

**PHYSICAL ANALYSIS OF COLLAGEN-GAG COMPOSITE SCAFFOLDS FOR  
NUCLEUS PULPOSUS TISSUE REGENERATION**

by

Jacob A. Simson

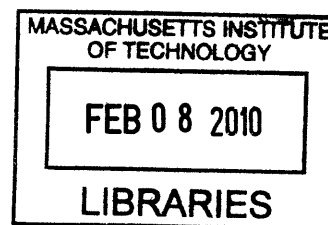
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May 8, 2008

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## **ABSTRACT**

In this study biomaterial scaffolds for regeneration of nucleus pulposus were developed by freeze drying slurries with different proportions of collagen II (CII), chondroitin-6-sulfate (CS), and hyaluronic acid (HA). The scaffolds were analyzed using biochemical assays to determine final composition. Chemically cross-linked scaffolds were analyzed to determine pore size and cross-link density. It was determined that every material type contained large enough pore size (275  $\mu\text{m}$ ) to seed nucleus pulposus cells and mesenchymal stem cells. The addition of CS to the scaffold increased pore size. It was also found that increasing levels of CS and HA resulted in lower cross-link density. These materials will be used next in *In Vitro* studies to determine their viability as regenerative tissue engineering constructs.

Thesis Supervisor: Myron Spector

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## **BACKGROUND INFORMATION**

The intervertebral disc plays a crucial role alongside vertebral bodies in the spine. Whereas the vertebral bodies are stiff, the discs are slightly flexible allowing for slight bending. The summation of these slight movements along the length of the spinal column can result in significant bending of the back. These bending motions are associated with compressive and tensile stresses in the disc, and thus the discs must be capable of bearing mechanical loads. Additionally, the disc must act as a shock absorber to deal with the compressive loads associated with activities such as walking, running, or lifting. The disc is comprised of two main components that work in tandem to distribute the loads equally<sup>1</sup>.

Two distinct tissues make up the intervertebral disc. The inner tissue, nucleus pulposus, is a composite material consisting of a randomly oriented matrix of about 35 percent collagen and 65 percent glycosaminoglycan (GAG) molecules. GAG molecules are highly hydratable, resulting in a gelatinous tissue that is nearly 90% water. This high water content gives the nucleus tissue a nearly incompressible characteristic. Thus when a load is applied the nucleus disc is shortened in height, and as a result it bulges radially towards the outer tissue of the disc, the annulus fibrosis. The annulus fibrosis is responsible for containing this outward bulge of the nucleus in order to maintain the structure of the disc. To satisfy this requirement, the annulus is composed of concentric rings of highly fibrocartilage fibers that connect directly to the vertebral body. As the nucleus bulges outward, the annulus bulges as well, but is able to contain the nucleus. These two tissues work in tandem to distribute the load evenly among the disc.

Two GAG molecules of interest are CS and HA, which are two prominent GAG types present in natural human intervertebral disc. The ratio of sulfated GAG to HA in the

disc is 10:1<sup>2</sup>. CS molecules are covalently bonded to long HA chains<sup>3</sup>, which are necessary for GAG aggregation, and these complexes attract water, making the tissue highly hydrated and gelatinous. The sulfated GAGs are able to attract water due to ionic carbonyl and sulphate groups within their structure<sup>4</sup>. The combination of high water content and high negative charge density makes the nucleus material highly incompressible, and thus allows the nucleus to bulge outwards under loading, distributing the forces horizontally to the annulus<sup>4</sup>.

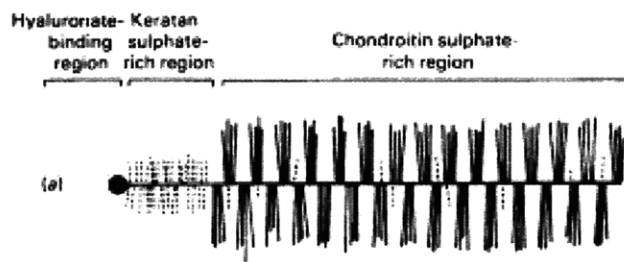


Fig. 1. Model of GAG structure<sup>5</sup>

This is a model of proteoglycans typical of those in intervertebral disc. It contains sulfated GAG molecules attached to a core protein. Many of these proteoglycan aggregates attach to long HA backbones to form large complexes.

At times the disc may bare extremely high loads, resulting in high pressures within the disc. Under these stresses, the disc may fail, allowing the nucleus to protrude into a tear in the annulus fibrosis<sup>6</sup>. This injury is known as spinal disc herniation, and it is very common. In fact, it imposes the greatest economic burden of any illness studied, totaling 100 billion dollars in the United States<sup>7</sup>. The injury often occurs at the rear portion of the disc, where the annulus fibrosis is slightly narrower and not supported by posterior longitudinal ligaments, and thus weaker<sup>8</sup>. At this location, herniations may impinge on the spinal cord or nerve roots, causing discomfort in the back and leg regions. Another problem with nucleus

pulposus is proteoglycan degradation. This results in dehydration of the nucleus, causing it to become more fibrous and lose its ability to distribute loads properly and resulting in degeneration throughout the disc<sup>8</sup>. This can also lead to herniation. One method of treatment for this injury is a discectomy, where the protruding nucleus material is surgically removed, which leaves behind a void where this nucleus material used to exist. The lack of a nucleus results in improper load distribution, and can lead to further degeneration of the joint. Some disc replacement prosthetics have been developed to treat herniated disc, but nucleus regeneration has not yet been achieved.

The goal of this thesis is to develop composite materials that can be used in a tissue engineering scaffold to regenerate natural nucleus pulposus. The success of tissue engineering constructs depends in part on cell signaling that results from interactions between ECM ligands and seeded-cell surface integrins. Therefore, this study is investigating the potential success of nucleus regeneration by using a scaffold with material and structure properties that mimic natural tissue. The presence of natural matrix polymers may direct seeded nucleus cells or stem cells to behave as they would in their natural environment. Additionally, the presence of HA and CS due to the break down of the scaffold prior to tissue formation may allow the cells to incorporate these scaffold materials into the new ECM. This could prevent the cell from having to synthesize these proteins from scratch.

The desired physical structure of the porous scaffold is a randomly oriented matrix of the collagen II and GAG molecules. The material must be highly porous in order to accommodate cell seeding. Natural disc tissue has a cell density of 6,000 cells/cm<sup>3</sup>, but it is common practice to seed millions of cells into a tissue engineering construct in order to accomplish regenerative results<sup>9</sup>. In previous work at the Tissue Engineering Laboratory, a

pore diameter in excess of 250  $\mu\text{m}$  is large enough to seed stem cells, and it is the goal of this project to create scaffolds with pore diameters close to this size. This will allow for future studies to use these scaffolds as cell seeding scaffolds for both nucleus cells and stem cells. It is critical that the pore size is not only large enough to seed the cells, but also small enough to allow enough surface area for sufficient cell and matrix interactions, which are responsible for directing the unit cell processes.

## **MATERIALS AND METHODS**

Chondrocell type II collagen from Geistlich Pharma AG (Wolhusen, Switzerland; Batch 014424), chondroitin-6-sulfate sodium salt from shark cartilage from Sigma (C4384-5G), and non-sterile sodium Hyaluronate from Genzyme Corp (Cambridge, MA: lot 05NP299D) were used in the scaffold preparation without further purification. JB-4 A monomer solution (Cat. # 0226A-800), JB-4 A catalyst (Benzoyl Peroxide, plasticized, Cat. # 02618-12), and JB-4 B embedding solution (Cat. # 0226B-30) were obtained from Polysciences. Aniline blue (Cat. # A-967), glacial acetic acid (Cat. # A A507-500), NaCl (S642, lot # 028434), and ethanol (lot # 100098) were obtained from Fisher. Cytoseal 60 for cover slipping was obtained from Electron Microscopy Sciences (Cat. # 18006). HCl (435570, batch # 00659BH), sodium tetraborohydrate (59640, batch # 074K0211), sulfuric acid (320501, batch # 07666PH), and proteinase-K (P6556, batch # 036K8604) were obtained from Sigma-Aldrich. EDAC (E7750, batch # 03K0753), glycine (G8895, lot # 51K0041), sodium bicarbonate (S5761, batch # 047K0072), carbazole (C5132, batch # 084K0125), and lysine (L5501, batch # 106K2511) were obtained from Sigma. NHS (130672, batch #

0672042) and DMMB (341088, batch # 2902BB) were obtained from Aldrich. TNBSA (28997, lot # JB121704) was obtained from Thermo Scientific, Rockford, IL.

### **AVERAGE SCAFFOLD MASS**

For each material type, three cross-linked scaffolds discs (8mm diameter) were massed on an electronic balance to determine the average scaffold mass.

### **PREPARATION OF SLURRIES**

Slurries were prepared containing 1 wt % collagen II, CS, or HA. Collagen II was dissolved hydrochloric acid at pH 3. CS and HA were dissolved in distilled water. Both the collagen II and CS slurries were blended at 15,000 RPM for 30 minutes to form uniform slurries and break apart large chunks. The HA slurry required 45 minutes of blending at 15,000 RPM to completely dissolve the powder. After blending, the slurries were centrifuged at 1000 rcf for 3 minutes at 4°C to remove trapped air.

Portions of the pure biomaterial slurries were mixed in order to prepare for fabrication of composite scaffolds. In total, six different scaffolds were prepared. The ratios of each material can be seen in Table 1. The slurries were mixed in these ratios and mixed for 5 minutes at 8,000 RPM. They were then centrifuged 3 minutes at 1000 rcf at 4°C to remove trapped air. After centrifuging they were mixed by hand with a metal spatula to ensure homogeneity, but prevent introduction of air into the slurries.



**Table 1. Material Proportions in Slurries for Scaffold Fabrication**

<u>Slurry</u>	<u>% Collagen II</u>	<u>% Hyaluronic Acid</u>	<u>% Chondroitin-6-Sulfate</u>
CII	100	0	0
CII/CS	66.67	33.33	0
CS/CII	33.33	66.67	0
CII/HA	66.67	0	33.33
HA/CII	33.33	0	66.67
NP	34	6	60

### **FABRICATION OF POROUS SCAFFOLDS**

For each slurry, approximately 65 ml was poured into a 5x5 inch metal pan. Visible air bubbles were removed using a 200  $\mu$ m pipette. The pans were then placed into a freeze dryer where they were held at 20°C for 5 minutes before being ramped to -10°C over a 20 minute period. The slurry was held at -10°C for approximately 2.5 hours in order to allow ice crystals to nucleate and anneal. At this time a vacuum was created within the chamber. When the vacuum pressure reached 700 mTorr, the temperature was raised to 0°C and the sample was left 17 hours to allow the ice to sublime.

### **CROSS-LINKING OF SCAFFOLDS**

A water soluble carbodiimide was used to chemically crosslink the scaffolds. Discs with 8 mm diameter and about 2 mm thick were punched from the scaffold sheets. The cross-linking agents were 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS; Sigma) at a 5:2 molar ratio in 100% ethanol. These reagents form amide bonds between glutamic or aspartic acid and lysine residues, resulting in collagen-collagen cross-linking or collagen-GAG cross-linking (Tang Et Al.). The cross-linking was carried out for 4 hours at room temperature and on a rotator to ensure

infiltration of the cross-linking solution into the scaffold discs. After cross-linking the EDAC/NHS solution was poured off and the scaffolds were rinsed in 100% ethanol to remove any residual EDAC. The scaffolds were then lyophilized at -40°C and 300 mTorr overnight.

### **SWELLING RATIO TEST FOR WATER UPTAKE ABILITY**

Six scaffolds from each cross-linked, dried sponge were placed in distilled water at 90°C for two minutes each. This treatment denatures the collagen into gelatin and allows for water uptake. Water was expelled from the pores by placing the scaffold between filter paper and placing a 1.0 kg weight on top for 20 seconds. The sample was then immediately massed wet (WM). The samples were placed in DHT at 110°C overnight. The dried samples were removed from the oven and their masses recorded again (DM). The swelling ratio was then calculated from the following equation

$$r^* = [(DM/\rho_{\text{scaffold}}) + ((WM-DM)/\rho_{\text{water}})] * \rho_{\text{scaffold}} / DM.$$

$\rho_{\text{scaffold}}$  was calculated by adding the weighted densities of the components in the scaffold, where  $\rho_{\text{water}} = 1.00 \text{ g/cm}^3$ ,  $\rho_{\text{CII}} = 1.32 \text{ g/cm}^3$ ,  $\rho_{\text{HA}} = 0.83 \text{ g/cm}^3$ , and  $\rho_{\text{CS}} = 1.44 \text{ g/cm}^3$ <sup>10</sup>.

### **JB-4 EMBEDDING OF SCAFFOLDS**

For analysis of pore size and porosity, 6 scaffolds from each material were embedded in a plastic resin, JB-4. The scaffolds were first dehydrated through three 10-minute soaks in 100% ethanol. The samples were then equilibrated at 4°C in a rotator for approximately 18 hours in a solution of 50% ethanol and 50% catalyzed JB-4 solution. The catalyzed solution

consisted of 0.625g of JB-4 A catalyst (Benzoyl Peroxide, plasticized) in 50 ml JB-4 A monomer solution. Following equilibration, the scaffolds were soaked in 100% catalyzed JB-4 solution at 4°C in a rotator for four days. The solution was changed every 24 hours.

After the fourth day in 100% catalyzed solution, the samples were ready for embedding. Embedding solution was prepared at a ratio of 25:1 of JB-4 catalyzed solution: JB-4 solution B. Scaffold samples were placed face down in plastic embedding molds and the molds were filled with embedding solution. Plastic block holders were placed into each well and the molds were placed in 4°C storage overnight to allow the samples to harden. The blocks were then removed from the mold and allowed to dry at room temperature overnight.

## **SECTIONING, STAINING, AND PORE ANALYSIS OF SCAFFOLDS**

For each material, only 3 scaffolds could be analyzed due to difficulties encountered in the JB-4 embedding procedure. A 5 µm thick section was cut from each scaffold using a microtome and stained with aniline blue to analyze porosity of the scaffolds. Aniline blue is a stain used to identify fibers in connective tissue. The aniline blue solution was prepared with 2.5g aniline blue, 2 ml glacial acetic acid, and 100 ml distilled water, and then filtered. Sections were cut and placed in a water bath with a few drops of ammonium hydroxide. The sections were then set on glass slides and allowed to dry overnight before staining. To stain, the slides were dipped in aniline blue solution for 2 minutes, and then placed in 1% acetic acid solution for 1 minute. They were then dipped in 95% alcohol 5-10 times, followed by 5-10 dips in 100% alcohol in order to remove background staining. The samples were mounted with cyto seal and coverslipped. A technique from O'Brien et al<sup>11</sup>. was used to analyze average pore size for each stained sample.

## **DIMETHYLMETHYLENE BLUE ASSAY FOR CHONDROITIN-6-SULFATE QUANTIFICATION**

In this experiment, scaffolds were digested using a proteinase-K protocol. For each material type, six scaffolds were randomly selected and each was digested in 1 ml of proteinase-K solution. The proteinase-K solution was prepared at a concentration of 0.5 mg/ml, or 0.05 wt%. The scaffolds were placed in 65°C water bath, shaken 40 times per minute, and left overnight to digest. Digested samples were diluted with TE buffer. Scaffolds CII, CII/CS, and CS/CII were diluted by a ratio of 10:1, scaffold CII/HA was diluted by a ratio of 40:1, and scaffolds HA/CII and NP were diluted by a ratio of 80:1. All dilutions are in terms of TE buffer: scaffold digest. Additionally, standard solutions were made over the range of 1.563µg/ml to 200µg/ml. CS stock solution (2mg/ml) is diluted at a 10:1 ratio in TE buffer to make a working solution (200µg/ml). Seven subsequent 2:1 dilutions in TE buffer are then made to produce the standard solutions. 20µL of each standard and sample are added to a 96-well plate. 200µL of DMMB dye solution are then added to each well used. DMMB is a dye that binds selectively to sulfate groups, causing a “metachromatic shift” and allowing the density of sulfate groups to be quantified using absorbance spectroscopy<sup>12</sup>. Readings were taken at 530nm using the WALLAC VICTOR<sup>2</sup> microplate reader and compared against the standard to determine sample concentration.

## **TRINITROBENZENE SULFONIC ACID ASSAY FOR COLLAGEN II QUANTIFICATION**

The quantification of collagen II in each sample was modified from a protocol in Choi et al<sup>13</sup>. Free amino groups will be labeled with TNBSA, thus hydroxyproline present in Collagen II should be easily identified. For each material six scaffolds were chosen at random, and each was placed in a solution containing 1 ml 4% NaHCO<sub>3</sub> and 1 ml 0.5% TNBSA. The solutions were heated at 37°C for 4 hours. 1.5 ml 6N HCl were added and the solutions were vortexed, and then placed in a 65°C water bath until the entire scaffold was broken down. The solution was diluted with 6.5 ml distilled water and 5 ml of the solution was removed from each sample and heated at 37°C for 30 minutes. 5 ml of distilled water was added to the solution and absorbance measurements were taken at 345 nm.

Lysine was used to create a standard curve because it has a free amino group for TNBSA to bind. 8 mg of Lysine were added to a solution containing 2 ml of 4% NaHCO<sub>3</sub> and 2 ml of 0.5% TNBSA. As in the DMMB protocol, seven subsequent 2:1 dilutions were made in solutions containing 1 ml of 4% NaHCO<sub>3</sub> and 1 ml of 0.5% TNBSA. These solutions were then put through the same steps as the digested scaffolds, and absorbance measurements were taken at 345 nm to create a standard curve.

### **CARBAZOLE ASSAY FOR QUANTIFICATION OF HYALURONIC ACID**

The protocol used was modified from Bitter and Muir<sup>14</sup>. 191 mg of sodium tetraborahydrate was dissolved in 20 ml of sulfuric acid to make 0.955 wt % solution. 0.125 wt % carbazole solution was made by adding 0.2083 g of carbazole to 166.4583 g of ethanol. Test tubes were filled with 3 ml sodium tetraborahydrate solution and placed in 4°C to cool. While in the chamber, 0.5 ml of HA solution was added to each test tube from both the scaffold digests and standard solutions. The tubes were shaken gently, then vigorously. The

tubes were then heated in boiling water for 10 minutes and then cooled to room temperature. 0.1 ml of carbazole solution was then added to each solution and shaken. The carbazole interacts with glucuronic acid to form a complex that can be detected using absorption<sup>15</sup>. The tubes were heated again in boiling water for 15 minutes and then cooled to room temperature. 300 µl of each solution were added to a 96-well plate and absorption measurements were taken at 530 nm on the WALLAC VICTOR<sup>2</sup> microplate reader. The standard curve was plotted and used to identify HA concentrations in each scaffold.

## RESULTS AND DISCUSSION

### AVERAGE SCAFFOLD MASS

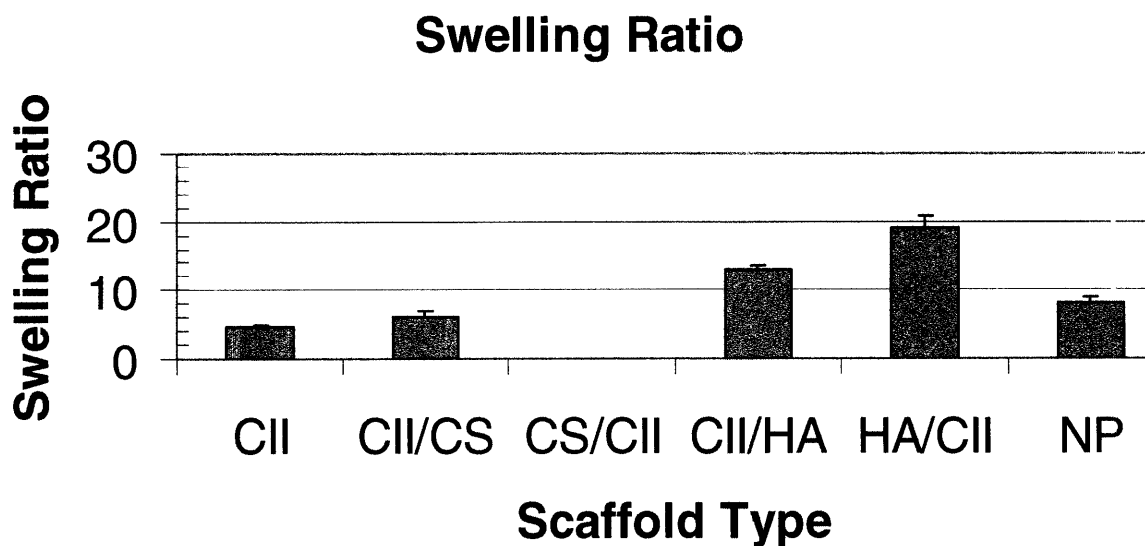
The average mass of each scaffold type can be found in Table 2. The average scaffold mass ranges from 2.37 g (HA/CII) to 3.1 g (CS/CII), which is not unexpected considering HA is the least dense material and CS is the most dense. These average masses are important in determining the compositional make-up of each scaffold type through the three assays.

**Table 2. Average Mass of Cross-linked Scaffolds with 8mm Diameter (n=3)**

Scaffold Type	Average Mass	Standard Deviation
CII	2.77	0.06
CII/CS	2.87	0.06
CS/CII	3.1	0.10
CII/HA	2.47	0.06
HA/CII	2.37	0.12
NP	2.73	0.15

## SWELLING RATIO

For an ideal randomly coiled polymer, the cross-link density is inversely proportional to swelling ratio<sup>16</sup>. Here, the heating of the scaffold gelatinizes the collagen, and it is assumed to behave as a randomly coiled polymer. All of the scaffolds are assumed to behave this way, and the CII scaffold is considered the control. It can be seen in Figure 2 that the addition of GAG to the scaffold increases the swelling ratio, indicating a decrease in cross-link density. One factor for this trend is that the GAG is interfering with the amide bond formation. If collagen-collagen cross-linking is most responsible for cross-linking, lower collagen content would result in lower cross-linking. A second factor to explain this trend is that the GAG molecules are much more capable of holding water molecules, and thus when the scaffolds are pressed with the 1 kg mass to expel water from the pores, the GAG-rich scaffolds still retain more water than CII rich scaffolds. Thus we would see a higher difference in wet mass and dry mass after DHT treatment, and this would imply lower cross-linking.



### *Fig. 2. Swelling Ratios of Cross-Linked Scaffolds*

Swelling ratio is inversely related to cross-link density, and is used to determine the density of cross-linking in a biomaterial scaffold. The material is denatured into a gelatinous, random coil in order to mimic an idealized rubber, and the wet and dry masses of the scaffold are used to compute swelling ratio.

There are two factors that may be affecting the results in this experiment as well. First, it is unclear whether or not these GAG molecules can be treated as randomly coiled polymers. If they do not behave as such on a molecular level, then the swelling ratio data becomes less relevant because the model becomes less applicable. More investigation should be done to determine if the swelling ratio can be feasibly applied to GAG molecules.

The second issue that may be affecting the accuracy of the data is that the CS/CII, CII/HA, HA/CII, and NP scaffolds were crushed under the weight of the 1kg mass. As a result, it was difficult to recover the entire sample, and difficult to ensure accurate massing. Additionally, some samples could not be recovered after DHT because they were not in a robust scaffold, and thus the 'n' value for some material types is less than 6. CII, CII/CS, and CII/HA have an n of 6, HA/CII has an n of 4, and NP has an n of 2. No samples were recovered for CS/CII. If it is determined that these materials require higher mechanical stability for future studies, a different cross-linking method should be considered.

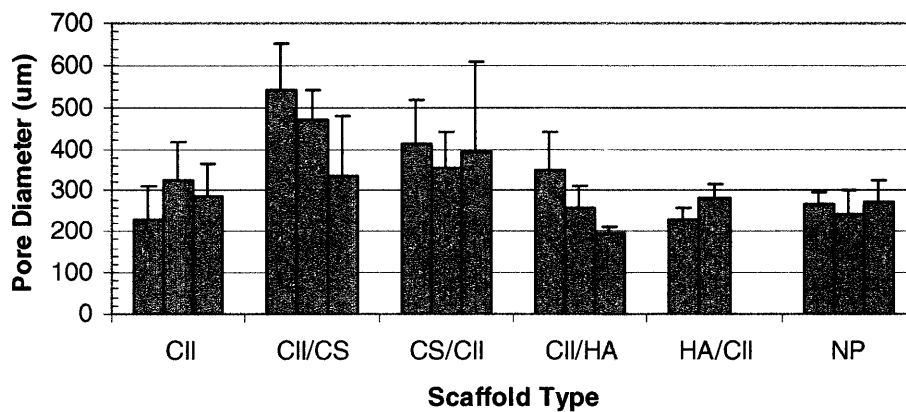
### **PORE ANALYSIS**

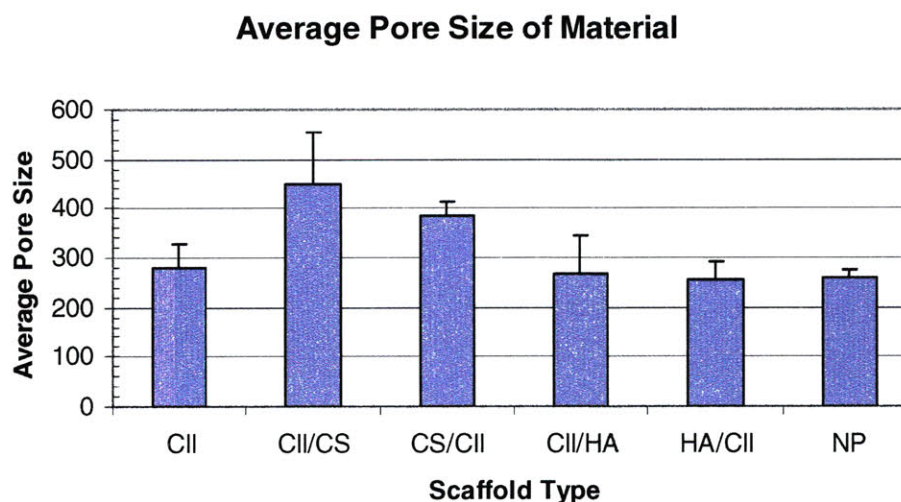
For each scaffold analyzed, six pores were measured to compute the average pore diameter, and for each material type, n=3, except for CII/HA which had an n of 2. The CII, CII/HA, HA/CII, and NP scaffolds were determined to have pore diameters of approximately 275  $\mu\text{m}$ . This result is encouraging because these scaffolds have suitably large pores for the seeding and culturing of mesenchymal stem cells, which is an intended future project using



these materials. CII/CS and CS/CII had slightly larger pore diameters of 450  $\mu\text{m}$  and 380  $\mu\text{m}$ , respectively. The presence of CS in the slurry affects the pore size. Pore size is affected by the rate of freezing. Rate of freezing is controlled by under cooling below the freezing point. Under cooling causes nucleation of ice crystals, which then grow into larger crystals. During the sublimation process, these crystals convert to gas, leaving behind the porous structure of the scaffold. It is the difference between freezing point and freezing temperature that controls ice crystal size. It is possible that the addition of CS is lowering the freezing point of water, essentially decreasing the difference between the freezing point of the slurry and the freezing temperature, which in this case is  $-10^{\circ}\text{C}$ . Decreasing the degree of under cooling would result in less nucleation, larger crystals, and thus larger pore size. To test this supposition, the affect of CS on the freezing point of water should be investigated.

**Average Pore Size of Scaffold**

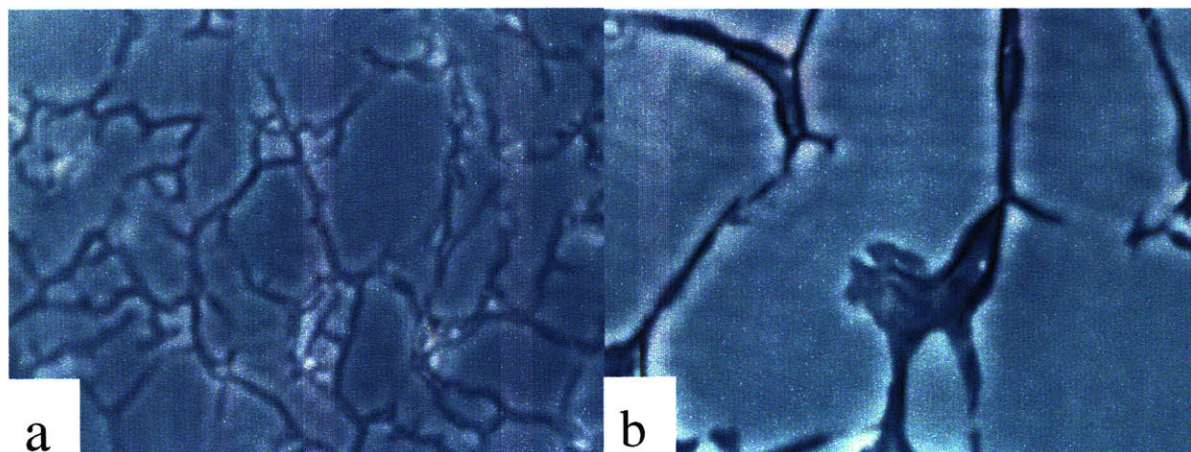




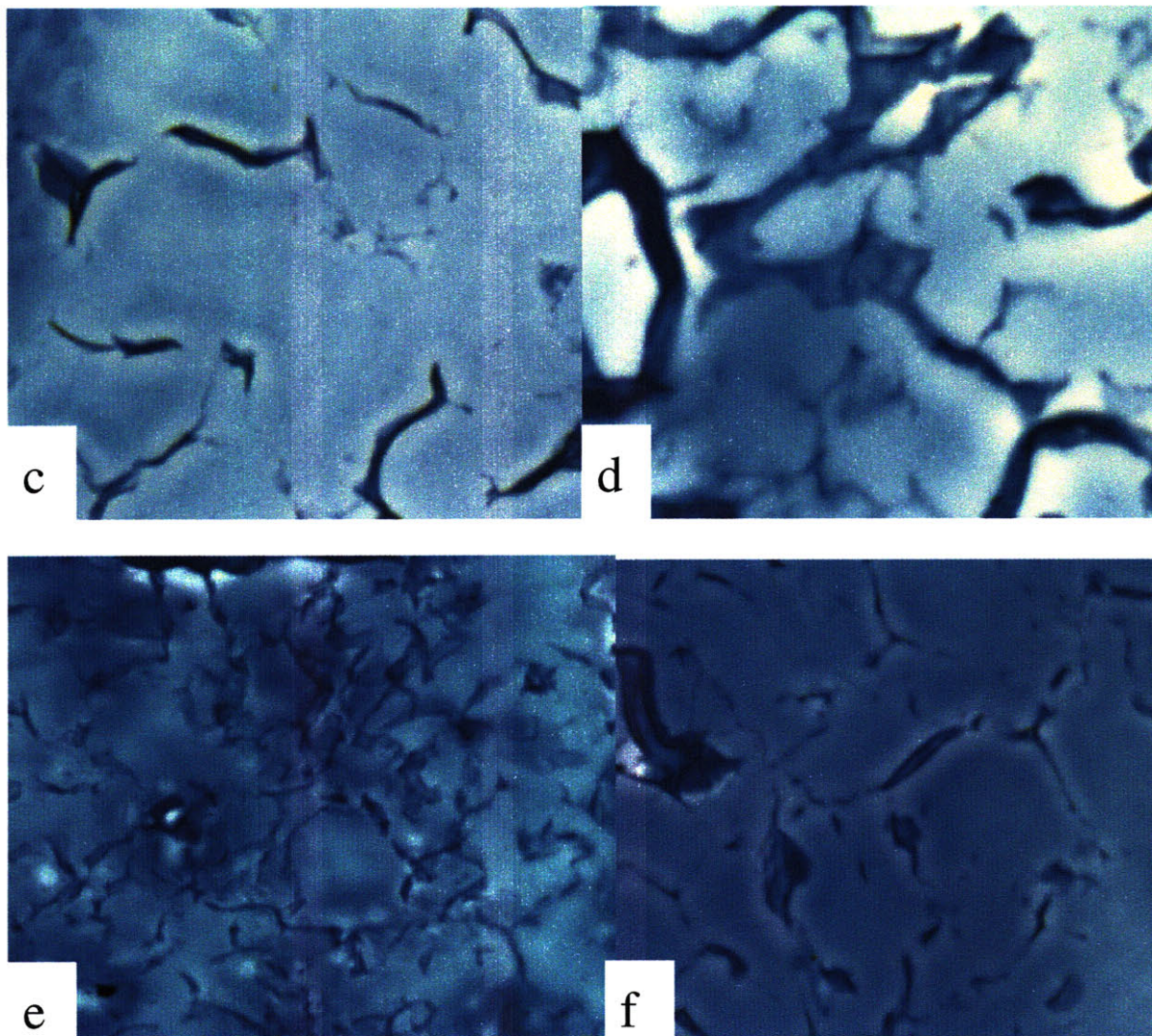
*Fig. 3. Pore Size in Composite Scaffolds*

(Top) The average pore size for individual scaffolds is indicated in this graph. For each scaffold, six pores were analyzed and the pore diameters were averaged. (Bottom) The average pore size for material type is indicated in the graph. The materials without CS are relatively constant around 275  $\mu\text{m}$ , while the scaffolds containing CS have considerably larger average pore diameter.

Figure 4 shows the porous structure of each material type. Notice that the samples including CS, 4a and 4b, have visibly larger pores than the other four materials. It is interesting to consider that we may be able to control pore size not only by controlling the degree of under cooling, but also by changing the GAG concentration in the scaffold. The dark blue staining in the figures are the collagen fibrils, with the background JB-4 polymer staining blue.







*Fig. 4. Aniline Blue Stained Sections of Porous Scaffolds, 4x*

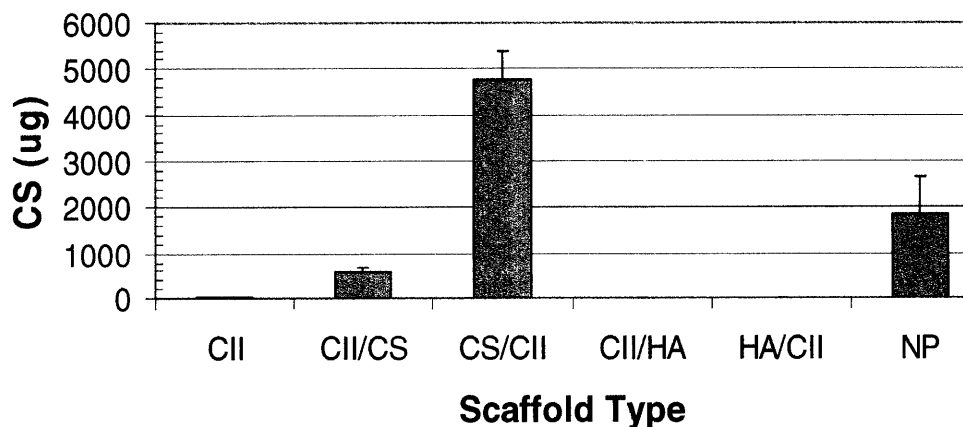
(a) CII - average pore diameter = 280  $\mu\text{m}$  (b) CII/CS – average pore diameter = 450  $\mu\text{m}$  (c) CS/CII – average pore diameter = 386  $\mu\text{m}$  (d) CII/HA – average pore diameter = 269  $\mu\text{m}$  (e) HA/CII – average pore diameter = 255  $\mu\text{m}$  (f) NP – average pore diameter = 261  $\mu\text{m}$ .

#### **DIMETHYLMETHYLENE BLUE ASSAY**

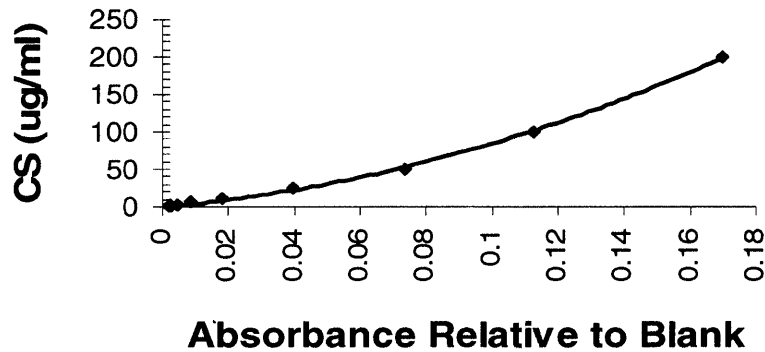
The DMMB assay indicated that materials CII/CS, CS/CII, and NP contained significant amounts of CS, while the other three contained either zero or trace amounts of the GAG. Figure 5a below shows the average CS concentration in each material. The CII/CS

scaffolds started with 33.3 mass percent CS in the slurry, and contained 20.0% CS after freeze drying and cross-linking. The NP slurry contained 60% CS, and in the scaffold form it contained 67% CS. According to the results, CS accounted for 153% of the mass in the CS/CII scaffold. This result is physically impossible and must be the result of an error in sample preparation. These results indicate that, with the exception of the CS/CII material, the CS quantities in the scaffolds are similar to the concentrations that were originally in the slurry. These results indicate that the current method of fabrication and cross-linking is successfully incorporating the desired amount of GAG into the scaffold. Regarding the erroneous CS value in CS/CII, it is possible that performing the assay again could reveal a more realistic CS concentration for CS/CII.

### Chondroitin-6-Sulfate per Scaffold



## Chondroitin-6-Sulfate Standard Curve



*Fig. 5. DMMB Sample and Standard Result*

(Top) DMMB assay results indicate the average amount of chondroitin-6-sulfate in a scaffold.

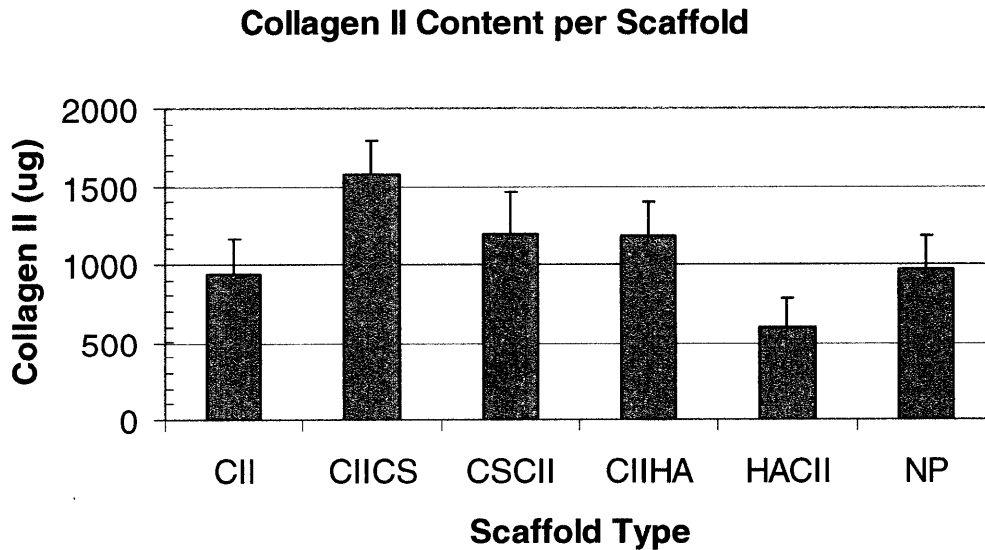
(Bottom) This standard of known CS concentrations vs. absorbance was used to calculate CS concentrations in samples.

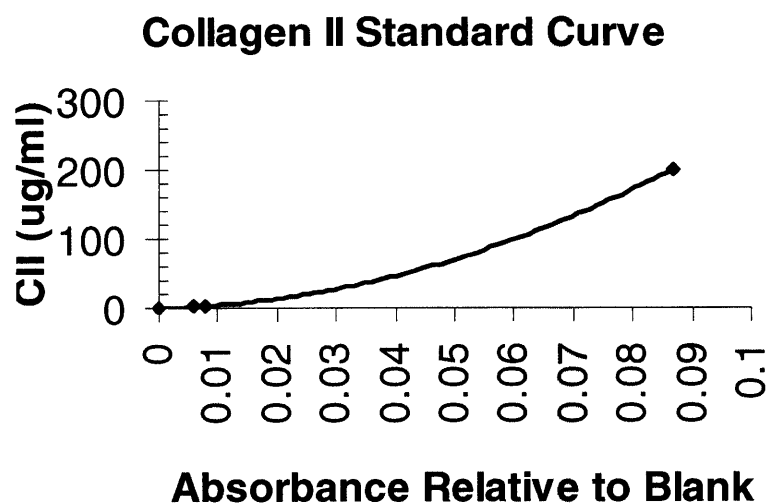
## TNBSA ASSAY

The TNBSA assay produced reasonable results for most of the samples, as seen below in Figure 6. The CII results for CII/CS and CS/CII are 55% and 39%, respectively. These values are close to the 67% and 33% CII proportions in the slurries. Also, the assay results for CII/HA and HA/CII indicate 48% and 25% of mass to be CII. While these values are lower than initial CII concentrations, they again show a 2:1 CII ratio for CII/HA: HA/CII. Lastly, the results of the assay show that 36% of the NP mass is collagen, nearly identical to the 34% of collagen in the NP slurry. For the most part, the results of this assay show that most of the CII in the slurries is being incorporated into the cross-linked scaffold. The lone exception is that of the pure CII scaffold. The results showed that only 34% of the CII scaffold is composed of collagen. Since collagen was the only material in this slurry, this result is most likely inaccurate, so the assay should be performed again to verify the true

composition of the CII scaffold. Additionally, the chemistry of the TNBSA assay should be investigated further to understand if there are other factors that may be affecting the results of any or all of these materials.

One other factor to consider while analyzing these results is that the standard curve could only be generated from three points: 1.5626  $\mu\text{g/ml}$ , 3.125  $\mu\text{g/ml}$ , and 200  $\mu\text{g/ml}$ . The concentration values for the other samples were significantly lower than these three because much of the lysine, which was being used as the standard, was found undissolved and deposited on the inside of the test tubes. Additionally, the standard curve was created at a separate time from the scaffold sample analysis, which could have resulted in differences in sample preparation. This assay should be performed again to verify the results.





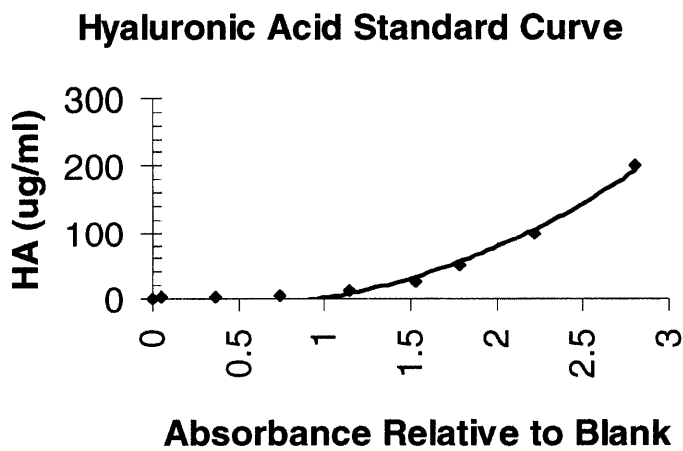
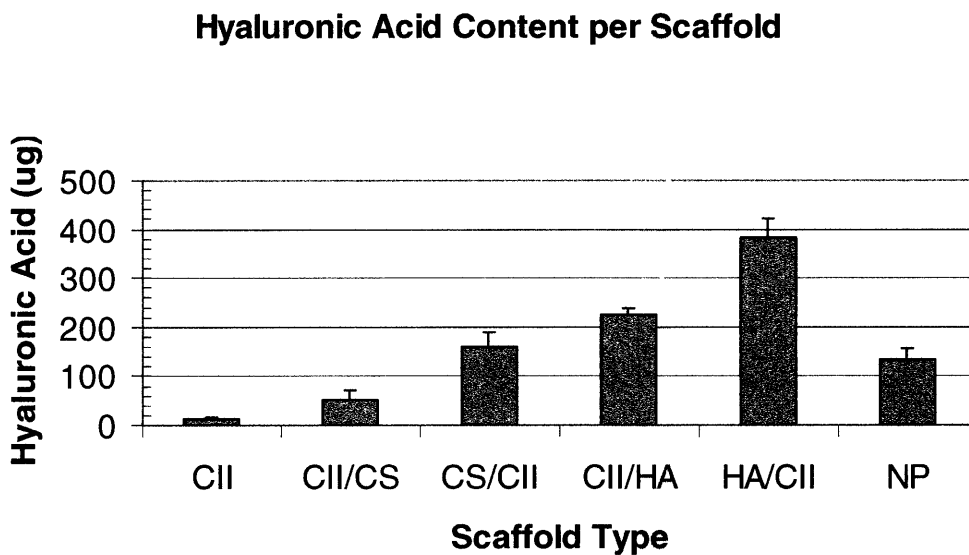
*Fig. 6. TNBSA Sample and Standard Result*

(Top) TNBSA assay results indicate the average amount of Collagen II in a scaffold. (Bottom) This standard of known CII concentrations vs. absorbance was used to calculate CII concentrations in samples.

## CARBAZOLE ASSAY

The carbazole assay indicates that all samples have measurable amounts of HA present. Figure 7 shows the HA sample concentrations determined by the carbazole assay. The CII/HA and HA/CII samples contain 9% and 16% HA by mass, respectively. These scaffold HA concentrations are lower than the slurry HA concentrations, 33% and 67%, but they do resemble a 1:2 ratio of HA in CII/HA: HA/CII. This result indicates that HA is being incorporated into the scaffolds at the same rate in both materials. The CII/CS and CS/CII samples contain 1.8% and 5.2% HA by mass, respectively. This trend indicated that CS was being recognized by the carbazole. Through literature search it was found that CS is composed of alternating repeat units of N-acetylgalactosamine and glucuronic acid<sup>17</sup>. As mentioned in the experimental methods for the carbazole assay, carbazole is known to activate with glucuronic acid. Therefore, the absorbance in the scaffolds containing CS is due not to the presence of HA, but to CS. As a result, we cannot be sure how much of the

absorbance in the NP sample (The HA mass percent would be 4.9% if absorbance was due to HA) is due to CS and how much is due to HA. The presence of HA in the CII scaffold is minimal, at 0.5% of total mass.



*Fig. 7. Carbazole Sample and Standard Result*

(Top) carbazole assay results indicate the average amount of hyaluronic acid in a scaffold. (Bottom) This standard of known HA concentrations vs. absorbance was used to calculate HA concentrations in samples.



## CONCLUSION

This work shows that scaffolds including as much as two-thirds GAG can be fabricated and cross-linked using an EDAC method. The scaffolds are robust enough to undergo JB-4 embedding and microtoming for the purpose of pore size analysis. It has also been shown that increasing levels of GAG in the scaffolds decrease cross-link density. It is unclear at this point whether CII, CS, and HA components in the scaffold can be quantified using the DMMB, carbazole, and TNBSA assays since these methods may detect the presence of more than one of these components. It is important going forward that a method of quantification can be developed in order to identify the exact composition of each scaffold type. Additionally, it will be desirable to perform mechanical testing on these cross-linked scaffolds to get an idea of the strength of these materials, which could be important if these materials are ever developed for an *In Vivo* model. Finally, these scaffolds will likely be used in cell seeding studies to observe the ability of the scaffold to facilitate nucleus regeneration *In Vitro*.

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