Efficient *In-Vitro* Chondrocyte Secretion of Sulphated Glycosaminoglycans

Jake Bewick

***Abstract*—Chondrocytes extracted from a bovine metacarpal-phalangeal joint have been embedded in 4% and 6% agarose constructs. Mechanical testing and sGAG measurements were taken at Day 0 and Day 7. There was no statistically significant difference when measuring the the tangent modulus at 15% strain (F(3,16) = 2.424, p = .104). Recordings of sGAG concentration were more favourable, yielding a statistically signifcant increase in sGAG concentration after a week of growth and within the 4% agarose explant (F(3,72) = 131.393, p = .000).**

I. Introduction

Due to the challenge in restoring *"full tissue function in damaged or diseased articular cartilage"* [1] tissue engineering therapies such as Autologous Chondrocyte Implantation (ACI) grow tissue *in-vitro* to be later implanted into the damaged joint, restoring mechanical function [2]. Chrondocyte expansion can take 3 to 5 weeks [3], optimising the growth medium can speed up this process. To this end we introduce basic cartilage biology and describe two methods of assessing healthy chondrocyte development.

Three varieties of cartilage exist within the human (and bovine) body: hyaline cartilage, fibrocartilage, and elastic cartilage [1]. Articular cartilage is found within the bovine metacarpal-phalangeal joint, this type of cartilage is *"is a soft and specialized hyaline cartilage that exhibits superior lubrication, wear, and low friction properties"* [1].

The chondrocyte is the only cell typical found in articular cartilage, and they are responsible for maintaining the extracellular matrix (ECM) - the source of cartilage's mechanical properties [4]. The ECM consists of three types of structural macromolecule: [4]

- *Collagens* - Provides form and tensile strength
- *Proteoglycans*
  - *Aggrecans* - Stiffness, compression resistance and resilience
  - *Smaller proteoglycans* - Connective tissue, matrix stability
- *Non-collagenous Proteins* - Organise, stabilise and bind

Proper proteoglycan production is important in healthy chondrocytes, and one way of measuring this is to monitor the concentration of sulphated glycosaminoglycan (sGAG) in the system[5].

As sGAG contributes to the compressive resistance of cartilage [4] we should also be able to observe a correlation between sGAG concentration and the mechanical properties of the chonrocyte complex.

Cartilage is a non-vascular tissue and as such nutrients are derived from the synovial fluid via diffusion [6]. As such we must chose a suitable culture medium for chondrocyte development. Agarose is ideal as its open-cell structure allows for unopposed diffusion [7] and the chondrocyte is allowed to remain spherical in shape [8].

Other possible techniques that effect chondrocyte ECM development have been discussed, focusing mainly on the chondrocytes ability to detect and respond to mechanical stimulus [5].

II. Method

1) Preparation of the Agarose/Chondrocyte Cell Culture: Articular cartilage was removed from a bovine metacarpal-phalangeal joint. Enzymes (pronase and collagenase) isolated the chondrocytes by digesting the surrounding extracellular matrix [8]. Cells were washed in phosphate buffered saline and suspended as explants in 10ml of Dulbeccos Minimal Essential Medium (DMEM) supplemented with 20% Foetal Calf Serum (FCS), as described by Lee and Bader [9]. The suspension was stained with Trypan Blue and cell viability was assesesed with the Trypan Blue Exclusion Method [10]. Cells were resuspended at 8×10^6cells ml^{-1} in a DMEM+20%FCS solution. An ultra-low gelling and Earle's Balanced Salt Solution (EBSS) mixture was autoclaved and then cooled to 37°C. The cell solution was mixed with the prepared agarose into 5mm height and diameter moulds, which were then gelled at -4°C for 10 minutes. Damaged specimens were rejected as faults could concentrate stress and affect mechanical testing. Cultures were produced at 4% and 6% agarose with a cell density of 8×10^6cells ml^{-1}.

2) Mechanical Testing of the Agarose/Chondrocyte Cell Culture: Cell culture samples of 4% and 6% agarose concentration were mechanically tested at Day 0 and Day 7 of development. An MTS machine was used to asses the stress relaxation of the cultures.

Specimens were loaded into the MTS and hydrated with EBSS. The machine was aligned and zeroed when a touch load of 0.003N was recorded, and a cross-head speed was set corresponding to a strain rate of $0.334\%s^{-1}$. The machine began compression to a strain of 20%, which was held at 10 minutes to measure stress relaxation. Data was acquired at 10Hz during compression and 1Hz during relaxation. The experiment was repeated five times for each sample preparation method.

3) Determination of Glycosaminoglycan Concentration of the Agarose/Chondrocyte Cell Culture: Spectrophotometric methods were used to determine sGAG concentration. 1-9-dimethylmethylene blue (DMB) "*binds to sulfate and carboxyl groups present in GAG chains*" [11] causing "*metachromatic changes*" [11] which are then detected by a ascent spectrophotometer.

Different concentrations of bovine chondroitin-4-sulphate were mixed with deionized water and DMB. These samples are chromatically analysed in the spectrophotometer to produce a sGAG standard curve, which is used to determine the concentration of sGAG in each sample [8].

4) Statistical Analysis: A one-way ANOVA was used to compare means of the sGAG concentration and the tangent modulus at 15% strain for the four different samples groups. Using ANOVA over the t-test removes the need for multiple comparision - a large source of Type 1 error [12]. Post-hoc analysis was preformed using Tukey's honest significance test. For both cases a confidence interval of $p < 0.05$ was used to indicate statistical significance.

III. Results

1) Mechanical Testing: Chondrocyte-agarose complexes were compressed and held at 20% strain as shown if Figure 1 and then allow to relax.

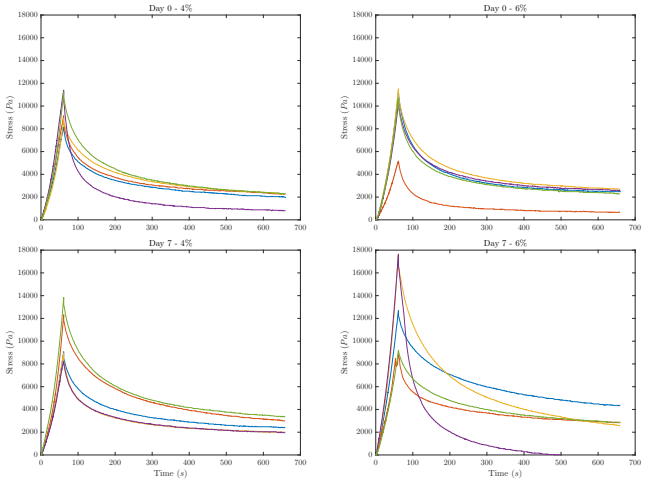

Fig. 1. Stress against time for the compression for chondrocyte-agarose constructs. The different colours correspond to the different samples tested - as show the experiment was repeated five times for each sample permutation. Stress relaxation is observed after strain is held at 20%.

Stress-strain graphs of the compression have been plotted in Figure 2. From this the average tangent modulus at 15% strain was calculated. This data can be found in Table I:

TABLE I
Mean Tangent Modulus at 15% Strain

Sample Preparation	Mean (Pa)	SD (Pa)
Day 0 - 4% Agarose	42720	6612
Day 0 - 6% Agarose	41385	10358
Day 7 - 4% Agarose	44281	8377
Day 7 - 6% Agarose	58080	16498

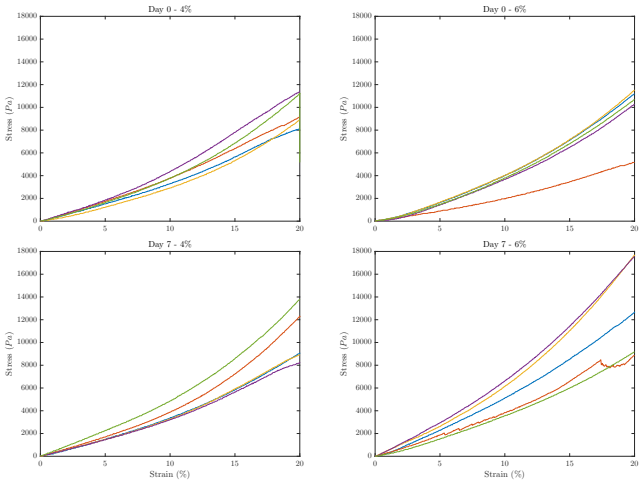

Fig. 2. Stress against strain for the compression for chondrocyte-agarose constructs. The different colours correspond to the different samples tested - as show the experiment was repeated five times for each sample permutation. Anomalous results can easily be identified (e.g. for Day 7 - 4% one sample slips from the machine).

This information is presented graphically in Figure 3:

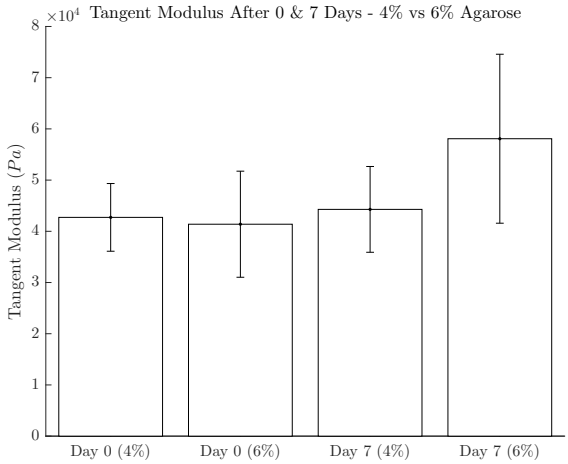

Fig. 3. Mean tangent modulus at 15% strain. Five samples were tested for each group.

Unfortunately, a one-way ANOVA test found no statistically significant difference between any of the groups ($F(3,16) = 2.424$, $p = .104$).

2) GAG Concentration: Mean sGAG concentration was calculated across the five samples using the DMB spectral method. This can be found in Table II:

TABLE II
Mean sGAG Concentration

Sample Preparation	Mean (ug/ml)	SD (ug/ml)
Day 0 - 4% Agarose	8.77	8.47
Day 0 - 6% Agarsoe	8.46	7.76
Day 7 - 4% Agarose	66.16	13.71
Day 7 - 6% Agarose	55.20	14.64

And has been plotted graphically in Figure 4. There was a statistically significant difference between groups as determined

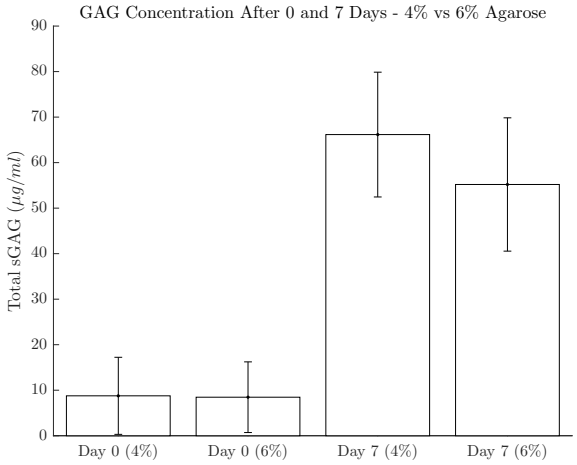

Fig. 4. Mean sGAG concentration for four different chondrocyte-agarose constructs.

by a one-way ANOVA (F(3,72) = 131.393, p = .000). Tukey's HSD post-hoc analysis was preformed to determine between which grous these differences exist. There was no statistically significant difference between Day 0 (4%) and Day 0 (6%), p = 1.000. There was a statistically significant difference between all other groups, $p < 0.050$.

IV. Discussion

Unfortunately we detected no statistically significant mechanical differences among the tested chondrocyte-agarose constructs. This is counter intuitive as the constructs did produce statistically unique levels of sGAG, and sGAG gives "the tissue its ability to resist compression" [6]. We should expect that complexes denser in sGAG would have a higher tangent modulus for this reason. While this looks to be the case (Table I shows the highest modulus is found in the Day 7 samples, where the chondrocytes have had an entire week to develop an sGAG rich ECM) in reality there was far to much variation in recorded results to draw any statistically significant trend.

So why do some samples develop better mechanical properties after one week despite all samples significantly increasing sGAG concentration over the same period of time (Figure 4)?

One possible theory is that the sGAG infused chondrocyte-agarose construct is anisotropic. During gelling it is possible the chondrocytes were not uniformly dispersed into the agarose, or maybe a cell gradient formed under gravity. When uniaxially loaded the constructs would display different mechanical properties depending on their orientation. However this theory fails to describe why the samples mechanically tested on Day 0 have a lower standard deviation.

So we need a theory that describes the large variation in tangent modulus after a week of significant sGAG development. One possible explanation involves the hydration of samples with EBSS prior to mechanical testing. The day 0 samples would stand to already be better hydrated then the day 7 samples, and so if manual hydration was insufficient it could explain the mechanical differences. One possible solution to this is to fully submerge all samples in EBSS prior to testing.

Why do the 4% agarose constructs produce more sGAG than the 6% constructs? Chondrocytes use diffusion for nutrient transfer [6]. The passive diffusive flux of a molecule can be described by Fick's first law [13]:

$$J = -\alpha D \nabla c \tag{1}$$

where J is the diffusive flux, α is the surface area, D is the diffusivity and ∇c is the concentration gradient [13]. A larger percentage of agarose in the explant reduces the effective surface area of the chondrocyte, α. This is counter-intuitive as agarose was only chosen in the first place: because it has an *"open lattice structure when polymerized, minimizing diffusional distances but maintaining the three-dimensional structure necessary for the fixation of cells"* [7].

We can conclude a lower percentage agarose leads to greater diffusion, and as such better nutrient utilization and sGAG production. Other researchers take this further, using an even lower 3% agarose gel [9], [5], [14].

We can turn again to Equation 1 to find other ways to speed metabolite diffusion into a chondrocyte. A large concentration gradient leads to faster diffusion, however over time the metabolite flux into a cell decreases as localised gradients approach equilibrium. To model this a numerical simulation of molar flux into a chondrocyte was programmed with MATLAB:

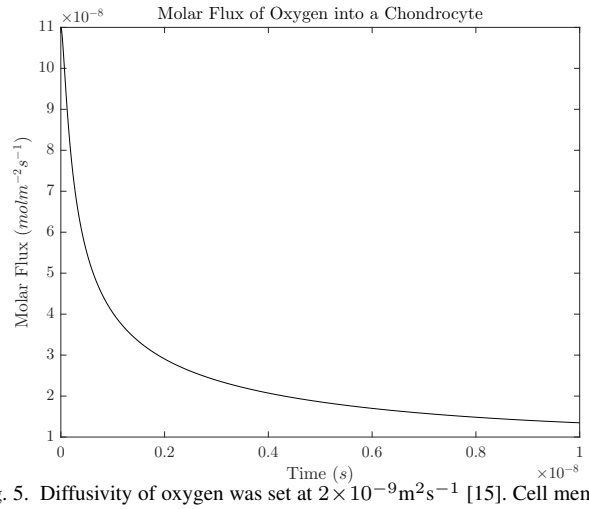

Fig. 5. Diffusivity of oxygen was set at $2\times10^{-9}\mathrm{m}^2\mathrm{s}^{-1}$ [15]. Cell membrane thickness was set at 10nm [16].

We can see from Figure 5 that the majority of metabolite transfer happens very quickly. Beyond that a shallow concentration gradients prevents speedy diffusion. The environment outside the cell should be agitated in some way to ensure fresh solute is delivered to the cell. Use of a bioreactor is one such solution. A simple spinner flask can introduce mechanical agitation, circulating the surrounding medium and preventing stagnation [17]. However this

movement can introduce shear forces onto the chondrocyte, deforming it into a non-spherical shape. One of the primary advantages of using agarose is that chondrocytes remain spherical in agarose [8].

In-vitro growing mediums are designed to simulate *in-vivo* conditons [17], and the knee is regularly subjected to periodic compressive forces which increase proteoglycan production [18]. Mechanical stimulation of the cartilage may produce *"electrical and physicochemical effects"* [4] and may even help with the previously mentioned diffusion problem by changing charge density around the membrane, altering the Donnan osmotic gradients [4].

However our chondrocyte-agarose complex was not loaded in anyway whatsoever. A bioreactor which can dynamically load the chondrocyte complex will increase proteoglycan synthesis [5], [17].

V. Conclusion

A statistically significant difference in sGAG concentration has been found between 4% and 6% agarose-chondrocyte constructs suggesting lower agarose concentrations better facilitate proteoglycan production. A diffusion-surface area based theory has been used to explain this.

There was also a statistically significant difference between in sGAG content between Day 0 samples and Day 7 samples. Our current explant preparation method does allow for proteoglycan synthesis.

Unfortunately there was no statistically significant difference in tangent modulus for any tested samples, despite being expected. Large variations within repeat measurements explain this.

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