TUNABLE HYDROGEL SUBSTRATES FOR STUDYING PATHOGEN-HOST CELL INTERACTIONS

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

Tunable Hydrogel Substrates for Studying Pathogen-Host Cell Interactions

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Extracellular matrix elasticity has been shown to affect a variety of cellular properties. However, the effect it has on pathogen-host cell interaction has yet to be thoroughly evaluated. Furthering our understanding of pathogenic infection mechanisms could be beneficial to the development of vaccinations for poorly understood pathogens as well as new therapies to combat a rapidly growing antibiotic-resistant pathogen population. We evaluated two polymer systems in this study: a polyacrylamide hydrogel system that was functionalized for cell culture and a poly(ethylene glycol)-norbornene hydrogel system that utilizes thiol-ene click chemistry for simple and rapid functionalization and tunability. Our pathogen of interest is *Brucella melitensis*, a bacteria that affects millions of people worldwide, through livestock death, zoonotic infection, and bioterroristic threat. Since *Brucella melitensis* primarily targets phagocytes, macrophages were utilized for evaluating the success of the hydrogels in hosting tissue culture. It was determined that the poly(ethylene glycol)-norbornene system had the most success in culturing healthy cells and easily tuning mechanical characteristics. Based on these results, future studies in the Alge Lab will involve seeding macrophages onto poly(ethylene glycol)-norbornene gels of varying elastic moduli and infecting them with *Brucella* to investigate the influence of extracellular matrix elasticity on *Brucella* infection rate.

DEDICATION

I would like to dedicate this work to my parents who have always provided me the support needed to chase the opportunities in my life, as well as my sister Rachel, who inspires me to try and be the person I would have never thought myself capable of being.

ACKNOWLEDGMENTS

First and foremost I would like to thank Dr. Daniel Alge, who not only has given me the opportunity to work in his lab, but has mentored me with more patience than I even give myself, as I try to find my path in research and in life. I would also like to thank Kirstin Brink for her work on this project, as well as the friendliness, generosity, and fruit snacks she brought with her every day. Finally, I would like to thank the entire Alge Lab Group for both setting an extremely high bar for me to constantly strive to meet and for creating the type of community that I am so grateful to be a part of.

NOMENCLATURE

DTT - Dithiothreitol

ECM – Extracellular matrix

LAP – Lithium acylphosphinate

PAAm – Polyacrylamide

PBS – Phosphate-buffered saline

PEG – Poly(ethylene glycol)

PEG-NB – Poly(ethylene glycol)-norbornene

UV – Ultraviolet

CHAPTER I

INTRODUCTION

Most human cells require anchorage to a solid substrate in order to survive and reproduce (Ruoslahti, 1987). This substrate functions similar to the extracellular matrix (ECM), which serves not only to support and organize tissue, but also can communicate with and influence cells. The mechanical properties of the ECM, specifically, have been shown to influence a multitude of factors in local cells, including gene expression (Discher, 2005), stem cell fate (Engler, 2006), and durotaxis (Lo, 2000). However, the effect of ECM elasticity on pathogen-host cell interaction has not been rigorously studied, and there are reasons to believe that such studies would reveal the presence of a significant effect. For instance, it has been documented that ECM elasticity has an effect on cytoskeletal organization (Discher, 2005). Many intracellular pathogens, including *Brucella melitensis*, the bacteria to be used in this study, rely on actin filaments for movement and organization within the host cell (de Figueiredo, 2015). Therefore, we have hypothesized that varying ECM elasticities will change the cytoskeletal organization of the cells, and thus change the pathogenic infection rate. This may be critical when developing vaccinations for high-risk pathogens, such as *Brucella melitensis*, a bioterrorism threat (Yagupsky, 2005). Furthermore, strengthening our fundamental understanding of pathogenic infection mechanisms and their dependencies could facilitate the development of new therapies to treat the growing population of antibiotic resistant pathogens (World Health Organization [WHO], 2014).

The pathogen of interest in this study is *Brucella melitensis*, a gram-negative, intracellular bacteria. It is part of the genus *Brucella* which includes ten different species, but we will herein refer to *Brucella melitensis* solely as *Brucella*. *Brucella* is a common zoonotic pathogen in third world countries that infects sheep and goats before transferring to humans through the milk and cheese of the livestock (de Figueiredo, 2015). Its infection mechanism is representative of a large group of intracellular pathogens, which will allow the results of the future study to be used for appropriate predictions on the behavior of a wide selection of pathogens.

Soft tissue substrates can be composed of natural biopolymers or functionalized synthetic polymers for *in vitro* study. While the use of natural material, such as collagen, is beneficial in terms of promoting cell adhesion and proliferation the inability to completely control mechanical properties, particularly on a batch-by-batch basis, are less than optimal (Kim, 1998). Thus, soft tissue scaffold material has been primarily centered on synthetic polymers, specifically in the form of hydrogels. Hydrogels are cross-linked polymer networks that have been extensively swollen with water. They are the current gold standard amongst scaffold materials, as their material properties, including stiffness, mesh size, and water content, resemble natural ECM. Furthermore, they can be easily functionalized with ECM proteins and growth factors to facilitate cell adhesion and survival (Hoffman, 2012). In this study, we evaluate two hydrogel systems for use as a soft tissue substrate in pathogen-host cell studies. The first is a polyacrylamide (PAAm) system covalently functionalized with fibronectin for cell adhesion. The second is a poly(ethylene glycol)-norbornene (PEG-NB) system that makes use of thiol-ene click chemistry, a rapid, specific, and high-yielding reaction. CGRGDS cell adhesion peptide is also “clicked” into place utilizing thiol-ene reactions, functionalizing the PEG-NB gels for cell

culture. The success of these hydrogel systems was evaluated by two criteria: their ability to host and maintain a healthy macrophage culture, and the tunability of their elastic modulus. If the hydrogel system were capable of meeting those two criteria, it could be readily applied to the study of *Brucella* and many other pathogens and would meet the ultimate objective of this study.

CHAPTER II

METHODS

Gel preparation

The hydrogels were adhered to 15 mm glass coverslips to prevent damage to the gels during the production and cell application processes. Coverslips were first cleaned through flame-treatment before applying either a (3-Aminopropyl)triethoxysilane (for the PAAm gels) or a (3-mercaptopropyl)trimethoxysilane (for the PEG-NB gels) solution. This reaction left either an available amine or thiol group respectively. The thiol group reacted with the PEG-NB via a thiol-ene click reaction, binding the gel to the coverslip. The amine group was reacted once more with 2% glutaraldehyde, leaving an available aldehyde group. This remaining available aldehyde group then reacted with the amide groups in the PAAm gels, completing the covalent chain between the PAAm gel and the coverslip.

The PAAm hydrogels were formed through a redox copolymerization of 40% acrylamide and a 2% bis-acrylamide cross-linker. 20 μL of 0.1 g/mL ammonium persulfate was used as a source of free radicals in conjunction with an accelerator, tetramethylethylenediamine (TEMED). The concentration of monomer to cross-linker was varied to tune the elasticity, according to Table 1A. Cells will not naturally adhere to polyacrylamide, so the PAAm gels were functionalized with fibronectin, a cell adhesion protein, using two different methods. The first was through the use of sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH), as outlined in Tse et al., 2010. A .2 mg/mL sulfo-SANPAH solution was applied to the PAAm gel containing a nitrophenyl azide group which was activated through ultraviolet irradiation for 10

minutes at 10 mW/cm^2. When activated, the nitrophenyl azide group reacted with the amide groups in the PAAm gels. The sulfo-SANPAH was then left with an available NHS ester group, which reacts with amine groups. We evaluated both a single dose of sulfo-SANPAH as well as a second dose, which was applied identically to the first dose after briefly washing the gels. After incorporation of the sulfo-SANPAH, a 20 μg/mL solution of fibronectin was applied to the surface of the gels and allowed to react for one hour. This process is summarized in Figure 1. The gels were then quenched in a 1% ethanolamine solution to neutralize any remaining NHS ester groups and sterilized in 5% isopropyl alcohol.

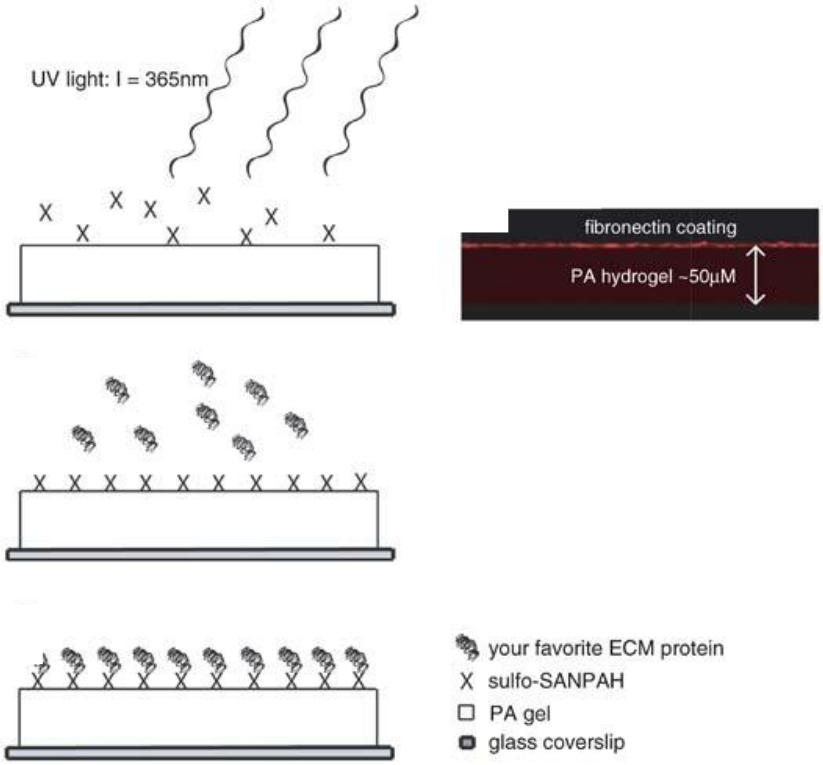

Figure 1: Functionalizing polyacrylamide gels using sulfo-SANPAH. Any protein containing an amine group could react with the sulfo-SANPAH, creating a versatile platform. Adapted from Tse et al., 2010.

The second method evaluated utilized a 20 mg/mL solution of N-succinimidyl acrylate dissolved in toluene, as described in Kandow et al., 2007. The initial formulation of the PAAm polymer solution was kept the same, as outlined in Table 1A. The toluene solution was then added to the polymer solution prior to polymerization, allowing the acrylate to be copolymerized. The toluene had a lower density than the PBS in the polymer solution, so the NHS ester containing N-succinimidyl acrylate was polymerized only at the surface of the gels, leaving available NHS ester groups affixed to the gel surface. This process is summarized in Figure 2. The protocol for applying the fibronectin, quenching the gels, and sterilizing was identical to that for the sulfo-SANPAH method.

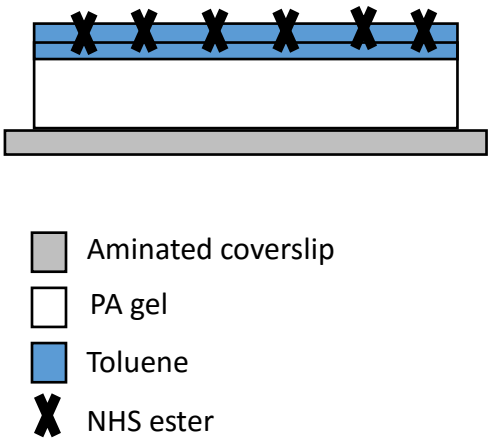

Figure 2: Comonomer method of functionalizing polyacrylamide hydrogels.

The PEG-NB hydrogels were polymerized through the use of thiol-ene click chemistry. A polymer solution consisting of PEG-NB, dithiothreitol (DTT) cross-linker, CGRGDS cell adhesion peptide, the photoinitiatior lithium acylphosphinate (LAP), and PBS was prepared. The molecular weight of the PEG-NB macromer, as well as the weight percent of PEG-NB, was

varied to tune the elasticity of the gels, while the concentrations of CGRGDS and LAP were kept constant across all gels. Differing formulations utilized in both the cell studies and the swelling studies are specified in Tables 3A-8A. The gels were then polymerized via a one minute dose of 10 mW/cm^2 ultraviolet light. The thiol groups at either end of the DTT reacted with the alkene of the norbornene in the PEG-NB macromers to cross-link the polymer network. Similarly, the thiol groups in the cysteines of the CGRGDS peptides would also react with the alkenes present in the PEG-NB, functionalizing the network for cell adhesion. This process is summarized in Figure 3.

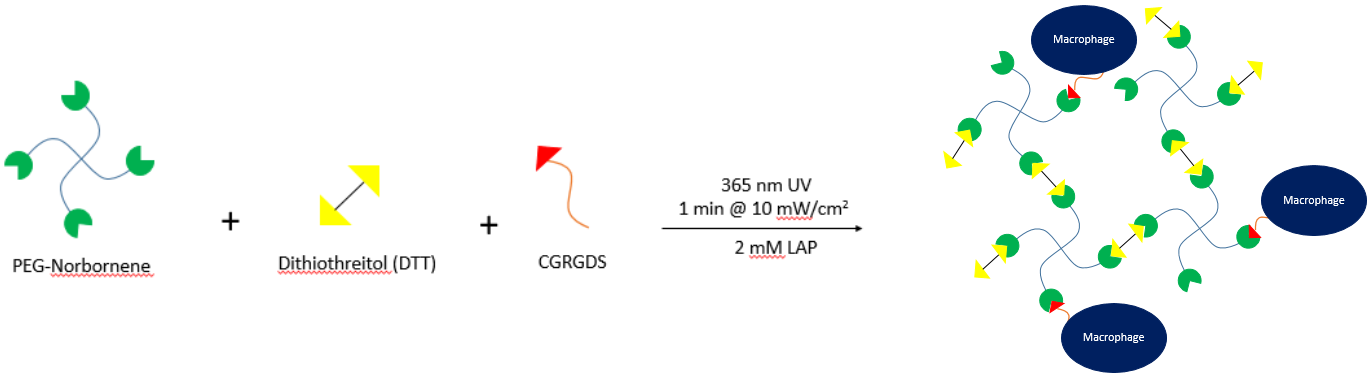

Figure 3: Thiol-ene polymerization of PEG-NB hydrogels. A solution of PEG-NB macromer, DTT cross-linker, and CGRGDS is irradiated with UV light in the presence of a photoinitiator and forms a hydrogel network through a series of thiol-ene click reactions.

Ellman's assay

In order to quantify the number of available NHS ester groups before the fibronectin was added and ensure that our protocols for either method of fibronectin addition were working properly, an Ellman's assay was performed on the PAAm gels. A 10 mM cysteine solution was added to the gels in place of the fibronectin. The amine group in cysteine reacted with the NHS esters as the amine groups in the fibronectin would. The remaining thiol groups on the cysteine were then at a

1:1 ratio with the NHS esters, and could be representative of the number of available NHS esters and quantified via the Ellman's assay.

Cell studies

To evaluate the efficacy of the hydrogels as a substrate for tissue culture, a series of cell studies were performed. RAW 264.7 macrophages and 3T3 fibroblasts were seeded onto PEG-NB and PAAm gels of varying fibronectin application methods at 250,000 cells per well. Due to the low adherent nature of the macrophages, the fibroblasts were included as a more adherent control. The cells were cultured in Dulbecco's Modified Eagle Medium treated with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. They were allowed to culture for 24 hours before being imaged.

Initially an alamarBlue assay was performed by culturing the cells for 24 hours, then replacing their media and adding 10% by volume alamarBlue. The cells were then incubated for 3 hours before samples were removed from each well and measured for absorbance. After initial conflicting results, a standard curve for an effective incubation time was developed for both cell types, as cell type can play a role in necessary incubation times. This was done by sampling the alamarBlue at different incubation time points over the course of 24 hours. However, after it became clear that neither absorbance nor fluorescence was providing much success in differentiating cell density, the alamarBlue assay was replaced with qualitative analysis through the use of 4',6-diamidino-2-phenylindole (DAPI) and rhodamine phalloidin staining. After 24 hours of culture, the cells were fixed using a 2% glutaraldehyde solution and then stained with

both stains. At 24, 48, and 76 hours the cells were fluorescently imaged and evaluated for cell density and adhesion.

Swelling studies

After the cell studies determined that the PEG-NB gels were the most successful at hosting tissue culture, the modulus of elasticity of PEG-NB gels of varying PEG-NB molecular weight and weight percent was evaluated through analysis of the swelling ratio of the gels. This is based on the relationship between elastic modulus, swelling ratio (via polymer volume fraction), and crosslink density demonstrated by Flory and Rehner, 1943. Five gels of 7.5, 10, and 12.5 wt% using either 10 or 20 kDa PEG-NB were created and left to swell for 24 hours in PBS. After 24 hours, their mass was measured before being placed in a desiccator for 48 hours. Their mass was then measured once more and the q (swelling) ratio of wet mass to dry mass was calculated. The higher the q ratio, the more swollen the gels were capable of becoming, which corresponded to a lower elastic modulus.

CHAPTER III

RESULTS

Ellman's assay

After initial use of the Ellman's assay it became apparent that the assay would not be useful in determining the number of available cell binding sites on the gel surface. Table 1 shows the calculated concentration of bound cysteine, and thus available NHS ester groups, for each gel type.

Table 1: Results of the Ellman's Assay

Type of Gel	Raw Absorbance	Calculated mM of Diluted Supernatant	Calculated mM of Bound Cysteine
1 kPa, Control	0.358756	0.690799	4.47361
34 kPa, Control	0.361278	0.697109	4.423131
1 kPa, Sulfo-SANPAH	0.358311	0.689687	4.482505
34 kPa, Sulfo-SANPHAH	0.360011	0.69394	4.448482
1 kPa, Comonomer	0.3574	0.687408	4.500739
34 kPa, Comonomer	0.331178	0.621807	5.02554

As one can see, there is no difference in calculated bound cysteine concentration between the NHS ester groups and the control groups, for which no NHS esters were applied. There are a few possible explanations for this. First, the cysteine to NHS ester reaction may not occur as efficiently as we had predicted, and thus would not be discernable from the gels where that reaction did not take place. Alternatively, it is possible that there was significant non-specific

binding between the cysteine and the polyacrylamide, so that significant cysteine binding would occur even without the presence of the NHS ester groups. Either way it was clear to us that the Ellman's assay could not provide us with a significant conclusion to which method of fibronectin application would be more successful, so it was decided to use the cell studies as an alternative assessment.

AlamarBlue assay

During the initial cell studies, alamarBlue was used as a means to quantify cellular viability. However, when comparing initial images after 24 hours with measured absorbances, the sensitivity of the assay for our experiment became suspect. Figure 4 shows 3T3 cells on a polyacrylamide gel treated with one dose of sulfo-SANPAH compared to 3T3 cells on tissue culture plastic.

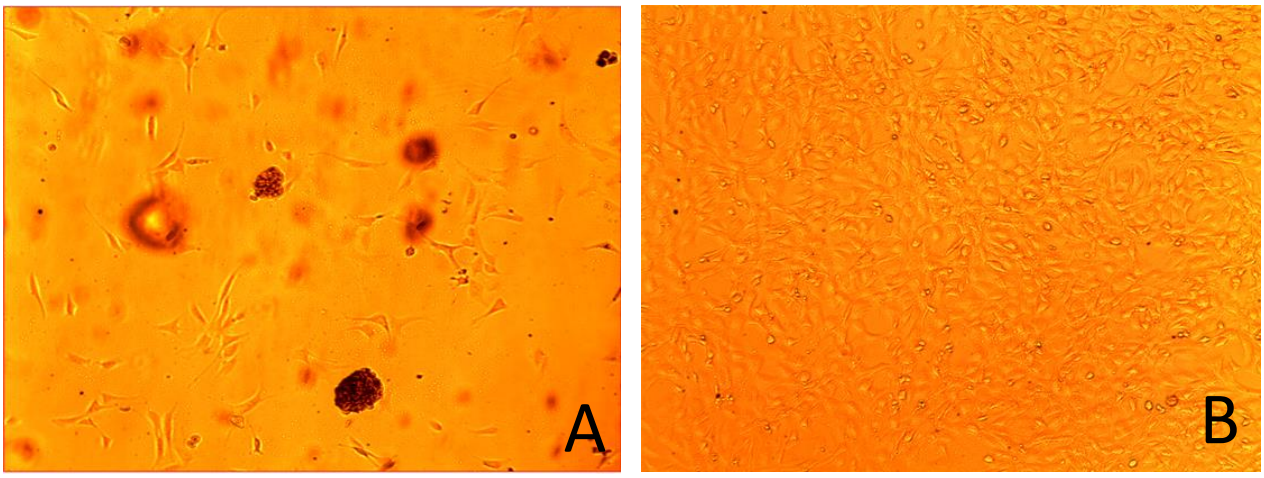

Figure 4: 3T3 cells on polyacrylamide hydrogels and tissue-culture plastic. (A) 3T3 cells on 34 kPa polyacrylamide treated with a single dose of sulfo-SANPAH. (B) 3T3 cells on tissue-culture plastic.

While it is clear from the microscopic images that there is a stark difference between the two in terms of cell density, this large difference was not reflected in the absorbance values from the assay, as can be seen in Figure 5.

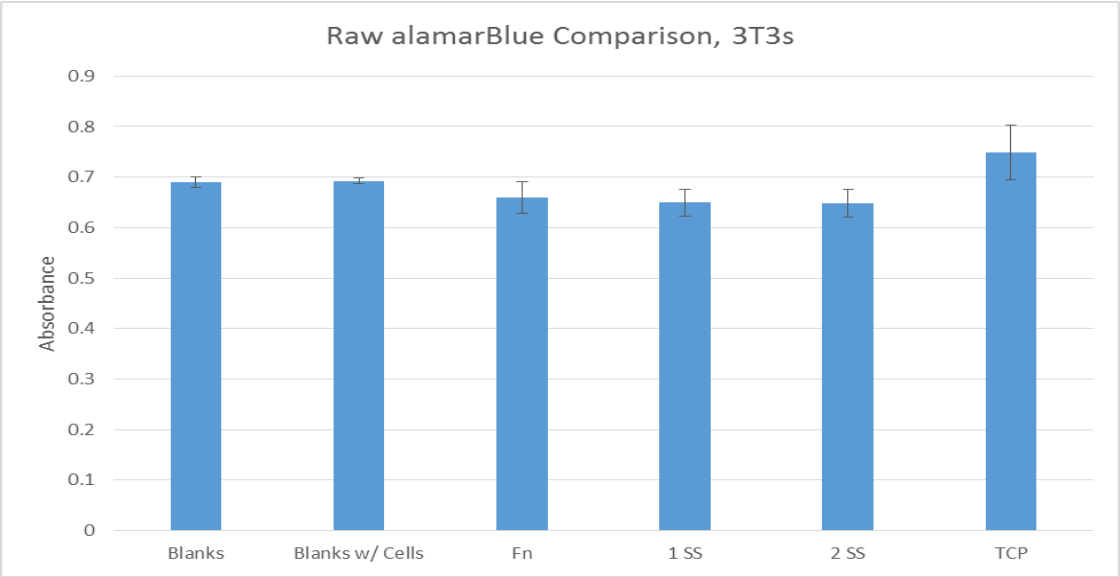

Figure 5: Absorbance values from alamarBlue assay. Blanks were untreated polyacrylamide gels, Fn were gels not treated with an NHS ester solution but were treated with fibronectin, 1 SS and 2 SS were one and two doses of sulfo-SANPAH respectively and TCP was tissue culture plastic. The comonomer method was not yet included in this study.

We hypothesized that this inefficiency may be due to an ineffective incubation time. A standard curve was developed using different incubation times in an attempt to determine the most effective one. However, as seen in Figure 1A, the standard curves did not yield a clear result. The fluorescence curves provided a difference for a cell density of 50,000 cells per well, however our working concentrations for the macrophages were 25,000 cells per well, more comparable to the 20,000 cells per well plots, which did not show a clear difference from the less dense plots. Since we are unable to see a clear distinction between the plots for the varying cell

densities, it was unclear if alamarBlue would be sensitive enough for our purposes. Therefore, we decided to rely on imaging to determine the adhesion and health of the cells.

Cell studies

There was some success seeding macrophages on the fibronectin-coated gels. First, wide-spread cell death was observed amongst the blank and NHS ester-less gels, as expected. Amongst the one and two doses of sulfo-SANPAH and the comonomer method varying degrees of success was observed, as seen in Figures 6, 7, and 8.

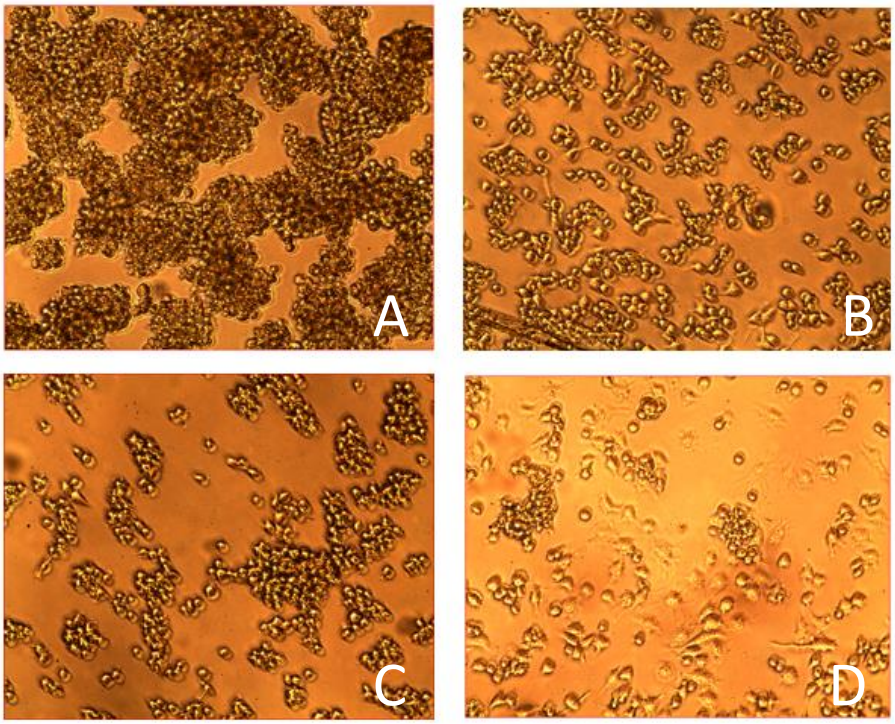

Figure 6: Macrophages seeded on gels with one dose of sulfo-SANPAH. Cell survival ranged from significant cell death (A), moderate cell survival and attachment (B) and (C), and significant cell adhesion and spreading (D).

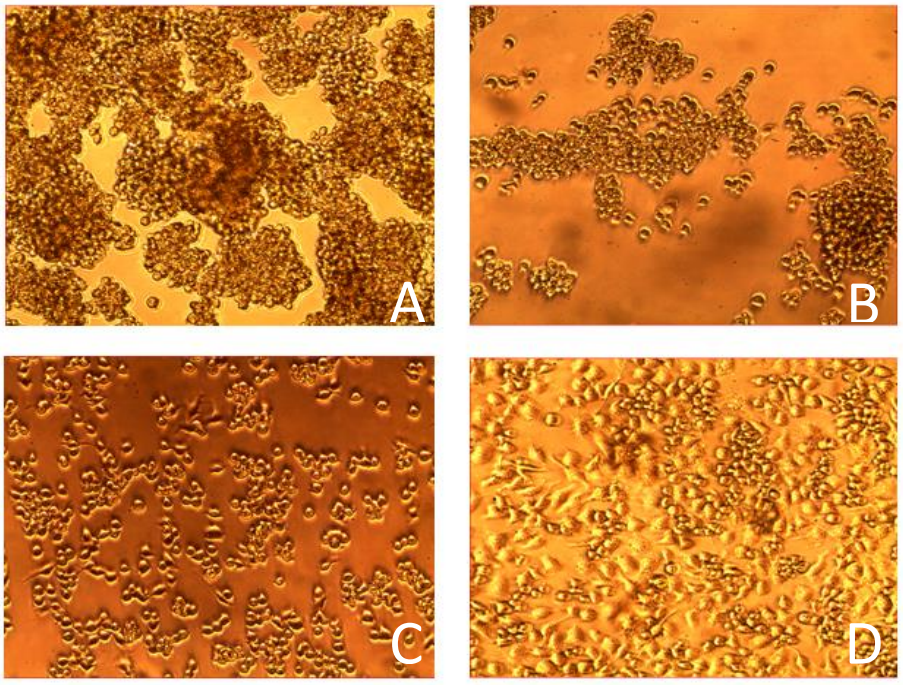

Figure 7: Macrophages seeded on gels with two doses of sulfo-SANPAH. Similar to the single dose cells seen in Figure 6, success of cell attachment and survival ranged from significant cell death (A), to moderate cell survival and attachment (B) and (C), to significant cell attachment and spreading (D).

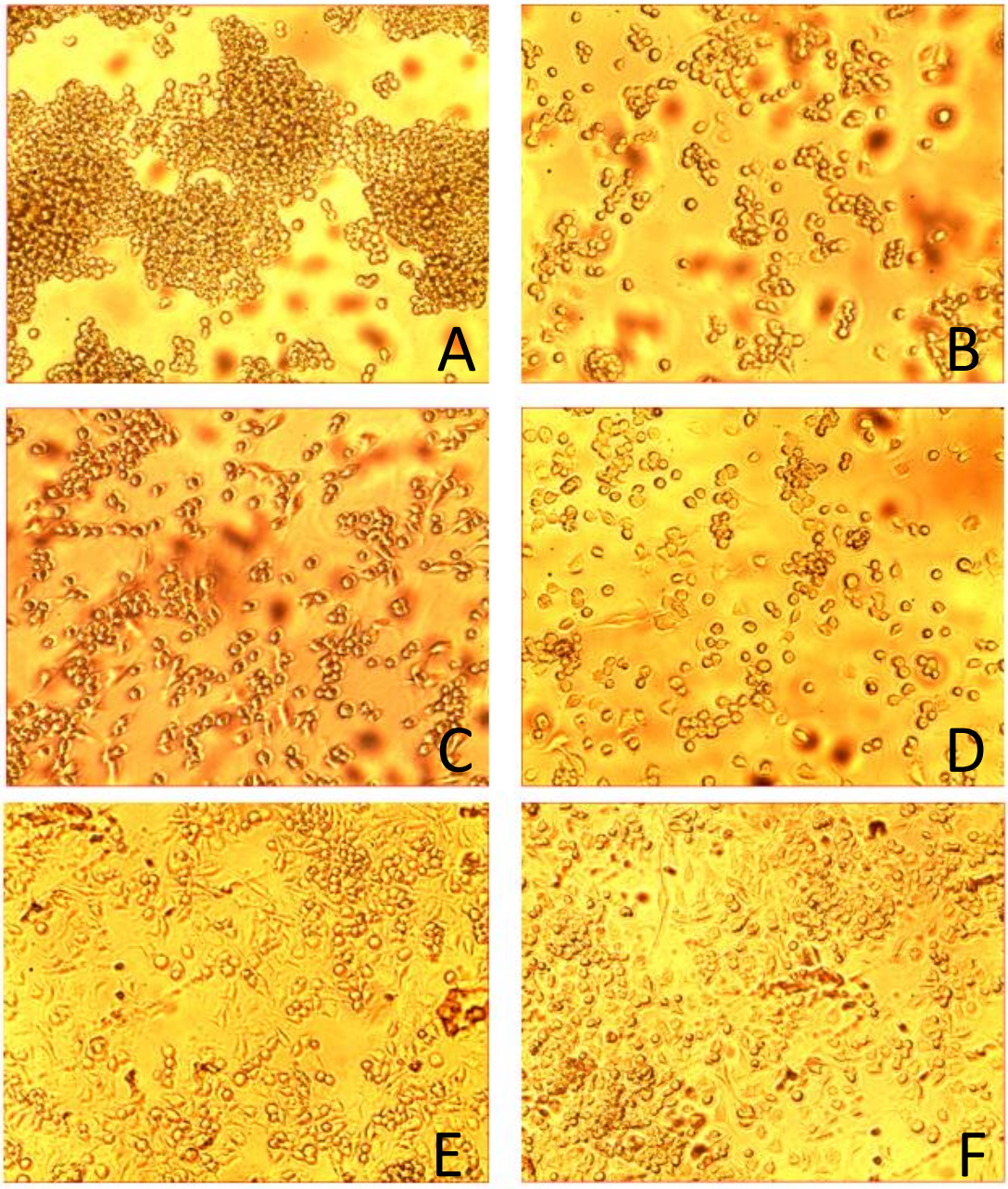

Figure 8: Cells seeded on gels treated with the comonomer method. Success again ranged from significant cell death (A), moderate cell survival and attachment (B), (C), and (D), and significant cell adhesion and spreading (E) and (F).

As can be seen, the macrophages adhered to each of the treated gel types with comparable amounts of success. There were still significantly large portions of the gels that had no cells adhered at all, but there were also sections that had a high cell density, possibly suggesting that the fibronectin distribution was not homogenous. Fluorescent imaging with DAPI false-stained green showed similar results, as seen in Figures 9 and 10.

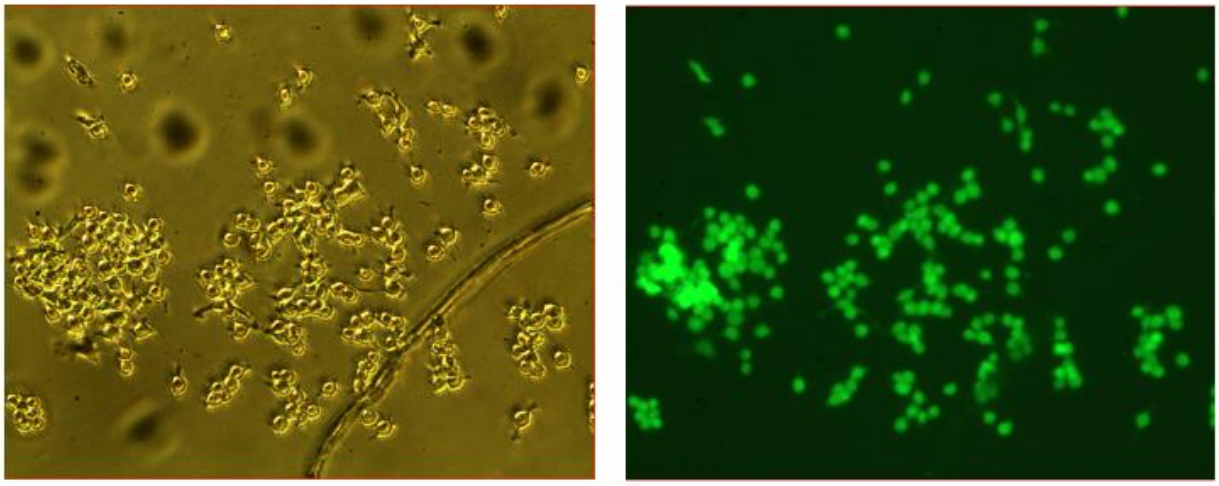

Figure 9: Brightfield and fluorescent image of macrophages on gel treated with one dose of sulfo-SANPAH. The fluorescent staining confirmed that the pictured cells were alive and attached to the polyacrylamide gel. Utilized a false-green DAPI nuclei stain.

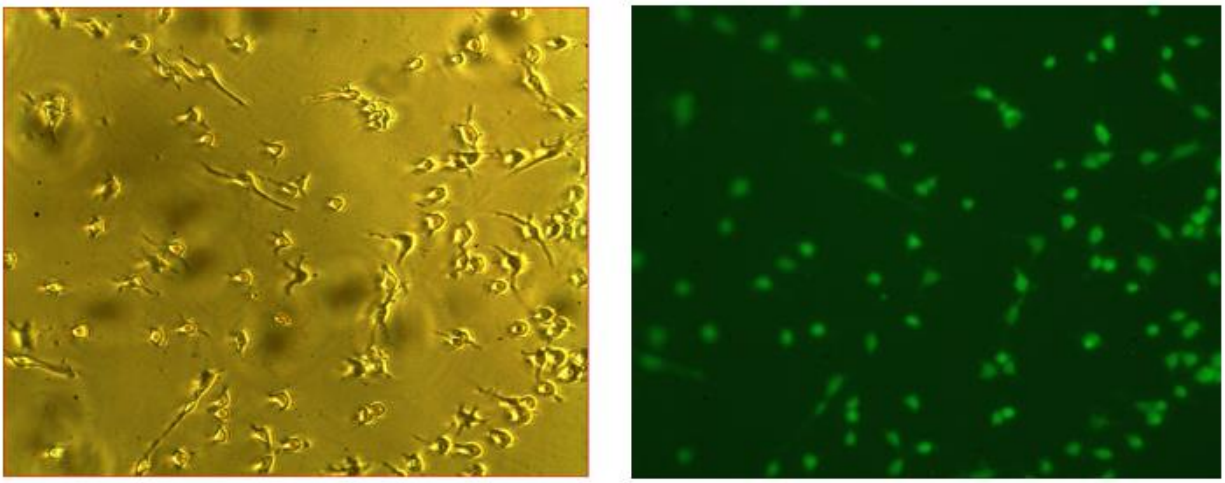

Figure 10: Brightfield and fluorescent image of macrophages on gel treated with comonomer method. The fluorescent staining confirmed that the pictured cells were alive and attached to the polyacrylamide gel. Utilized a false-green DAPI nuclei stain.

After monitoring the gels for an excess of 24 hours, however, it became clear that the PAAm gels were not capable of maintaining a healthy cell culture over a period longer than 24 hours. This is not preferable, as many studies would necessarily need to last for over 24 hours.

Initial cell studies of the PEG hydrogels provided results superior to the polyacrylamide, with higher cell density, longer cell life, and a surface with less physical defects. This was evaluated through qualitative fluorescent imaging using a DAPI nuclei stain and rhodamine phalloidin actin staining, such as that used in Figure 11.

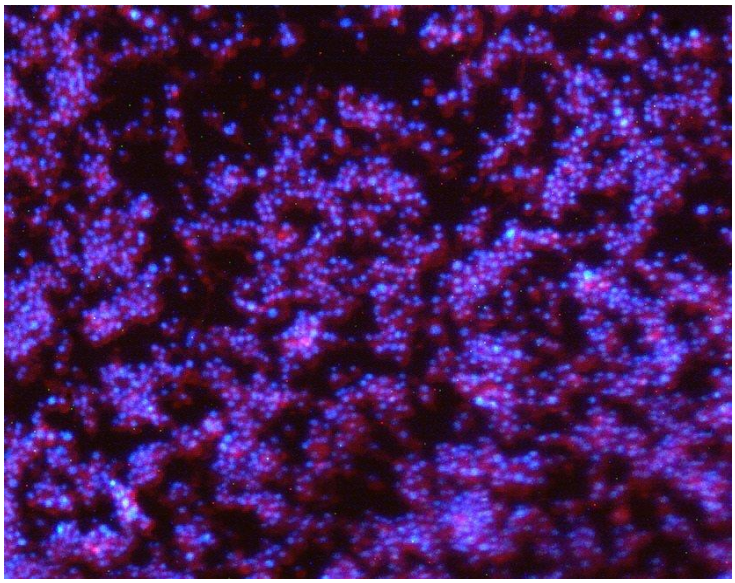

Figure 11: DAPI and rhodamine phalloidin staining of 10 kDa PEG-NB hydrogel after 72 hours. Both the 10 kDa and the 20 kDa showed successful cell adhesion up to at least 72 hours for all weight percents. The actin morphology was not distinguishable, however, preventing analysis of actin filament organization.

Due to the resolution of the initial photos, we were unable to phenotype the actin organization of the cells, which is necessary for us to analyze the effect matrix elasticity has on cell phenotype.

However, the success of the PEG-NB gels in culturing healthy macrophages demonstrated the potential of the PEG-NB system, and was selected for use in continued studies.

Swelling studies

The results of the study on the swelling ratio of 7.5, 10, and 12.5 weight percent of both 10 and 20 kDa PEG-NB hydrogels was as expected. As molecular weight of the PEG-NB macromer increased, cross-link density decreased, resulting in a lower modulus of elasticity and higher q ratio. Alternatively, as weight percent of the PEG-NB was increased, the concentration of macromer increased, which raised modulus of elasticity and lowered the q ratio. Both of these trends were observed during the swelling study, as seen in Figure 12.

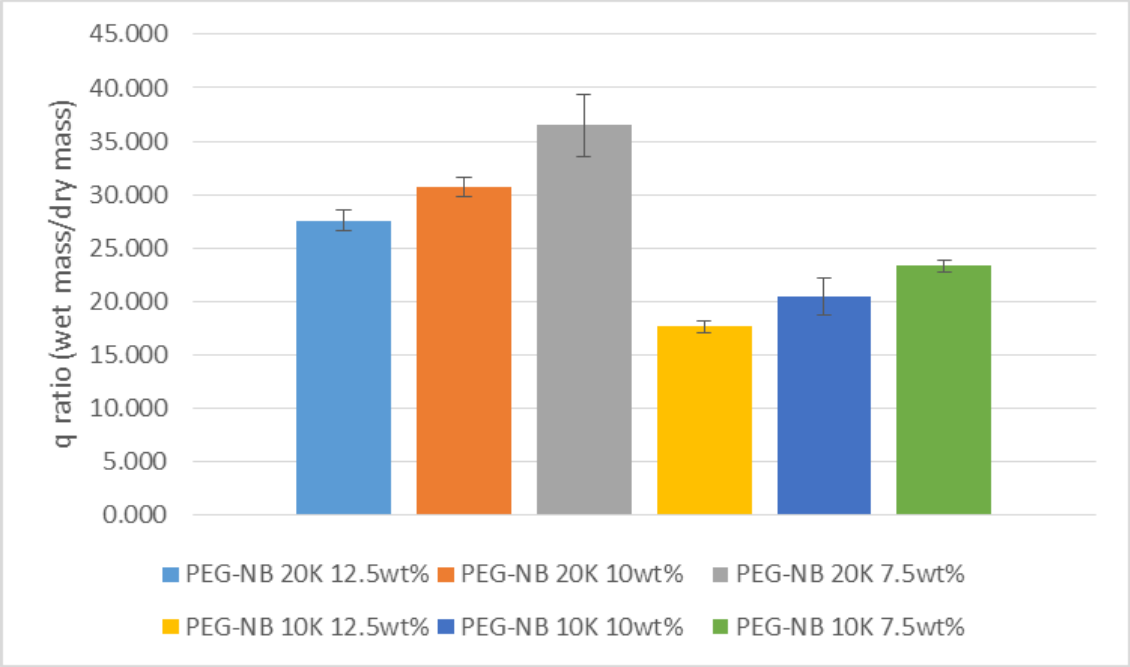

Figure 12: q ratio of each PEG-NB gel formulation. The q ratio varied as predicted, increasing as molecular weight increased and weight percent decreased. The q ratio is inversely proportional to the modulus of elasticity, and thus tunability of q ratio represents the tunability of elastic modulus.

Since the q ratio varied in a predictable manner as suspected, we concluded that the elastic modulus of the PEG-NB hydrogels could also be predictably varied, representing the tunability of our PEG-NB platform.

CHAPTER IV

CONCLUSION

We have been able to demonstrate the successful development of a poly(ethylene glycol)-norbornene hydrogel platform capable of hosting pathogen-host cell interaction studies. This conclusion was drawn from the length of time with which the PEG-NB gels could host a healthy macrophage culture as well as the ease with which the elastic modulus of the gels could be tuned. In a simple, one-step polymerization and functionalization, the PEG-NB gels can be formed with a specific modulus, based off of the molecular weight and weight percent of the macromer, and can host a macrophage culture for at least 72 hours. This is in contrast to the PAAm hydrogels, which required lengthy fabrication and functionalization processes. We believe it was due to this extended fabrication, which had plenty of opportunities for damage to the gel or its functionalized surface, that we observed underwhelming results from the PAAm gels.

Current studies utilizing the PEG-NB gels for study of *Brucella* are currently ongoing. In addition to these infection rate studies, we intend to pursue further characterization of the hydrogel elasticity through rheology. Furthermore, we intend to thoroughly phenotype the macrophages on gels of varying elasticity. This will include a more in-depth look at actin filament morphology, as well as analysis of reactive oxidative species and phosphoinositide 3-kinase, to assess macrophage health, and tumor necrotic factor alpha to assess macrophage function. With these studies we intend to thoroughly evaluate the factors involved in pathogen-host cell interaction.

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APPENDIX A

Table 1A: Formulations of varying elasticity for polyacrylamide hydrogels. Adapted from Tse et al., 2010

	Modulus	**Acrylamide**	**Bis-Acrylamide**	**PBS**
3 µL TEMED	1.19 kPa	100 µL	30 µL	870 µL
	8.73 kPa	125 µL	150 µL	725 µL
2 µL TEMED	19.66 kPa	200 µL	132 µL	668 µL
	34.88 kPa	250 µL	150 µL	600 µL

Table 3A: Gel table for 20 kDa PEG-NB gels at 7.5 wt%

PEG-NB 20K
7.5wt%

	thiol-ene	thiols	alkenes	Gel	Gels
	1	14.64844	14.64844	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 20K)	20480	9.765625	3.662109	18.75	300
DTT		100	6.324219	3.162109	50.59375
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				25.08789	401.4063
				50	800

Table 4A: Gel table for 20 kDa PEG-NB gels at 10 wt%

PEG-NB 20K
10wt%

	thiol-ene	thiols	alkenes	Gel	Gels
	1	19.53125	19.53125	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 20K)	20480	9.765625	4.882813	25	400
DTT		100	8.765625	4.382813	70.125
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				17.61719	281.875
				50	800

Table 5A: Gel table for 20 kDa PEG-NB gels at 12.5 wt%

PEG-NB 20K
12.5wt%

	thiol-ene	thiols	alkenes	Gel	Gels
	1	24.41406	24.41406	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 20K)	20480	9.765625	6.103516	31.25	500
DTT		100	11.20703	5.603516	89.65625
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				10.14648	162.3438
				50	800

Table 6A: Gel table for 10 kDa PEG-NB gels at 7.5 wt%

PEG-NB 10K
7.5wt%

	thiol-ene	thiols	alkenes	Gel	Gels
	1	28.62595	28.62595	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 10K)	10480	19.08397	7.156489	18.75	300
DTT		100	13.31298	6.656489	106.5038
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				21.59351	345.4962
				50	800

Table 7A: Gel table for 10 kDa PEG-NB gels at 10 wt%

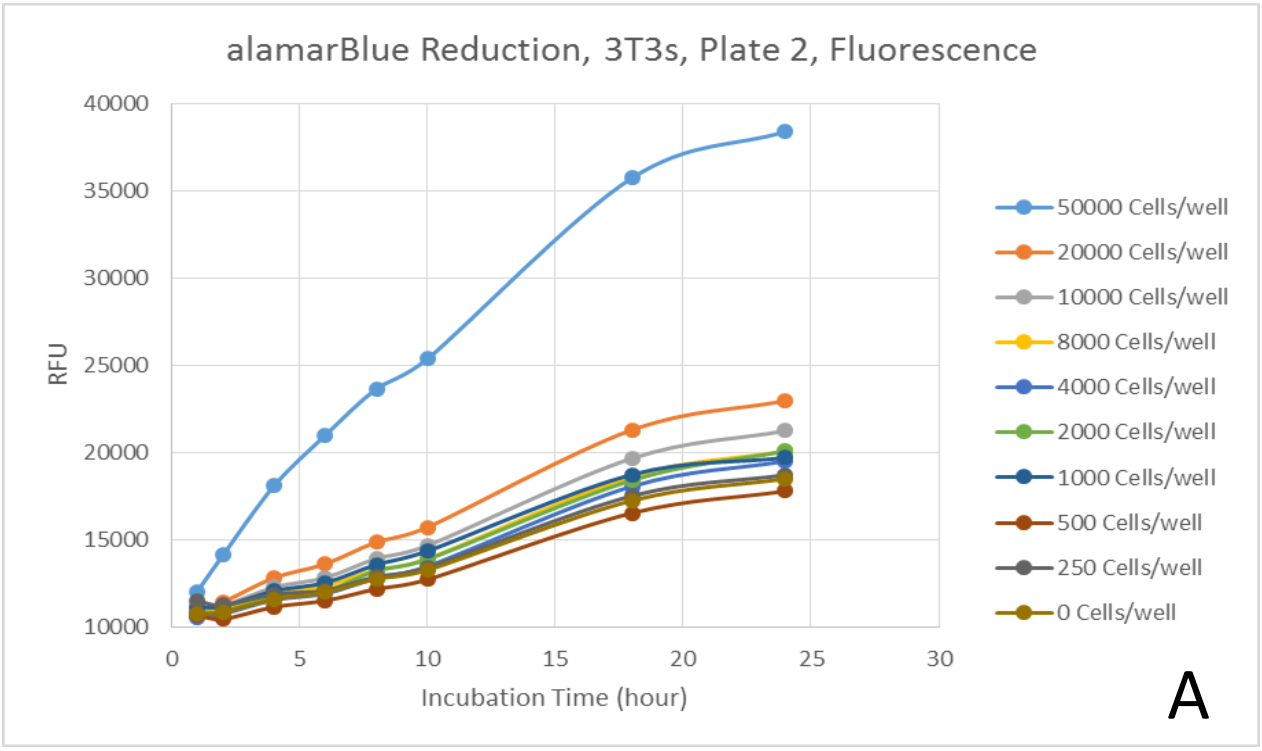
PEG-NB 10K
10wt%

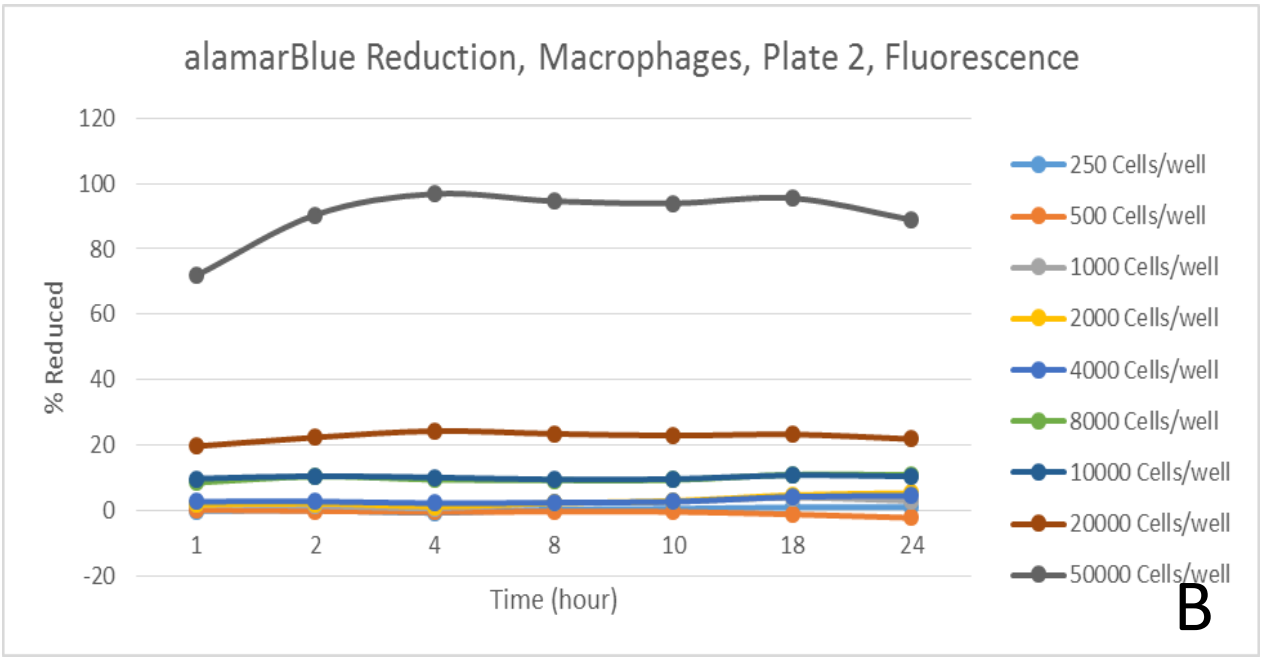
	thiol-ene	thiols	alkenes	Gel	Gels
	1	38.16794	38.16794	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 10K)	10480	19.08397	9.541985	25	400
DTT		100	18.08397	9.041985	144.6718
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				12.95802	207.3282
				50	800

Table 8A: Gel table for 10 kDa PEG-NB gels at 12.5 wt%

PEG-NB 10K
12.5wt%

	thiol-ene	thiols	alkenes	Gel	Gels
	1	47.70992	47.70992	1	16
Chemical	MW	Stock (mM)	Working (mM)	Volume (uL)	Volume (uL)
PEG- NB (4 arm, 10K)	10480	19.08397	11.92748	31.25	500
DTT		100	22.85496	11.42748	182.8397
CGRGDS	593.62	50	2	2	32
LAP		100	2	1	16
PBS				4.322519	69.16031
				50	800





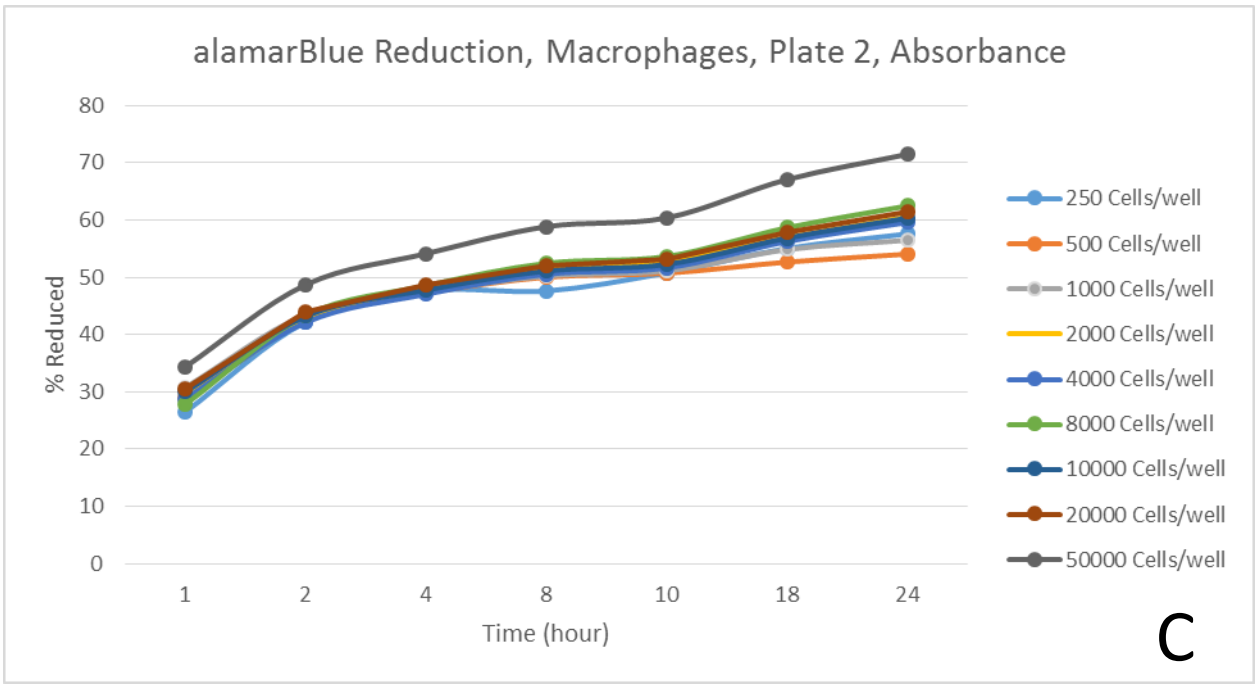


Figure 1A: AlamarBlue standard curves for both 3T3s and macrophages. (A) Graph of the 3T3 standard curve developed. Each plot represents the number of cells per well. (B) Graph of the macrophage standard curve measuring fluorescence. (C) Graph of the macrophage standard curve measuring absorbance.