, and

the dissection path is predetermined by the presence of integrins expressed by these cells. It is required that the ECM degrades for the migration of the epidermal cells, and this is dependent on the production of collagenase.

The inflammatory stage is followed by the production of fibroblasts and blood vessels at the site of the wound. The fibroblasts start forming ECM to support cell growth. The ECM elements basically provide a framework for cellular migration.

A few days after the appearance of the wound, fibroblasts assume a myofibroblast phenotype and collagen remodeling starts to take place, and the degradation of collagen is controlled by the MMPs, as mentioned earlier. The presence of collagen helps to connect the newly formed tissues with those already present. The schematic in **Figure 4** gives a brief idea about the mechanism behind the wound repair process. In uninjured cells, epithelial cells in the skin are present in multiple layers. Upon injury, these layers are disrupted creating gaps in the tissue. Upon continuous secretion of collagen aided by MMP-2/MMP-9 expression, new tissue is formed in the injured region and the area begins to heal. Finally a new layer of epithelial cells is formed.

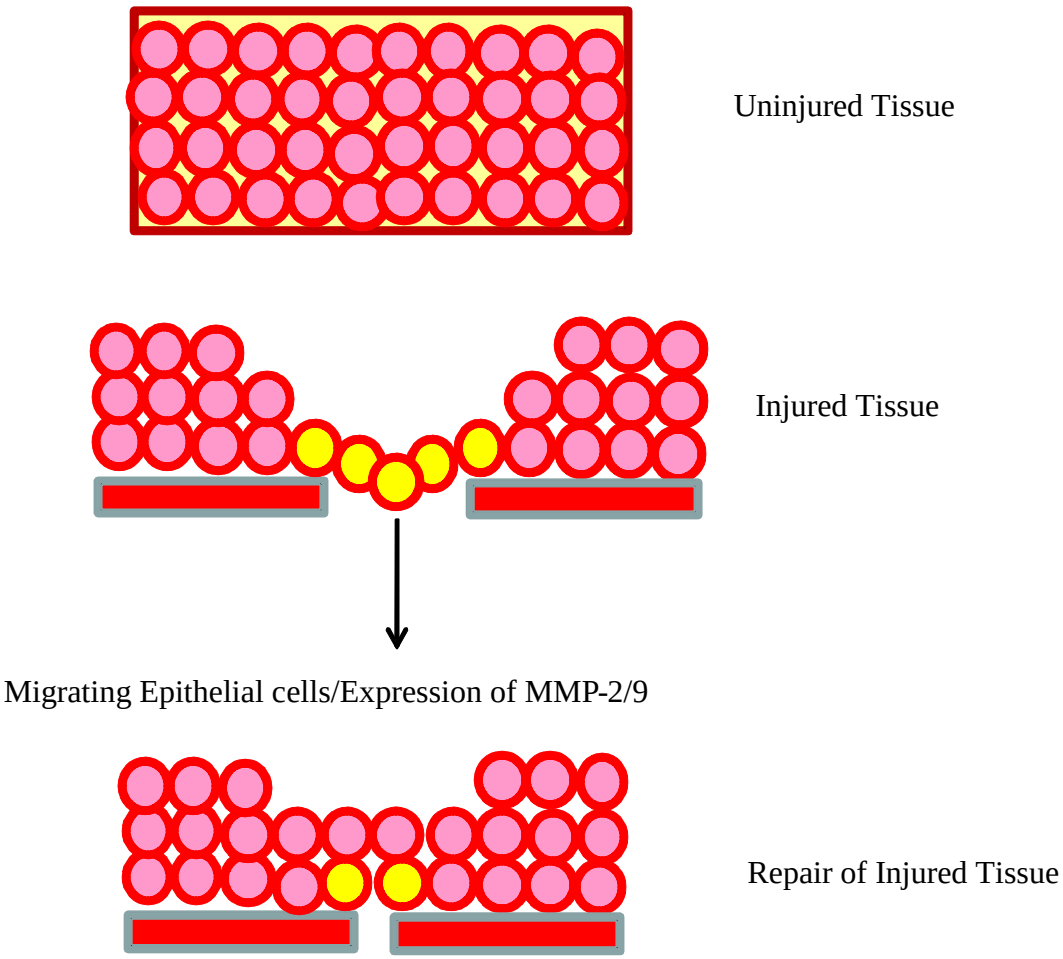

Figure 4: Basic wound repair process

Correlation of Mechanical Stiffness and Cellular Response

The question now arises, why do we need to grow cells on structures that have some mechanical stiffness associated with them? Why not just grow them as such in a fluid environment, with the necessary proteins? Most viable cells need adhesion to extracellular structures. They are not viable upon disassociation in a fluid (Discher, Janmey et al. 2005). Apart from applying force, a normal tissue cell responds through cytoskeleton organization to the resistance sensed by the cell, whether derived from

normal tissue matrix, synthetic substrate or an adjacent cell. Also, physical properties of the tissues can change in diseased state, and their response to the matrix also changes. Muscle cells, neurons and many other tissue cells have been shown to sense substrate stiffness (Wang, Dembo et al. 2000; Deroanne, Lapiere et al. 2001; Engler, Bacakova et al. 2004).

Cells adhere to substrates that range in stiffness from soft to rigid and vary in topography and thickness. The resistance of a substrate to stress is given by the elastic modulus, E, which is obtained by applying a force to the material of interest, and then measuring the relative change in length, or strain. **Figure 5** shows an example of substrate strain and tissue stiffness (Discher, Janmey et al. 2005).

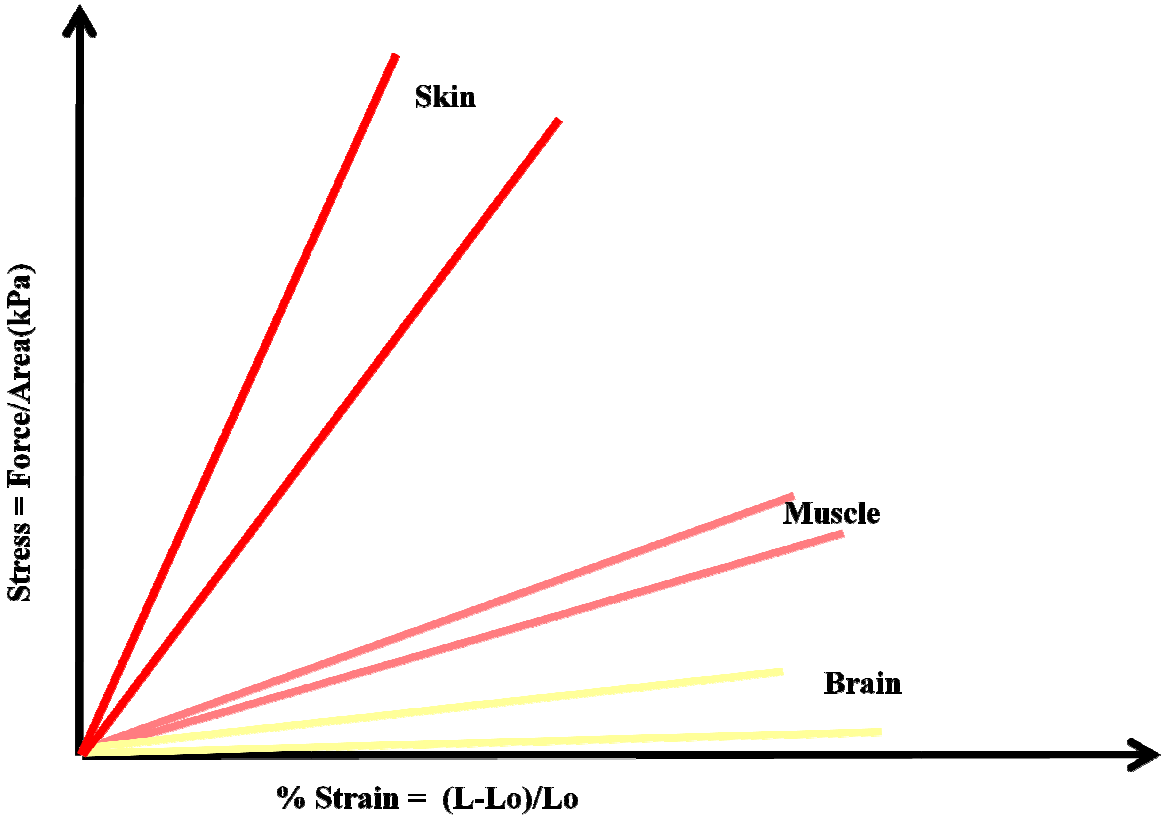

Figure 5: Correlation between mechanical stiffness and biological tissue, inferred from Discher, Janmey et al. 2005

It has been shown in previous studies by our group (Yan Huang 2006) that spatial architecture influences cell shape and colonization. This further cemented the belief that substrate stiffness plays a vital role in dictating cellular response to the structure. Therefore, this study evaluated structures with varying mechanical stiffness values and varying spatial architecture. Before doing so, it was necessary to review reseach carried out on individual matrices – two dimensional (2D) membranes, three dimensional (3D) porous structures and hydrogels.

Biodegradable Templates

2D films: It has been shown that cells can guide their movement by exploring the substrate rigidity (Lo, Wang et al. 2000). Also, there has been a study showing that cells respond to matrices of diverse biochemical and biophysical properties by using the focal adhesion as a combinatorial site for creating different signaling complexes (Wozniak, Modzelewska et al. 2004).

When a 2D surface such as tissue culture plastic is used, the responses of the cell might be influenced by the stiffness and properties of the material. This is not representative of cellular behavior in a 3D environment, such as the human constitution. Also culturing cells on flat substrates induces an artificial polarity between the lower and upper surfaces of these normally non-polar cells. Due to this, it has been shown that fibroblast morphology and migration differ, once suspended in collagen gels (Elsdale and Bard 1972; Friedl and Brocker 2000). Therefore, it was necessary to evaluate cellular colonization of materials that could mimic the ECM of the body.

3D porous structures: To better mimic the ECM, three dimensional scaffolds were considered as an alternative, as they provided the required support, in addition to promoting cell adhesion and migration. The 3D matrix affects both solute diffusion and effector protein binding, such as growth factors and enzymes, thereby establishing tissue-scale solute concentration gradients, as well as local pericellular gradients (Griffith and Swartz 2006). Also, study in 3D environments challenge the use of traditional 2D tissue culture conditions for understanding in vivo structure, functions and migration (Cukierman, Pankov et al. 2001). There are several key advantages to 3D cell cultures. First, the movements of cells in the 3D environment of a whole organism typically follow a chemical signal or molecular gradient and it is impossible to establish a 3D gradient in a 2D environment. Cells isolated from higher organisms have to significantly adapt themselves to the 2D environment, possibly altering their gene expression patterns and metabolism. In addition, cells in 2D culture are prone to morphological changes, and they alter their own production of ECM proteins. The importance of the 3D ECM is recognized for epithelial cells where 3D environments provide epithelial polarity and differentiation (Roskelley and Bissell 1995). There have been a number of studies in the past decade which suggest that the use of 3D scaffolds fabricated from certain biocompatible materials is not cytotoxic to cellular growth. For example, a study (Yoon Sung Nam 1999) suggested that macroporous open cellular scaffolds could be potentially used for tissue regeneration using efficient cell seeding techniques. In one study, it was proposed that the controlled growth and proliferation of human embryonic stem (hES) cells can be achieved by culturing them in a 3D environment (Levenberg, Huang et al. 2003). Their results showed that complex structures with features of embryonic tissues

can be generated, *in vitro*, by using early differentiating hES cells and inducing their subsequent growth in a supportive 3D environment such as poly(lactic-*co*-glycolic acid) (PLGA) and poly(l-lactic acid) (PLLA) polymer scaffolds.

Injectable Hydrogels: There are certain soft tissues such ad cartilage where transplanting porous structures may need intensive surgical procedure. For this purpose, hydrogels, which have high water content, can be considered. Hydrogels are cross-linked hydrophilic polymers that contain large amounts of water without dissolution (Ma 2004). They offer a minimally invasive alternative for procedures such as arthroscopic surgeries and ease of incorporation of cells and bioactive agents (Burdick, Peterson et al. 2001; Kuo and Ma 2001; Mann, Gobin et al. 2001). There are various methods and materials by which hydrogels are fabricated. Some studies have employed the use of Polyaxmers (copolymers of poly ethylene oxide and poly propylene oxide) (Malmsten and Lindman 1992). In one particular study, polyvinyl alcohol was blended with chitosan in different ratios and the attachment and growth of fibroblasts on these structures were investigated (Tomoe Koyano 1998). There has also been a study (Mann, Gobin et al. 2001) on photopolymerizable hydrogels where various blends of bioactive polyethylene glycol (PEG) derivatives have been used in order to create a matrix substitute. In another study where polyethylene glycol was used, it was shown that the incorporation of a phosphoester endgroup between PEG and methacrylate provides a photopolymerizable hydrogel that is degradable and could be used in cartilage or bone tissue engineering. However, most Polyaxmers have been shown to lack physiological biodegradability characteristics. An alternative method which utilized chitosan as the base material for preparing the hydrogel was investigated (Chenite, Chaput et al. 2000) without the

presence of a cross link or without any chemical modification. In this study glycerol phosphate was added to chitosan solution, to form a gel at body temperature. Essentially the solution remained as a liquid outside the physiological temperature. **Figure 6** shows specific applications of different kinds of structures for hard and soft tissue replacement:

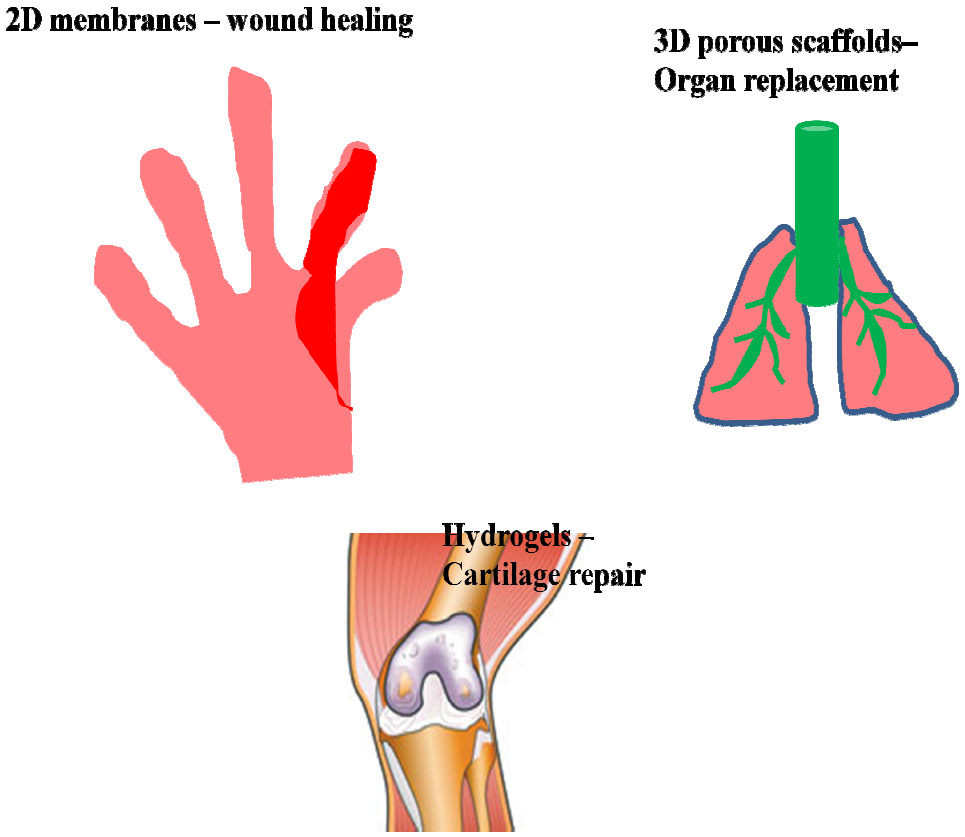

Figure 6: Examples of applications for biomaterial structures

Properties Affecting Cellular Colonization – Past Studies

Past studies have investigated the effect of optimizing physiostructural properties of the scaffolds like pore size, porosity and stiffness to improve their ability to match natural tissues. For example, (Hollister, Maddox et al. 2002) have developed a computational procedure to design scaffold microstructure such that the scaffold and the regenerated

tissue would match the mechanical stiffness of the host tissue. Their study considered mechanical stiffness values from 2 to 15 GPa, covering the range of biopolymer and ceramic scaffolds. Their procedure produced a one to one match of scaffold stiffness to target tissue stiffness. In a study by (Dietmar W. Hutmacher 2001), a technique called *Fused Deposition Modeling* (FDM) to produce 3D scaffolds with an interconnecting pore network. Polycaprolactone scaffolds designed with this model showed good biocompatibility when used with human fibroblast and periosteal cell culture systems. Woodfield *et al* used (Woodfield, Malda et al. 2004) Rapid Prototyping to produce 3D scaffolds with a range of mechanical properties, to study their potential use in articular cartilage tissue engineering. The scaffolds were shown to support rapid attachment of bovine chrondocytes and evaluated for the presence of articular cartilage ECM elements. Similar results were achieved for human articular chrondocytes.

Although each one of these studies and others has looked at the effect of varying mechanical properties on scaffold efficiency, they have not evaluated the relative advantages of one type of scaffold over others. For example, as mentioned in the previous section, there has been no systematic study comparing the differences in mechanical properties playing a role in cellular colonization. An exception to this would be a previous study by our group (Yan Huang 2006) where it was revealed that the differences in spatial architecture of the scaffolds, in particular between 2D and 3D scaffolds influences cellular colonization on these structures. This study was the motivation for this work where more detailed analyses has been carried out, expanding the scope for studying the differences in both the structural and chemical variations of the matrices and the effect that these variations have on cellular colonization. The flow

diagram in **Figure 7** gives an idea about the design of this study. 2D, 3D and hydrogel structures are studied because they have varying mechanical stiffness values. Also, these structures are made from two different materials – chitosan, and chitosan-gelatin, to evaluate the effect of the cell binding domain which is contributed by the presence of gelatin.

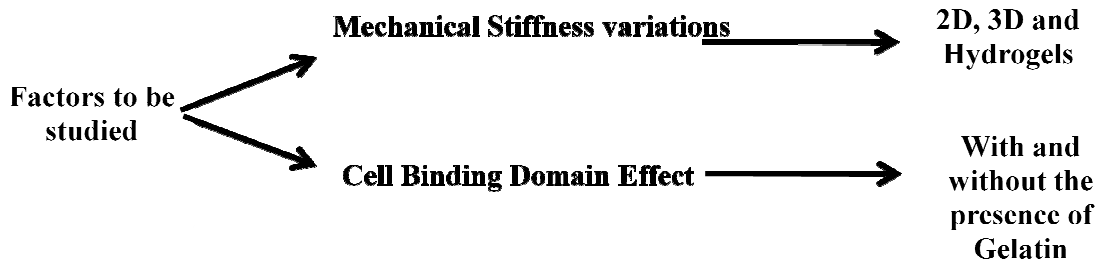

Figure 7: An overview of this study

CHAPTER III

METHODOLOGY

Sources for material

Chitosan (200-300 kDa molecular weight, Mw, 85% degree of deacetylation), Gelatin type – A (300 Bloom) and 2-Glycerol phosphate (2-GP) were obtained from Sigma Aldrich Chemical (St. Louis, MO). Ethyl Alcohol, 200 proof, absolute, anhydrous was obtained from Pharmaco. Matrix metalloprotease 2 (MMP-2) and matrix metalloprotease 9 (MMP-9) fluorogenic peptide (DNP-Pro-Leu-Gly-Met-Trp-Ser-Srg-OH) was purchased from CalBiotech (Spring Valley, CA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Protein Research Products (Rockford, IL). Alexa Fluor 546 phalloidin were obtained from Molecular Probes (Eugene, OR). 4', 6-diamidino-2-phenylindole (DAPI) and (carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) were obtained from Invitrogen Corp., (Carlsbad, CA, USA).

Scaffold, membrane and hydrogel fabrication

To prepare sterile chitosan solution, 100 mL deionized water containing 0.5% w/v chitosan was autoclaved and 200 µL of 0.1 N HCL was added to dissolve the solution overnight. One milliliter of 0.56 gm/mL 2-GP was added drop wise in an ice bath to 9 mL of chitosan solution for pH adjustment. To prepare chitosan-gelatin solution, 1% w/v

sterile chitosan solution was prepared, and 1 mL of 2-GP was added drop-wise to 4.5 mL of the chitosan solution. Then, 4.5 mL of 1% sterile gelatin solution was added drop wise to this mixture, to form a 0.5%w/v chitosan-gelatin solution.

To prepare 3D porous structures, 400 µL of solution was frozen in 24-well plates overnight at -20°C. The samples were then lyophilized using a Benchtop 6Kl lyophilizer (VirTis, Gardiner, NY) overnight.

To prepare 2D membranes, 10 mL of solution was air dried on Teflon sheet. The air dried samples were then cut into 14 mm diameter sizes and transferred to a 24 well plate, precoated and air-dried with 100 µL of the same solution.

To prepare hydrogel samples, first 400 µL of the solution was mixed with 25,000 stained fibroblasts, as described in the cell culture section, incubated for two hours at 37°C in a 24 well plate and then supplemented with 0.5 mL serum free growth medium. This was done to ensure that the fibroblasts would infiltrate the hydrogel structures, and avoid growth only on the surface, which might also result in them getting washed away during medium changes.

Cell culture

Human Foreskin Fibroblasts (HFF-1, cell line) was purchased from American Type Culture Collection (Walkersville, MD) and maintained in Dulbecco's modified Eagle medium supplemented with 4 mM L-glutamine, 4.5 g/L sodium bicarbonate, 100 U/mL penicillin-streptomycin, and 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) initially. Cells were maintained at 37 °C, 5% CO2/95% air, and fed with fresh medium every alternate day. Four days prior to seeding on different surfaces, incubation medium

was changed to serum free FGM medium (Lonza, Walkersville MD), supplemented with L-Glutamine, Insulin and Human Fibroblast Growth Factor. All subsequent experiments were performed using serum free medium.

When confluent or for seeding on different matrices, Cells were detached with TRYPLE Express (Invitrogen Corp., Carlsbad, CA). Cells were centrifuged at 1200 rpm for five minutes and dispersed in growth medium. Viable cells were counted using Trypan blue dye exclusion assay. Cells were then incubated in growth medium containing 2 µM CFDA-SE at 37°C for 20 min followed by washing the excess stain with growth medium. 10,000 cells were seeded onto tissue culture plastic (TCP) surface, and 2D membranes and 25,000 cells were seeded onto the 3D matrices and hydrogels.

To test the binding of proteins from the culture medium and stability of immobilized gelatin, few wells were incubated in growth medium without cells and analyzed at the end of culture period.

Flow cytometry analysis

After 4 and 10 days (with medium change on the second day), TRYPLE Express was used to detach cells from different surfaces. Cells were centrifuged at 1200 rpm for five minutes and dispersed in 300 µL Phosphate buffered saline (PBS) solution containing 0.1% bovine serum albumin obtained from Sigma Aldrich Chemical (St. Louis, MO). Cells were analyzed in FACSCalibur (Becton-Dickinson, San Jose, CA) flow cytometer. Stained and unstained cells on Day Zero were used as controls.

Quantification of cell seeding efficiency

100 µL spent media collected on Day 2 and Day 4 were used to analyze for cellular viability in an indirect manner. The collected medium was assessed for CFDA-SE fluorescence intensity using Gemini XS spectrofluorometer (MDS technologies, Santa Clara, CA) at excitation and emission wavelengths of 485nm and 525 nm respectively. A calibration line between CFDA-SE fluorescence intensity and number of cells was developed by seeding known number of cells (from zero to 10,000 cells) and killing them by repeated freezing and thawing. This calibration was used to determine the seeding efficiency on day 2, and number of dead cells on day 4. Since this is an indirect quantification of viability, the schematic below (**Figure 8**) would give a clearer idea about the whole process.

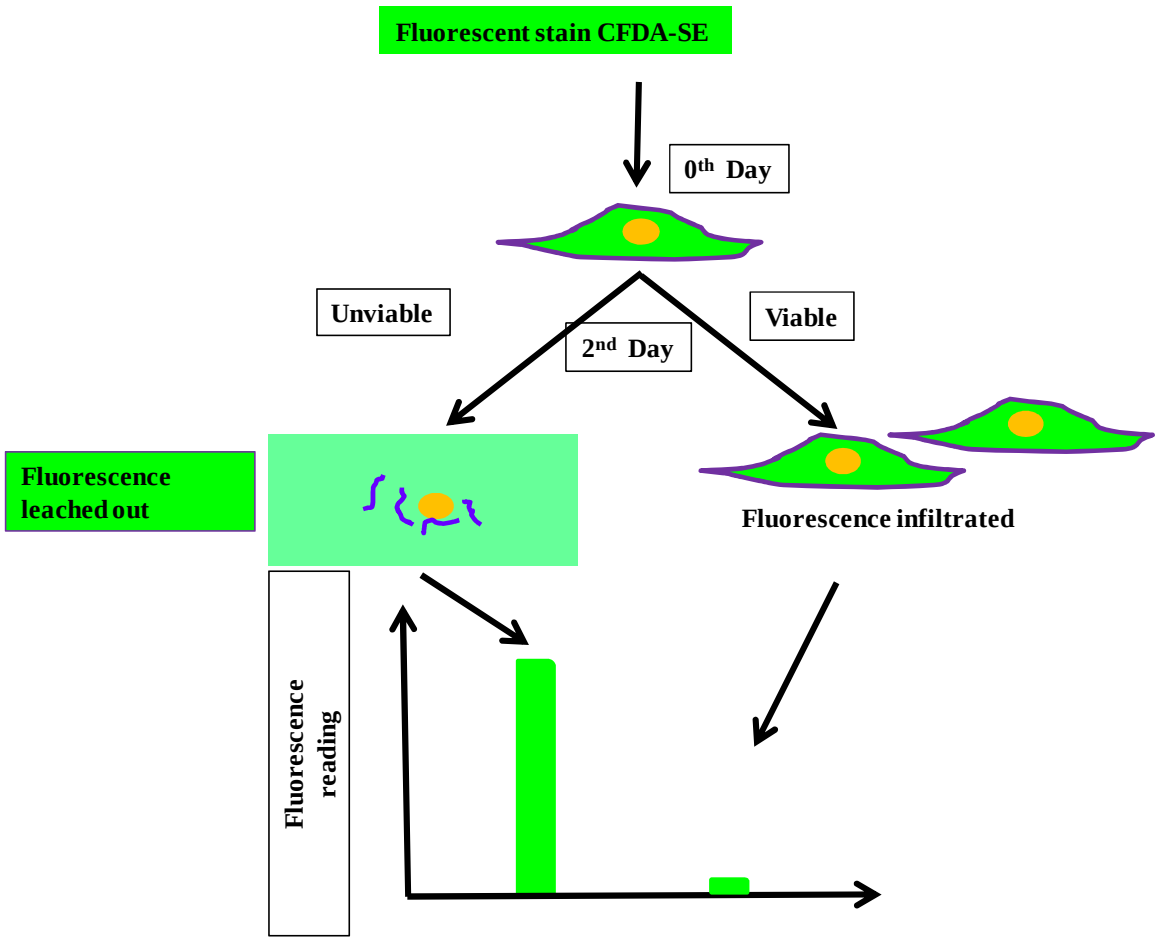

Figure 8: Indirect quantification of cellular viability

Evaluation of cell morphology

At the end of the incubation period, samples were fixed in 3.7% formaldehyde for 30 min at room temperature. Samples were washed three times with PBS, and permeabilized with -20°C ethanol overnight at 4°C. They were stained with Alexa Fluor 546 phalloidin (Molecular Probes, Eugene, OR) for 3h at -4°C in the dark. Samples were counterstained with DAPI following vendor's protocol (Invitrogen Corp, Carlsbad, CA, USA), observed under an inverted fluorescence microscope (Nikon TE2000, Melville, NY) and digital

micrographs were collected using the attached CCD camera.

Total Protein Content in the Medium

The total amount of protein present in the medium exposed to cells was assessed using a standard BCA assay kit (Fisher Scientific) following vendor's protocol. To assess the concentration of total protein due to cellular secretion (Cp), concentration of total protein in the medium exposed structures without cells ($C_{2,\ 0}$) was subtracted from the concentration of total protein in the medium exposed structures with cells (C_2).

Characterization of Collagen Content

The amount of acid soluble collagen secreted into the spent medium was assessed using the Sircol™ Assay (Accurate Chemicals, Westbury,NY) using vendors protocol. In brief, 1000 µL of dye solution was added to 100 µL of spent medium (volume was made to 100 µL using 50 µL of spent medium + 50 µL of DI water, according to protocol by vendor) and incubated for 30 min on a shaker at room temperature. Then samples were centrifuged at 14000rpm for 10 minutes and room temperature, supernatant was drained and 1000 µL of alkali reagent was added. After 10 min of vortexing, 200 µL of the solution was pippetted into a 96 well plate and absorbance was measured at 540 nm using Spectramax Emax spectrometer (Molecular Devices, Sunnyvale, CA). To determine the collagen secreted by the cells, collagen content in the medium exposed to structures were subtracted from the collagen content in the medium exposed to structures containing cells.

To determine the collagen content deposited in the matrix, samples were first digested

using Pepsin (Worthington Biochemical Corp., Lakewood, NJ) and 0.5 N Acetic acid, for 16 hours at 4°C. The supernatant obtained after the digestion process was evaluated using the Sircol Assay.

MMP-2/MMP-9 activity

To understand the phenotypic changes in cells, the amount of MMP-2/MMP- 9 secreted into the growth medium was monitored using a fluorogenic substrate (DNP-Pro-Leu-Gly-Met-Trp-Ser-Srg-OH) specific for MMP-2/MMP-9 (Lauer-Fields, Broder et al. 2001; Waas, Lomme et al. 2002). In brief, 100 µL of cell supernatant was incubated with 100 µL of a 100 M solution of fluorogenic peptide. After 20 min, at room temperature, fluorescence measurements were taken at 320 nm excitation and 405 nm emission. The amount of fluorescence was then normalized using the total protein content of the samples.

Characterization of Elastin

The amount of elastin secreted into the medium was analyzed using the Fastin Elastin™ Assay (Accurate Chemicals, Westbury, NY) using vendors protocol. In brief, 50 µL of spent medium was added to equal amount of precipitating reagent and incubated for 10 min on a shaker at room temperature. Then samples were centrifuged at 14000 rpm for 10 minutes and room temperature. Supernatant was drained and 1000 µL of dye reagent was added. After 90 min, samples were centrifuged at 14000 rpm for 10 minutes and room temperature. Supernatant was drained and 250 µL of the dye dissociation reagent solution was added. After mixing the contents, solution was pippetted into a 96 well

plate and absorbance was measured at 540 nm using a Spectramax Emax spectrometer (Molecular Devices, Sunnyvale, CA).

To determine the elastin secreted by the cells, elastin content in the medium exposed to structures were subtracted from the elastin content in the medium exposed to structures containing cells.

Statistical Analysis

All experiments were repeated three or more times with triplicate samples. Significant differences between two groups were evaluated using a one way analysis of variance (ANOVA) with 99% confidence interval. When $p<0.05$, differences were considered to be statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

Analysis of cell proliferation on matrices

The structures were investigated for viability and proliferation potential after an incubation period of four days, with a medium change on the second day.CFDA-SE is appropriate for the analysis of cell division at the level of the individual cell and permits a distinction between progeny of cells that have undergone a single round of division versus those that have undergone several rounds. The label is inherited by daughter cells after cell division, with subsequent halving of fluorescence (Lyons and Parish 1994). CFDA-SE is inherited equally by daughter cells after division, resulting in the sequential halving of CFDA-SE fluorescence with each generation. Flow cytometry histogram analysis of cells cultured on tissue culture plastic (TCP) (**Figure 9A**) showed distinct fluorescence peaks (indicated by red color) from the day zero stained (indicated by black bold line) samples. Interestingly, fibroblasts on chitosan-gelatin 3D porous structure showed a prominent peak (indicated by green color) near the vicinity of day zero stained samples. This suggests that there was no significant proliferation in those samples although all the cells were viable. Note that if the cells were not viable then the fluorescence signal would be near zero. This indicates that gelatin blended with chitosan, in the form of 3D porous structures is not toxic to cell growth. For the other structures,

the histogram analysis did not show significant peaks or proliferation activity, similar to that obtained for chitosan-gelatin 3D structures. After 10 days (**Figure 9B**), a shift in fluorescent intensity was observed for all the structures, with negligible fluorescence for TCP. Also, chitosan-gelatin 3D structures showed slightly decreased intensity compared to the fourth day samples. Presence of such an intense signal suggests reduced proliferation of HFF-1 cells on 3D chitosan-gelatin structures.

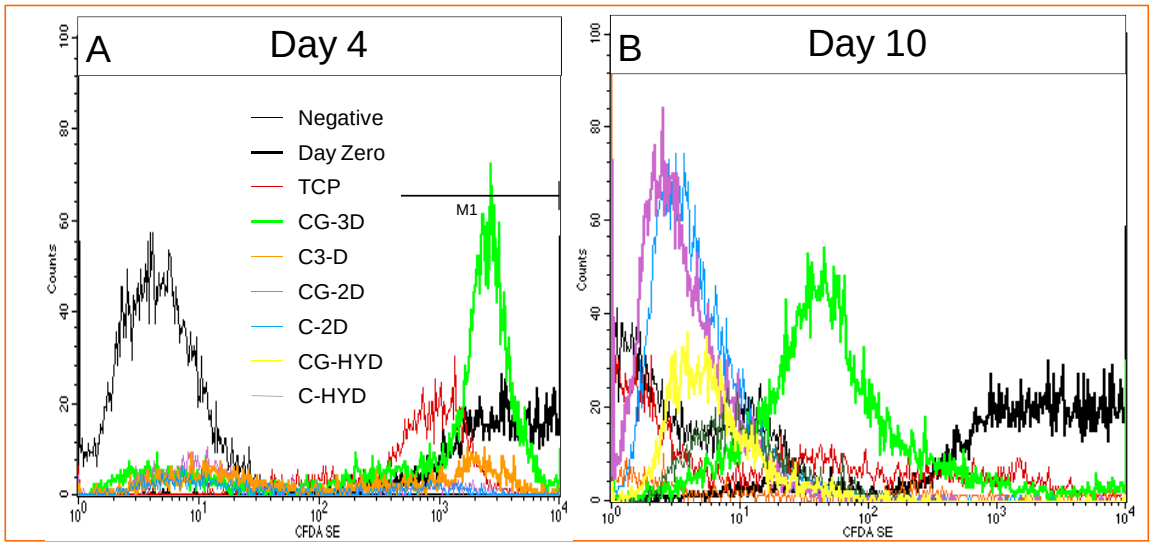

Figure 9: (A) Flow cytometric histograms of CFDA-SE stained fibroblasts on Day 4 (B) Flow cytometric histograms of CFDA-SE stained fibroblasts on Day 10

Evaluation of cellular morphology on matrices

Cell morphologies were evaluated to understand how different structures supported cell colonization. Cytoskeletal organization of HFF-1 was probed via actin staining. These results (**Figure 10A**) showed that HFFs had well spread spindle shape on TCP surface and peripheral distribution of actin filaments, similar to previous publications (Huang, Onyeri et al. 2005; Lawrence, Maase et al. 2008). Counterstaining with DAPI confirmed

the presence of nuclei. Similar morphologies were also observed on chitosan-gelatin 3D structures. However, cells on all other conditions showed significant reduction in spreading and also changed shape of the cells. The lowest spreading was observed on hydrogels and 2D membranes despite the presence of gelatin. After continued incubation for four days, the hydrogel structures show spindle shaped cells just beginning to form. According to a previous study (Weng, Romanov et al. 2008), cell spreading on the hydrogels are significant on the seventh day of incubation, whereas on the fourth day, spindle like cells are just beginning to form. This is similar to the results seen on the hydrogel structures of containing gelatin in this study. The cells speared to be rounded on the 2D membranes, confirming the minimal spreading characteristics of these structures. To confirm that they were cells, samples on 3D porous structures were analyzed by scanning electron microscopy. These results (**Figure 10B**) confirmed that cell attachment mimicked the pore morphologies of the chitosan-gelatin scaffold, showing that cell spreading and adhesion appeared to be guided by the porous structure. Also, matrix elements were observed on these structures, similar to the actin and DAPI stained images.

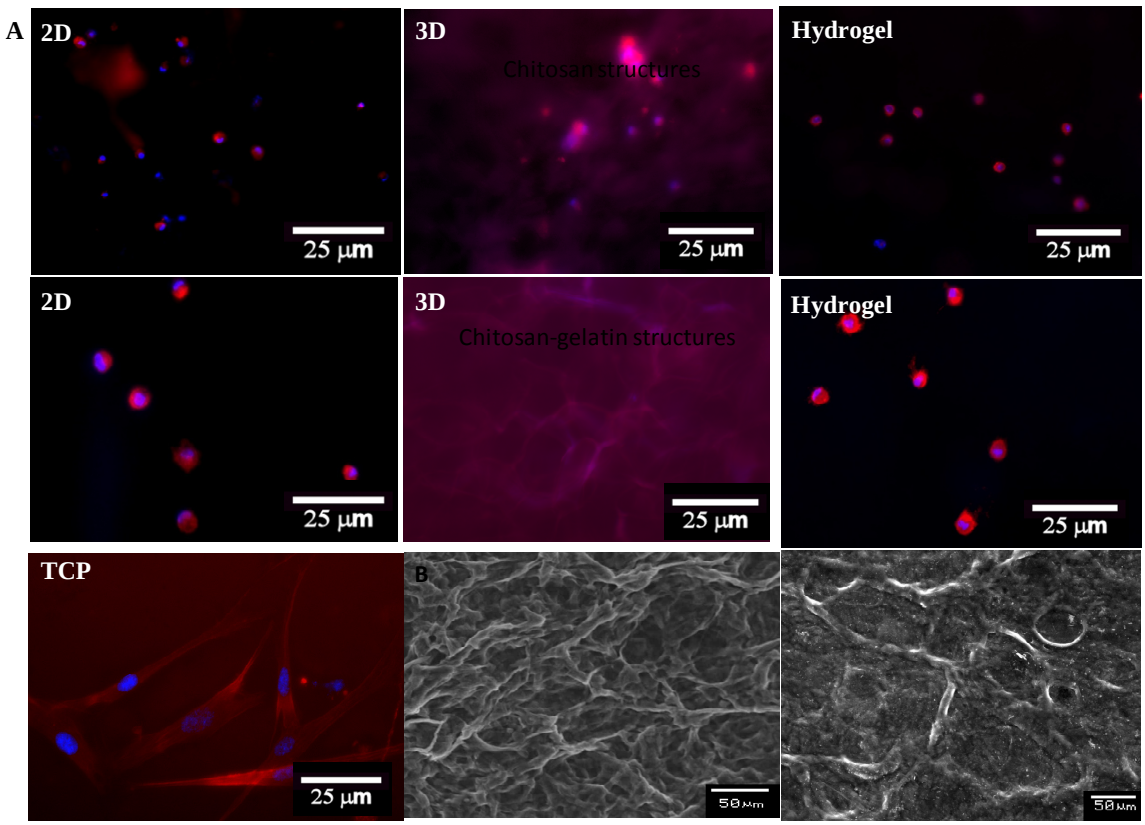

Figure 10: Morphology of cells on different structures. (A) Micrographs of cells stained for actin using Alexa phalloidin 546 and nuclei using DAPI after four days of incubation in serum free medium. (B) Scanning Electron Micrograph images of chitosan 3D and Chitosan-gelatin 3D structures

Characterization of cell viability

The dye carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) passively diffuses into cells. It is non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent and membrane non-permeable carboxyfluorescein succinimidyl ester, spontaneously and irreversibly coupling to cellular proteins by reaction with lysine side chains and other available amines (Weston and Parish 1990). The dye has been shown to be non-toxic enough to be widely used *in vivo* for visualizing cells (Weston and Parish 1990) and studying uptake of labeled substrates by cells (Iyoda, Shimoyama et al. 2002; Kulprathipanja and Kruse 2004).

To assess the efficiency of seeding, amount of fluorescence leached out into the medium due to dead cells was measured. These results **(Figure 3)** showed that the seeding efficiency was greater than 94% in all conditions on day 2. There was no significant difference in the cell death in all cases. Even on day 4, no significant difference was observed.

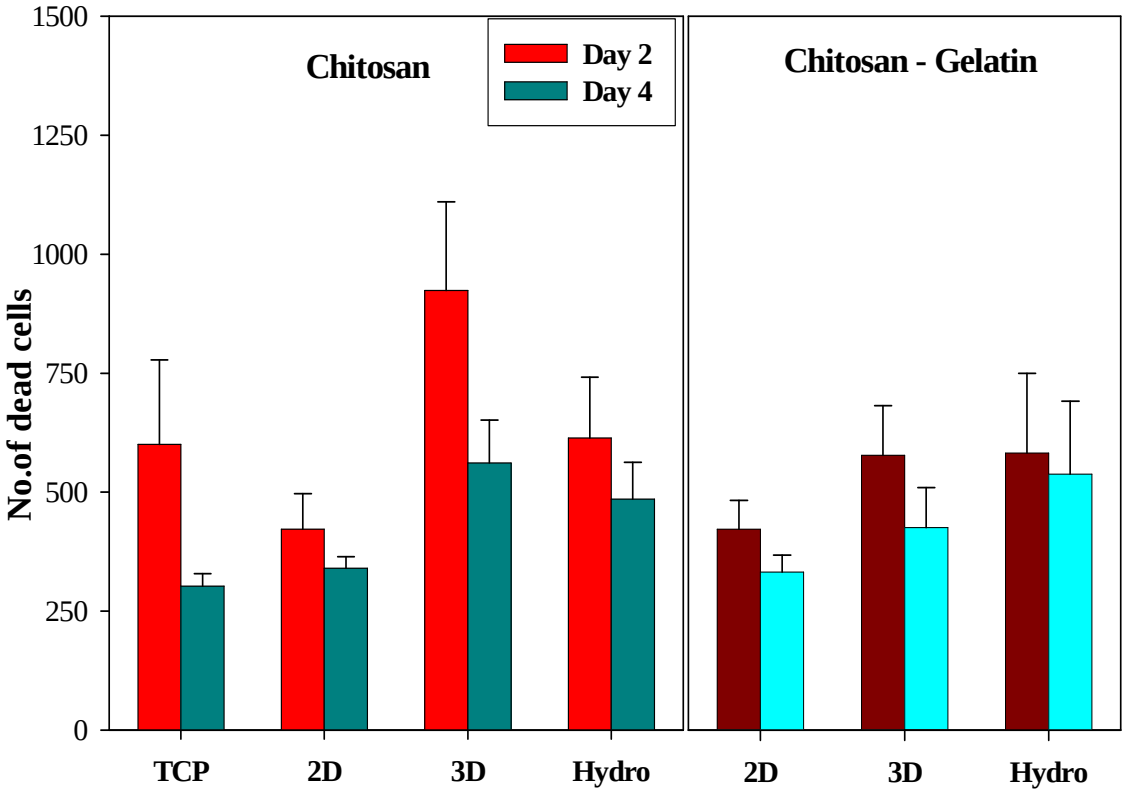

Figure 11: Amount of CFDA-SE present in the spent medium containing pre-stained fibroblasts on Day 2 and Day 4

Characterization of Extracellular Protein

To understand the implications of these changes on other observables, the total protein content in the spent medium was studies using a commercially available BCA assay (**Figure 12**). These results showed a significant increase in the total protein content in

the spent media from chitosan-gelatin 3D scaffold. This implies that cells, dead or alive, have secreted more protein into the spent media on this structure. This is in contrast to the increased proliferation observed on TCP which suggests there are more cells on day four relative to non-proliferative cells on chitosan-gelatin 3D scaffold. A possibility of gelatin (a form of denatured collagen, which in turn is a protein) being leached into the spent medium was considered. Therefore, a negative control experiment was carried out, where the structures were incubated in growth medium, without exposing them to cells, for the incubation period of four days. The spent medium collected was analyzed for total protein content. This was subtracted from the protein content values obtained for the structures exposed to cells. Interestingly, chitosan-gelatin 2D structures showed higher protein content due to leaching in the negative control experiment. For total protein content analysis, data points from three different experiments showed a significant difference ($p<0.05$) between TCP and chitosan-gelatin 3D structures, with the latter showing significantly higher protein content. Similar trends were observed for other analysis like collagen content and elastin content.

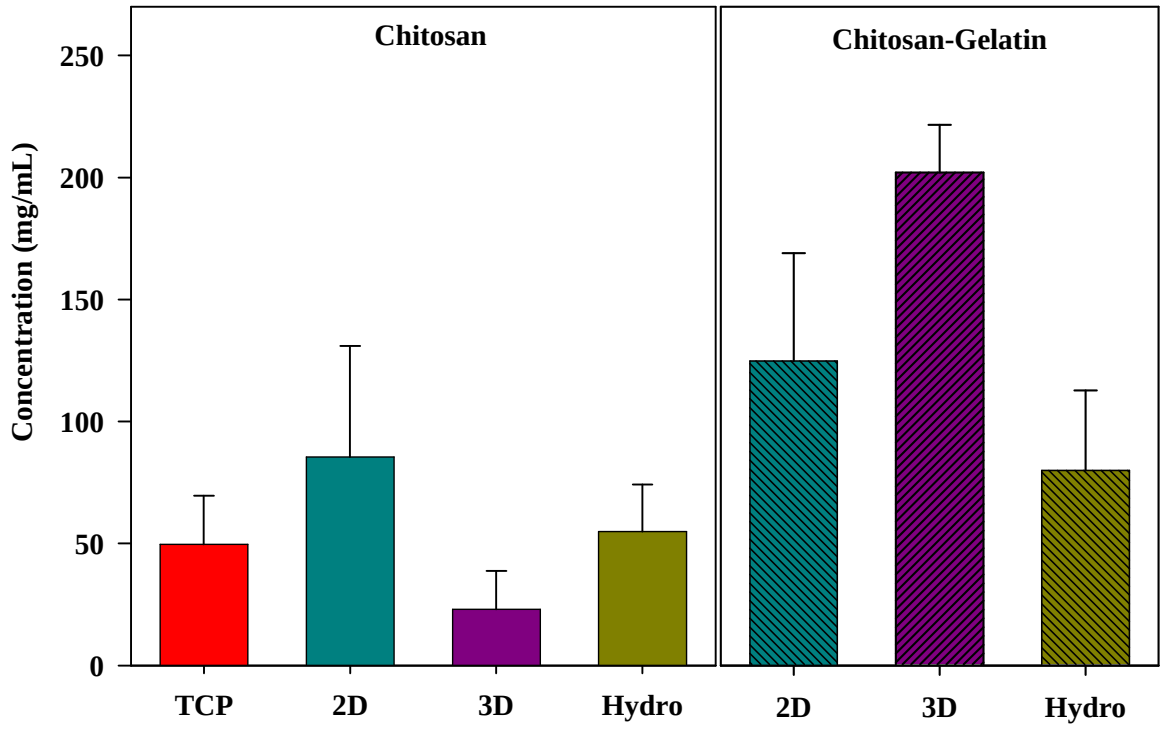

Figure 12: Total protein content in the spent medium on day 4

Dynamics of Collagen Synthesis

Collagen secreted into the medium: To understand what increased protein content corresponds to, analysis of collagen in the spent medium synthesis on day four is necessary. A similar negative control was carried out to analyze collagen content. There was a significant increase in the soluble portion of collagen in the medium for chitosan-gelatin 3D structures. However, there was no difference between chitosan 2D, 3D and TCP. Secretion was significantly less for chitosan-gelatin 2D structures and hydrogels. The inference that we can draw from here is that, collagen, one of the important

extracellular matrix elements, is secreted in significantly higher amount ($p<0.05$) by chitosan-gelatin 3D structures, compared to the others. (**Figure 13A**).

Analysis of Collagen Synthesis and Degradation.

To better characterize the functionality of the structures, it was necessary to analyze the secretion characteristics of extra cellular matrix elements that are synthesized by the ECM in human fibroblast cells. Since collagen is readily synthesized by fibroblasts, it was necessary to understand the synthesis and degradation of collagen as a dynamic process. There are three different aspects of the system that needs to be taken into account – cell-matrix interactions, cell-cell interactions and cell-matrix-nutrient interactions. The assumptions for the derivation are as follows:

1. Binding sites on the structures remain unchanged with or without medium
2. Degradation characteristics of the structures remains constant from day zero to day four

According to the manufacturers, there is no collagen in fresh growth medium. Hence, any collagen (or gelatin) in the medium (C_{C0}) without cells is due to the leaching of gelatin out of the structures. Hence, collagen remaining in the matrix can be (C_{m0}) calculated knowing the initial amount of collagen added in each matrix. Assuming the same behavior to follow in presence of cells (i.e., neglecting the reduction in leaching due to cell adhesion and spreading), secreted collagen can be assessed by measuring the collagen content in the medium (C_{C2}) and the matrix (C_{m2}) exposed to cells.

Collagen synthesized by cells into medium, $C_C = C_{C2} - C_{C0}$

Collagen synthesized by cells deposited in the matrix, $C_m = C_{m2} - C_{m0}$

Then total collagen secreted by cells can be calculated by $C_{Ct} = C_m - C_C$

From day 4 analysis (**Figure 13B**) we observed that the collagen content in the matrix structure was not significantly different for any of the structures. This was in contradiction to the collagen secreted into medium on the fourth day (**Figure 13A**). This raised a question – How could the cells be excreting more collagen but not contain more collagen in the matrix? Was this due to strong binding of collagen to the matrix that it was not detached easily during analysis? To explain this phenomenon, we carried out a separate experiment on structures after 10 days of incubation. Interestingly from **Figure 13D**, we can see that collagen synthesized on the structures has increased significantly (at least two fold or about 200%) for chitosan-gelatin 3D structures, whereas it has remained almost constant for the other structures. This hints at the possibility that chitosan-gelatin 3D structures might support better functionality when cultured with cells for a longer period of time. Also, culturing for an increased time period could have resulted in better detachment of collagen from the structure, thereby helping the analysis better on day 10. Further, day 10 analysis of collagen content is in line with the flow cytometer studies discussed in the earlier section, where it was shown that after 10 days, chitosan-gelatin structures show significantly higher viability than other structures.

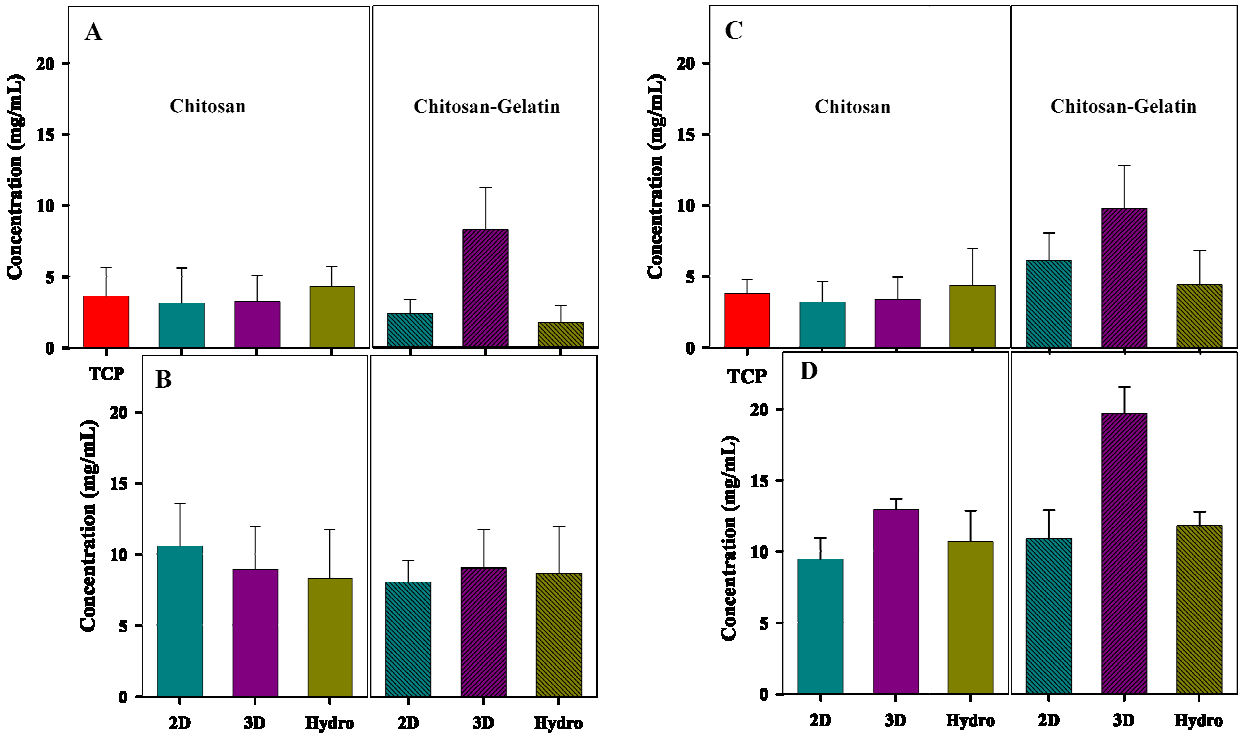

Figure 13: (A) Total amount of collagen synthesized by cells into the medium on day 4 (B) Total amount of collagen synthesized by cells into the matrix on day 4 (C) Total amount of collagen synthesized by cells into the medium on day 10 (D) Total amount of collagen synthesized by cells into the matrix on day 4

MMP-2/MMP-9 Activity

The amount of MMP-2/MMP-9 secreted into the growth medium was monitored using a fluorogenic substrate. MMPs facilitate degradation of ECM molecules such as native and denatured collagens, elastin, laminin and fibronectin (Agren M.S, Jorgensen et al. 1998). The relative fluorescence units (RFU) per mg total protein (RFU/mg) were significantly higher in all gelatin containing and chitosan 3D structures than TCP (**Figure 14**). On an average, these structures showed two times more enzyme activity of the enzymes than TCP, while there was no significant difference between chitosan 2D, chitosan hydrogel and TCP. According to a study by Agren (M.S. ÅGREN 1994), gelatinases are present a

considerable amount of time during the wound repair process. In wound repair, the synthesis and degradation of collagen is a dynamic process, which is facilitated by the presence of MMP-9. MMP-9 is involved in degradation of collagen, and increased levels of MMP-9 indicate high collagen levels. In addition, an increase in MMP-2 might be important from the perspective of remodeling wounded tissue. Therefore, the increased levels of MMP-2/9 activity in the gelatin containing structures and chitosan 3D structures might be associated with the increased ability of these templates to aid in the wound repair process. For MMP-2/9, all the chitosan-gelatin structures and chitosan 3D structure showed a significantly higher ($p<0.05$) gelatinase activity than TCP. Other structures were comparable to TCP.

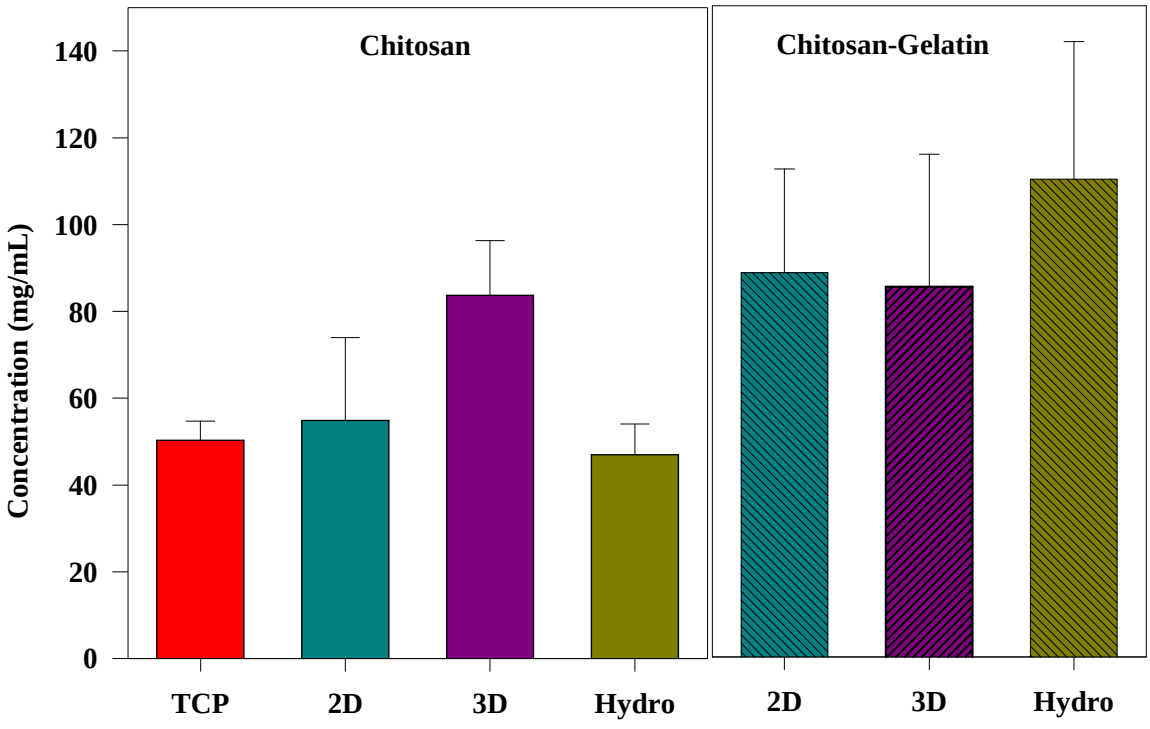

Figure 14: MMP-2/MMP-9 activity in the spent medium on day 4

Accumulation of Elastin in the Extracellular Matrix

Elastin content in the spent medium collected from the samples and TCP was analyzed. Elastin, another extracellular matrix protein like collagen, contributes elastic characteristics to the tissue. In other words, it helps to restore tissues to their original shape when they stretch. It is found as elastic fibers in the ECM and comprises an important fraction of the dry weight of the ECM. In this study, it is observed that gelatin-containing 3D structures have released more elastin into the medium than the other structures (**Figure 15**), which is similar to the results obtained for total protein content and collagen. It needs to be asserted here that before various secretions from the structures could be accounted for, 2D chitosan gelatin structures showed similar secretion values like chitosan gelatin 3D structures. There was a significant reduction, however, in the elastin secretions from the 2D structures when the miscellaneous secretions from the structures (negative control) were taken into the calculations. This could be due to the ready leaching of elements from the surface of the relatively less porous 2D membranes, compared to the porous 3D structures and cell embedded hydrogels. Also, chitosan-gelatin 3D structures secrete five times more elastin into the medium than other structures (**Figure 15**), in particular chitosan 3D and TCP. For all other structures, secretion of elastin was similar or not significantly greater than TCP. However, for chitosan gelatin hydrogels, the secretion of elastin was at least two times greater than TCP.

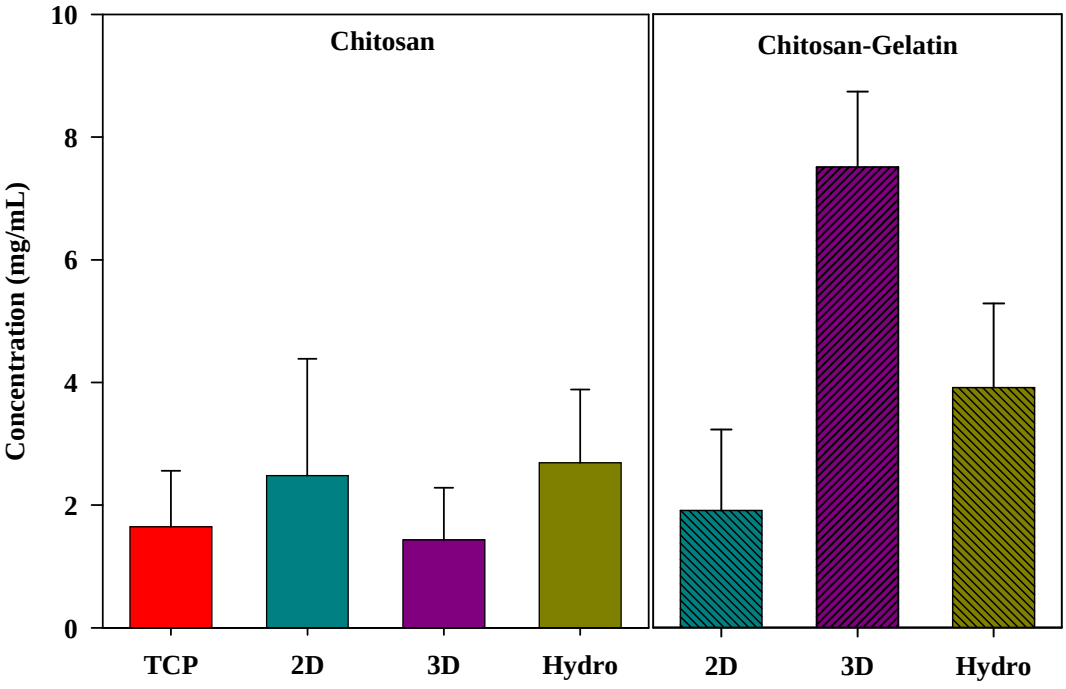

Figure 15: Elastin content in the spent medium on day 4

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

There were two objectives to this study:

1.The first objective was to understand the influence of a matrix composed of a polymer without cell binding domain (chitosan) and which had varying mechanical properties (2Pa to 2MPa) on cellular activity. From viability and proliferation studies, it was observed that chitosan structures demonstrated reduced viability and proliferation as they did not have a cell binding domain. This was supported by morphology studies where reduced cell spreading was observed on all the chitosan structures. Assays for ECM elements like collagen and elastin were carried out. Collagen content in the spent medium was significantly less, and collagen content in the matrix remained the same on the 4^{th} and 10^{th} days of analysis. Similar results were observed for elastin secreted from the spent medium. From an assessment of MMP-2/MMP-9 activity, it was observed that chitosan 3D structures have higher enzymatic activity than 2D and hydrogel structures.

2.The second objective was to understand the influence of a matrix composed of a polymer with cell binding domain (chitosan-gelatin) and which had varying mechanical properties (2Pa to 2MPa) on cellular activity. From viability and proliferation studies, it was observed that chitosan-gelatin 3D structures exhibited significantly higher viability

than other structures. Morphology studies also showed greater cell spreading on these structures. Matrix elements like collagen and elastin secreted from the spent medium were significantly higher for gelatin-containing 3D structures. Collagen content in the matrix for the 10^{th} day of analysis was higher than the 4^{th} day and significantly higher than the other structures. MMP-2/MMP-9 activity was higher for all the gelatin containing structures, indicating either the process of tissue repair if MMP-2 was high or inflammation if MMP-9 was high.

Overall summary: In summary, it can be stated that chitosan-gelatin 3D matrices, which contain a binding domain, and have optimum mechanical stiffness values (2KPa), exhibit better cell colonization, and significantly better functionality than the other structures which included 2D structures, hydrogel structures and chitosan 3D structures.

Recommendations

1. This study is an insight into the relation between physical (mechanical characteristics) previously established and chemical characteristics (presence of a binding domain) of biomaterial structures with their biological responses (*in vitro* studies). It has been demonstrated that functionality of the structures is an important parameter when evaluating their potential as implants. In future, this could be supplemented by histology studies which would confirm the results in a more qualitative manner. Cell to Matrix composition could be analyzed to ascertain if viability is completely supported by functionality in the form of synthesized ECM elements.

2. The increase in secreted matrix elements, like collagen and elastin, could be due to either protein secretions at the cellular level or gene behavior at the genetic level. For

this purpose, it is suggested that gene behavior be explored to completely understand the implications of increased functionality in the presence of cellular viability and in the absence of cellular proliferation. Also, other matrix elements like fibronectins, laminins and proteoglycans should be studied to understand their contribution to the overall ECM composition. In the current work, collagen and elastin analysis of the spent medium was evaluated. Further, collagen in the matrix/structure was assessed. This analysis should be extended to include elastin and other matrix elements like laminins, fibronectins and proteoglycans.

3. This study has looked at combined MMP-2/MMP-9 activity. While MMP-2 is constitutively expressed by fibroblasts, increased MMP-9 levels are associated with inflammatory activity. In future, MMP-2 and MMP-9 should be studied individually to understand if an increased level of the enzymatic activity is because of increased cellular viability or due to inflammatory responses. Inflammatory responses may arise due to damaged tissues, and this type of analysis would help us understand if there is some damage involved in the structures, for both chitosan and chitosan-gelatin. Also in future, the actual amount of enzymes should be evaluated instead of looking only at the intensity values. Since MMP-2/MMP-9 activity is an indicator of the synthesis and degradation of collagen, similar studies could be extended to involve enzymes that are associated during the synthesis and degradation of elastin.

4. The current study explored chitosan-based structures, and it was observed that they did not support cellular colonization. However, chitosan-gelatin structures which contain a cell binding domain show increased viability and functionality. Therefore, future studies would benefit from studying chitosan-gelatin 3D structures further. If these structures

were blended with synthetic polymers, they would open the possibility of structures with varied mechanical characteristics. Chitosan-gelatin structures have a cell binding domain whereas synthetic polymers do not. Blending the two could result in a versatile structure that has optimal mechanical characteristics. For example, mechanical characteristics can be manipulated to explore cellular behavior on the resulting structures. The chemical features of chitosan-gelatin structures could also be altered, by varying weight/weight ratios of chitosan and gelatin to ascertain if this has an effect on the resulting cellular activity of these structures. The *in vitro* studies done in this study could be supported by *in vivo* studies, to explore potential in a clinical scenario.

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VITA

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Thesis: BIOPHYSICAL EFFECTS OF MATRICES ON CELLULAR COLONIZATION

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Major Field: Chemical Engineering

Scope and Method of Study: The objective of this study was comparing the cell colonization characteristics on 2D membranes, 3D porous structures and hydrogels made from same materials but with varying mechanical properties.

Findings and Conclusions: Chitosan-gelatin 3D matrices exhibit better cell viability, and significantly better functionality than the other structures which included tissue culture plastic, 2D structures, hydrogel structures and chitosan 3D structures. This can be attributed to the presence of a cell binding domain in chitosan-gelatin 3D structures. The mechanical stiffness values of these matrices were 2KPa which was inside the range of the matrices analyzed – 2Pa to 2MPa. Total protein content, collagen content and elastin content in the spent medium was high for chitosan-gelatin 3D matrices. The amount of collagen in chitosan-gelatin 3D matrices increased from day 4 to day 10, while it remained the same on the other structures. This could be attributed to the increase in incubation time. Though there is reduced proliferation on these structures, there was significant increase in functionality.

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