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Evaluation Of Chitosan Gelatin Complex Scaffolds For Articular Cartilage Tissue Engineering

Harshal Prabhakar Mahajan

EVALUATION OF CHITOSAN GELATIN COMPLEX SCAFFOLDS FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

By

Harshal Prabhakar Mahajan

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Harshal Prabhakar Mahajan

Approved:

Steven H. Elder
Associate Professor of Biological Engineering
(Director of Thesis)

James N. Warnock
Assistant Professor of Biological Engineering
(Committee Member)

Ronald M. McLaughlin
Associate Professor
Department of Clinical Sciences
(Committee Member)

Dwayne A. Wise
Professor
Department of Biological Sciences
(Committee Member)

Gary McFadyen
Assistant Research Professor
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(Committee Member)

Kirk H. Schulz
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Kirk H. Schulz
Dean

College of Engineering

Name: Harshal Prabhakar Mahajan

Date of Degree: December 9, 2005

Institution: Mississippi State University

Major Field: Biomedical Engineering

Major Professor: Dr. Steven H. Elder

Title of Study: EVALUATION OF CHITOSAN GELATIN COMPLEX SCAFFOLDS FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

Pages in Study: 84

Candidate for Degree of Master of Science in Biomedical Engineering

In search of better scaffolding materials for *in vitro* culture of chondrocytes, the combination of chitosan (similar to glycosoaminoglycans) and gelatin (denatured collagen) was tested due to its resemblance to cartilage extra-cellular matrix (ECM).

Porous scaffolds were fabricated from chitosan gelatin blends (1:1, 2:1, and 3:1). The response of chondrocytes to them was evaluated from the amount of sulphated GAG and collagen type 2 secreted after 3 and 5 weeks. The effect due to static (transwell inserts) and dynamic (rotating bioreactor) culture methods was analyzed.

Results indicate that 1:1 chitosan gelatin blends showed the best chondro-conductive potential. The rotating bioreactor facilitated better cell distribution across scaffold but did not show higher ECM secretion compared to transwell culture after 3 weeks. Gelatin leeched out by dissolution in culture media and left an open and interconnected chitosan network. Chitosan gelatin scaffolds show a potential for use in cartilage tissue engineering applications.

DEDICATION

To “The Purpose of Existence”, “Swami”, Aai, Baba, the whole family, and some unknowns.

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CHAPTER I

INTRODUCTION AND BACKGROUND REVIEW

Arthritis and Cartilage Repair

Prevalence

According to The Centers for Disease Control and Prevention, in 2002 1 out of 5 (i.e. about 43 million) American adults reported doctor-diagnosed arthritis and another 23 million reported symptoms similar to chronic arthritis. In 1997 the medical care for arthritis cost $51 billion. Arthritis occurs irrespective of racial and ethnic groups, and about two-thirds of people with arthritis are below 65 years (The Center for Disease Control and Prevention, 2005).

Arthritis is comprised of a group of over 100 diseases and conditions of which osteoarthritis, gout, Rheumatoid arthritis, and fibromyalgia are the most common. These conditions follow a certain sequence of events like proteoglycan washout from the extracellular matrix, disruption of the collagen network, and metaplasia, and the superficial focal regions of the defect gradually progress deep into the cartilage tissue (Hunziker, 2001). Unlike other tissues like bone and skeletal muscles the lesions in

cartilage generally do not heal as effectively. Since cartilage acts as a lubricating membrane between the ends of moving bones, loss in its consistency eventually causes severe wear and tear of contacting bones, accompanied by pain. Other orthopaedic complications become prominent eventually.

Repair of Cartilage Lesions

The avascularity, low metabolic activity, and catabolic activity of chondrocytes are reasons that may have lead to the 200 year old dogma "Cartilage once destroyed never heals" (Hunter, 1743). Current research shows these lesions in cartilage do heal under some biological circumstances, which include untreated cartilage defects. Articular cartilage repair and/or regeneration may occur only in the region of defect and only if there are adequate "stimuli" for such activity. This repair process is initiated when the defect in cartilage reaches subchondral bone and bone marrow and causes bleeding from blood vessels (Hunziker, 2001). This leads to formation of hematoma and fibrin clot and encourages accumulation of certain morphogenetic proteins, and glycoproteins, growth factors released by the bone (Reddi, 1999). Progenitor cells like mesenchymal stem cells and fibroblast migrate from the surrounding cartilage, bone, underlying bone marrow, and synovium into the repair site in about two to five days. These play an important role in initiating and maintaining the repair process (Shapiro *et al*., 1993, Hunziker, 2001). After about a week of infiltration of stem cells, the fibrin network from the blood clot serves as a scaffold for growth and differentiation of mesenchymal stem cells. The repair process is limited by the diameter of the full thickness defect created in experimental models. Varying from species to species, small defects of about 1-3 mm diameter are

seen to heal spontaneously. Large defects approaching 6 mm in diameter were not seen to heal well in a goat model (Jackson *et al*., 2001). The depth and location of the defect, age, and body mass of the animal are other considerations that may affect the repair process.

In a small defect spontaneously generated repair tissue fills it completely in few months, but its integration with the native cartilage is questionable. Shapiro *et al*., (1993) report, in their rabbit model, full thickness defects (3mm diameter) were filled with loosely integrated repair tissue, in four weeks. But even after eight weeks, the lacunae at the boundary of the defect remained empty with low or no chondrocytic activity seen in a layer about three to eight cells deep. In fact, chondrocytes in adjacent layers of the defect die within few hours of drilling the defect. Eventually, signs of degeneration of the repair cartilage were seen after 12 weeks and these remained a prominent feature thereafter. The heterogeneous biochemical composition of the repair tissue and its inferior biomechanical properties may lead to micromotion between the boundaries of both cartilages, and this may initiate a degradation response (Shapiro *et al*., 1993, Hunziker, 2001).

Surgical Interventions

Surgical techniques like Pridie debridement (Insall, 1974), Abrasion chondroplasty (Chen *et al*., 1999), and Microfracture (Stedman *et al*., 1999) are based on the principle of causing forced bleeding in the cartilage defect by drilling deep into the defect until the subchondral bone is reached. The microfracture technique has shown encouraging results. Since it is less expensive, yet effective, it is a widely adopted clinical procedure especially for young individuals (Kinner and Spector, 2004). Extensive surgeries like arthroplasty replace the weakened joints or their components with artificial

joints or parts. Osteotomy involves improving the biomechanical load transfer and alignment at the joint to achieve pain relief (Hunziker, 2001).

Long term clinical trials have shown that cell based therapies using autologous chondrocyte implantations are effective to help regenerate tissue close to healthy hyaline cartilage (Peterson *et al*., 2000). Therapies like autologous periosteal grafting (O'Driscoll, 1999), autologous perichondrial grafting (Bouwmeester, *et al*. 1997), and osteochondral transplantation (Hangody *et al*., 1998) have also attained clinical significance due to various reasons. The results of autologous periosteal grafting look promising from short term clinical study reports, but there are no controlled trials performed to prove their effect.

The quality of the repair tissue developed in the cartilage lesion is influenced by type, location, and number of lesions worked on in a single joint. The source of implanted cells, alignment and loading at the joint, and disease conditions may also have an influence on the repair process (Kinner and Spector, 2004). Although the associated pain relief and fair mechanical properties of the resultant tissue make these procedures clinically acceptable, the histological integrity of the tissue may be considered a better marker for the evaluation of joint and cartilage health.

Tissue Engineering Based Approaches for Cartilage Repair

Tissue engineering may be defined as the application of principles of cell biology, material science, engineering, and surgery to the regeneration and implantation of functional tissue or its components either *in vitro*, *in vivo* or both (Goldberg and Caplan, 2004). Given the low activity of chondrocytes and the avascularity and complexity of the

cartilage extra-cellular matrix, the process of regeneration of articular cartilage becomes complicated and challenging. Even for a surgical defect that penetrates the subchondral bone, the resulting repaired cartilage tissue is closer to a fibrocartilagenous phenotype and may have significantly different mechanical properties compared to the native articular cartilage (Kinner and Spector 2004). Since the repaired fibrocartilage is not very well integrated with the surrounding tissue, it has a possibility to rupture eventually. Also the loading causes fibrocartilagenous tissue to have a tendency towards ossification.

These limitations may be circumvented, (at least partially if not fully) using novel biomaterials modified by sophisticated tissue-engineering techniques that allow precise control over cellular behavior both *in vitro* and *in vivo*. Customized biomaterial implants may prove to be beneficial if used in combination with conventional surgical and cell based therapies. The overall goal during this process is to bring the phenotype of repair cartilage closer to that of a healthy articular cartilage.

Even if such trend is achieved from *in vitro* culture, the *in vivo* performance of the engineered tissue and its integration with the native tissue posseses further challenges. These practical limitations restrict the use of only tissue engineering techniques as a clinically suitable treatment option. Using engineered tissue along with surgical procedures like debridement or osteochondral drilling seems a viable alternative at this stage.

Biomaterials and Tissue Engineering Techniques

As a first step, the selection biomaterial for the implant is important. Apart from being less cytotoxic (more biocompatible), specific requirements on the material may be

based on the conditions at the implant site. Table 1 summarizes some of the important requirements. If the material needs to be implanted temporarily, it should be biodegradable; if used for long-term implantation, it should be less susceptible to mechanical degradation. The substances released from the degradation should not be harmful to the body and must be easily converted and/or transported. The material should be easier to fabricate into implants of required shapes and its mechanical properties should be controllable and customizable as required for the implant site (Hunziker 1999). The bonding capabilities, surface characteristics, remodeling capacity and overall architecture are other considerations (Goldberg and Caplan, 2004).

Table 1: Biological effects corresponding to properties of matrix. Modified from Hunziker and Ernt (1999)

Matrix Characteristics	**Biological response or effect**
Porosity	Cell migration inside matrix hence uniform distribution of newly grown tissue
Chemical properties of component biomaterial(s)	Aids in cell attachment and signaling in cell environment Aids accumulation and organization of ECM components Allows release of bioactive substances
Biocompatibility	Cell viability and tissue response
Biodegradability	Aids tissue remodeling
Gross mechanical properties	Affects cell growth and proliferation response In-vivo load bearing capacity
Structural anisotropy	Anisotropic growth & tissue organization

Considering cell loss due to the massive apoptotic response (Mao *et al*., 2004) to cells in the first few hours of contact with the material, a high cell seeding density is required. The cell seeding technique should be such that it would give a uniform distribution in the loading matrix.

Generally there are two strategies followed for tissue engineered implants. A near mature cartilage tissue may be cultured *in vitro* and later implanted in the cartilage defect. This method is demands considerable amount of time and poses concerns on the integration of the implanted tissue with surrounding tissue. Lack of proper binding between these tissues possibly leads to micromotion between them and hence failure of the implant. Another strategy would be to implant the defect with tissue construct that has an immature tissue grown on it. Since this demands lesser amount of time in *in vitro* culture there is a better possibility of maintaining chondrocyte phenotype. Chondrocytes from the immature tissue may migrate and bridge the surrounding tissue and integrate well.

In a healthy cartilage the chondrocytes monitor the extra cellular matrix and control and regulate its secretion in response to mechanical and chemical stimuli (Caterson *et al*., 2004). Improper signaling in the cartilage environment causes them to change their phenotype to more of a fibrocartilage type. For efficient *in vitro* culture that would preserve chondrocyte phenotype, the required chemical and mechanical signals must be supplied. The requirements of a high seeding density are met by using a porous 3D scaffold matrix for cell culture. The porous structure gives a high surface area to volume ratio and keeps the cells attached in a very close proximity. Further, an

interconnected porous structure facilitates cell migration deep inside the matrix. For a uniform filling of the scaffold with newly grown tissue the viability of cells attached deep inside is maintained. These mass transport and diffusional limitations of the porous structure may be eliminated by selection of proper culture conditions.

Aerobic culture conditions were seen to enhance chondrogenesis in 3D polymer constructs developed by Obradovic *et al*. (1999). Vunjak-Novakovic *et al*. (1999) report cell-seeded polymer constructs cultured in a rotating cell culture bioreactor showed much higher secretion of collagen and glycosoaminoglycans (GAG) as compared to those cultured in other culture techniques like spinner flask, orbital mixing, and solid body rotation. Chondrocytes cultured on PGA constructs, in a rotating bioreactor, for six weeks had composition and mechanical properties comparable to an immature fetal cartilage while these values were comparable to the native cartilage after seven months. A gradient in concentration of sGAG and a uniformly proliferated cartilaginous matrix was observed after six weeks (Vunjak-Novakovic *et al*., 2004). This makes the selection of culture conditions important. Intermittent hydrostatic loading on chondrocytes is seen to modulate Agrecan and type II collagen expression (Smith *et al*., 2004, Ikenoue *et al*., 2003). These factors demonstrate the importance of culture techniques.

Typically the evaluation of a biomaterial implant is preceded with an extensive confidence from its *in vitro* cellular response to the tissue of interest. In order to access the *in vivo* biocompatibility of the material, a heterotopic model may be employed. Here the implant performance is evaluated by placing the implant in a subcutaneous pouch generally in a small animal model (mice, rabbit etc.). If an acceptable response is seen a

large animal model (goat, dog etc) may be employed to simulate conditions similar to those in a human body. Here the biomaterial is implanted into a full thickness defect drilled deep up-to subchondral bone. Practical and fiscal limitations prevent usage of large animals for preliminary studies (Reinholz *et al*., 2004). Extensive clinical trials may be performed in animals and the same procedure repeated with human cells.

Chitosan as a Biomaterial

Chitin is a linear polysaccharide found in marine crustacean shells and the cell walls of bacteria and fungi (Mi *et al*., 2001). It is the second most abundant natural polymer after cellulose. Chitosan (with repeating units of β (1-4) 2-amino-2-deoxy-D-glucose) is a deacetylated (40-98%) derivative of chitin having molecular weight about 300 KDa to 2000 KDa (Nettles *et al*., 2002, Di Martino *et al*., 2005). Chitosan is known to have some unique physiochemical, biomedical and pharmaceutical properties, which have wide range applications in tissue engineering and orthopedics, pharmaceutics, food industry, cosmetics, agriculture, and waste management etc. Chitosan is generally insoluble at neutral and alkaline pH but forms salts with organic acids at low pH (Illeum, 1998). The degree of deacetylation and preparation methods influence the crystalanity and molecular weight of chitosan obtained.

Chitosan is structurally similar to glycosaminoglycans (GAGs), which are long unbranched polysaccharides found in extra cellular matrices (ECM) of some connective tissues. (Hamilton *et al*., submitted). Chitosan and its derivatives are known to be bacteriostatic (Yang *et al*., 2005), fungistatic, antimicrobial, antiinflammatory, antioxidant and antimutagenic (Mi *et al*., 2001, Seo *et al*., 2003, Tomihata *et al*., 1997,

Kogan *et al*., 2004). The antiinflamatory properties of chitosan may be attributed to its inhibition of over-production of prostoglandins E2 and pro-inflammatory cytokines, such as tumor necrosis factor-alpha produced by macrophages (Chou *et al*., 2003).

The protonation of amine group of chitosan on its dissociation imparts it a cationic nature (Illeum 1998), which helps cell adhesion by allowing attachment of anionic cell membrane proteins and growth factors (Chatelet *et al*., 2001) as seen from the hemagglutination response on contact with blood (Lee *et al*., 1995, Onishi and Machida 1999, Tomihata and Ikada, 1997, Rao and Sharma, 1997). Higher the degree of deacetylation higher is the number of amide groups available for protonation and hence better is cell adhesion (Mao *et al*., 2004).

Due to its hemostatic properties (Rao and Sharma 1997) chitosan is reported to have many wound healing applications like wound and burn dressing material (Kato *et al*., 2003, Muzzarelli *et al*., 1999), and fluid absorbing chitosan beads (Yusof 2001). It has numerous pharmaceutical applications like tablets, gels, oral mucoadhesive, drug delivery microgranules (Gupta and Ravi Kumar, 2000), non-viral gene delivery transfection vehicle (Borchard, 2001) and many more as reported by Illeum (1998) and Kato *et al*. (2003).

Biodegradation of Chitosan

Since chitosan is a polysaccharide, its primary mechanism of *in vivo* degradation is through enzyme hydrolysis that is triggered by lysozyme and not by human chitinase (Varum *et al*., 1997). The degradation is dependant on the pH, type and method of preparation of chitosan (Nettles *et al*., 2002). Lysozyme is an enzyme abundantly found

in blood and cartilage extra cellular matrix and is also secreted by chondrocytes (Moss *et al*., 1997).

Degree of deacetylation (DDA) has a notable effect on the rate of degradation of chitosan (Hirano *et al* 1989, Shigemasa st al.1994, Tomihata and Ikada 1997). Higher the DDA slower is the rate of degradation. The cationic nature of chitosan is chemotractant to negatively charged cell surface proteins (Chung *et al*., 2003) and so higher the DDA higher is the cell attachment and severe is the tissue response and subsequent cell apoptosis. Tomihata and Ikada (1997) observed that chitosan films with high DDA (>73%) showed less degradation by lysozyme *in vitro* and *in vivo*, when implanted subcutaneously in rats. This implies material properties like hydrophilicuty and molecular weight also play a role in the *in vivo* degradation and overall performance of chitosan. Although not much consistent data is reported, the lysozyme-induced depolymerization process yields mostly non-toxic oligosaccharide residues, which are easily excreted by the body (Nettles 2001, Onishi and Machida 1999). Overall, these finding suggest better biocompatibility of chitosan.

3-D Scaffolds from Chitosan —What Have We Learned?

Each tissue has its own specific requirements from the implanted biomaterial. A versatile polysaccharide like chitosan presents promising possibilities for customization to cell and tissue specific culture conditions. Due to its low cytotoxicity, biodegradability, cationic nature, and chemical similarity to GAG and hyaluronic acid, chitosan is blended with other ECM components to simulate a more cartilage-like environment in the scaffold/construct. Although using chitosan alone may seem to be promising in some *in*

vitro models, its limitations to degradation and specific chemotaxic effects (Peluso *et al*., 1994) may be of concern for long-term *in vivo* implantation.

Traditionally, porous scaffolds from chitosan are fabricated by dissolving it in an organic acid such as acetic acid (1 to 3% v/v) followed by freezing and lyophilizing. In lyophilization the liquid components of the solution, in form of ice crystals, are removed by subjecting it to vacuum. The dissolved chitosan is left behind in a highly interconnected porous matrix structure. As would be expected, the pore size of the resulting scaffold depends on the size of nucleated ice crystals during freezing of the solution. Lower the freezing temperature, faster is the rate of ice formation and smaller is the size of ice crystals that are formed resulting in a small scaffold pore size (Nettles, 2001). Porous scaffolds may also be constructed by internal bubbling process (Chow and Khor, 2000). Chitin solution was loaded with Calcium carbonate ($CaCO_3$) to form gels. These gels were then submerged in HCl to give a highly porous matrix. Release of CO_2 on the reaction of $CaCO_3$ and HCl helps to form bubbles in the gel matrix. This process resulted in scaffolds with larger pores diameter (100-500 and 500-1000μm) as compared to those from lyophilization (40-100μm) by Nettles *et al*. (2002). Geng *et al*., (2005) manufactured chitosan scaffold using a desktop rapid prototyping system. Here, the solution of NaOH in ethanol was used to neutralize acetic acid from chitosan solution to form porous scaffolds of about 90% porosity and pore diameter 200-500μm. The system seems promising to develop highly ordered porous chitosan scaffolds of required shape and porosity.

3D Scaffolds from Chitosan Gelatin Blends

Chitosan based scaffolds and/or films were constructed by blending it with molecules like collagen (Ma *et al*., 2003), GAG (Madihally and Mathew, 1999), alginate (Iwasaki *et al*., 2004), lactose (Donati, 2005), poly(L-lactic acid) (Cui *et al*. 2003), hyaluronic acid (Yamane *et al*., 2005), calcium phosphate (Zang and Zang, 2001), and gelatin (Mao *et al*., 2004, 2003, 2002, Xia *et al*., 2004, Zhao *et al*., 2001). Chitosan is also used extensively for surface modification of biomaterials and implants (Bumgardner *et al*., 2003). These chitosan based/modified constructs are explored for *in vitro* and *in vivo* tissue engineering applications for different tissues including cartilage, bone, ligament and skin.

Gelatin is basically partially denatured collagen obtained by breaking its triple helix structure mostly by hydrolysis. If used in its pure form it is known to be hemostatic as seen from the presence of an acute *in vivo* inflammatory response and increased neutrophil activity (Burugapalli *et al*., 2003, Rose *et al*., 1989). These characteristics may be attributed to the anionic nature of gelatin. This charge may be balanced by complexing it with cationic molecules like chitosan. Moreover, the hydrophilicity of gelatin due to its amino and carboxyl groups helps in improving water retention and oxygen and nutrient transfer throughout the scaffold architecture (Xia *et al*., 2004).

Zhao *et al*., (2002) prepared a 3-D hydroxyapatite chitosan gelatin network scaffold by cross-linking the components with glutaraldehyde. After 7-day culture of osteoblast on these scaffolds good cell attachment and proliferation was seen, and osteoid formation and mineralization of scaffolds was seen after 21 days.

Mao *et al*., (2003) prepared monolayered and bilayered scaffolds by setting a thermal gradient in the chitosan and gelatin mixture while freezing. Glutaraldehyde was used as a cross-linking agent. The mixture was brought in contact with a pre-cooled plate and frozen for few hours followed by lyophilization. The mean pore size in the bilayered scaffold varied from about 30µm (freezing plate interface) to 110µm (air interface) and resulted in highly ordered cylindrical pores. Reducing the initial freezing temperature from –20º C to –60º C for the monolayered scaffolds decreased the mean pore size from 210µm to 115µm. Scaffolds with lesser pore size (-60ºC) degraded faster due to lysozyme activity. Similarly, membranes (films) prepared from chitosan gelatin polyelectrolyte complex showed improved hydrophilicity as compared to pure chitosan films (Mao *et al*., 2004). A polyelectrolyte complex is a loose association between two or more ionic or covalent molecular entities. Since chitosan has a tendency to form weak hydrogen bonds with gelatin, its high positive charge density due to its DDA may be shielded by complexing it with gelatin. The cell cycle analysis performed through flow cytometry indicated that chitosan gelatin film is more biocompatible to trigger L929 cell proliferation and results in lesser number of apoptic cells as compared to pure chitosan. DDA was not seen to affect cell cycle progression (Mao *et al*., 2004). In another study, Mao *et al*. (2003) reported chitosan gelatin membranes modified by hyaluronic acid showed better fibroblast proliferation compared to pure chitosan and pure gelatin membranes, after 11 days of culture. Also blending hyaluronic acid in the chitosan gelatin scaffold made it slightly less degradable and showed higher GAG concentration after *in vitro* fibroblast culture. As a potential artificial skin substitute, chitosan gelatin

scaffolds (Liu *et al*., 2004) and chitosan gelatin hyaluronic acid scaffolds (Mao *et al*., 2002) supported growth of fibroblasts co-cultured with keratinocytes. The modified scaffolds showed better cell proliferation and penetration of spherical keratinocytes into scaffold pores and secretion of matrix after culturing for 4 weeks. The hydrophilicity of hyaluronic acid seems to help the binding of peptides and growth factors to culture substrate. Similarly, chitosan gelatin membranes prepared by Cheng *et al*., (2003) showed higher neural cell affinity and proliferation as compared to pure chitosan membranes but were about the same as pure gelatin films.

Xia *et al*. (2004) prepared porous scaffolds with equal parts of chitosan and gelatin solutions. The resultant bilayered scaffold had a mean pore size ranging 60-200μm in diameter. After *in vitro* culture of auricular chondrocytes on them for 7 days, they were subcutaneously implanted into pig abdomens. These cell seeded constructs, when harvested after 10 and 16 weeks showed generation of ECM and neochondrogenesis, while the chitosan gelatin controls scaffolds without chondrocytes were completely degraded in 16 weeks. The cartilage tissue developed after 16 weeks had GAG levels 89% of native auricular cartilage and similar biomechanical properties.

Risbud *et al*., (2001) cultured human nasal septal cartilage on cover slips coated with freeze-dried chitosan gelatin hydrogels and films (3:2 w/w). The cells maintained chondrocytic phenotype and secreted ECM after 14 days of culture. This was confirmed from RT-PCR analysis, which showed significant expression of type II collagen compared to type I and III. Elcin *et al*. (1998) showed chitosan gelatin membranes to support higher cell attachment as compared to chitosan collagen and pure chitosan

membranes. Similarly others have reported pure collagen to be more cytotoxic as compared to pure gelatin (Burugapalli *et al*., 2003).

In a more recent study on chitosan gelatin blended scaffolds (1:3, 1:1, and 3:1) cultured with Human umbilical vein endothelial cells (HUVEC) and Mouse Embryonic Fibtoblasts (MEF), no significant stimulatory effect was observed due to the presence of gelatin in scaffolds (Huang *et al*., 2005). Cross-linking of scaffolds with 0.25% glutaraldehyde significantly decreased their degradation rate and made them harder to handle after 24 hours. These cells when cultured on chitosan gelatin (1:1) membranes for 2 days had higher cell spreading area as compared to pure chitosan membranes as seen from their fluorescence micrographs stained for actin filaments. The amount of gelatin in chitosan scaffolds did not appear to affect cell viability on 3D scaffolds (Huang *et al*., 2005). Also, the compressive elastic moduli of chitosan gelatin scaffolds with 25% and 50% gelatin were not significantly different from that of pure chitosan scaffolds.

For potential use of chitosan gelatin scaffolds for cartilage repair applications it may be useful to control the rate of degradation of the implant depending on the species, pathological condition of cartilage, and the age of the patient. Glutaraldehyde cross-linking prolongs degradation of chitosan (Jameela and Jayakrishnan, 1995, Huang *et al*. 2005). Pure gelatin, as well as gelatin films cross-linked with glutaraldehyde more than 66%, showed persistent *in vivo* acute tissue response after 3 months (Burugapalli *et al*., 2003). This response may be due to cytotoxicity of glutaraldehyde released out of the implants and its degradation products (Jayakrishanan and Jameela, 1996). The cross linking scheme may be useful to extend the *in vivo* degradation rate of chitosan based

drug delivery systems (Li *et al*., 2004), which are subjected to high enzymatic activity from the systemic system. The relatively low metabolic activity of cartilage demands scaffolds that are easily degradable by low enzymatic activity of regenerating chondrocytes. Glutaraldehyde cross-linking cleaves away acetyl groups from chitosan. These play an important role in lysozyme mediated enzymatic degradation of chitosan. Since articular cartilage is avascular, the toxic glutaraldehyde degradation products may not be effectively transported away from the implant site and their accumulation would cause more inflammatory response, which may interfere with the process of chondrogenesis. Hence having the scaffold to degrade faster would balance the degradation rate to the rate of regeneration of new cartilage by cultured cells.

The studies cited above show adequate evidence of biocompatibility and chondrogenic characteristic of the combination of chitosan and gelatin. Gelatin is known to be less antigenic compared to its precursor collagen. Since chitosan and gelatin are chemically similar to two most essential components of cartilage ECM, viz. GAG and collagen, their combination in a 3D scaffold may effectively simulate ECM for an *in vitro* culture of articular chondrocytes.

Chitosan Scaffolds – Future Directions

Cell adhesion and proliferation on the scaffold are influenced not only by the DDA but also the surface structure of the scaffold. Wang *et al*. (2003) report that surface modification of chitosan films using specific lectin molecules was seen to improve cell adhesion and the resulting oligosaccharide mediated cell adhesion may also help in cell proliferation.

Chitosan protects the activity of Fibroblastic Growth Factor-2 (Masuoka 2005), and hence may act conductive for tissue generation. Chitosan is seen to increase activity of growth factors like Transforming growth factor (TGF)-β, Platelet derived growth factor (PDGF) (Ueno *et al*., 2001), and Interleukin-1, which provide necessary signaling for growth and proliferation of chondrocytes (van der Kraan *et al*., 2002).

Typically, any biomaterial implant, which is to be used for *in vitro* cell culture, must be fabricated in such a way that it adequately replicates the conditions of native tissue environment, as closely as possible. The porosity of 3-D scaffolds facilitates cell attachment and hence maintains a high chondrocyte density, which in turn helps to conserves their phenotype.

Articular cartilage chondrocytes rely on cell signaling mechanisms through integrins, growth factors (TGF-β, PDGF, BMP-2) and interleukins, for their growth and differentiation (van der Kraan *et al*., 2002). These mechanisms enable them to transduce the mechanical environment of the joint and maintain ECM as required. These growth factors may be supplemented along with the culture medium used for the cell-seeded scaffolds or may be integrated in the scaffold matrix itself in the form of microspheres (Kim *et al*., 2003). Lee *et al*. (2004) report that the scaffolds constructed by complexing chitosan with other components of extra cellular matrix, like chondroitin sulphate, GAGs, collagen and growth factor TGF, showed significant high proliferation rate and secretion of GAGs.

Instead of adding growth factors to the culture medium, gene encoding for these growth factors may be incorporated in the cells itself. Transfection of TGF-β1 by

retroviral vectors has been reported to increase matrix synthesis for meniscal cells (Goto *et al*., 2000). Similar encouraging results were reported for nonviral transfection of TGF-β1 in osteochondral defect in a rabbit model (Goomer *et al*., 2000). Chitosan is shown to have a potential of non-viral gene transfection vector (Borchard, 2001, Mansouri *et al*., 2004). Non viral transfection using chitosan DNA complexesmay be a better way to supply regulatory molecules for *in vitro* culture. Currently used gene transfection methods may not deliver regulatory signals in specific spatio-temporal patterns as required (Madry and Trippel, 2000). This limitation may be overcomed, at least partially, by employing matrix based transfection vectors that may be released to the chondrocytes at a controlled rate. Non-viral gene transfection vectors do not pose the risks of toxicity and immunogenenic response, which are common for viral vectors (Lieberman, 2004). However, their performance for long term *in vitro* culture is limited by the fast release characteristics of these agents.

Chondrocytes, once harvested from the patient may be cultured *in vitro* on these modified 3-D scaffolds along with necessary chemical and mechanical stimuli from culture environment. An immature neo-cartilaginous tissue that may result this way may be implanted in a freshly created subchondral bone defect. The porous scaffolds would absorb and retain the blood oozing from the defect. This would accelerate fibrin clot formation in the defect and initiate a local immunological response. This clot would serve to be chemo-attractant to the stem cells from bone marrow and necessary growth factors for chondrogenesis. Electrochemical charges on the matrix components play an important role to either hold or repel matrix growth factors and proteins. The cationic nature of

chitosan would not only aid in initial cell adhesion, but it may also hold anionic growth factors like Bone Morphogenic Proteins from the matrix. This would help the attached cells to preserve their chondrocyte phenotype. With a carefully selected post surgery protocol followed a fairly good integration of newly growing tissue may be achieved.

Statement of Hypothesis

The combination of chitosan and gelatin in form of a porous 3D scaffold helps to simulate the structure and chemical composition of the environment in native articular cartilage *in vitro*. It was hypothesized that gelatin blended chitosan scaffolds combined with a dynamic culture technique would show a higher secretion of cartilage extra cellular matrix components and hence, help to maintain chondrocytic phenotype, more effectively as compared to pure chitosan scaffolds in an *in vitro* static monolayer culture. The amount of GAGs and collagen secreted by the cultured chondrocytes was used to quantify the extent of growth and proliferation of chondrocytes in response to the variation in concentration of gelatin in the 3D chitosan scaffold. Further, the effect due to *in vitro* static and dynamic culture methods was evaluated using the same parameters along with the compressive resilience elastic modulus.

Aims

To evaluate the chondrogenic potential of chitosan gelatin scaffold for *in vitro* culture of chondrocytes.

Objectives

- Prepare porous scaffolds from chitosan gelatin blends.
- Evaluate the long-term cell viability and proliferation of chondrocytes cultured on chitosan gelatin scaffolds.
- Evaluate the effect due to static and dynamic culture methods on chondrocytes cultured on these scaffolds.

CHAPTER II

EXPERIMENTAL DESIGN & METHODS

Experimental Design

The overall goal of this project is to design constructs for the repair of cartilage lesions. For this purpose, the scaffolds should support chondrogenesis and secretion of ECM and degrade at a rate similar to the rate of growth of native cartilage tissue. This ensures better integration of the developing new tissue with the existing native cartilage. The *in vivo* degradation rate of chitosan depends on factors like degree of deacetylation (DDA), preparation and pretreatment methods of chitosan along with the porosity and preparation method of implanted constructs.

Since the pretreatment of chitosan, such as the sterilization method, may alter its properties care was taken of not exposing it to strong chemicals or ionizing radiation. Scaffolds were prepared by freezing and lyophilizing blends with varying concentrations of chitosan and gelatin solutions in acetic acid.

In order to test the effect due to gelatin concentration in the blend, three scaffolds each were prepared having chitosan to gelatin ratio 1:1, 2:1 and 3:1 (w/v) while pure chitosan scaffolds served as controls. Since, the compressive elastic moduli of chitosan

gelatin scaffolds with 25% and 50% gelatin were not significantly different from that of pure chitosan scaffolds (Huang et al., 2005), using these combinations avoided the variability in chondrocyte response due to inherent stiffness of scaffold. Adult porcine knee chondrocytes were seeded on all of these scaffolds placed in transwell inserts and cultured for 3 weeks. Transwell inserts help to eliminate the diffusional limitations of static culture on tissue culture plate. Due to the easy availability of cells pig model was selected for initial *in vitro* pilot studies. One scaffold from each of these was analyzed for neochondrogenesis and secretion of extra cellular matrix (ECM) components by immuno-histochemistry procedures. The remaining two scaffolds were divided into four equal parts and used to quantification of amounts of DNA and sulphated glycosaminoglycans (sGAG) secreted by cells after 3 weeks in culture. This data from sGAG (µg) normalized to amount of DNA (µg) was analyzed by One-way ANOVA analysis using the GLM procedure of SAS (SAS Institute, Inc. NC, USA). Duncan's Multiple Comparison test was performed to indicate the differences in groups if significant differences were detected from ANOVA.

In order to quantify the effect of culture techniques on ECM secretion by cells and chondrogenesis, a large batch (22 numbers) of 1:1 (w/w) chitosan gelatin scaffolds was prepared. Ten scaffolds each were cultured in static (transwell inserts) and dynamic (Rotating wall bioreactor) cultures for 3 weeks. Six scaffolds from each of these groups were qualitatively analyzed for neochondrogenesis and ECM secretion by immuno histochemical staining. The other four were used for DNA and sGAG quantification. The mechanical properties of nine scaffolds were evaluated by testing them for compressive

resilience. Two additional scaffolds were cultured in for a total of five weeks in rotating wall bioreactor and analyzed thereafter similarly. In order to quantify scaffold degradation and/or dissolution due to culture conditions only, the mechanical properties of cell seeded cultured scaffold after 3 and 5 weeks were compared to those of no cell-seeded scaffolds left in similar culture conditions for 3 and 5 weeks.

Experimental Methods

Selection of Chitosan

The selection of chitosan for orthopedic purposes is based on factors like the biocompatibility, degradation residues, solubility, and mechanical properties of chitosan constructs. There should be a balance between the *in vivo* degradation of chitosan and the regeneration of new tissue or matrix. The degree of deacetylation (DDA) of chitosan influences its *in vivo* degradability (Tomihata and Ikada, 1997). Higher the DDA, slower is the *in vivo* degradation. Since, the lysozyme mediated degradation of chitosan involves acetyl groups their loss during the process of deacetylation is one reason for the slower rate of degradation. As little as 10% change in DDA relation has a significant effect on cell attachment and cell viability after as little as 2 days of culture (Prasitsilp *et al.*, 2000). This makes the selection of chitosan as one of the deciding factors for effectiveness of the implant. Chitosan with DDA in the range of 80 to 90% was seen to be biocompatible and showed adequate cell attachment and proliferation (Nettles *et al.*, 2002). Chitosan used for this project was a kind gift from AgraTech International, Inc. (Denville, NJ). The chitosan manufactured by them was either ungraded or near to an industrial grade rather

than medical or pharmaceutical grade. Since, the DDA of a specific batch of chitosan was not reported earlier it was empirically quantified during or after its use to make scaffolds. The DDA values were not similar (± 10%) among different batches. Due to availability of chitosan, the batch with 84% DDA was used to test the effect of gelatin concentration while the batch with DDA (between 50-60%) was used to test the effect of culture technique.

Sterilization Procedure for Chitosan

The required amount of chitosan was taken in sterile centrifuge tubes and boiled with excess amounts of cell biology grade water for 2-3 hours. This was followed by its dehydration in graded series of alcohol solutions (10% to 100% in increasing steps of 10%) in cell biology grade water. Chitosan was left in 70% alcohol for atelast 8 hours for optimum sterilization (Marreco *et al.*, 2004). Later, chitosan was left in a high temperature enclosure for few hours or until completely dry of alcohol. According to Lim *et al.* (1999) dry heat (oven) and saturated steam (autoclave) reduces solubility of chitosan in acetic acid. Since large amounts of insoluble residues were obtained when chitosan subjected to dry heat was dissolved in acetic acid, the drying step was discontinued.

Boiling chitosan in cell biology grade water didn't seem to have much effect on its solubility or physical characteristic like color and mechanical integrity. Required amount of chitosan was taken in a pre weighed and autoclaved beaker along with cell biology grade water and boiled for at least 3 hours. The beaker was reweighed and acetic

acid and cell biology grade water were added to have 1% (w/v) solution of chitosan in 1% (v/v) acetic acid solution.

Preparation of Porous Chitosan Gelatin Scaffolds

Undissolved components from above chitosan acetic acid solution were filtered through a gauze filter whenever required. Calfskin gelatin (G-9382, Type B, 225 bloom, Sigma-Aldrich St. Louis, MO) was dissolved at 10mg/ml in 1% acetic acid and added to the chitosan solution to have final proportion of chitosan to gelatin as 1:1, 2:1 and 3:1. These mixtures were put in to wells of a 96-well plate tissue culture plate (100 µL per well) and frozen at -20º C for at least 24 hours. Care was taken that the frozen scaffolds did not thaw while transferring and handling. The scaffolds were equilibrated to low temperature in an -80º C freezer for about an hour and lyophilized in a Flexi-Dry Freeze Dryer (FTS Systems, Inc. Stone Ridge NY) at temperature between -70º C to -80º C.

For preparing the large batch of 1:1 chitosan gelatin scaffolds, 1% w/v solutions of chitosan (DDA between 50-60) and porcine skin gelatin (G-1890, Type A, 300 bloom, Sigma-Aldrich St. Louis, MO) in 1% aqueous acetic acid were mixed in equal amounts and vortexed to give a highly viscous mixture, which was used to cast the scaffolds. Porcine skin gelatin was used instead of calfskin gelatin in order to avoid any immune response to it from porcine knee chondrocytes.

Cell Isolation

Due to easier availability and fairly high cell to matrix ratio, the pig model was selected to check the *in vitro* response of chondrocytes to chitosan gelatin scaffolds.

Chondrocytes were isolated from adult female porcine knee cartilage maintaining aseptic techniques. The pieces of cartilage from knee were minced and incubated in a solution containing 1mg/ml type 2 collagenase in Dulbecco's Modified Eagle Medium (DMEM), 5% adult bovine serum and 1.5% antibiotics-antimycotics (10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 µg/ml amphotericin B). With most of the cartilage pieces dissolved this solution after about 12 hours was filtered through a sterile 100µm filter to remove undissolved clumps. The filtrate was centrifuged at 3000 rpm for 8 minutes and the cell pellet obtained was re-suspended in cell culture medium containing DMEM, 10% fetal bovine serum, 1.5% antibiotics-antimycotics, and 50µg/ml ascorbic acid and seeded on scaffolds.

Scaffold Pretreatment and Cell Culture

As mentioned above, after re-hydration in graded series of alcohol with 8 hours 70% alcohol, the scaffolds were disinfected in a solution of phosphate buffer saline (PBS) with 2% antibiotics-antimycotics for 1-2 hours. In order to observe the response of cells to the varying degree of chitosan and gelatin the aforesaid 1:1, 2:1, 3:1 and pure chitosan (control) scaffolds were infiltrated with DMEM at 37º C and seeded with chondrocytes at 25 x 10^3 cells/scaffold.

Nettles *et al.* (2002) report that the cells grown in static culture mainly colonize and proliferate at the boundaries of the cell-seeded scaffold. In order to get an even cell distribution and attachment, 5 aliquots, each of 5µl of concentrated cell suspension, were pipetted on 4-5 physically different locations on the scaffolds. The scaffolds were placed in transwell inserts (12µm pore size and 12 mm diameter) kept in the wells of a 48 well

tissue culture plate with just air interface and cells were allowed to attach to them. After about 3 hours, 800µl cell culture medium was added to each of the wells (600µl outside the transwell insert and 200µl directly on the scaffold). These cell-seeded scaffolds were cultured for 3 weeks in a humidified, 37º C incubator containing 5% CO_2. The medium was changed every 3 days and ascorbic acid (50µg/ml) was supplemented daily in to each well.

A large batch of 1:1 chitosan to gelatin scaffolds was prepared using the similar protocol to check the effect of cell culture method. A set of 12 scaffolds each was cultured in transwell inserts and in a rotating wall bioreactor. The cell seeding protocol was modified in order to facilitate deeper penetration of cells into the scaffolds. The wet scaffolds infiltrated with DMEM (37º C) were lightly blotted off extra medium by an autoclaved filter paper and placed in a 48 well tissue culture plate without any medium. This was done to ensure that the scaffold holds most of the cell suspension. 12.5µl of cell suspension (17.4 x 10^6 cells/ml) was added to each scaffold and cells were left to attach for about 3 hours.

A high number of apoptotic cells are seen after about an hour of seeding cells on the scaffolds. This implies the physiochemistry of implant material surface may have an influence on cell signaling (Mao *et al.*, 2004). Hence to facilitate adequate cell attachment and to replenish lost cells, 12.5µl of cell suspension was added per scaffold after 3 hours followed by another 25µl after a gap of one hour. This added to a total of about 870 x 10^3 cells/scaffold. Zhao *et al.* (2002) followed similar cell seeding strategy. Scaffolds to be cultured in transwell inserts placed in them with 800µl culture media.

The laminar flow profile setup in the rotating wall bioreactor helps to overcome mass transport limitations of static culture and is seen to stimulate faster and more uniform tissue generation and ECM secretion by chondrocytes seeded on Polyglycolic acid (PGA) scaffolds (Vunjak-Novakovic *et al.*, 2002, Vunjak-Novakovic *et al.*, 2004). The scaffolds to be cultured in the bioreactor were left in 48 well tissue culture plate for 24 hours with about 200µl/scaffold growth media to support them and allow cell adhesion. In order to evaluate efficacy of this culture technique they were then carefully transferred into a 50 ml rotating wall bioreactor (RCCS-D, Synthecon, Inc., Houston, TX). The cell culture medium in bioreactor was partially replaced (25ml each time) with fresh medium every 7 days for a total culture time of 21 days. In order to maintain sterility of the apparatus 50µg/ml ascorbic acid was added only during medium change. The bioreactor was allowed to rotate in a humidified, 37º C incubator with 5% CO_2. The speed of the rotating bioreactor was adjusted such that the scaffolds suffered minimum shearing force from the rotating medium and were in a constant state of free fall. Ten scaffolds each from transwell and bioreactor were used for further analysis and other two were left in culture for two more weeks. With half of the media in bioreactor replaced at the end of 3 weeks no further replacement was performed up to the end of five weeks. Since, two scaffolds in transwell had to be removed from culture due to infection; they were not available for analysis at the end of 5 weeks.

Histology and Immunohistochemistry

The cell-seeded scaffolds cultured for 3 weeks were fixed in Carnoy's solution (3:1 solution of 95% ethanol to glacial acetic acid) for 3 hours. This fixative was

recommended by Developmental Hybridoma Bank, Iowa City, Iowa, for use with the II-II6B3 anti-type II collagen antibody, used for immuno-histochemical procedures.

These scaffolds were washed in 95 % ethanol followed by 100 % alcohol (3 washes each) for complete dehydration. The scaffolds were then left in 1:1 solution of 100% ethanol and infiltration solution (Immuno-Bed Kit, Polysciences, Niles, IL) for 8-10 hours and subsequently infiltrated for 5-6 hours before embedding into glycol methacrylate embedding media (Immuno-Bed Kit, Polysciences, Niles, IL). After the overnight polymerization the glycol methacrylate blocks were mounted on a rotary microtome and 5µm sections were cut. These sections were mounted on charged slides and allowed to dry. They were then stained with hematoxylin followed by 1% toluidine blue to selectively stain nuclei (purple) and ECM (blue) respectively.

The sections were analyzed for immunohistochemistry for the generation of type II collagen using a chick anti mouse monoclonal primary antibody against type II collagen (II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and a streptavidin-peroxidase immunohistochemical staining kit (Histostain-SP DAB, Zymed Laboratories, South San Francisco, CA) according to the manufacturer's instructions as follows. The sections were incubated with 2500 IU/ml hyaluronidase (H6254, Sigma-Aldrich, St. Louis, MO) for 45 min to digest hyaluronic acid in ECM that may be blocking antigen binding sites. After washing in PBS, they were incubated in non-immune goat serum for 20-30 min to avoid any nonspecific staining. The experimental controls were then incubated with II-II6B3 type II collagen primary antibody for 90-120 min at 37º C in a CO_2 incubator. Controls were left with PBS during

this time. After 3 x 2min washes the sections were incubated with broad-spectrum biotinylated secondary antibody for 30 min. This was followed by washing in PBS and incubation with streptavidin-peroxidase enzyme conjugate and DAB cromogen for 20 min each. The sections were counter stained with hematoxylin to stain cell nuclei and mounted using Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA).

Quantification of DNA on Scaffolds

After culturing the scaffolds for 3 weeks they were taken out and divided in 4 smaller pieces to have more number of samples for DNA quantification. The cells on each of these were lysed in 300µl of NP-40 cell lysis solution (0.5% Igepal CA-630, 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40). After vortexing for about 30 min they were stored at 4º C before DNA could be measured. The DNA was quantified through bisbenzimide fluorometry assay based on the Hoescht dye procedure using DNA-Quantification Kit (DNA-QF, Sigma-Aldrich St. Louis, MO). The fluorescent dye bisbenzimide binds primarily to the AT sequences in the minor groove of double stranded DNA. When excited at 360nm the fluorescence emission at 460nm gives a measure of DNA present. Calf thymus DNA was used for constructing the standard curve.

Quantification of Sulphated Glycosaminoglycan on Scaffolds

The cell lysate on the scaffolds was replaced by 250µl/sample of 1% v/v papain and 1 mg/ml cysteine in 50 mM sodium acetate buffer (pH 6) and digested at 60ºC for 8-10 hours. This solution dissolves GAGs in the ECM secreted by the cells. For the

experiment to test effect of gelatin concentration on response to seeded chondrocytes 1,9-dimethyl-methylene blue procedure from Farndale *et al*. (1986) was followed to measure sGAG. The intensity of bound dye was read on a μQuant spectrophotometer (Biotek Instrument, Inc.) at 525nm. In this method the glycosaminoglycan-dimethylmethyleneblue complexes eventually start to aggregate and precipitate immediately on mixing. Hence, the method may show some variability in its readings over time. Hence for all further analysis the 1,9-dimethylmethylene blue (DMMB) Blyscan sGAG Assay (Biocolor Ltd., North Ireland) was used. Known quantity of aliquot from papain digestate was mixed with 1ml of DMMB dye in an inorganic buffer. The mixture was vortexed for 30-45 min and the formed complexes were centrifuged into a pellet. After draining off the supernatant fluid the pellet was dissociated with a chaotropic salt solution in aqueous propan-1-ol by vortexing for 30-45 min. The absorbance of dye released from pellet was read at 656nm on a μQuant spectrophotometer (Biotek Instrument, Inc.) at 656nm. Bovine trachea chondroitin 4-sulphate was used to prepare the standard curve.

Cell Attachment Kinetics

Freeze dried scaffolds were re-hydrated in graded series of alcohol and equilibrated in culture media at 37º C. Chondrocytes were isolated from hip and knee cartilages of 4 adult rabbits using similar protocol as mentioned above. High-density cell suspension (5 x10^6 cells/ml) in culture media was added in 2 ml XPERTEK glass crimp vials (P. J. Cobert Associates, Inc., MO, USA) containing 2 scaffolds each. These vials, when capped with gas permeable PTFE/Silicone rubber crimp caps, were externally

attached to the rotor of Synthecon bioreactor and set in rotary motion at very low speeds. The silicone rubber caps are gas permeable and hence provide for the required CO_2 exchange. Everyday the cells from old culture media were isolated by centrifugation at about 2000 rpm for 8 min, resuspended in fresh media with 50µg/ml ascorbate, and replaced on scaffolds. At intervals of 1, 4 and 7 days scaffolds were fixed, embedded in polymethylmethacrylate, sectioned (5µm), and stained with hematoxylin to evaluate cell viability and penetration inside the matrix of scaffold.

Mechanical Testing of Scaffolds

Mechanical testing of scaffolds was performed only for the large batch of 1:1 chitosan gelatin scaffolds to see the effect of culture technique on mechanical properties of scaffolds. After 3 weeks of culture compressive resilience test was performed on 9 scaffolds from both groups using MACH-1 Mechanical tester (Biosyntech, Inc., Quebec, Canada). Wet scaffolds were placed submerged in a testing dish filled with cell culture medium. One kg load cell was be used to detect 0.5 grams of contact force from most of the scaffolds. Since, the scaffolds were much thicker before culture than after, mechanical testing on wet uncultured scaffolds was performed with 1gm force as the threshold for detecting contact. This was followed by a delay step of 30 seconds and five subsequent compressions on scaffolds (100µm each covered in 2 seconds). The MACH-1 system measures amount of reaction force exerted by the scaffold on the indenter head, amplifies, and streams the data to the connected computer. The scaffolds were allowed to relax between adjacent compressions till they exerted an almost constant force on the indenter. If the slope of indenter load-data points (relaxation rate) fell below 0.5 g/min in

a window of 5 seconds a constant force was assumed and a new compression routine initiated. ImageJ (Image processing and analysis in Java, Wayne Rasband, NIH, USA) was used to analyze the approximate contact area of the scaffolds from their digital images. The stress relaxation response of scaffolds was evaluated from this data by plotting equilibrium stress achieved by the scaffolds after stress relaxation, against the corresponding constant strain applied to the scaffolds. Slope of this curve (line) gave the wet compressive elastic modulii of the scaffolds. Of the five stress strain data points obtained for each test, first point was neglected from slope calculation so as to achieve better linearity for some of the non linear stress strain curves.

In order to observe the change in mechanical properties of scaffolds due to culture technique alone, 3 scaffolds with no cells were rehydrated in graded series of alcohol and left in static culture and bioreactor culture for 3 and 5 weeks with PBS. These were similarly tested mechanically and the data compared with that of cell seeded scaffolds.

Statistical Analysis

SAS system (SAS Institute, Inc. NC, USA.) was used to perform One-way Analysis of Variance (ANOVA) procedure for comparing different groups in the experiment for their sGAG/DNA values and compressive resilience. If any statistically significant ($p<0.05$) difference was detected from ANOVA Duncan's multiple range test was performed to detect specific differences within the compared groups. Fisher's least significance difference (LSD) performs all possible comparison between the compared groups to detect if any differences exist. In LSD the experiment-wise error rate tends to

be much higher compared to comparison wise error rate. On the other hand Tukey's honestly significant difference (HSD) procedure gives a very conservative experiment-wise error rate causing to loss of power. Hence Duncan's multiple range test is a good compromise between LSD and HSD as it helps to control experiment-wise type I error rate without excessive loss of power (Freund and Wilson 2003).

CHAPTER III

RESULTS

Objective 1: Preparation of Porous Chitosan Gelatin Scaffolds

Porous chitosan scaffolds were prepared from chitosan and gelatin blended in proportions 1:1, 2:1, 3:1, and 100% chitosan. Freeze dried scaffolds were about 5 mm in diameter and 2-3 mm in thickness. When dry the scaffolds were spongy and fragile and shrunk in their physical dimensions when hydrated. Wet scaffolds before and after culture were strong enough to be handled using forceps.

The 100% gelatin scaffolds did not sustain the alcohol sterilization step and dissolved completely. Pure chitosan scaffolds served as controls for all further analysis.

Objective 2: Effect of Gelatin Concentration on Chondrocyte Response

Variation in the gelatin concentrations in scaffolds did have a notable effect on the physical appearances as well as chondrocyte growth and the amount of extra-cellular matrix (ECM) secreted on them.

The scaffolds with higher amounts of gelatin in them were less opaque than pure chitosan scaffolds. After 3 weeks in culture the scaffolds were mechanically intact and those with gelatin showed a notable decrease in their circumference and thickness.

One-way ANOVA analysis on the amount of sulphated glycosaminoglycans (sGAG) normalized to the amount of DNA (μg/μg) for different blends showed significant difference among the groups (p-value 0.0035). Since, at α=0.05 a significant difference existed between these groups, Duncan's multiple comparison was performed to identify these differences. The sGAG/DNA values from 50% chitosan gelatin scaffolds were significantly higher as compared to other groups. Since, the DNA quantified from the different scaffolds types was not statistically different, it may be concluded that the amount of sulphated GAG secreted by cells on the 50% gelatin blend was higher as compared to that on other blends and pure chitosan.

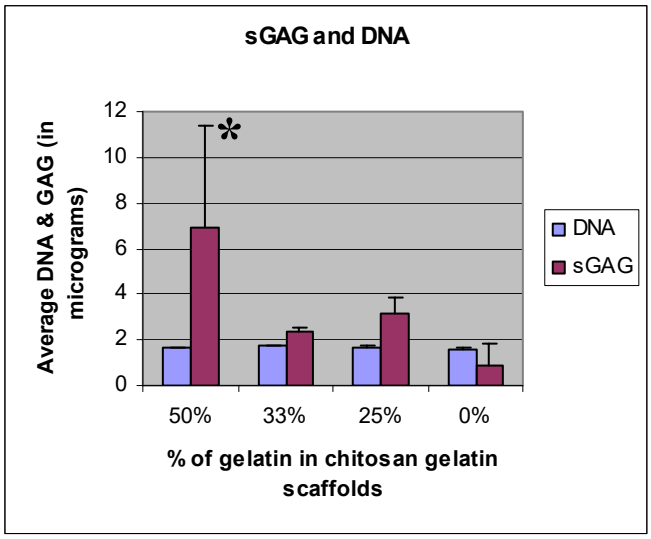

Figure 1: sGAG and DNA for different blends of chitosan and Gelatin after 3 weeks culture in transwell inserts. Bars indicate one standard deviation and * indicates statistically significant values among groups

The GLM Procedure

Dependent Variable: value sGAG normalised to DNA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	60.6809903	20.2269968	5.72	0.0035
Error	28	98.9689637	3.5346058		
Corrected Total	31	159.6499540			

The GLM Procedure

Duncan's Multiple Range Test for value

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	4.246	8	50%Gelatin
B	1.924	8	25%Gelatin
B			
B	1.380	8	33%Gelatin
B			
B	1.066	4	100%Chitosan

Figure 2: ANOVA comparison of sGAG/DNA (μg/μg) values obtained from different chitosan gelatin blends.

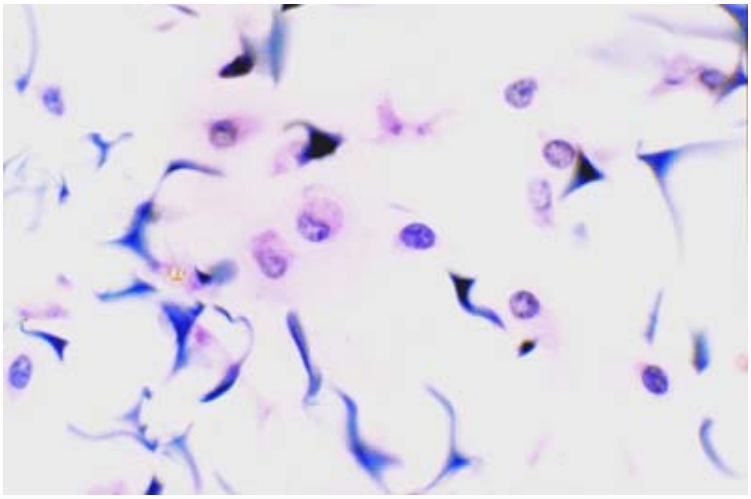

Figure 3: Hematoxylin and toluidine blue staining of section through interior of chitosan gelatin sponge showing round cell morphology. Cells are violet; chitosan is blue. (40x objective)

Overall chondrocytes cultured on chitosan gelatin scaffolds showed a circular morphology (Figure 3). Figure 4 shows hematoxylin (nuclei blue) and toluidine Blue

(proteoglycan ECM purple) staining of cross sections through scaffolds cultured in transwell inserts for 3 weeks. A general gradient was seen in the amount and penetration of proteoglycan rich ECM deep inside the scaffold as the amount of gelatin in the scaffold increased. Of these blends, those with 50% chitosan and gelatin showed much dense and deep penetration of ECM in scaffold. Toluidine staining of sections from cultured scaffolds showed metachromatic staining of ECM. Immunostaining of sections through the cultured scaffolds indicated the presence of Type II collagen. Especially, the 50% gelatin scaffolds showed dense deposits spanning deep into the interior of scaffolds (Figure 5). The ECM was predominantly seen near the boundaries and surfaces of scaffolds and was localized in few patches as seen in the sections. Compared to other groups, the ECM for the 50% chitosan gelatin blends was prominently seen to span over fairly large distributed regions on the scaffold surface.

This confirms with the above result that 50% gelatin scaffolds were able to support neochondrogenesis helped to maintain a chondrocytic phenotype.

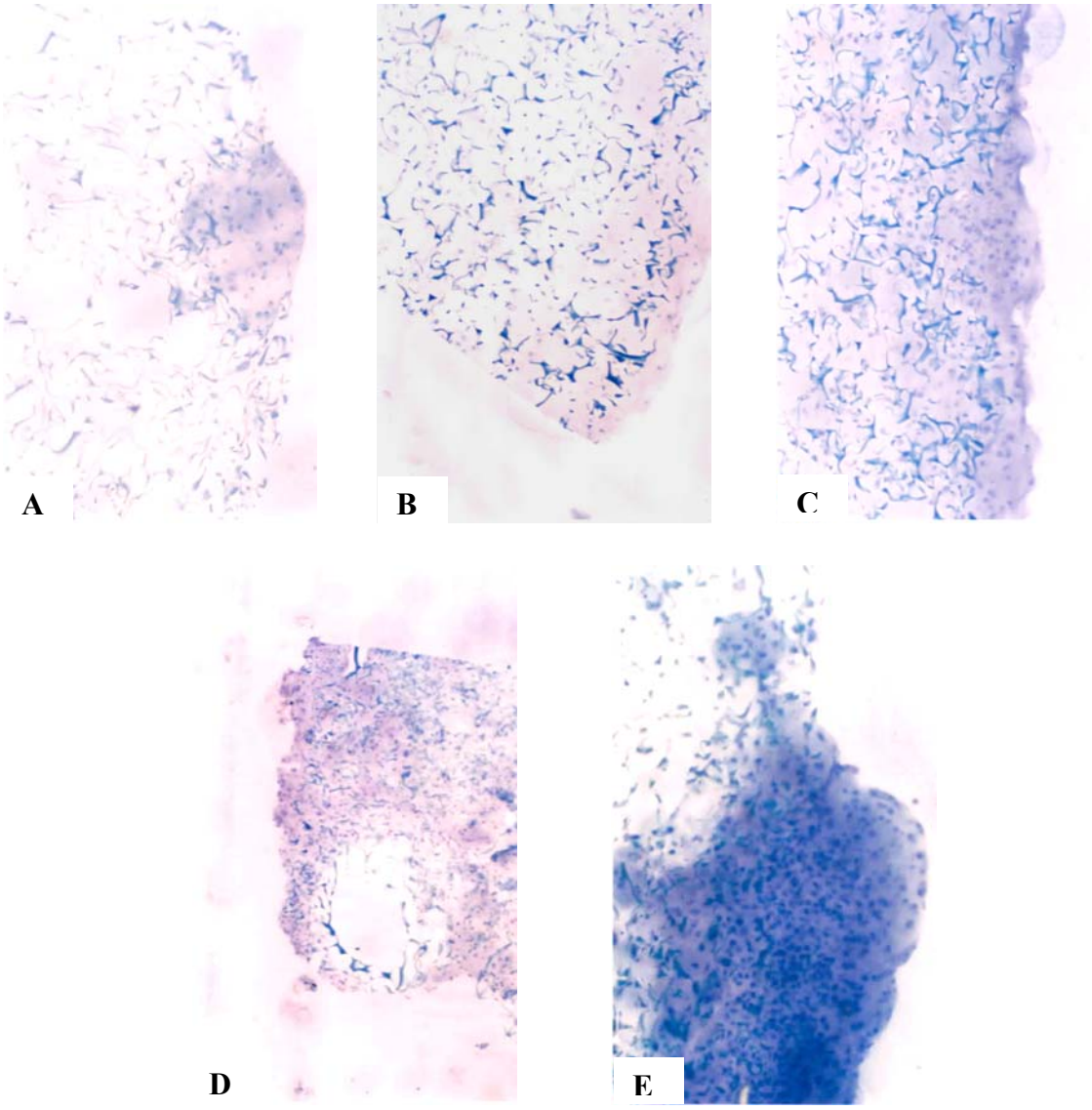

Figure 4: Hematoxylin and toluidine blue staining of chitosan scaffolds with (a) 100% Chitosan (b) 25% Gelatin (c) 33% Gelatin all at 10x (d) 50% Gelatin at 4x (e) 50% Gelatin at 10x after 3 weeks of culture in transwell inserts.

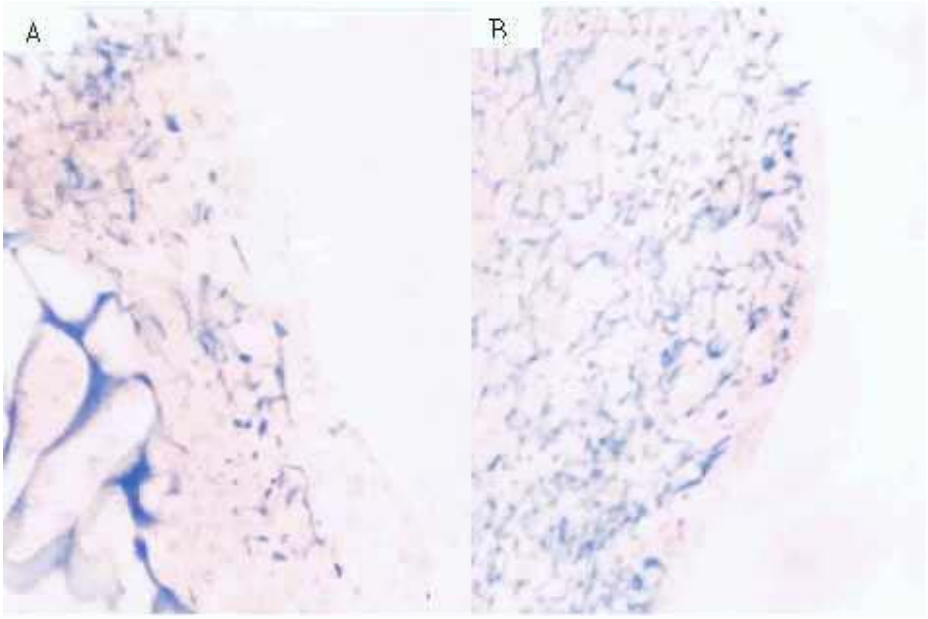

Figure 5: Immuno staining for Type II collagen in (a) 1:1 chitosan gelatin and (b) 100% chitosan scaffolds counterstained with hematoxylin. Chitosan is stained blue and collagen indicated by reddish brown deposits. (10x objective)

Objective 3: Test the Effect of Culture Technique on Generation of ECM

A large batch of 1:1 chitosan gelatin scaffolds was cultured in trans-well inserts and in a rotating cell culture system to test the effects of culture technique on growth and proliferation of chondrocytes.

Table 2 Sulphated GAG (µg) and DNA (µg) for four scaffolds each cultured in transwell inserts and Bioreactor for 3 weeks

Scaffolds in transwell for 3 weeks				Scaffolds in Rotating Bioreactor for 3 weeks			
Scaffold	sGAG(µg)	DNA(µg)	sGAG/DNA (µg / µg)	Scaffold	sGAG(µg)	DNA(µg)	sGAG/DNA (µg / µg)
T1	1.135446	31.059596	0.036557	R1	0.042683	29.25959	0.001459
T2	1.626234	27.978788	0.058124	R2	0.191057	39.31823	0.004859
T3	1.245984	31.640404	0.03938	R3	0	25.31842	0
T4	0.918791	28.736364	0.031973	R4	0.028455	25.38421	0.001121

Table 3 Sulphated GAG (µg) and DNA (µg) for one scaffold cultured in Bioreactor for 5 weeks

R11 scaffold in bioreactor for 5 weeks	sGAG(µg)	DNA(µg)	sGAG/DNA (µg/µg)
	1.311535	19.21026	0.068273

The amount of DNA quantified after 3 weeks of culture from trans-well and bioreactor scaffolds was not significantly different (p value 0.1973). However, the amounts of sGAG/DNA were significantly different between these groups (p value 0.0004) indicating differences in sGAG content secreted on scaffolds.

Dependent Variable: dna DNA in micrograms

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	100.3814383	50.1907191	2.15	0.1973
Error	6	139.8811045	23.3135174		
Corrected Total	8	240.2625428			

Figure 6: ANOVA comparison for the amounts of DNA (µg) obtained from scaffolds after culturing 3 weeks in transwell inserts and Bioreactor (n=4 each) and 5 weeks in Bioreactor (n=1)

Dependent Variable: sgagbydna sGAG normalised to DNA (µg /µg)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.00507336	0.00253668	37.19	0.0004
Error	6	0.00040920	0.00006820		
Corrected Total	8	0.00548256			

The GLM Procedure

Duncan's Multiple Range Test for sgagbydna

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6

```
Error Mean Square           0.000068
Harmonic Mean of Cell Sizes        2

  NOTE: Cell sizes are not equal.

Number of Means           2           3
Critical Range       .02021      .02094

Means with the same letter are not significantly different.

Duncan Grouping          Mean      N    group

               A      0.068273      1    r_5w

               B      0.041508      4    t_3w

               C      0.001860      4    r_3w
```

Figure 7: ANOVA and Duncan's Multiple comparisons for sGAG/DNA values obtained after culturing scaffolds for 3 weeks in transwell inserts and bioreactor (n=4 each) and 5 weeks in bioreactor (n=1)

Duncan's multiple comparison test indicated that scaffolds cultured in transwell inserts had higher amounts of sGAG/DNA as compared to those in Bioreactor. The single scaffold in bioreactor cultured for 5 weeks had higher values of sGAG/DNA compared those from 3 weeks. This indicates that culturing in bioreactor did not have an extremely significant stimulatory effect on ECM secretion for short culture time.

Gross observation of scaffolds cultured in bioreactor showed a filmy/fibrous tissue surrounding them. Although not analyzed separately, this may be a mass of fibrous tissue as a result of high amount of shearing from culture media. The decrease in the circumferential area and thickness of scaffolds may be attributed either to metabolic activity of seeded cells or to the weak adhesion in chitosan gelatin blend, which did not sustain the dissolving effect of surrounding fluid. A noticeable degradation was observed after 5 weeks in bioreactor.

In order to check the contribution of culture technique alone to scaffold degradation scaffolds (3 each) were left in static petri dishes and rotating bioreactor without any cells on them for 3 and 5 weeks. Compressive resilience testing on them after 3 and 5 weeks showed that the mechanical properties of scaffolds did change due to culture conditions. The average elastic modulus of scaffolds without any cells gradually decreased, while it remained same or improved for cell-seeded scaffolds after culture.

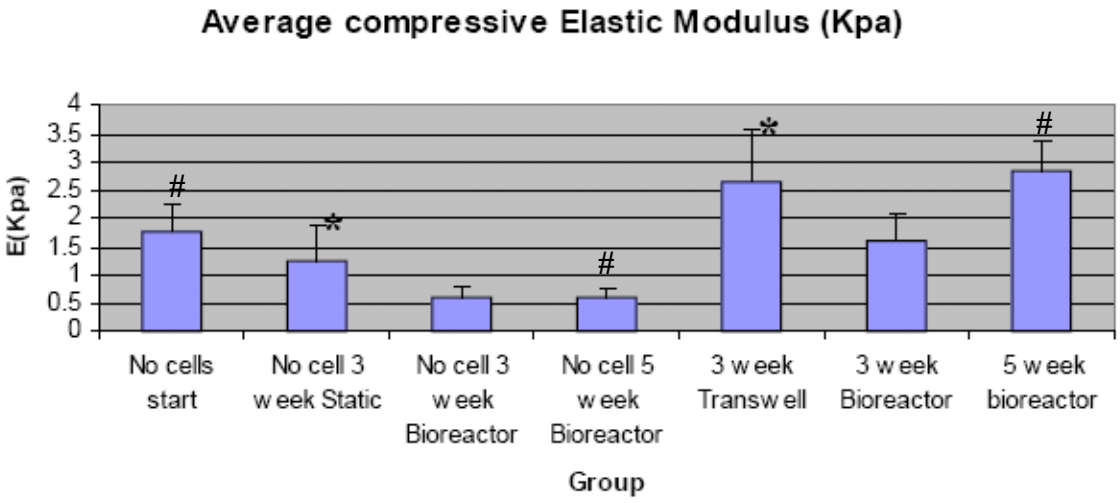

Figure 8: Average compressive elastic modulus (KPa) of scaffolds from different groups. Those marked with * & # are significantly different

Scaffolds with no cells and in static conditions, similar to transwell inserts, showed a slight decrease (Figure 9 not statistically significant at $\alpha = 0.05$) in their elastic modulus after 3 weeks. But, these values were significantly lower as compared to elastic moduli of cell-seeded scaffolds after 3 weeks (Figure 8 marked as *), thus suggesting

secretion of ECM components by cultured cells had an effect on scaffold mechanical properties.

Compressive elastic modulus
The GLM Procedure
Dependent Variable: value Youngs modulus

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	5.35715891	2.67857946	4.23	0.0365
Error	14	8.86079589	0.63291399		
Corrected Total	16	14.21795480			

Compressive elastic modulus

The GLM Procedure

Duncan's Multiple Range Test for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.632914
Harmonic Mean of Cell Sizes	4.655172

NOTE: Cell sizes are not equal.

Number of Means	2	3
Critical Range	1.118	1.172

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	2.6214	9	celltrans3
A			
B A	1.7493	5	nocellbefore
B			
B	1.2242	3	nocelltrans3

Figure 9: ANOVA and Duncan's Multiple comparisons of Elastic moduli of scaffolds cultured in static (transwell) conditions with and without cells for 3 weeks.

Similarly, comparing scaffolds from rotating bioreactor (RCC) without cells showed that their elastic moduli significantly decreased after 3 and 5 weeks as compared to initial values. Elastic modulus of cell-seeded scaffolds after 5 weeks in bioreactor was higher than that obtained after 3 weeks (cell seeded scaffolds) and from no cell seeded scaffolds in bioreactor (Figure 8 marked as # also see Figure 10). Since the sGAG values

obtained after 5 weeks were also significantly higher than that after 3 weeks (Figure 7), this improvement in mechanical properties should be due to secretion of ECM by cultured cells.

Compressive elastic modulus
The GLM Procedure

Dependent Variable: value Youngs modulus

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	8.94819878	2.23704970	11.61	<.0001
Error	17	3.27651088	0.19273593		
Corrected Total	21	12.22470966			

Compressive elastic modulus
The GLM Procedure

Duncan's Multiple Range Test for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	17
Error Mean Square	0.192736
Harmonic Mean of Cell Sizes	3.383459

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5
Critical Range	.7121	.7470	.7689	.7840

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	2.8415	2	cellrcc5
B	1.7493	5	nocellbefore
B B			
B	1.6029	9	cellrcc3
C	0.5932	3	nocellrcc3
C C			
C	0.5885	3	nocellrcc5

Figure 10: ANOVA and Duncan's Multiple comparisons of Elastic moduli of scaffolds cultured in rotating bioreactor conditions with and without cells for 3 and 5 weeks.

The above two comparisons indicate that culturing chondrocytes on 1:1 chitosan gelatin scaffolds improved their mechanical properties from activity of cultured chondrocytes viz. secretion of ECM. To determine which of the two culture techniques

was more effective in doing so, the elastic moduli of cell seeded scaffolds in transwell and bioreactor was compared with that of no cell seeded scaffolds after 3 and 5 weeks.

Dependent Variable: value Elastic Modulus (KPa)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	18.30186400	3.66037280	8.19	0.0001
Error	23	10.28455481	0.44715456		
Corrected Total	28	28.58641881			

The GLM Procedure

Duncan's Multiple Range Test for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	23
Error Mean Square	0.447155
Harmonic Mean of Cell Sizes	3.483871

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5	6
Critical Range	1.048	1.101	1.134	1.158	1.176

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	2.8415	2	rcell5
A			
B A	2.6214	9	tcell3
B			
B C	1.6029	9	rcell3
C			
C	1.2242	3	nocelltrans3
C			
C	0.5932	3	nocellrcc3
C			
C	0.5885	3	nocellrcc5

Figure 11: ANOVA and Duncan's Multiple Comparison test on compressive elastic moduli of scaffolds cultured with cells for 3 weeks in transwell inserts and bioreactor (n=9 each), 5 weeks in bioreactor (n=2) and without any cells static and bioreactor

ANOVA comparisons indicated differences between compared groups (p value 0.001). Moduli of scaffolds after 3 weeks in transwell and bioreactor indicate no statistically significant difference. However, after 5 weeks in bioreactor the elastic

moduli of scaffolds were not different from 3 weeks in transwell group. Values from these 2 groups were significantly higher than those obtained from no cell seeded scaffolds after 3 and 5 weeks. This confirms enhancement in mechanical properties of scaffolds due to the activity of seeded chondrocytes. Regarding the effectiveness of culture techniques, bioreactor culture helped to improve scaffold mechanical properties over 3 to 5 weeks but showed marginal (not statistically significant at α=0.05) improvement over transwell culture.

Cultured scaffolds were embedded in polymethylmethacrylate, sectioned, and stained with hematoxylin and tolluidine blue to observe cell penetration and proteoglycan rich extracellular matrix. Sections from these embedded scaffolds were further stained treated with II-II6B3 type II collagen primary antibody and counterstained with to show collagen (Type II) stained yellow.

As seen in figure 12 (A & B) scaffolds in transwell showed cells localized near one of its surfaces and more prominently near the edges of the scaffold. These dense cell layers showed secretion of ECM components but these did not appear to penetrate deep inside the scaffold. There were few instances of large interconnecting pores were seen localized near one surface of the scaffold. It was not possible to track whether the surface with dense cell deposition was at top or bottom when cells were seeded on the scaffold.

Sections from bioreactor scaffolds after 3 weeks (Figure 14) showed cells to be distributed deep inside the porous scaffold. The scaffold structure was notably open and interconnected with large pores as compared to those from transwell. Groups of cells were seen to line pores and crevices deep inside the scaffold. Few cell groups were seen

attached to the surface of the scaffold. Secretion of ECM components was seen to span fairly well across some cross sections and the cells in these groups appeared to maintain a circular morphology. This indicates the cultured chondrocytes maintained their chondrocytic phenotype.

After 5 weeks in bioreactor (Figure 15) the ECM components stained denser than that was seen after 3 weeks. Also less number of cells were seen to be dispersed across cross section of these scaffold as compared to earlier.

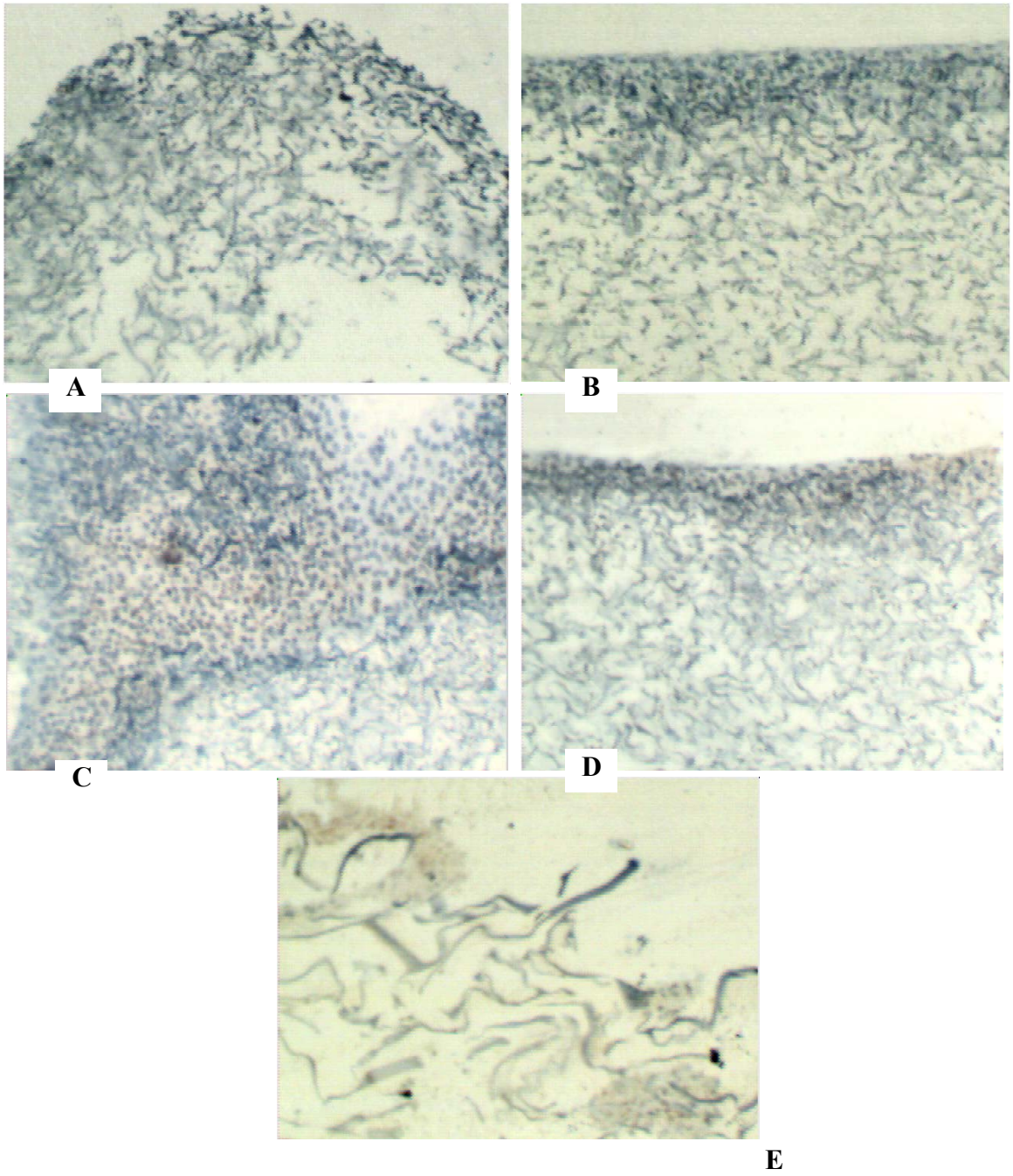

Figure 12: Scaffolds after 3 weeks in transwell. Hematoxylin and Tolluidine blue staining of sections through surface (A) and cross section (B). Immuno-staining for collagen type II of section through surface (C), cross section (D & E) all with 10x objective.

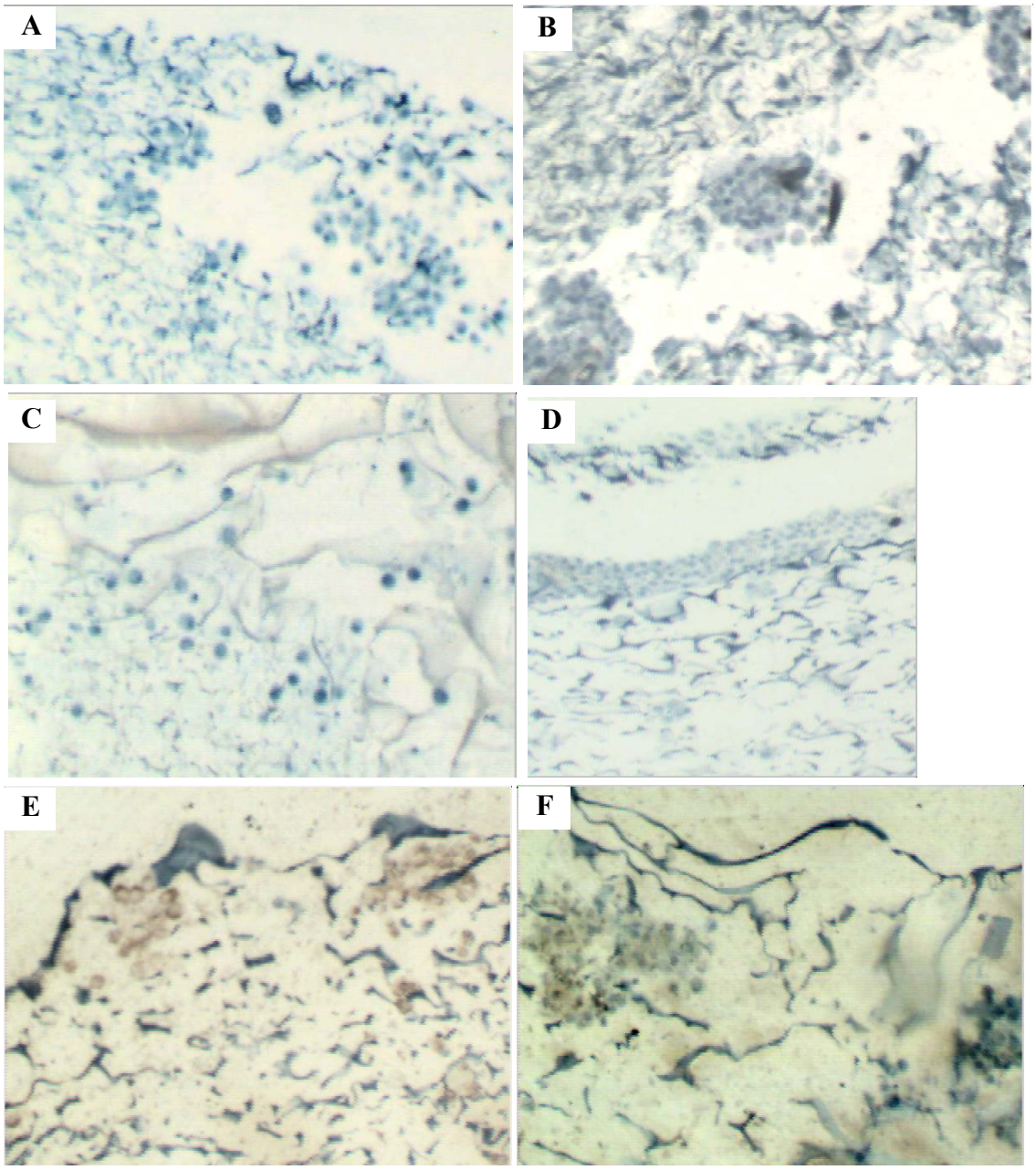

Figure 13: Scaffolds after 3 weeks in bioreactor. Hematoxylin and Tolluidine blue staining of sections through surface (A) and cross section (B, C, & D). Immuno-staining for collagen type II of section through cross section (E & F) all with 10x objective.

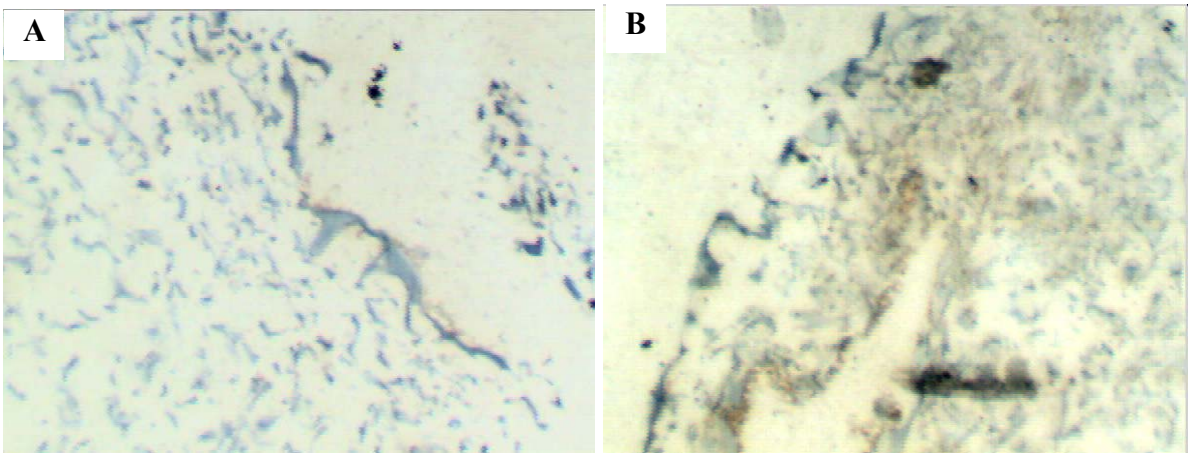

Figure 14: Scaffolds after 5 weeks in bioreactor. Immuno-staining for collagen type II of section through cross section (A negative control & B positive control) all with 10x objective.

Cell Attachment Kinetics

Immuno histochemical analysis of scaffolds from bioreactor and transwell showed concentration of ECM components in small groups/patches localized near the scaffold boundaries and surfaces. Such a trend was expected from static transwell culture due to the absence of any mixing regimen. In order to determine cell penetration and viability that is achieved after a dynamic cell seeding technique, similar to a bioreactor, cell attachment was studied over upto a week. Results after day 1 showed almost uniform cell loading across smaller cross sections (Figure 12A). Some of the larger cross sections through the central region of scaffolds did not show such uniform cell loading but they showed dense groups of cells in patches and clumps (Figure 12B). These dense cell colonies showed fairly deep penetration inside the porous scaffold matrix.

After day 4 similar trend was seen with almost uniform cell distribution across small cross sections (Figure 12C). The larger cross sections from central regions of scaffolds had better or at least maintained deeply penetrated cell colonies (Figure 12D). Scaffold structure appeared to become more open, and this may be due to dissolution of gelatin predominantly near the surfaces. Gelatin component was seen in form of elongated fibers entangled with the chitosan matrix (Figure 12E). Cells appeared to maintain a circular to ellipsoidal morphology (Figure 12F). Due to infection of these scaffolds after 5 days, the presence of chondrocytes in scaffold sections could not be well quantified. After 7 days in culture the scaffold pore structure became more open and an interconnected chitosan matrix was predominantly seen (Figure 12G).

These results from analysis of cultured scaffolds grossly (comparing their elastic modulus) and microscopically (from immuno-histochemical staining) give enough evidence to confirm that chitosan gelatin scaffolds support chondrogenesis, while those containing 50% showed better results compared to other blends and pure chitosan. For short culture periods, up to 3 weeks, the scaffolds cultured in rotating bioreactor did not outperform those cultured in transwell inserts.

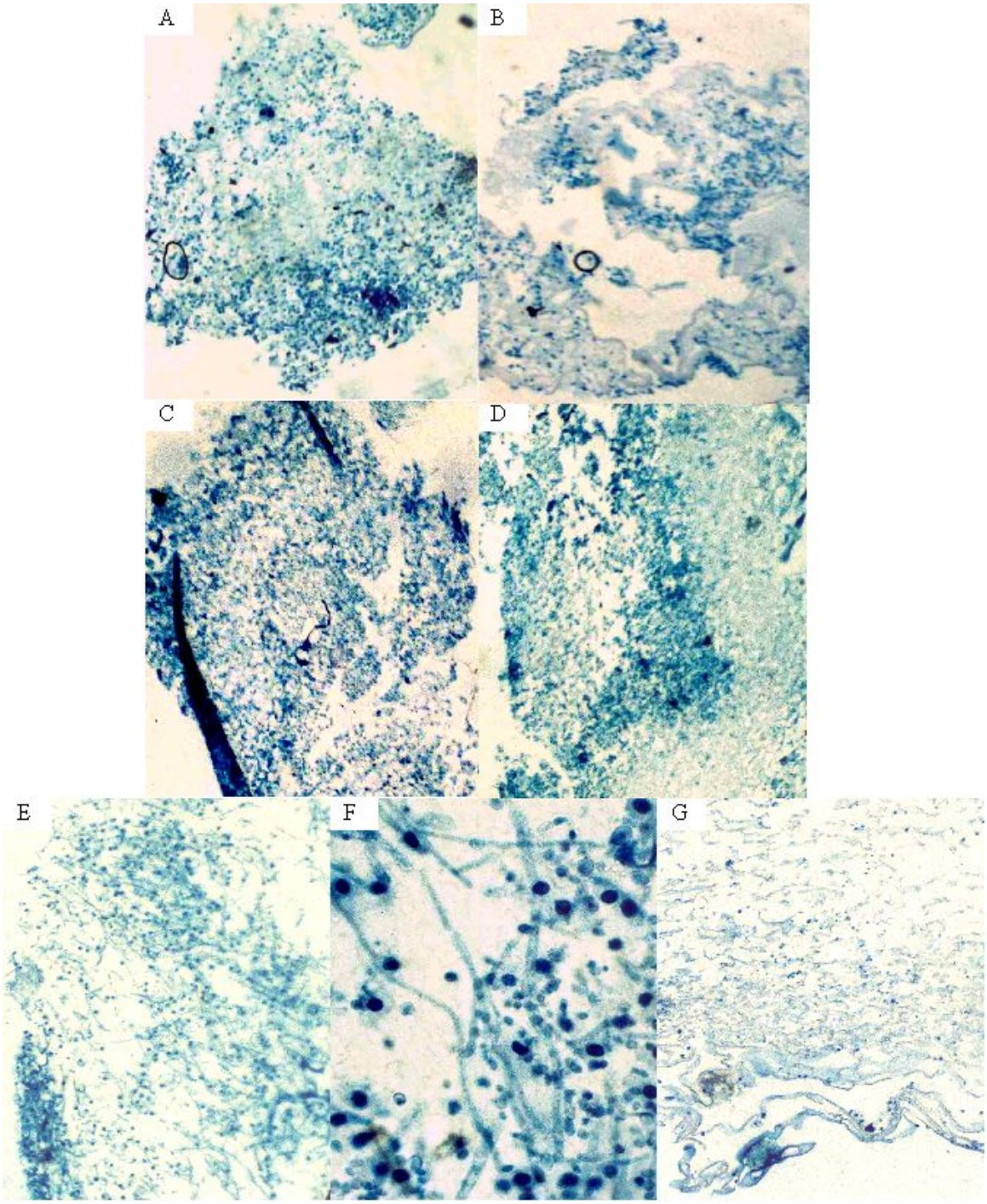

Figure 15: Cell attachment kinetics. Hematoxylin staining of cross sections through scaffold showing cultured chondrocytes after 1 day (A and B), 4 days (C, D, E) all with 4x objective; gelatin network after 4 days (F with 10x) and scaffold structure after 7 days (G with 40x objective).

CHAPTER IV

DISCUSSION

Objective 1: Preparation of Porous Chitosan Gelatin Scaffolds

Porous 3D scaffolds were prepared from blends of chitosan and Gelatin by simple freezing and lyophilizing these mixtures, maintaining aseptic techniques. Due to the different types of materials used in preparation, slight structural dissimilarities were observed between the scaffolds prepared for preliminary tests and large batch. Calfskin gelatin (Type B, 225 bloom) was used in preliminary tests to check the effects of gelatin concentration. For the large batch of 1:1 chitosan gelatin scaffolds, porcine skin gelatin (Type A, 300 bloom) was selected to have better cytocompatibility with porcine knee chondrocytes. Type A gelatin is derived from acid cured tissue while type B is obtained from lime treated tissue. Higher the bloom number stronger gel is formed when gelatin goes into solution. Mississippi Agricultural and Forestry Experiment Station (MAFES) primarily funded this project in order to support AgraTech International, Inc. (Denville, NJ) for experimenting with improvement of the quality of chitosan produced by them. Collaborating with the Biomaterials laboratory at Mississippi State University, chitosan was made available by the manufacturer for its chemical analysis to determine

DDA, moisture content, ash content, and protein content for the different batches of chitosan. Hence the type of chitosan used for this project was dependant on its availability from the manufacturer for testing purposes. Although the grade and purity of chitosan was not evaluated by the researcher, it was known that no special manufacturing conditions were maintained to produce medical or pharmaceutical grade chitosan thus making the product more of an industrial grade. Also, since the manufacturer was in the process of refining their chitosan production protocol the DDA of chitosan used for the large batch tests was not known precisely before starting experiments. Based on earlier batches it was not expected to be as low as 50-60%. This low DDA and extraneous impurities in chitosan would have had some effect on cell attachment and overall biocompatibility of scaffolds used for the large batch.

Scaffold sections from preliminary tests generally did not show much variation in their pore sizes across a cross section. However, similar sections from scaffolds from large batch showed large elongated pores localized near one surface. Since the freezing rate and temperature were maintained same in both studies, the only explanation to this may be the inhomogeneous mixture of chitosan and gelatin solutions in acetic acid for the large batch scaffolds. Gel clumps that were formed in gelatin acetic acid solution remained un-dissolved even after 30 min of vortexing. Loosely packed gelatin may have tended to dissolve away from the scaffold while in culture and left behind large voids. This would also have contributed to faster decrease in scaffold mass from dissolution and this rate of dissolution was not well balanced with the rate of growth of new tissue. Heating solutions to about 50º C is reported to form uniform chitosan gelatin

polyelectrolyte complexes (Yin *et al.*, 1999). Also aqueous gelatin solutions may be used, if desired.

Objective 2: Effect of Gelatin Concentration on Chondrocyte Response

Scaffolds were prepared by blending chitosan and gelatin (1:1, 2:1, and 3:1 w/w) and from 100% chitosan (control) solutions in 1% acetic acid. Hematoxylin and toluidine blue staining showed a general gradation in the amounts of ECM secreted as the amount of gelatin in scaffolds increased, having its maximum for 50% chitosan gelatin blends. Yin et al (1999) observed similar trend from X-Ray diffraction analysis of chitosan gelatin blends. As the gelatin proportion in chitosan scaffold approached 50% there was a gradual smoothening of crystalline peaks in the diffraction patterns and better tensile properties of films were seen. This trend was speculated to indicate optimum compatibility between chitosan and gelatin material phases at room temperature. Further Cheng *et al.* (2003) report an increase in the wettability of chitosan gelatin blends with concentrations approaching 50% gelatin. Films made from these solutions showed better elastic properties as compared to films from pure chitosan and pure gelatin.

The cell seeding density used for this study (25 x 10^3 cells/scaffold) was much lower compared to that used in culturing the large batch (870 x 10^3 cells/scaffold) of 1:1 chitosan gelatin scaffolds and so results of sGAG/DNA from these studies may not be directly compared to each other. These values for 1:1 chitosan gelatin scaffolds from preliminary tests and that from the large batch are somewhat different due to different sGAG quantification methods and types of component materials used in blends.

To maximize cell loading and considering the initial apoptotic response of cells when seeded on scaffold, care was taken to supplement fresh cells to the large batch scaffolds, at intervals of few hours. Although, this may have facilitated initial loading of cells into the scaffold, there were limitations to the depth of penetration of cells that could be achieved from this static cell seeding technique. Introducing cells to the scaffold by static techniques like pipetting would have caused many of them to fall off through the scaffold pores and stick to the polystyrene well. Scaffolds having a more closed pore structure may be constructed by increasing the freezing rate (decreasing freezing temperature below -20º C) of solutions. On the other hand scaffolds that were set rotating in a dense cell suspension showed almost uniform cell loading across cross sections after 1 day and was maintained until 3 days. Such cell seeding techniques may be followed in future studies (Figure 12).

Objective 3: Test the Effect of Culture Technique on Generation of ECM

While culturing the large batch scaffolds in a bioreactor, the speed of rotation was varied to maintain scaffolds in a constant state of drag/flow along with culture media. This is recommended in order to avoid shearing effects from surrounding fluids. Although, a state of free fall is recommended to have maximum benefits of mass transport capabilities of the bioreactor, scaffolds set up in orbiting motion showed no statistically significant difference in terms of the amount of cells, GAG, and collagen secreted after 1 week (Freed and Vunjak-Novakovic 1995). In order to maintain sterility of apparatus, half of the culture medium was changed once every week and ascorbate was supplemented then. As indicated by Obradovic *et al.* (1999) such a protocol did not have

any significant effect on the number of cells, GAG content, and wet weight of polymer constructs after 5 weeks in culture as compared to those with typically 3 medium changes per week.

Biochemical analysis after 3 weeks of culture revealed that transwell and bioreactor scaffolds have nearly the same DNA content and GAG content of transwell scaffolds is higher than those in bioreactor. Since, much of the sGAG secreted by the growing chondrocytes are released into the culture media (Obradovic *et al.*, 1999) higher flow rates in bioreactor may be responsible for hindering the organization of ECM in the scaffold. One important source of variability in data may be the sGAG quantification methods. sGAG were estimated from each of the 4 quarters of scaffolds from transwell (4x4 = 16) and summed up to give an estimate for that specific scaffold. On the other hand, whole of the bioreactor scaffolds (n = 4) were used for this purpose. However, as seen from histological staining, scaffolds in bioreactor generally appeared to have deeper cell penetration and cells were viable after 3 weeks. Similarly, stained sections of scaffolds in transwell constructs showed ECM concentrated mainly at surfaces and boundaries with little to no penetration. Bioreactor scaffolds, on the other hand, had fairly deep dispersion of cells and ECM secretion.

Cells that represent growth response or apoptosis response secrete certain chemical factors in culture media that by either aid or hinder the growth of new cells. It was observed that the amount of DNA obtained after 5 weeks in bioreactor culture was slightly lesser (not statistically significant) than that obtained after 3 weeks (Figure 6). Although this observation is limited by a small sample size (n=1), such a trend may

suggest concentration of cell apoptosis factors in the culture media. Given that the half of the bioreactor culture media was changed once a week (not replaced between 3rd and 5th weeks) the possibility of initiation of an apoptotic cell response may not be ruled out.

There are certain limitations to the analysis of collagen content of cultured scaffolds solely through immuno-histochemistry methods. This method gives insights into the amount and extent of penetration of matrix contents across a specific section, it is more often dependant on the site and orientation of the section. These limitations may be overcomed, at least partially, by the use of quantitative assays for collagen measurement (for example chloramine-T hydroxyproline assay).

Hu and Athanasiou (2005) report of a similar study involving 3D PGA scaffolds that were cultured in static and bioreactor cultures in a rotating regimen with cell seeding density comparable to this study. Bioreactor cultures did not appear to outperform static cultures after 4 weeks of culture time, as indicated from the amount of sGAG and collagen per dry weight of scaffolds. Similarly, scaffolds in static cultures were stiffer than those in bioreactor. Factors like type of bioreactor, maintenance of gas and nutrient perfusion levels, and hydrodynamic/flow parameters are to be judiciously selected and controlled to have maximum benefit from this culture technique.

Testing the compressive elastic modulus of scaffolds seemed a good way to analyze bulk properties of cultured constructs. Due to differences between individual specimens, all mechanical tests were performed at a different starting thickness of scaffolds. The reaction force encountered by the indenter compressing on scaffolds served as a contact detection criterion. While studying stress relaxation response of

scaffolds the indenter step size (constant strain) and speed of compression (strain rate) were fixed. Hence the elastic moduli of scaffolds are a reliable measure to be compared directly. Results show that the scaffolds left into static and dynamic culture conditions without any cells on them, degraded in their mechanical properties between 3 and 5 weeks. However, culturing chondrocytes on them resulted in higher elastic moduli, suggesting that cell culture resulted in growth of a mechanically strong neocartilagenous tissue and/or at least prevented scaffold degradation from dissolution of gelatin.

Transwell scaffolds after 3 weeks of culture were mechanically stiffer as compared to those in bioreactor (Figure 11). This may be attributed to low amounts of ECM secretion/organization on bioreactor scaffolds. A bioreactor is expected to have higher amounts of ECM secretion as a result of better mass and nutrient transport that is achieved. However, hydrodynamic factors in culture may wash away newly secreted ECM components and thus prevent its early organization in scaffold. Eventually as the rate of matrix synthesis exceeds that of its dissolution the accumulation of ECM is seen. With a control on gas and nutrient transfer and other hydrodynamic factors of bioreactor culture, this study indicates that noticeable deposition of ECM may be seen after 5 weeks or more. Consistently throughout the 5 weeks culture period the bioreactor culture showed more uniform and deep penetration of ECM compared to static transwell culture.

Comparing the elastic moduli of scaffolds with and without cells from transwell and bioreactor shows that culture of chondrocytes does enhance the mechanical properties of scaffolds. In agreement with the results of Hu and Athanasiou (2005), this study also indicates that bioreactor cultures did not outperform static cultures in terms of

amount of sGAG quantified after culture. Whether this was due to the rotating regimen of the scaffolds in bioreactor or degradation activity of chondrocytes or due to any other factor, needs to be evaluated by further controlled studies.

Overall the mechanical properties of cultured chitosan gelatin scaffolds in this study were not better to than those obtained from other studies involving chondrocyte culture on polymer scaffolds in bioreactor (Vunjak-Novakovic *et al.*, 2004). Differences between both studies are apparent due to factors like high initial cell seeding density (3-5 million or more cells/scaffold in other studies versus less than 1million in this study), age and species of animal source (calf versus adult pigs in this study). While some of these other studies focus on in vitro growth of a nearly mature cartilaginous tissue, this study was aimed at designing a biocompatible yet easily degradable cell culture construct. Such a strategy would help in better and uniform integration of the construct with native cartilage when implanted *in vivo*.

Cell Attachment Kinetics

In order to study the time course of cell attachment and viability into the scaffold, they were set into a rotating motion in a dense cell suspension (5×10^6 cells/scaffold) with conditions similar to a small volume bioreactor. Short-term evaluation, up to a week, showed almost uniform cell dispersion across the cross section of scaffolds. Once such uniform cell loading is achieved, the true potential of these 3D scaffolds to support neochondrogenesis and ECM secretion may be evaluated from long culture times.

Gelatin filaments were seen entangled along with chitosan matrix after 4 days and were almost extinct after 7 days in cell suspension to have a more open pore structure

(Figure 12G). This correlates well with the observation that mechanical properties of scaffolds with no cells in bioreactor did not show a noticeable degradation after 3 weeks upto 5 weeks (Graph Figure 8). This indicates most of the gelatin component in scaffolds was washed away by dissolution between 1 and 3 weeks. This degradation rate may be slightly faster as compared to the rate of regeneration of new tissue. The inhomogeneous mixture of chitosan gelatin in their blends may be responsible for weak binding between them and hence faster washout of gelatin.

The tendency of gelatin of dissolve away from the blended scaffold may be encouraging as it would leave behind a more open and interconnected chitosan network. Pure chitosan is superbly biocompatible and supports chondrocyte growth and proliferation (Nettles *et al.*, 2002). Gelatin too does not pose hazards of any adverse tissue response.

High positive charge density of chitosan is sometimes held responsible for the severe cell apoptosis response on their contact with the biomaterial. Combination of chitosan and gelatin is aimed to reduce the possibility of such response. By the time most of the gelatin component of scaffolds would dissolve away chondrocyte colonies would have started differentiating. The resultant open structure of the scaffold would help in better gas and nutrient transfer to the growing cells that would eventually fill up the pores. Such strategy may be advantageous as it would facilitate uniform distribution of ECM once the rate of dissolution of gelatin is balanced with that of chondrocyte proliferation.

Chitosan (with specific DDA) and gelatin (from a source compatible to cells used and with specific molecular weight) may be selected to control degradation of chitosan gelatin scaffolds from cellular enzymatic activity. In order to control degradation from dissolving, better initial binding between chitosan and gelatin should be achieved during fabrication of polyelectrolyte complex from them. Heating the mixture of solutions (low temperature around 50º C) and/or use of a carefully selected and biocompatible cross-linking agent may be helpful in this regard. Stiffer scaffolds or culture matrices are reported to keep chondrocytes in a proliferative rather than differentiating phenotype, thus resulting in less type II collagen secretion (Drury and Mooney, 2003). In order to develop a better culture environment for chondrocytes in chitosan gelatin scaffolds, the cross-linking density, scaffold composition, pore size and connectivity may be altered to control their gross mechanical properties.

Future Direction of Work

The ultimate objective of this project is to build a construct for culturing chondrocytes *in vitro* and potentially implanting it into a cartilage defect so that it may be healed. As a first step towards this it is important to have confidence from the *in vitro* response of cells to this composite biomaterial. Factors like selection and processing of component materials to form scaffolds and the hydrodynamic and mechanical stimulations obtained from the culturing technique may be optimized and controlled to have the desired tissue growth. The *in vivo* biocompatibility could be evaluated by implanting scaffolds in subdermal pouches in a small animal model (such as nude mice). This may be followed by an evaluation of the *in vivo* chondrogenic efficacy and long-

term performance of construct to fill in cartilage defects by implanting the cultured scaffolds in an artificially created subchondral bone defect in a large animal (such as rabbit) model.

CHAPTER V

CONCLUSION

This study demonstrated the potential of 3D chitosan gelatin complex scaffolds for culturing articular chondrocytes. Selecting chitosan and gelatin from proper source and with known chemical properties,porous scaffolds can be easily constructed from their blends. Of the different combinations of chitosan and gelatin blends that were tested the ones with 50% gelatin showed maximum biosynthetic activity of the seeded chondrocytes after 3 weeks in culture. The presence of gelatin in chitosan scaffolds had a small but noticeable effect on growth of chondrocytes. Moreover, gelatin served as a leeching agent that dissolved in culture media leaving behind a more open and interconnected chitosan scaffold structure. Overall chitosan gelatin scaffolds showed better performance in culturing chondrocytes as compared to pure chitosan scaffolds.

Evaluating the effect cast due to the culturing technique, the scaffolds in bioreactor culture for small period up to 3 weeks did not outperform those in transwell culture in terms of the amount of sulphated glycosaminoglycans secreted and the overall mechanical properties of scaffolds. Results indicate that longer culture periods (5 weeks or more) may be required to see a noticeable effect on extra cellular matrix secretion in the bioreactor culture.

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APPENDIX A

ARTICULAR CARTILAGE TISSUE ENGINEERING CONSIDERATIONS

Structure

Articular cartilage is an aneural, avascular, and alymphatic tissue covering the interfacing ends of bones in diarthrodial joints. It is the smooth, pearly white layer that lubricates joint motion by preventing direct rubbing of the hard bony tissue against each other. Moreover it plays a vital role in load bearing and load transfer across the joints. Its thickness and quality varies from joint to joint and from the location in a single joint. Other factors like the age and species of animal source, body mass, physical activity and presence of diseased conditions that influence the quality of cartilage tissue.

Essentially, articular cartilage is made up of a single type of cells, called chondrocytes, embedded in a sea of extra-cellular matrix that is secreted by them. The variation of the chemical composition of the matrix (with age, physical activity, location in tissue etc.) and the changes in morphology and activity of the chondrocytes makes the tissue biologically more complex than expected. The matrix mainly consists of water (65-80% of wet weight), proteoglycans, collagens (50% dry weight) and some other proteins and glycoproteins. The unique biomechanical characteristics of the tissue are attributed to its viscoelasticity, anisotrophy, inhomogeneity and nonlinearity in mechanical properties (Guilak, 2004) of the matrix.

Articular cartilage may be divided into 4 distinct zones according to the morphology of chondrocytes in different layers and the variation in chemical composition of these layers. Going from superficial to deep into the tissue the four layers are surface layer, middle layer, deep layer and finally the zone of calcified cartilage at the interface of cartilage with subchondral bone (Poole, 1993). A detailed study of the chondrocyte

morphology across the thickness of cartilage reveals that they oriented along the lines of maximum force that is subjected at that region in the matrix.

Table 4: Summary of different zones in articular cartilage. Modified from Poole A.C. (1993) and Buckwalter J.A. (2004)

Zones / Properties	Zone I (Superficial)	Zone II (Middle)	Zone III (Deep)	Zone IV (Calcified Cartilage)
Volume	5-10%	40-45%	40-45%	5-10%
Chondrocyte morphology	Thin and discoid	Round	Spheroidal	Small & rounded
Chondrocyte orientation	Parallel to surface	Disperse	Arranged in columns	Random & encased in calcified cocoon
Collagen fibers	Uniform sheet tangential to surface	Amorphous network of oblique fibrils	Radially aligned fibers Large diameter	Radialy aligned
Water content	Highest (80%)	High	Lowest (65%)	
Proteoglycan	Lowest	High	Highest	Absent

Chondrocytes: Biomechanical Response

Chondrocytes are the only type of cells present in the cartilage but account for less then 10% of the total matrix volume. A balance between their anabolic and catabolic activities helps to maintain the cartilaginous matrix. Since there are no inter or intra cellular vascular connections between them most of the transfer of gasses and nutrients takes place by diffusion through the synovium and bone vasculature. Their anaerobic

metabolism renders them to be metabolically less active and less sensitive to loading and injury compared to bone and muscle cells (Buckwalter, 2004).

The stress environment within the joint and the ability of chondrocytes to regulate their metabolism provides them a means to regulate the structure and composition of cartilage matrix depending on the requirements of the body. The chondrocytes respond to hydrostatic loading effect on the matrix and its components due to externally applied load. Their metabolic activity and cell differentiation are modulated by integrin and other adhesion mediators. High frequency oscillating loads stimulates matrix synthesis as compared to static loads or low frequency hydrostatic loading (Elder at al., 2005, Caterson *et al.*, 2004). Poole (1993) emphasizes on chondron, the periarticular environment of chondrocytes, to be the functional and metabolic unit acting as a transducer for maintaining of cartilage homeostasis.

Under certain abnormal conditions like disease or excessive tissue injury may lead to initiation of inflammation and degradation process eventually leading to joint degeneration (Guilak, 2004).

APPENDIX B

MECHANICAL TESTING OF CHITOSAN GELATIN SCAFFOLDS

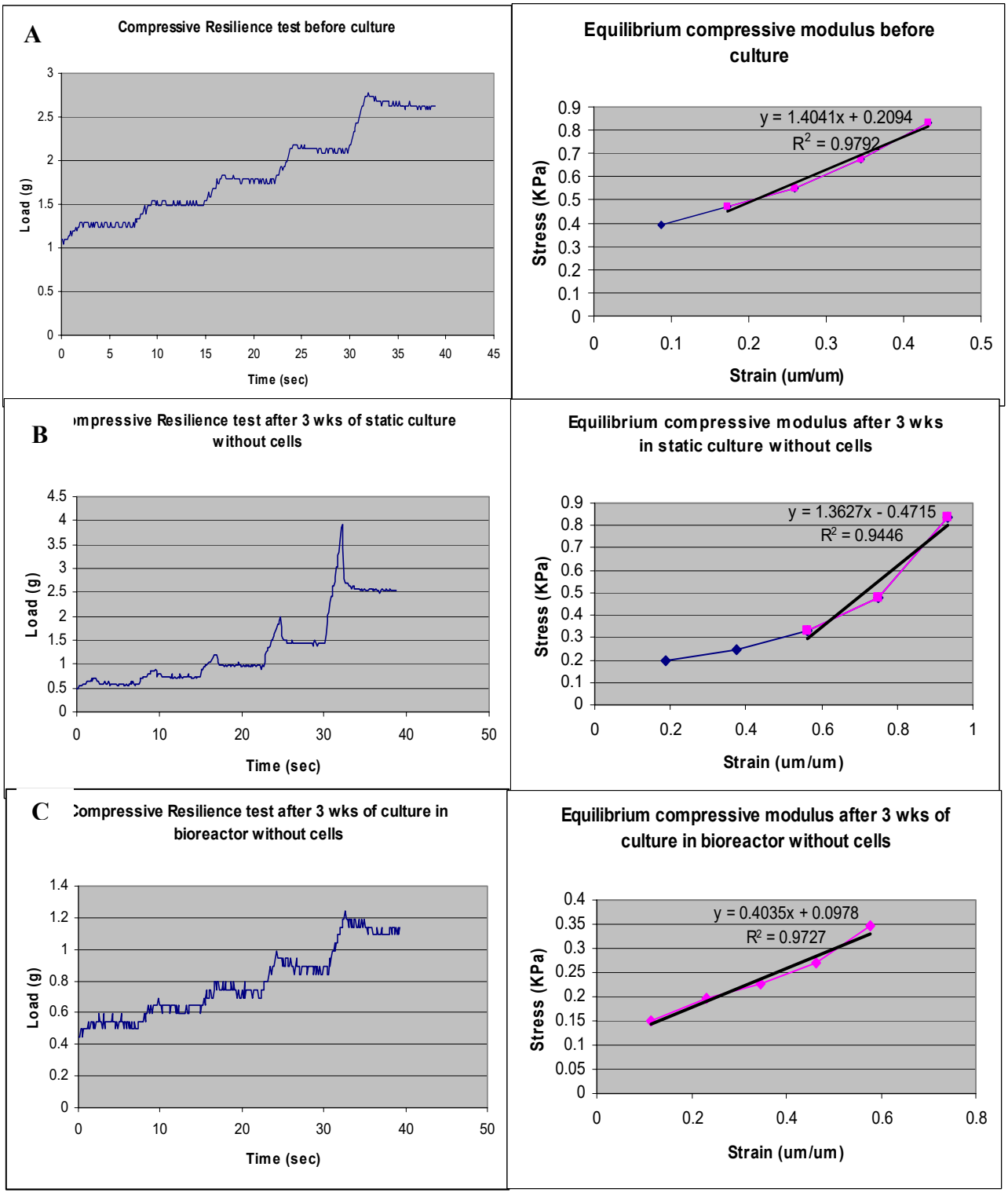

Figure 16: Compressive resilience testing on scaffolds before (A) and after 3 weeks in static culture (B) and bioreactor (C) without cells.

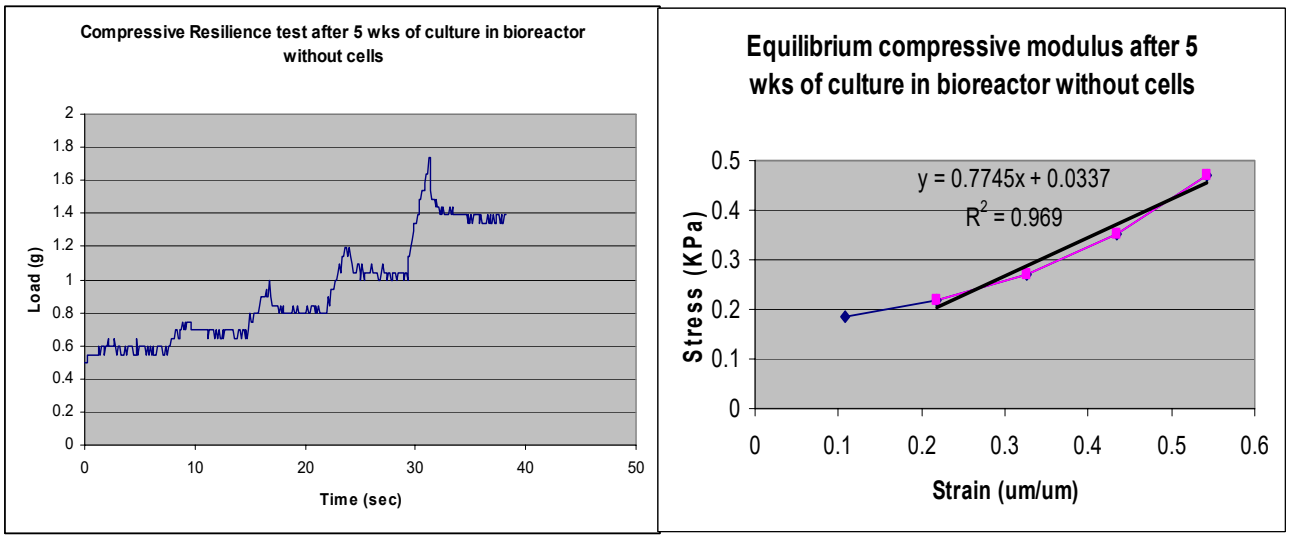

Figure 17: Compressive resilience testing on scaffolds after 5 weeks in bioreactor without cells.

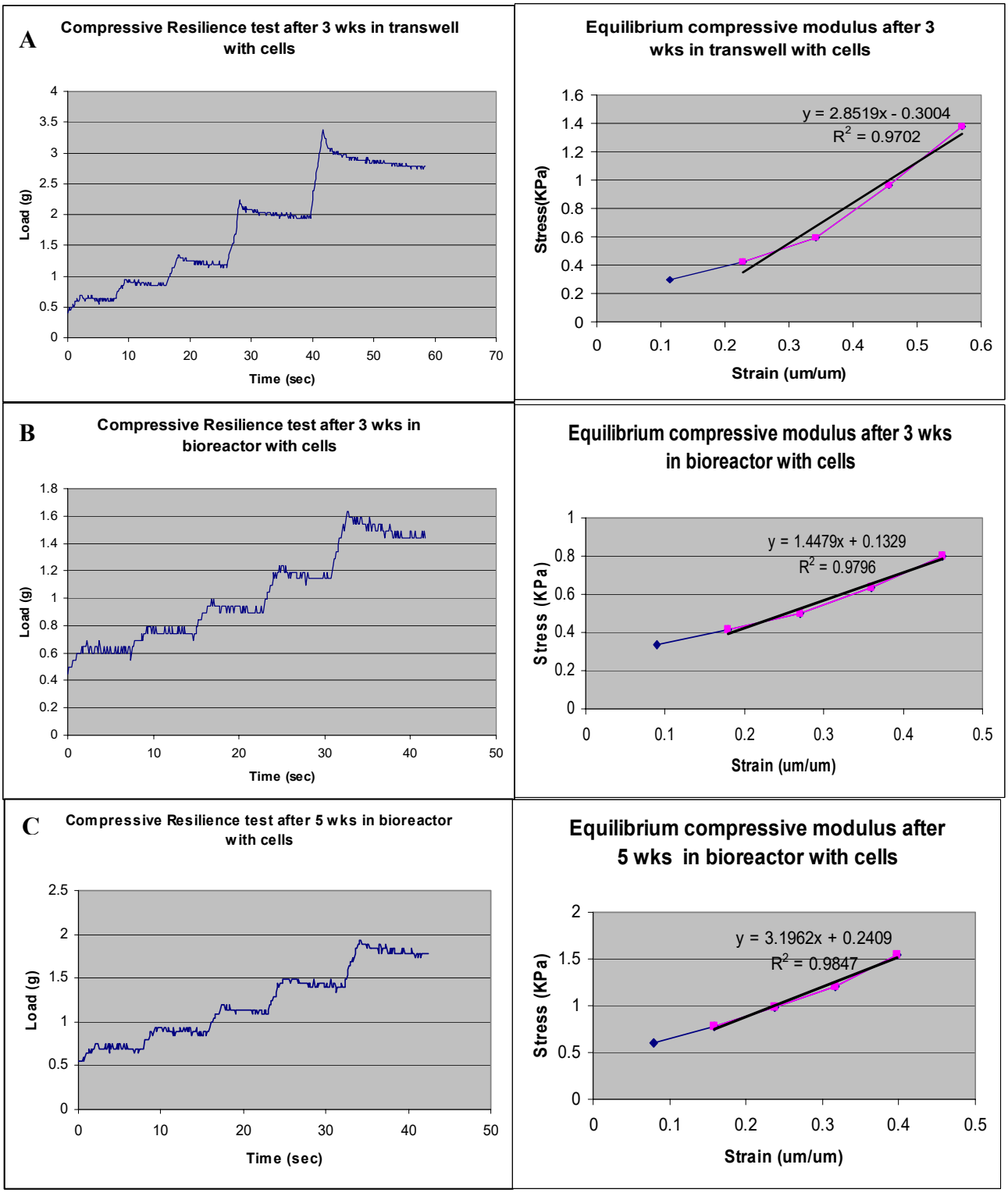

Figure 18: Compressive resilience tests after culturing chondrocytes on scaffolds for 3 weeks in transwell (A) and bioreactor (B) and after 5 weeks in bioreactor (C).