5-2024

Optimization of Collagen and Cell Concentrations for Engineered Tissues

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An Undergraduate Honors College Thesis in the Department of Chemical Engineering

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Spring 2024

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Abstract

Improvements regarding the drug discovery process are necessary due to less than 1% of drugs making it from the initial discovery phase to patient treatment in clinics. One method of enhancing this process involves improving the relevance and dependability of in vitro testbeds utilized during pre-clinical testing. Improvement along these factors can provide a more relevant view for eventual drug behavior in patients. This additional relevance can allow for a reduced timeline in drug development for improving patient care. A consistent and morphologically sound baseline engineered tissue is vital to the creation of successful experimental models. Parameters such as cell and collagen concentration in engineered tissue fabrication affect the eventual mature morphology of the engineered tissue. Characteristics impacted by these parameters include tissue compaction, activity, uniformity, and functionality. These characteristics can inform optimal conditions. The aim of this study is to determine which cell and collagen concentrations yield a superior testbed for future in vitro testing of collagen based engineered tissues utilized in mechanical stretch studies where morphology is critical. Optimal behavior was found in condition A, possessing the lowest cell (1.75E+06 cell/ml) and collagen (1 mg/ml) concentration combination, due to its ideal compaction results not requiring a mechanical release. Lower protein concentrations were found to increase compaction, along with activity, uniformity, and functionality results. Future experiments will utilize condition A as the superior testbed for applying mechanical conditions in strives to improve the drug delivery process for the goal of patient benefit.

Keywords: collagen, engineered tissue, gel fabrication, optimize, cell concentration, protein concentration

I. Introduction

Personalized medicine is becoming more vital than ever as one American loses their life every minute due to a coronary event.[1] Less than 1% of drugs are able to make it from the initial discovery phase into clinical use for patients.[2] Due to this minimal number, advancements of in vitro models can enhance improvements in drug development, yielding more successful trials. With reduced drug development timelines, an improvement in patient care can be achieved.

Improved in vitro models can provide the advantage of greater predictivity in the development process. A method of improving these models is to incorporate greater physiological relevance through the customizability that can be applied with engineered tissues.[3] Achieving repeatable quality and morphology in the formation of customizable in vitro models will improve the reliability and precision of future experimentation by having a strong and dependable baseline.[4] Precise in vitro models can be used for testing purposes to discover successful results through realistic tissues that properly reflect characteristics in potential human tissue.[5-6]

Appropriate replication of tissue properties when studying in vitro models is vital in order to obtain transferrable results.[7] Physiological relevance and consistency are fundamental for in vitro models to be effective during the drug discovery phase.[8] Compaction, activity, uniformity and functionality are characteristics of 3D in vitro tissue constructs which influence the experimental results. Optimizing these conditions can allow for the fabrication of useful engineered tissues. Assessing these factors in the context of cell concentration and collagen concentration can provide valuable insights into the effect of initial cell culture parameter conditions on the ultimate experimental outcome.[9] Therefore, determining optimal parameters is essential to producing both legitimate and consistent results. The purpose of this research was to improve

engineered tissues for future experimentation by optimizing cell and collagen concentrations. Value can be provided through improving in vitro testbeds with relevant engineered tissues for more effectively bringing discovered drugs to patient treatment.[10]

II. Methods

a. Cell Culture Protocol

The cells utilized during this experiment were from a green fluorescent protein transfected NIH Swiss mouse embryo fibroblast cell line, NIH3T3/GFP (Cell Biolabs Inc, CAT#: AKR-214). T75 flasks were seeded with 1M cells and cultured under standard culture conditions (5% CO_2, 37℃) with fibroblast media (DMEM, 10% FBS, 0.1% penstrep, 0.05% ampho-B, L-abscorbic acid). Cells were passaged until experimental utilization at passage 6. Media was changed every other day during initial culture.

b. Experimental Equipment

A custom culture plate was designed in AutoCAD Inventor and then 3D printed from Biomed Clear v2 resin with the Form 3B SLA Printer (Formlabs). Prior to use, the culture plate was sterilized via autoclave. The wells of the custom plate contained stationary grip mounts which collagen gels would form around to anchor them in place. This anchoring maintained their length and allowed compaction across their width during the experimental protocol.

c. Gel Fabrication

Rat Telocollagen Type I From Tail (Advanced Biomatrix Cat#: 5153-100MG) served as the collagen source for the gels. The kit provided neutralization solution (Advanced Biomatrix Cat#: 5155-10ML) was utilized in conjugation with the kit provided collagen solution at a 1:9 ratio to form the desired 3D gels. This solution was beneficial as it eliminated the need for

additional titrations and errors, providing a proper collagen solution, ready to be utilized for gel formation.

To begin the gel fabrication process, cells harvested after passage 6 were counted and suspended in media at appropriate concentrations. The cells were then mixed first with the neutralization solution and then with the collagen solution at volumes that satisfy the desired experimental conditions presented in Table 1. These experimental conditions are the varied concentrations of cells and collagen utilized during the experiment.

Table 1: Experimental Conditions.

Condition	Protein Concentration (mg/ml)	Cell Concentration (cells/ml)
A	1	1.75E+06
B	1	3.50E+06
C	2	1.75E+06
D	2	3.50E+06

Shown in Table 1, the A and B conditions possessed a consistent protein concentration with differing cell conditions. Conditions C and D were introduced in order to present higher protein concentration conditions with cell concentrations matching respectively both A and B. These conditions enabled analysis of effects resulting from cell concentration and protein concentration variations. The control group for this experiment was condition A, as it was the concentration pair currently utilized by the lab.

Once the conditions were prepared, the 85uL of solution would be transferred into each well of the sterile well plate to allow gel formation to begin. The gels were incubated at 37 ℃, 5% CO_2 for the duration of the experiment with daily media changes using a gel media prepared

by adding 0.02 mg/ml of aprotinin to fibroblast media. At Day 3, a mechanical release technique was implemented to dislodge any gels that did not self-detach from the walls of the well. A syringe was moved along the sides of all gels in the well plate in order to mechanically dislodge them and remove the wall attachment influence on morphology for the remaining days of the time course. After this procedure, the gels continued to remain in the incubator for the remainder of the time, except when images were captured, without any physical adjustments.

d. Data Analysis Tools

Images of all gels were captured daily over the 6-day period. Images were then uploaded to an in-house custom machine learning (ML) pipeline, where gel tracing software analyzed the gel morphologies. The ML pipeline was utilized to accurately trace the gel images and produce measurements of length and width in pixels. Data was outputted in percentiles ranging from the 10^{th} to 80^{th} percentile in increments of tens.

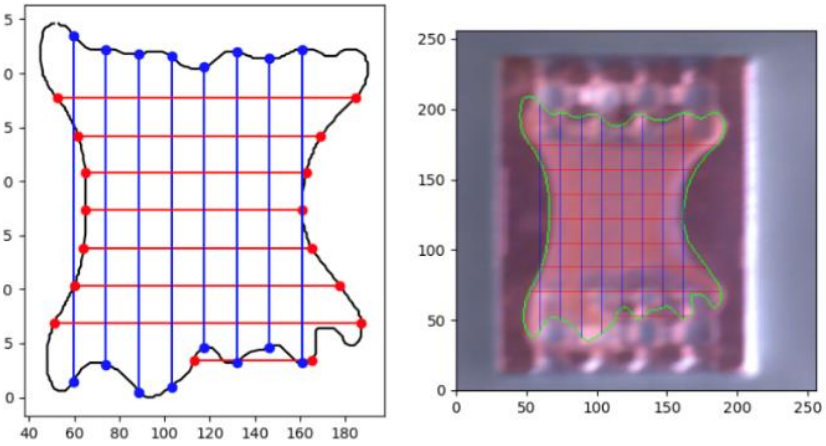

Figure 1. Example of an accurately traced gel.

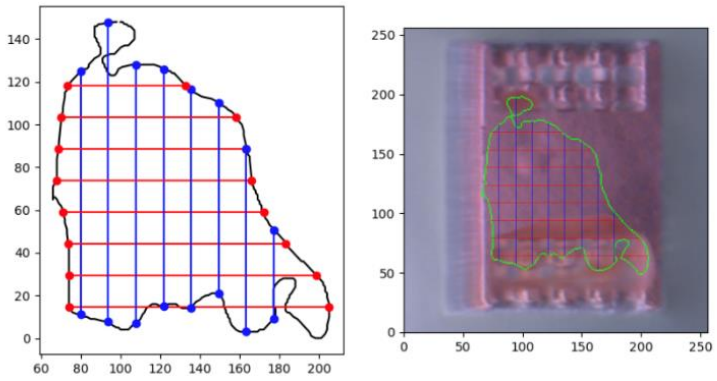

Figure 2. Example of an inaccurately traced gel.

Figures 1 and 2 present example images and measurements captured for each individual gel, showing both an accurate and inaccurate tracing. These tracings were reviewed in order to ensure the most accurate readings were utilized. An accurate tracing would be classified as outlining the edge shape of the gel, as seen in Figure 1; therefore, inaccurate tracings were not utilized in order to prevent unrealistic outcomes of gel morphology. Figure 2 highlights a gel that failed to form and was inaccurately traced. From the length and width pixel values determined, the morphology of the gel was able to be analyzed. The analysis was based upon the percentile calculations and yielded grip width, middle width, boundary length and middle length values.

III. Results and Discussion

a. Graphical Representation and Interpretation

Analysis of the gel morphology, based on calculations from the ML pipeline data, yielded results regarding the characteristics of compaction, activity, uniformity and functionality. The boundary length and middle length value calculations indicated results regarding uniformity of the gels. Meanwhile, grip width and middle width values were used for results pertaining to compaction, activity, and functionality. Since condition A is the literature informed control

group, alternative collagen and cell concentration conditions were used experimentally to determine the superior testbed.

The statical significance was assessed for the results regarding compaction, activity, uniformity, and functionality using ANOVA or t-tests. An n-way ANOVA and multiple comparisons analysis was conducted through MATLAB software. This enabled the evaluation of the relationships between the variables of day, cell, and protein concentration with the middle width data. An analysis of variance yielded results regarding compaction.

Compaction results were investigated through the middle width values of the gel to determine the size of the central region over the time course. Middle width values were calculated by averaging together the widths located at the 40^{th}, 50^{th}, and 60^{th} length percentiles of each individual sample over the 6-day period. The middle width value for the 5 samples for each condition at each day, provided results regarding compaction of the gels over the 6-day period, as presented in Figure 3.

The analysis of variance results indicated protein concentration directly impacted the middle width value while cell concentration alone did not. However, the interaction of cell concentration and day of analysis yielded a p-value of 0.0489 from the results. This value being less than 0.05 indicated the interaction of cell concentration and day of observation played a role in the variance of collagen gel middle width value across the time course.

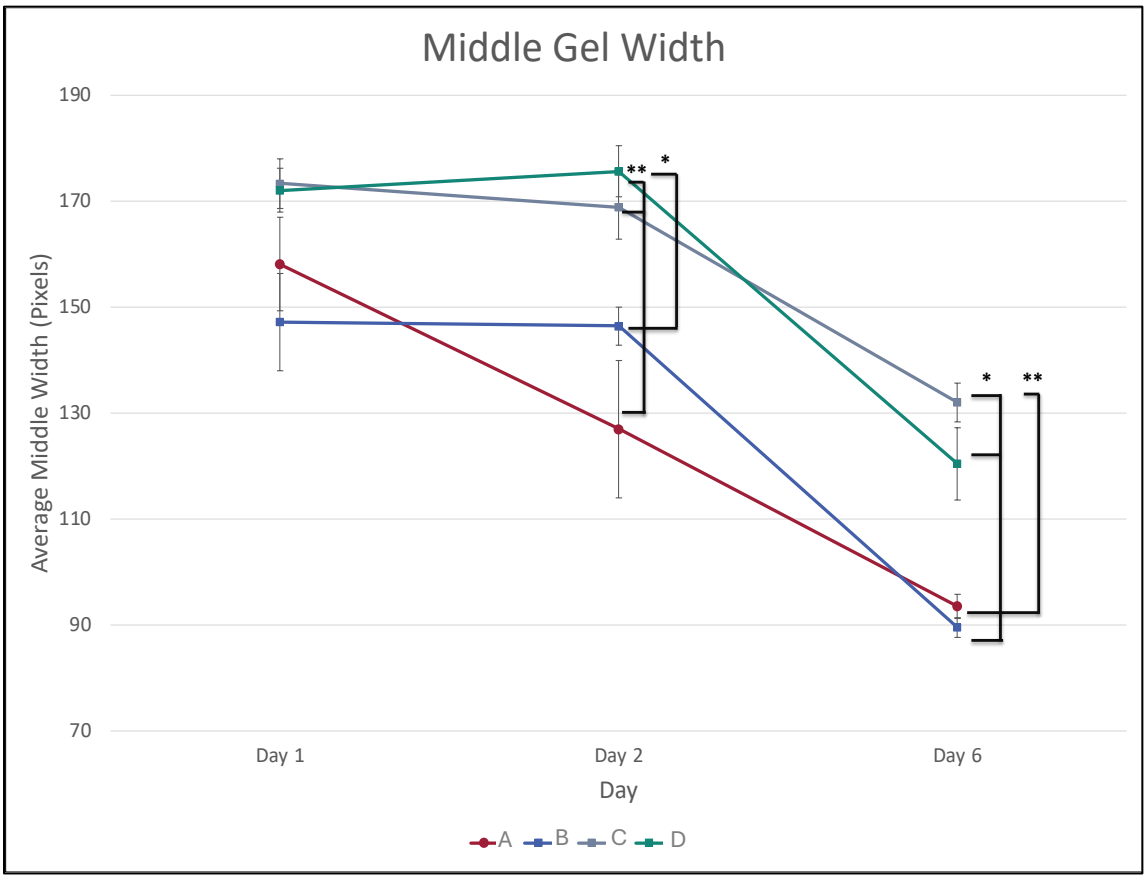

Figure 3. Compaction. Middle width values were calculated using the 40th, 50th, and 60th length percentiles of each sample over the 6-day period. Condition A appears to linearly decline over the time course as opposed to the alternative conditions. Statistical significance between conditions A&C as well as B&D depicts the effect of protein concentration on morphology. (Sample size: n=5, ** $p < 0.01$, * $p < 0.05$)

Based on Figure 3, Condition A appeared to linearly decline over the time course as opposed to the alternative conditions. This linear decline suggests condition A had a steady middle width compaction over the 6-day period. Statistical significance between conditions A and C as well as B and D highlighted the effect of protein concentration on morphology. It is apparent the lower protein concentration conditions possessed overall lower average middle widths than higher protein concentration conditions over the time course.

Another vital characteristic for achieving ideal morphology is uniformity. Length values were evaluated to determine the consistency in the length of the samples over the time course. Figure 4 displays gel edge length differences which were determined by calculating the absolute value of the difference between the length of each 20^{th} & 80^{th} width percentile for all conditions across the sample short axis.

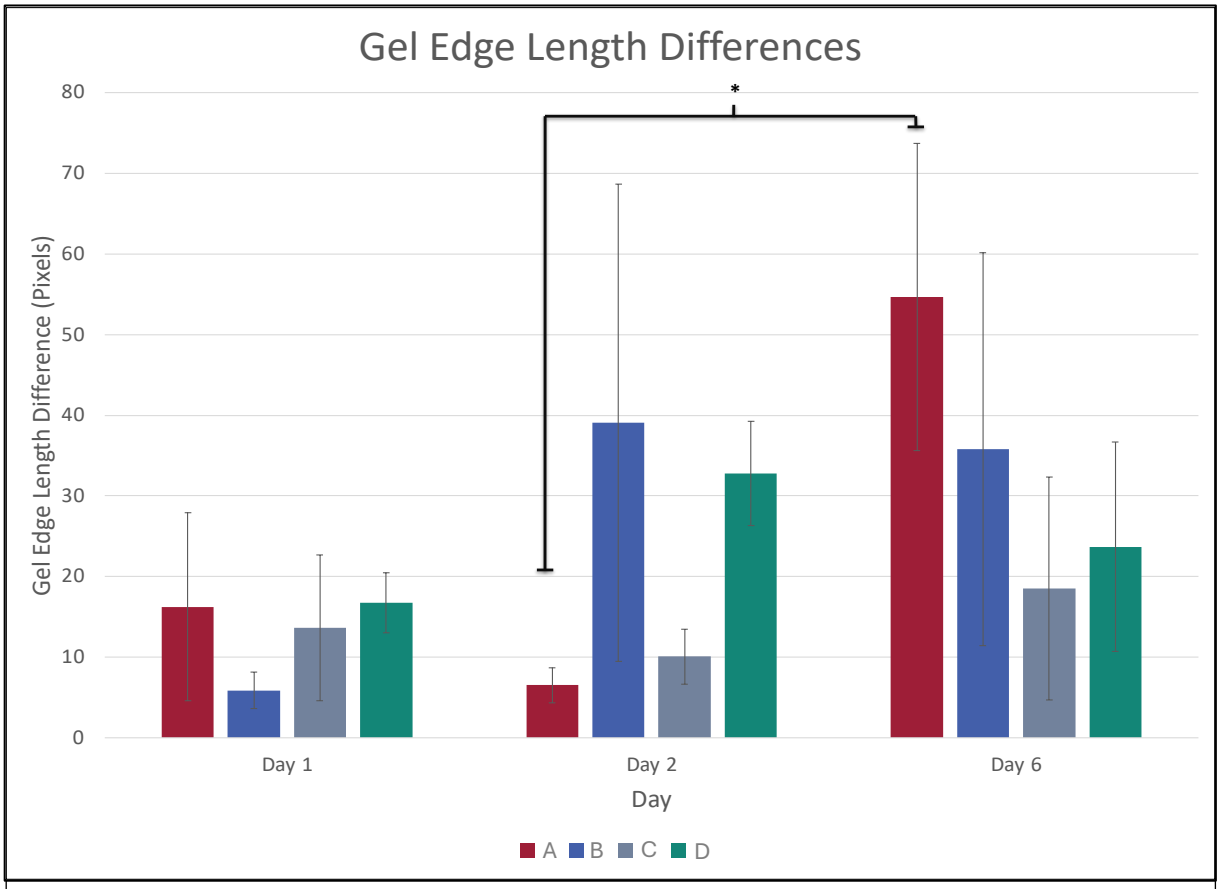

Figure 4. Uniformity. Gel edge length difference was found by calculating the absolute value of the difference between the length of each 20^{th} & 80^{th} width percentile across the sample short axis. The process of mechanical release disrupted the degree of uniformity based on the visible increase of boundary length difference for gels with lower cell concentration. (Sample size: n=5, * $p < 0.05$)

Ideal uniformity for the gel was represented by a constant gel edge length difference value over the time course. An increase in gel edge length difference was not desirable as it indicated the potential ripping of the gel from the grip, resulting in a non-uniform gel. The process of mechanical release disrupted the degree of uniformity based on the visible increase of

boundary length difference for gels with lower cell concentrations, conditions A and C, in Figure 4. Regarding the conditions B and D which possessed higher cell concentration, the mechanical release had less of an evident effect on the uniformity of these samples. Due to the mechanical release causing non-uniformity to occur in gels, this physical adjustment was not preferred for selecting the optimal condition.

The percent change from the baseline middle width, pre- and post- mechanical release, was calculated considering the average middle width values. Day 2 and day 6 middle width values for each condition were determined against the day 1 baseline value to calculate the percent change, as displayed in Figure 5. Activity of the gels during the time course was depicted by the middle width percent change from baseline, before and after mechanical release. Condition A achieved the greatest percent of compaction compared to the other conditions, yielding the largest middle width change percent values for both day 2 and day 6.

The lower protein concentrations yielded more substantial and effective compaction rates than concentrations containing higher protein concentrations. This was noticeable in the greater value differences at both day 2 and day 6 for the A and B conditions, having a protein concentration of 1 mg/ml, compared to that of the C and D conditions, possessing a concentration of 2 mg/ml. It appeared the compacting activity was found more in condition A, followed closely by condition B.

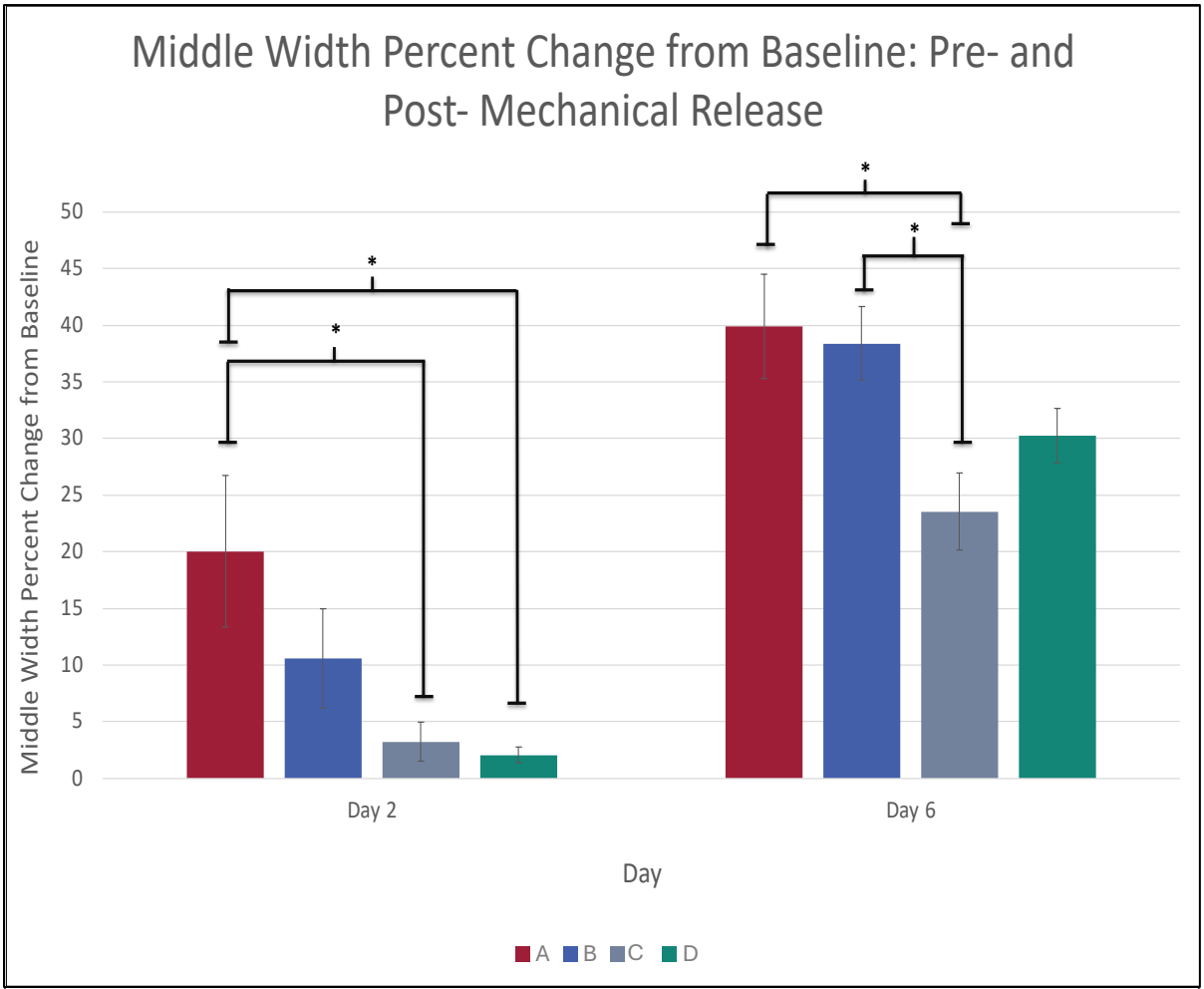

Figure 5. Activity. Percent change in the middle width value for day 2 and day 6 determined against the day 1 baseline value. Condition A achieved the greatest percent of compaction, yielding the largest values, for both day 2 and 6 compared to other conditions. Lower protein concentrations yielded more substantial and effective compaction rates than concentrations containing higher protein value. (Sample size: n=5, * $p < 0.05$)

Although conditions C and D were able to achieve greater compaction following the mechanical release, their middle width percent change values were still unable to surpass the A and B conditions. The minimal percent change for conditions C and D prior to the mechanical release indicates the inability for these conditions to compact without receiving physical assistance. The larger values post-release for the percent changes of conditions A and B

indicated the mechanical release was a requirement to assist these lower protein concentration conditions in their compaction. Mechanical release was found to increase the percent change for all conditions.

The rate of change for middle width values before and after mechanical release displayed the functionality of the gels. Middle width values were evaluated in the context of pre- and post-mechanical release, illustrated in Figure 6, comparing the effects of intervention on gel compaction by the embedded cell population.

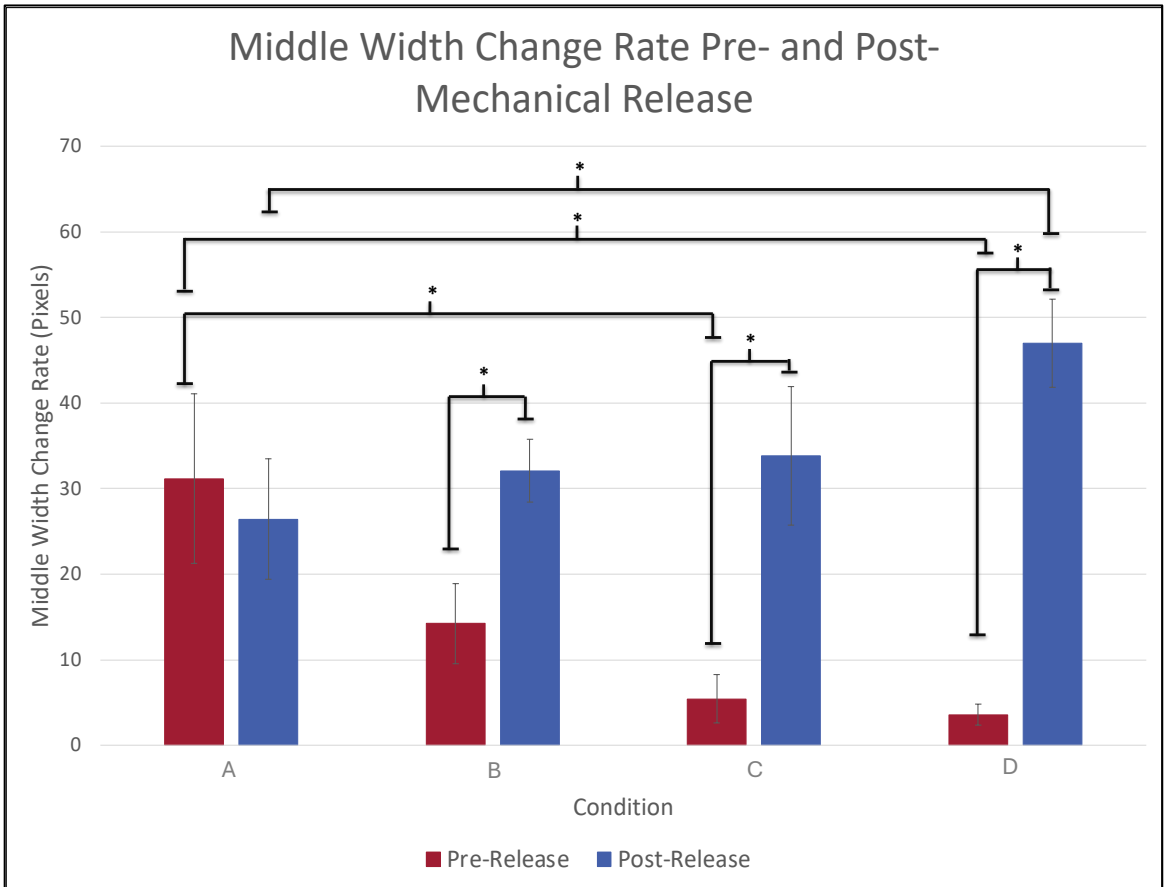

Figure 6. Functionality. Middle width evaluated in the context of pre- and post- mechanical release comparing effects of intervention on gel compaction by the embedded cell population. All conditions, apart from the control group, required mechanical release. Gels with lower protein concentrations possessed a greater compaction rate pre-release. (Sample size: n=5, * $p < 0.05$)

Based on Figure 6, all conditions, apart from the control group, required mechanical release. Gels with lower protein concentrations, conditions A and B, possessed a greater compaction rate pre-release. This initial compaction rate displayed an independence from the physical adjustment, as opposed to this technique being necessary for success in conditions C and D. Therefore, lower collagen concentrations were found to compact greater prior to release. Since mechanical release was positively affected by all conditions except condition A, condition A revealed superior uniformity, as applying a mechanical release was not desirable.

b. Statistical Analysis

The statical significance was assessed for the results regarding compaction, activity, uniformity, and functionality using ANOVA or t-tests. For uniformity and functionality, a paired t-test was utilized for comparing intra-condition values across daily measures. Meanwhile, intra-day comparisons were tested through an unpaired t-test to evaluate statistical significance between conditions on specific days for compaction, activity and functionality. Statical significance, as well as error bars representing standard error, are visible in Figures 3,4,5 and 6. Based on the results from the n-way ANOVA and t-tests, significance levels were denoted by using a single asterisk (*) for p-values less than 0.05 or double asterisk (**) for p-values less than 0.01. The standard error for error bars was calculated by dividing the standard deviation by the square root of the sample size.

c. Limitations

Although the findings of this study provide insight into the effects of cell and collagen concentration on gel morphology, the parameters optimized may not be applicable to all engineered tissues. In order to customize specific experimental models, repeating this experiment

utilizing the desired cell type paired with the extracellular matrix protein would be necessary due to the variability in engineered tissues. Additionally, improvements to the tracing model through the machine learning could aim to increase the population pool of included gels during the analyzation process. Regarding nonmechanically dynamic tissues which focus on a greater degree of mechanical independence, this optimization may not be necessary. Lastly, the mechanical intervention executed in this study was found to affect the morphology of the gels. Therefore, repeating this experiment without a physical adjustment through the mechanical release of the gels could yield unexpected results in a longer timeframe.

IV. Conclusion

In conclusion, experimentation showed that collagen concentration directly affected morphology of collagen gels while the cell concentration had an effect once the multi-day nature of the time course was considered. Based on Figures 3,4,5 and 6, Condition A exhibited optimal behavior as mechanical release was unnecessary for compaction and did not alter trends; therefore, proving this condition to be the desired parameter set as it reached desired compaction results without the need of physical assistance. Lower protein concentrations, represented with conditions A and B, were found to increase compaction along with ideal activity, uniformity, and functionality compared to the conditions of higher collagen concentration. In future experimentation, mechanical conditions and testing would be performed using condition A, with the overall goal of improving the drug discovery process to reveal more success in patient treatment.

V. Acknowledgements

I would like to thank Dr. William Richardson for providing me with the opportunity to be part of his laboratory group in the Department of Chemical Engineering. Secondly, I want to

thank Dr. Jake Potter for his help, support, and encouragement throughout my research. This research was supported by an Honors College Research Grant for the Terms Fall 2023 and Spring 2024.

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