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Evaluation of Chitosan as a Cell Scaffolding Material for Cartilage Tissue Engineering

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EVALUATION OF CHITOSAN AS A CELL SCAFFOLDING MATERIAL FOR CARTILAGE TISSUE ENGINEERING

By

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Current articular cartilage tissue engineering endeavors, using synthetic polymers as scaffolds, have been somewhat successful. However, the use of these materials has not yielded a satisfactory, functional replacement for articular cartilage. Therefore, this project focuses on an alternative to these materials, chitosan, which is a naturally occurring biopolymer.

The first project objective was to fabricate and analyze bulk, porous chitosan scaffolds, based on total porosity, average pore diameter, mechanical integrity, and degradation susceptibility. Secondly, scaffolds were evaluated in terms of their ability to support neochondrogenesis, including assessments of cell attachment and viability, cell morphology, and the biosynthesis of proteoglycan and type-II collagen-rich extracellular matrix.

Results indicated that chitosan scaffolds possessing an interconnecting, porous structure could be easily created through a simple freezing and lyophilization process,

and these scaffolds did support neochondrogenesis. Results suggest chitosan may be a useful alternative to synthetic polymers for use in cartilage tissue engineering applications.

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ABBREVIATION	DEFINITION
ECM	extracellular matrix
MSC	mesenchymal stem cells
PGA	poly (glycolic acid)
PLLA	poly (L-lactic acid)
PLGA	poly (DL-lactic-co-glycolic acid)
LPS	lipopolysaccharide
LPPS	lipopolysaccharide-protein complex
MIP	mercury intrusion porosimetry
SEM	scanning electron microscope
PBS	phosphate buffered saline
FBS	fetal bovine serum
DMEM	Dulbecco's Modified Eagles Medium
GAG	glycosaminoglycan
HMDS	hexamethyldisilazane

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Tissue and organ failure accounts for nearly one half of the total annual healthcare expenditure in the United States, or approximately $400 billion (Lewis, 1995). Injury and disease can affect something as simple as skin or can affect the more complex organs like the kidneys, liver, or heart. Treatment options for these diseases and injuries include transplants, surgical repair, prosthetic devices, mechanical support, and in some cases, drug therapy; however, these treatments often provide unsatisfactory repair or maintenance. In the case of transplantation, there are donor shortages and problems with immunosuppression. Though the probability of rejection declines with each passing month following a transplant, the possibility persists throughout the patient's life. In the case of surgery, repairs are often imperfect and require follow-up procedures. Prosthetic and mechanical support devices do not replace the full and proper function of the damaged tissue or organ, which often leads to continued disease progression. Drug therapy is only available for some diseases, and can be associated with unwanted side effects such as heart rate and blood pressure fluctuations or nausea and loss of appetite for example. Because these options do not provide satisfactory restoration of organ and/or tissue function, other avenues to restore, repair, and/or maintain tissues and organs must be pursued. One of these avenues is the emerging science of tissue engineering.

Tissue Engineering

"Tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions" (NSF Workshop on Tissue Engineering, 1988). In other words, tissue engineering involves the use of cells and extracellular components, either synthetic or natural, to create replacement, implantable parts to restore, maintain, or repair the function of damaged/diseased tissues and organs (Nerem, 1991). This approach may be applied toward several goals. Among these goals are providing replacement parts or cellular prostheses for the human body, providing vehicles to deliver engineered cells to an organism, creating tissue models with cells to study the states of diseases using aberrant cells, surfacing non-biological apparatuses, and providing acellular parts capable of regenerating tissues.

The first important phase of any tissue engineering endeavor is to evaluate the types, ratios, and organization of the cells necessary to produce the tissue or organ of interest. In general, one chooses a substrate material upon which to culture the cells that will enhance their organization in three dimensions and possibly provide initial mechanical integrity to the cell-polymer construct. This material should be biocompatible, sterilizable, and have a large surface area on which new tissue can grow (Paige and Vacanti, 1995). Applications may also require the scaffold material to be biodegradable. Secondly, the cells of the tissue or organ of interest are seeded onto the

substrate and cultured for a period of time *in vitro*. Finally, the cell-substrate construct may be implanted at the site of tissue damage. *in vivo*.

This general approach is currently being used in many tissue engineering research labs around the world and has proven clinically successful, as evidenced by commercially available tissue engineered skins. Other engineered tissues and organs such as cartilage, heart valves, blood vessels, bone, intestine, and tendon, among many others, also have promise for clinical success. Obviously the more complex the tissue or organ, the more complex the engineering process, but the same general methods are still employed. One tissue that is very well suited for tissue engineering methods is articular cartilage because of its fairly simple composition, one cell type, and lack of vascularity.

Articular Cartilage

Articular cartilage of the knee is a highly specialized connective tissue responsible for cushioning and lubricating. It is avasular, aneural, alymphatic, is composed mostly of water, and contains only one cell type, the chondrocyte. Articular cartilage has four distinct zones or areas. The first is called the superficial zone, which is the thinnest zone and forms the gliding surface of the joint. The second zone, the transition zone, lies just beneath the superficial zone and is a much more voluminous zone than the superficial zone. The third zone is called the deep or radial zone. This zone occupies the largest part of articular cartilage. The last zone, the zone of calcified cartilage, lies just above the subchondral bone and acts to separate the softer cartilage of the radial zone from the hard bone beneath. The zones are oriented in such a way to

compensate for different forces at the surface in relation to forces deeper in the tissue. Thus, the variation in the morphology of the zones reflects their structural role in withstanding the different forces within the joint.

The dry weight cartilage as a whole is composed of chondrocytes and the extracellular matrix (ECM) they synthesize. This extracellular matrix contains cartilage-specific proteoglycans, type II collagen, and other noncollagenous proteins, whose purpose is to provide the overall cushioning and lubricating properties of articular cartilage (Buckwalter, 1983).

The proteoglycans found in the ECM consist of a protein core composed of three regions and are responsible for the resiliency and stiffness to compression of articular cartilage. The three regions of the core protein include a hyaluronic acid binding region, a keratin sulfate-rich region, and a chondroitin sulfate-rich region. Hyaluronic acid, keratin sulfate, and chondroitin sulfate are glycosaminogylcans, which are repeating disaccharide units usually containing glucosamine or galactosamine and hexuronic acid or galactose. Proteoglycans either exist as monomers, one unit (Figure 1A), or aggregates, which are groups of monomers connected via link proteins to a hyaluronic acid filament (Figure 1B).

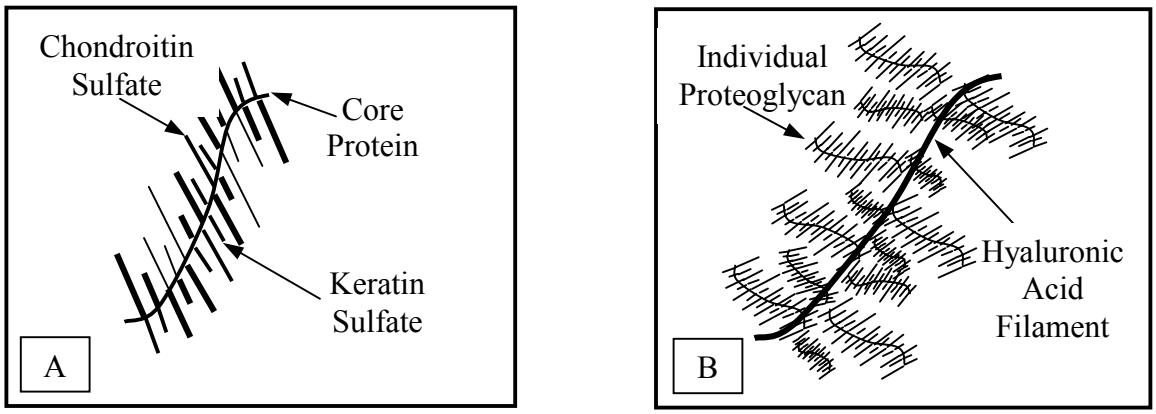

Figure 1A. Proteoglycan Monomer. B. Proteoglycan Aggregate

Type II collagen provides the form and tensile strength of cartilage. Collagen, in general, is composed of three alpha chains consisting of about 1000 amino acids each. The three alpha chains are tight left-handed helices that are bound together in a right-handed helix to form what is called tropocollagen. The spontaneous, but specific association of these tropocollagen molecules in the extracellular matrix of cartilage forms collagen microfibrils. Type II collagen can be distinguished from other types of collagen by its high hydroxylysine content, the high content of carbohydrates bound to the hydroxylysine, and its small diameter fibrils (Buckwalter, 1983).

Articular cartilage is particularly vulnerable to injury by trauma, disease, or congenital abnormalities. Because cartilage is avascular, alypmhatic, and aneural (Buckwalter, 1983, Huber et al, 2000), disease and injury often go undetected until severe damage has already occurred (Buckwalter, 1983). Once the tissue is damaged, it has little capacity for intrinsic repair. Repair that does occur often results in fibrocartilaginous areas at the injury site consisting of clumps of cells bound together by sheets of collagen that do not exhibit the structural organization or strength for healthy

articular cartilage function (Huber, 2000, Buckwalter, 1983). For this reason, disease often progresses to severe degenerative osteoarthritis. Osteoarthritis affects an estimated 20.7 million Americans and accounts for over $67 million in lost wages and productivity annually.

Articular Cartilage Tissue Engineering

Articular cartilage is a good candidate tissue for tissue engineering. Its lack of vasculature and its single cell type allow the engineer to create a construct consisting simply of polymer and chondrocytes. Chondrocytes are easily obtained from native cartilage, preferably from a young source, through a simple enzymatic digestion procedure. These cells are considered primary cells and already possess the chondrocyte phenotype. Chondrocytes also proliferate rapidly in monolayer, which allows the polymer substrate to be covered in a relatively short time period. Another attractive quality of articular cartilage is that chondrocyte metabolism *in vivo* is anaerobic, using the glycolitic pathway, which is the usual mechanism of cell metabolism *in vitro* (Muir, 1995; Lane et al, 1977); therefore, culture conditions do not create metabolic stresses on the chondrocytes.

Mesenchymal stem cells (MSC's) may also be used to engineer articular cartialge. Mesenchymal stem cells have the potential to become one of many different types of cells, including muscle, fat, bone, cartilage, tendon, and marrow stroma. The exact environment needed for MSC's to differentiate into each different cell type is not well understood, but includes varying combinations of nutrients, growth factors, spatial organization, and mechanical stimulus (Pittenger et al, 1999).

Once the type of cell to be used has been chosen, the next most important decision becomes the choice of the polymer substrate material. The most extensively investigated materials for this application are the synthetic polymers poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and copolymers of the two, poly(DL-lactic-co-glycolic acid) (PLGA) (Freed et al., 1993; Sittinger et al., 1996; Baker and Goodwin, 1997; Grande et al., 1997). Using these polymers, neocartilage has been engineered that resembles native cartilage in morphology and composition; however, there have been no reports of tissue engineered cartilage that meet the functional requirement to replace native cartilage (Dounchis et al., 2000; Chu et al., 1995). Therefore, alternative polymers are currently being investigated, including the natural polymers collagen and hyaluronic acid (Hutmacher, 2000, Vaissiere et al 1999, Chevallay and Herbage, 1999). These are very attractive materials because they are major constituents of the extracellular matrix of native articular cartilage and have been implicated in important cell signaling events that occur during chondrogenesis (Allemann et al., 2001). Another attractive material is chitosan, a derivative of a naturally occurring biopolymer, whose chemical structure resembles that of hyaluronic acid. Chitosan has been suggested as a bipolymer for use in many medical and non-medical applications (Suh and Matthew, 2000) and is the topic of this research.

Chitosan

Chitosan is a modified carbohydrate polymer derived from chitin, a structural, linear polysaccharide found in the exoskeletons of crustaceans. Chitin is the second most

abundant biopolymer found in nature, second only to cellulose, and is an abundant waste product of the seafood industry. It accounts for approximately one third of the dry weight of the waste shells. Chitin can be easily processed into chitosan, typically by an initial decalcification in dilute, aqueous hydrochloric acid solution, and then deproteination in dilute aqueous sodium hydroxide. The product is subsequently decolorized in 0.5% potassium permanganate (aqueous) and oxalic acid (aqueous) or sunshine, and finally deacetylated in hot, concentrated (40-50%) sodium hydroxide solution to produce the final product, chitosan. Chitosans are a family of poly-D-glucosamine units that vary in their degree of deacetylation and molecular weight.

There are two broad categories to explain chitosan. It is either a random-type copolymer of GlcNAc and D-glucosamine termed heterogeneous chitosan, or it is sequenced blocks of acetylated glucosamines termed homogeneous chitosan (Aiba, 1993). These chitosans not only have different physical forms, but they can also act quite differently under certain environmental conditions.

Both types of chitosans have been shown to possess characteristics that make them suitable as a tissue engineering cell substrate. Certain chitosans are antimicrobial, biocompatible, and degrade to absorbable oligosaccharides (Denuziere, 1998). Chitosan can be easily processed into porous scaffolds, films, and beads (Jarry et al, 2001). Especially important for cartilage tissue engineering, is that chitosan is polycationic, and its structure is similar to hyaluronic acid, an important molecule in the extracellular matrix of articular cartilage (Suh et al, 2000). Two-dimensional substrates of chitosan have been shown to support the synthesis of cartilaginous extracellular matrix

components in monolayer culture (Lahiji et al, 2000), and have been shown to enhance osteogenesis *in vitro* (Klokkevold et al, 1996).

Antimicrobial Action of Chitosan

Some chitosans have been shown to have antimicrobial and bacteriostatic potential. These chitosans inhibit microbial activity by attacking the microbial cell wall. Chitosan has been observed to act more quickly on fungi and algae than on bacteria (Cuero, 1999); however, like other properties of chitosan, this activity may be dependent on the type of chitosan, chitosan molecular weight, and degree of deacetylation, among other factors influencing the environment in which the chitosan is placed. This antimicrobial activity of chitosan has been investigated for use as an anti-spoilage mechanism in food research. Davydova et al (1999) found mildy hydrolyzed chitosan to exhibited antimicrobial activity against spoilage organisms. Chitosan has also been investigated in medical applications as a possible mechanism to prevent secondary infections or to avert immunological rejections of implants and/or transplants. Felt et al (2000) studied the antibacterial effects of chitosan against E. coli and S. aureus, in a study on the efficacy of using chitosan as a tear substitute in cases of dry eye to prevent secondary infections. They found that the minimal inhibitory concentration of chitosan against E. coli was as low as 0.375 mg/ml and as low as 0.15 mg/ml against S. aureus (Felt et al, 2000). In another study by Rhoades et al (2000) the interactions of bacterial endotoxins, lipopolysaccharide (LPS) and lipopolysaccharide-protein complex (LPPC), with chitosan were investigated. These endotoxins are a major component of the outer

membranes of gram-negative bacteria. The interaction between these endotoxins and chitosan was determined to be quite complicated and depended not only on the molecular organization of the endotoxin, but also on the degree of polymerization of the chitosan, with the highest affinity being between longer O-chain endotoxins and lower molecular weight chitosans. They concluded this binding between chitosans and LPS decreased acute toxicity (Rhoades et al, 2000).

Biodegradation of Chitosan

An important consideration in cartilage tissue engineering applications is the rate at which the polymer matrix used to organize the cells *in vitro* biodegrades, if at all. The presence of non-biodegradable foreign materials in soft tissue often causes acute foreign-body reactions elicited by the body's immune system that can result in severe inflammation and soreness around the implant site. However, while biocompatible, biodegradable polymers may elicit an initial immune response, it is often possible to control their degradation rate to decrease the length of time during which foreign material is in contact with living tissue.

Many studies have shown that chitin and chitosan are biodegradable polymers, and that they degrade *in vivo* mainly through their susceptibility to enzymatic hydrolysis mediated by lysozyme, which is ubiquitous in the human body; however, this action has been determined to be dependent on factors including pH, type of chitin or chitosan, and chitosan preparation method. Davies et al (1969) determined that chitosan is most susceptible to hydrolysis by lysozyme at pH 5.2, and the optimum range of pH values has

been determined to be between pH 5.2 and 8.0 (Davies et al, 1969 and Shigemasa et al 1993).

Pangburn et al. (1982) studied the effect of deacetylation on the susceptibilities of chitin and chitosan to lysozyme and concluded that pure chitin (0% deacetylation) is most susceptible to lysozyme, while pure chitosan (100% deacetylation) cannot be degraded by lysozyme. In 1990, Sashiwa et al. (1990) studied the relative rates of degradation of six chitosans varying in degree of deacetylation (45%, 66%, 70%, 84%, 91%, and 95%), but and found that 70% deacetylated chitosan degraded most quickly.

Shigemasa et al. (1993) investigated the effects preparation methods had on degradation. They found that for the same molecular weight and degree of deacetylation, homogeneously prepared chitosans were more susceptible to hydrolysis by lysozyme than heterogeneously prepared chitosans (Shigemasa et al, 1993). These data were consistent with results from Lee et al. (1995). Their findings suggest that there must be segments consisting of at least three consecutive N-acetyl sugar residues for lysozyme to be effective. Block segments of N-acetyl sugars are commonly found in homogeneous chitosans. Segments consisting of only glucosamine were not susceptible to hydrolysis by lysozyme (Lee, 1995).

In a more recent study Varum et al. (1996) noted that the specificity for most enzymatically catalyzed reactions usually included the number of sugar residues recognized by the active site of the enzyme, and found that this was in fact true for the lysozyme-chitosan enzyme-substrate complex as well. The lysozyme active cleft consists of six subsites, $A_L - F_L$. They found a high affinity of the D_L and E_L subsites of the

active cleft of the lysozyme for A-units of chitosan (oligosaccharides with acetylated reducing ends) rather than D-units (oligosaccharides with deacetylated reducing ends). The F_L subsite showed no selectivity for A- or D-units. Their results also suggested that the nearest neighbor to the A-unit in the D_L subsite should also be an A-unit for hydrolysis to occur at an appreciable rate (Varum et al., 1996). Figure 2 depicts the lysozyme-chitosan complex and shows the specificity of these lysozyme subsites for certain chitosan subunits.

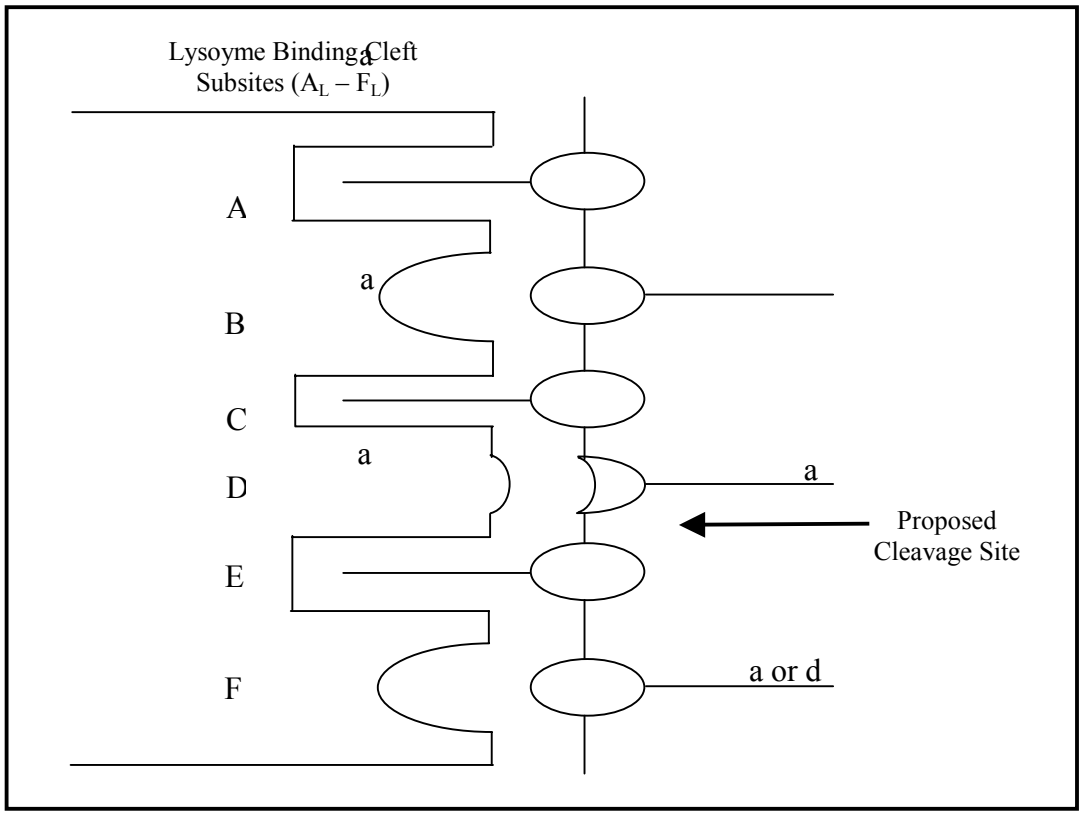

Figure 2. Schematic Illustration of Lysozyme Binding Cleft Subsites and Their Specificity for a- and /or d-units of the Chitosan Chain. The Arrow Illustrates the Proposed Cleavage Site of the Chitosan Chain.

While there is still conflicting data as to the exact products of chitosan degradation *in vivo*, the products have been identified as simple oligosaccharides that are not harmful and are often further degraded by β-glucosaminidases before being easily flushed from the body (Onishi and Machida, 1999). These oligosaccharides are usually tetramers, pentamers, and hexamers, which suggests that lysozyme acts as an endozyme. If chitosan were acting as an exozyme, oligomers resulting from enzymatic degradation would include the monomers and dimers of D-glucose.

Chitosan Biocompatibility and Charge

Tomihata and Ikada (1997) studied how degradation rate related to biocompatibility of chitosan films. Results showed a marked decrease in the degradation of deacetylated films when the degree of deacetylation increased from 69% to 73%. The more rapidly degrading 69% deacetylated chitosan showed strong cell adhesion properties, but elicited an acute inflammation reaction when implanted subcutaneously in rats. This was most likely due to the accumulation of degradation products at the implant site, caused by rapid degradation of this film. On the other hand, implanted films of 73% deacetylation or more did not elicit a strong foreign-body reaction, but still displayed very strong cell adhesion properties. While cell adhesion properties of chitosan are attributed in part to its cationicity, other implantable materials with a positive charge often elicit acute foreign-body reactions *in vivo*. Tomihata and Ikada believe the lack of severe reaction to more highly deacetylated chitosan was related to the zeta potential of these films. Zeta potential is a measure of the potential at the boundary between a

particle and a continuous layer, or slipping plane. More highly deacetylated chitosans, while still possessing a strong cationic charge due to their primary amines, display lower zeta potentials than their less highly deacetylated counterparts. The lower zeta potential of more highly deacetylated chitosans in concert with their slower degradation are believed responsible for the lack of an acute inflammation reaction at the implant site (Tomihata and Ikada, 1997).

Cellular and Tissue Responses to Chitosan

Chitosan's cationic nature appears to be the main mechanism by which cells are attracted to this polymer; however, the degree of cell attachment has also been shown to be related to the percent deacetylation of the chitosan. Prasitsilp et al (2000) studied how degree of deacetylation affected *in vitro* cellular responses to chitosan from two different sources, shrimp and cuttle fish. They tested four chitosan substrates, two from each source, differing by about 10% in deacetylation and ranging between 76% and 90% deacetylation. Results indicated that cells more readily attached to more highly deacetylated chitosans from both sources. Their results also indicate that adherence and growth of cells cultured in extract mediums from the more highly deacetylated chitosans was greater than that of cells cultured in extract mediums from lesser deacetylated chitosans. When cultured in the presence of discs made from the four different substrates, cells readily attached to and grew on discs of higher deacetylation while forming a ring around discs of lesser deacetylation; however, cells in the presence of discs of lesser deacetylation eventually began to migrate onto the discs when cultures

were allowed to become overgrown (Prasitslip et al, 2000). This suggested the lack of an inhibition zone around the chitosan, which indicated that the chitosan was not cytotoxic.

The *in vivo* chitosan-tissue biocompatibility studies tend to be in agreement with the *in vitro* cell response studies. *In vivo* studies of tissue response to films of chitosan by Tomihata et al (1997) showed a marked increase in chitosan-tissue biocompatibility as chitosan deacetylation increased (Tomihada and Ikada, 1997). Hidaka et al. (1999) studied the histopathological and immunohistochemical effects of chitosan membranes differing in degree of deacetylation implanted over rat calvaria. Their findings suggested that chitosan membranes with percentages of deacetylation ranging between 65% and 80% enhanced osteogenesis at the site of implantation, while those membranes of 94% or 100% deacetylation showed only minimal osteogenesis; however, membranes of lesser deacetylation elicited a more marked inflammation response than membranes of higher deacetylation. Hidaka et al suggested the need to control the inflammation response before chitosan could be considered in clinical applications (Hidaka et al, 1999).

Muzzarelli et al (1988) studied the biological activity of chitosan from an ultrastructural standpoint by investigating tissue repair induced by chitosan. They used 86.8% deacetylated chitosan in direct contact with or in substitution of dura mater, a tough, fibrous tissue found in the brain and spinal cord. Results indicated that chitosan seemed to mimic the function of the dura mater and had a significant inductive and stimulatory effect on the rebuilding of this connective tissue. Muzzarelli et al suggested that chitosan may be considered as a structural biomaterial for the repair of connective tissues because of its structural similarity to glycosaminoglycans found in the matrix of

these tissues (Muzzarelli et al, 1988). Others have proposed using chitosan in substrates used to treat burns and other severe skin lesions. These research teams have found that areas treated with substrates incorporating chitosan demonstrated an early return to normal skin color, facilitated rapid wound epithelializaiton and regeneration of nerves within the vascular dermis (Stone et al, 2000), and promoted skin with morphological equivalence to normal skin (Shahabeddin et al, 1990). After their first clinical uses of a dermal substrate incorporating chitosan, Damour et al believed chitosan was a promising candidate as an alternative to a skin homograft because of its availability and its exemption from micro-organism transmission (Damour et al, 1994).

Chitosan has also been shown to have great potential to enhance osteogenesis when used as a film or two-dimensional culture substrate. In a study to evaluate the effect of chitosan on osteoblast differentiation and bone formation *in vitro*, Klokkevold et al (1996) cultured mesenchymal stem cells from fetal Swiss Webster mice calvariae in the presence of 200 µl of a 2mg/ml chitosan/acetic acid solution or in control wells that were either untreated or treated only with 0.2% acetic acid. Results indicated a significantly higher number of osteoblast colonies in chitosan-treated wells as compared to control wells, while bone formation was also higher, but not statistically significant in the chitosan treated wells. These results suggested that chitosan did potentiate the differentiation of osteoprogenitor cells and may have also enhanced bone formation (Klokkevold et al, 1996). In a study using methylpyrrolidinone chitosan in an animal model of a bone defect, Muzzarelli et al (1993) found that the cationic nature and chelating ability of this chitosan favored mineralization of neoformed bone tissue, while

control defects showed only minimal bone formation originating only from pre-existing bone or from the periosteum (Muzzarelli et al, 1993).

Studies exploring the use of chitosan to potentiate neochondrogenesis have shown similar trends, such as the ability of chitosan to promote the maintenance of the chondrocyte phenotype and biosynthesis of extracellular matrix components, glycosaminoglycans and type II collagen, when grown on two-dimensional substrates of chitosan (Lahiji et al, 2000) or chitosan hydrogels (Sechriest et al, 1999).

Potential Advantages of Chitosan to Synthetic Polymers

Chitosan may have certain advantages over the more widely studied synthetic polymers used in cartilage tissue engineering applications. One advantage may be the relatively easy preparation of chitosan substrates. Chitosan powder is soluble in mildly acidic solutions, below pH 6.5, except in solutions containing sulfuric acid or phosphoric acid, and is most commonly dissolved in formic acid or acetic acid. Once dissolved, chitosan solutions can be either centrifuged or filtered to remove any undissolved particles, after which it can be easily formed into bulk porous scaffolds through a simple freezing and lyophilization process. Ice crystals that nucleate during freezing are removed from the frozen solutions by lyophilization, which creates an interconnecting, porous matrix. Furthermore, there is evidence that microstructural properties of porous scaffolds can be controlled by varying the chitosan concentration, molecular weight, percent deacetylation, degree of crystallinity, and the rate of freezing of the solution. For example, the pores become larger as the rate of freezing decrease (Madihally and

Matthew, 1999). Chitosan crystallinity can be controlled by reacting dissolved chitosan with aqueous, dilute nitrous acid, which depolymerizes chitosan chains by reacting with amino groups and cleaving the polymer at the β-glycosidic lingages. The rate of this depolymerization reaction was found to be independent of molecular weight (Allen and Payron, 1995). Controlling the crystallinity of chitosan may provide another means by which to control the microstructural properties of porous chitosan scaffolds and the rate at which they degrade. Furthermore, chitosan's free hydroxyl and amino groups provide an easy means of derivitization of this polymer and easy attachment of other bioactive side groups.

Processing procedures involved in creating synthetic polymer matrices for tissue engineering often include time, labor, and money intensive methods whose results are not always reproducible. Synthetic polymer processing can also involve the extensive use of toxic solvents. In the case of solvent casting used to prepare thin membranes, results are often less than desirable with respect to pore interconnectivity and can only be used to produce very thin membranes. Other processes including emulsion freeze-drying and thermally induced phase separation technology are very dependent upon the user and the technique employed. Emulsion freeze-drying is a process by which an emulsion, which consists of small monomer particles (0.05 - 0.5 μm) surrounded by a surfactant in water to which an initiator is introduced to start the polymerization process. The end product is a stable latex in water, which is freeze dried to recover the polymer (Tadmor and Gogos, 1979). Thermally induced phase separation technology involves using a latent solvent , one that is a high-boiling, low molecular weight solvent for the polymer at hight

temperatures, but a nonsolvent at lower temperatures, to cool a polymer mixture to a point of thermal separation. At this point the solvent phase can be extracted to recover the polymer phase (Fried, 1995).

Though some groups have been very successful in precisely controlling the properties of their polymers by using rapid prototyping and fused depositing modeling techniques, these are also extremely time consuming and expensive (Hutmacher, 2000). Therefore, the simplicity involved in producing chitosan matrices helps make it an attractive alternative to synthetic polymers used in tissue engineering.

Chitosan matrices also display strong cell adhesion properties without further processing, due to its cationic nature and high charge density. Often times, synthetic polymers require the blending of two or more polymer types, such as poly-L-lactide (PLLA) and poly (D,L-lactide co-glycolide) (PLGA) to induce optimal cell adhesion (Cohen et al, 1993). Furthermore, given that the chemical structure of chitosan is similar to that of glycosaminoglycans and hyaluronic acid, which have been implicated as important stimulators of chondrogenesis, chitosan may also share this and other characteristics with these molecules. Chitosan is also able to form insoluble ionic complexes with these molecules, which may aid in the integration of chitosan implants with native tissues (Suh and Matthew, 2000). In light of these promising characteristics of chitosan, the present research is aimed at an initial investigation of using three-dimensional, porous chitosan scaffolds as cell substrates for cartilage tissue engineering.

Statement of Hypotheses

The primary hypothesis was that the simple freezing and lyophilization of a chitosan-acid solution would produce bulk chitosan matrices possessing an interconnecting porous structure. To test this hypothesis chitosan scaffolds were fabricated through the simple freezing and lyophilization of a chitosan-acid solution. Chitosan-acid solutions were analyzed for viscosity, and completed scaffolds were analyzed by mercury intrusion porosimetry (MIP), scanning electron microscopy (SEM), mechanical compression, and in degradation tests to determine optimal conditions for producing these matrices. The secondary hypothesis was that porous chitosan scaffolds would support neochondrogenesis (i.e. the synthesis of a cartilaginous matrix by chondrocytes) *in vitro*. This entailed evaluations of chondrocyte attachment, viability, morphology, and biosynthesis of cartilaginous extracellular matrix (ECM) components.

CHAPTER II

METHODS AND MATERIALS

Experimental Design

To address the first hypothesis, chitosan scaffolds fabricated for cartilage tissue engineering must be highly porous and degrade at a rate consistent with the rate of new tissue formation. The goal was to produce degradable scaffolds with a target overall porosity between 90%-95%, consisting of pores with an average diameter between 100-300 μm. In an effort to find the best process for creating such scaffolds, parameters including the acid used as the solvent for the chitosan powder, the size and shape of the container used to freeze the solutions, and the rate of freezing of the solutions were varied. The viscosities and pH's of these solutions were also determined and compared. Solutions were frozen in either 35mm petri dishes or plastic vials and lyophilized to form bulk, porous scaffolds. Once lyophilized, chitosan scaffolds were analyzed for surface morphology by scanning electron microscopy (SEM). Scaffolds were scanned and evaluated for surface pore size and the appearance of an interconnecting pore structure. Scaffolds were also analyzed in two-dimensional sections after being embedded, sectioned, stained and photographed under a bright field microscope. The images were then digitized and evaluated for pore fraction, average pore diameter, and overall two-dimensional porosity.

Representative scaffolds were sent to Micrometertics, Norcross, GA, for three-dimensional evaluation by mercury intrusion porosimetry.

An additional ten scaffolds having a thickness of 6.35 mm and diameter of 12.7 mm, underwent mechanical evaluation. These scaffolds were assessed using a stress relaxation test to determine an equilibrium compression modulus.

Finally, scaffold degradation was analyzed. Methods were adapted from ASTM F-1635-95, Standard Test Method for Testing the *In Vitro* Degradation of Poly (L-lactic Acid) Resin and Fabricated Form for Surgical Implants. Eight test scaffolds were incubated in complete culture medium containing 500 μg/ml chick egg white lysozyme, while eight control scaffolds were incubated with complete culture medium only. Test and control scaffolds were divided into four groups corresponding to four time periods of incubation in test and control solutions. The dry weights of the scaffolds were measured before the beginning of the incubation period, and the dry weights of two test and two control scaffolds were measured at one, four, seven, and fourteen days following the start of the test. Scaffolds were 5 mm in diameter × 2mm thick (0.0125 cm^2). Two milliliters of solution was used for each scaffold, which yielded a ratio of solution volume to scaffold surface area of 160:1. A ratio of at least 100:1 is suggested by the standard.

To address the second hypothesis ten chitosan scaffolds (5 mm in diameter × 2mm thick) were seeded with porcine chondrocytes from three-week old pigs (5×10^5 cells/ml) and cultured in a 50 ml rotary bioreactor vessel for up to 28 days. After 48 hours, two scaffolds were harvested, and analyzed for cell attachment using SEM or viability using the LIVE/DEAD cell viability/cytotoxicity assay (LIVE/DEAD Cell

Viability/Cytotoxity kit, Molecular Probes, Eugene, OR). After one, two, three, and four weeks, two of the remaining scaffolds were harvested, fixed, embedded, and analyzed for the presence of proteoglycan and type II collagen-rich ECM by histological and immunohistochemical methods.

In a later test, three 5 mm diameter × 2mm thick scaffolds were seeded with three-week porcine chondrocytes (16×10^6 cells/ml) and cultured in a 10 ml rotary bioreactor vessel. All three scaffolds remained in culture for the 28-day period and were then analyzed as described earlier.

Experimental Methods

Scaffold Fabrication and Analysis

To begin, 86% deacetylated, powdered chitosan with a molecular weight of 200,000 g/mol (Vanson, Inc., Redmond, WA) was dissolved in 0.2 M acetic acid and 1% solutions of acetic, formic, and propionic by stirring for 48 hours. Once dissolved, the pH of each solution was tested with a pH meter, and their viscosities were tested using a Brookfield viscometer. The #2 spindle was used to test the solutions at motor speeds ranging from 5 to 30 rpm. Viscosities were then plotted as a function of shear rate to evaluate the validity of comparing the viscosities of the different solutions to one another within this range of shear rates.

Chitosan-acid solutions were poured into 35mm polystyrene petri dishes to a depth of approximately 5 mm and frozen at -27°C or into 12.7 mm diameter plastic vials to a final volume of 7 ml and frozen at -9°C. The solutions were allowed to freeze for 48

hours. Solutions in 35 mm petri dishes were then moved to a -80°C freezer where they were kept until lyophilization. The frozen chitosan cores from the plastic vials were removed from the vials by inversion after brief submersion in a 37°C water bath before being moved to the -80°C freezer. Frozen solutions were then lyophilized at -40°C for 24 hours or until completely dry. Lyophilization of scaffolds causes the removal of ice crystals from the frozen solutions, which produces the interconnecting, porous structure of the scaffolds.

To evaluate the time needed to freeze chitosan solutions, a thermocouple was inserted into one of the solutions frozen in a 35 mm petri dish during the freezing process. Temperatures were recorded every 10 minutes and a graph was constructed of temperature versus time using the collected data.

Microstructural Characterization

Lyophilized scaffolds were evaluated for surface morphology by examination under SEM, for three-dimensional microstructural properties using mercury intrusion porosimetry (MIP), and for two-dimensional properties using embedding, staining, and imaging techniques.

To analyze the surface characteristics of lyophilized scaffolds using SEM, they were first mounted on specimen holders with carbon tape and then sputter-coated with gold palladium to make them conductive. One three-minute coating was sufficient to eliminate charging inside the scope. Specimens were then simply placed in the SEM scope and visualized using secondary electron detection mode.

One scaffold from each fabrication technique was sent for three-dimensional evaluation by MIP on an Autopore IV 9500 (Micromeritics, Norcross, GA). MIP is based on the premise that a non-wetting liquid, such as mercury, will only intrude pores under pressure. Pore diameter is calculated according to the Washburn equation (Washburn, 1921):

$$p = (-4\gamma\cos\theta)/d$$

Where p is the pressure required to force mercury into a pore of entry diameter d, γ is the mercury surface tension, and θ is the contact angle between mercury and the sample. The contact angle of mercury on chitosan is 130 degrees, and mercury surface tension is 485 dynes/cm. A total of 266 low- and high-pressure data points in the range of 1.2 kPa to 413.7 MPa are collected to provide a high-resolution profile of mercury uptake.

Scaffolds from each fabrication technique were embedded in glycolmethacrylate (Immunobed, Polysciences, Niles, IL), sectioned to 7 μm using a rotary microtome, and mounted on slides to be stained and evaluated in two-dimensions. Chitosan's polycationic nature allows it to absorb anionic dyes, such as eosin, which was used to highlight the chitosan on the sections. Sections were mounted with glass coverslips using Permount (Fisher Scientific, Fair Lawn, NJ) and photographed with bright-field microscopy. Once digitized, the images of sections were analyzed using NIH Image for overall porosity and pore diameter. The results from two-dimensional and three-dimensional analyses were compared.

Mechanical Testing

Ten scaffolds from each fabrication technique having a thickness of 6.35 mm were mechanically tested in compression. These scaffolds were fully rehydrated before being tested. They were compressed in 5% increments to 50% of their initial height using the fixture shown in figure 3. It consists of a micrometer head connected in series with a ± 50 N load cell. The micrometer head was used to precisely control each step of compression. Scaffolds were held at each step for five minutes and then compressed to the next strain level. Stress relaxation was monitored and recorded using LabVIEW software. The data were then used to compute an equilibrium compressive modulus for each type of chitosan scaffold tested.

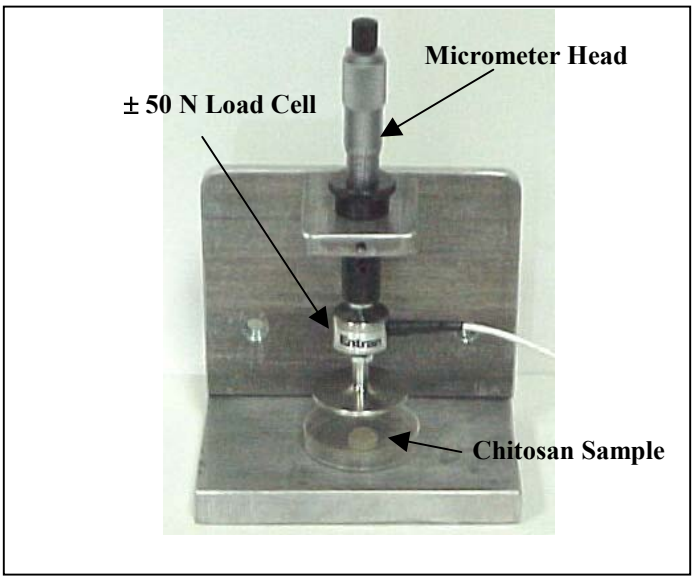

Figure 3. Mechanical Testing Fixture Used to Compress Chitosan Scaffolds

Degradation

To assess degradation of chitosan scaffolds *in vitro*, eight fully rehydrated scaffolds were incubated in 2 ml of complete culture medium containing 500 ug/ml chick

egg white lysozyme. This concentration of lysozyme was chosen in order to produce an effect, and is not necessarily the concentration present *in* vivo. Eight control scaffolds were incubated in complete culture medium without lysozyme. Incubations were done in a 37°C humidified 5% carbon dioxide (CO_2) incubator throughout the two-week study. The dry weight of each scaffold was determined before incubation. The dry weights of two control and two test scaffolds were measured again after one, four, seven and fourteen days of incubation. Scaffolds were dehydrated in a graded ethanol series before being measured. Scaffold weights were then recorded and compared to their initial dry weight. Degradation was determined as the percentage of weight loss as described by Section 8 of ASTM F-1635, and according to the formula:

$$\% WL = [(W_0 - W)/W_0] * 100,$$

where WL was weight loss, W_0 was initial weight, and W was weight after degradation. Statistical significance for main effects was determined using a two way ANOVA at the 95% confidence level, and treatment effects were determined using Tukey's honestly significantly different test also at the 95% confidence level.

Solutions Viscosities

The viscosities of chitosan-acid solutions were tested using a Brookfield Viscometer. The #2 spindle was used to test the solutions at motor speeds ranging from 5 to 30 rpm. Viscosities were then plotted as a function of shear rate to evaluate the validity of comparing the viscosities of the different solutions to one another within this

range of shear rates. Statistical significance was determined for the viscosities using a one way ANOVA at the 95% confidence level.

Cell Isolation and Culture

Initially, ten scaffolds were prepared for cell culture. They were partially rehydrated in a graded ethanol series (100%, 95%, 80%, 70%) to a 70% ethanol solution at which time they were cored using a 5 mm diameter core punch. These cores were then added to a fresh 70% ethanol solution and remained there overnight. This 24-hour incubation facilitated scaffold being bacteriostatic. After this 24 hour incubation, rehydration was completed with a solution of 50% ethanol to a final solution of phosphate buffered saline (PBS) containing 2× antibiotics-antimycotics. The scaffolds were incubated for at least 24 in this PBS solution to help ensure sterility. Before being seeded with cells, scaffolds were incubated with sterile fetal bovine serum (FBS) for twenty-four hours to coat the scaffolds with cell-essential proteins and facilitate cell attachment.

Chondrocytes were isolated from native cartilage harvested from the femoral chondyles of three-week-old pigs, using aseptic technique. Cartilage was minced with a scalpel, and the pieces were incubated with 1% type 2 collagenase dissolved in a 5% fetal calf serum solution in Dulbecco's Modified Eagles Medium (DMEM) and stirred overnight. This enzymatic solution digested the cartilaginous matrix components, releasing the cells into the medium. The solution was filtered through a 100 μm sterile filter and subsequently centrifuged at 1000 rpm for eight minutes to produce a cell pellet.

After the medium was removed from the pellet, the cells were resuspended at 5×10^5 cells/ml in complete cell culture medium (DMEM, 1% L-glutamine, 1% antibiotic-antimycotic, 10% FBS, and 50 ng/ml ascorbate) in a 50 ml rotating-wall bioreactor vessel (Synthecon, Houston, TX). The chitosan scaffolds were added to the vessel, where they remained in culture with the chondrocytes for up to 28 days. Cultures were kept in a 37°C humidified CO_2 incubator.

The rotating-wall bioreactor vessel system has been shown to be the optimal method of culturing three-dimensional cartilage constructs. Freed and Vunjak-Novakovic, 1995, showed that when rotating such that constructs are in a state of constant freefall, that bioreactor culture conditions most closely mimic *in vivo* chondrogenesis conditions (Freed and Vunjak-Novakovic, 1995). They also showed that this method of culture helps modulate the composition and mechanical properties of tissue engineered cartilage. Vunjak-Novakovic et al, 1998, found that cartilage constructs cultured in microgravity (in free-fall in a bioreactor) resulted in the highest fractions of regenerated tissue and glycosaminoglycans (GAG), when compared to constructs grown in other culture conditions including orbital mixing cultures, solid-body rotation, and spinner-flask culture. This environment also helps promote the maintenance of the chondrocyte phenotype throughout extended culture times (Freed and Vunjak-Novakovic, 1997).

The speed of our bioreactor was adjusted to 17.5 rpm, which allowed constructs to stay in a state of constant free-fall throughout the duration of the study. As scaffolds rotated in this low shear environment, nutrients were able to reach cells that have

attached to the interior of the scaffolds, and at the same time, metabolic waste products were moved away from the cells and out into the medium. In other words, this environment allows efficient mass transfer. The medium was changed every three days, at which time, the cultures were supplemented with L-ascorbic acid to a final concentration of 50 ng/ml. L-ascorbic acid is necessary for cartilaginous matrix synthesis.

Another trial was performed with three scaffolds fabricated using 1% formic acid as the solvent. These scaffolds were chosen based on results from the degradation study. Because higher initial cell density has been shown to increase the quantity and quality of engineered cartilage (Puelacher et al, 1994), this trial was performed with an initial cell density of 16 million cells/ml of culture medium. Scaffolds were seeded in a 10 ml culture vessel and after 24 hours were moved to a 50 ml culture vessel at which point 20 million more cells were added. In this trial, all scaffolds remained in culture for four weeks.

Cell Attachment and Viability

Cell attachment was evaluated after 48 hours for the first trial, by performing a hemocytometer count of cells remaining in the medium. The number counted was subtracted from the initial cell density to determine the number attached to each scaffold. One scaffold from the first trial was also fixed in 2.5% glutaraldehyde, dehydrated in a graded ethanol series, and chemically dried with Hexamethyldisilazane (HMDS) for viewing by SEM. This construct was mounted with carbon tape on a specimen holder

and sputter-coated with gold-palladium to render it conductive. Two, three minute coatings was necessary to prevent charging in the scope. The specimen was viewed in secondary electron detection mode on a LEO Stereoscan 360 (MSU Electron Microcopy Center) and its surface evaluated visually for cell attachment.

Cell viability was also evaluated after the 48 hour seeding period for the first trial and was confirmed by intracellular esterase activity as indicated by the green fluorescence of calcein that is enzymatically converted from calcein AM in live cells. Dead cells were simultaneously recognized by their red fluorescence, due to the binding of nucleic acids to ethidium homodimer-1 that enters the cells through damaged membranes. Samples were viewed qualitatively on a LEICA TSNSC Confocal Laser Scanning Microsope (MSU Electron Microsopy Center). The 48 hour cell attachment and viability analyses were not performed on scaffolds from the second trial because the scaffolds were moved to the larger volume vessel after 24 hours. Also there were only three scaffolds, which were all needed at the 4-week end point for other analyses.

Histology and Immunohistochemistry

The remaining scaffolds in the first trial were maintained in culture for 10, 18, and 28 days. All scaffolds from the second trial were harvested after 28 days. Scaffolds from first trial were processed for either histology or immunohistochemistry, while scaffolds from the second trial were processed only for histology. All samples were fixed in Carnoy's solution, which is a 3:1 solution of 95% ethanol to glacial acetic acid. This fixative was recommended by the Developmental Hybridoma Bank, Iowa City, Iowa, for

use with the II-II6B3 anti-type II collagen antibody, which was used for immunohistochemical procedures. After at least two hours in the fixative, samples were washed extensively with 95% ethanol to eliminate residual acid, and were then washed several times in 100% ethanol to complete dehydration. Samples were cut in half and were embedded in glycolmethacrylate (Immunobed, Polysciences, Niles, IL) in a vacuum chamber.

For routine histology, glycolmethacrylate blocks containing samples were mounted in the specimen holder of a rotary microtome and sectioned to 7 μm. Sections were placed on charged glass slides and allowed to dry for 24 hours. Sections were stained with hematoxylin and eosin and counter stained with toluidine blue to highlight proteoglycan-rich ECM (Kiernan, 2000). Slides were subsequently dehydrated, cleared in xylenes, and mounted with glass coverslips using Permount. Using this staining technique, cell nuclei stain blue, and areas of ECM that contain proteoglycans stain metachromatically.

Samples that were to be analyzed immunohistochemically for type II collagen, using a HistoStain SP Kit (Zymed, San Francisco, CA) were cut to 8 μm. After being placed on charged slides and allowed to dry, sections were rehydrated in a graded ethanol series to PBS and incubated with 2500IU/ml hyaluronidase (H6254, Sigma, St. Louis, MO) for 5 minutes. This enzyme digests any hyaluronic acid in the extracellular matrix that may be blocking antigenic-binding sites. After washing in PBS, section were incubated for 10 min with a peroxidase solution (1 part 30% hydrogen peroxide to 9 parts 100% methanol) and washed in PBS (5 × 5 min). Sections were then incubated with 10%

non-immune goat serum to block non-specific antigen binding sites. At this point, the blocking solution remained on negative control sections, while experimental sections were incubated for 120 min with a monoclonal antibody to type II collagen in a 37°C humidified CO_2 incubator. After another 5 × 5 min washing in PBS, all sections were incubated for 30 min with a biotinylated secondary antibody at 37°C and subsequently washed. Sections were then incubated for 30 min with a streptavidin-peroxidase conjugate, 10 min with a substrate-chromagen (hydrogen peroxide-DAB) mixture, and rinsed for 2 min with distilled water. Finally, sections were stained for 3 min with hematoxylin, and underwent a final rinsing for 30 sec in PBS and 2 min in distilled water. The sections were then dehydrated in a graded ethanol series, cleared in Xylene, and mounted with glass coverslips using Histomount (Zymed Laboratories, San Francisco, CA). Areas where type II collagen was present on experimental sections stain orange-brown, and the cell nuclei stain blue. Sections were viewed under bright-field microscopy.

CHPATER III

RESULTS

Experimental Results

Scaffold Fabrication and Analysis

Bulk, porous chitosan scaffolds were easily fabricated using a simple freezing and lyophilization process. Scaffolds fabricated by dissolving 86% deacetylated chitosan powder in 0.2 M acetic acid and freezing in 35 mm petri dishes at –20°C, revealed a what appeared to be an interconnecting porous structure when viewed under SEM (Figure 4A); however, when visually insepected, scaffold surfaces that had been in contact with polystyrene, displayed a very different surface structure that was less porous than scaffolds surfaces that did not contact petri dish surfaces (Figure 4B). Upon visual inspection of SEM microphotographs of scaffolds fabricated by dissolving 86% deacetylated chitosan powder in 0.2 M acetic acid and subsequently freezing in 12.7 mm diameter vials at –9°C, appeared to possess a more porous structure than scaffolds frozen in 35 mm petri dishes. A representative microphotograph is shown in figure 5.

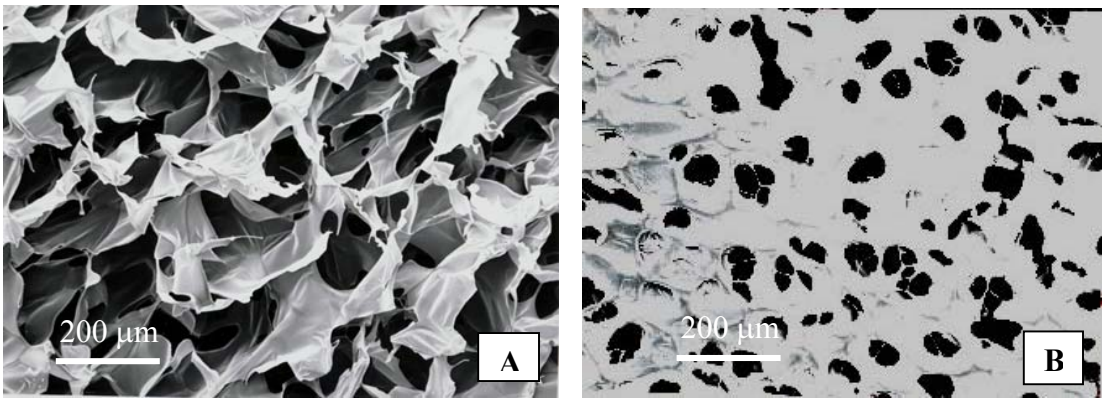

Figure 4 A. 100X SEM of Porous Chitosan Scaffold Surface B. 100X SEM of Porous Chitosan Scaffold Surface that was in Contact with Polystyrene Surface

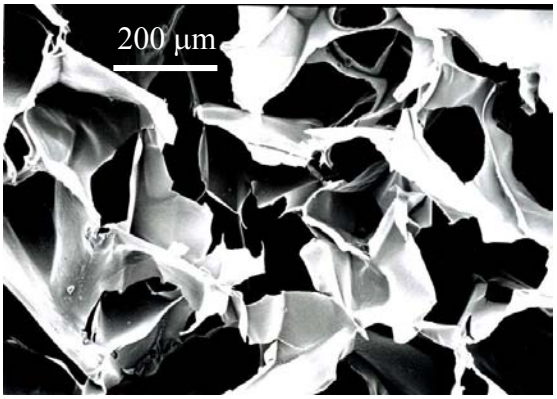

Figure 5. 100X SEM of Porous Chitosan Scaffold Frozen in Plastic Vial at –9 C

Figure 6 represents that temperature verses time data for the freezing of chitosan solutions in 35 mm petri dishes in a -27°C freezer. The temperature of the solutions dropped in a parabolic fashion until the solution reached 0°C, at which point the temperature remained constant until the entire volume of solution was frozen. The temperature then began to decrease further to reach the temperature of the freezer. The time taken from the solutions to reach equilibrium at 0°C was approximately 2 hours and 20 minutes for this solution.

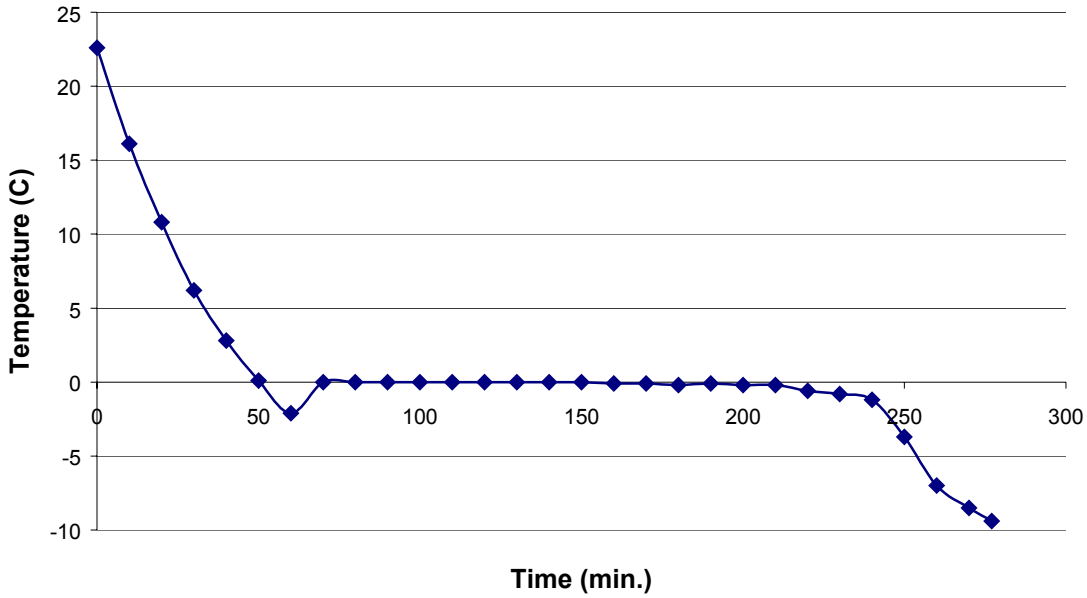

Figure 6. Temperature Vs. Time for Freezing of Chitosan Solutions in 35 mm Petri Dishes at -27°C

Microstructural Characterization

Three-dimensional microstructural properties, as determined by MIP analysis, for one scaffold made by freezing in a 35 mm petri dish and one scaffold made by freezing in a 12.7 mm vial is shown in Table 1. Both scaffolds were made using acetic acid as the solvent.

Table 1. Scaffold Properties Determined Using MIP

Freezing Container	Bulk Density	Total Pore Area	Total Porosity
35 mm petri dish	0.06 g/ml	0.8 m^2/g	80.70%
12.7 mm vial	0.17 g/ml	0.702 m^2/g	94.2%

The overall porosity of the scaffold frozen in the 12.7 mm plastic vial was 94%, while the overall porosity of the scaffold frozen in the 35 mm plastic petri dish was only 80%. By visual inspection, scaffolds frozen in vials appeared to have much larger pores in interior regions (the region used for cell culture), while those pores closer to the exterior where much smaller. Note, only one scaffold of each type was sent for analysis because of cost constraints; hence, the lack of statistical analysis.

Mechanical Testing

The compressive modulus of porous chitosan scaffolds frozen in 35mm polystyrene petri dishes was found to be 3.5 ± 0.4 kPa in the 50% compression range. While this is similar to the compressive modulus of other common culture mediums such as alginate or agarose, it is much softer than healthy hyaline cartilage, which has a compressive modulus of 1 MPa in healthy individuals (Mow et al, 1991). Figure 7 shows the stress vs. strain curves for three porous chitosan scaffolds that were representative of those used in this study.

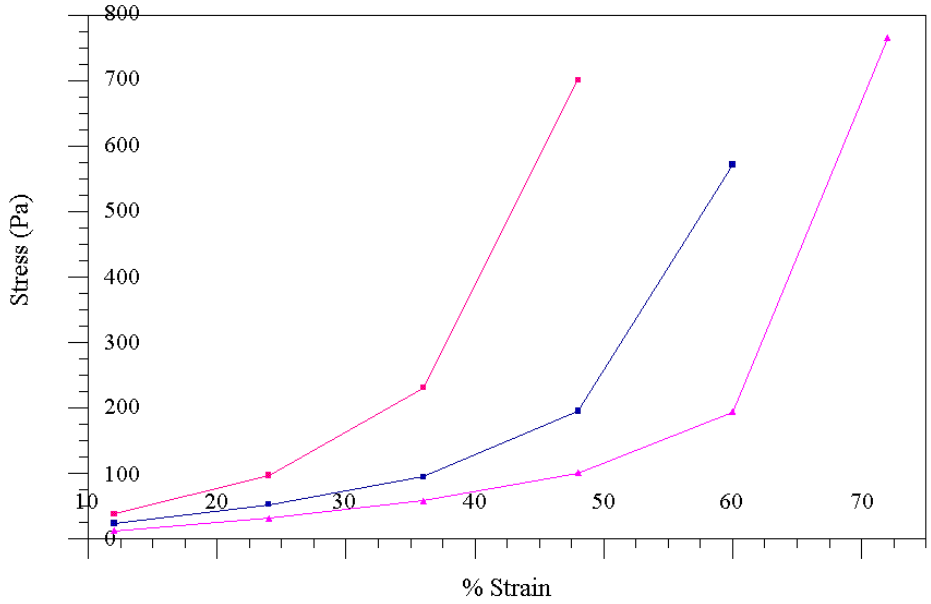

Figure 7. Engineering Stress Vs. Strain Curve for Chitosan Scaffolds

Solution Viscosities

The viscosities of chitosan-acid solutions were determined to be Newtonian, as determined by their plots of viscosity versus shear stress being linear (Figure 8). In this plot, viscosity does not change significantly as shear stress increases for any solution. Figure 9 compares shear rate versus shear stress for three different solutions. Because solutions viscosities were not determined to be significantly different from one another (p-value > 0.05), this property was determined not to be an indicator of degradation rate of the scaffolds created using these solutions.

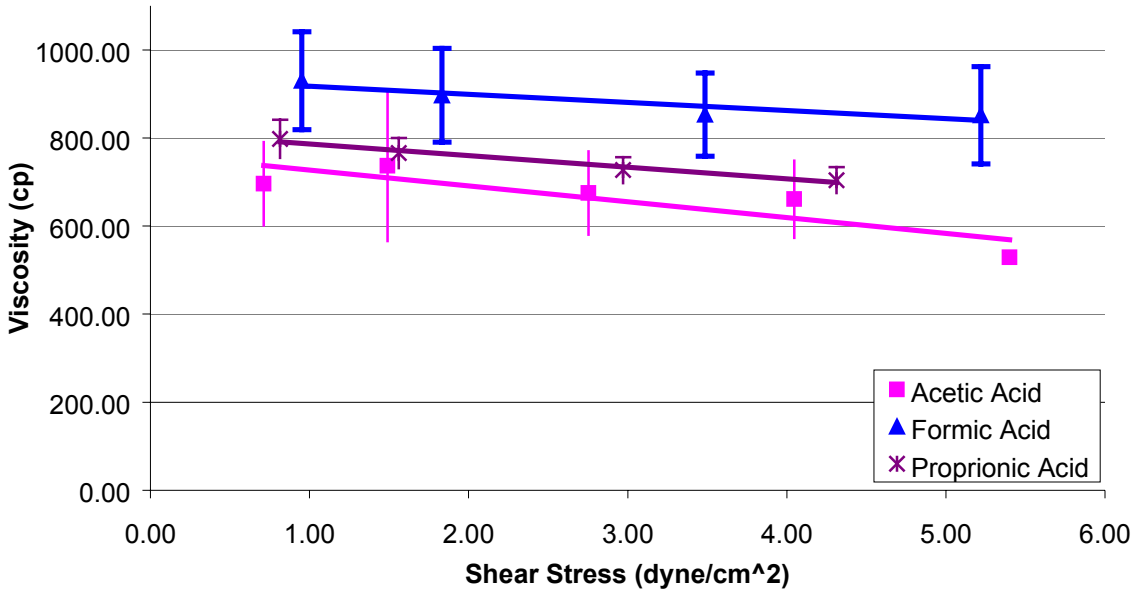

Figure 8. Viscosity Vs. Shear Rate for Chitosan-Acid Solutions

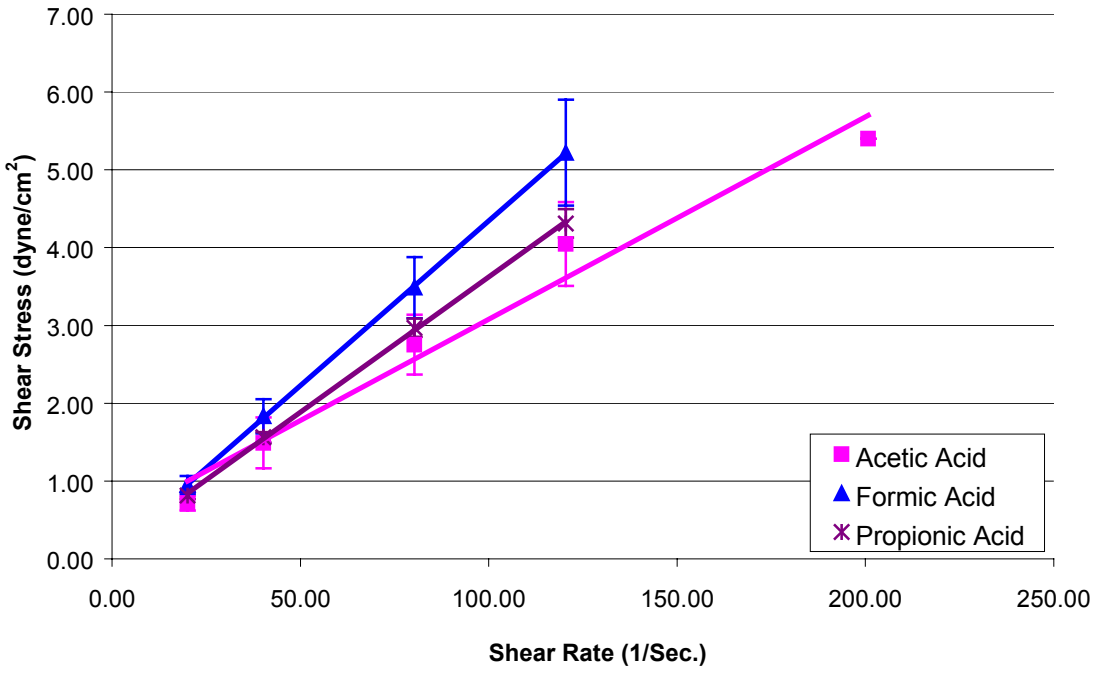

Figure 9. Shear Stress Vs. Shear Rate for Chitosan/Acid Solutions.

Degradation

Upon visual inspection of scaffolds exposed to lysozyme for four days, it was evident that formic acid scaffolds degraded most quickly, followed by propionic acid scaffolds. Acetic acid scaffolds did not appear to have degraded at all and looked similar to control scaffolds at this time point (Figure 10).

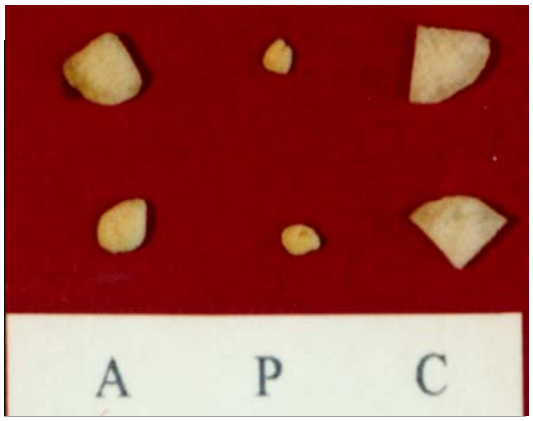

Figure 10. Acetic Acid (A), Propionic Acid (P), and Acetic Acid Control Scaffolds (C) After 4 Days

Scaffolds incubated with lysozyme degraded significantly faster than control scaffolds (p-value > 0.05). Formic acid scaffolds were determined to degrade significantly faster than both propionic acid scaffolds and acetic acid scaffolds, while there was no significant difference between the rate of degradation of propionic acid scaffolds and acetic acid scaffolds. Figure 11 shows the means plot for the mean weight loss with respect to acid type of the scaffolds. The asterisks represent significant differences in weight loss.

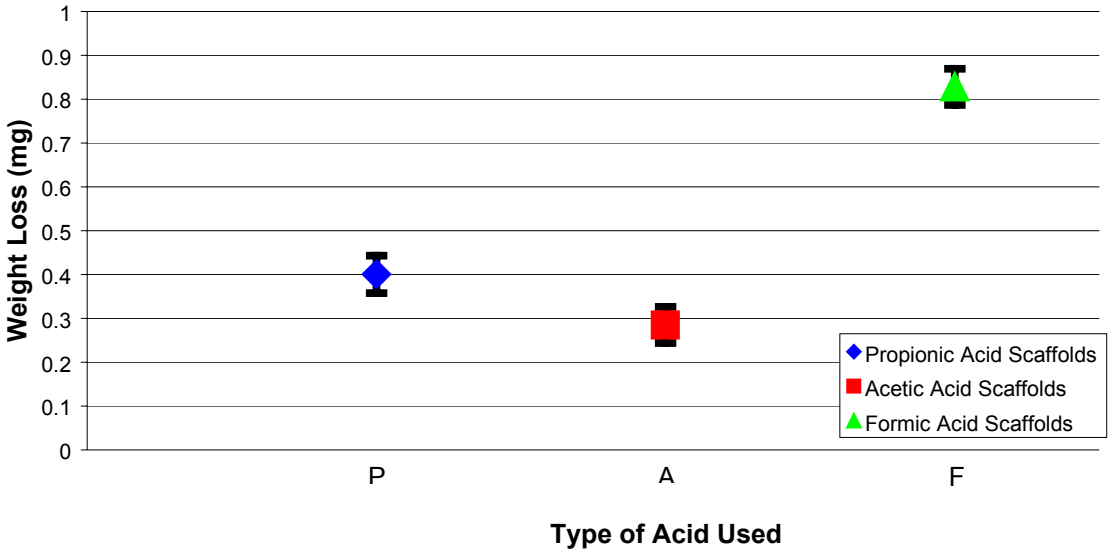

Figure 11. Means Plot for Weight Loss Vs. Acid Type (P = Propionic Acid Scaffolds, A = Acetic Acid Scaffolds, F = Formic Acid Scaffolds)

Cell Attachment and Viability

From the hemocytometer count of unattached cells from the first cell culture trial, it was determined that 2.5×10^5 cells attached to each of 10 scaffolds after 48 hours. SEM microphotographs showed a fairly uniform covering of cells on the scaffold surface (Figure 12A) and the maintenance of the spherical morphology characteristic of a chondrocyte by attached cells (Figure 12B). The cell viability assay also showed a fairly uniform covering of cells on the scaffold and a large number of viable cells. Though some dead cells were visible, though some dead cells were visible, it was difficult to estimate their numbers due to the chitosan displaying the same red color as the dead cells.

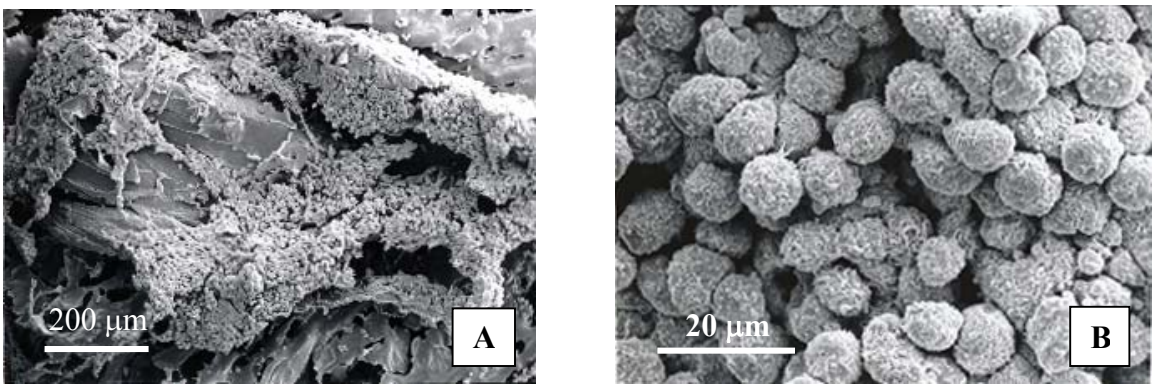

Figure 12 A. 100X SEM Micrograph of Cells Attached to Chitosan Scaffolds After 48 Hours B. 1500X SEM Micrograph Showing the Spherical Morphology of Cells Attached to Chitosan Scaffolds After 48 Hours

Histology and Immunohistochemistry

Histological sections from day 18 scaffolds from trial one showed that cells were attached to the chitosan and had begun to fill pores. Some cell-dense areas stained metachromatically with toluidine blue, indicating the presence of proteoglycan-rich ECM (Figure 13A and B). These biosynthetic phenomena were mostly confined to the periphery of the scaffolds because those cells on the exterior of the scaffolds tended to form a "shell" and effectively starved any cells that had attached or migrated to the interior of the scaffolds. It was also evident from histological sections than some closed pore volume existed within the scaffold microstructure preventing cells from attaching to all interior surfaces. Scaffolds from 28 days showed similar trends (Figure 14A).

Histological sections from 28 day scaffolds from the second trial revealed a larger number of cells in interior pores of the scaffolds. These cells as well as those closer to the exterior of the scaffolds did produce proteoglycan-rich ECM, which is highlighted by the metachromatic staining shown in figure 14B. These histological sections both

imaged at 40X were also used to compare the microstructures of the scaffolds at the 28 day time point. The microstructure of the scaffold shown in figure 14A and used in the first trial was clearly denser than that of the scaffold shown in figure 14B, used in the second trial.

The cell clusters that displayed methachromasia with toluidine blue from the first trial were the same areas that stained positively (orange-brown) for type II collagen (Figure 15 A and B). Most collagen synthesis appeared to be pericellular, or confined to those regions immediately adjacent to cells. Constructs analyzed after 28 days showed similar trends.

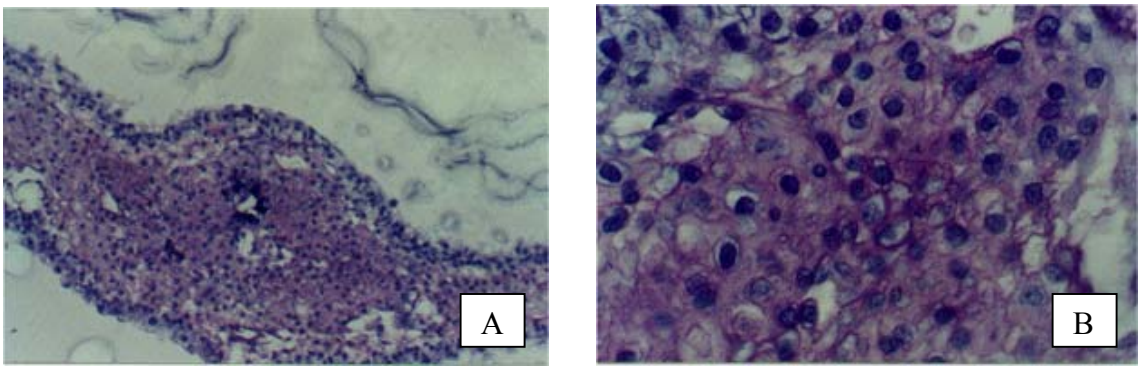

Figure 13A. And B. Histology Sections from Day 18 Scaffolds Stained for Proteoglycans

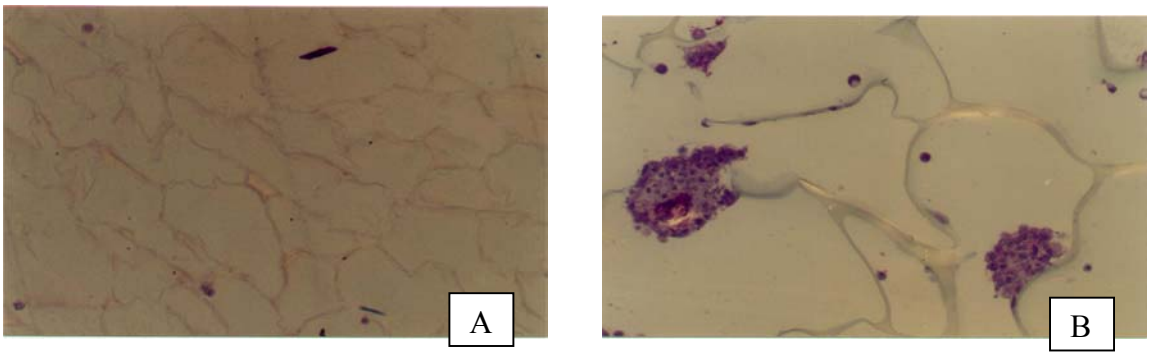

Figure 14 40X Histology Sections from Day 28 Acetic Acid (A) and Formic Acid (B) Scaffolds

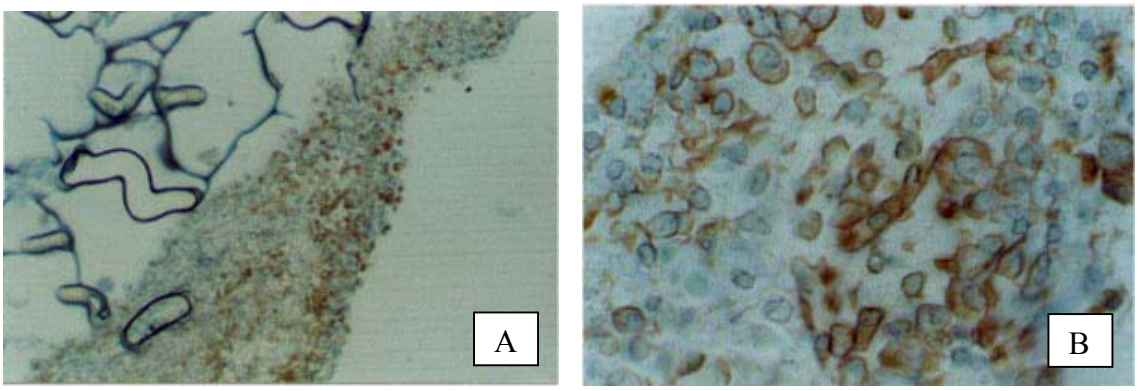

Figure 15A. and B. Immunohistochemical Histology Sections from Day 18 Scaffolds Stained for Type II Collagen

CHAPTER IV

SUMMARY AND DISCUSSION

Summary

Chitosan scaffolds possessing an interconnecting porous structure were easily fabricated using a simple freezing and lyophilization process. The scaffolds that resulted from freezing chitosan solutions in 35mm petri dishes at –20°C appeared to limit scaffold performance because of their low porosity. This problem was addressed by altering scaffold fabrication parameters including the shape and size of the container used to freeze the solutions and the temperature at which the solutions were frozen. By freezing the chitosan in cylindrical vials at –9°C, porosity was increased to 94%; however, results from MIP suggest that the average pore diameter was smaller in these scaffolds. It is believed that this was due to the average pore diameter being an average of all pores and not just those contained within the area used for culture, which were cut from the center most portion of the larger cylindrical chitosan plugs. Visually, pores in this area were obviously larger than those in the areas not used in culture and larger than those in scaffolds frozen in 35 mm petri dishes at –20°C.

Scaffolds showed minimal mechanical integrity before cells were added to them, but were strong enough to support cell attachment and growth and remained in tact through the culture period in the rotating bioreactor.

Scaffold performance was also measured by their ability to biodegrade during the 28-day culture period. Degradation tests conducted in the absence of cells suggested that scaffolds fabricated using formic acid as the solvent would degrade an average of 67% faster than their acetic acid counterparts. This may lead one to believe that acetic acid may somehow inhibit the action of the lysozyme, the enzyme responsible for breaking down chitosan, while formic acid may not. It is possible that perhaps acid salts remained in the scaffold microstructure after volatile acid was removed during lyophilization. This suggests than a relationship may exist between these salts and the lysozyme's ability to attack key complexes in the chitosan structure. This proposed relationship between the acid salts and lysozyme should be investigated further before any strong conclusions are drawn concerning lysozyme inhibition. It should also be noted that when used in culture both scaffold types remained largely unchanged.

In terms of their ability to truly organize the cells in three dimensions, the more porous scaffolds were more successful. When cultured on the less porous scaffolds, large numbers of cells were unable to penetrate to the interior of the scaffolds, and those that did, appeared to be effectively starved by cells forming a shell around the exterior of the scaffolds. When more porous scaffolds were used in culture, cells were able to penetrated further into the scaffold microstructure; however, this may have been due in part to the higher cell seeding density. Both scaffold types were judged equally as capable of supporting the biosynthesis of a proteoglycan and type II collagen-rich extracellular matrix.

Future Directions

It appeared that by changing the morphology of the freezing container and the rate at which solutions were frozen, that porosity was fairly easily altered. It would be useful to know exactly how changing each parameter affected the porosity. Using the thermocouple to monitor the freezing rates of solutions over a range of temperatures would be one way of answering at least the question of how temperature affects the porosity. However, perhaps a more versatile method would be to create a finite element model of the system in which one would have more freedom in varying parameters and would be able to visualize the effects more quickly.

Perhaps other parameters such as crystallinity of the chitosan solutions and the physical state of the solutions before freezing are also responsible for not only affecting final porosity, but also affecting degradation rate and overall scaffold performance. With this in mind, current research is being conducted using Nuclear Magnetic Resonance to determine exactly what is present in chitosan solutions made with different acids. This information may be useful in determining what inhibits or enhances degradation of scaffolds. Solutions are also being cast as films for viewing under a polarizing light microscope to determine the crystallinity of solutions, as crystallinity may play a role in porosity and degradation rate. There has also been some evidence that by allowing a chitosan solution to gel before freezing may have an effect on porosity. Tests to evaluate this theory are currently being conducted.

Still another avenue being investigated is the attachment of side groups to the chitosan structure using available amino groups as reactive sites. Possibilities for

attachment include proteins that may enhance chondrogenesis, or other inert groups that may serve to help control or predict the rate of degradation of the polymer during culture. By further understanding the chemistry responsible for the scaffold's physical properties and performance, both chemically and in-culture, it may be possible to create specially tailored chitosan scaffolds for specific applications.

Chitosan is a biopolymer with many promising characteristics including its antimicrobial activity, its ability to bind cells and sustain their life, and the availability of reactive groups for modifying its structure. Chitosan may prove to be a useful biomaterial for applications other than tissue engineering, as is currently being investigated by others, including applications in artificial skin, nerves, would healing, biological coatings, and drug delivery. The present research showed chitosans usefulness for cartilage tissue engineering, and also began to show its versatility by discovering the ease with which this material can be manipulated to fit certain circumstances. Given that chitosan is an abundant waste product of the shellfish industry, is relatively inexpensive to researchers, and is fairly easily processed, this polymer should be further investigated for wide use as a biopolymer in medical applications and may provide a useful and less expensive alternative to conventional materials currently being used.

Conclusions

This discovery-based research has opened several avenues of thought concerning chitosan as a biopolymer for tissue engineering applications. In this work, two different chitosan scaffold types were generated that were able to support the attachment and viability of chondrocytes, and help to maintain their spherical morphology characteristic

of the chondrocyte phenotype. Cells attached to both types of scaffolds also showed a biosynthetic profile including proteoglycans and type-II collagen. However, scaffolds frozen at a slower rate, and therefore possessing greater total porosity, supported this activity through a greater volume than scaffolds frozen more quickly and possessing a denser microstructure. Through both trials, scaffold microstructure and degradation characteristics seemed to be the limiting factors. Though scaffolds frozen at a slower rate were more porous, it is possible that both scaffold types possess a certain amount of closed pore volume that prevents complete penetration of cells into all void areas; however, results were promising and did indicate that given optimal chitosan scaffolds, that this polymer might prove to be a useful alternative to synthetic polymers in cartilage tissue engineering applications.

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