EMS617U – Tissue Engineering and Regenerative Medicine

Individual Conference Paper

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Abstract

The aims of the experiments carried out in the lab were to determine the GAG concentrations in 4% and 6% agarose constructs tested after zero and seven days via spectrophotometry, alongside testing the mechanical properties of each construct via a compression test. Graphs of absorbance against GAG concentration and stress against strain were plotted to evaluate the presence of GAG and the compressive strength of each agarose construct. It was concluded that 4% agarose tested on day zero had the highest concentration of GAGs at 67.2 µg/mL, and 6% agarose tested on day seven was the stiffest construct due to having the highest tangent modulus of 0.495 N/mm^2 and, as a result, exhibited the highest compressive strength.

Introduction

Glycosaminoglycans (GAGs) play a critical role in the extracellular matrix of tissue through cell signalling processes. GAGs modulate various cellular processes, including cell proliferation and differentiation [1]. Additionally, GAGs are highly beneficial in skin wound repair, reinforcing the importance of GAGs in tissue engineering applications [2]. GAGs have a polysaccharide-based structure consisting of uronic acid and hexosamines. By interacting with proteins such as growth factors and cell adhesion molecules, GAGs can be effectively used in healthcare products and medicine. Typically, GAGs are produced via extraction from animal tissue, with the exception of hyaluronic acid. However, this method is not robust due to the primary issue of limited availability of tissue to extract glycosaminoglycans. Alongside this drawback, other factors, including impurities, contamination and poor control of animal tissue, greatly affect the sustainability of conventional GAG production. Therefore, alternative approaches have been studied, namely chemical synthesis, chemoenzymatic synthesis and metabolic engineering, in order to increase the longevity of GAG production [3].

A crucial factor to consider when culturing cells is the surrounding environment, as cells are extremely sensitive to changes in temperature, pH, nutrient availability, and oxygen diffusion. Coupled with these components, the mechanical properties of the construct in which cells are cultured have a significant impact on the rate of cell growth and GAG presence. A stiffer structure may provide enhanced mechanical support to the extracellular matrix, as opposed to a more elastic construct, where deformation under an applied force is likely to be more prominent. In addition, a stiffer construct is inclined to be more resistant to temperature change due to tighter bonds between molecules, increasing the energy required to break down the structure. Conversely, a more elastic structure may increase the rate of nutrient and oxygen diffusion due to a less compact construct, allowing space for an efficient diffusion pathway. A construct of higher stiffness may obstruct the diffusion pathway due to a more tightly packed, denser structure, reducing the rate of oxygen and nutrient diffusion. Therefore, a balance of the mechanical properties of a construct must be achieved to cater to structural support, rate of diffusion and resistance to changes in the environment as effectively as possible.

Two experiments will be carried out in this lab. The first involves a GAG assay, where the main objective is to use spectrophotometry to determine the glycosaminoglycan concentration in 4% and 6% agarose constructs, tested at zero days and seven days, to study the effect of time on GAG presence in addition to the result of using different agarose concentrations. By creating a series of dilutions with increasing GAG concentrations and determining the absorbance value

for each solution, a graph of absorbance against GAG concentration can be plotted, which can then be used to calculate the GAG concentration in any unknown samples.

The second experiment involves exploring the mechanical properties of 4% and 6% agarose constructs at zero days and seven days, specifically the compressive strength of each structure. This will be conducted by applying an increasing compressive load on the agarose constructs and recording values for the load and extension over time, which can later be used to calculate the stress and strain for each construct. A graph of stress and strain can be used to investigate the viscoelasticity of 4% and 6% agarose and how this property changes from zero to seven days.

Methods

The primary aim of the GAG assay experiment was to quantify the amount of glycosaminoglycans produced by chondrocytes in 4% and 6% agarose constructs, testing at zero and seven days. Using a pipette, the initial step was to dilute 1 mg/mL of bovine chondroitin-4-sulphate stock solution to 100 µg/mL using deionised water. Using 11 Eppendorf tubes, a series of standard dilutions from 0 to 50 µg/mL of GAG concentration were prepared, each with a total volume of 200 µL. Following this, 20 µL of each standard was pipetted into a 96-well plate, along with four unknown samples. This was repeated on a second row, leaving four blank solutions corresponding to 0 µg/mL of GAG concentration, two of each standard solution, and two of each unknown solution.

The latter portion of the experiment was to determine the absorbance of each standard at 525 nm. This was achieved by adding 250 µL of 1-9-dimethyl methylene blue (DMB) reagent to each well and mixing thoroughly. The plate was then transferred to the ascent spectrophotometer, where results were obtained for the absorbance of each well. Using these values, a graph of absorbance against GAG concentration was plotted. A linear trendline was determined from the graph, and the GAG concentration in the unknown samples was calculated using the formula:

$$\text{GAG Concentration (µg/mL)} = \frac{Absorbance - b}{m} \quad (1)$$

Finally, the GAG concentrations and standard deviations of 4% and 6% agarose at day zero and day seven were calculated and compared.

In the mechanical testing experiment, the overarching goal was to calculate and compare the tangent modulus values of 4% and 6% agarose constructs cultured for 0 and 7 days. Using a micrometer, the diameter and height of each cylindrical agarose construct were measured to be 5 mm. Next, a construct was mounted onto the Instron machine, ensuring suitable alignment to avoid uneven compression. The following step involved hydrating the construct with EBSS solution to mimic physiological conditions, recording any changes in the appearance of the construct during the process. The subsequent stage was running the compression test, which was done by setting the Instron machine to compress the construct, using a speed of 0.0167 mm/s, until 20% strain was achieved. Any changes in the shape or behaviour of the agarose construct were noted. After reaching 20% strain, the stress relaxation values were recorded. The Instron machine's software provided data for the load and extension for each construct, which were also recorded.

In the data analysis section of this experiment, the first step was to calculate the cross-sectional area, stress and strain for each agarose construct using the formulas listed below:

$$\text{Area (mm}^2\text{)} = \pi \times \left(\frac{Diameter}{2}\right)^2 \quad (2)$$

$$\text{Stress (N/mm}^2\text{)} = \frac{Load\ (N)}{Cross\text{-}section\ (mm^2)} \quad (3)$$

$$\text{Strain (\%)} = \frac{Extension\ (mm)}{Original\ length\ (mm)} \times 100 \quad (4)$$

Using the recorded data, a graph of stress against strain was plotted for each construct. The tangent modulus was calculated at 15% strain using the formula:

$$\text{Tangent Modulus at 15\% (N/mm}^2\text{)} = \frac{Stress\ (N/mm^2)}{Strain\ at\ 15\%} \quad (5)$$

The standard deviations for each dataset were determined, and these parameters were compared for the 4% and 6% agarose constructs at day zero and day seven.

Results

GAG Determination

As there were two absorbance values for each GAG concentration, an average was taken for each sample. The values for absorbance, along with GAG concentration, can be seen in Table 1.

Table 1 – Absorbance values for each solution.

GAG Concentration (µg/mL)	0	0	5	10	15	20	25	30	35	40	45	50
Absorbance	0.825	0.771	0.721	0.736	0.764	0.748	0.744	0.752	0.804	0.876	0.791	0.820

Figure 1 below shows the graph of absorbance at 525 nm against GAG concentration for the standard solutions.

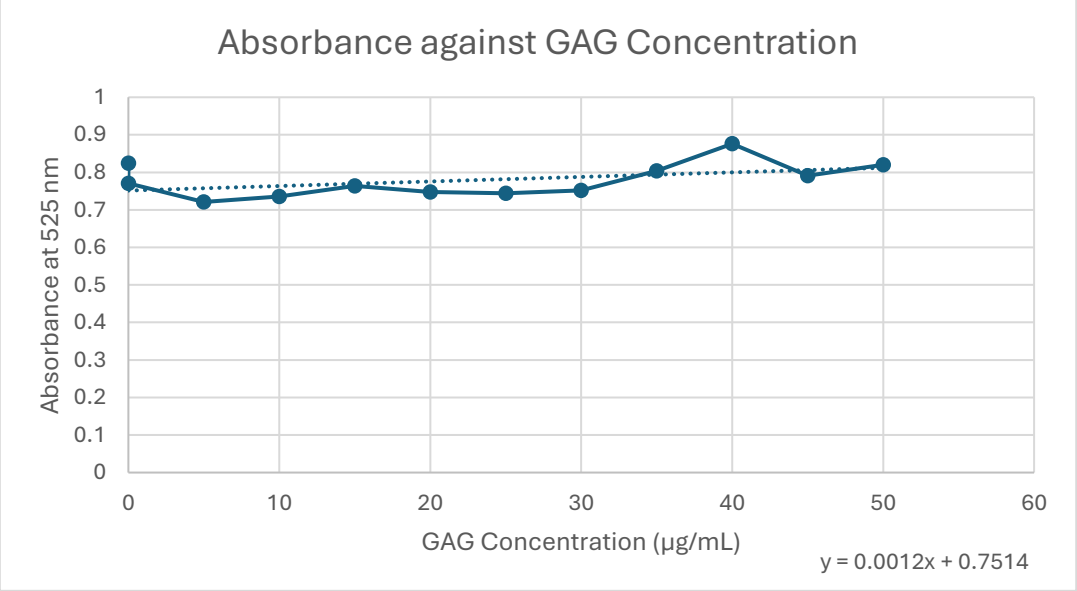

Figure 1 – Graph of Absorbance against GAG Concentration

This graph exhibits a very gradual increase in absorbance in proportion to the GAG concentration, evidenced by a positive gradient. However, though this is the expected result, it can be seen in some portions of the graph that the absorbance is inversely proportional to GAG concentration. These fluctuations in absorbance may have resulted from errors during the experiment.

The standard deviation of this dataset was determined as 0.04507, indicating low variability in the data.

The GAG concentrations for the 4% and 6% agarose constructs at day zero and day seven were calculated using equation (1). Again, an average of the absorbance values was taken for these samples to obtain one absorbance that could be used for each solution. These results have been tabulated below in Table 2.

Table 2 – Results for GAG Concentration of the Agarose Samples.

	6% Day 7	4% Day 7	6% Day 0	4% Day 0
Absorbance	0.733	0.746	0.824	0.832
GAG Concentration (µg/mL)	-15.8	-4.92	60.1	67.2

An immediate anomaly is seen in 4% and 6% agarose on day seven, where the GAG concentration is negative, which is impossible. This may have occurred due to various errors in the experiment. There is a trend in which both day zero agarose constructs have a significantly higher GAG concentration than those tested on day seven. This could be because of a limited nutrient supply for cells, reducing the presence of GAG. When comparing 4% and 6% agarose directly, it can be seen that there is a higher GAG concentration in both types of 4% agarose constructs, possibly due to a lower concentration of agarose in the gel, allowing for a higher uptake of glycosaminoglycans.

Mechanical Testing

Using equation (2) to calculate the area of the cross-section of the agarose constructs and using the load and extension values recorded from the software in equations (3) and (4), the stress and strain values up to 20% strain were obtained for 4% and 6% agarose at day zero and day seven. These are displayed in Figures 2, 3, 4 and 5 below.

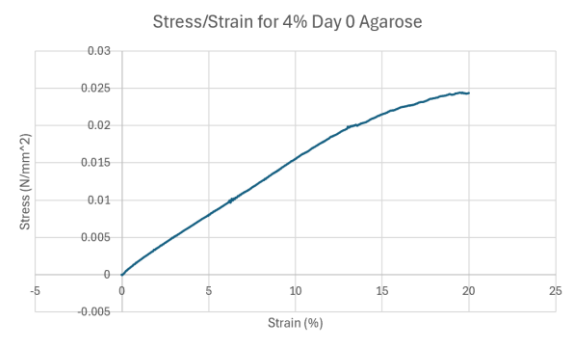

Figure 2 – Graph of Stress against Strain for 4% Day 0 Agarose.

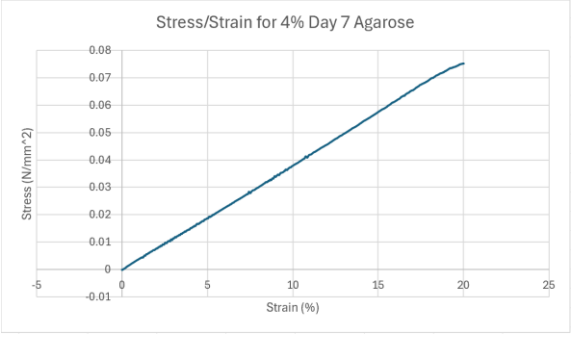

Figure 3 – Graph of Stress against Strain for 4% Day 7 Agarose.

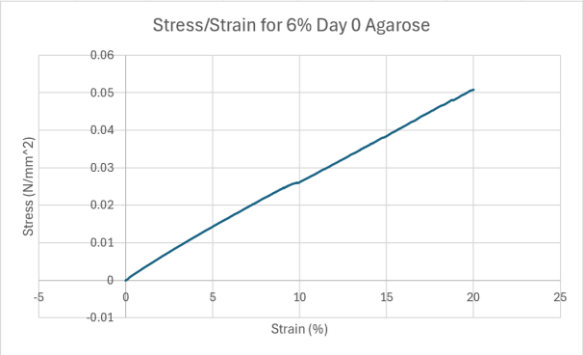

Figure 4 - Graph of Stress against Strain for 6% Day 0 Agarose.

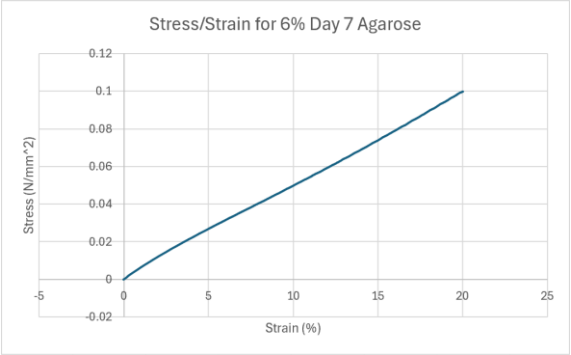

Figure 5 - Graph of Stress against Strain for 6% Day 7 Agarose.

After initial observations, all four graphs demonstrate an increase in stress directly proportional to strain and eventually plateau. As the trend remains consistent for all of the constructs, it can be assumed that all possess viscoelastic properties.

Comparing the stress-strain graphs for day zero and day seven, it can be seen that the stress reaches a higher value before plateauing for the day seven constructs. Another key observation is that when analysing the 4% agarose constructs, the construct tested on day zero reached maximum stress relaxation more gradually than the day seven construct, as indicated by the diminished slope in Figure 2, in contrast to the steeper transition in Figure 3. Therefore, an argument could be made that the constructs tested on day seven display higher compressive strength and stiffness than those on day zero due to withstanding higher stress and experiencing a faster transition into deformation.

When directly comparing the 4% and 6% agarose constructs, the 6% agarose constructs experience higher stress before deforming than the 4% agarose constructs, signifying that the 6% agarose constructs have higher compressive strength. Similarly to comparing days zero and seven, the 4% agarose construct has a slower transition into maximum stress relaxation, which can be seen when observing the results for the constructs tested on day zero. Furthermore, there is a more subtle occurrence of this when comparing the constructs on day seven, reinforcing this trend in the data. Therefore, evidence suggests that the compressive strength of the 6% agarose constructs is greater than that of the 4% agarose constructs. Moreover, the results reflect that the 6% agarose constructs have a higher stiffness and, consequently, are less viscoelastic.

Table 3 below shows the calculated tangent modulus at 15% strain for each agarose construct, using equation (5), as well as the standard deviation for each dataset.

Table 3 – Tangent Modulus at 15% strain for each agarose construct and Standard Deviations for each Dataset.

	4% Day 0	**6% Day 0**	**4% Day 7**	**6% Day 7**
Tangent Modulus (N/mm^2)	0.139	0.258	0.382	0.495
Standard Deviation	0.00749	0.0145	0.0223	0.0282

The tangent modulus for both day seven constructs is noticeably higher than for day zero. A similar point can be made when comparing the 4% and 6% agarose constructs, where both 6% agarose constructs experience a higher stress, thus implying a higher compressive strength. These outcomes may have been due to the stiffness of each construct, where the stiffer the structure is, the higher the resistance to compression.

Discussion

The expected result for the GAG assay experiment was a directly proportional relationship between absorbance and GAG concentration. Generally, the amount of light that gets absorbed is affected by the number of molecules that it interacts with. As a result, a more concentrated solution would have a higher absorbance [4]. Given this, it can be inferred that the decrease in absorbance at certain points in Figure 1 may have been due to errors in the experiment. One potential source of error could have been the insufficient mixing of each solution. If the solutions are not mixed thoroughly enough, the glycosaminoglycans may settle in one area of the solution, thus decreasing the absorbance due to allowing light to pass through parts of the solution with fewer GAGs. Another cause of error could have been the resolution of the pipette, which may have deviated the intended volume of the solution, resulting in an inaccurate volume in each well and, in turn, decreasing the absorbance. In connection with this, a parallax error may have occurred when setting up the pipette, resulting in the incorrect volume being added to the wells. The final source of error could have resulted from either the DMB reagent or the standard solutions spilling on the walls of the well. This might have reduced the number of GAGs the light interacted with, allowing more light to pass through and thus decreasing the absorbance value.

Another unusual set of results in the GAG assay experiment was determining the GAG concentration in the unknown samples. Particularly for the 4% and 6% agarose constructs on day seven, the GAG concentrations were calculated as negative values, which, practically, is not possible. However, the source of this inconsistency was likely due to the errors stated above.

In the mechanical testing experiment, it was seen from the tangent modulus values that the constructs tested on day seven could tolerate higher stress than those tested on day zero, suggesting that the day seven constructs have greater compressive strength. As the constructs are given more time to solidify, they can withstand a higher load due to forming a stiffer structure through agarose gelation, hence becoming more difficult to deform initially. Another factor contributing to this result may have been the hydration of the agarose construct. Before beginning the compression test, the construct was hydrated with EBSS solution, which may have caused loose bonding in the agarose constructs tested on day zero, as opposed to day seven, where the constructs may have dehydrated, leading to the agarose becoming denser and more compact, ultimately increasing the compressive strength of the construct [5]. Another finding from the results suggested that the stiffness of the agarose constructs tested on day seven was greater than those tested on day zero. As evidenced by the points mentioned previously, allowing time for the agarose constructs to solidify tightens the bonds between molecules to form a stiffer structure.

The comparison of the 4% and 6% agarose constructs showed that the 6% agarose construct exhibited higher compressive strength, parallel to when comparing the day zero and day seven structures. This difference may have been caused by the variation in mechanical properties in 4% and 6% agarose. In 6% agarose, there is a higher agarose concentration in the gel, meaning that the gel is more densely packed, allowing for greater resistance to compression, whereas, in 4% agarose, a less dense gel is formed, lowering the compressive strength. When analysing the end portion of each graph for the 4% and 6% agarose constructs, there is a sharper transition to maximum stress relaxation displayed in the 6% agarose graphs. For a similar reason to the

distinction in compressive strength, the cause of this steep decline in the slope is due to the properties of the 6% agarose construct, in that it has a higher stiffness, so the rate of change of deformation is rapid, provided a high enough stress is applied to the structure. This contrasts greatly with the 4% agarose construct, where the rate of change of deformation is much slower due to possessing lower stiffness.

Conclusion

In conclusion, the results of the GAG assay experiment show that over a series of solutions with increasing GAG concentrations, the absorbance value increases proportionally. The results show that glycosaminoglycan presence in both agarose constructs at day zero was significantly higher than in the agarose constructs tested at day seven, possibly due to the lack of nutrients and hydration in the day seven constructs, causing GAG concentration to decrease. Comparing the 4% and 6% agarose constructs on both day zero and day seven, it was clearly shown that GAG presence was more prominent in 4% agarose, potentially due to the lower density of the gel, allowing greater uptake of glycosaminoglycans.

To conclude the results of the mechanical testing experiment, there was a clear distinction between the 4% and 6% agarose, but alongside this, the day on which they were tested also played an important in the compressive strength of each construct. On day zero, the constructs were freshly hydrated and not given enough time for gelation to occur and stiffen the structure, causing the day zero constructs to deform at a quicker rate relative to the day seven constructs, where time was given for the gel to solidify, hence increasing the resistance to compression. Comparing the 4% agarose and 6% agarose constructs, there was a clear difference in that the 6% agarose exhibited a higher compressive strength than 4% agarose, likely due to a denser gel, allowing a tighter and more rigid structure, which could withstand greater stress.

Deciding on the optimal construct for cell culturing can be challenging due to the various factors. While stiffer structures are more suited to provide support to the extracellular matrix and resist changes to the environment, elastic structures provide a nutrient and oxygen diffusion pathway that is more efficient. Setbacks such as these highlight the core obstacles in tissue engineering, where there is currently no ideal solution in a situation.

References

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[5]