

The Effects of Glycosaminoglycan Content on the Compressive Modulus of Chondrocyte Seeded Type II Collagen Scaffolds

by

Emily Pfeiffer

SUBMITTED TO THE DEPARTMENT OF MECHANICAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

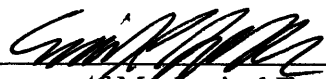
BACHELOR OF SCIENCE  
AT THE  
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2007

© 2007 Emily Pfeiffer. All Rights Reserved

The author hereby grants to MIT permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part in any medium now known or hereafter created.

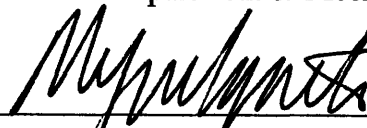
Signature of Author: \_\_\_\_\_



Department of Mechanical Engineering

February 5, 2007

Certified by: \_\_\_\_\_



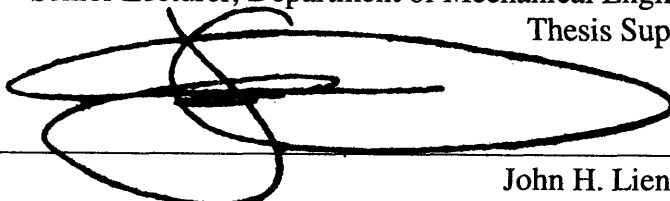
Myron Spector

Professor of Orthopaedic Surgery (Biomaterials), Harvard Medical School

Senior Lecturer, Department of Mechanical Engineering

Thesis Supervisor

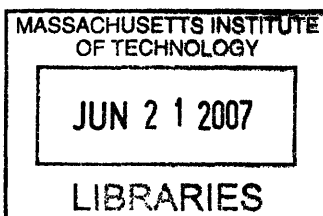
Accepted by: \_\_\_\_\_



John H. Lienhard V

Professor of Mechanical Engineering

Chairman, Undergraduate Thesis Committee



ARCHIVES

The Effects of Glycosaminoglycan Content on the Compressive Modulus of Chondrocyte Seeded Type II Collagen Scaffolds

by

Emily Pfeiffer

Submitted to the Department of Mechanical Engineering on  
February 5, 2007 in Partial Fulfillment of the Requirements for the  
Degree of Bachelor of Science in Engineering as  
Recommended by the Department of Mechanical Engineering

ABSTRACT

This study examines glycosaminoglycan (GAG) density and aggregate compressive modulus  $H_A$  of engineered cartilaginous implants. Culture parameters were developed to cause the goat articular chondrocyte seeded type II collagen scaffolds to generate 25 and 50% of the natural biochemical content of articular cartilage, with an overall goal of identifying construct compositions that might provide the most favorable response when implanted into defects in articular cartilage. Several scaffold cross-link densities were compared across constructs cultured *in vitro* to several time-points. The compressive modulus  $H_A$  was measured through unconfined compression. One group of scaffolds averaged a compressive modulus one order of magnitude below that of natural tissue. Histological analysis verified that a chondrogenic phenotype was maintained and revealed a concentration of tissue development in the center of most scaffolds. This work includes a design for an original mechanical test apparatus for measuring the Poisson's ratio of the samples, enabling meaningful interpretation of indentation test results.

Thesis Supervisor: Myron Spector

Title: Senior Lecturer, Mechanical Engineering,

Professor of Orthopaedic Surgery (Biomaterials), Harvard Medical School

## TABLE OF CONTENTS

|                                                        |    |
|--------------------------------------------------------|----|
| <b>1.0 Introduction</b>                                | 6  |
| <b>2.0 Background</b>                                  | 7  |
| <b>3.0 Materials and Methods</b>                       | 9  |
| 3.1 <i>Type II Collagen – GAG Scaffold Fabrication</i> | 9  |
| 3.2 <i>Chondrocyte Cell Culture</i>                    | 9  |
| 3.3 <i>Culture Parameter Determination Pilot Study</i> | 10 |
| 3.4 <i>Controls</i>                                    | 12 |
| 3.5 <i>Mechanical Analysis</i>                         | 12 |
| 3.6 <i>Biochemical Analysis</i>                        | 12 |
| 3.7 <i>Measurement Apparatus</i>                       | 13 |
| <b>4.0 Results</b>                                     | 13 |
| 4.1 <i>GAG Density</i>                                 | 13 |
| 4.2 <i>Parameter Selection</i>                         | 16 |
| 4.3 <i>Compressive Modulus</i>                         | 17 |
| 4.4 <i>ANOVA Analysis of Variance</i>                  | 19 |
| 4.5 <i>Regression</i>                                  | 20 |
| 4.6 <i>Controls</i>                                    | 22 |
| 4.7 <i>Histological Analysis</i>                       | 22 |
| <b>5.0 Discussion</b>                                  | 27 |
| 5.1 <i>GAG Density</i>                                 | 27 |
| 5.2 <i>Compressive Modulus Measured</i>                | 28 |
| 5.3 <i>Parameter Dependence</i>                        | 28 |
| <b>6.0 Device Design</b>                               | 30 |
| <b>7.0 Conclusion</b>                                  | 31 |
| <b>ACKNOWLEDGMENTS</b>                                 | 33 |
| <b>REFERENCES</b>                                      | 34 |
| <b>APPENDICES</b>                                      | 37 |

|                                                                                                    |    |
|----------------------------------------------------------------------------------------------------|----|
| <b>Appendix A: Temporal distribution of GAG and <math>H</math> for E1.5 Cross-linked Scaffolds</b> | 37 |
| <b>Appendix B: Cell-Seeded scaffolds GAG content descriptive statistics</b>                        | 37 |
| <b>Appendix C: Cell-Seeded scaffolds volume descriptive statistics</b>                             | 38 |
| <b>Appendix D: Cell-Seeded scaffolds GAG density descriptive statistics</b>                        | 39 |
| <b>Appendix E: Cell-Seeded scaffolds compressive modulus descriptive statistics</b>                | 40 |
| <b>Appendix F: Linear regression relations split by cross-link group and set</b>                   | 41 |

## FIGURES AND TABLES

|                                                                                                                                                                                         |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1: Pilot study comparing the effect of cross-link group and time in culture on GAG content.                                                                                      | 11 |
| Figure 2: Mean GAG per specimen for each time point and cross-link combination.                                                                                                         | 14 |
| Figure 3: Mean volume per scaffold for each time point and cross-link combination.                                                                                                      | 15 |
| Figure 4: Mean GAG density for each time point and cross-link combination.                                                                                                              | 16 |
| Figure 5: Stress relaxation of scaffold 1 of the Week 9 E1.5 set 149A.                                                                                                                  | 17 |
| Figure 6: The compressive modulus <i>was</i> calculated as the slope of a line of best fit through the equilibrium stress and strain values of successive unconfined compression steps. | 18 |
| Figure 7: Mean compressive modulus from each time point and cross-link combination.                                                                                                     | 19 |
| Figure 8: Scaffold compressive modulus plotted with respect to GAG density.                                                                                                             | 21 |
| Figure 9: Pilot study E1.5 scaffold terminated at week 3, stained red to indicate GAG.                                                                                                  | 23 |
| Figure 10: A section of E1.5 scaffold terminated at week 4 of the pilot study.                                                                                                          | 24 |
| Figure 11: Increased magnification of a week 3 E1.5 scaffold stained for GAG.                                                                                                           | 25 |
| Figure 12: Type II Collagen stain (red) with chondrocyte nuclei highlighted in dark blue.                                                                                               | 26 |
| Figure 13: A selection of scaffolds fold in culture.                                                                                                                                    | 27 |
| Figure 14: Poisson's ratio measurement apparatus.                                                                                                                                       | 31 |
| <br>                                                                                                                                                                                    |    |
| Table 1: Cross-linking agent molar ratios in cross-link groups for the culture parameter determination pilot study.                                                                     | 10 |
| Table 2: Fisher's PLSD tests with $\alpha$ of 5% determining effect of culture factors on GAG, volume, GAG density and compressive modulus.                                             | 17 |
| Table 3: ANOVA for GAG, Volume, GAG density and compressive modulus.                                                                                                                    | 20 |
| Table 4: Linear regression equations relating H to GAG density and content for E1.5 groups with correlation coefficients better than 0.4.                                               | 22 |

## 1.0 Introduction

Degeneration and loss of articular cartilage caused by injury, disease or aging is a clinical problem currently without effective treatment. Untreated defects in articular cartilage are a source of patient pain and disability, and are capable of debilitating a joint to the point of requiring total joint replacement.<sup>1</sup> Tissue-engineered cartilaginous implants may prove to be a viable clinical solution.<sup>2</sup> These constructs are typically composed of a biodegradable polymeric scaffold seeded with cells and cultured *in vitro* before implantation. The proposed work contributes to the development of a chondrocyte-seeded type II collagen-glycosaminoglycan (GAG) scaffold that is designed to treat defects in articular cartilage by facilitating endogenous tissue regeneration. The goal of this treatment is to block and reverse painful joint degeneration, preventing joint replacement in many patients.

Criteria of successful tissue regeneration include the production of tissue with histological, biochemical and mechanical properties that match those of native articular cartilage.<sup>3</sup> *In vivo*, these tissue-engineered implants should undergo cell-driven remodeling (*i.e.*, a synchronous degradation of the tissue-engineered implant and formation of new cartilage), and integrate well with the surrounding native tissue.<sup>4,5</sup> The amount of cartilaginous matrix produced during *in vitro* culture and mechanical properties of the resulting construct may influence the success of these remodeling and integration processes. While an undeveloped construct might result in ingrowth of fibrous tissue rather than regeneration of the damaged hyaline cartilage, a sample fully developed *in vitro* to the biochemical composition of healthy tissue may not integrate well with neighboring tissue, possibly requiring additional degradation and remodeling and resulting in delayed or reduced healing.

The proposed work will select culture conditions to generate constructs to 25, 50 and 75% of the GAG content per volume of natural articular cartilage, and will compare the compressive aggregate modulus  $H_A$  of these constructs to that of healthy tissue. These findings will influence subsequent work that will investigate the *in vivo* integration of scaffolds prepared *in vitro* with varying GAG content per volume. This thesis will also address the design of an apparatus to

measure the Poisson's ratio of the tissue-engineered constructs, necessary for one method of mechanical testing (the indentation test).

## 2.0 Background

Cartilage is a non-regenerative tissue incapable of full restoration of a functional reparative tissue in defects in the post-natal individual. Observations of limited reparative response under certain conditions have prompted development of therapies to facilitate complete regeneration. While no treatment to date has resulted in regeneration of tissue matching the functional properties of natural cartilage, levels of repair achieved have reduced pain in some patients, a clinically significant result.<sup>6,7</sup> The promise of cartilage tissue engineering is based in part on the success of similar techniques in regenerating other tissues such as dermis.<sup>8</sup>

Collagen is a promising biomaterial for scaffold construction. Collagen sponges are implantable *in vivo* without toxic or inflammatory effects, can be degraded and remodeled by cellular processes, and have been engineered to have architectures conducive to chondrocyte adhesion and migration.<sup>4,9</sup> Chondrocytes maintain their rounded morphology and the ability to synthesize GAG and type II collagen characteristic of the phenotype when cultured in collagen gels (i.e., gelatin) and scaffolds.<sup>3,10,11</sup> Reviews of articular cartilage tissue engineering find the use of collagen scaffolds to be advantageous, generally successful in maintaining chondrocyte phenotype and facilitating biosynthesis.<sup>12</sup> Type II collagen scaffolds have been found to be more effective than those made of type I collagen in maintaining the chondrocyte phenotype.<sup>4,13</sup> Freeze-drying and cross-linking methods have been developed to produce collagen scaffolds of pore size and stiffness that result in desired chondrocyte proliferation and construct contraction.<sup>14,15</sup> *In vitro* studies have suggested that the degradation rate of these scaffolds *in vivo* may be independent of the rate of tissue remodeling, warranting investigation into optimal implant parameters.<sup>16</sup>

Studies have investigated an array of culture conditions for the preparing tissue-engineered constructs. Caprine chondrocytes have begun to be used for these studies because goats are a

comparatively favorable animal model for *in vivo* study of engineered cartilage implants.<sup>3</sup> Autologous cells are preferred for implantation into the subject from which they have been harvested to prevent transmission of disease or immunological rejection.<sup>8</sup> Cells can be harvested from a site less functionally critical than that of the defect, as this region will also not be capable of natural regeneration.<sup>17</sup> Expansion in the number of harvested cells through two subcultures (*i.e.*, to passage 2) is beneficial for obtaining high cell counts from limited harvest numbers, and generates cells that are more prolific than those originally harvested.<sup>13,18</sup> It is preferable not to carry cultures through confluency.<sup>19</sup> While the presence of growth factors in the culture medium has a complex and sometimes contradictory effect on monolayer proliferation and chondrogenesis in 3D cultures, some factors have been shown to have significantly beneficial effects. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), for instance, encourages collagen and proteoglycan synthesis, inhibits matrix breakdown, and helps to maintain the chondrocyte phenotype under some culture conditions.<sup>2,20</sup>

Testing of constructs cultured *in vitro* focuses on three areas: biochemical content, mechanical properties, and histological qualities.<sup>3</sup> Specific quantities commonly studied include: GAG content, a primary biochemical component of cartilaginous tissue; the aggregate modulus  $H_A$ , a measure of tissue stiffness; and quality and distribution of GAG and type II collagen.

Several methods of mechanical testing can be implemented to characterize the compressive aggregate modulus. Unconfined compression testing is one effective method.<sup>21,22</sup> Indentation testing is a second method, but is utilized with more difficulty in interpretation of results. Calculation of modulus from these tests relies on independent knowledge of Poisson's ratio. Some implementations of this test method cite Poisson's ratio values characteristic of articular cartilage.<sup>17,23,24</sup> Other studies suggest that depth dependent properties of cartilage may cause Poisson's ratio to vary with engineering strain.<sup>25,26</sup> Unsuccessful efforts to calculate Poisson's ratio systematically by comparing tests with different indenter radii have been attributed to inhomogeneities in the biphasic composition of the tissue.<sup>27</sup> These studies suggest that Poisson's ratio must be measured independently for each sample at each strain level tested.



### 3.0 Materials and Methods

This study examined the GAG density and aggregate compressive modulus  $H_A$  of cartilaginous implants constructed from type II collagen scaffolds seeded with caprine chondrocytes. Culture parameters that generate implants with 25, 50 and 75% the GAG density of native tissue were explored and the compressive moduli for these groups were compared to that of natural tissue. Histological analysis was conducted to qualitatively characterize the developing tissue. Laboratory equipment to benefit future work was also designed.

#### 3.1 Type II Collagen – GAG Scaffold Fabrication

Collagen scaffolds were prepared using a protocol observed to generate scaffolds with porosity greater than 95% and pores with a diameter of  $95.9 \pm 12.3 \mu\text{m}$ .<sup>28</sup> A 1% slurry of porcine type II collagen (Geistlich Biomaterials, Wolhusen, Switzerland) in 0.001 N HCl was blended for 5 minutes while the temperature was maintained at 4°C to prevent denaturation. The slurry was poured into trays 3 mm deep, where visible air bubbles were removed by pipette to avoid blistering during lyophilization. The slurry trays were placed in a freeze-dryer and the temperature brought from 20°C to -40°C at a constant ramp rate over 15 minutes. The temperature was held at -40°C for one hour, then the pressure was dropped to 200 mTorr. The temperature was raised to 0°C and the chamber was held at that state for 17 hours, sublimating the frozen solute and leaving the collagen matrix.<sup>28</sup> The scaffolds were transferred to aluminum envelopes and sterilized by 17 hours of dehydrothermal treatment (DHT). The scaffolds were stored in a desiccator in the aluminum packets.

#### 3.2 Chondrocyte Cell Culture

Caprine articular chondrocytes were expanded in monolayer through two subcultures in chondrocyte expansion medium containing 0.1% TGF- $\beta$ 1, 0.1% PDGF- $\beta\beta$  and 0.05% FGF-2. Cultures were not permitted to reach 100% confluence. Chondrocytes had been harvested from three animals referred to as goats 149 (g149), 52 and 43 and stored at -80°C. Cells from g149

were separated into the three sets g149A, g149B and g149C, g52 into two sets, and g43 constituted one set. The six sets were cultured independently.

### 3.3 Culture Parameter Determination Pilot Study

Chondrocyte-seeded collagen type II scaffolds were grown according to culture parameters selected from a pilot study to target varying GAG content. The pilot study compared GAG synthesis in scaffolds of a range of cross-link densities and cultured to three time points after seeding with chondrocytes harvested from g149 and expanded through the second passage. The scaffolds were cut into disks of 8.5 mm diameter (and 3 mm thickness, dry dimensions) and prepared using six cross-linking protocols described in Table 1.

Table 1: Cross-linking agent molar ratios in cross-link groups for the culture parameter determination pilot study. Cross-linking agents employed are 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS). Ratios are shown relative to the carboxyl contained in the collagen scaffold (1.2 mM COOH per mole of collagen).<sup>29</sup> Five groups received EDAC (E) treatment and a sixth group was cross-linked by DHT alone (0).

| Cross-link Group | Molar Ratios |     |      | Swelling Ratio |
|------------------|--------------|-----|------|----------------|
|                  | EDAC         | NHS | COOH |                |
| 0                | 0            | 0   | 1    | 8.18 ± 2.49    |
| E1               | 1            | 1   | 5    | 7.36 ± 0.67    |
| E1.5             | 2.5          | 1   | 5    | 5.73 ± 0.29    |
| E2               | 5            | 2   | 5    | 5.30 ± 0.12    |
| E2.5             | 10           | 4   | 5    | 5.06 ± 0.44    |
| E3               | 10           | 4   | 1    | 4.36 ± 0.33    |

After treating scaffold disks in sterile cross-linking solution for 30 minutes, the scaffolds were rinsed thoroughly in sterile distilled water to remove the cross-linking reagents. Excess water was then removed by placing scaffolds on sterile filter paper, and 2 million cells in highly concentrated solution were delivered to each side of the disk, for a total of 4 million cells per scaffold. Disks were incubated at room temperature for one hour in well plates lined with

agarose gel. The cell-seeded scaffolds were cultured *in vitro* for 2, 3, 4 and 6 weeks in 1.5 ml of chondrogenic culture medium containing 1% TGF- $\beta$ 1. Medium was changed every 2-3 days. GAG content (Figure 1) was reported as percent of that in healthy cartilage tissue ( $27 \mu\text{g}/\text{mm}^3$ ).<sup>30</sup>

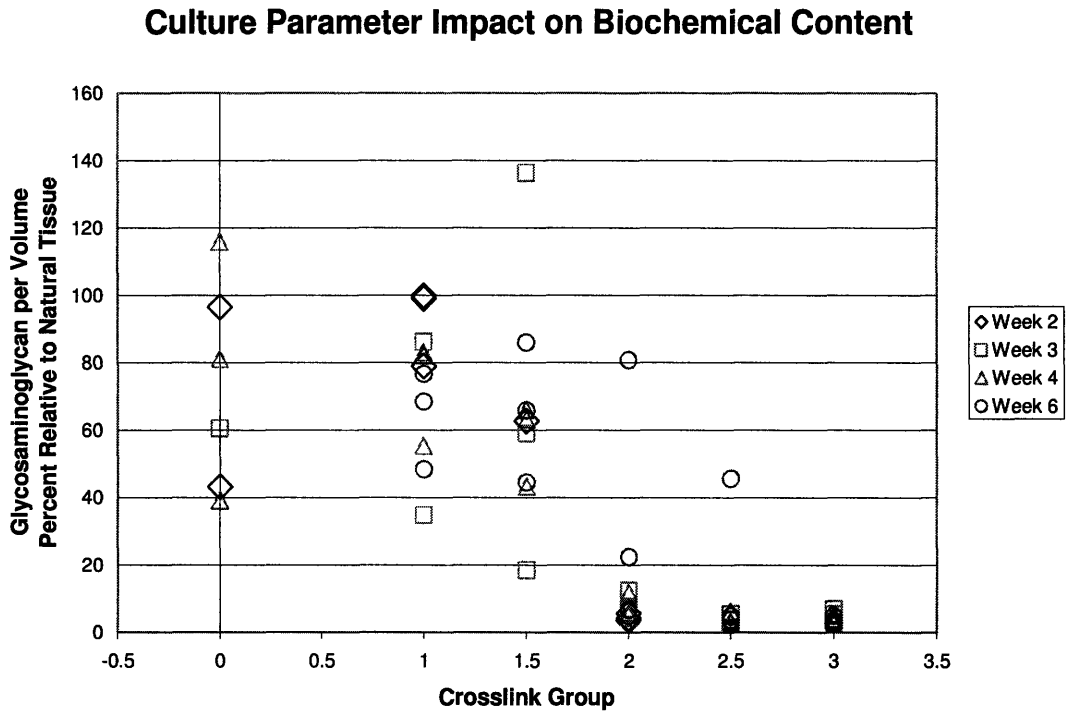


Figure 1: Pilot study comparing the effect of cross-link group and time in culture on GAG content. Type II collagen scaffolds were cross-linked according to six protocols and seeded with chondrocytes, then cultured *in vitro* to four timepoints. GAG content is shown as percent of that in healthy articular cartilage.

Cross-link groups E1.5 and E2 were selected to be cultured for 3, 5 and 9 weeks based on the results of this pilot study. A sample size of  $n=6$  was chosen, using cells harvested from three animals as described in 3.2. The sample size was determined from a power calculation using mean and standard deviation values of GAG content and Young's modulus generated by the pilot study. The sample size determination was based on detecting as significant a difference between 2 groups of 30%, with  $\alpha=0.05$  and  $\beta=0.05$ . One E2 and four E1.5 scaffolds were terminated at three time points for each of the six sample sets, with an objective of generating a sample size of at least 6 constructs per time and cross-linking group for biochemical GAG densities of 25, 50

and 75% relative to normal tissue. An analysis of variance (ANOVA) test was carried out to determine the significance of the effects of cross-link density, animal source, and time in culture on GAG content and compressive modulus. Fisher's protected least squares differences (PLSD) post-hoc testing was performed to determine the significance of differences between selected groups. Regression analysis was used to demonstrate relationships between GAG content and modulus.

### *3.4 Controls*

A set of unseeded scaffolds including cross-link groups E1.5 and E2 was tested mechanically and analyzed for GAG content. Mechanical analysis of native caprine articular cartilage tissue was also conducted.

### *3.5 Mechanical Analysis*

Mechanical testing of each sample was conducted in order to determine the compressive aggregate modulus of elasticity,  $H_A$ . Stress relaxation tests were conducted under unconfined compression to steps of 2, 4, 6, 8, 10, 15 and 20% engineering strain, each applied at a constant rate over five seconds and held for a subsequent 300 seconds. The compressive modulus is taken to be the slope of a linear regression line fit to the equilibrium stress and strain calculated at the completion of each step. Tests were accepted if the linear regression coefficient of determination was at least 0.9. Scaffolds were submerged in phosphate buffered saline (PBS) during testing to emulate physiological levels of hydration.

### *3.6 Biochemical Analysis*

Each sample was sliced in half after mechanical testing for biochemical analysis. GAG content was measured by spectrophotometry in one half. The second half was examined by histological staining for GAG and type II collagen to verify that a rounded chondrocyte phenotype was maintained and to examine the distribution and type of tissue (hyaline cartilage, fibrocartilage or fibrous tissue). Specimens for histological analysis were embedded in paraffin and sliced into 6

$\mu\text{m}$  sections. Sections were stained with Safranin O for GAG or by immunohistochemistry with an aminoethyl carbazole (AEC) substrate chromogen immunostain for type II collagen. All histological samples were stained with hemotoxylin to highlight cell nuclei.

### 3.7 Measurement Apparatus

An indentation test method is anticipated to be used for the mechanical testing of reparative tissue *in situ* in the goat model in future work. Indentation testing is of interest as it allows measurement of Young's modulus, a more universally applicable parameter than the aggregate compressive modulus. Accurate calculation of Young's modulus requires an accurate measurement of Poisson's ratio. This value is reported to range from 0.05 to 0.26 in natural articular cartilage.<sup>31, 23</sup> This thesis will include the design of an apparatus to measure Poisson's ratio through the observation of lateral expansion of matrices under unconfined compression. This ability is not included in the current testing equipment.

## 4.0 Results

This study examines chondrocyte-seeded type II collagen-GAG scaffolds *in vitro*, specifically comparing the tissue culture parameters of scaffold crosslink density and time in culture with an aim of producing engineered cartilage with GAG densities of 25, 50 and 70% that of native tissue. The compressive aggregate modulus of elasticity  $H_A$  is measured and compared to that of native articular cartilage.

### 4.1 GAG Density

GAG content per specimen is grouped here according to time point (weeks 3, 5 and 9) and cross-link group (E1.5 and E2). The average content per specimen for each group was  $74.9 \pm 27.0$ ,  $78.3 \pm 29.3$ ,  $246.9 \pm 93.8$ ,  $316.1 \pm 113.3$ ,  $368.7 \pm 175.1$  and  $370.1 \pm 161.2$   $\mu\text{g}$  for Week 3 E1.5, Week 3 E2, Week 5 E1.5, Week 5 E2, Week 9 E1.5, and Week 9 E2 respectively. These measurements are displayed in Figure 2.

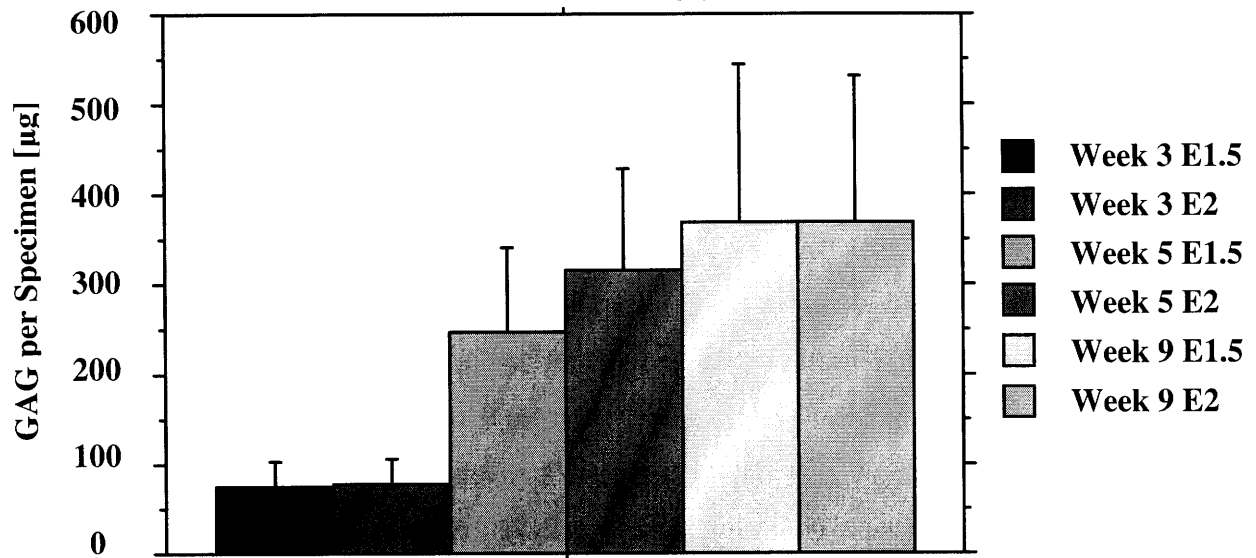


Figure 2: Mean GAG per specimen for each time point and cross-link combination. Error bars represent one standard deviation.

Scaffolds contracted visibly after as little as one week in culture, so scaffold dimensions were measured at the time of culture termination in order to estimate GAG density. The average volume at termination, shown in Figure 3, was  $52 \pm 52$ ,  $306 \pm 115$ ,  $68 \pm 63$ ,  $292 \pm 110$ ,  $65 \pm 44$ , and  $277 \pm 105$  mm<sup>3</sup> for Week 3 E1.5, Week 3 E2, Week 5 E1.5, Week 5 E2, Week 9 E1.5, and Week 9 E2 respectively.

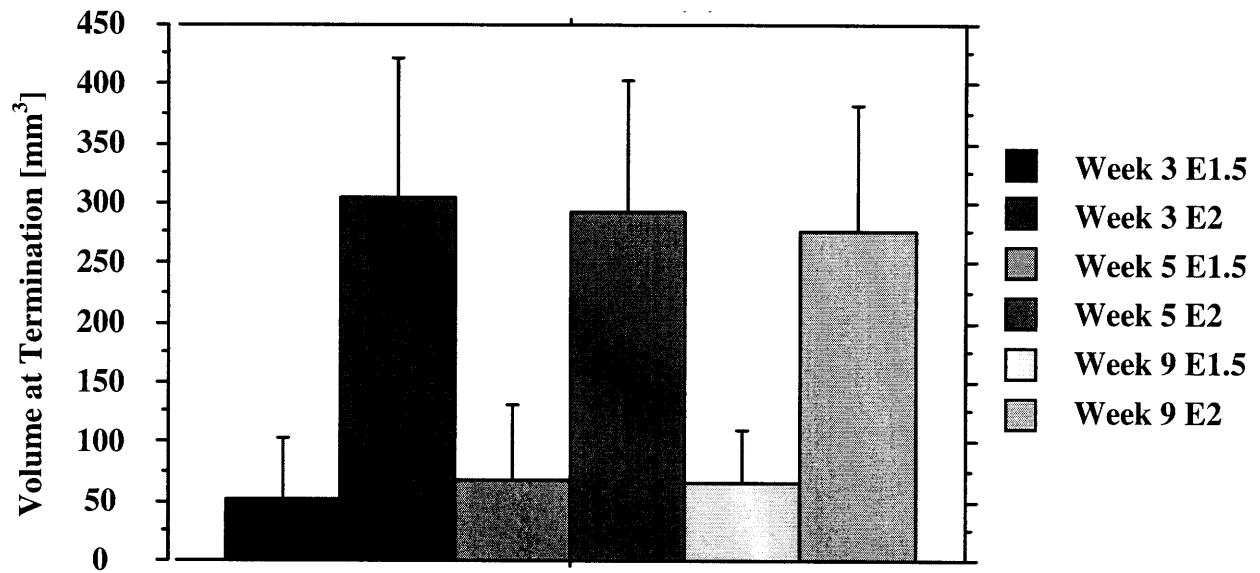


Figure 3: Mean volume per scaffold for each time point and cross-link combination. Error bars represent one standard deviation.

The GAG density per scaffold was calculated to be  $6.2 \pm 4.3$ ,  $0.6 \pm 0.4$ ,  $12.3 \pm 7.5$ ,  $2.7 \pm 2.0$ ,  $14.9 \pm 9.8$  and  $2.7 \pm 0.8$   $\mu\text{g}/\text{mm}^3$  for Week 3 E1.5, Week 3 E2, Week 5 E1.5, Week 5 E2, Week 9 E1.5, and Week 9 E2 respectively. These values are displayed in Figure 4. The mean thickness for each group was  $1.995 \pm 0.291$ ,  $1.750 \pm 0.413$ ,  $2.547 \pm 0.330$ ,  $1.86 \pm 0.157$ ,  $2.726 \pm 0.437$  and  $2.207 \pm 0.188$  mm.

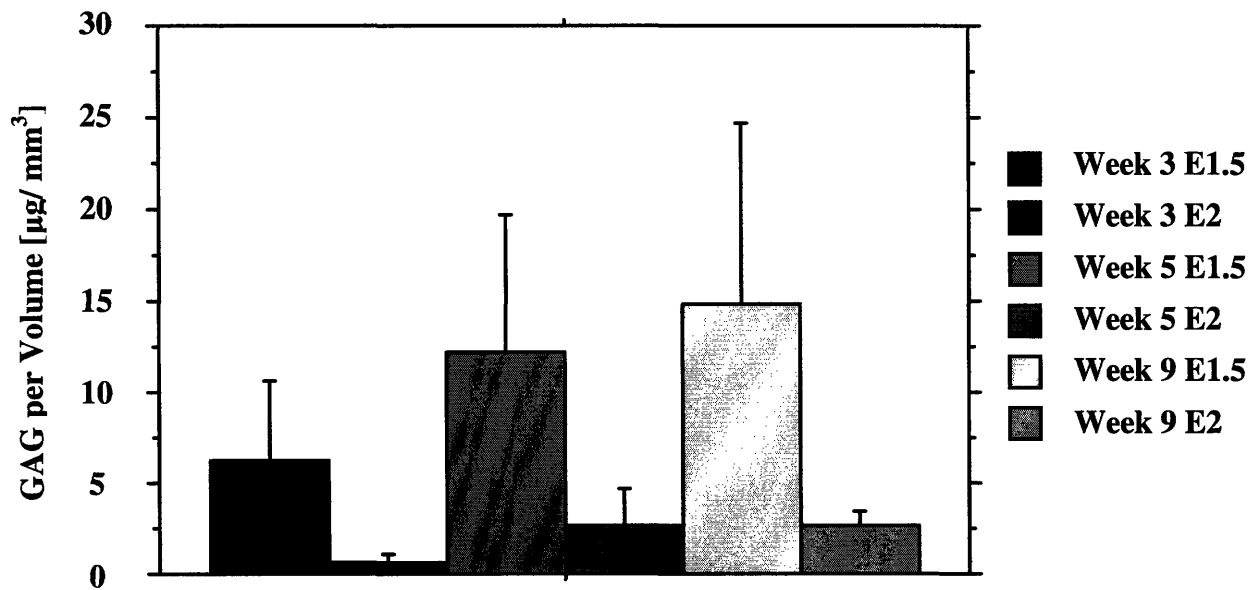


Figure 4: Mean GAG density for each time point and cross-link combination. Error bars represent one standard deviation.

#### 4.2 Parameter Selection

One aim of this study was to select culture parameters of cross-link treatment and time in culture that generate scaffolds with GAG densities of 25, 50 and 75% of that of native articular cartilage ( $27 \mu\text{g}/\text{mm}^3$ ). The groups Week 3 E1.5, Week 5 E1.5 and Week 9 E1.5 generated  $23.0 \pm 16.1$ ,  $45.4 \pm 27.7$  and  $55.2 \pm 36.2$  percent respectively. The groups Week 3 E1.5 and Week 5 E1.5 may be accepted as producing 25% and 50% of the native GAG density, as demonstrated by one-sample Student's t-test analysis generating p-values of 0.9692 and 0.4657 respectively. A Fisher's PLSD test with  $\alpha$  of 5% judging the difference between E1.5 Weeks 3 and 5 showed a p-value of 0.0018. Further Fisher's PLSD analysis of the difference between groups generated the p-values listed in Table 2.



Table 2: Fisher's PLSD tests with  $\alpha$  of 5% determining effect of culture factors on GAG, volume, GAG density and compressive modulus.

| Fisher's PLSD, $\alpha = 5\%$ | GAG<br>P-value | Volume<br>P-value | GAG/V<br>P-value | H<br>P-value |
|-------------------------------|----------------|-------------------|------------------|--------------|
| E1.5 compared to E2           | 0.4087         | < 0.0001          | < 0.0001         | 0.0006       |
| Week 3 compared to Week 5     | < 0.0001       | 0.3330            | 0.0082           | 0.0027       |
| Week 3 compared to Week 9     | < 0.0001       | 0.7618            | < 0.0001         | < 0.0001     |
| Week 5 compared to Week 9     | 0.0011         | 0.4979            | 0.1901           | < 0.0001     |

### 4.3 Compressive Modulus

The compressive modulus  $H_A$  was calculated from the equilibrium stress  $\sigma_{EQ}$  and strain  $\epsilon_{EQ}$  measurements of successive 2, 4, 6, 8, 10, 15 and 20% strain steps under unconfined compression. An example of the stress profile of these steps is shown in Figure 5.

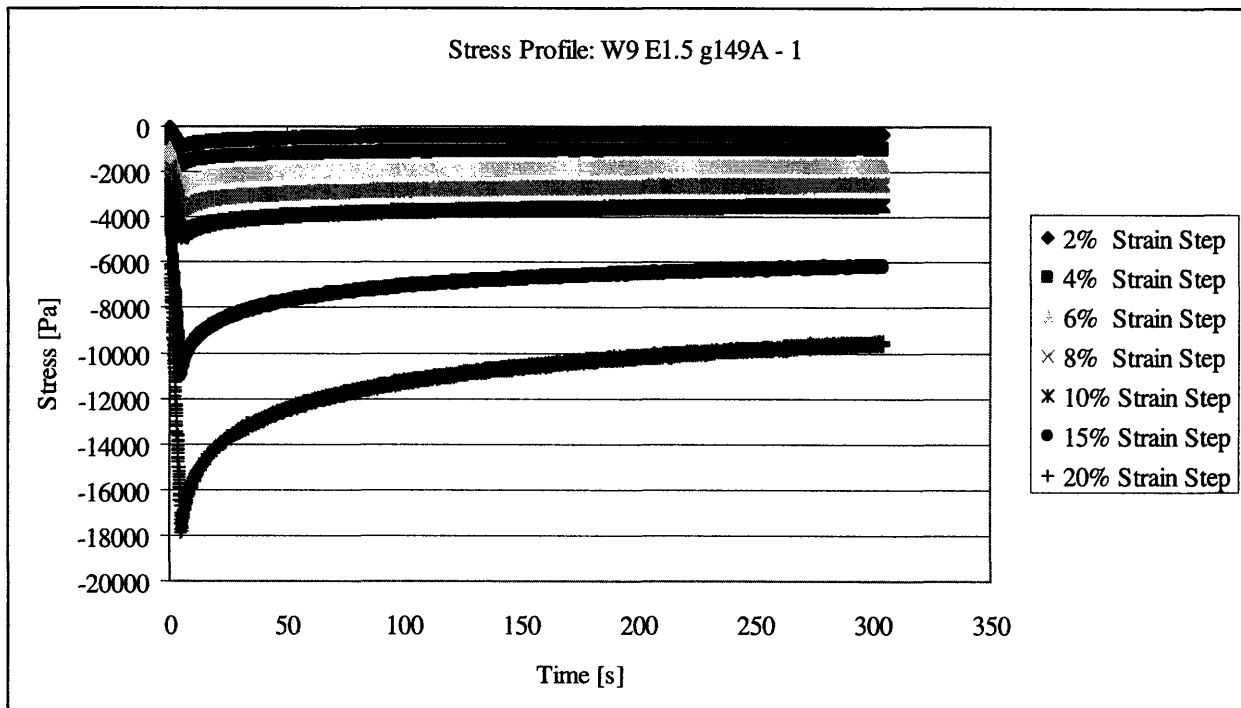


Figure 5: Stress relaxation of scaffold 1 of the Week 9 E1.5 set 149A. Strain steps are successive and shown here with respect to the beginning of each step.

Equilibrium stress and strain values were fit with first order equation to generate a linear relationship as shown in Figure 6, the slope of which was taken as the compressive modulus  $H_A$ .

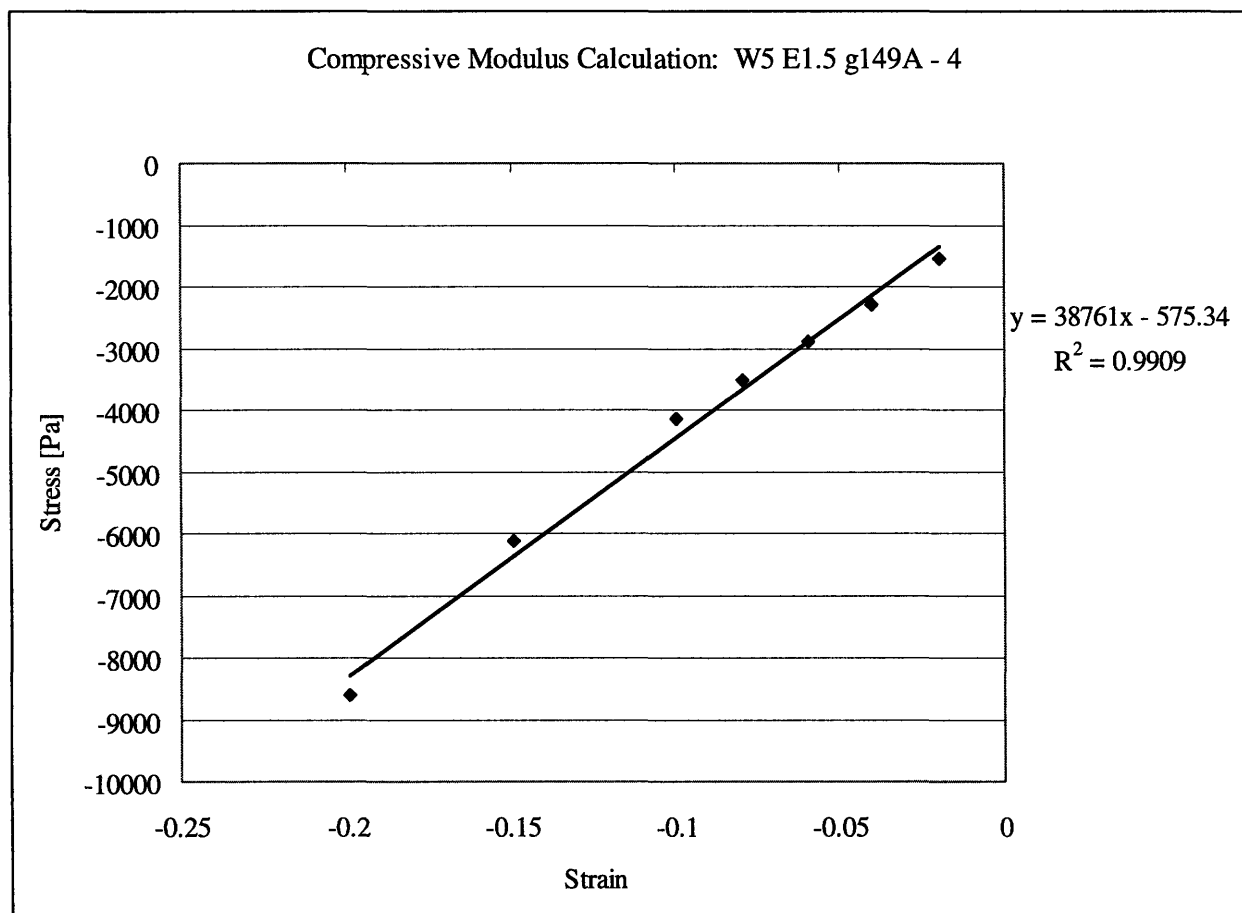


Figure 6: The compressive modulus was calculated as the slope of a line of best fit through the equilibrium stress and strain values of successive unconfined compression steps.

The mean compressive modulus  $H_A$  per time point and cross-link group was  $6131 \pm 9351$ ,  $2838 \pm 2431$ ,  $16733 \pm 7281$ ,  $9297 \pm 7770$ ,  $31463 \pm 16059$ , and  $10718 \pm 11866$  Pa for Week 3 E1.5, Week 3 E2, Week 5 E1.5, Week 5 E2, Week 9 E1.5, and Week 9 E2 respectively.

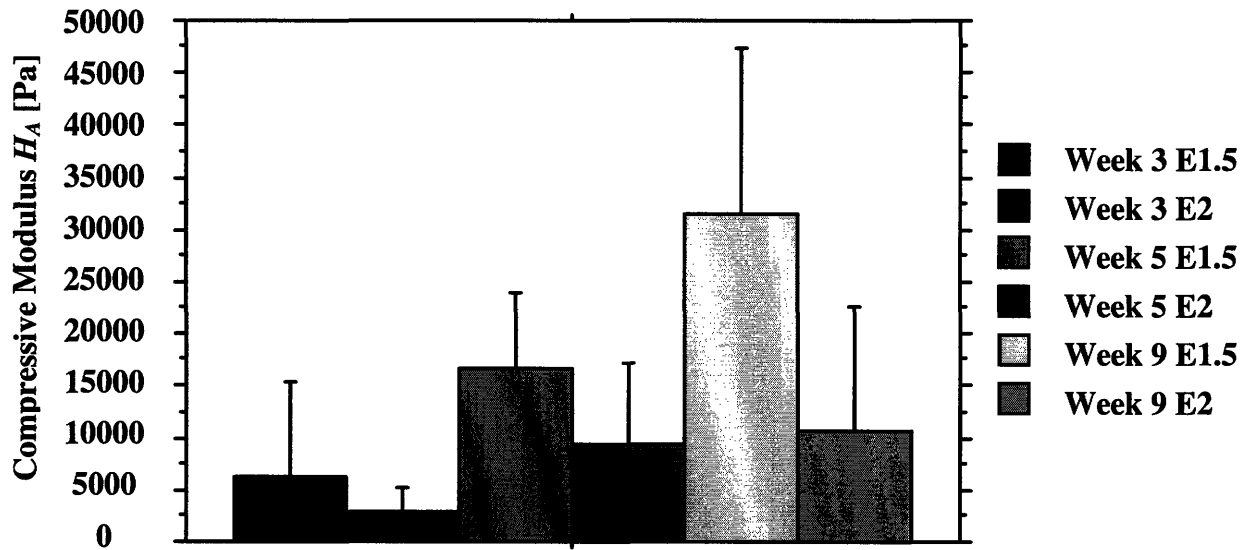


Figure 7: Mean compressive modulus from each time point and cross-link combination. Error bars represent one standard deviation.

#### 4.4 ANOVA Analysis of Variance

ANOVA was conducted to determine the influence of each independent factor on the dependent parameters GAG content, volume, GAG density and compressive modulus  $H_A$ . Tests for cross-link group, week, set, animal, were each conducted separately from tests for the combined effects of several factors. The p-values calculated are displayed in Table 3. P-values less than 0.05 are considered to indicate a significant effect of the factor or combination of factors on the dependent parameter.

Table 3: ANOVA for GAG, Volume, GAG density and compressive modulus concerning the effects of cross-link group, week, set, animal, and the combined effects of several of the parameters.

| ANOVA, $\alpha = 5\%$            | GAG<br>P-value | Volume<br>P-value | GAG/V<br>P-value | H<br>P-value |
|----------------------------------|----------------|-------------------|------------------|--------------|
| Cross-link Group                 | 0.5700         | < 0.0001          | < 0.0001         | 0.0076       |
| Week                             | < 0.0001       | 0.9403            | 0.0008           | < 0.0001     |
| Set                              | 0.2778         | 0.0007            | 0.0048           | 0.1879       |
| Animal                           | 0.0546         | < 0.0001          | 0.0002           | 0.3245       |
| Cross-link Group & Week          | 0.8175         | 0.3489            | 0.1470           | 0.0194       |
| Cross-link Group & Week & Set    | 0.5113         | 0.0002            | 0.9524           | 0.4201       |
| Cross-link Group & Week & Animal | 0.2184         | 0.5650            | 0.6283           | 0.1262       |

Of the p-values less than 0.05, only two had powers less than 0.80. Those were H dependence on cross-link group, with power 0.783, and H dependence on cross-link group and week, with a power of 0.65.

#### 4.5 Regression

The effect of GAG density on compressive modulus  $H_A$  was examined and a nonlinear relationship was calculated. Correlation coefficients  $r^2$  of 0.39 and 0.29 were calculated for E1.5 and E2 scaffolds, respectively.

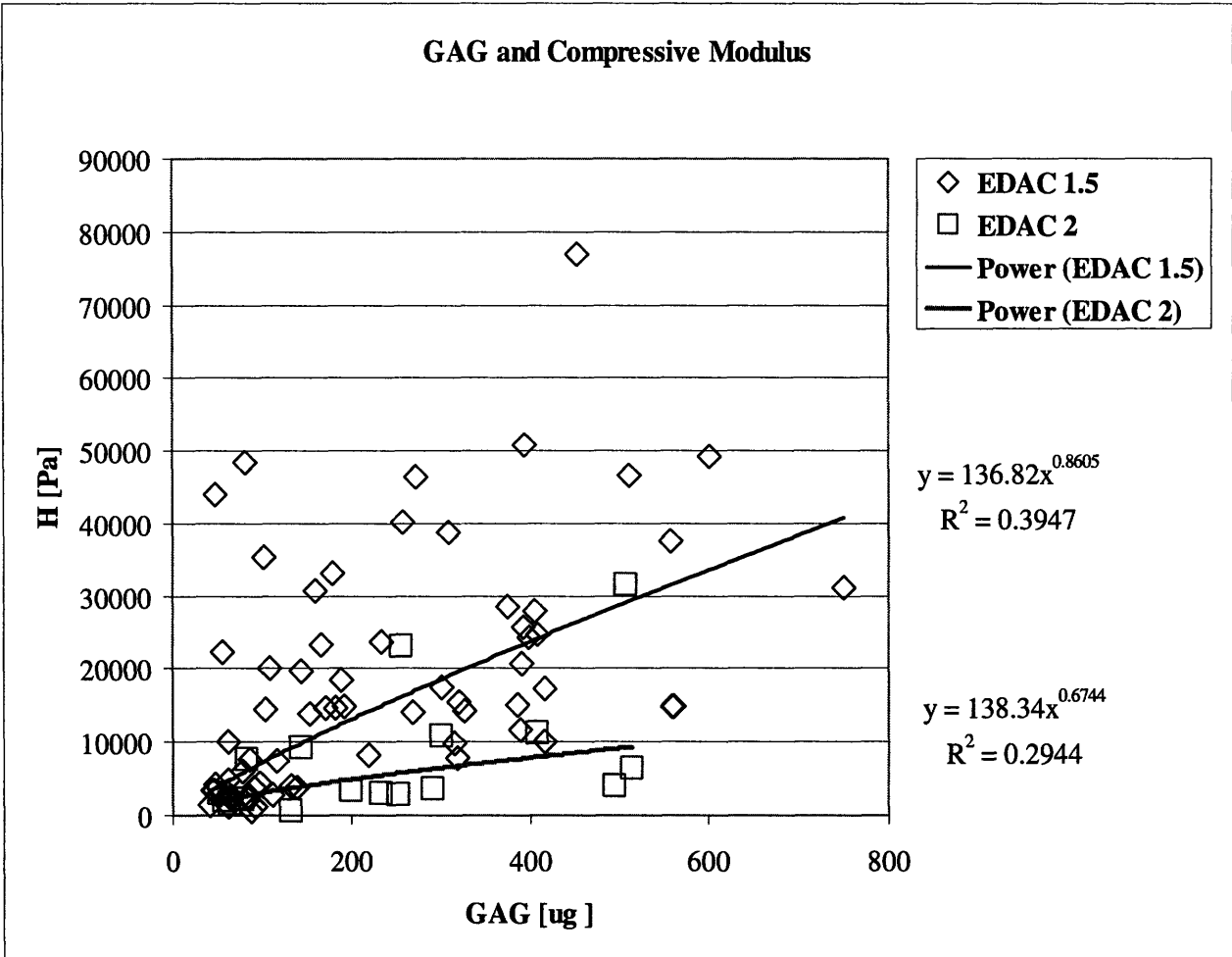


Figure 8: Scaffold compressive modulus plotted with respect to GAG density.

ANOVA indicated that the culture parameters of set and animal as well as cross-link density and week played a significant role in determining GAG density so the data were examined further within the groups defined by cross-link treatment and culture set. Of E1.5 relations, only those for sets 149B and 52A had correlation coefficients greater than 0.4. These are listed in Table 4.

Table 4: Linear regression equations relating H to GAG density and content for E1.5 groups with correlation coefficients better than 0.4.

| Linear Regression Equation        | Correlation Coefficient R <sup>2</sup> | Group     |
|-----------------------------------|----------------------------------------|-----------|
| H = -5379.22 + 1658.152 * GAG / V | 0.54                                   | E1.5 149B |
| H = 2185.12 + 1832.041 * GAG / V  | 0.66                                   | E1.5 52A  |
| H = -7.804 + 107.677 * GAG        | 0.76                                   | E1.5 149B |
| H = 1371.367 + 32.2 * GAG         | 0.54                                   | E1.5 52A  |

#### 4.6 Controls

Unseeded scaffolds were tested for GAG content and compressive modulus. Scaffolds in the E1.5 cross-linking group had a modulus of 883±380 Pa while scaffolds in the E2 group had a modulus of 1450±836 Pa. Unseeded scaffolds were found to have a GAG density of 0.1±0.1 µg/mm<sup>3</sup>. Native caprine articular cartilage had a modulus of 313897±220690 Pa.

#### 4.7 Histological Analysis

Histological analysis revealed GAG distribution throughout the scaffold. GAG tended to be dense throughout the bulk of the scaffold, but scarce along the outer rim. This band of relatively GAG-free scaffold generally decreased with time as illustrated in Figure 9 and Figure 10. In Figure 9 the GAG-free zone appears to be approximately 700 µm and in Figure 10 it appears approximately 70 µm wide.

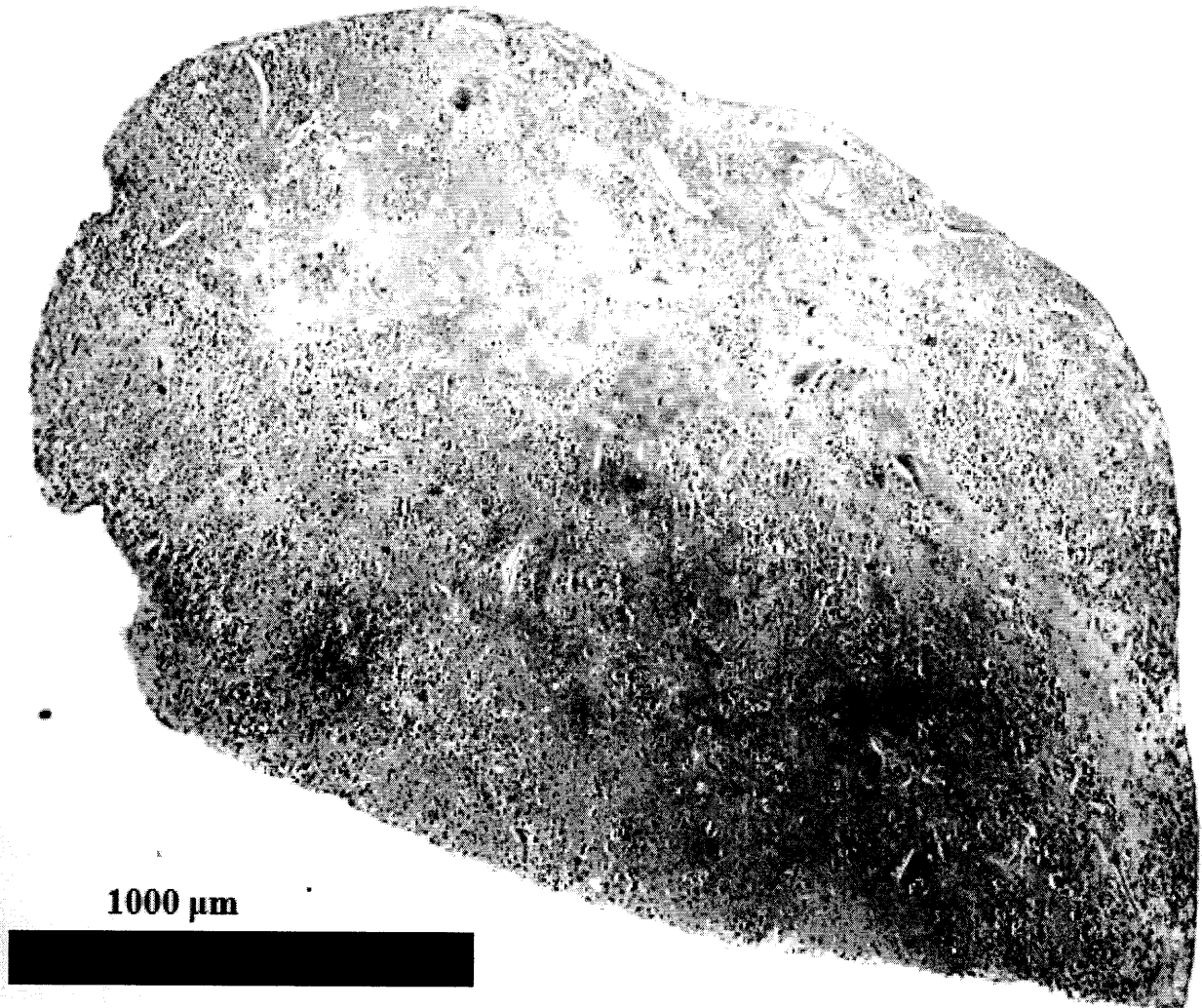


Figure 9: Pilot study E1.5 scaffold terminated at week 3, stained red to indicate GAG. The specimen was cut in half and sliced into 6  $\mu\text{m}$  cross-sections.

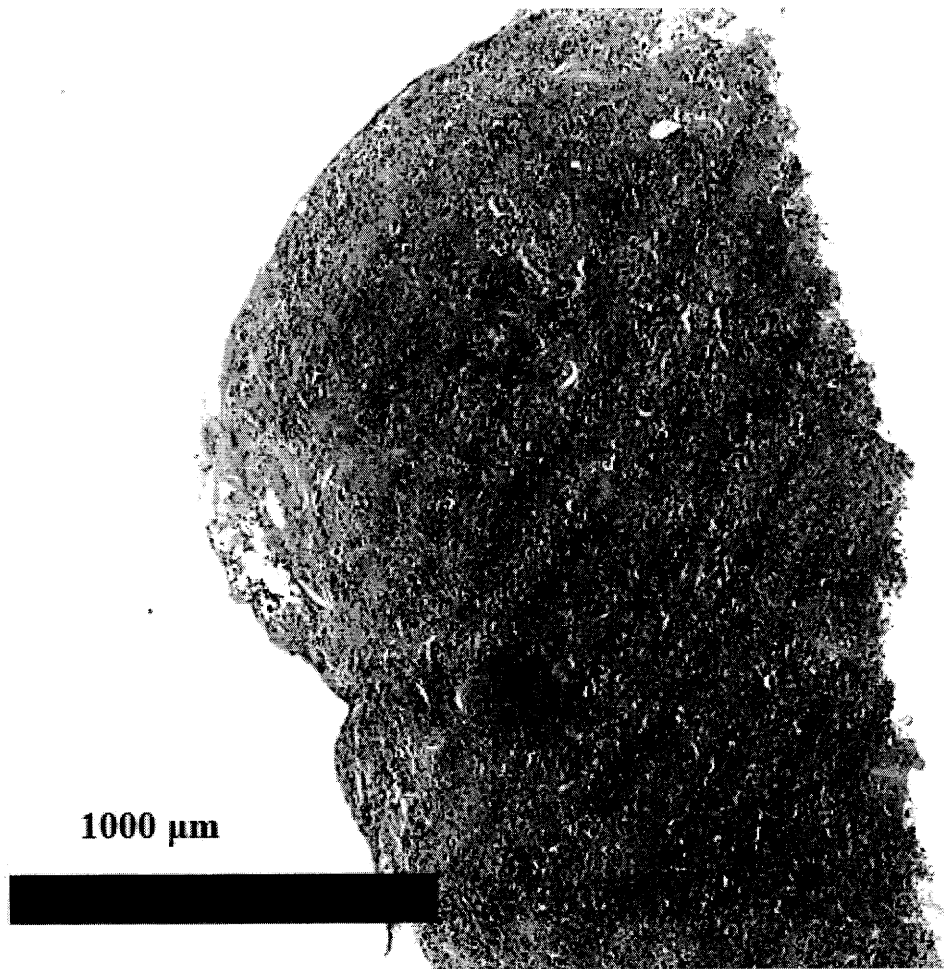


Figure 10: A section of E1.5 scaffold terminated at week 4 of the pilot study. Red stain indicates GAG.

The week 3 E1.5 scaffold shown in Figure 9 is repeated in Figure 11 at a higher magnification. Chondrocytes appear to inhabit the scaffold at a uniformly high population density, though are obscured from view in areas of GAG deposit.



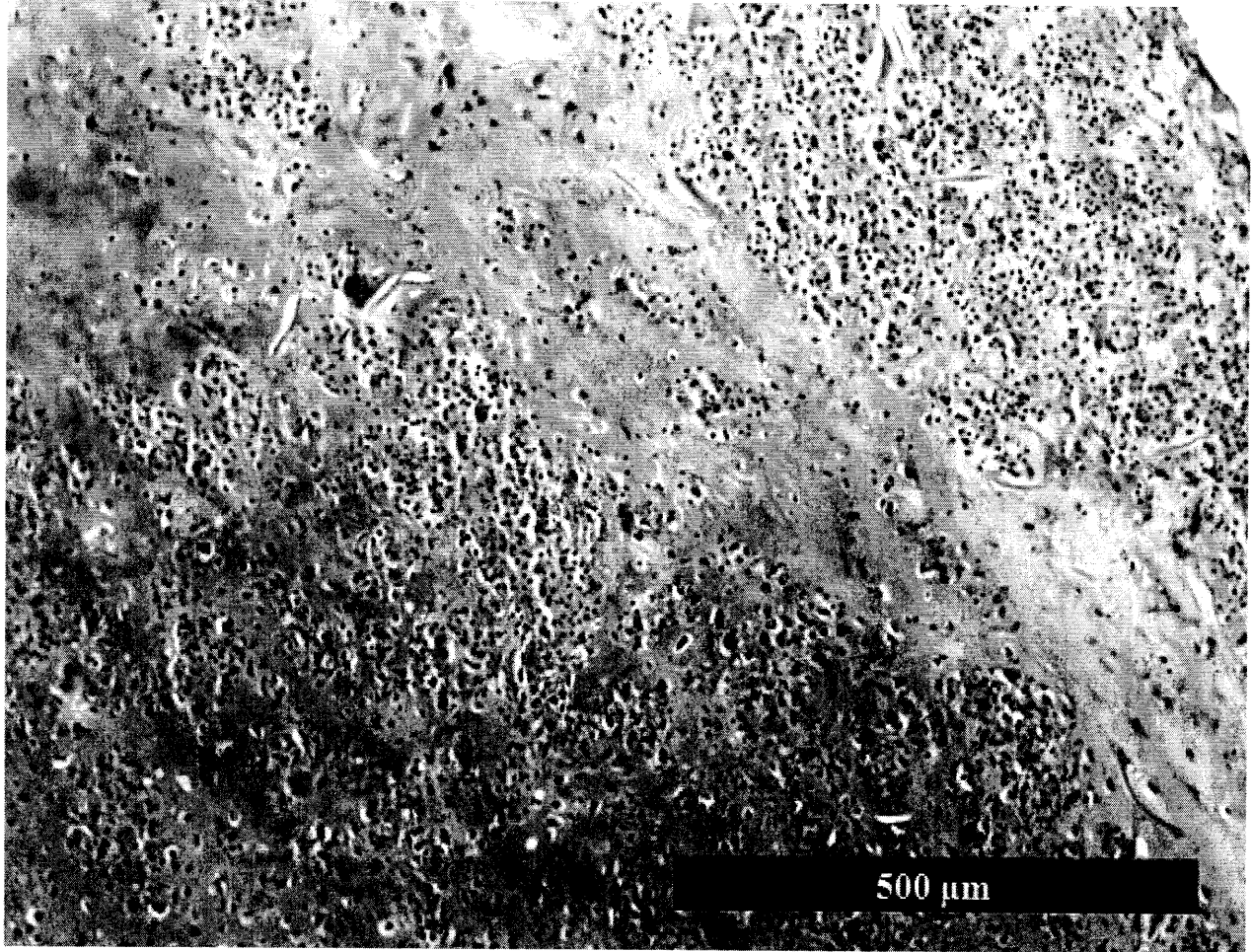


Figure 11: Increased magnification of a week 3 E1.5 scaffold stained for GAG. The top right corner is the outer rim of the scaffold.

The type II collagen stain in Figure 12 shows an E1.5 scaffold terminated after 9 weeks in culture. Chondrocyte nuclei are highlighted with hematoxylin (dark blue). Here, most chondrocytes appear randomly distributed indicating hyaline cartilage, although rows of cells that may indicate the presence of fibrocartilage can be detected. Cell morphology appears consistent with the chondrocyte phenotype.

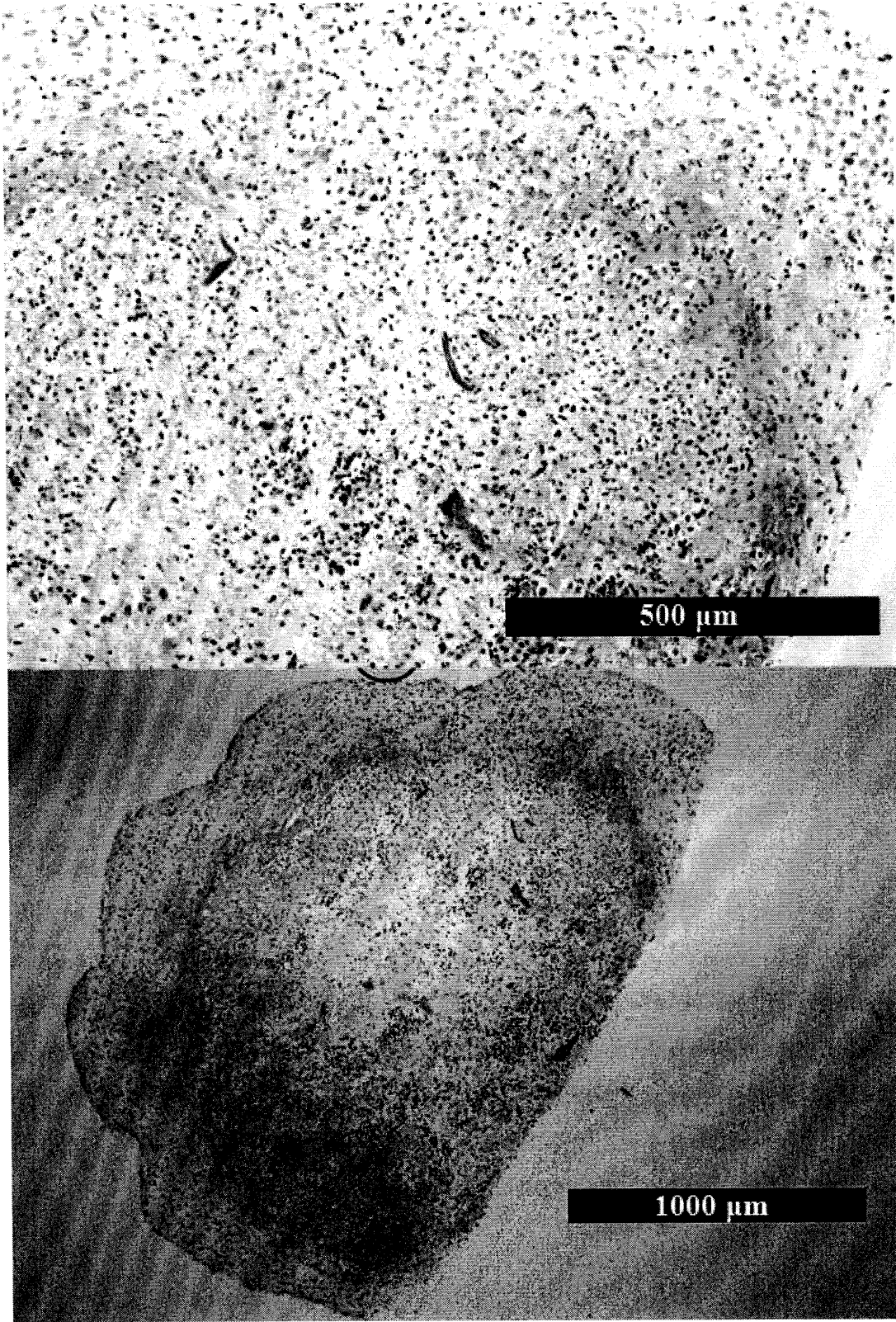


Figure 12: Type II Collagen stain (red) with chondrocyte nuclei highlighted in dark blue. Week 9 E1.5 scaffold seeded with g43 chondrocytes. A: 10x magnification. B: 4x magnification.

While all E2 and many E1.5 scaffolds remain in the disk shape throughout the culture periods used in this study, less cross-linked groups often distort to a more stable conformation. Figure 12 shows two E1 scaffolds in the folded state.

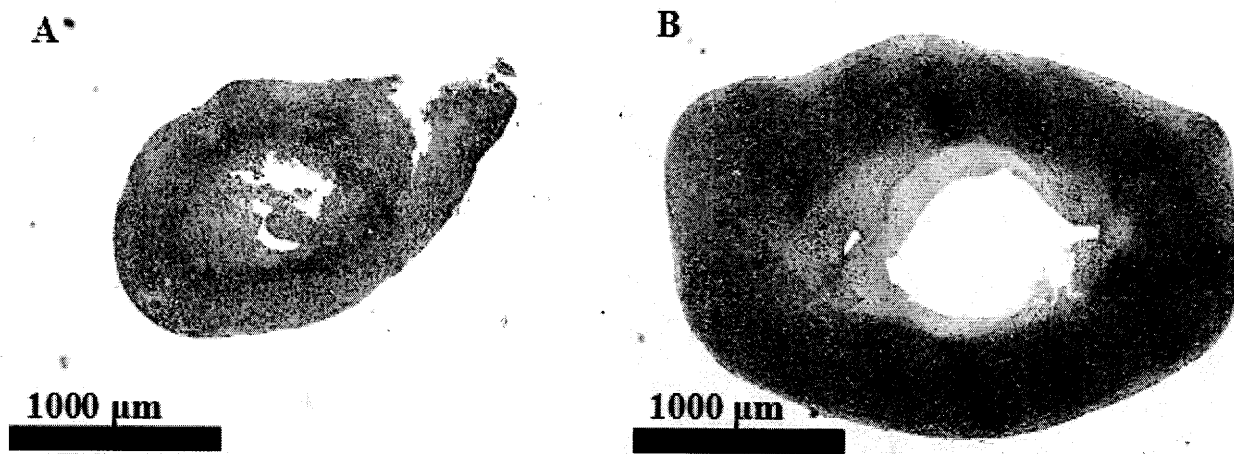


Figure 13: A selection of scaffolds fold in culture. A: Cross-section of an E1 scaffold terminated after 3 weeks and stained for GAG. B: E1 scaffold terminated after 4 weeks and stained for GAG.

## 5.0 Discussion

This study sought to control the GAG density of chondrocyte-seeded collagen scaffolds through the cell culture parameters of cross-link density and time in culture, and to characterize the aggregate compressive modulus  $H_A$  of these engineered cartilaginous implants. Statistical assays were conducted to determine the relationship between these parameters and their dependence on the culture conditions.

### 5.1 GAG Density Produced

While scaffolds with 25 and 50% of native articular cartilage GAG density were produced, this study was not able to produce seeded scaffolds with 75% of the natural GAG density. Taking

clinical limits into account, increased time in culture beyond 5 weeks does not appear to be an effective method of reaching this goal, suggesting that using a lighter cross-linking treatment such as “E1” in the pilot study might be a better approach to reach higher GAG densities. This cross-linking treatment was not pursued in the full study due to high loss of volume and fragility at early time points.

### *5.2 Compressive Modulus Measured*

The compressive modulus measurements compare well to native tissue. The mean modulus measured for the week 9, E1.5 group was one order of magnitude smaller than that of compressive modulus, with an outlier value approaching 30%. This better than a common approach using gels rather than collagen scaffolds for which moduli were two orders of magnitude smaller than native tissue have been reported from tests conducted under unconfined compression.<sup>21</sup>

The mechanical measurements of natural caprine articular cartilage reported in this study are consistent with previous work, which report  $H = 0.8 \pm 0.33$ ,  $0.57 \pm 0.17$  and  $0.31 \pm 0.18$  MPa for bovine articular cartilage tested under unconfined compression.<sup>32</sup>

### *5.3 Parameter Dependence*

GAG content, volume, GAG density and compressive modulus were all examined for dependence on parameters including scaffold cross-link density, time in culture, cell animal source and culture set. The Fisher’s PLSD analysis compared cross-link group E1.5 to E2, week 3 to week 5, week 3 to week 9 and week 5 to week 9. This examination revealed that GAG content was significantly affected by time in culture but not cross-link group and that volume at termination was significantly affected by cross-link group but not time in culture. The resultant GAG density was found to vary significantly between cross-link densities and between weeks 3 and 5 but not between weeks 5 and 9. The compressive modulus was found to vary significantly between groups divided by cross-link density or by week.

ANOVA tests were applied to further elucidate the extent to which scaffold cross-link density, time in culture, cell animal source and culture set contributed to the variation in GAG content, volume, GAG density and compressive modulus. The combined effects of cross-link and week, cross-link and week and set, and cross-link and week and animal were also examined. GAG content was determined to be significantly affected by week but not by any other variable or variable combination. GAG density was found to be significantly influenced by cross-link group, week, set and animal. The dependence of GAG density on animal and set is attributable to the significant dependence of volume on these factors and implies that chondrocytes from different animal source generate different stresses within their matrix but produce the same amount of GAG. The effect of animal was not great enough, however, to cause combinations of this factor with cross-link group and week to be significant. The compressive modulus was not affected by animal, set or combinations but was significantly influenced by both cross-link group and week.

The influence of set over GAG density suggested a regression analysis split by that parameter as well as cross-link group. The resultant correlation coefficients remained low, with only the sets 149B and 52A having  $r^2 > 0.4$ . These sets also had strong GAG content and H correlations, deemphasizing the importance of set in determining the relation between GAG density and compressive modulus.

#### *5.4 Histological Analysis*

One constant trend among histological examination of GAG distribution is the late development of GAG along the outside rim of the scaffold suggests. A functional outer zone is important to the clinical success of the treatment as this will be the region to integrate with the surrounding native tissue. While a lack of cartilaginous tissue close to the implies improper seeding, the high cell density in this area shown in Figure 11 suggests that cells are thriving in that area but the matrix molecules they produce are being swept away during media changes. One solution may be to replace the traditional well plate set up. Wells positioned in a trough, for example, would allow the removal and replacement of media in the chamber while lessening the disturbance of

fluid around the scaffold. This approach would mandate a single cell type per continuous apparatus.

Another trend was observed that may impact the clinical success of the cartilaginous implants. While relatively heavily cross-linked scaffolds such as E1.5 and especially E2 maintained a disk geometry throughout the study, less stiff scaffolds would often buckle as shown in Figure 13. This rearrangement frequently led to a spherical scaffold with a hollow center, a configuration which might disrupt assimilation with surrounding tissue and alter mechanical properties. It is recommended that cross-link densities at least as high as those in the E1.5 group continue to be used, or that if less cross-linked scaffolds are desired, geometries with aspect ratios close to one be employed, i.e. cubic.

## **6.0 Device Design**

This thesis suggests designs for a laboratory apparatus. This device permits calculations of Poisson's ratio for individual scaffolds and the second ensures uniform seeding of scaffolds with cells.

The apparatus for determining Poisson's ratio must produce measurable deformation in a scaffold of small dimensions, and allow the experimenter access to those measurements. The design proposed in Figure 14 allows a user capable of detecting differences of 0.01" with calipers (the human eye is capable of detecting 0.001" differences) to measure Poisson's ratio in increments of 0.02 for a scaffold as small as 3 mm in diameter.

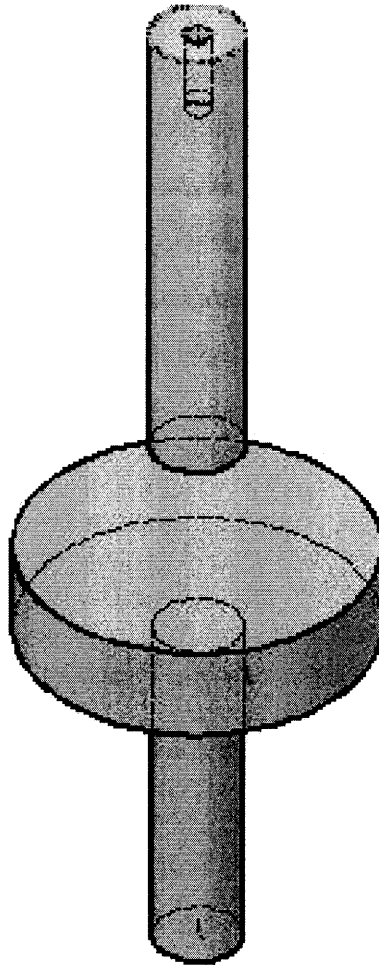


Figure 14: Poisson's ratio measurement apparatus.

## 7.0 Conclusion

This study examined the GAG density and aggregate compressive modulus  $H_A$  of cartilaginous implants constructed from type II collagen scaffolds seeded with caprine chondrocytes. Culture parameters were developed to generate implants with 25, 50 and 75% the GAG density of native tissue and the compressive modulus for these groups were compared to that of natural tissue. Histological analysis was conducted qualitatively characterize the developing tissue. Laboratory equipment to benefit future work was also designed.

Scaffolds in the E1.5 Weeks 3 and 5 groups represented 25 and 50% of the natural GAG density well. Scaffolds carried through 9 weeks did not show significant additional development of GAG density, although the compressive modulus did increase during this period to an average of one order of magnitude below that of natural tissue. Strong dependence of these parameters on cross-link group and time point were observed, with some effect of animal chondrocyte source. Histological examination revealed a concentration of GAG in the center of the scaffold and tendency of scaffolds with cross-linking treatments of E1 and below to fold in culture, creating a potentially discontinuous implant.



## **ACKNOWLEDGMENTS**

The author would like to thank Professor Myron Spector, Dr. Scott Vickers and Alix Weaver for their enthusiastic support and guidance throughout the research and analysis of this thesis. Gratitude also goes to Professor Alan Grodzinsky and Elliot Frank for facilitation of the mechanical testing. Cell culture aspects of this work were made possible by the Veterans Administration Boston Healthcare System. The financial support from the Department of Veterans Affairs is appreciated.

## REFERENCES

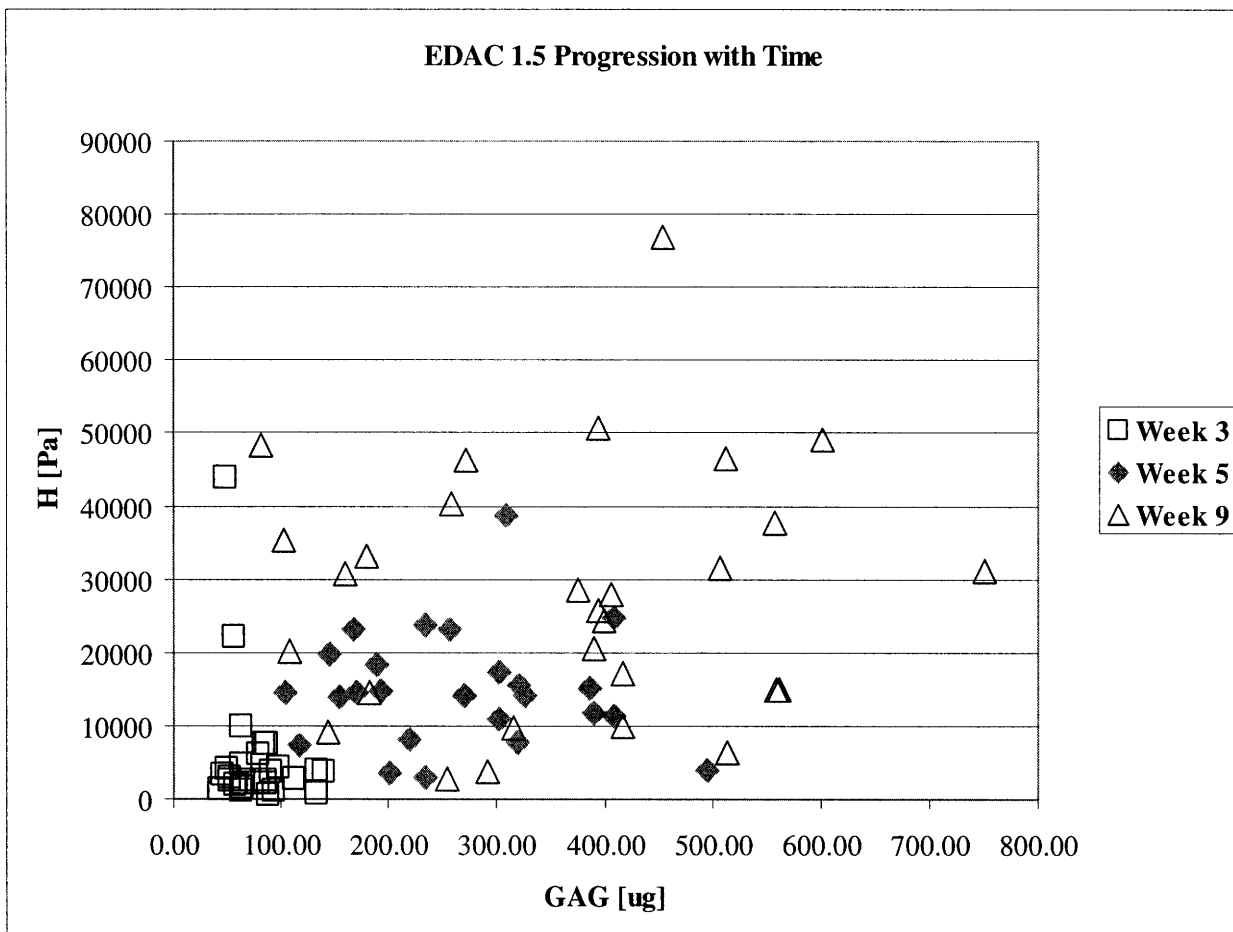
1. Setton, L. A., D.M. Elliott, V.C. Mow. Altered mechanics of cartilage with osteoarthritis: human osteoarthritis and an experimental model of joint degeneration. *OsteoArthritis Cartilage* **7**, 2-14 (1999).
2. Capito, R. M., M. Spector. Scaffold-Based Articular Cartilage Repair: Future Prospects Wedding Gene Therapy and Tissue Engineering. *IEEE Engineering in Medicine and Biology Magazine*, 42-50 (2003).
3. ASTM. F 2451-05 Standard Guide for *in vivo* Assessment of Implantable Devices Intended to Repair or Regenerate Articular Cartilage. *Annual Book of ASTM Standards* (2005).
4. Nehrer, S., Howard A. Breinan, Arun Ramappa, Sonya Shortkroff, Gretchen Young, Tom Minas, Clement B. Sledge, Ioannis V. Yannas, Myron Spector. Canine Chondrocytes Seeded in Type I and Type II Collagen Implants Investigated *In Vitro*. *Journal of Biomedical Materials Research* **38**, 95-104 (1997).
5. Schaefer, D., I. Martin, P. Shastri, R.F. Padera, R. Langer, L. Freed, G. Vunjak-Novakovic. In vitro generation of osteochondral composites. *Biomater.* **21**, 2599-2606 (2000).
6. Kinner, B., R. M. Capito, M. Spector. Regeneration of Articular Cartilage. *Adv Biochem Engin/Biotechnol* **94**, 91-123 (2005).
7. Lee C.R., A. J. G., H.-P. Hsu, M. Spector. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. *Journal of Orthopaedic Research* **21**, 272-281 (2003).
8. Yannas, I. V., J. F. Burke, D. P. Orgill, E. M. Skrabut. Wound Tissue Can Utilize a Polymeric Template to Synthesize a Functional Extension of Skin. *Science* **215**, 174-176 (1982).
9. Speer, D. P., M. Chavapil, R.G. Volz, M.D. Holmes. Enhancement of healing in osteochondral defects by collagen sponge implants. (1979).
10. Kimura, T., N. Yasui, S. Ohsawa, K. Ono. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. *Clin Orthop Relat Res.* **186**, 231-9 (1984).
11. Grande, D. A., C. Halberstadt, G. Naughton, R. Schwartz, and Ryhana Manji. Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. *Journal of Biomedical Materials Research* **34**, 211-220 (1997).
12. Temenoff, J., A. Mikos. Review: tissue engineering for regeneration of articular cartilage. *Biomater.* **21**, 431-440 (2000).
13. Veilleux, N. H., I.V. Yannas, and M. Spector. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. *Tiss. Engr.* **10**, 119-127 (2004).
14. Lee C.R., A. J. G., M. Spector. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. *Biomater.* **22**, 3145-3154 (2001).

15. Vickers, S. M., L.S. Squitieri, M. Spector. Effects of Cross-linking Type II Collagen-GAG Scaffolds on Chondrogenesis In Vitro: Dynamic Pore Reduction Promotes Cartilage Formation. *Tiss. Engr.* **12**, 1345-1355 (2006).
16. Gordon, T. D., L. Schloesser, D.E. Humphries and M. Spector. Effects of the Degradation Rate of Collagen Matrices on Articular Chondrocyte Proliferation and Biosynthesis in Vitro. *Tiss. Engr.* **10**, 1287-1295 (2004).
17. Lee C.R., A. J. G., H.-P. Hsu, S.D. Martin, M. Spector. Effects of Harvest and Selected Cartilage Repair Procedures on the Physical and Biochemical Properties of Articular Cartilage in the Canine Knee. *Journal of Orthopaedic Research* **18**, 790-799 (2000).
18. Marijnissen, W. J. C. M., Gerjo J. V. M. van Oschb, Joachim Aignerc, Henriette L. Verwoerd-Verhoefb and Jan A. N. Verhaara. Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM). *Biomat.* **21**, 571-580 (2000).
19. Hendriks, J., Jens Riesle and Clemens A. Vanblitterswijk. Effect of Stratified Culture Compared to Confluent Culture in Monolayer on Proliferation and Differentiation of Human Articular Chondrocytes. *Tiss. Engr.* **12**, 2397-2405 (2006).
20. Luyten, F., P Chen, V Paralkar, AH Reddi. Recombinant bone morphogenetic protein-4, transforming growth factor-beta 1, and activin A enhance the cartilage phenotype of articular chondrocytes in vitro. *Exp Cell Res* **210**, 224-9 (1994).
21. LeRoux, M. A., Farshid Guilak, Lori A. Setton. Compressive and shear properties of alginate gel: Effects of sodium ions and alginate concentration. *Journal of Biomedical Materials Research* **47**, 46-53 (1999).
22. Pek, Y. S., M. Spector, I.V. Yannas, L.J. Gibson. Degredation of a collagen-chondroitin-6-sulfate matrix by collagenase and by chondroitinase. *Biomaterials* **25**, 473-482 (2004).
23. Wong, M., M. Ponticiello, V. Kovanen, J.S. Jurvelin. Volumetric changes of articular cartilage during stress relaxation in unconfined compression. *Journal of Biomechanics* **33**, 1049-1954 (2000).
24. Schaefer, D., I. Martin, G. Jundt, J. Seidel, M. Heberer, A. Grodzinsky, I. Bergin, G. Vunjak-Novakovic, L. Freed. Tissue-Engineered Composites for the Repair of Large Osteochondral Defects. *Arthritis & Rheumatism* **46**, 2524-2534 (2002).
25. Chen, C. S., I.V. Yannas and M. Spector. Pore strain behaviour of collagen-glycosaminoglycan analogues of extracellular matrix. *Biomat.* **16**, 777-783 (1995).
26. Mow, V. C., and X.E. Guo. Mechano-Electrochemical Properties of Articular Cartilage: Their Inhomogeneities and Anisotropies. *Annu. Rev. Biomed. Eng.* **4**, 175-209 (2002).
27. Jin, H. a. J. L. L. Determination of Poisson's Ratio of Articular Cartilage by Indentation Using Different-Sized Indenters. *Transactions of the ASME* **126**, 138-145 (2004).
28. O'Brien, F. J., B.A. Harley, I.V. Yannas, and L.J. Gibson. Influence of Freezing Rate on Uniformity of Pore Structure in Freeze-Dried Collagen-GAG Scaffolds. *Biomaterials* **25**, 1077-1086 (2004).
29. Olde Damink, L. H. H., P. J. Dijkstra, M. J. A. van Luyn, P. B. van Wachem, P. Nieuwenhuis, and J. Feijen. Cross-linking of dermal sheep collagen using a water-soluble carbodimide. *Biomat.* **17**, 765-773 (1996).
30. Sah, R. L., A.C. Chen, A.J. Grodzinsky, S.B. Trippel. Differential effects of bFGF and IGF-1 on matrix metabolism in calf and adult bovine cartilage explants. *Arch. Biochem. Biophys* **308**, 137-147 (1994).

31. Likhitpanichkul, M. G., X E; Lai, W M; Mow, V C. in *50th Annual Meeting of the Orthopaedic Research Society* (Orthopaedic Research Society, San Francisco, CA, 2004).
32. Korhonen, R. K., M.S. Laasanen, J. Toyras, J. Rieppo, J. Hirvonen, H.J. Helminen, J.S. Jurvelin. Comparison of the equilibrium response of articular cartilage in unconfined compression, confined compression and indentation. *Journal of Biomechanics* **35**, 903-909 (2002).

## APPENDICES

### Appendix A: Temporal distribution of GAG and $H$ for E1.5 Cross-linked Scaffolds



### Appendix B: Cell-Seeded scaffolds GAG content descriptive statistics

#### Descriptive Statistics: GAG

Split By: Timepoint, Crosslink Group, Set

|            | All     |
|------------|---------|
| Mean       | 234.564 |
| Std. Dev.  | 169.116 |
| Std. Error | 18.236  |
| Count      | 86      |
| Minimum    | 42.22   |
| Maximum    | 750.24  |

| E1.5 |      |      |     |     |    |
|------|------|------|-----|-----|----|
| 149A | 149B | 149C | 52A | 52B | 43 |

| <b>Week 3</b>     |        |        |         |         |        |       |
|-------------------|--------|--------|---------|---------|--------|-------|
| <b>Mean</b>       | 61.222 | 64.235 | 100.763 | 106.785 | 62.3   | 54.06 |
| <b>Std. Dev.</b>  | 17.537 | 18.603 | 22.003  | 25.602  | 17.413 | 8.153 |
| <b>Std. Error</b> | 8.768  | 9.301  | 11.001  | 12.801  | 8.706  | 4.077 |
| <b>Count</b>      | 4      | 4      | 4       | 4       | 4      | 4     |
| <b>Minimum</b>    | 45.89  | 42.22  | 85.32   | 78.51   | 45.29  | 48.42 |
| <b>Maximum</b>    | 85.51  | 87.65  | 133.36  | 139.27  | 86.25  | 65.87 |

| <b>Week 5</b>     |        |         |        |         |         |         |
|-------------------|--------|---------|--------|---------|---------|---------|
| <b>Mean</b>       | 238.2  | 136.683 | 181.62 | 361.135 | 305.325 | 182.103 |
| <b>Std. Dev.</b>  | 67.069 | 45.764  | 15.274 | 44.607  | 71.336  | 46.471  |
| <b>Std. Error</b> | 33.534 | 26.422  | 10.8   | 22.303  | 35.668  | 26.83   |
| <b>Count</b>      | 4      | 3       | 2      | 4       | 4       | 3       |
| <b>Minimum</b>    | 153.81 | 103.75  | 170.82 | 319.64  | 213.09  | 144.89  |
| <b>Maximum</b>    | 309.16 | 188.94  | 192.42 | 408.15  | 386.09  | 234.19  |

| <b>Week 9</b>     |         |         |         |         |         |         |
|-------------------|---------|---------|---------|---------|---------|---------|
| <b>Mean</b>       | 268.603 | 264.55  | 352.102 | 421.337 | 552.788 | 352.97  |
| <b>Std. Dev.</b>  | 160.302 | 176.156 | 198.714 | 102.703 | 155.673 | 163.197 |
| <b>Std. Error</b> | 80.151  | 88.078  | 99.357  | 51.351  | 77.836  | 81.598  |
| <b>Count</b>      | 4       | 4       | 4       | 4       | 4       | 4       |
| <b>Minimum</b>    | 103.08  | 108.23  | 81.19   | 316.13  | 406.26  | 182.24  |
| <b>Maximum</b>    | 416.86  | 512.19  | 559.11  | 561.4   | 750.24  | 557.95  |

| <b>E2</b> |             |             |             |            |            |           |
|-----------|-------------|-------------|-------------|------------|------------|-----------|
|           | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>W3</b> | 79.09       | 132.77      | 83.83       | 57.71      | 64.11      | 52.26     |
| <b>W5</b> | 494.7       | 408.52      | 301.92      | 200.83     | 234.29     | 256.57    |
| <b>W9</b> | 292.1       | 143.75      | 253.91      | 514.23     | 507.52     | 509.32    |

**Appendix C: Cell-Seeded scaffolds volume descriptive statistics**

**Descriptive Statistics: Volume**  
**Split By: Timepoint, Crosslink Group, Set**

| <b>All</b>        |       |
|-------------------|-------|
| <b>Mean</b>       | 109.6 |
| <b>Std. Dev.</b>  | 115.0 |
| <b>Std. Error</b> | 12.4  |
| <b>Count</b>      | 86    |
| <b>Minimum</b>    | 9.7   |
| <b>Maximum</b>    | 449.1 |

| <b>E1.5</b>       |             |             |             |            |            |           |
|-------------------|-------------|-------------|-------------|------------|------------|-----------|
|                   | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>Week 3</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 17.1        | 14.8        | 17.0        | 135.5      | 106.1      | 19.1      |
| <b>Std. Dev.</b>  | 2.0         | 3.5         | 8.0         | 28.7       | 17.0       | 1.6       |
| <b>Std. Error</b> | 1.0         | 1.8         | 4.0         | 14.3       | 8.5        | 0.8       |
| <b>Count</b>      | 4           | 4           | 4           | 4          | 4          | 4         |
| <b>Minimum</b>    | 15.8        | 9.7         | 12.4        | 97.0       | 91.8       | 17.2      |
| <b>Maximum</b>    | 20.0        | 17.6        | 29.0        | 166.3      | 130.7      | 21.0      |
| <b>Week 5</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 26.5        | 20.0        | 24.7        | 134.0      | 138.4      | 21.1      |
| <b>Std. Dev.</b>  | 4.8         | 8.1         | 2.2         | 61.2       | 23.1       | 0.1       |
| <b>Std. Error</b> | 2.4         | 4.7         | 1.5         | 30.6       | 11.6       | 0.1       |
| <b>Count</b>      | 4           | 3           | 2           | 4          | 4          | 3         |
| <b>Minimum</b>    | 19.8        | 12.7        | 23.2        | 80.5       | 110.4      | 21.0      |
| <b>Maximum</b>    | 30.3        | 28.7        | 26.2        | 220.7      | 166.3      | 21.2      |
| <b>Week 9</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 35.9        | 30.3        | 51.9        | 135.5      | 108.9      | 30.4      |
| <b>Std. Dev.</b>  | 8.6         | 3.6         | 14.2        | 18.8       | 17.6       | 3.8       |
| <b>Std. Error</b> | 4.3         | 1.8         | 7.1         | 9.4        | 8.8        | 1.9       |
| <b>Count</b>      | 4           | 4           | 4           | 4          | 4          | 4         |
| <b>Minimum</b>    | 27.2        | 25.3        | 34.5        | 113.7      | 93.5       | 27.0      |
| <b>Maximum</b>    | 46.6        | 32.8        | 69.2        | 154.8      | 127.1      | 35.4      |
| <b>E2</b>         |             |             |             |            |            |           |
|                   | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>W3</b>         | 263.7       | 279.9       | 123.0       | 406.7      | 447.5      | 314.4     |
| <b>W5</b>         | 157.2       | 226.8       | 228.9       | 449.1      | 388.7      | 301.8     |
| <b>W9</b>         | 228.9       | 126.4       | 263.7       | 403.6      | 391.7      | 245.9     |

**Appendix D: Cell-Seeded scaffolds GAG density descriptive statistics**

**Descriptive Statistics: Volume**  
**Split By: Timepoint, Crosslink Group, Set**

| <b>All</b>        |       |
|-------------------|-------|
| <b>Mean</b>       | 109.6 |
| <b>Std. Dev.</b>  | 115.0 |
| <b>Std. Error</b> | 12.4  |
| <b>Count</b>      | 86    |
| <b>Minimum</b>    | 9.7   |
| <b>Maximum</b>    | 449.1 |

| <b>E1.5</b>       |             |             |             |            |            |           |
|-------------------|-------------|-------------|-------------|------------|------------|-----------|
|                   | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>Week 3</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 17.1        | 14.8        | 17.0        | 135.5      | 106.1      | 19.1      |
| <b>Std. Dev.</b>  | 2.0         | 3.5         | 8.0         | 28.7       | 17.0       | 1.6       |
| <b>Std. Error</b> | 1.0         | 1.8         | 4.0         | 14.3       | 8.5        | 0.8       |
| <b>Count</b>      | 4           | 4           | 4           | 4          | 4          | 4         |
| <b>Minimum</b>    | 15.8        | 9.7         | 12.4        | 97.0       | 91.8       | 17.2      |
| <b>Maximum</b>    | 20.0        | 17.6        | 29.0        | 166.3      | 130.7      | 21.0      |
| <b>Week 5</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 26.5        | 20.0        | 24.7        | 134.0      | 138.4      | 21.1      |
| <b>Std. Dev.</b>  | 4.8         | 8.1         | 2.2         | 61.2       | 23.1       | 0.1       |
| <b>Std. Error</b> | 2.4         | 4.7         | 1.5         | 30.6       | 11.6       | 0.1       |
| <b>Count</b>      | 4           | 3           | 2           | 4          | 4          | 3         |
| <b>Minimum</b>    | 19.8        | 12.7        | 23.2        | 80.5       | 110.4      | 21.0      |
| <b>Maximum</b>    | 30.3        | 28.7        | 26.2        | 220.7      | 166.3      | 21.2      |
| <b>Week 9</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 35.9        | 30.3        | 51.9        | 135.5      | 108.9      | 30.4      |
| <b>Std. Dev.</b>  | 8.6         | 3.6         | 14.2        | 18.8       | 17.6       | 3.8       |
| <b>Std. Error</b> | 4.3         | 1.8         | 7.1         | 9.4        | 8.8        | 1.9       |
| <b>Count</b>      | 4           | 4           | 4           | 4          | 4          | 4         |
| <b>Minimum</b>    | 27.2        | 25.3        | 34.5        | 113.7      | 93.5       | 27.0      |
| <b>Maximum</b>    | 46.6        | 32.8        | 69.2        | 154.8      | 127.1      | 35.4      |
| <b>E2</b>         |             |             |             |            |            |           |
|                   | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>W3</b>         | 263.7       | 279.9       | 123.0       | 406.7      | 447.5      | 314.4     |
| <b>W5</b>         | 157.2       | 226.8       | 228.9       | 449.1      | 388.7      | 301.8     |
| <b>W9</b>         | 228.9       | 126.4       | 263.7       | 403.6      | 391.7      | 245.9     |

**Appendix E: Cell-Seeded scaffolds compressive modulus descriptive statistics**

**Descriptive Statistics: H**

**Split By: Timepoint, Crosslink Group, Set**

| <b>All</b>        |         |
|-------------------|---------|
| <b>Mean</b>       | 16150.0 |
| <b>Std. Dev.</b>  | 15277.7 |
| <b>Std. Error</b> | 1676.9  |
| <b>Count</b>      | 83      |



**Minimum** 568.3  
**Maximum** 76919.2

| <b>E1.5</b>       |             |             |             |            |            |           |
|-------------------|-------------|-------------|-------------|------------|------------|-----------|
|                   | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>Week 3</b>     |             |             |             |            |            |           |
| <b>Mean</b>       | 3376.0      | 1341.7      | 2769.9      | 4268.6     | 10810.0    | 16915.1   |
| <b>Std. Dev.</b>  | 1078.7      | 680.3       | 1378.6      | 1383.8     | 8088.3     | 23381.1   |
| <b>Std. Error</b> | 539.4       | 340.1       | 689.3       | 691.9      | 4044.2     | 13499.1   |
| <b>Count</b>      | 4           | 4           | 4           | 4          | 4          | 3         |
| <b>Minimum</b>    | 2529.5      | 568.3       | 1139.0      | 2790.0     | 3420.3     | 2681.3    |
| <b>Maximum</b>    | 4869.8      | 2225.2      | 4053.9      | 6086.8     | 22229.7    | 43899.8   |

| <b>Week 5</b>     |         |         |         |         |         |         |
|-------------------|---------|---------|---------|---------|---------|---------|
| <b>Mean</b>       | 18770.9 | 13432.4 | 14704.2 | 14633.9 | 16013.2 | 22189.9 |
| <b>Std. Dev.</b>  | 13602.0 | 5587.9  | 178.9   | 7264.3  | 1225.3  | 2177.4  |
| <b>Std. Error</b> | 6801.0  | 3226.2  | 126.5   | 3632.1  | 707.4   | 1257.1  |
| <b>Count</b>      | 4       | 3       | 2       | 4       | 3       | 3       |
| <b>Minimum</b>    | 8259.8  | 7395.2  | 14577.7 | 7830.8  | 15128.8 | 19689.7 |
| <b>Maximum</b>    | 38761.4 | 18422.9 | 14830.7 | 24800.1 | 17411.8 | 23670.0 |

| <b>Week 9</b>     |         |         |         |         |         |         |
|-------------------|---------|---------|---------|---------|---------|---------|
| <b>Mean</b>       | 33500.0 | 35003.4 | 29391.2 | 13845.8 | 46295.8 | 30740.1 |
| <b>Std. Dev.</b>  | 13832.3 | 11353.4 | 13953.3 | 5086.8  | 22445.5 | 14025.7 |
| <b>Std. Error</b> | 6916.2  | 5676.7  | 6976.7  | 2543.4  | 11222.7 | 7012.9  |
| <b>Count</b>      | 4       | 4       | 4       | 4       | 4       | 4       |
| <b>Minimum</b>    | 17252.4 | 20060.7 | 14905.5 | 9867.7  | 28018.8 | 14651.5 |
| <b>Maximum</b>    | 50787.0 | 46508.7 | 48364.8 | 20628.6 | 76919.2 | 46313.7 |

| <b>E2</b> |             |             |             |            |            |           |
|-----------|-------------|-------------|-------------|------------|------------|-----------|
|           | <b>149A</b> | <b>149B</b> | <b>149C</b> | <b>52A</b> | <b>52B</b> | <b>43</b> |
| <b>W3</b> | 2198.4      | 699.1       | 7552.6      | 1910.3     | 1634.3     | 3030.8    |
| <b>W5</b> | 3927.7      | 11288.5     | 10901.9     | 3515.2     | 2962.4     | 23188.4   |
| <b>W9</b> | 3708.2      | 9274.1      | 2760.9      | 6392.1     | 31452.6    |           |

**Appendix F: Linear regression relations split by cross-link group and set**

| <b>Linear Regression Equation</b>  | <b>Correlation Coefficient R<sup>2</sup></b> | <b>Group</b> |
|------------------------------------|----------------------------------------------|--------------|
| H = 6336.217 + 867.317 * GAG / V   | 0.214                                        | E1.5 149A    |
| H = -5379.22 + 1658.152 * GAG / V  | 0.537                                        | E1.5 149B    |
| H = 26564.749 - 759.957 * GAG / V  | 0.089                                        | E1.5 149C    |
| H = 2185.12 + 1832.041 * GAG / V   | 0.665                                        | E1.5 52A     |
| H = 11683.347 + 2373.533 * GAG / V | 0.302                                        | E1.5 52B     |

|                                       |       |           |
|---------------------------------------|-------|-----------|
| $H = 14840.563 + 553.668 * GAG / V$   | 0.173 | E1.5 43   |
| $H = 2424.444 + 271.146 * GAG / V$    | 0.694 | E2 149A   |
| $H = -1989.16 + 3989.929 * GAG / V$   | 0.886 | E2 149B   |
| $H = 1125.838 + 3009.388 * GAG / V$   | 0.221 | E2 149C   |
| $H = 1542.921 + 1929.253 * GAG / V$   | 0.991 | E2 52A    |
| $H = -6423.439 + 13546.496 * GAG / V$ | 0.871 | E2 52B    |
|                                       |       |           |
| $H = 4532.892 + 74.025 * GAG$         | 0.357 | E1.5 149A |
| $H = -7.804 + 107.677 * GAG$          | 0.756 | E1.5 149B |
| $H = 11252.389 + 20.936 * GAG$        | 0.054 | E1.5 149C |
| $H = 1371.367 + 32.2 * GAG$           | 0.536 | E1.5 52A  |
| $H = 7869.791 + 54.747 * GAG$         | 0.367 | E1.5 52B  |
| $H = 15389.046 + 40.722 * GAG$        | 0.196 | E1.5 43   |
| $H = 2069.886 + 4.186 * GAG$          | 0.854 | E2 149A   |
| $H = 1548.701 + 24.255 * GAG$         | 0.454 | E2 149B   |
| $H = 6242.82 + 3.888 * GAG$           | 0.012 | E2 149C   |
| $H = 1437.134 + 9.713 * GAG$          | 0.998 | E2 52A    |
| $H = -6981.795 + 70.72 * GAG$         | 0.882 | E2 52B    |