Viability of Murine Fibroblasts in Sodium Alginate Hydrogels using a Live-Dead Assay

Abstract

The objective of this study was to analyze the viability of marine fibroblasts cells in sodium alginate hydrogels under different aquatic conditions. Sodium alginate has unique properties that allow for its polysaccharide chains to react to form ionic crosslinks, which creates a hydrogel structure. Three different aquatic conditions were used in this study, which included hypertonic, hypotonic, and a control (growth media) treatment. To determine the cell viability under these conditions, a live-dead assay was conducted using fluorescent dyes. To count the number of viable and non-viable cells a MATALB script was used, which allowed for the automation of the process. In this MATLAB script, separate channels were created depending on color, and object centroids were plotted to count the number of cells. It was observed that control group had the highest cell viability with 5.1698×10^8 cells/cm^3, while the hypotonic group has the lowest cell viability with 3.2407×10^8 cells/cm^3. Although, a one-way ANOVA was conducted, which found a p-value of 0.2154, indicating that no statistical significance was found between any of the treatment groups. These results indicate that variation in salinity conditions have no effect on the viability of NIH 3T3 cells in sodium alginate hydrogels.

Introduction

In this experiment, the goal was to act in place of a bioengineer at a company that is developing cell-embedded hydrogels, specifically sodium alginate hydrogels, for wound healing in aquatic animals. Sodium alginate, which is derived from brown seaweed, has garnered interest in the bioengineering community due to its unique crosslinking properties. When sodium alginate is exposed to divalent cations such as Ca^{2+} and Mg^{2+} in aqueous solutions, the polysaccharide chains react to form ionic crosslinks. This creates a hydrogel structure between the chains, which is capable of containing 99% water. These remarkable abilities makes sodium alginate hydrogels extremely useful for biomedical applications.

Among its various uses, cell-embedded hydrogels stand out for their potential uses in 3D cell culture and wound care. For example, in a study conducted by Sung et al., 3D hydrogels were used to provide an environment for cell culture to analyze the effects of anti-cancer drugs[1]. These hydrogel-based cell cultures were able to simulate drug metabolism and its impact on different cell types, such as the liver and pancreas. Moreover, in a study conducted by Dong et al., human adipose-derived stem cells were encapsulated into crosslinked hydrogels and cultured in 3D topography[2]. The encapsulated cells were attached and spread inside the hydrogel, allowing for the maintenance of cell viability and regeneration. These cells were then able to be delivered to diabetic wounds through the hydrogel as a vehicle, which produced accelerated wound closure.

In this experiment, NIH 3T3 murine fibroblasts and fluorescent live-dead dyes were mixed into a sodium alginate solution to form hydrogels. These hydrogels were dealt with three different treatments: hypotonic, low salt concentration, hypertonic, high salt concentration, and a control group, standard growth media. After incubation, fluorescent micrographs were taken of the hydrogels, and cell viability was analyzed using MATLAB, where the number of non-viable and viable cells were counted. MATLAB offers various tools for processing these images, however, both advantages and disadvantages are present. The advantages of the software include the ability to handle large datasets and to automate tedious tasks, which reduces human error. For example, in a study conducted by Courtney et al., MATtrack was developed as open-source computational platform to process multi-Tiff files for live cell fluorescent microscopy[3]. The platform automates several steps of image processing, including red and green classification, noise filtering, contrast stretching, and temporal smoothing. On the other hand, the use of MATLAB requires sufficient programming knowledge and the potential for overlooking unique nuances that only a human observer can catch.

Ultimately, the purpose of this experiment was to assess the viability of NIH 3T3 murine fibroblasts in sodium alginate hydrogels when exposed to aquatic environments of varying salt content. These differing aquatic conditions are meant to replicate the different conditions hydrogels may face in

freshwater and marine environments. The hypothesis in this study is that hydrogels in hypertonic solutions will experience decreased cell viability when compared to hypotonic solution and the control group. This is since cells in the hypertonic solution may experience cell dehydration due to the high salt concentration, which can lead to death. On the other hand, the hypotonic solution may also have decreased cell viability due to cell swealing that can occur as water flows into the cells. Ultimately, the control group, the standard growth media, should have the highest cell viability as it offers the most ideal conditions to the cells.

Materials and Methods

Preparation of Hydrogels

In this experiment, each group was assigned one treatment: control, hypertonic, or hypotonic. First, 4mL of sodium alginate, 100uL of cell suspension (NIH 3T3 murine fibroblasts), and 30uL of pre-mixed live and dead fluorescent dyes were pipetted into a test tube. Then, the test tube was mixed using a vortex, where the mixture was pipetted onto a petri dish and swirled. Next, 5mL of 5% $CaCl_2$ was pipetted onto the gel and the solution was swirled until a crosslinked hydrogel was formed. The $CaCl_2$ was then removed from the dish and deionized water was used to wash the gel. Afterwards, 10mL of either 5% NaCl (hypertonic), deionized water (hypotonic), or NIH 3T3 growth media (control) was pipetted on the gel depending on which treatment group. Then, the plates were incubated for 10 minutes in the dark at 5% CO_2 and 37 C. Once the plates were finished incubating, the thickness of the hydrogel was measured using calipers, which was found to be approximately 1.5mm. Once the plates were parafilmed, they were ready for imaging.

Fluorescent Imaging

Using a ThermoFisher Scientific EVOS FL Fluorescence Inverted Microscope, images were captured of the gels. On the gel, three different locations were viewed, which were evenly spaced out. The images were able to be captured at three different magnifications: 4x, 10x, and 20x. For this lab, images

were only taken at 10x magnification. When capturing images, an initial image was captured using the Texas Red filter, and then the same image was captured using the Green Fluorescent Protein (GFP) filter. The brightness and contrasts of these images were adjusted, and then overlayed to create one image for analysis. Additionally, on this microscope, the 10x objective has a field of view of 432 um^2. Using this value and the average thickness of the hydrogel, the volume of the hydrogels used were found.

Image Analysis in MATLAB

For image analysis, MATLAB R2023a and its Image Processing Toolbox was utilized. In MATLAB, a script was created to count the number of red (dying) and green (viable) cells. First, the images were imported into MATLAB using the function *imread*(). Then, the images were separated into three color bands: red, green, and blue. To do so, *img*() was used, where red was color band #1, green color band #2, and blue color band #3. Next, histograms were created for each channel using the function *imhist*(). Using these histograms, the distribution of the pixel intensity was able to be seen, which was used to apply thresholds to the color channels. To apply thresholds, a mask was created for each color channel which had a lower threshold of 40 and an upper threshold of 255. Then, these masks were cleaned using the command *bwareopen*() to remove objects too small to be cells.

Next, the individual cells were identified and counted. To do so, *regionprops*() identified the centroid of each object and then overlayed a marker. Once all centroid coordinates were found, they were converted into matrix format using *cat*(). Finally, the number of green and red cells were counted using the lengths of the matrices previously created. Then, the number of viable and nonviable cells were determined. Viable cells were the number of green cells minus the number of red cells, while nonviable cells were only the number of red cells. This process was repeated for each image.

Statistical Analysis in MATLAB

Next, statistical analysis was completed to determine the difference in cell viability across treatment groups. Prior to statistical analysis, the cell viability was determined by diving the number of

viable cells by the volume of the hydrogels. Then, the descriptive statistics were found for each treatment group using *mean*(), *median*(), and *std*(). Afterwards, the data was prepared for a one-factor ANOVA. To do so, all viability data was combined into a single matrix and grouping labels were created using *repmat*(). Then, a one-factor ANOVA was performed using *anonan*(). To check the assumptions of the ANOVA, equality of variances was tested, and a probability plot of the residuals was made. For the variance test, *vartest*() was used with the same matrix used in the ANOVA. Then, the residuals were taken from the ANOVA test using *stats.resid*, and *probplot*() was used to create a normal probability plot.

Results

After incubation of each hydrogel, fluorescent images were taken at each location for each hydrogel. Then, the images were placed into MATLAB and several micrographs were created from the initial image. First, the image, which is the control group at 10x magnification in this example, was separated into red and green RGB channels (Figure 1). Then, thresholds were applied for each channel (Figure 2), which included adjusting the pixel intensities for each channel. Finally, the masks were cleaned, and object centroids were plotted to determine the non-viable and viable cells (Figure 3).

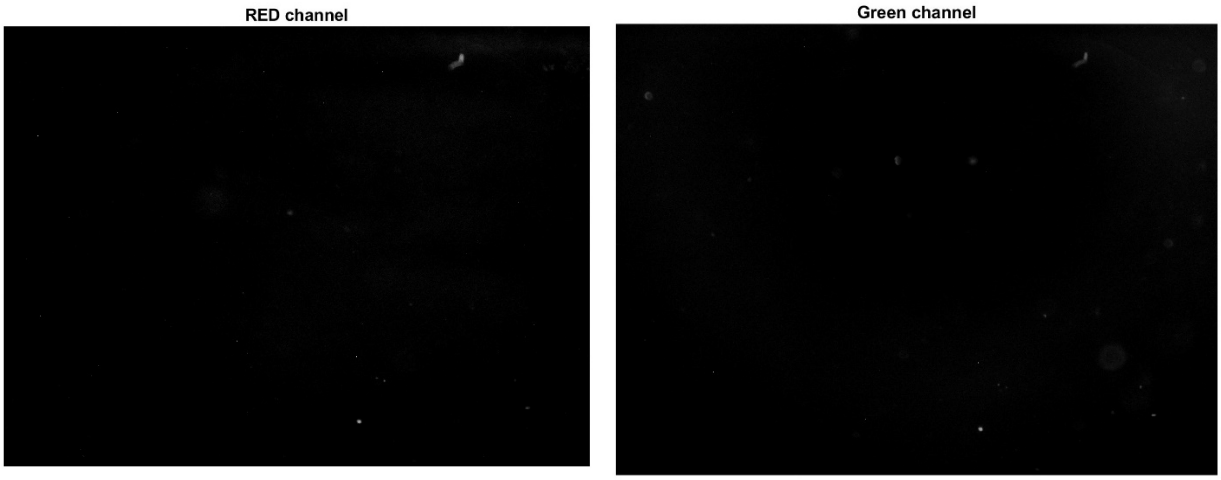

Figure 1. Red and green channels for initial image (Control 10x). The red channel indicates non-viable cells, while the green channel indicates viable cells.

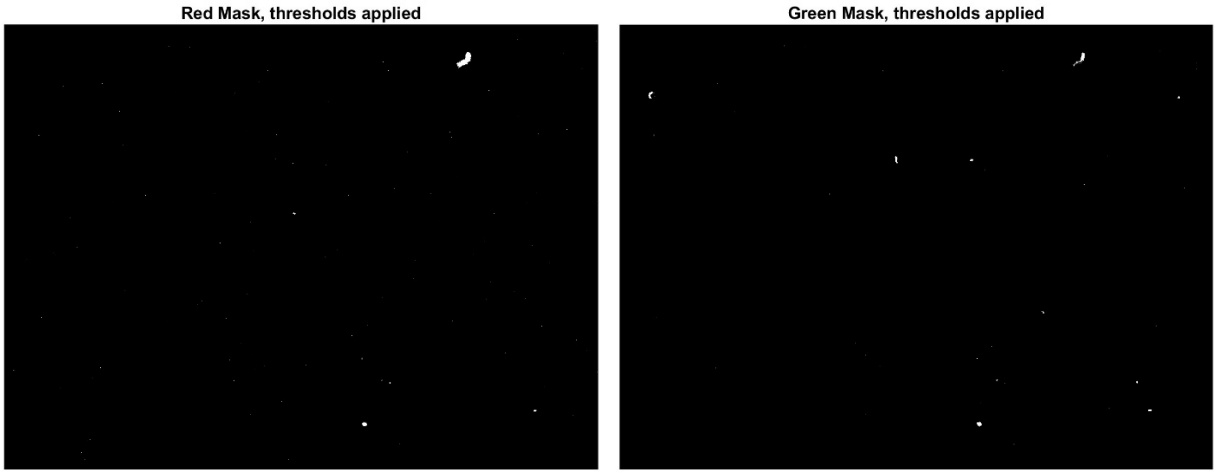

Figure 2. Red and green Masks with thresholds for initial image (Control 10x). Thresholds were dependent on pixel intensity of original image.

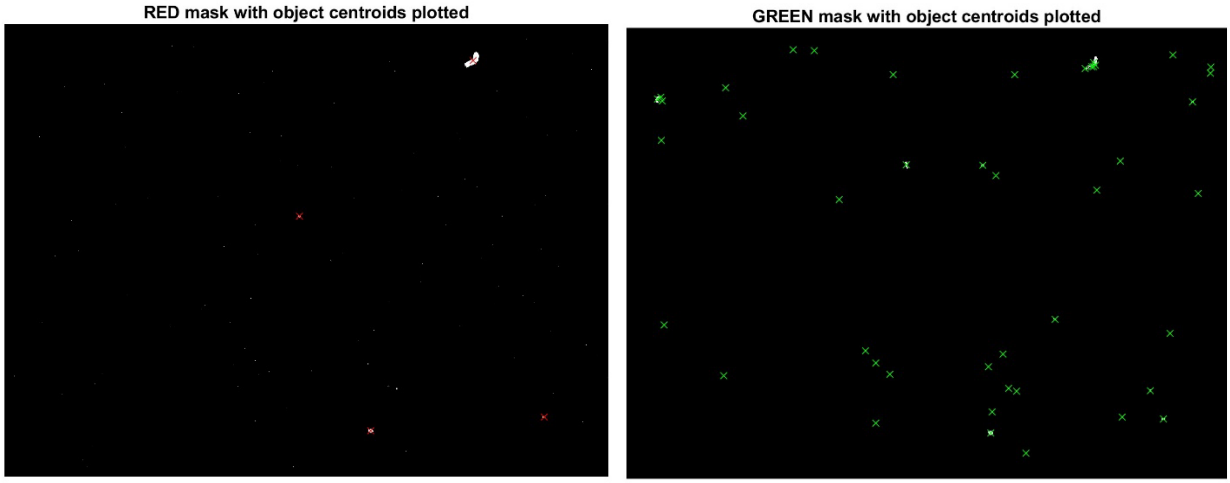

Figure 3. Masks showing non-viable (Left) and viable (Right) cells for control 10x group. Object centroids are plotted to count the number of cells for each mask.

Table 1: Descriptive statistics for cell viability for each treatment group.

Group	Control	Hypertonic	Hypotonic
Mean (cells/cm^3)	5.1698e+08	3.8580e+08	2.8807e+08
Median (cells/cm^3)	4.6296e+08	3.7037e+08	3.2407e+08
Standard Deviation (cells/cm^3)	2.1355e+08	7.0719e+07	1.3483e+08

After MATLAB was used to count for the non-viable cells and viable cells for each hydrogel sample, the cell viability for each hydrogel sample was found. Then descriptive statistics were found for each treatment group (Table 1). It was found that the control group had the highest cell viability with 5.1698×10^8 cells/cm^3, while the hypotonic group has the lowest cell viability with 3.2407×10^8 cells/cm^3. Similarly, the median of the control group was the highest, while the median of the hypotonic groups was the lowest. In terms of standard deviation, a larger standard deviation was found for the control and hypotonic groups compared to the hypertonic groups.

Next, a one-way ANOVA was used to determine if there was statistical significance present between the treatment groups. For the test, an overall p-value of 0.2154 was found. With an alpha value of 0.05, this indicated that the null hypothesis failed to be rejected. This meant that no statistical significance was present between the three treatment groups. Additionally, a multi-comparison test was conducted to determine if any statistical significance was present between any two treatment groups (Table 2). It was found that no statistical significance was present between any pair of treatment groups as each had a p-value greater than 0.05.

Table 2: Multi-comparison test for different treatment groups.

Group 1	Group 2	P-Value
Control	Hypotonic	0.2073
Control	Hypertonic	0.5591
Hypotonic	Control	0.7775

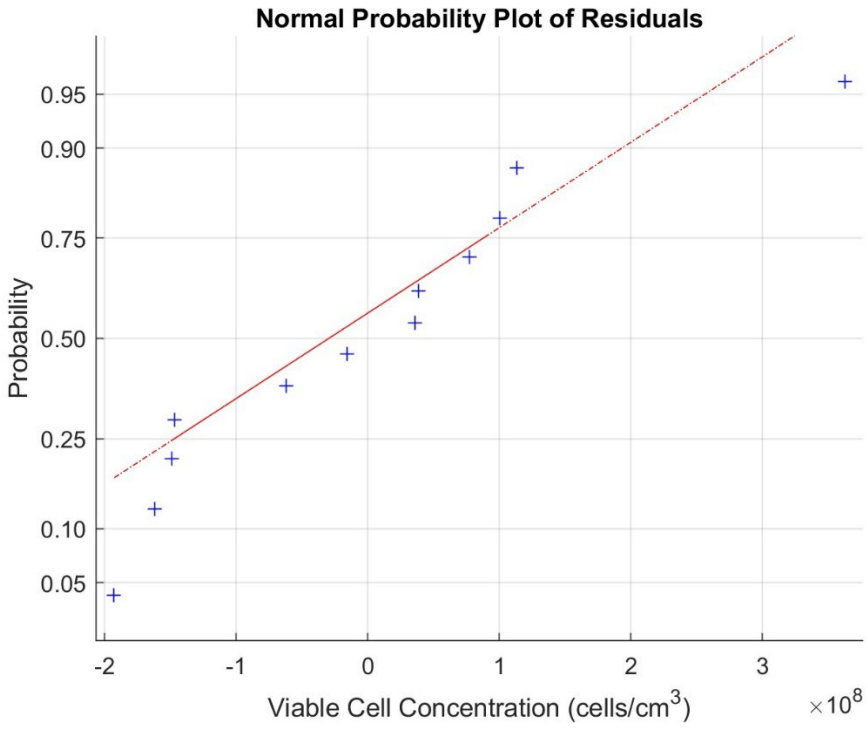

Figure 4. Normal Probability Plot of Residuals from ANOVA Test

A normal probability plot was created for the residuals of the ANOVA test to determine if the assumptions of the test were met. (Figure 4). This plot demonstrates that a majority of the points closely align with the central line of distribution, which indicates that a normal distribution is present. Additionally, a test for equal variances was completed in MATLAB (Figure 5), where a p-value of 0.20936 was found. With an alpha value of 0.05, the results indicate that the null hypothesis failed to be rejected, and that the variance across treatment groups was equal.

Group Summary Table

Group	Count	Mean	Std Dev
Control	6	5.16975e+08	2.13555e+08
Hypotonic	3	2.88066e+08	1.34829e+08
Hypertonic	3	3.85802e+08	7.07188e+07
Pooled	12	4.26955e+08	1.74607e+08

Levene's statistic (absolute)	1.86978
Degrees of freedom	2, 9
p-value	0.20936

Figure 5. Results of Test for Equal Variances in MATLAB

Discussion

As stated previously, a one-way ANOVA test was conducted to determine the statistical significance between the treatment groups used in this study. The two main assumptions of this test include normally distributed residuals and groups of equal variances. Using a probability plot (Figure 4), it was found that the residuals did not significantly deviate from the central line of distribution, indicating a normal distribution. Then, it was found that an equal variance test (Figure 5) yielded a p-value of 0.20936, indicating that the null hypothesis failed to be rejected, suggesting homogeneity of variances across the groups. Therefore, the assumptions of the one-way ANOVA test were met, suggesting that it was an appropriate test for analyzing the differences in cell viability.

Using the one-way ANOVA test, it was found that the results had no statistically significant differences in cell viability across control, hypertonic, and hypertonic groups, with a p-value of 0.2154. This suggests that the changes in aquatic conditions had no significant effect on the viability of NIH 3T3 murine fibroblasts in sodium alginate hydrogels. These results do not support the initial hypothesis that the control group would have the highest cell viability, followed by the hypotonic and hypertonic groups. While the control group did have the highest viability with 5.1698×10^8 cells/cm^3 and the hypotonic group had the lowest viability with 3.2407×10^8 cells/cm^3, a multi-comparison test demonstrated a p-value of 0.2073, indicating no significant difference was present. There may have been several factors leading to there being no differences between the groups, including the cells used being extremely resilient to osmotic stress or that the salinity variations were not extreme enough. Additionally, there may have been errors with the MATLAB script, leading to incorrect data calculations.

To redesign this experiment for a two-way ANOVA test, the experiment could be modified to include additional variables. For example, different cell lines could be introduced to the study, which would lead to an understanding of how different types of cells react to changes in aquatic conditions. Additionally, there could have been varying concentrations of salinity for the hypotonic and hypertonic

groups. This would allow for an understanding of how extreme the salinity conditions could be before cell viability is affected. Finally, different concentrations of sodium alginate could be used to create the hydrogels, which would lead to a comparison of matrix density to cell viability.

In this experiment, a MATLAB script was developed to automate the cell counting process for the live-dead assay. This script was able to easily separate the non-viable and viable cells from each other using color thresholding, however, the script did run into performance errors when actually counting the number of cells. In some cases, especially for images with low quality, the script would overcount the number of cells as due to the smearing of the green and red fluorescent dyes. For example, in one image, the green florescent dyes was extremely smeared, leading to a viable cell-count of nearly 8000, which was clearly incorrect. Additionally, the script would sometimes count the same cell twice if it was slightly larger than the other cells in comparison. Due to these errors in the script, it would have most likely been more accurate to count the cells manual. Manual counting is better in scenarios that are ambiguous as a human has a more nuanced understanding of discernment. Moreover, since the amount of data was relatively small in this study, manual counting would not have been very time-consuming.

Finally, in this study, the cells were in a 3D sodium alginate hydrogel matrix. If the cells were cultured in a 2D adherent culture instead, the observed results may have been different. In a 2D culture, the cells are spread over a flat surface, which may lead to changes in growth patterns and cellular functions when compared to a 3D gel. On the other hand, 3D cultures are more similar to the in-vivo environments that cells experience, which may have made the cells more resistant to the osmotic stress. Thus, using a 2D culture would have most likely adjusted the results of this study, as it is worse replication of the cell's true environment, leading to inaccurate results.

Conclusion

The objective of this study was to analyze the viability of marine fibroblasts cells in sodium alginate hydrogels under different aquatic conditions, which included hypertonic, hypotonic, and a control

(growth media) treatment. To determine the cell viability under these conditions, a live-dead assay was conducted, and a MATALB script was used to count the number of viable and non-viable cells. It was observed that control group had the highest cell viability with 5.1698×10^8 cells/cm^3, while the hypotonic group has the lowest cell viability with 3.2407×10^8 cells/cm^3. Although, a one-way ANOVA was conducted, which found a p-value of 0.2154, indicating that no statistical significance was found between any of the treatment groups. The results indicate that variation in salinity conditions have no effect on the viability of NIH 3T3 cells in sodium alginate hydrogels.

In the future, this experiment can be expanded upon in several ways to produce more results. To begin, more trials can be conducted to further support or reject the results found in this experiment. This can include testing more samples of sodium alginate hydrogels under the salinity conditions used. Additionally, the experiment can be expanded to include more concentrations of salinity as the salinity levels in this study may not have been extreme enough. Finally, the MATLAB script can be modified to produce more accurate cell-counting results. This may include using different functions or further automating the process.

References

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