Effects of Biochemical and Mechanical Stimulation of Articular Chondrocytes in Collagen-GAG Scaffolds: Extracellular Matrix Biosynthesis and Scaffold Stiffness

by

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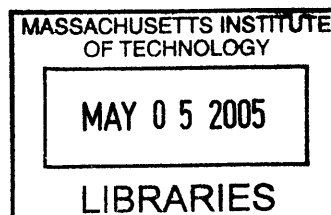September 2004

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EFFECTS OF BIOCHEMICAL AND MECHANICAL STIMULATION OF ARTICULAR CHONDROCYTES IN COLLAGEN-GAG SCAFFOLDS: EXTRACELLULAR MATRIX BIOSYNTHESIS AND SCAFFOLD STIFFNESS

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ABSTRACT

As the incidence of osteoarthritis and other degenerative joint conditions continues to grow, rehabilitation via tissue engineering is becomingly increasingly attractive as an alternative to traditional surgical interventions.

Chapters 2 and 3 of this thesis are specifically concerned with cartilage tissue engineering, while chapter 4 is relevant to bone and osteochondral tissue engineering.

The cartilage tissue engineering sections focus on the effects of two different classes of regulators of chondrocyte behavior: chemical growth factors and mechanical loading. In chapter 2, FGF-2, a chemical regulator, was supplied to chondrocyte-seeded constructs over a 4 week culture period. Afterward, these constructs were subjected to sequential ramp and hold compressive strains on a Dynastat mechanical testing apparatus, and the unconfined elastic moduli were calculated. These data were compared to the values for scaffolds receiving no FGF. The results indicate that FGF-2 induced a significant increase in the modulus of chondrocyte-seeded scaffolds.

Numerous reports indicate that certain types of mechanical loading can increase chondrocytes' ECM biosynthesis in particular cell-scaffold systems *in vitro*. Few if any loading experiments have been done, however, with type II collagen-GAG scaffolds cultured in serum-free medium. Chapter 3 describes a series of experiments in which chondrocyte-seeded scaffolds were subjected to dynamic compression and the effects of this treatment on the proliferation of the chondrocytes, their synthesis of ECM, and the stiffness of the scaffolds were measured. The results of these experiments were inconclusive. Analysis indicated that very few chondrocytes were retained in the scaffolds. A post hoc investigation of the scaffolds revealed that they were biologically inactive due to their oversize pores. The low cell density was reflected in unusually low biosynthesis values and no significant differences in stiffness post-loading.

The mechanical properties of implantable constructs such as stiffness and compressive strength are likely to significantly affect the clinical outcome. The fourth chapter describes measurements of the elastic modulus and ultimate compressive strength

of a bone scaffold material. Five different scaffold formulations were tested, and the mechanical properties correlated with the variations in their composition.

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1. GENERAL INTRODUCTION

1.1 Articular Cartilage Composition, Function and Degeneration

Articular cartilage is an exquisitely tuned composite of cells (chondrocytes), extracellular matrix (ECM) molecules, and water which sustains the tremendous forces experienced by knees, hips and the other diarthroidal joints. (Note that ECM, scaffold, matrix, and substrate are used interchangeably in this thesis.) The chondrocytes manufacture and remodel the ECM which simultaneously provides the 3-dimensional structure housing and protecting the cells as well as the mechanical integrity of cartilage. Chondrocytes will only carry out their normal metabolic functions if they are able to attach to a substrate; thus, the production and maintenance of the substrate (i.e., ECM) depends on the prior existence of a substrate. We will return to this consideration later in the section on tissue engineering.

The other primary component of cartilage, water, comprises approximately 80% of the wet weight of cartilage. The water contains electrolytes and cations which balance the negatively charged proteoglycans within the ECM, and this molecular interaction also contributes to cartilage's stiffness and resilience (Temenoff and Mikos 2000).

At densities of approximately 10,000 chondroctyes/mm^3 in adult humans, cartilage is relatively acellular. It is, furthermore, completely lacking in vascular connection; thus, chondrocytes rely on diffusion from the synovial fluid within the joint capsule through the dense ECM for their nutrient supply and waste removal (Buckwalter 1998). Due to its low metabolic activity and proliferation (for many years it was believed that chondrocytes, like nerve cells, do not reproduce at all), damage to cartilage – whether through disease or acute trauma – generally does not heal. Thus for millions of osteoarthritis patients, the prognosis is usually poor: increasing pain and decreasing joint function as focal lesions expand into the neighboring healthy cartilage.

1.2 Cartilage Repair: Traditional Approaches

Numerous techniques for treating damaged joints have been proposed. Although some studies have shown a reduction in pain after certain superficial treatments, there is little evidence that such treatments actually improve the condition of the cartilage. One class of these treatments currently in use involves little more than arthroscopic lavage of the joint and/or removal of fibrillated tissue (Kim, Moran et al. 1991; Altman, Kates et al. 1992). In osteotomy, a more invasive procedure, the tibia or femur (in the case of damage to the knee) is surgically reshaped, realigning the axis of the limb away from the diseased area. In severe cases either part or the entire joint may be surgically replaced by unicompartmental joint arthroplasty or total joint arthroplasty (TJA), respectively {AAOS Online, 2001 #196}.

Recognizing the critical role of the extracellular matrix, other interventions endeavor to create a natural matrix by penetrating the subchondral bone, inducing clot formation from blood which then flows into the defect from the marrow space. Numerous techniques exist for creating these penetrations. Another treatment, referred to as mosaicplasty, is the transplantation of autologous cartilage from non-weight bearing sections of a damaged joint to the weight bearing sections that are more critical to joint function and biomechanical load transfer (Buckwalter 1998; Buckwalter 1998). In contrast to transplanting cartilage plugs as in mosaicplasty, in autologous chondrocyte transplantation cells are excised and allowed to multiply *in vitro* and then implanted (usually under a periosteal flap) in the cartilage defect (Brittberg, Lindahl et al. 1994; Brittberg, Nilsson et al. 1996; Breinan, Minas et al. 1997; Brittberg 1999).

There are a host of problems with these surgical interventions. For example, mosaicplasty produces significant morbidity at the donor site, and techniques employing subchondral penetration have been shown to produce functionally inferior repair tissue (fibrocartilage) instead of true hyaline cartilage, especially at longer post-operative times (Breinan, Minas et al. 2001).

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1.3 Cartilage Repair: Tissue Engineering Approaches

Cartilage treatments exist along a continuum with classical and usually highly invasive surgical interventions such as TJA on one end and less invasive tissue engineering interventions on the other, but many of the treatments currently in use or under investigation possess attributes from both ends of the spectrum. Tissue engineering typically involves three primary components: cells, three-dimensional scaffolds which mimic the cells' extracellular matrix and regulators that affect cellular processes. Many diverse examples from each of these categories are currently under investigation.

Tissue engineering approaches circumvent the previously mentioned problem by introducing a temporary matrix that will provide the cells in the cartilage (or those added during the treatment) with the substrate that they require to begin their metabolic activities. In this respect, the surgical techniques employing subchondral penetration and fibrin clotting may be considered to possess some of the attributes of a tissue engineering approach. Usually TE substrates are designed to biodegrade as a part of the remodeling process, to be replaced eventually by newly synthesized ECM. The kinetics of this process are important yet little understood (Gordon TD 2004).

1.3.1 Cells

While chondrocytes are an obvious choice of cells to be used in a cartilage TE system, many studies have been performed with cells from synovium, perichondrium (Dounchis, Goomer et al. 1997), periosteum (O'Driscoll 1999), and bone marrow derived stem cells.

1.3.2 Scaffold Materials

There are a wide range of materials currently employed as matrices – collagen (Grande, Halberstadt et al. 1997; Nehrer, Breinan et al. 1997), fibrin (Ameer, Mahmood et al. 2002; Hunter, Mouw et al. 2004), polylactic (Chu, Coutts et al. 1995; Dounchis, Goomer et al. 1997; Saldanha and Grande 2000; Chen, Sato et al. 2003) and polyglycolic acid (Freed, Vunjak-Novakovic et al. 1994; Vunjak-Novakovic, Obradovic et al. 1998;

Chen, Sato et al. 2003), agarose, self assembling peptide hydrogels (Kisiday, Jin et al. 2002) and alginate – to name a few. And much attention has been given to the proper modification (Lee, Grodzinsky et al. 2001) of these materials to ensure their biocompatibility and ability to induce and/or maintain chondrocytic phenotype when seeded with cells.

1.3.3 Regulators

The literature on the effects of chemical regulators is vast and includes discussion of growth factors such as insulin-like growth factor (IGF), fibroblast growth factor (FGF), etc.; factors promoting differentiation of chondroprogenitor cells such as dexamethasone and transforming growth factor (TGF); and serum (Hascall, Handley et al. 1983; McQuillan, Handley et al. 1986; McQuillan, Handley et al. 1986). While the biochemical pathways of mechanotransduction are still incompletely understood, there is a similarly large literature concerning the effects of mechanical regulators on cells (Gray, Pizzanelli et al. 1989; Sah, Kim et al. 1989; Larsson, Aspden et al. 1991; Sah, Doong et al. 1991; Greco, Specchia et al. 1992; Guilak, Meyer et al. 1994; Kim, Sah et al. 1994; Lee and Bader 1997; Lee, Noguchi et al. 1998; Grodzinsky, Levenston et al. 2000; Lee, Noguchi et al. 2000; Mauck, Soltz et al. 2000; Bonassar, Grodzinsky et al. 2001; Kisiday, Jin et al. 2002; Lee, Grodzinsky et al. 2003).

1.4 Tissue Engineering Subchondral Bone

Cartilage defects often may be connected – causally and/or temporally – with defects in bone (Gao, Dennis et al. 2002; Schaefer, Martin et al. 2002; Sherwood, Riley et al. 2002; Cao, Ho et al. 2003). Consequently, there is increasing interest in osteochondral tissue engineering in which specific factors from the three TE components (cell, scaffold, and regulator) conducive to the development of bone are employed to produce constructs that can repair simultaneous defects in cartilage and the underlying bone. The molecular level explanation for the stiffness of bone differs from that of cartilage, but in the final analysis any successful scaffold must possess sufficient mechanical integrity to sustain the loads it will experience *in vivo*. Critical properties of such scaffolds include stiffness

or modulus and ultimate strength. In addition to the investigation of the biochemical and mechanical factors affecting chondrocytes, a study was performed to determine critical mechanical properties of a novel bone scaffold material and to compare them to native bone. In the case of bone, it is particularly important to employ a scaffold that is comparable in stiffness to bone. If the construct's stiffness is significantly greater than the surrounding bone, stress shielding and concomitant bone density loss may occur; thus, it is important to design an implant that will either degrade relatively rapidly or that will be of comparable stiffness to native bone.

1.5 Specific Aims

This thesis investigates the effects of two different classes of regulators of chondrocyte behavior: chemical growth factors and mechanical loading. The overall aim of the work is to determine to what extent these regulators influence the maturation of chondrocyte-seeded constructs. In addition to the cartilage related work, a study of the compressive mechanical properties of several different bone scaffold materials was performed in anticipation of the clinical demand for composite bone-cartilage scaffolds.

This thesis attempts to answer the following questions:

1. FGF Treatment (chapter 2)

a. Does the addition of 5 ng/ml fibroblast growth factor (FGF-2) to canine articular chondrocyte-seeded type II collagen-GAG scaffolds cause these scaffolds to attain higher moduli after 28 days of culture than untreated controls?

2. EDAC Cross-linking Treatment (chapter 2)

a. What is the relationship between the duration of EDAC cross-linking treatment and the modulus of unseeded type II collagen-GAG scaffolds?

b. What is the optimum cross-linking time?

3. Dynamic Compression (chapter 3)

a. In serum-free medium, does the application of 3% dynamic compressive strain to canine articular chondrocyte-seeded type II collagen-GAG scaffolds increase the rates of protein and proteoglycan biosynthesis?

b. Are the results in serum-free medium comparable to those performed in serum-supplemented medium (comparison with (Lee, Grodzinsky et al. 2003))?

c. Does such compression increase the absolute amount of GAG and protein deposited in the scaffolds?

d. Does such compression increase the modulus of the scaffolds?

4. Orthoss Mechanical Properties (chapter 4)

a. Do the ultimate compressive strength and modulus vary with chemical composition for Orthoss bone scaffold?

b. Which formulation produces the greatest strength and stiffness?

2. THE EFFECTS OF FGF-2 AND CROSS-LINKING TIME ON THE COMPRESSIVE MODULUS OF ADULT CANINE CHONDROCYTE-SEEDED TYPE II COLLAGEN-GAG SCAFFOLDS GROWN IN SERUM-FREE MEDIUM

2.1 Introduction

In one approach to articular cartilage tissue engineering an immature cartilaginous construct, developed from a chondrocyte-seeded scaffold, is implanted into a defect, where it will then mature and remodel *in vivo*. While these maturation processes are complex, primary features include the synthesis of extracellular matrix (ECM) components such as proteoglycans (PG) and type II collagen by the chondrocytes and the proliferation of the cells themselves. The synthesized ECM is deposited into the scaffold, gradually replacing the biodegrading original scaffold. The result of this process is a stiffer, more tissue-like material than the original scaffold.

The mechanical properties of a construct at the time of implantation are likely to significantly affect the clinical outcome: a stiff scaffold well-populated with ECM is much more likely to sustain the biomechanical forces it will experience *in vivo* than a compliant scaffold containing less PG and collagen. Furthermore, an implant's rapid integration with the healthy surrounding cartilage tissue depends on its ability to actively synthesize ECM.

One way to assess the rate of ECM deposition is to measure the mechanical stiffness of the scaffold. Hence, the objective of this study is to evaluate the compressive modulus of canine articular chondrocyte-seeded type II collagen-GAG scaffolds after 28 days of culture with and without fibroblast growth factor (FGF-2) in order to determine whether this regulator can facilitate scaffold maturation. Elevated modulus values after treatment with FGF would be interpreted as a positive finding.

Prior investigations have demonstrated that the addition of FGF-2 during monolayer *in vitro* expansion stimulates chondrocytes' proliferation and later, their GAG production in 3-dimensional culture (scaffolds) (Martin, Vunjak-Novakovic et al. 1999;

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Martin, Suetterlin et al. 2001). Other researchers have also found that FGF-2 in monolayer culture increases chondrocyte differentiation while increasing proliferation. It should be noted that some researchers have found that when FGF was used in 3-dimensional culture a downregulation of GAG, type II collagen, wet weight and stiffness were observed (Jakob, Demarteau et al. 2001; Pei, Seidel et al. 2002).

Type II collagen scaffolds have yielded promising results for articular cartilage tissue engineering. In a recent canine study type II collagen-GAG scaffolds facilitated the increased production of viable repair tissue in cartilage defects, as demonstrated by comparison of their histomorphometric results with those from autologous chondrocyte implantation. These constructs (cultured without FGF-2) yielded better histomorphometric results than autologous chondrocyte implantation (Lee, Grodzinsky et al. 2003).

In addition to increasing scaffold stiffness via chemical regulator-mediated increases in ECM deposition, the *intrinsic* stiffness of a scaffold material is an important consideration when choosing a biomaterial for tissue engineering. A stiffer (unseeded) starting material will likely produce a stiffer, more physiologically appropriate implant than a more compliant scaffold. Prior studies of the effects of various cross-linking treatments on collagen scaffolds concluded that dehydrothermal treatment (DHT) followed by immersion in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) produced scaffolds with the greatest degree of cross-linking, and hence the lowest contraction and highest modulus (Weadock, Olson et al. 1983; Weadock, Miller et al. 1995; Olde Damink, Dijkstra et al. 1996; Weadock, Miller et al. 1996; Lee, Grodzinsky et al. 2001).

In order to optimize the EDAC cross-linking process a study of the kinetics of the reaction was performed. It was hypothesized that greater cross-linking times would significantly increase the stiffness of unseeded scaffolds, and that a more mechanically robust tissue-engineered construct could be produced by treating these maximally cross-linked cell-seeded scaffolds with FGF-2. The ultimate goal of these studies is to shorten the pre-implantation culture period and increase the robustness of such constructs in clinical applications.

2.2 Methods

2.2.1 Scaffold Fabrication and Cross-Linking

Sheets of the scaffold, 3-mm thick, were produced by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland) as previously reported for type I collagen-glycosaminoglycan matrices (Yannas, Lee et al. 1989). Eight-mm diameter samples punched from sheets of the material were cross-linked by dehydrothermal treatment followed by immersion in an aqueous solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC) (Olde Damink, Dijkstra et al. 1996). After cross-linking the scaffolds were transferred to 50 ml centrifuge tubes and were rinsed four times with sterile distilled water. During each rinse stage, the centrifuge tubes were nutated for approximately 30 minutes before siphoning off the water. After the final rinse, the scaffolds were stored in fresh, sterile water.

Comparable type II collagen scaffolds were previously (Nehrer, Breinan et al. 1997) found to have an average pore size of 86 μm and 93% porosity. A prior study employing SDS-polyacrylamide gel electrophoresis (Nehrer 1998) confirmed that the scaffolds contained primarily type II collagen. In the prior study there were no significant differences in these properties anticipated among the specimens.

2.2.2 Cell Isolation and Culture

Articular cartilage was harvested from the knee (stifle) joints of five adult mongrel dogs. Chondrocytes were isolated from the cartilage by digesting the tissue first with pronase (20 U/ml, 1hr) and subsequently with collagenase (200 U/ml), overnight, as previously described (Kuettner, Pauli et al. 1982). After isolation, the cells were washed several times in a serum-free base (SFB) culture medium, adapted from a formulation from Jakob (Jakob, Demarteau et al. 2001) (see Appendix) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA). The cells were suspended in medium and 10% dimethyl sulfoxide (DMSO) at a concentration of 10^6 cells/ml and stored at -80°C.

After cells from all five animals had been collected they were thawed by adding drops of SFB medium supplemented with 10% FBS, and the cells were washed several

times using fresh medium. The cells were counted using a hemacytometer; typically 50% of the initial number of cells was viable upon thawing.

Keeping each animal separate, the cells were suspended in a growth factor and FBS supplemented expansion medium (see Appendix) and then plated in 75-cm^2 flasks (15 ml of cell suspension/flask) at a concentration of approximately 2 million cells/flask. The cells were incubated at 37°C and 5% CO_2. Once the chondrocytes reached confluence, which was after 8-9 days for the first subculture, they were trypsinized, resuspended and replated into 75-cm^2 flasks at the same concentration. The cells reached confluence (passage 1) after 4-5 days.

2.2.3 Cell Seeding and Culture of Collagen Matrices

After the first passage the chondrocytes were transferred to serum-free medium containing ITS and then dynamically seeded (1.5 hours on a nutator) into scaffolds (2x10^6 cells/scaffold) that had been EDAC cross-linked for 10 min. It has been estimated based on our prior studies that this dynamic seeding method results in approximately 50% of the chondrocytes being attached to the scaffolds, yielding a cell density approximately equal to that in articular cartilage (10,000 cells/mm^3) (Venn and Maroudas 1977). After seeding, the matrices were incubated in 1.5 ml of medium per agarose-coated well in 24-well plates. Medium was exchanged every two to three days.

Half of the scaffolds received FGF-2 (5 ng/ml) with each medium change. Scaffolds were incubated for 28 days and subsequently terminated by placing them in PBS.

2.2.4 Mechanical Testing of Scaffolds

Within 48 hours of sacrifice, the thicknesses of the scaffolds were measured with a custom fabricated micrometer with an LED indicating the jaw-to-jaw distance when an electrical circuit was first formed by the micrometer's two platens' contact with the scaffold. Scaffolds were then placed in a PBS-filled polymethylmethacrylate (PMMA) chamber mounted in the lower jaw of a Dynastat Mechanical Spectrometer (IMASS, Hingham, MA). A 50-gram load cell (Sensotec, Cleveland, OH) fitted with a 9.5-mm diameter PMMA cylindrical plunger was fixed in the upper jaw of the Dynastat and the

distance between the plunger and the lower chamber set to the thickness of the hydrated scaffold.

The scaffolds were then immediately subjected to uniaxial compression and their unconfined stress-strain responses were measured. The Dynastat was then programmed to compress the specimen at a rate of approximately 2.5 μm/sec while force and displacement data were recorded at a sampling rate of 0.1 / second. The compression was terminated at 60% engineering strain ($\Delta L/L_0$). The recorded force/displacement data were converted to engineering stress/strain and plotted in EXCEL. The equilibrium modulus was defined as the slope of the stress-strain data over the 20-50% strain range.

The moduli of unseeded scaffolds after six different durations (n=2-3 for each time) of EDAC treatment (0, 1, 5, 10, 60, and 120 minutes) were measured.

2.2.5 Statistical Analysis

Data from all assays are reported as the mean ± standard deviation. Analysis of variance (ANOVA) and Fisher's protected least squares differences (PLSD) post-hoc testing were performed using StatView (SAS Institute Inc, Cary, NC). The criterion for statistical significance was set at $p = 0.05$.

2.3 Results

2.3.1 Effect of EDAC Cross-linking Time On Modulus of Unseeded Scaffolds

The data (Fig. 1) indicate that scaffold modulus is related exponentially to EDAC treatment time. For the EDAC protocol used in this study, modulus *M(t)* vs. EDAC time was described ($r^2 = 0.74$) by a kinetics equation of the form

$$M(t) = M_o + a*(1-e^{-t/\tau}),$$

where the time constant, $\tau = 6.07$ min., $a = 3.43$ kPa, and the non-EDAC cross-linked stiffness, $M_o = 0.83$ kPa. The average value for the equilibrium modulus of the unseeded scaffolds EDAC cross-linked for 10 min (n=5) was 3.1 kPa with a 95% confidence interval of 2.7-3.5 kPa (data not shown).
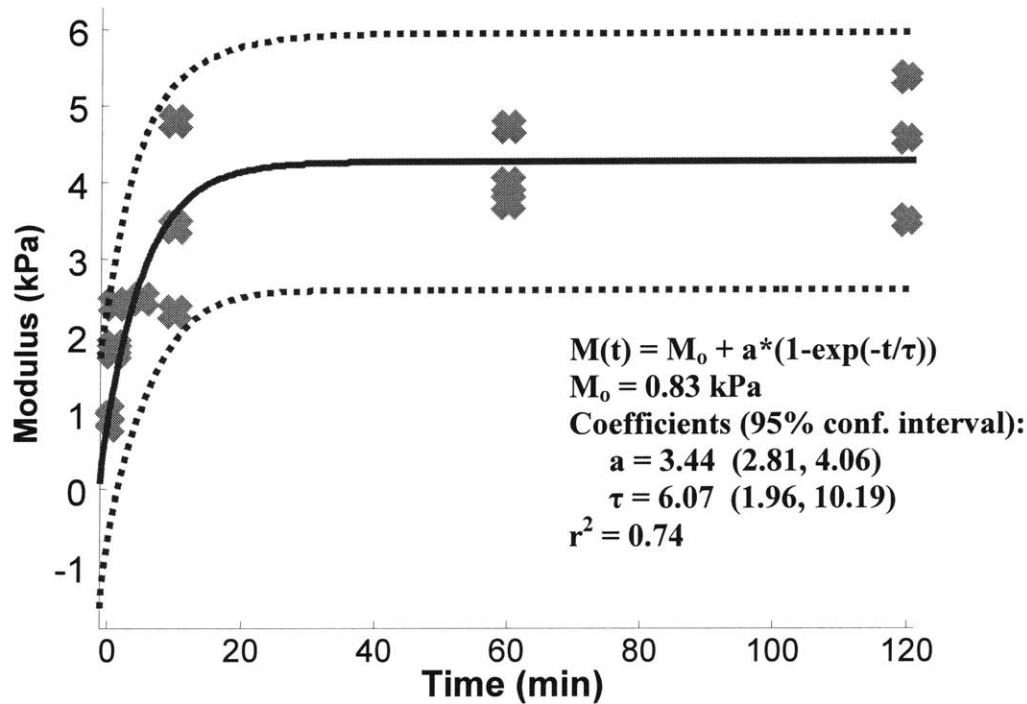
Figure 1. Effects of EDAC cross-linking time on the unconfined compressive modulus of unseeded type II collagen-GAG scaffolds.

2.3.2 Effect of FGF-2 on Modulus of Seeded Scaffolds

The correlation coefficients for the individual linear regressions over the 20-50% strain range were between 0.98 and 1.0; however, there was considerable variation in the modulus of constructs prepared with cells from the different animals. The mean value for the modulus of the cell-seeded constructs cultured in medium without FGF-2 supplementation for 28 days was almost 2-fold higher than the non-seeded controls (EDAC cross-linked for 10 min). However, the high level of inter-animal variation prevented this result from achieving statistical significance (unpaired Student's t test; p=0.09).

Treatment of the constructs with FGF-2 resulted in a statistically significant (paired Student's t test; p=0.02) doubling of the modulus, compared to the non-treated cell-seeded constructs: 17.4 ± 9.0 versus 8.8 ± 5.5 kPa (Fig. 3). A typical stress-strain curve for FGF-treated and control scaffolds from animal B is shown in Fig. 2. The

linear-fit equations are displayed in the figure and the high degree of linearity over this strain range is evident from the high values for the goodness of fit parameter.

During weeks 2 to 4 of culture the coloration of the medium of the non-FGF-treated and FGF-treated groups differed dramatically, reflecting the effects of the growth factor on the metabolism of the cells.
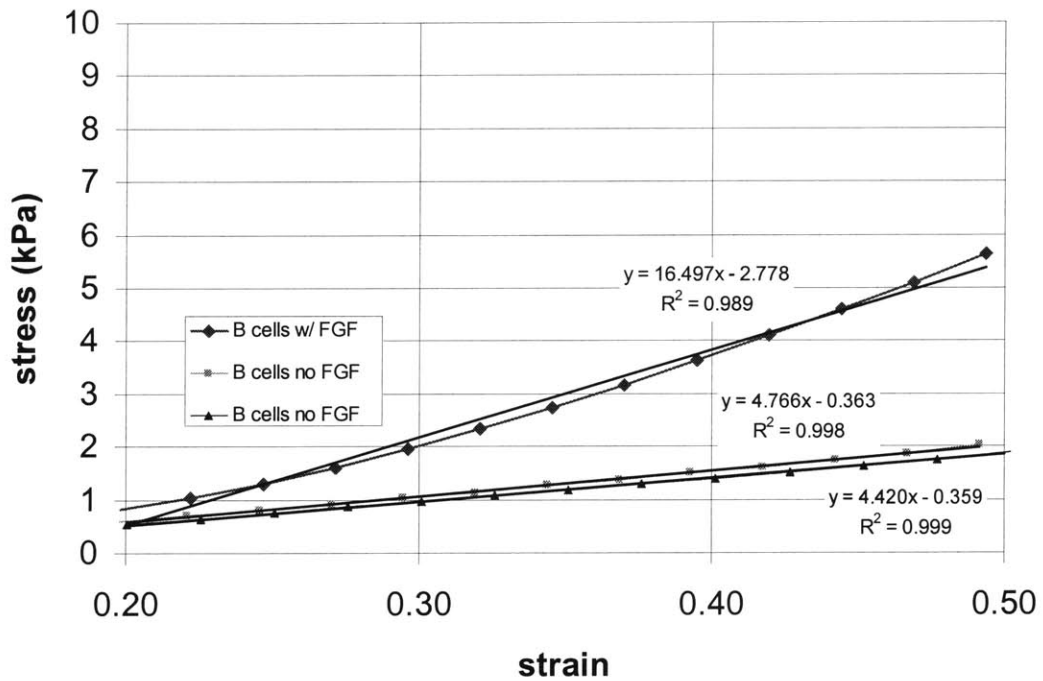
Figure 2. Stress-strain response of cell-seeded (from animal B) collagen-GAG scaffolds, indicating the higher modulus of FGF treated scaffold (upper curve) when compared to scaffolds receiving no FGF (lower 2 curves).
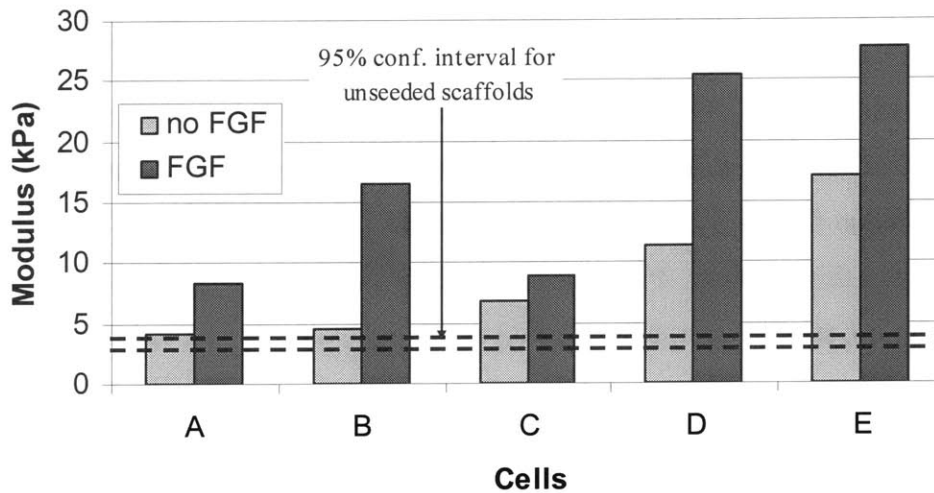
Figure 3. Equilibrium unconfined compressive moduli for constructs prepared using chondrocytes from 5 animals (A-E).

2.4 Discussion

FGF-2 treatment over a 4-week culture period resulted in constructs that were markedly stiffer than untreated scaffolds. The high degree of inter-animal variation was not anticipated, but may be a result of differences in age, incipient osteoarthritis or other degenerative joint conditions, degree of exercise provided to different animals, or other factors.

Since biochemical assays were not performed with these scaffolds, it was not possible to determine the origin of the modulus increase. It is believed that the increased stiffness was due to upregulated ECM production and deposition by the cells. However, it is also possible that the per cell rate of ECM production was not changed but the proliferation of cells was increased, leading to a net increase in ECM deposition. Either of these situations could lead to the observed variation in medium coloration; elevated levels of metabolic byproducts discharged to the medium could be caused by either more cells working at average rates or by fewer cells working at greater rates. Future work will be required to distinguish between these or other possible explanations of the effects of FGF on the stiffness.

The kinetics data for the EDAC cross-linking indicate that 20 minutes of treatment (*i.e.*, approximately 3τ) increased the intrinsic modulus to approximately 95% of the maximum obtainable by this method. Thus, we may have realized slightly stiffer seeded matrices if we had started with matrices that had received 20 minutes of cross-linking rather than 10 minutes. The difference in stiffness would likely have been small.

During EDAC cross-linking the scaffolds tend to trap air bubbles internally, preventing the solution from fully penetrating the scaffold. A pilot-study revealed the following solution to this problem. Place the scaffolds in a solution (either the EDAC cross-linking solution, sterile water, ethanol, etc.) under light vacuum in a vacuum flask. Then very rapidly allow the pressure to rise (by quickly removing the vacuum tube from the flask in a sterile biosafety cabinet). This shock dislodges the bubbles from the scaffolds very effectively.

Future *in vivo* work will be required to determine if FGF-2 treatment can reduce the culture period required before implantation of the constructs and the relationship between the compressive modulus of the construct and treatment outcome.

3. DYNAMIC MECHANICAL STIMULATION OF ARTICULAR CHONDROCYTES IN COLLAGEN-GAG MATRICES AND SERUM-FREE MEDIUM

3.1 Introduction

In addition to biochemical stimulation, it is well-known that mechanical loading is an important regulator of the metabolic processes of chondrocytes *in situ*. During normal physical activities articulating joints are subjected to a wide range of stresses, and sensing the induced strains, the chondrocytes modify their biosynthetic behavior. Static strains reduce the cellular production of proteins and proteoglycans that constitute the ECM. Dynamic strains, however, increase the production of ECM molecules in an amplitude and frequency dependent manner. Thus, it appears that the effects of "exercise" can be traced to cellular and molecular levels. Chondrocytes receiving such mechanical loading produce augmented levels of ECM molecules relative to static conditions, thus strengthening their ability to withstand the strains they experience.

Recent investigations have also shown that dynamic loading can increase chondrocytes' ECM biosynthesis in cell-scaffold systems *in vitro* (Gray, Pizzanelli et al. 1988; Gray, Pizzanelli et al. 1989; Sah, Kim et al. 1989; Larsson, Aspden et al. 1991; Greco, Specchia et al. 1992; Guilak, Meyer et al. 1994; Kim, Sah et al. 1994; Lee and Bader 1997; Bonassar, Grodzinsky et al. 2000; Grodzinsky, Levenston et al. 2000; Lee, Noguchi et al. 2000; Mauck, Soltz et al. 2000; Wilkins, Browning et al. 2000; Bonassar, Grodzinsky et al. 2001; Gooch, Blunk et al. 2001; Mauck, Seyhan et al. 2002; Lee, Grodzinsky et al. 2003; Mauck, Nicoll et al. 2003; Hunter, Mouw et al. 2004). Since the ECM is responsible for the high compressive modulus of cartilage *in vivo*, we hypothesized that increased ECM production within these scaffolds due to dynamic loading would be reflected in increased scaffold stiffness. In this study, chondrocyte-seeded scaffolds were subjected to dynamic compression and the effects of this treatment on the proliferation of the chondrocytes, their synthesis of ECM, and the stiffness of the scaffolds were measured.

3.2 Materials and Methods

3.2.1 Scaffold Fabrication and Cross-linking

Sheets of the scaffold, 3-mm thick, were produced by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland) as previously reported for type I collagen-glycosaminoglycan matrices (Yannas, Lee et al. 1989). Eight-mm diameter samples punched from sheets of the scaffold were cross-linked by dehydrothermal treatment followed by immersion in a solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC) (Olde Damink, Dijkstra et al. 1996). Comparable type II collagen scaffolds were previously (Nehrer, Breinan et al. 1997) found to have an average pore size of 86 μm and 93% porosity. A prior study employing SDS-polyacrylamide gel electrophoresis (Nehrer 1998) confirmed that the scaffolds contained primarily type II collagen. In the prior study there were no significant differences in these properties anticipated among the specimens.

3.2.2 Cell Isolation, Culture and Seeding

Articular cartilage was harvested from the stifle joints of six adult dogs. Chondrocytes were isolated from the cartilage by digesting the tissue first with pronase (20 U/ml, 1hr) and subsequently with collagenase (200 U/ml), overnight, as previously described (Kuettner, Pauli et al. 1982). After isolation, the cells were washed several times in a serum-free base (SFB) culture medium, adapted from a formulation from Jakob (Jakob, Demarteau et al. 2001) (see Appendix) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA). The cells were suspended in and 10% dimethyl sulfoxide (DMSO,) at a concentration of 10^6 cells/ml and stored in liquid nitrogen.

After cells from all six animals had been collected they were thawed by adding drops of SFB medium supplemented with 10% FBS, and the cells were washed several times using fresh medium. The cells were counted using a hemacytometer; typically 50% of the initial number of cells was viable upon thawing.

Keeping each animal separate, the cells were suspended in a growth factor and FBS supplemented expansion medium (see Appendix) and then plated in 75-cm^2 flasks (15 ml of cell suspension/flask) at a concentration of approximately 2 million cells/flask. The cells were incubated at 37°C and 5% CO_2. Once the chondrocytes reached confluence, which was after 8-9 days for the first subculture, they were trypsinized, resuspended and replated into 75-cm^2 flasks at the same concentration. The cells reached confluence (passage 1) after 4-5 days.

After the first passage the chondrocytes were transferred to serum-free medium containing ITS and then dynamically seeded into scaffolds (2×10^6 cells/scaffold) that had been EDAC cross-linked for 10 min. Medium (1.5 ml medium/scaffold) was changed every 2-3 days. Scaffolds were incubated for 19 days and subsequently terminated by placing them in PBS.

3.2.3 Dynamic Mechanical Compression

The thicknesses of the scaffolds were measured with a custom fabricated micrometer with an LED indicating the jaw-to-jaw distance when an electrical circuit was first formed by the micrometer's two platens' contact with the scaffold. Scaffolds were placed in polysulfone chambers with 0.6 ml of radiolabeled serum-free medium and transferred to an incubator-housed mechanical spectrometer. The platens of the loading chamber were individually adjusted so that they would just touch the surface of each scaffold. Scaffolds were subjected to 24 or 96 hours of 0.1 Hz, 3% sinusoidal compressive strain superimposed on a 10% offset strain (10%/60 sec) (Frank, Jin et al. 2000; Lee, Grodzinsky et al. 2003).

Since all the scaffolds were compressed by the spectrometer the same absolute distance, only scaffolds of exactly the same thickness would experience identical strains. Although the tested scaffolds were not identical in thickness, individual scaffolds did not typically differ by more than 10% from the average thickness. Thus, the dynamic and offset strains were based on the average thickness of all the scaffolds in the polysulfone loading chambers.

3.2.4 DNA Analysis

The DNA content of the matrices was measured using the Hoechst 33258 dye method (Kim, Sah et al. 1988). A 20 μl aliquot of the proteinase K digest mixed with 180 μl of Hoechst dye solution (10% Hoechst dye in 10 mM Tris, 1mM Na_2EDTA and 0.1 M NaCl, pH 7.4) was assayed fluorometrically. The results were extrapolated from a standard curve established using calf thymus DNA. The DNA contents of unseeded matrices were measured as controls, and subtracted from values obtained for the cell-seeded samples.

3.2.5 GAG Analysis

The GAG content of the matrices was determined by the dimethylmethylene blue (DMMB) dye assay (Farndale, Sayers et al. 1982). A 100 μl aliquot of the proteinase K digest was mixed with 3 ml of the DMMB dye and the absorbance at 535 nm was measured with a spectrophotometer (Ultrospec 4050, LKB Biochrom, Cambridge, England). The GAG content of the matrices was derived from comparisons with a standard curve produced from measurements of shark chondroitin-6-sulfate standards.

3.2.6 Proline and Sulfate Radiolabel Incorporation to Evaluate Protein and Glycosaminoglycan Synthesis Rates

After 18-19 days of culture in serum-free medium the cell-seeded matrices were transferred to the polysulfone loading chamber and incubated in medium containing 10 μCi/ml of ^{3}H-proline and 20 μCi/ml of ^{35}S-sulfate during the loading to determine protein (assumed to be primarily type II collagen) and GAG synthesis rates, respectively. At the end of the 24 or 96 hour radiolabeling period, the matrices were washed (5 x 15 min at 4°C) in phosphate buffered saline (PBS) supplemented with unlabeled proline (1.0 mM) and sulfate (0.8 mM).

Scaffolds were lyophilized overnight and then solubilized for at least 24 hours at 60°C with 1 ml of proteinase K solution (100 μg in 1 ml 50 mM Tris-HCl buffer with 1 mM $CaCl_2$). In order to determine the radioactivity content, 200 μl aliquots of the digest were mixed with 2 ml scintillation fluid (CytoScint ES, ICN Biomedicals Inc., Irvine,

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CA) and counted in a liquid scintillation counter (Rack-Beta 1211, LKB, Turku, Finland). The ^{3}H and ^{35}S counts per minute were recorded (channel A recorded activity from 0.5 – 18.6 keV and channel B recorded activity from 18.6 – 156 keV) with corrections for spillover and then converted to nanomoles of incorporated radiolabel. The counting period for each sample was 3 minutes. Quenching effects were assumed to be constant throughout all measurements and were, therefore, not directly treated. Counts were normalized both to DNA content and radiolabeling period.

3.2.7 Mechanical Testing

A random selection of scaffolds was allotted for mechanical testing after the dynamic compression. These scaffolds were not lyophilized or treated with Proteinase-K. Their moduli were determined by 5% sequential ramp (30 seconds) and hold (90 seconds) displacements from 0-55% strain. The sampling time was 0.1 seconds for force and displacement measurements. The data were processed by averaging the last 10 force and displacement data points for each of the 11 ramp sequences. After multiplying by the appropriate factors, the data were transformed into 11 points on a stress vs. strain graph and a line was fitted to these data. The slope of the initial linear region was taken as the modulus.

3.2.8 Statistical Analysis

Data from all assays are reported as the mean ± standard error of the mean (± SEM). Analysis of variance (ANOVA) and Fisher's protected least squares differences (PLSD) post-hoc testing were performed using StatView (SAS Institute Inc, Cary, NC). The criterion for statistical significance was set at $p = 0.05$.

3.3 Results

3.3.1 Radiolabel Incorporation

The power of the ANOVAs comparing the effects of 24 hours of loading on synthesis of proline and sulfate in the scaffolds and the medium were all very low (between 0.05 and 0.297), indicating that these assays were inconclusive. The low power values signify

that the experiment was unable to detect a difference between the dynamically loaded and the free-swelling specimens even if one did in fact exist (type II error). This was due to different factors, but in all cases the differences between the means (experimental vs. control treatments) were small. In some comparisons the variance was also quite large and the sample size small. The net effect is that while trends may be noted, statistically significant conclusions are impossible with the current data set.

The averages of the total newly synthesized proline ($\pm$ SEM) in the media with and without 24 hours of dynamic loading were 26.897 (2.188) and 26.301 (4.532) nmol, respectively ($p = 0.7017$, power $= 0.065$). Average proline levels in the scaffolds were 0.01485 (0.00528) and 0.01474 (0.00207) nmol, respectively ($p = 0.9592$, power $= 0.05$). No proline synthesis data were recorded for the specimens dynamically compressed for 96 hours.

The amounts of sulfate in the loaded specimens and media were elevated, but as with total proline synthesis, these results were not significant. The averages of the total newly synthesized sulfate ($\pm$ SEM) in the media with and without 24 hours of dynamic loading were 227.179 (11.98) and 214.392 (24.194) nmol, respectively ($p = 0.139$, power $= 0.297$). Average sulfate levels in the scaffolds were 0.058 (0.029) and 0.053 (0.013), respectively ($p = 0.6547$, power $= 0.071$). No sulfate synthesis data were recorded for the specimens dynamically compressed for 96 hours.

The rates of biosynthesis (normalized by the radiolabeling period and the mass of DNA) were calculated for the scaffolds with and without loading. Proline synthesis rates were 0.00146 (0.00178) and 0.00109 (0.00144) nmol, respectively ($p = 0.6437$, power $= 0.072$). Sulfate synthesis rates were 0.00594 (0.00793) and 0.00360 (0.00452) nmol, respectively ($p = 0.4875$, power $= 0.100$).

3.3.2 GAG Content

The net GAG contained in the matrices was measured, but again, the data were inconclusive, as indicated by low power values. The averages (Fig. 4) of the total GAG ($\pm$ SEM) in the scaffold with and without 24 hours of loading were 0.387 (0.153) and 0.844 (0.530) µg, respectively ($p = 0.3032$, power $= 0.164$). The averages (Fig. 5) of the

total GAG (± SEM) in the scaffold with and without 96 hours of loading were 0.185 (0.340) and 0.347 (0.101) µg, respectively ($p = 0.6855$, power = 0.066).
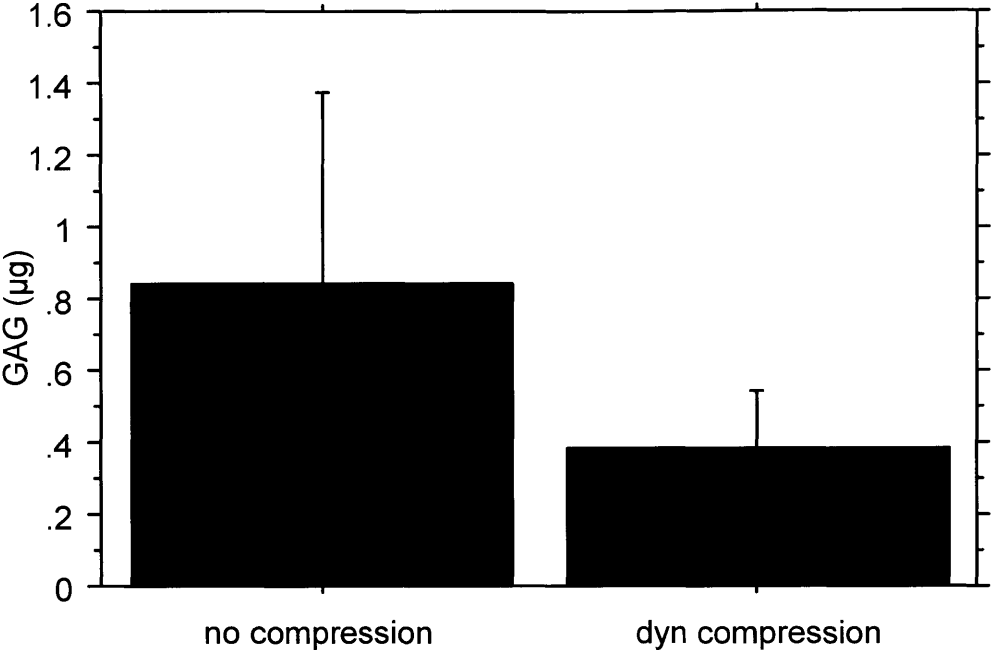
Figure 4. Average (± SEM) net GAG content for seeded matrices dynamically compressed for 24 h is lower than for uncompressed matrices.
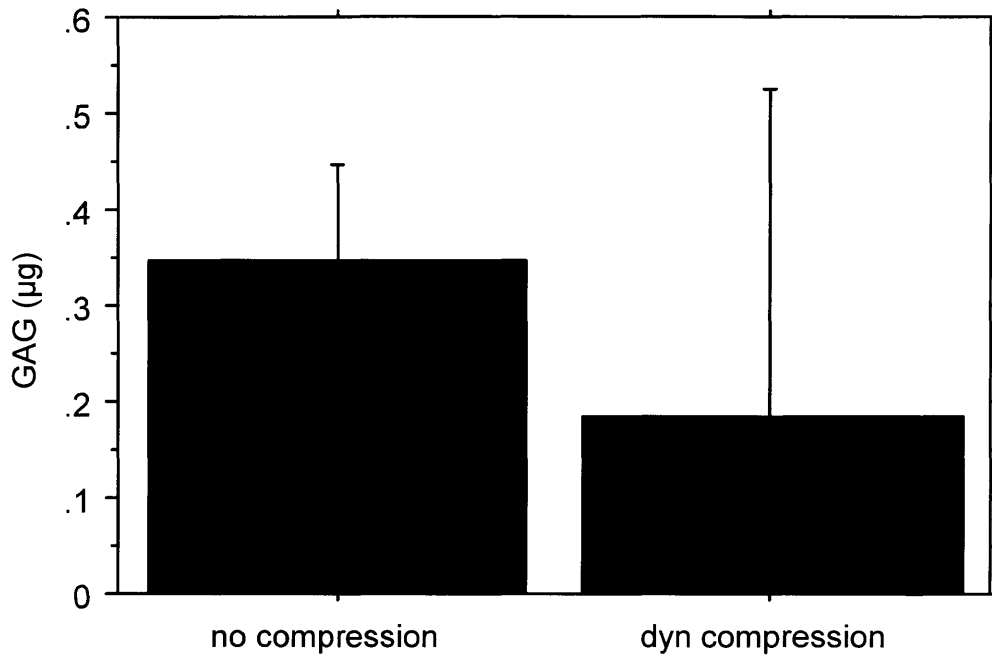
Figure 5. Average (± SEM) net GAG content for seeded matrices dynamically compressed for 96 h is lower than for uncompressed matrices.

3.3.3 DNA Content

The net DNA contained in the matrices was measured, but the data were inconclusive, as indicated by low power values. The averages (Fig. 6) of the total DNA (± SEM) in the scaffold with and without 24 hours of loading were 1.107 (0.270) and 1.423 (0.372) µg, respectively (p = 0.498, power = 0.099). The averages (Fig. 7) of the total DNA (± SEM) in the scaffold with and without 96 hours of loading were 0.227 (0.052) and 0.264 (0.028) µg, respectively (p = 0.573, power = 0.081).
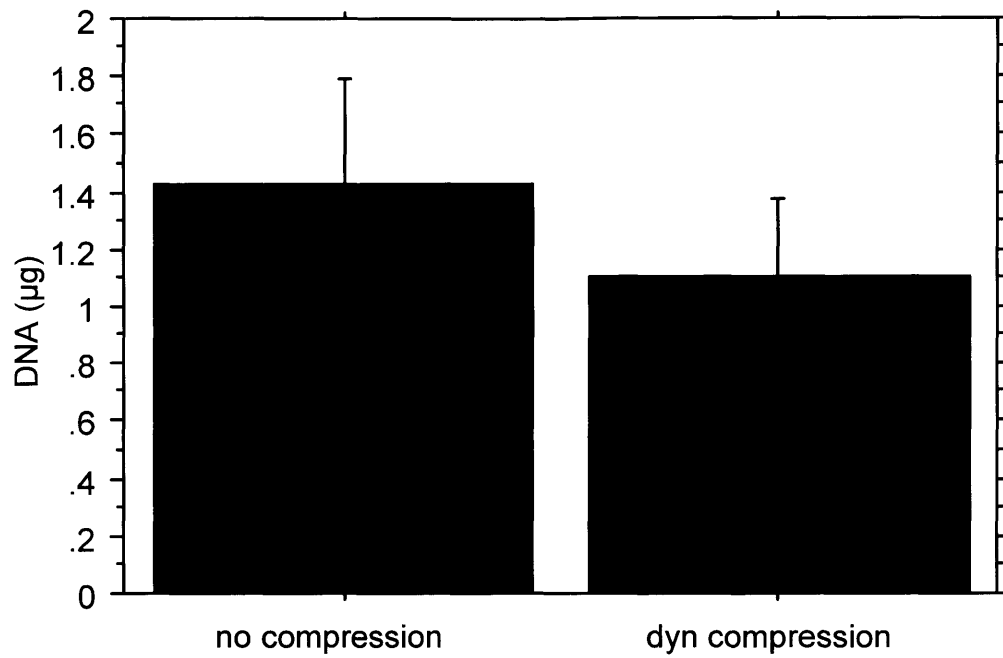
Figure 6. Average (± SEM) net DNA content for seeded matrices dynamically compressed for 24 h is lower than for uncompressed matrices.
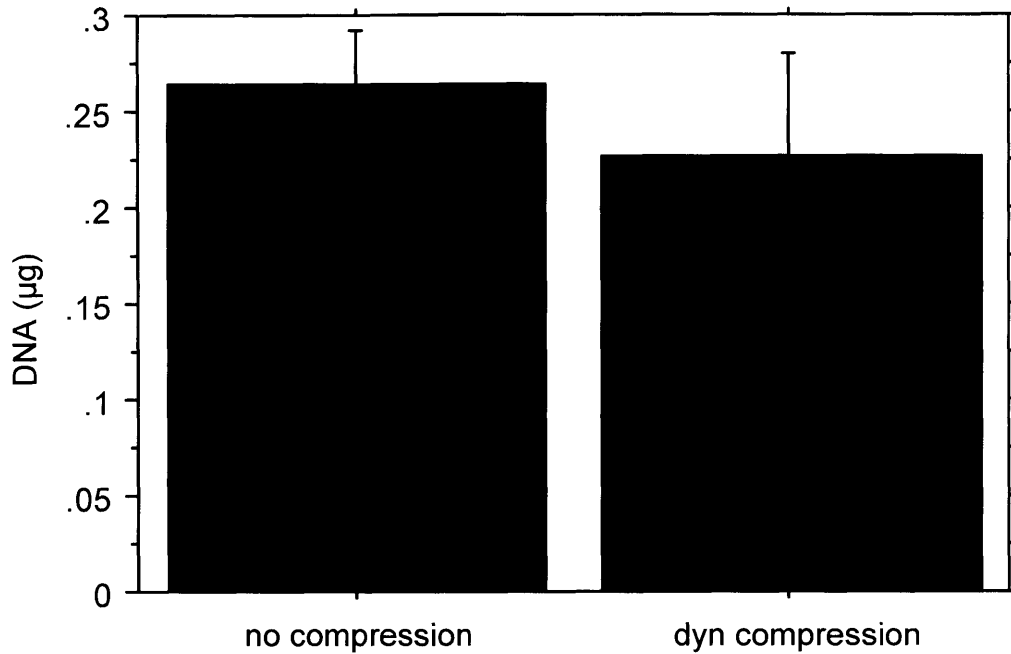
Figure 7. Average (± SEM) net DNA content for seeded matrices dynamically compressed for 96 h is lower than for uncompressed matrices.

3.3.4 Modulus Measurements

The moduli of dynamically compressed (96 hours) cell seeded matrices were compared to free-swelling specimens and to unseeded matrices. The average (± SEM) moduli for unseeded matrices, seeded without dynamic compression, and seeded with dynamic compression were 2.147 (0.310), 2.245 (0.105), 2.823 (0.130), respectively. These data (Fig. 8) were inconclusive ($p = 0.0522$, power = 0.322).

In light of the low statistical power of these comparisons, any conclusions regarding the modulus data are likely to be spurious. Nonetheless, the post-hoc analysis was performed for completeness. The results suggest that the dynamically compressed

scaffolds may have a greater modulus than the unseeded scaffolds (p = 0.0776). The other comparisons were not meaningful.
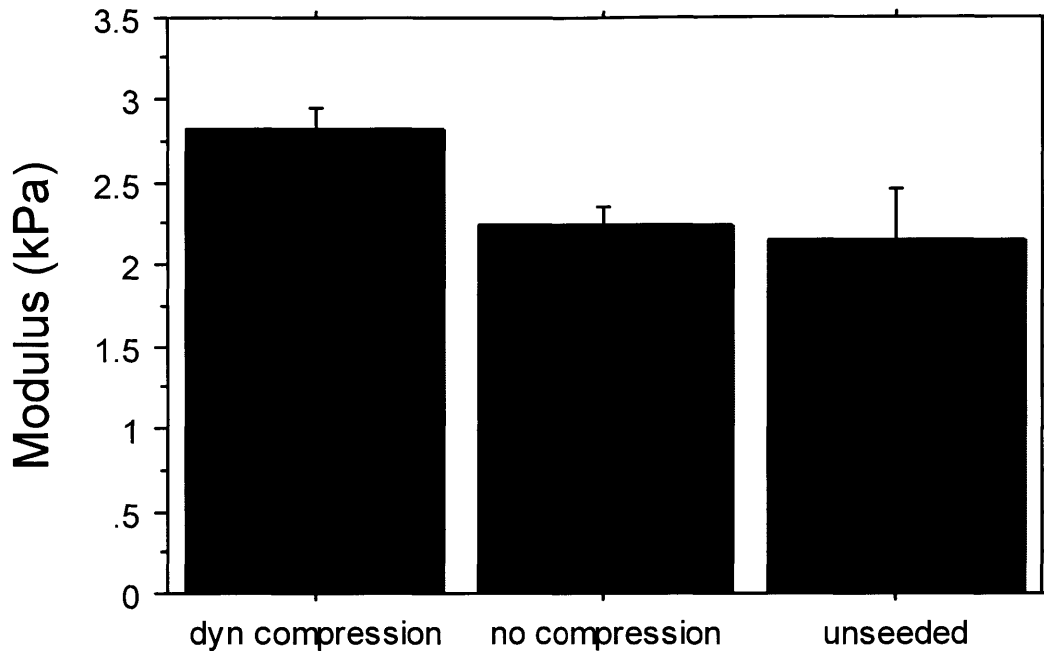
Figure 8. Comparison of moduli from unseeded scaffolds, free-swelling cell-seeded scaffolds, and dynamically compressed (96 h) cell-seeded scaffolds (n=3, n=2, n=3).

3.4 Discussion

The matrices used in this study were fabricated in a previously untested set of polysulfone molds. Preliminary pore-size analysis performed *after* the experiments described in this chapter suggests that the diameter of the pores of the matrices used in these experiments was much larger (300-400 μm) than the original estimate (90 μm). Although it was not anticipated at the outset of the experiment, it is now believed that differences in the geometry and material of the molds used to make the matrices for these experiments had a profound effect on the freeze-drying process. Further work will be required to determine the most important factors, but these may include a retardation of

the heat transfer rate due to increased mold wall-thickness relative to the molds used for scaffold production in the experiments in chapter 2 of this thesis.

Although the origin of the drastic increase in pore diameter is unclear, the effect on cell culture was abundantly clear. The very low levels of biosynthesis, GAG, and DNA present at the conclusion of the culture period confirm the results of other researchers who have found that outside a narrow range of pore diameters the effectiveness of scaffolds decreases dramatically (Yannas 2001). During cell culture there was no change observed in the medium coloration – another indicator of low metabolic activity. Thus, the efforts of these experiments are believed to have been thwarted by a scaffold microstructure that was incapable of supporting and promoting a large and active chondrocyte population. While it is difficult to make further conclusions based on experiments with a biologically inactive matrix, the GAG and DNA levels within the scaffolds appeared to decrease substantially from 24 to 96 hours of dynamic compression. This observation is supported by numerous other studies which found that in compressing the scaffolds, the DNA and GAG were expelled to the medium. For longer compression periods a corresponding greater amount of DNA and GAG should be expelled to the medium.

Radiolabel analysis indicated that the vast majority of newly synthesized protein and GAG was found in the medium and the levels retained in the matrices were essentially zero.

In addition to the unwitting use of biologically inactive matrices, another reason for the inconclusive results is that the procedure for dynamic loading was not optimized. The extreme compliance of the matrices made it difficult to accurately determine their thickness without compressing them. Variations in the thickness resulted in different applied strains during the dynamic loading. In some of the early data the individual polysulfone platens would stick, and it is not clear that they were in contact with the matrices during the entire loading cycle. This was later corrected, but the experiment should be repeated in order to produce a consistent data set.

The modulus data were inconclusive for several reasons. First, several of the samples were infected by the time they were removed. This was due to the fact that the polysulfone chamber was not designed to maintain sterility. Previous use of this fixture

never exceeded 24 hour compression periods, so sterility was not an issue. Second, a significant amount of the medium in the polysulfone chambers evaporated during the 96 hour compression period. Again, this was not anticipated since the fixture had never been used for such an extended period.

In addition to the EDAC treated scaffolds, earlier experiments were initiated using DHT treated matrices. It was not possible to collect data from these specimens because during culture the matrices' mechanical properties degraded drastically. Some matrices dissolved in the media and others assumed the consistency of a highly viscous liquid rather than a solid capable of sustaining compression. This result was observed in independent experiments by other researchers in the same lab, but the reason is unclear.

4. MECHANICAL PROPERTIES OF ORTHOSS BONE SCAFFOLD IN UNIAXIAL COMPRESSION

4.1 Introduction

Many different materials and processes for the production of osteo- and osteochondral implants are found in the literature. These include nanofibers, poly-D,L-lactide (PDLLA), poly(lactide-co-glycolide) (PLGA), bioglass®, poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), poly(α-hydroxyesters), collagen and chitin. Most engineered bone scaffold systems contain at least some hydroxyapatite (HAP) – the primary constituent of normal bone and teeth.

Chemical composition, porosity and mechanical properties are interdependent parameters. Thus, changing the porosity of the scaffold, for example, will affect its mechanical properties. It has often been suggested in the literature that a suitable scaffold should be biomimetic; that its properties should closely approximate those found *in vivo*. In the case of bone tissue, it has been shown that the introduction to a defect site of materials which are either much stiffer than native bone or much more compliant will produce negative results. In the former case, stress shielding and subsequent bone resorption will occur in the surrounding tissue; while in the latter case, fibrous tissue will form instead of bone.

In one HAP scaffold study researchers found that the compressive strength and compressive modulus of their 40% porous scaffolds were 30+/-8 MPa and 1.4+/-0.4 GPa (Chu, Orton et al. 2002). However, the scaffolds tested in those experiments had a highly ordered and closed-cell microstructure, making it difficult to directly compare or even to scale between that material and scaffolds such as Orthoss with a more random and open-cell architecture.

For scaffolds similar in microstructure to Orthoss, the elastic modulus and modulus of rupture (related to the ultimate compressive strength) should scale with the

ratio of their densities. (Relative density is equal to 1 – % porosity.) Elastic moduli and moduli of rupture are related as

$$E^* = E_s \left(\frac{\rho^*}{\rho_s} \right)^2$$

$$\sigma^* = 0.2\sigma_s \left(\frac{\rho^*}{\rho_s} \right)^{3/2}$$

where the subscript denotes the properties of the solid (non-porous) material and the superscript asterisk denotes the porous properties.

From these relations it should be possible to compare the mechanical properties of Orthoss found in the current work with the properties of other scaffolds as reported in the literature. Lin et al. used a poly (L-lactide-co-DL-lactide) scaffold to obtain structures with 99% interconnectivity of the scaffold porosity and six different porosity levels (between 58.3% and 80.3%). The scaffold with maximum porosity had an average compressive modulus and ultimate strength of 43.5 MPa and 2.7 MPa, respectively (Lin, Barrows et al. 2003). The literature supplied by the manufacturer of the scaffolds used in the current study claim that they are 60% porous – similar to that of the scaffolds used in the study by Lin et al.

4.2 Methods

4.2.1 Scaffold Preparation

Porous hydroxyapatite bone scaffold was obtained (Geistlich, Switzerland) and tested in uniaxial unconfined compression. Five batches of Orthoss© scaffold, differing only in chemical composition, were tested. The irregularly shaped samples were machined to cubes of appropriate size for testing in the Dynastat. The as received specimens were affixed to a precision-machined aluminum block with a small volume of epoxy. The block was used both for holding the sample during the milling process and as a reference surface. The irregularly shaped specimens were then milled to cubes

approximately 6 mm on each edge. Care was taken to remove all the epoxy from the specimen during the milling process. Any traces of epoxy that remained on the specimen were restricted to a single face of the specimen and this face was positioned on the bottom during the testing. Thus, if any epoxy remained in the scaffold after machining, the reinforced face was not placed parallel to the direction of loading, where the measurements could have been affected by the mechanical properties of the epoxy rather than of the scaffold.
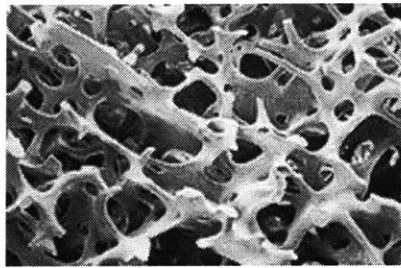
Figure 9. SEM of Orthoss scaffold (40x) from http://www.geistlich.com/biomaterials/en/ortho/index.html.

4.2.2 Dynastat Compression Protocol

After measuring the cross-sectional area with a micrometer, the dry scaffold specimen was placed between two stainless steel platens mounted in the Dynastat Mechanical Spectrometer (IMASS, Hingham, MA) at a separation of 10 mm. A 10 kg load cell (Sensotec, Cleveland, OH) was fixed in the stationary upper platen and directly coupled to the Dynastat.

The thickness of the specimen was measured by raising the Dynastat's bottom platen (approximately 25 µm/sec) until the load cell measured a 30 gram compressive force; the distance between the platens at this nominal load was taken as the thickness of the specimen.

The Dynastat was then programmed to compress the specimen at a rate of approximately 2.5 µm/sec while force and displacement data were recorded at a sampling rate of 0.1 / second. The 600 second compression was terminated at 25% engineering strain ($\Delta L/L_0$). The recorded force/displacement data were converted to engineering stress/strain and plotted in EXCEL.

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4.2.3 Analysis of Dynastat Data

The linear elastic region was estimated visually from the stress-strain curve. Typically, the linear region began after approximately 0.5-2.0% strain. This initial non-linear region was assumed to be due to slight irregularities in the specimens' shape. The linear region usually extended an additional 0.5-2.0%. A trend line was fitted to the linear region from which the modulus (i.e., slope) and coefficient of determination were recorded. In addition the ultimate compressive strength was noted. In several instances it was not possible to measure an exact value for the ultimate compressive strength since it exceeded the load cell's capacity. For such specimens the highest stress achieved before exceeding the Dynastat's capacity was recorded.

4.2.4 Statistical Analysis

Data from all assays are reported as the mean ($\pm$ SEM). Analysis of variance (ANOVA) and Fisher's protected least squares differences (PLSD) post-hoc testing were performed using StatView (SAS Institute Inc, Cary, NC). The criterion for statistical significance was set at $p = 0.05$.

4.3 Results

The modulus, the coefficient of determination for the modulus, and the ultimate strength values are listed in table 1 for all of the specimens tested. There was considerable scatter in the data for both the ultimate strength and the modulus. Strength measurements were further complicated by the fact that almost a third of the trials were aborted before the ultimate strength was achieved due to loads exceeding the Dynastat's limits.

Batch	Sample	Modulus (kPa)	r^2	Ultimate Strength (kPa)
1332	1	82.1	0.9803	~~1450~~
	2	44.7	0.9770	750
	3	41.8	0.9709	500
	4	17.6	0.9666	450
	5	20.7	0.9739	450

	6	~~184.3~~	0.9683	>1700
6993	1	47.5	0.9637	800
	2	90.2	0.9554	>1050
	3	31.3	0.9249	500
	4	58.7	0.9785	1100
	5	121.5	0.9836	~~1950~~
	6	27.4	0.9519	550
7093	1	102.6	0.9889	1550
	2	109.0	0.9850	>1600
	3	149.2	0.9310	>1600
	4	85.1	0.9867	>1600
	5	72.0	0.9602	850
	6	113.6	0.9752	1500
	7	68.0	0.9050	800
	8	59.8	0.9412	800
7193	1	345.7	0.9554	>3400
	2	181.9	0.9674	2400
	3	75.7	0.9314	~~900~~
	4	298.4	0.9349	>2300
	5	177.1	0.9722	2200
	6	221.9	0.9721	>2400
	7	198.9	0.9670	>2000
7293	1	59.4	0.9189	900
	2	72.8	0.9620	950
	3	97.5	0.9757	>2200
	4	123.6	0.9587	>2500
	5	92.7	0.9724	1300
	6	88.8	0.9483	1100
	7	130.6	0.9705	1300
	8	~~290.9~~	0.9655	>2500

Table 1. Modulus, coefficient of determination of modulus and ultimate strength values for non-hydrated Orthoss samples tested in uniaxial, unconfined compression. Values with the strikethrough symbol were deemed outliers according to the analysis described in the Results. Values with the "greater than" symbol are the highest recorded values before the load cell limits were exceeded.

Two different statistical approaches to data analysis were undertaken. In the first approach, it was assumed that there were no outliers and all the data were included in the ANOVA and post hoc tests. In the second approach, outliers (determined as described in section 4.3.2) were culled from the data prior to performing the analyses.

4.3.1 Analysis of Entire Data Set

It was first assumed that all data were meaningful, and no outliers were removed from the values. For those specimens whose ultimate strength could not be precisely determined due to the limitations of the load cell, the highest recorded values for the stress were taken as the ultimate strength; these values are preceded by the ">" symbol in Table 1. The results of the strength measurements are indicated in the interaction bar plot in Fig. 10, and the box plot in Fig. 11 gives some measure of the degree of scatter in the data. The results of the modulus measurements appear in Fig. 12, and the box plot in Fig. 13 indicates the scatter in the modulus data.

ANOVA indicated both ultimate strength and modulus were significantly affected by Orthoss batch (Fig. 10, $p < 0.0019$, power = 0.959 and Fig. 12, $p < 0.0006$, power = 0.986). Batch 7193 was significantly stiffer and stronger than all other batches. There were no other statistically significant differences in the modulus measurements, although the trend of the data suggests 1332 possessed the lowest modulus. A similar trend was observed for the ultimate strengths. Batch 1332 had a lower ultimate strength ($p = 0.0345$) than 7293, but 1332 did not differ significantly in strength from 6993 or 7093.

Figure 10. Average (± SEM) values for ultimate compressive strength of non-hydrated Orthoss specimens. Outliers are not removed.

Figure 11. Box plot for ultimate compressive strength of non-hydrated Orthoss specimens. Outliers are not removed. The middle horizontal line is the median; the upper and lower ends of the notches are the 95% confidence limit about the median; the upper and lower horizontal ends of the boxes are the 75th and 25th percentile of observations about the median; and all dots beyond the extended lines represent observations that are either in the top or bottom 10 percent.
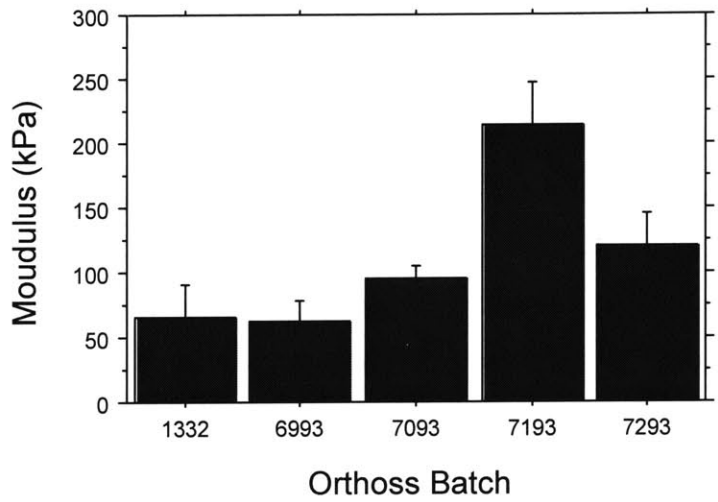
Figure 12. Average (± SEM) values for compressive moduli of non-hydrated Orthoss specimens. Outliers are not removed.
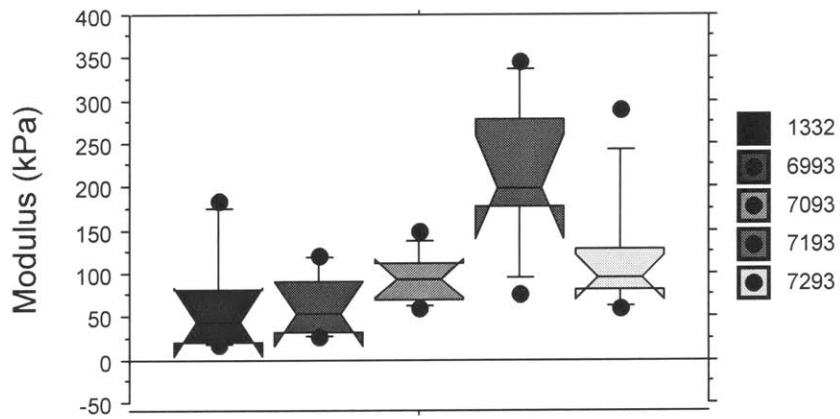
Figure 13. Box plot for compressive moduli of non-hydrated Orthoss specimens. Outliers are not removed.

4.3.2 Elimination of Outliers from Data

A simple statistical test was used to minimize the impact of outliers on the results. To determine whether a particular value should be considered an outlier, the average and standard deviation were calculated for all the measurements of the same type (i.e., modulus or compressive strength) and from the same batch (i.e., 1332, 6993, 7093, 7193, and 7293) *excluding* the measurement in question. Then the value in question was compared to the average value for that batch, and if it differed from the average by more than three times the standard deviation, it was considered an outlier. The outliers are indicated in the table by the strikethrough symbol superimposed on the value. For example, in batch 1332 the modulus of sample 6 is an outlier since the average ($\pm$ standard deviation) modulus value for samples 1-5 was 41.4 ($\pm$ 25.8) kPa, and as shown below this average was greater than three sigma:

$$\text{value} - \text{average value} >? \ 3\sigma$$

$$184.3 - 41.4 = 142.9 > 3 \ x \ 25.8$$

Therefore, only modulus values from samples 1-5 for batch 1332 were used in the ANOVA and post hoc testing.

It is important to note regarding the analysis of the ultimate strengths that those values that are preceded by the ">" symbol were ignored in the calculation of the average and standard deviation. Thus, for example in batch 7193, the determination of outliers was severely limited by the fact that four out of the seven data points were ignored since no final values were recorded. After ignoring those four data points, the statistical test indicated that the low data point was an outlier. Therefore, only two data points for 7193 were actually used in the inter-batch statistical comparison. The fact that the ultimate strength values that were ignored for 7193 were all much higher than the 900 kPa value substantiates the conclusion that this value is in fact abnormally low.

After the removal of outliers ANOVA indicated both ultimate strength and modulus were significantly affected by Orthoss batch (Figs. 14 and 16, $p < 0.0001$, power = 1.000). Batch 7193 was significantly stiffer and stronger than all other batches ($p <$

0.0001). Although 1332 appeared to possess the lowest modulus ($p = 0.0626$ for 1332, 7093 and $p = 0.0687$ for 1332, 7293), these results did not achieve statistical significance. However, 1332 did have the lowest ultimate strength ($p = 0.0065$ for 1332, 7093 and $p = 0.0058$ for 1332, 7293).
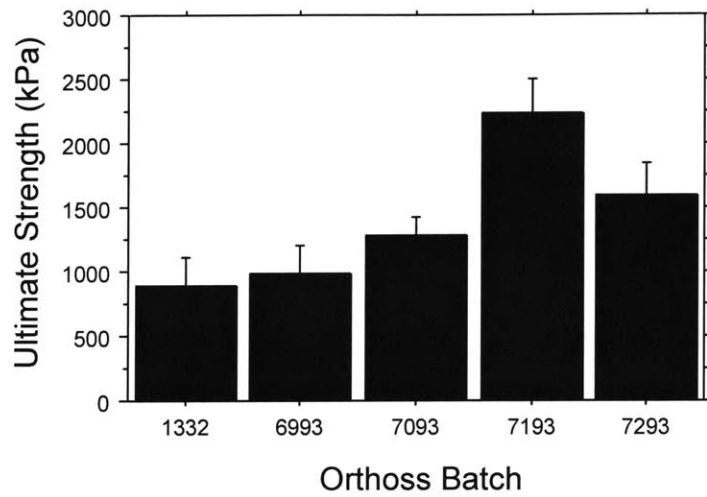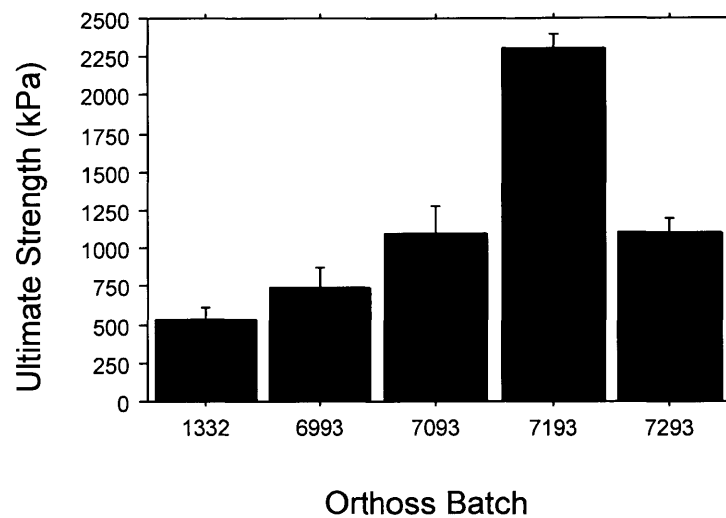

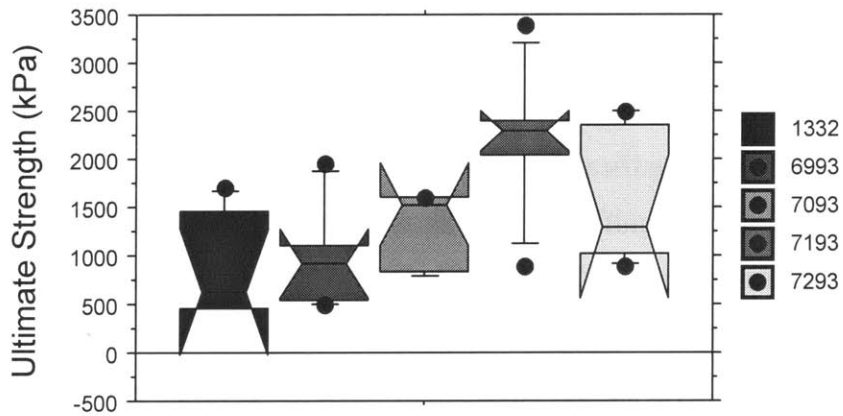**Figure 14.** Average ($\pm$ SEM) values for ultimate compressive strength of non-hydrated Orthoss specimens. Outliers (greater than 3σ, see description above) are not included.
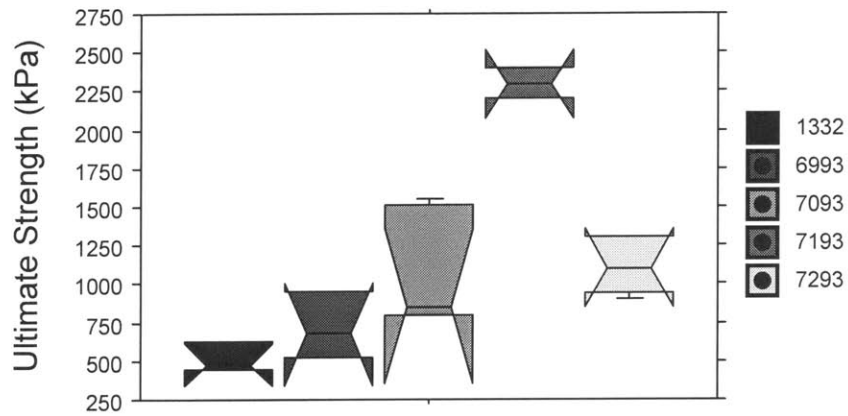
Figure 15. Box plot for ultimate compressive strength of non-hydrated Orthoss specimens. Outliers (greater than 3σ, see description above) are not included.
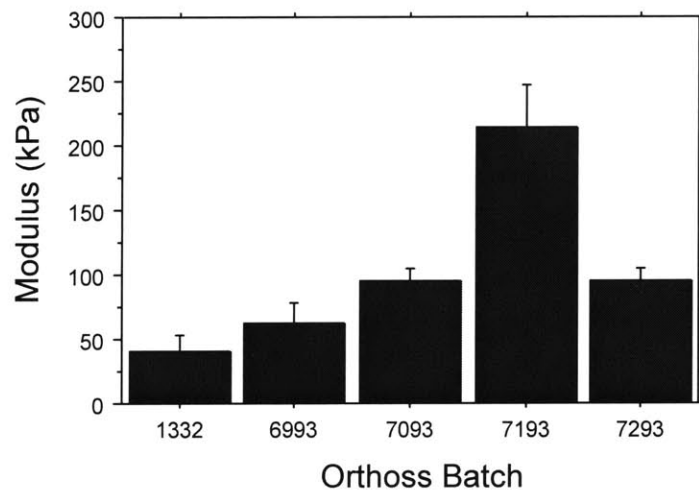
Figure 16. Average (± SEM) values for compressive moduli of non-hydrated Orthoss specimens. Outliers (greater than 3σ, see description above) are not included.
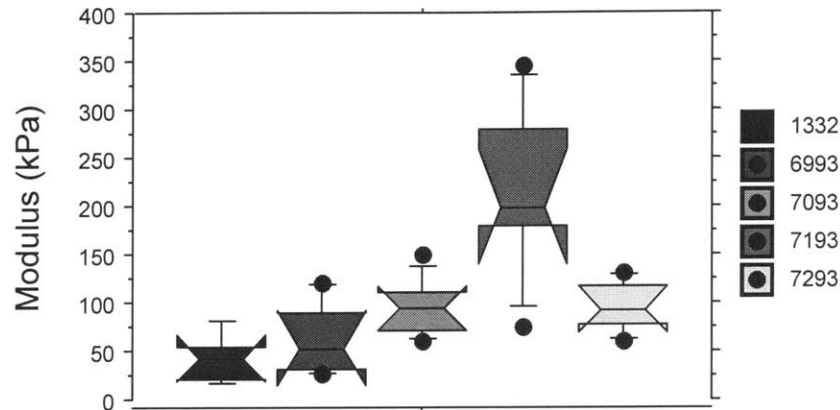
Figure 17. Box plot for compressive moduli of non-hydrated Orthoss specimens. Outliers (greater than 3σ, see description above) are not included.

4.4 Discussion

The high values for the coefficient of determination (0.91 – 0.99) confirmed that the chosen strain region was highly linear.

The same trends were observed for both strength and modulus; in terms of increasing values 1332 < 6993 < 7093 = 7293 < 7193.

By assigning the ultimate strengths for a number of the specimens to be equal to the highest recorded value prior to overload, we have introduced artifacts to the statistical analysis. First, we have in certain cases artificially lowered the apparent SEM. For example, with batch 7093, we have assumed that three of the specimens had precisely the same value for UTS, namely, 1600 kPa, whereas, it is highly unlikely that the true values are so closely grouped. The effect of this artifact cannot be judged either uniformly conservative or non-conservative. Such a determination will depend on what the other values in the data set happen to be. Considering batch 7093 once again, we find that the SEM of all eight UTS values is 138 kPa, while when the three approximated values are removed from the data set, the SEM is 174 kPa. Thus, in the case of batch 7093 if we remove the three values we are actually increasing the variability within that batch, so

that it is less likely to appear to be significantly different from the other batches. This is because the ANOVA and post hoc tests are sensitive to the variability within and between batches. At the same time as we are increasing the variability (by removing the three approximated values) we are reducing the mean UTS value from 1288 kPa to 1100 kPa. Thus there is no simple method for determining which of the two methods is more conservative.

Fortunately, both methods of analysis yield quite similar results. In fact the only significant difference between the two methods is that when the outliers are removed the UTS for 1332 is not different than that of 7093. Otherwise, the trends are the same, and 7193 is clearly stronger and stiffer according to either method of analysis.

In natural bone, there is a distinct orientation of the porous structure which has a profound effect on the mechanical properties. It is as yet unclear whether the specimens tested in the current study possess similar anisotropy. In these mechanical tests, no attempt was made to orient the specimens with respect to the average axis of the pores.

5. CONCLUSIONS

1. FGF Treatment (chapter 2)

    a. Treating canine articular chondrocyte-seeded type II collagen-GAG scaffolds with 5 ng/ml fibroblast growth factor (FGF-2) does result in higher moduli after 28 days of culture when compared with untreated controls.

2. EDAC Cross-linking Treatment (chapter 2)

    a. EDAC cross-linking increases the modulus of unseeded type II collagen-GAG scaffolds exponentially with time as described ($r^2 = 0.74$) by the kinetics equation

$$M(t) = 0.83 + 3.43*(1\text{-e}^{-t/\,6.07}).$$

The non-EDAC treated scaffold stiffness of 0.83 kPa increased to 3.1 kPa (95% c.i. of 2.7-3.5 kPa) after 10 minutes of EDAC cross-linking.

    b. The kinetics data for the EDAC cross-linking indicate that 18 minutes of treatment (i.e., approximately 3τ) increased the intrinsic modulus to approximately 95% of the maximum obtainable by this method, thus rationalizing this as the optimum cross-linking treatment time.

3. Dynamic Compression (chapter 3)

These experiments were fraught with problems stemming from the unwitting use of scaffolds with oversized pores. The large pore size (along with possible decreased pore interconnectivity) rendered the scaffolds ineffectual in promoting cell proliferation and ECM production.

Definitive conclusions based on the current data are not warranted, but the following observations are noted. GAG and DNA levels within the scaffolds decreased substantially from 24 to 96 hours of dynamic compression, as expected from reports in another similar study (Lee, Grodzinsky et al. 2003) which found that in compressing the scaffolds, the DNA and GAG were expelled to the medium. Radiolabel analysis indicated that essentially all of the newly synthesized protein and GAG was found in the medium. There may have been some increase in the modulus of the dynamically compressed (96 h) matrices, but the low sample size (due to infections with many of the scaffolds, which in turn was due to a loading chamber inadequate for long term compression studies) makes it impossible to be certain.

4. Orthoss Mechanical Properties (chapter 4)

a. Chemical composition does affect the mechanical stiffness of bone scaffold material. Differences in the scaffold composition (the details of which were unavailable at the time of writing) and source (bovine vs. porcine) can be related to both the modulus and the ultimate compressive strength.

b. Both modulus and ultimate strength were significantly affected by Orthoss batch. Batch 7193 (labeled "spongiosa, porcine, loaded with collagen S" by the scaffold manufacturer) was significantly stiffer and stronger than all other batches regardless of which method of statistical analysis was employed. Batch 1332 (no description available from the scaffold manufacturer) had the lowest ultimate strength.

c. Given the impact of both density and trabecular orientation upon the mechanical behavior, it would be useful in the future to correlate mechanical behavior with these parameters.

6. LIMITATIONS AND FUTURE WORK

The work presented in this thesis reveals the need for further efforts in each of the three primary areas of investigation.

1. **FGF treatment (chapter 2)**

   a. Repeat the experiment with a sufficient number of samples to perform histology and biochemical assays (radiolabel, GAG and DNA). This will help to determine the origin of the observed increase in scaffold modulus. Is it due to increased ECM synthesis per cell, cellular proliferation (with unchanged per cell synthesis rates) or something else?

   b. Perform an *in vivo* study of FGF treated scaffolds in order to determine if they reduce the pre-implantation time. Or, equivalently, how do treated and untreated scaffolds (cultured for the same time prior to implantation) compare (histologically, etc.) after a clinically relevant post-implantation period?

2. **Dynamic compression (chapter 3)**

   a. Repeat experiments using scaffolds with a biologically active (~ 90 μm) pore size. Compare results with others who have used collagen-GAG scaffolds (Lee, Grodzinsky et al. 2003) and hydrogel scaffolds (Mauck, Seyhan et al. 2002).

   b. Retain media used during dynamic loading and measure DNA and GAG lost from the scaffolds during loading.

   c. Perform experiments that combine growth factors and dynamic loading to determine whether a synergistic effect can be observed under the culture

conditions employed in this experiment. Compare to work with other scaffold materials (Mauck, Nicoll et al. 2003).

3. Orthoss mechanical properties (chapter 4)

a. Obtain SEMs of Orthoss specimens before and after machining to determine whether microstructural cracks were formed as a result of machining. Determine the effect of such cracks on mechanical properties.

b. Repeat the experiment using Orthoss specimens fabricated to a standard size and shape appropriate for testing in the Dynastat so that no machining of the specimens is required.

c. Repeat the experiments with smaller Orthoss samples, so that the capacity of the load cell is not exceeded.

d. Perform the experiments with Orthoss specimens that have been hydrated in a physiologically relevant solution.

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8. APPENDIX A

This appendix includes only those protocols that have been substantially changed or clarified during the work described in this thesis. Standard protocols are collected in a notebook in the orthopaedic research laboratory.

8.1 Serum-Free Chondrocyte Culture Media

8.1.1 Jakob Base Medium

*Used in both expansion and differentiation media

*Can be made in large batches (500 ml) and stored since it doesn't contain FBS

*All prices as of 10-28-03

1. **500 ml DMEM** (high glucose: 4.5%) w/o L-Glutamin w/ Sodium Pyruvate (GIBCO cat. no. 10313021, $16.40/bottle; this medium is not sold by the case)

2. **5 ml MEM Nonessential Amino Acids** (NEAA) (10mM solution, GIBCO cat. no. 11140 050, $12.20)

3. **5 ml sterilized Hepes Buffer** (1M solution, by GIBCO cat. no. 15630 056)
   - OR can make 1M solution w/ Hepes powder (238.3g/mol) in distilled water (0.2383 g Hepes powder/ml dH_2O—need to **sterile filter**)

4. **5 ml PSG** contains 10000U/ml penicillin, 10000µg/ml streptomycin glutamate (GIBCO, cat. no. 10378 016, $19.65/100 ml bottle)

8.1.2 Expansion Medium

*ADD growth factors at the last minute before use

*Avoid repeated freeze-thaw cycles for all growth factors

1. **515ml of Jakob base media**

2. **50 ml heat inactivated FBS** or ~ 0.1 ml FBS/ml of Jakob base (by GIBCO, cat. no. 16000 036, $81.44)

3. **TGFβ1— 1μl TGFβ1 stock/ml media**   (1 ng TGFβ1/ml medium)
   - Stock   (1 μg TGFβ1/ml buffer)
     - Buffer
       - Combine 2.5mg bovine serum albumin (BSA) + 0.1ml 0.1N HCl** + 2.4ml H_2O in 5ml centrifuge tube and vortex
         Calculation: [(0.1ml of HCl) / (2.5 ml HCl+H_2O)] x 0.1 moles HCl/liter = 4mM
     - Sterile filter 1ml of the buffer and add 1μg of TGFβ1
   - Aliquot 100μl stock/sterile 0.5ml eppendorf tube and store @ -70°C for three months. TGFβ1 from R&D Systems, cat. no. 240-B-002 (for 2μg @ $245), cat. no. 240-B-010 (10 μg @ $805)

   **We have "HCl plus" that is 12.1N HCl, so add 0.5ml of 12.1N HCl + 60.5ml H_2O to get 0.1N HCl

4. **FGF-2 — 0.5μl FGF-2 stock/ml media**   (5 ng FGF/ml medium)
   - Stock   (10 μg FGF/ml buffer)
     - Buffer
       - Combine 0.0025g BSA + 0.0004g DTT + 2.5 ml PBS in 5ml centrifuge tube and vortex
     - Sterile filter w/ syringe, and add to 25 μg of FGF-2
   - Aliquot 100μl stock/sterile 0.5ml eppendorf tube and store @ -70°C for three months. FGF-2 from R&D Systems, cat. no. 233-FB-025 ($175/25μg)

5. **PDGFββ— 1μl PDGF stock/ml media**   (10 ng PDGF/ml media)
   - Stock   (10 μg PDGF/ml
     buffer)
     - Buffer
       - Combine 2.5mg BSA + 0.1ml 0.1N HCl + 2.4ml H2O in 5ml
         centrifuge tube and vortex
     - Sterile filter 1ml of the buffer and add to 10μg of PDGFββ
   - Aliquot 100μl stock/sterile 0.5ml eppendorf tube and store @ -70°C for three
     months. PDGFββ from R&D Systems, cat. no. 220-BB-010 ($240/10μg)

8.1.3 Differentiation Medium

*ADD supplements at the last minute before use:

1. **5Medium for differentiation:**
2. **0015 ml Jakob base medium**
DMEM
3. **5 ml MEM Nonessential Amino Acids (NEAA)**
4. **5 ml Hepes Buffer**
5. **5 ml PSG (penicillin, streptomycin and glutamate)**
6.
7. *--Above is the Jakob base media*
8.
9. **5 ml ITS +1** Liquid Media Supplement (100X), by SIGMA, cat. I2521

10. **5 ml <u>STERILE</u> human Serum Albumin solution** (can useor BSA if not using
    human cells—Atala protocol says to use 1.25mg BSA/ml of media) <u>**MUST
    STERILE FILTER**</u>

11. **FGF-2 — if desired**

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12. --Add ITS and BSA in correct amounts based on how much base media used (ie. 5ml per 515ml of Jakob base media

13.

14. **TGFβ1 — 10μl TGFβ1 stock/ml media**   **(10** ng TGFβ1/ml

media)

> ***!!Note that this is 10x the amount used in the expansion medium!!***

15. **Dexamethasone (DM) — 10μl working solution/ml media**   (100 nM)

- Dexamethasone (100 nM) water soluble (~65mg dexamethasone/gram of powder—MW of dexamethasone = 392.5g/mol), by Sigma

- →10□m of a 1/100 dilution of 10-3 M dexamethasone stock solution

- per ml of media

- Make 10-3 M dexamethasone (DM) stock in 100% ethanol (stable for 1 yr, stored @ -20°C) (Calc: 1ml 10-3 M DM x 0.001mol DM/1000ml x 392.5g DM/1mol DM x 1g powder/ 0.065g DM = 0.00603g powder -- in 1ml of ethanol)

- Working solution: Make 10^{-5} M DM

    - 20μl of 10^{-3} M DM stock + 1.98 ml **LG-DMEM** (low glucose!!)

- Stock: 10^{-3} M DM in 100% ethanol

    - 6.03mg of DM powder per 1ml of 100% ethanol

        Calculation: 1ml 10^{-3} M DM x 0.001mol DM/1000ml x 392.5g DM/1mol DM x 1g powder/ 0.065g DM = 0.00603g powder -- in 1ml of ethanol

- 10^{-5} M dexamethasone in low glucose DMEM (LG) by adding 20□l of 10^{-3} M dexamethasone stock + 1.98 ml LG-DMEM

- Store in 100μl aliquots @ -20°C (stable for 1 yr)

    ~65mg DM/gram of powder
    molec. wt. of DM = 392.5g/mol
    Sigma

- Do not freeze/thaw cycle

6. **L-Ascorbic acid 2-Phosphate — 10μl stock/ml media**   (0.1 mM)

- → Stock: 37.5 mg ascorbate 2-phosphate + 10 ml Tyrodes solution

- See Tyrodes salt preparation below
- Sterile filter the solution and store frozen (-20°C) in 2ml aliquots
- It is ok to freeze/thaw cycle

molec. wt. of 10□m of a 1/100 dilution of ascorbate 2-phosphate = 289.54g/mol

Add 37.5 mg ascorbate 2-phosphate + 10 ml Tyrodes solution

♦ Tyrodes solution is made from Tyrodes salts and prepared following the instructions that camame w/ the Tyrodes bottle. For preparation of 100ml of Tyrodes solution:

- Add 1g of Tyrodes salt + 90ml of dH_2O (15-20°C)—gently stir the water while adding salt until completely dissolved.
- Add 0.1g sodium bicarbonate or 1.33 ml of sodium bicarbonate solution (7.5%w/v) for each liter of final volume of medium being prepared (i.e. 0.01g sodium bicarbonate for 100ml of Tyrodes solution). Stir until dissolved.
- While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH (8.0) since it may rise during filtration (desired pH is 8.0) using 1N HCl or 1N NaOH.
- Add additional water to bring the solution to the final volume (i.e., 100ml).
- Sterilize immediately using a 0.22 μm filter.
- Double check pH of the solution each time solution is used.
- Store the dry powdered salts at 2-8°C under dry conditions and liquid medium at 2-8°C in the dark.
- * *Discard dry salt if there is a color change, granulation/ clumping, or insolubility. Liquid mediumDiscard solution if there is a pH change, precipitate or particulate matter throughout the solution, cloudy appearance, or a color change.*

Sterile filter the ascorbate 2-phosphate solution and store frozen (-20°C) in 2ml aliquots

- To passage cells, before trypsinization, incubate cells with **STERILE FILTERED** solution of 0.15% collagenase type 2 in PBS for ~5 minutes (i.e., 0.0015 g collagenase/ml PBS). Some cells will detach and float in the PBS. Pipet out the PBS

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into a centrifuge tube and save. Add trypsin to flasks and incubate for 5 min. Deactivate the trypsin by adding medium **WITH FBS** and transfer the medium + trypsin + cells to the centrifuge tube with the PBS/collagenase solution. Spin down and remove the supernatant. Resuspend in medium with FBS. Spin down and remove the supernatant. Resuspend in a volume of **SERUM-FREE Jakob base** medium convenient for counting.

8.1.4 The Short List Of Media Ingredients

8.1.4.1 Base Medium
500 ml DMEM (**high glucose**)
5 ml MEM Nonessential Amino Acids
5 ml **sterile** Hepes Buffer
5 ml PSG

8.1.4.2 Expansion Medium
515ml of Jakob base
50 ml FBS or ~ 0.1 ml FBS/ml of Jakob base
1μl TGFβ1 stock/ml media
0.5μl FGF-2 stock/ml media
1μl PDGF stock/ml media

8.1.4.3 Differentiation Medium
5Medium for differentiation:
0015 ml Jakob base DMEM
5 ml MEM Nonessential Amino Acids (NEAA)
5 ml Hepes Buffer
5 ml PSG (penicillin, streptomycin and glutamate)

--*Above is the Jakob base media*

5 ml ITS or 10µl ITS/ml media

5 ml can useBSA or 10µl BSA/ml media

--Add ITS and BSA in correct amounts based on how much base media used (ie. 5ml per 515ml of Jakob base media

10µl TGFβ1 stock/ml media

10µl working dexameth. soln/ml media

10µl L-Asc. acid 2-Phosphate stock/ml media

FGF-2 — if desired

8.2 Scintillation Counting of Radiolabeled Matrices

(from C. Lee's dissertation Appendix E.5, modified by T. Gordon 4-27-04)

8.2.1 Summary

Radioactive counts of matrix digests and calibrated medium are measured with the Rack-Beta 1211, LKB (Turku, Finland) liquid scintillation counter.

8.2.2 Protocol

Combine 100 µl of sample digest or calibrated medium with 2 ml of scintillation fluid (CytoScint ES, ICN Biomedicals Inc., Irvine, CA). If the counts are too low or high, you may have to add more or less than 100 µl of digest; make up the balance with dH_2O.

Counts per minute (cpm) were measured with the Rack-Beta 1211, LKB (Turku, Finland) liquid scintillation counter in the 3rd floor of the Biomedical Research Department at 500 Tech Square, Cambridge, MA.

Channel 1 (also referred to as channel A) recorded activity from 0.5 – 18.6 keV, channel 2 (or, channel B) recorded activity from 18.6 – 156 keV and the machine counted for 3 minutes/sample (or less if it determines accuracy to 2σ before this period). After determining the spillover correction constants (discussed below), the cpmA and cpmB can be converted to nanomoles of incorporated radiolabel.

Quenching effects were assumed to be constant throughout all measurements and were, therefore, not directly treated in the calculations.

After samples are counted, the vials with scintillation fluid and radioactive digest should be dumped into the barrel for liquid scintillation vials.

8.2.3 Theory

It is not possible to isolate all the counts for both radionuclides into separate channels when counting a dual-labeled sample, since the energy vs. cpm curve for the radionuclides will overlap. However, it is possible to isolate tritium. The maximum energy of ^{3}H counts is 18.6 keV, so by setting the upper limit of channel 1 to this value, all ^{3}H counts will occur in channel 1 and none will spill over into channel 2. The lower limit of channel 1 is set at 0.5 keV to eliminate any low energy noise. Unfortunately, ^{35}S counts extend from 0 to 167 keV, so some of the counts in channel 1 will be due to ^{35}S and some will be due to ^{3}H.

In general, when there is spillover of counts from both radionuclides into the channels of each other, four constants are necessary to determine the concentrations ^{3}H and ^{35}S. These constants can be thought of as the instrument's counting efficiencies for the two isotopes in the two different energy regions. Two of the constants give the percentages of cpmA and cpmB values due to ^{3}H. The other two constants give the percentages of cpmA and cpmB values due to ^{35}S. In the calculations, the constants are defined as follows:

k_{11} = % cpm in channel 1 from ^{3}H, *not* ^{35}S.

k_{12} = % cpm in channel 2 from ^{3}H, *not* ^{35}S.

k_{21} = % cpm in channel 1 from ^{35}S, *not* ^{3}H.

k_{22} = % cpm in channel 2 from ^{35}S, *not* ^{3}H.

8.2.4 Calculations

8.2.4.1 Calculating the Counting Efficiency Constants

The concentration of ^{3}H and ^{35}S is directly proportional to the amount of radioactivity (as indicated by the measureable radioactive decay) in the matrix digests. These concentrations may be expressed as a function of the cpm from channels A and B (i.e., C_1 and C_2) as follows:

$$\begin{pmatrix} [^3H] \\ [^{35}S] \end{pmatrix} = \begin{pmatrix} k_{11} & k_{12} \\ k_{21} & k_{22} \end{pmatrix} \begin{pmatrix} C_1 \\ C_2 \end{pmatrix}$$

From the first vial (containing ^{35}S only, no ^{3}H), the equation becomes:

$$0 = k_{11}C_1^S + k_{12}C_2^S$$
$$[^{35}S] = k_{21}C_1^S + k_{22}C_2^S$$

where $[^{35}S]$ indicates the concentration of ^{35}S added to media; superscript "S" indicates that counts are from first media sample with ^{35}S only, subscripts denote the channel counted. The fact that there is no tritium in the first vial (or at least there shouldn't be any if you followed the radiolabeling procedure correctly) is reflected by the zero in the first of the above pair of equations.

From the second vial (conaining both ^{35}S and ^{3}H), the appropriate equations are:

$$[^3H] = k_{11}C_1^{S,H} + k_{12}C_2^{S,H}$$
$$[^{35}S] = k_{21}C_1^{S,H} + k_{22}C_2^{S,H}$$

where $[^{35}S]$ and $[^3H]$ indicate the concentrations of ^{35}S and ^{3}H, in μCi/ml, added to the medium, respectively; superscript "S,H" indicates that counts are from the second vial, containing both ^{35}S and ^{3}H; subscripts denote the channel counted.

Solving these 4 equations for the 4 unknown k's:

$$k_{11} = -k_{12}\frac{C_2^S}{C_1^S} = \frac{[^3H]}{C_1^{S,H} - \dfrac{C_2^{S,H}C_1^S}{C_2^S}}$$

$$k_{12} = \frac{[^3H]}{C_2^{S,H} - \dfrac{C_1^{S,H}C_2^S}{C_1^S}}$$

$$k_{21} = \frac{[^{35}S]\left(\dfrac{C_2^S}{C_2^{S,H}} - 1\right)}{\left(\dfrac{C_1^{S,H}C_2^S}{C_2^{S,H}}\right) - C_1^S}$$

$$k_{22} = \frac{[^{35}S]\left(\dfrac{C_1^{S,H}}{C_1^S} - 1\right)}{\left(\dfrac{C_1^{S,H}C_2^S}{C_1^S}\right) - C_2^{S,H}}$$

8.2.4.2 Calculating the Radioactive Proline and Sulfate Concentrations in the Samples

By using these four constants and the counts per minute for channels 1 and 2 for each sample, you can determine the concentration of radioactive proline and sulfate in the sample. For example, if one of your samples produces a cpmA value of 1000 and cpmB value of 3000, then the radioactive proline and sulfate concentrations for that sample are

$$[^3H] = 1000 \cdot (k_{11}) + 3000 \cdot (k_{12})$$

$$[^{35}S] = 1000 \cdot (k_{21}) + 3000 \cdot (k_{22}).$$

In order to get the actual values, substitute the values of the four constants found in section A of these instructions into the expressions above.

8.2.4.3 Calculating the Fractions of Available Proline and Sulfate Incorporated into the ECM

The percentage of the total available radiolabeled proline incorporated into the protein manufactured during the radiolabeling period $\alpha_{proline}$ is given by the ratio of the radioactive proline concentration in the digested sample (found in part B, above) to the proline concentration of the medium (the known amount of radioactive proline you added to the medium, usually in $\mu Ci/ml$). The calculation for $\alpha_{sulfate}$ is analogous. The expressions for these fractions are

$$\alpha_{proline} = \frac{C_1 k_{11} + C_2 k_{12}}{\left[^3 H\right]}$$

$$\alpha_{sulfate} = \frac{C_1 k_{21} + C_2 k_{22}}{\left[^{35} S\right]}.$$

Next, we assume that the same *percentage* of radiolabeled proline/sulfate and unlabeled proline/sulfate were incorporated into the ECM by the cells. This makes sense since if there are 1000 molecules of unlabeled proline in the medium and only 10 molecules of labeled proline and the cells use 101 molecules of proline total, they will use 100 molecules of unlabeled proline and 1 molecule of labeled proline. In other words, the only reason there is more unlabeled proline incorporation is because the actual number of available unlabeled molecules of proline is greater than the number of labeled molecules. But the *percentage* of radiolabeled and unlabeled molecules is the same because the cells do not inherently prefer one over the other:

(100 unlabeled molecules used) / (1000 unlabeled molecules available) = (1 labeled molecule used) / (10 labeled molecules available) = 10% of available molecules used

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Furthermore, assume that the amount of proline/sulfate added in radiolabeled form is insignificant compared to the concentrations of unlabeled proline/sulfate in the medium. Strictly speaking, the total number of moles of proline/sulfate incorporated into the ECM is the sum of the radiolabeled proline/sulfate and the unlabeled proline/sulfate, but if you assume the labeled molecules are negligible, you can ignore their contribution to the total number of moles of incorporation. Then the proline/sulfate incorporation is due only to the unlabeled proline/sulfate taken out of the medium by the cells in order to synthesize collagen and proteoglycans, respectively. The following calculation demonstrates the validity of the assumption that the amount of added radiolabeled proline/sulfate is insignificant compared to the concentrations of unlabeled proline/sulfate present in the medium: one recent bottle of proline had 31 Ci/mmol and the concentration of radioactive proline in the culture medium was $[^3H] = 10$ µCi/ml, so the molarity of radioactive proline in the medium is

$$\frac{10x10^{-6}\,Ci\,/\,ml}{31Ci\,/\,mmol} = 3.2x10^{-7}\,mmol\,/\,ml = 0.32nmol\,/\,ml\;.$$

This molarity value is much lower than the 150 nmol/ml proline in DMEM/F12 medium (this value is from the manufacturer's data sheet on the contents of DMEM/F12). The situation is similar for sulfate: there is 406 nmol/ml of unlabeled sulfate in DMEM/F12 medium.

IMPORTANT NOTE: if you use any medium other than DMEM/F12 during the radiolabeling period, you must look up the proline and sulfate molar concentrations for all of the components of that medium and add them together (multiplying each component by its volume fraction in the medium) to get the actual values for your calculations. For example, the non-essential amino acids (NEAA) used in the Jakob media has proline in it. You cannot use the values of 150 nmol/ml proline and 406 nmol/ml sulfate unless the only proline and sulfate containing ingredient in your media is DMEM/F12. Other media formulations will require different numbers. However in the calculations below I have used those values as an example. I have also assumed that 88%

of the volume of the medium was due to the DMEM and 12% of the medium was due to other ingredients which did not contain any proline of sulfate.

The amount of proline/sulfate incorporated into macromolecular form during the radiolabel period is then determined as follows:

$$moles\ of\ proline = V \cdot \alpha_{proline} \cdot (0.88) \cdot (150\ nmol/ml)$$

$$moles\ of\ sulfate = V \cdot \alpha_{sulfate} \cdot (0.88) \cdot (406\ nmol/ml)$$

where V is the volume of radiolabeled media fed to the cultures, in milliliters.

Incorporation data can be normalized to the time of radiolabel and the amount of DNA in the matrix to yield the rate of incorporation normalized to cell content (nmol/µg DNA/hr).

For the Jakob serum-free media formulation the high-glucose DMEM (GIBCO cat. no. 10313021) contains 97.67 mg/L of magnesium sulfate (molec. wt. = 120.37 g/mol) = $8.11\ x10^{-4}$ M. Approximately 95% of the differentiation medium is DMEM, so there is $7.71\ x10^{-7}$ mol/L = **771 nanomoles of sulfate per ml of differentiation medium**. Similarly, the 100x NEAA (10mM solution, GIBCO cat. no. 11140 050) contains 1150 mg/L of proline (molec. wt. = 115.13 g/mol) = 0.01 M. But only 0.95% of the differentiation medium is DMEM, so there are $0.95\ x10^{-4}$ mol/liter = $9.5\ x10^{-8}$ mol/L = **95 nanomoles of proline per liter of differentiation medium**. Thus with the Jakob media the amount of proline/sulfate incorporated into macromolecular form during the radiolabel period is

$$moles\ of\ proline = V \cdot \alpha_{proline} \cdot (95\ nmol/ml)$$

$$moles\ of\ sulfate = V \cdot \alpha_{sulfate} \cdot (771\ nmol/ml)$$

where V is the volume of radiolabeled media fed to the cultures, in milliliters. There is no proline or sulfate in any other Jakob media components.

9. APPENDIX B

This appendix includes data for the modulus vs. cross-linking time and modulus of FGF- and non-FGF treated scaffolds shown in Fig.1 and 3 in chapter 2 of this thesis.

9.1 Modulus vs.EDAC Cross-linking Time (chap. 2.3.1)

cross-link time (min)	modulus (kPa)
0	0.8304
0	1.0071
1	1.8691
1	1.7929
1	2.4242
5	2.4832
5	2.4947
10	4.7958
10	2.3142
10	3.4156
60	4.7202
60	3.9748
60	3.7239
120	3.4916
120	5.3429
120	4.5583

9.2 Modulus of FGF- and non-FGF Treated Scaffolds After Four Weeks of Incubation (chap. 2.3.2)

cells	modulus (kPa)		
	unseeded	control	FGF
A	3.098	4.134	8.440
B	3.885	4.593	16.497
C	2.906	6.741	8.842
D	2.899	11.367	25.465
E	2.661	17.169	27.794

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