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High-Throughput Assessment of a Novel, Thiol-Acrylate Hydrogel for Tumor Spheroid Synthesis in a Microfluidic Device

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HIGH-THROUGHPUT ASSESSMENT OF A NOVEL, THIOL-ACRYLATE HYDROGEL FOR TUMOR SPHEROID SYNTHESIS IN A MICROFLUIDIC DEVICE

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ABSTRACT

The extracellular environment plays an important role in regulating cell behavior. The mechanical, structural, and compositional properties of the extracellular matrix can determine the fate of the growing cells. Standard two-dimensional culturing in a flask fails to represent a native cellular environment. To create the native environment suitable for cells, hydrogels were previously studied. One of the most extensively studied synthetic hydrogels was a poly(ethylene glycol diacrylate) (PEGDA) hydrogel. However, the need for UV polymerization may introduce unwanted changes in cell morphology and viability. The hydrogel being proposed was a form of a thiol-acrylate hydrogel that can eliminate this problem and can easily be modified to approximate a native cellular environment. To be more specific, it was a PEGDA (700 MW) and ethoxylated trimethylolpropane tri(3-mercaptopropionate) (ETMPTMP 1300 MW) hydrogel, through thiol-Michael addition instead of thiol-ene radical polymerization. This thiol-acrylate hydrogel was evaluated for its swelling behavior, degradation behavior, and elastic modulus. The hydrogel was then used to encapsulate breast cancer cells to determine the effect on the viability of the cells and on-chip microfluidics. The breast cancer cell lines used for viability testing and microfluidics were MDA-MB-231 and MCF-7. All experiments were done at pH 7.4 and kept at 37.1°C.

CHAPTER 1. THIOL-ACRYLATE HYDROGEL FORMULATION AND CHARACTERIZATION

1.1. Chapter Summary

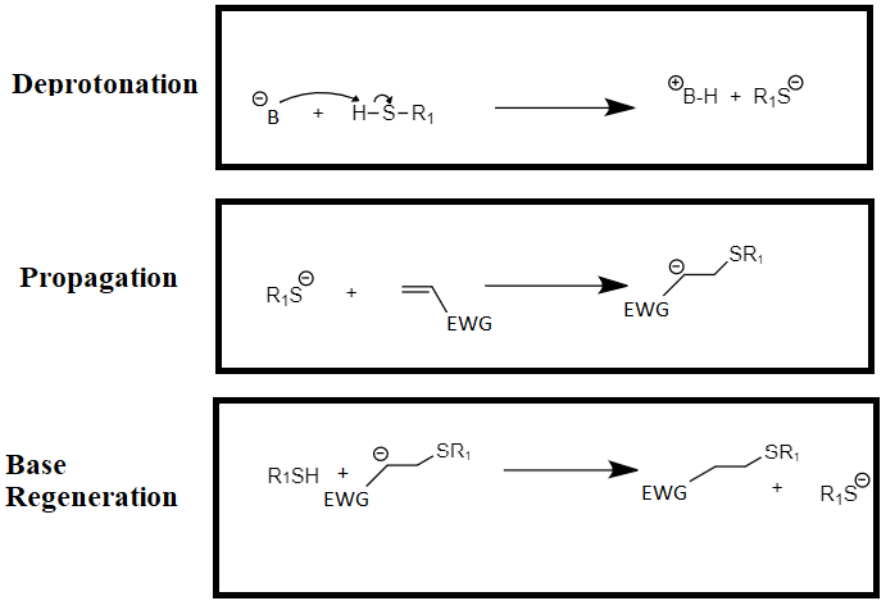
In this chapter, three thiol-acrylate polymer concentrations will be examined and characterized to look at each of their advantages and disadvantages they might offer towards tumor spheroid culturing. This particular thiol-acrylate recipe was designed by Dr. Tullier, but he focused on longer lasting hydrogels.[1] First, the motivation or the problem that this hydrogel will try to solve will be discussed. Next, the general curing procedure will be introduced, and some of the expertise needed to perfect the hydrogel making process. Following that, the characterization of each hydrogel, starting with the amount of swelling that occurs when adding growth media. Next, the degradation under *in vivo* physiological condition will be tested to determine gel life. The last characterization will focus on the elastic modulus and how the different polymer concentrations will affect the stiffness of the hydrogel. The final section will focus on advantages and disadvantages of each hydrogel and why the proposed polymer concentration can be further used in tumor spheroid testing. All testing was done under the body's physiological conditions, which was 37.1°C, 7.4 pH, and an open environment for oxygen intake.

1.2. Introduction

Thiol-ene chemistry is not a novel idea. Posner is accredited with reporting on the thiol-ene reaction first in 1905.[2] However, it was not until the late 1990s that a wider range of applications had been reported. A thiol-ene reaction is the formation of a carbon-sulfur bond between a thiol group (R-SH) and a carbon-carbon double bond (alkene). There are two different mechanisms by which a thiol-ene can react, the first being through free-radical additions. Free-radical additions can be initiated by light, heat, or radical initiators.[3] This step however could negatively impact the growth of the cancer cells because of the additional variable added into the

delicate growth of cells which can cause cell death or contamination. The thiol-acrylate that is being proposed is not through this mechanism, but through a Michael addition. This mechanism eliminates the use of an initiator and is catalyzed by either a base or a nucleophile.[4] Since cells are needed to be cultured in 7.4 pH adding a base to raise the pH is already required.

The Michael addition reaction was named after Arthur Michael who reported on them in 1887. A Michael addition is the addition of a nucleophile to an electron deficient carbon covalent bond.[5] The thiol-Michael addition requires the thiol to first be deprotonated in order to react, and was reported in 1947 by Hurd and Gershbein.[6] The general mechanism of base-catalyzed thiol-Michael addition reaction is shown in scheme 1. The initiation step deprotonates the thiol with a base to form a thiolate anion. The propagation step is when the thiolate adds to an electrophilic ene to form a stabilized carbanion. After, the carbanion deprotonates the base catalyst. This catalyst had been protonated in the initiation step in order to regenerate it.



Scheme 1. General base-catalyzed thiol-Michael addition mechanism

Both radical thiol-ene and thiol-Michael polymerize through a step-growth mechanism. The rate limiting step for thiol-Michael polymerization is the propagation step because of the proton needed for the transfer to a strong carbanion base.

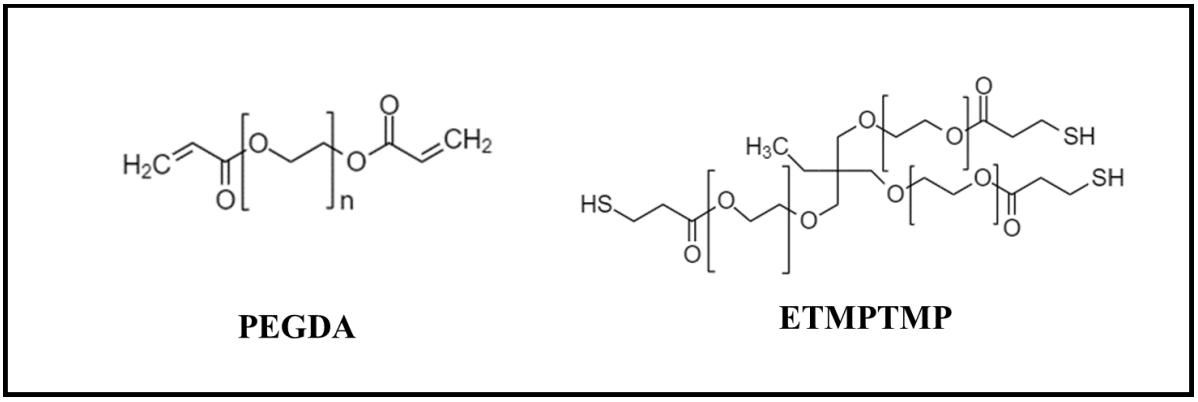
1.3. Motivation/Problem

A thiol-Michael addition hydrogel attempted to solve some possible drawbacks of the currently used radical thiol-ene seen in tumor spheroid culturing. Before this could be accomplished the thiol-Michael addition hydrogel had to be heat-resistant at 37.1°C, be permeable to oxygen, have a pH of 7.4 and stay solid for at least 3 days, preferably 7 days. These criteria, excluding time, allowed the cells to grow in semi-realistic setting. The reason this hydrogel was needed was because current hydrogel materials require harsh gelation strategies, which may affect long term cell growth in cultures. A poly(ethylene glycol) hydrogel was previously used before this thiol-acrylate hydrogel was tested. The materials and methods for that specific hydrogel were replicated from Krutkramelis et al.[7] and Yeh et al.[8] A possible drawback was that this specific hydrogel requires the use of UV light to cure the hydrogel, which may kill the cells already trapped inside. An avoidance of any initiator that may harm cell growth would be beneficial, so moving into thiol-Michael addition reactions would remove that initiator step. Another benefit of thiol-Michael addition was the fact that gelling occurs at room temperature.[9] Moreover, the thiol-acrylate hydrogel was highly tunable. The thiol-acrylate's stiffness can be easily changed with a change in the formulation of the hydrogel. The thiol-acrylate can also be conjugated with relevant biomolecules to better simulate the extracellular matrix of the specific cells to be studied.[10]

1.4. Hydrogel Formulation

1.4.1. Materials

Ethoxylated trimethylolpropane tri(3-mercaptopropionate) 1300 (ETMPTMP 1300) was generously donated by Evans Chemetics LP. Poly(ethylene glycol) diacrylate average Mn 700 g/mol (PEGDA 700) was purchased from Sigma Aldrich. The phosphate-buffered solution (PBS) was purchased from Amresco Inc in powdered form. See Scheme 1.2 for reagent structures.



Scheme 1.2. Reagent Structures

1.4.2. Methods

The acrylate and thiol were added sequentially to a plastic 15 mL centrifuge tube. Care was taken to mix each layer as little as possible during addition to prevent local gelation from occurring. It was possible that if they mix early, a majority of the mixture would be unreacted and the hydrogel would not form a homogenous mixture. Both the thiol and acrylate were extremely viscous at room temperature so a bulb pipette was used throughout the process. The acrylate was added first down the center of the centrifuge tube. Once a layer of acrylate was formed, the thiol was then added slowly, drop by drop down the side of the centrifuge tube. After all the thiol slid down the tube, the PBS was added the same way as the acrylate was. Finally, the base (5 M NaOH) was added to raise the pH to 7.4. The centrifuge tube was vigorously shaken

up and down, then vortexed at 20 seconds, and repeated once more. See table 1 for exact amount of reagents used.

Table 1. Thiol-Acrylate Formulation

Thiol-Acrylate Formulation				
Reagent	8.5%	9.5%	10.5%	units
PEGDA (700 MW)	0.42	0.48	0.53	g
THIOCURE® ETMPTMP (1300 MW)	0.52	0.59	0.66	g
PBS (1X)	10.12	10.23	10.11	g
NaOH (5M)	5	6.5	7	µl

1.4.3. General Curing Tips and Times

Before the hydrogel was prepared, chemical reagents were adjusted to room temperature. The ETMPTMP at room temperature was much easier to dispense than when it was not. There were three different formulation being tested. A higher polymer concentration [(PEGDA + ETMPTMP)/ (PEGDA + ETMPTMP + PBS + NaOH) in mass] had a shorter time to cure and become a solid hydrogel. A higher polymer concentration had a longer time where the hydrogel was solid. The 8.5% will take approximately 3 hours, 9.5% approximately 1 hour, and 10.5% approximately 30 minutes to gel. The addition of the base catalyzes the reaction and allowed for gelling in approximately 1 hour instead of 12 or more.[11]

1.5. Characterization of Thiol-Acrylate Hydrogel

1.5.1. Swell Test Material

All chemicals used in section 1.5 can be found in section 1.4.1, unless otherwise noted. The gravity convection incubator was purchased from VWR.

1.5.2. Swell Test Methods

See section 1.4.2 on how to make hydrogel. The vortexed hydrogel was then poured into petri dishes. One petri dish was the control, with no growth media (refer to section 2.3.3 for

specifics on growth media) added. Once the hydrogel was fully gelled, the mass of each petri dish was recorded (W_i). Next, the same amount of growth media was added on top of the non-control group petri dishes. The petri dish was then carefully placed into the incubator at 37.1°C until the next measurement. After 24 hours, the growth media in the petri dish was emptied out in a waste container. The petri dish was then weighed again (W_f). The same amount of growth media added the previous day was then added again. This step was repeated daily for 7 days or until the gel was degraded. Each day a measurement was taken. The new W_f measurement was compared to the initial W_i. The percentage of swelling, Q, was calculated from the equation:

$$Q = \frac{W_f}{W_i} \times 100$$

1.5.3. Swell Test Results

At 30 minutes the 8.5% hydrogel swelled by 8.6% mass, the 9.5% hydrogel by 8.4% mass, and the 10.5% hydrogel by 12% mass. Looking at figure 1, an immediate swelling was seen. This gave evidence that the hydrogel membrane was not impermeable to growth media, which meant that the hydrogel will allow growth media to get inside the membrane to feed the cells. There was an odd dip in percentage after 24 hours in the incubator for every trial run. After that initial dip at 24 hours, the initial swell doubled for both the 9.5% and 10.5% hydrogel before they fully degraded. The 8.5% nearly doubled, but degraded quicker than the other 2 formulations, so it only had a final swell of 14%. The 9.5% had a final swell of 19%, while the 10.5% had a final swell of 23%. It appeared that the higher formulation had a higher swell, but that was only because the 10.5% stayed in growth media for a longer period of time. If the figure was examined more closely, the swelling percentage was highest for the lowest polymer concentration on the final day before degradation. There was either very little difference between each polymer concentration or the lowest hydrogel concentration had the highest swelling

percentage. The pattern that the lower polymer concentration had a higher swelling percentage, was to be expected.[12]

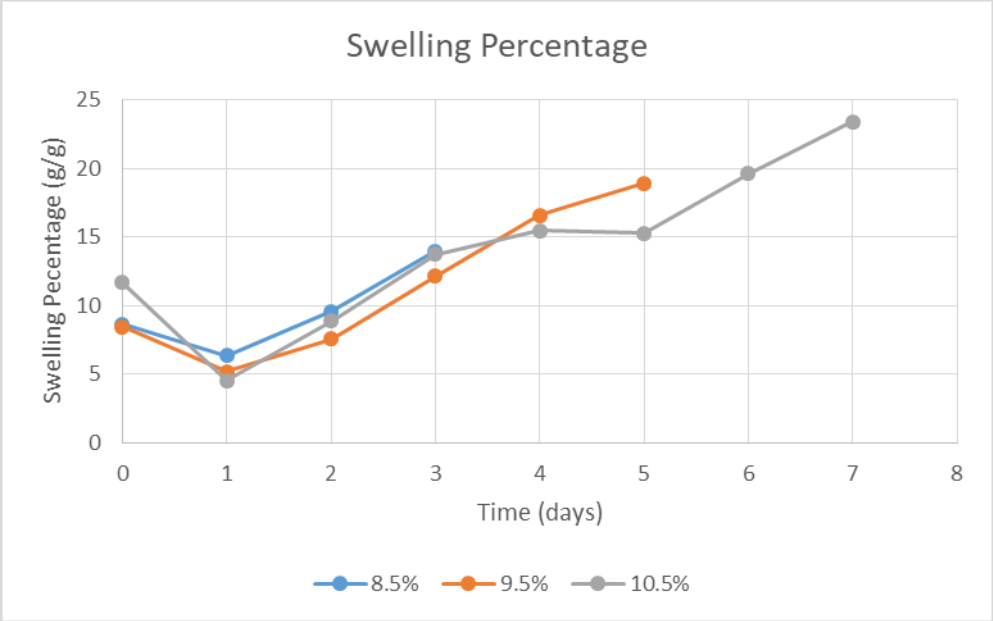


Figure 1. Swelling percentage of hydrogel formulation

1.5.4. Degradation Methods

Same steps as section 1.5.2, but instead of measuring mass, attention was given to the gel itself. This was continued until all gels degraded into a liquid form, and a record was taken once a day. Control was used as dry testing, while other petri dishes were used for wet degradation.

1.5.5. Degradation Results

The 8.5% hydrogel without growth media was a viscous liquid after approximately 3 hours. The 8.5% hydrogel never fully gelled, but it stayed in that form for about 48 hours. The 8.5% hydrogel with growth media lasted a bit longer. At 48 hours, the hydrogel was still a viscous liquid, but after 72 hours, it was entirely liquid. The 9.5% and the 10.5% hydrogel both gelled to a solid state. The 9.5% hydrogel without growth media lasted less than 3 days before initial signs of degradation occurred, and by 4 days, it was completely degraded. The 9.5%

hydrogel with growth media showed first signs of degradation at 4 days and lasted 5 days before complete degradation. The 10.5% hydrogel without growth media showed first signs of degradation at about 7 days and lasted 8 days before complete degradation. The 10.5% hydrogel with growth media lasted 8 days before first signs of degradation and about 10 days for complete degradation. The conversion back to liquid form is due to hydrolysis of ester bond present in polymer matrix. It was worth noting that the 10.5% hydrogel without growth media, in a sealed tube and not in an incubator, had shown no signs of degradation after 40+ days. Table 1.2 shows results with growth media.

Table 1.2. Approximate gel and degradation times of hydrogel

Gel Polymer Percentage	8.5%	9.5%	10.5%
Gelation Time	3 hours	1 hour	30 minutes
Gel Life before Degradation	3 days	5 days	10 days

1.5.6. Hydrogel Modulus Materials

DMA Q800 was provided by Polymer Analysis Lab (PAL) at Louisiana State University in the Department of Chemistry. The silica rectangular mold was provided by Dr. Pojman's lab.

1.5.7. Methodology for Measuring the Hydrogel Modulus

For modulus measurements, three samples were prepared and then used to pour into a silica mold to gel. Once gelled, the hydrogel was taken out of the mold and placed into a petri dish with growth media to prevent evaporation. The gel was then cut into a square (1 mm x 10 mm) within guidelines of DMA sample preparation for compression clamp. DMA was performed at 37.1°C and initial force applied was 0.0005 N. This force was increased by 1 N/min up to 18 N or until the gel deformed.

1.5.8. Hydrogel Modulus Results

All three samples were prepared, but because the 8.5% hydrogel formed a viscous liquid instead of a solid gel, DMA was not able to be used to characterize it. Figure 1.2 shows the Young's modulus of 9.5% and 10.5% hydrogel. The 10.5% hydrogel had an elastic modulus of 8400 Pa and the 9.5% hydrogel had an elastic modulus of 7800 Pa. The trend that the lower polymer percentage had the lower elastic modulus was to be expected.[13] Figure 1.3 illustrates the stiffness and relaxation modulus. This figure shows that the gel will revert back to its gel-like state after force was exerted upon it.

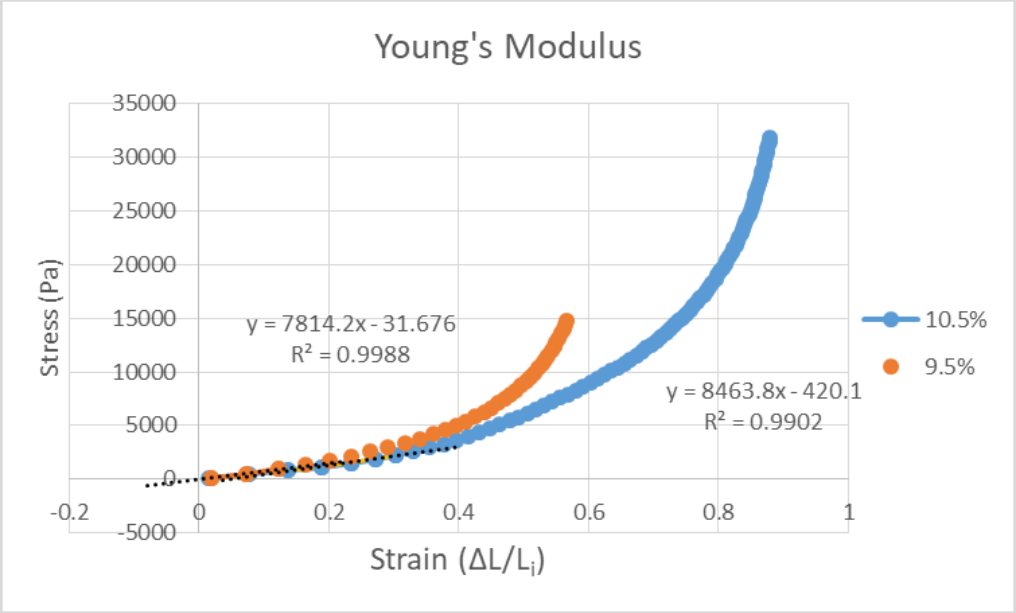


Figure 1.2. Young's modulus from raw data

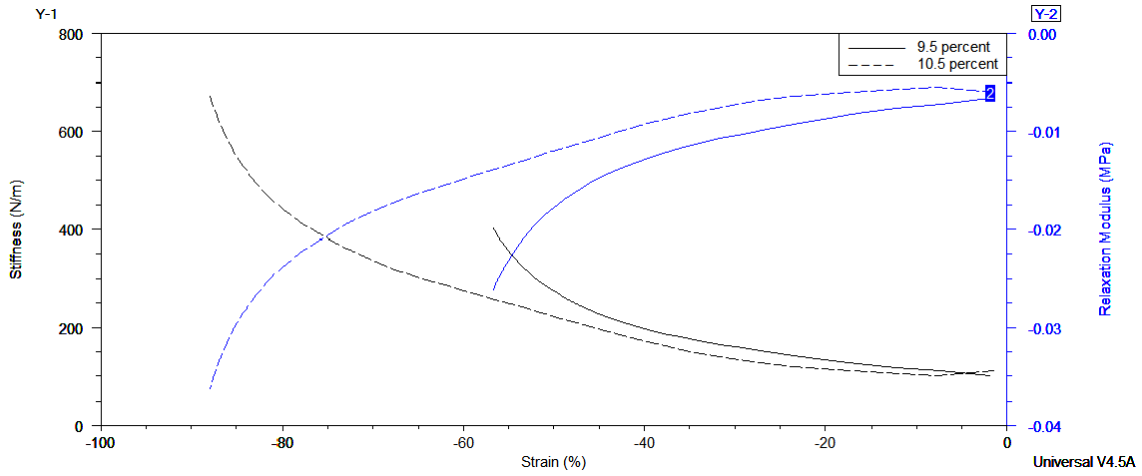


Figure 1.3. Stiffness and relaxation modulus for 9.5% and 10.5% hydrogel

1.6. Conclusion

Each hydrogel formulation has advantages and disadvantages depending on which experiment is being conducted. Each hydrogel concentration absorbs growth media fairly well. There is no reason to think that this would be the determining factor in choosing the specific hydrogel, because they would each be able to allow growth factor to feed the cells. The degradation time, however, could be a factor depending on the experiment that is being run. The longevity of the higher concentration of polymer is good, but the drawback is the much shorter gel time. If more time is needed for cell preparation, a lower concentration of the polymer may be needed. Finally, the elastic modulus was tested, and the higher the polymer concentration, the higher the stiffness. This again, could be good or bad depending on the stiffness that certain cells can tolerate. These advantages and disadvantages would need to be examined when doing cell testing to determine the best match for the specific experiment. With this in mind, initial hydrogel testing with breast cancer cells will be done with 10.5% hydrogel because of the longer lifespan of the hydrogel. This will allow more time to study cell growth.

CHAPTER 2. VIABILITY OF TUMOR SPHEROID WITH THIOL-ACRYLATE HYDROGEL THROUGH CONFOCAL MICROSCOPY AND MICROFLUIDICS

2.1. Chapter Summary

Viability testing with confocal microscopy was done to determine if the thiol-acrylate hydrogel was inherently cytotoxic to the breast cancer cells. After the gel was confirmed to not impact cancer cell viability, on-chip microfluidics experiments were done to test the growth rate of the cells inside the hydrogel. The first thing to be discussed will be how cells are prepared for each experiment. Next, the two cell lines that were used will be discussed, and how they differ from each other. After, the viability experiment with confocal microscopy will be discussed. Finally, this will lead into the growth testing discussion with on-chip microfluidics.

2.2. Introduction

Mass cell culturing is fundamental to the creation of viral vaccines. Cell culturing is the practice of growing cells under controlled conditions, usually outside their natural environment. The cells are obtained from living tissue that is maintained under certain conditions. Those conditions can differ from each cell type, but they generally need a growth media that supplies essential nutrients such as vitamins, minerals, amino acids, and growth factors. They also need to be in a well-regulated environment that controls the pH, the gradient pressure, and the temperature.[14] In this experiment, those factors will be controlled by a modified growth media, a thiol-acrylate hydrogel and an incubator.

One of the most well-known and common methods for culturing cells is the two-dimensional (2D) cell culture method. This method was first reported in 1885 by Wilhelm Roux, who maintained a medullary plate of an embryonic chicken on a flat glass plate.[15] With the advancement of technology came the petri dish from Julius Petri[16] and the laboratory flask. This

method of culturing maintains the cell line easily enough, but cannot be efficiently used for testing because it does not represent a similar environment in which the original cells are found.[17] Cells are now being grown on more flexible substrates to get a different phenotype response from the cells that cannot be replicated with a rigid flask.[17]

This relatively new method is three-dimensional (3D) culturing. There are a few ways that this can be accomplished, but primarily 3D culturing in hydrogels is the method that will be discussed. The hydrogel can be made to simulate the extracellular matrix, which can mimic *in vivo* like cell culturing. The extracellular matrix is vital in the survival, proliferation, differentiation, and migration of cells.[18] The closer the natural environment of the isolated cell can be exhibited, the higher the likelihood of being able to effectively test them.

Hydrogel polymers can be divided into two categories. Those two divisions are natural polymers and synthetic polymers. Natural polymers are usually derived from extracellular matrix proteins, like collagen, or biological polysaccharides, like alginate. Synthetic polymers, like PEG,[7-8] are beneficial because the gelation strategy can be tailored to not harm the cells when they are trapped within the hydrogel.[19] A synthetic polymer like the thiol-acrylate being introduced, had not yet been tested for cytotoxicity of the gelation as well as the hydrogel itself, and will be the focus of this chapter.

2.3. Cell Preparation

2.3.1. Materials

MDA-MB-231 and MCF-7 breast cancer cells were provide by Dr. Elizabeth Martin in the Department of Biological and Agricultural Engineering. Dulbecco's modification of Eagle's medium (DMEM), phosphate-buffered saline (PBS), 0.25% Trypsin with 2.21 mM

ethylenediaminetetraacetic acid (EDTA), sodium pyruvate, MEM nonessential amino acid, MEM amino acid, calf serum, insulin and 75 cm^2 flask was purchased from Corning Cellgro. The Symphony incubator was purchased from VWR.

2.3.2. Methods

2.3.3. Preparation of Media

50 mL of calf serum, 6 µl insulin, 5 mL of essential amino acids, and 5 mL of nonessential amino acids were added into a 500 mL container of DMEM. This was the growth media used for all culturing experiments conducted with MDA-MB-231 and MCF-7 cancer cells.

2.3.4. Tissue Culture

When cells were approximately 75% confluent in the flask, they were passed to extend the life of the cells. To do this, 15 mL of aqueous media containing confluent cells was aspirated. The flask was then washed with 5 mL of PBS, and aspirated again. 2 mL of trypsin was then added and put into an incubator at 37.1°C for 4 minutes to detach the cells. Once the cells were detached, 8 mL of DMEM was added to the flask. This flask was then used to prepare a new flask with the desired ratio. In general, a 1:5 ratio was used when no experiments were scheduled. This was done by putting 2 mL of solution from the old flask into a new empty flask. Lastly, 13 mL of fresh DMEM was added to the flask with 2 mL of cells to make a flask final volume of 15 mL.

2.4. What are MDA-MB-231 and MCF-7 cells?

MDA-MB-231 is an epithelial, human breast cancer cell line taken from a pleural effusion of a 51-year old Caucasian female with metastatic mammary adenocarcinoma.[20] MDA-MB-231 is a highly aggressive, invasive, and differentiated by being a triple-negative breast

cancer cell line, which means that MDA-MB-231 cells lack estrogen receptors, progesterone receptors, and human epidermal growth factor receptors.[20] In 3D culture, this cell line displays endothelial-like morphology and can be distinguished by the stellate projections that often bridge multiple cell colonies. MDA-MB-231 cells are one of the most commonly used breast cancer cell lines used in medical research laboratories.[20]

MCF-7 is a breast cancer cell line isolated from a 69-year old Caucasian woman.[21] This cell line had estrogen, progesterone receptors, but lacked human epidermal growth factor receptors.[21] This cell line is less aggressive when compared to the MDA-MB-231 cell line.

2.5. Viability with Confocal Microscopy

After the hydrogel was made, the practical function of the hydrogel could be studied. The hydrogel served as an *in vivo* environment for the cancer cells. The idea was that to better understand cancer growth, a more realistic environment was needed.[22] This was accomplished with cells encapsulated inside the hydrogel, all of which was placed inside a 96-well plate. Since the hydrogel formed a natural 3D shape while inside the 96-well plate, a bright field microscope could not be used to efficiently get a picture of all of the cells entrapped within the hydrogel. A confocal microscope was instead used to capture multiple 2D images at different depths that could be used to reconstruct a 3D structure. A 3D representation of the gel should be similar to the figure 2 layout shown, with live cells on the outside and dead cells in the core. Use of confocal microscopy was very common when assessing viability of tumor spheroid within hydrogels.[23]

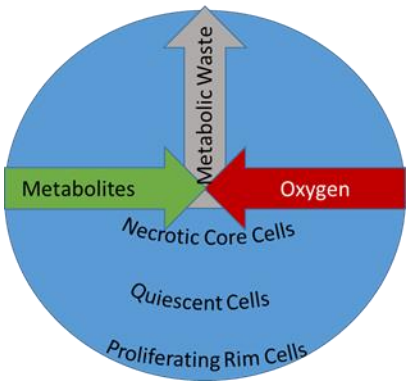


Figure 2. Tumor spheroid layout

2.5.1. Materials

The Leica SP8 confocal microscope was provided by the Shared Instrumentation Facility (SIF) at LSU. 96-well Ultra Low Attachment plates were purchased from Corning. MDA-MB-231 cells and MCF-7 cells were provided by Dr. Martin. Growth media for each cell line, PBS, and Trypsin was purchased from Corning Cellgro. Calcein AM and ethidium homodimer-1 (ethd-1) was purchased from Thermo Fisher Scientific. Bright-Line Hemacytometer was purchased from Hausser Scientific.

2.5.2. Methods

2.5.3. Cell Counting

After cells were detached with trypsin, growth media was added to inactivate the trypsin. The suspended cells were than used for counting with a hemocytometer. Once counted, a desired cell density, for example 5 x 10^5 cells/mL, was taken for subsequent experiments.

2.5.4. 96-Well Plate Preparation for Confocal Microscopy

The two cell density sizes that were used for MDA-MB-231 and MCF-7 cells were 1 x 10^5 and 5 x 10^5 cells/mL. These cell density values were calculated by taking into account that

they would be added into 1 mL of the hydrogel (before gelled). Once the cells were inside the gel, 20 µl droplets were added to each well of the 96-well plate, matching the schematic from table 2. Sufficient time was allowed for the hydrogel to fully gel. After the hydrogel was done gelling, on the days when the samples were not being imaged, 250 µl of growth media was added on top of the gel. On the days that cells were being imaged, the samples received 250 µl of calcein AM and ethd-1 dye mix instead of the growth media. See section 2.5.5 for details.

Table 2. 96 well plate schematic

Cell Density		A	B	C	D	E	F	G	H	I	J	K	L
100,000 MDA-MB-231	A	Day 0			Day 1			Day 2			Day 3		
	B	Day 4			Day 5			Day 6			Day 7		
500,000 MDA-MB-231	C	Day 0			Day 1			Day 2			Day 3		
	D	Day 4			Day 5			Day 6			Day 7		
100,000 MCF-77	E	Day 0			Day 1			Day 2			Day 3		
	F	Day 4			Day 5			Day 6			Day 7		
500,000 MCF-7	G	Day 0			Day 1			Day 2			Day 3		
	H	Day 4			Day 5			Day 6			Day 7		

2.5.5. Staining Procedure

Calcein and ethd-1 dye were used for a live/dead staining of the cells. The final volume of calcein was 5 µM and ethd-1 was 8 µM. Each dye was prepared in individual centrifuge tubes and diluted with PBS to correct concentration. Afterwards, the two tubes were mixed, 250 µl of stain was added for the days that were being imaged. The dye takes approximately 1 hour and 30 minutes to set after being added to the individual wells.

2.5.6. Preliminary Results of Viability Testing with Bright Field Microscopy

Figure 2.2 shows images of the 10.5% thiol-acrylate hydrogel with a cell density size of 6 $\times 10^5$ cell/ml. The day 0 imaging of MCF-7 shows clumping, while the MDA-MB-231 has distinguishable individual cells. This clumping was due to the natural endothelial nature of MCF-7 cells. The figures show that the hydrogel does not inherently kill the cells upon contact, when

looking at day 0 to day 2. The viable cells appeared as clear single cells when looking at the bright field image. The calcein dye showed green for live cells, while the ethd-1 dye showed red for dead cells. However, most of what was seen in the dead images does not have a distinct cell in the bright field, so this was most likely due to an unincorporated cell membrane being stained. There were dead cells seen in Day 0, but this was to be expected when using a confluent flask and most likely not due to the hydrogel killing them in the 2 hours before imaging. Since each well represented a different day, only cytotoxicity of the gel could be proven. The gel was not was not cytotoxic, but the growth of each individual cell could not be determined because each well had a different starting spot. This meant that once dyed by the calcein and ethd-1 the cells were dead after 6 hours, and could not be imaged again. Although there was no inherent toxicity of the hydrogel to the cells, the assessment of cell dispersion cannot be seen with a 2D microscope. Since each layer of the hydrogel contains cells it was hard to accurately quantify if there was growth, so a confocal microscope was used.

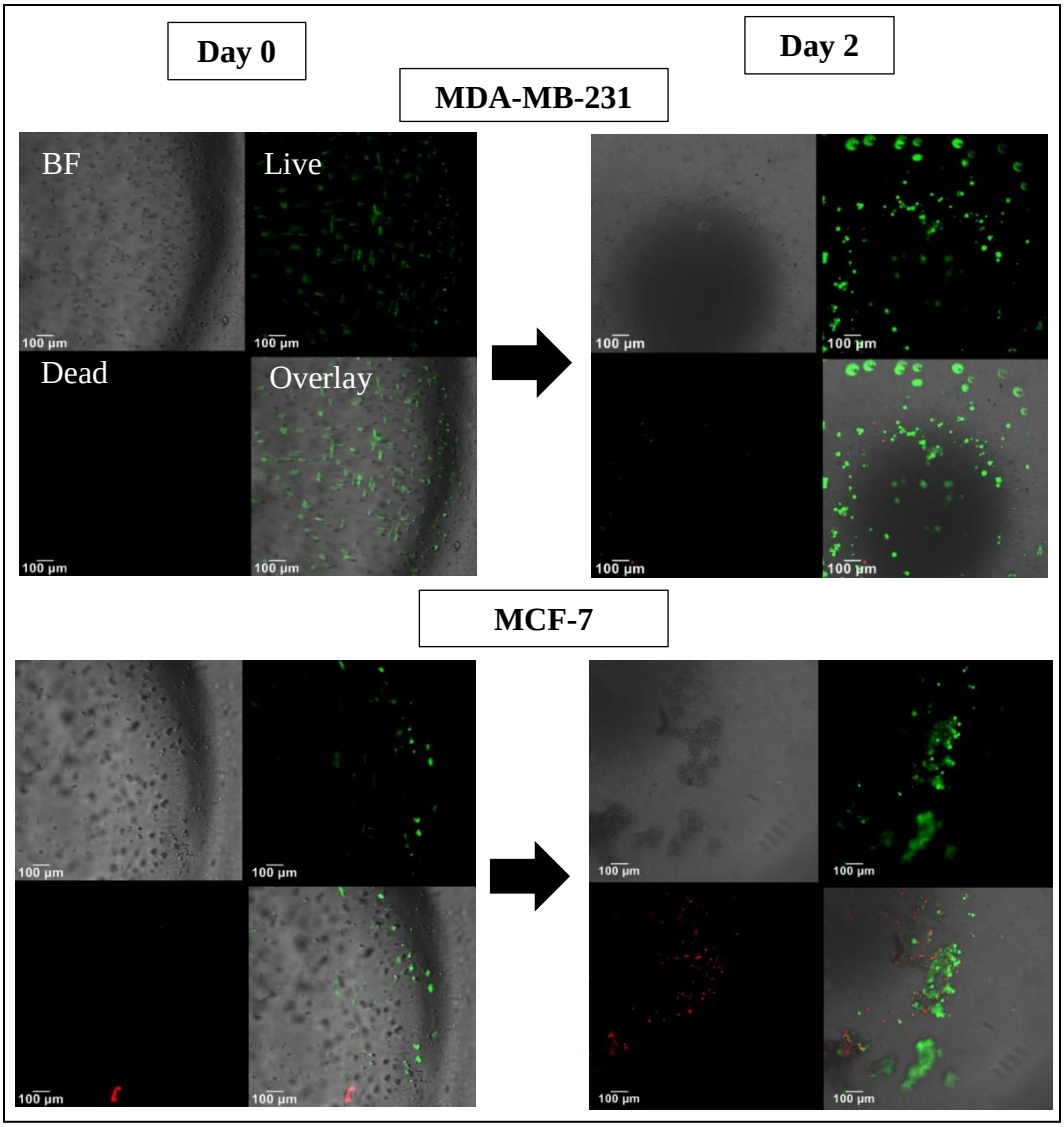


Figure 2.2. Bright field microscope of thiol-acrylate with live/dead stain of 600,000 cells/mL

2.5.7. Results of Viability with Confocal Microscopy

Confocal microscopy provided a 3D image for spatial arrangement of cells in the hydrogel in the 96-well plate. The 96-well plate shaped the hydrogel into a spheroid. This spheroid had multiple layers of cells that needed to be imaged to compare with the expected tumor spheroid from Figure 2.2. Figure 2.3 shows results of confocal imaging with a cell density size of 5×10^5 cells/mL. Clumping was still seen in MCF-7 due to the endothelial nature of the

cell line. The hydrogel was again shown to not be inherently toxic for at least 2 days. This was concluded due to the lack of red/dead cells seen, and the abundance of green/live cells. The red ring that was seen in Day 2 was most likely an overstain of the ethd-1 because most of the red does not correspond with a cell seen in the bright field image. The 96-well plate did not allow for quantification because each individual well represented a different starting population size. This problem can be fixed with droplet-based microfluidics. Droplet-based microfluidics allowed for identification of the same droplet over multiple days, which was used to see individual growth.

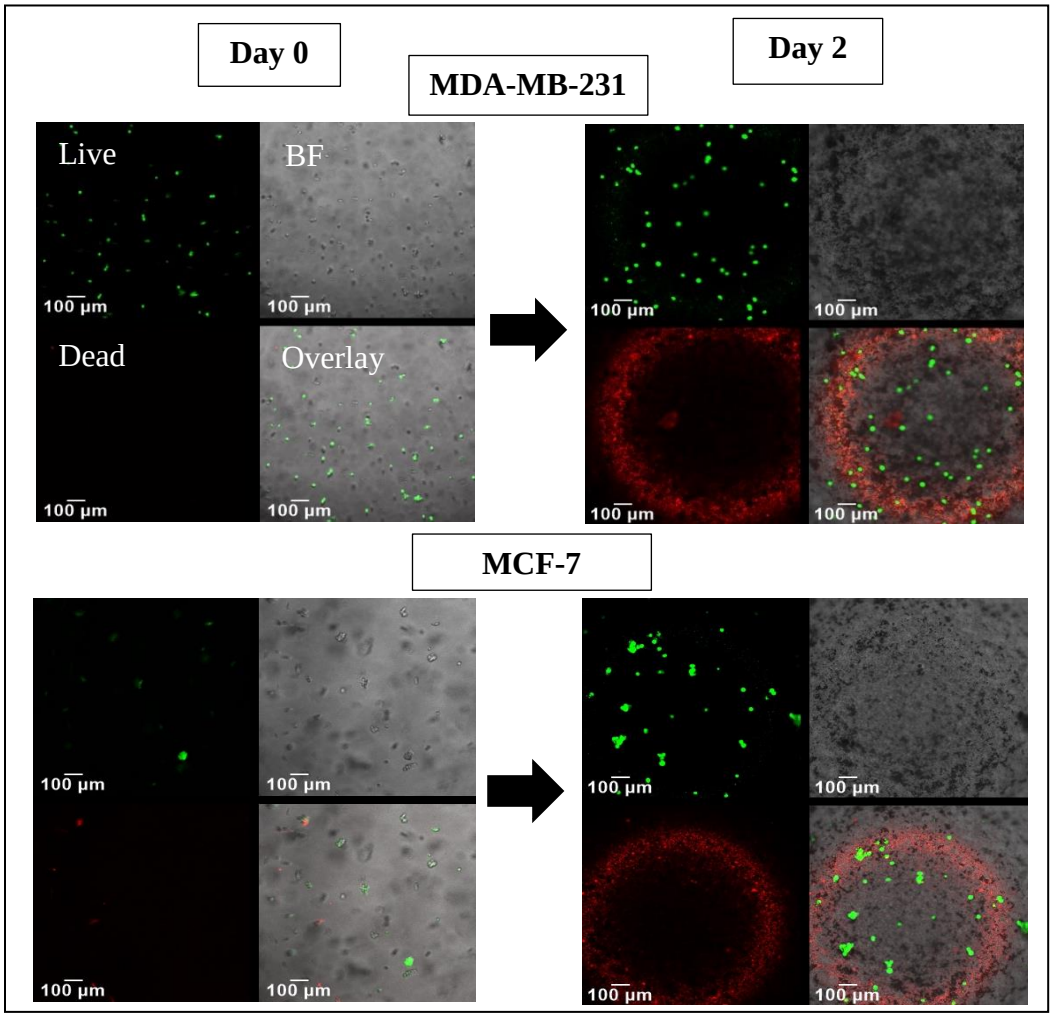


Figure 2.3. Confocal images of thiol-acrylate with live/dead stain of 500,000 cells/mL

2.6. On-Chip Microfluidic Analysis of hydrogel droplet cell encapsulation

The next step was on-chip microfluidics. This meant that instead of using the 96-well plate to study cancer cell growth the microfluidic device was used. Microfluidics as the name implied was in the sub-millimeter scale. There were micrometer-sized channels imprinted onto the device to guide the flow of liquids. Some advantages of using channels on this scale were the low volume of samples and reagents required and the ability to reproduce hundreds of homogenous cultures from the encapsulation of cells.[24-25] There are many types of microfluidics, but the focus was on droplet-based microfluidics. This type of microfluidics replicates the droplets of the hydrogel to be trapped and studied for growth. The device does this by using a constant flow of oil to form droplets of the hydrogel that also have a constant flow rate.[26] Droplet-based microfluidics allows for encapsulation of cells and was suitable for high-throughput experiments.[26] The traps designed to capture the droplets were on a second layer of the device, and droplets are trapped when they rise due to surface tension in the aqueous phase.[27] This allowed for very quick trapping, which provided hundreds of droplets with single cells to be made instead of having to do multiple individual trials as were done with the 96-well plate.

2.6.1. Materials

The silicon master wafer of the microfluidic device was provided by Dr. Melvin's lab in the Chemical Engineering Department at LSU. Imaging was done with a Leica DMi 8 microscope provided by Dr. Melvin. Polydimethylsiloxane (PDMS), a curing agent, Harrick PLASMA PDC-32G, Aquapel, Novec 7500 oil, nitrogen tank, 2% fluoro-surfactant, and Tygon tubing was provided by Dr. Melvin's lab.

2.6.2. Methods

2.6.3. Making the Microfluidic Device

PDMS replicates were created from the silicon master wafer by pouring a 1:10 mixture of curing agent to base over the wafer. This was then heated at 65°C for 6-8 hours, which solidified the PDMS and captured the features of the wafer. The hardened PDMS was peeled off the wafer and the six induvial devices were then cut and separated. The inlet and outlet ports on the device were then punched with a blunted 18-guage needle. Each individual device was then bonded to a glass slide using a Harrick Plasma Cleaner. The interior of the device was then treated by injecting hydrophobic Aquapel coating. This coating was then removed by blowing nitrogen through the inlet ports and washed using NOVEC 7500 oil.

2.6.4. Operation of Microfluidic Device

Tubing was inserted into the three ports (2 inlets and 1 outlet). The tubing connected to the outlet port was put into a waste disposal container. The oil inlet port tubing was connected to a 5 mL syringe of NOVEC oil and 2 wt% 008-Neat-fluoro-surfactant (Ran Biotechnologies). The fluoro-surfactant within the oil was used to stabilize the aqueous droplets from clumping. The other inlet port tubing was connected to a 5 mL syringe of pre-gelled hydrogel with cells. See sections 2.5.3 and 2.5.4 for more details on counting and preparing hydrogel with cells. The flows for the syringes were provided by individual syringe pumps which applied a steady pressure. The oil inlet flowed first to remove gas from the device. After the gas was removed, the gel inlet was then turned on. The flowrates of both the gel and oil were tweaked to get a droplet size of 70 µl that was trapped within the microfluidic device. Once the hydrogel droplets were trapped and fully gelled, a growth media syringe replaced the gel syringe. The growth media was then run through the device to wash out all oil that could be toxic to cells with prolonged

exposure. Imaging was done and then the growth media was added into a petri dish, with the trapping array being imaged once a day. Figure 2.4 shows a detailed image of a microfluidic device. In the figure, 1 is the oil inlet, 2 is the aqueous inlet for the hydrogel and the growth media, 3 is the flow junction that makes the hydrogel droplets, 4 is a section of a trapping array, and 5 is the fluid outlet. Figure 2.5 shows how a microfluidic device proceeds after droplets are made.

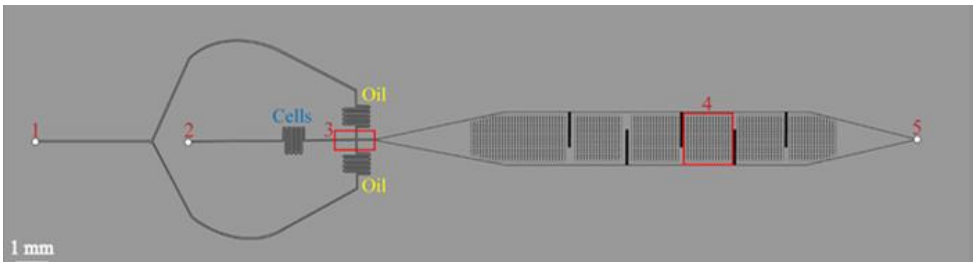


Figure 2.4. Overview of microfluidic device provided by Nora Safa[28]

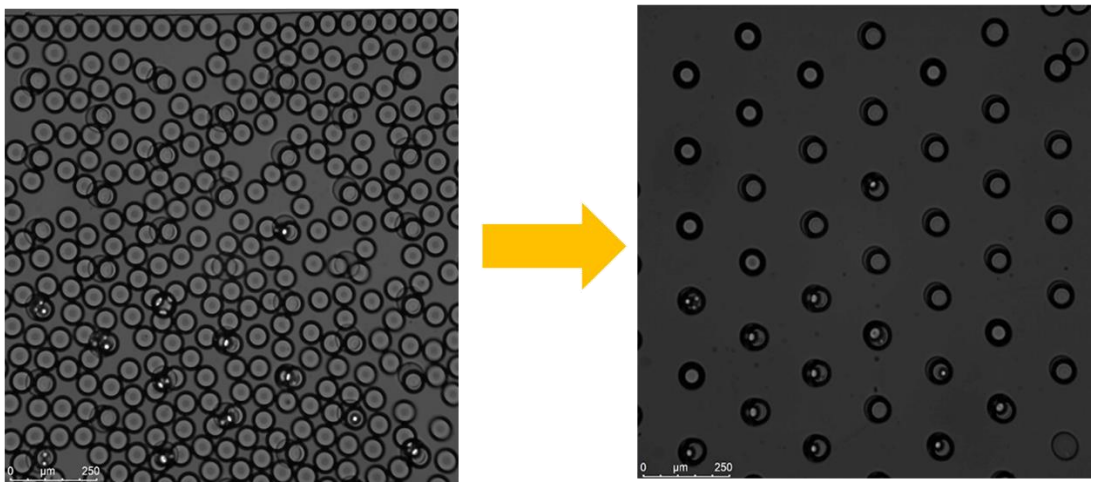


Figure 2.5. Microfluidic droplet generation before and after droplets were trapped

2.6.5. 10.5% Thiol-Acrylate Complication

Microfluidics was first done with 10.5% hydrogel and MDA-MB-231 cells. Images from figure 2.6 shows day 0 and day 2 images of droplets generated through microfluidics with a cell

density 3×10^6 cell/mL. Throughout this specific microfluidic device, there are approximately 20% single-celled droplets, 30% multiple-cell droplets, and the rest are empty droplets. The endothelial nature of the cells was causing clumping and preventing high single-celled encapsulation. The EDTA was added into PBS when passing cells to rectify this, but clumping of cells remained an issue.[29] This specific device had about 775 traps for droplets to be encapsulated. Cells would normally divide at least once in 2 days, but no division was seen in figure 2.6. The cells are not dead, which can be confirmed from previous viability testing. However, the lack of division could be due to the stiffness of the 10.5% hydrogel. Future microfluidic testing will be done with the 9.5% hydrogel because of the lower stiffness.

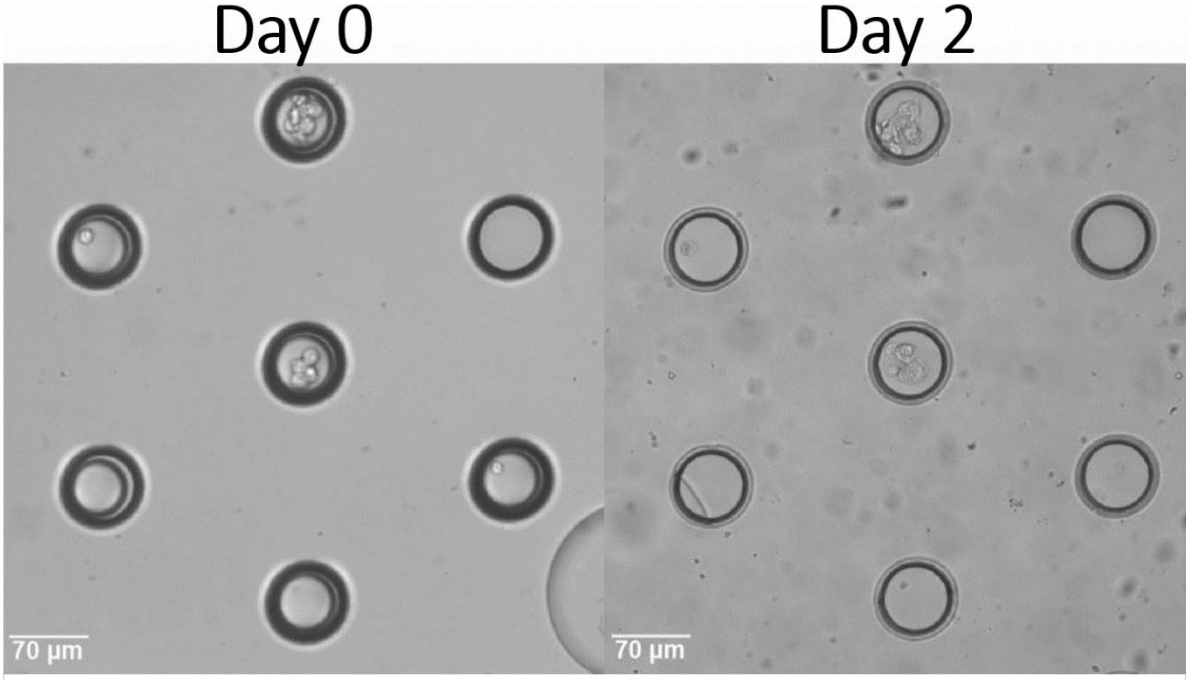


Figure 2.6. Microfluidic droplet images with cell density 3 x 10^6 cells/mL

2.6.6. 9.5% Thiol-Acrylate Microfluidic Results

The 9.5% hydrogel was used instead of the 10.5% hydrogel to confirm if stiffness of the gel was the problem with the lack of cell division. Figure 2.7 appears to show division of the

MDA-MB-231 cells, when comparing day 0 to day 3. The day 0 droplet has about 7 cells, while the day 3 droplet has about 13 cells. The Day 0 image appears to have either multiple gel droplets entrapped or what appears to be a cracked gel, but this image was taken during the gelling process so that should not be the case. This could mean that the gel was being formed, and this theory was likely because on day 3 there was one droplet per trap. Microfluidic encapsulation of single cells when using the 9.5% hydrogel was very low. This was again due to the endothelial nature of the cells that was causing the clumping of the cells.

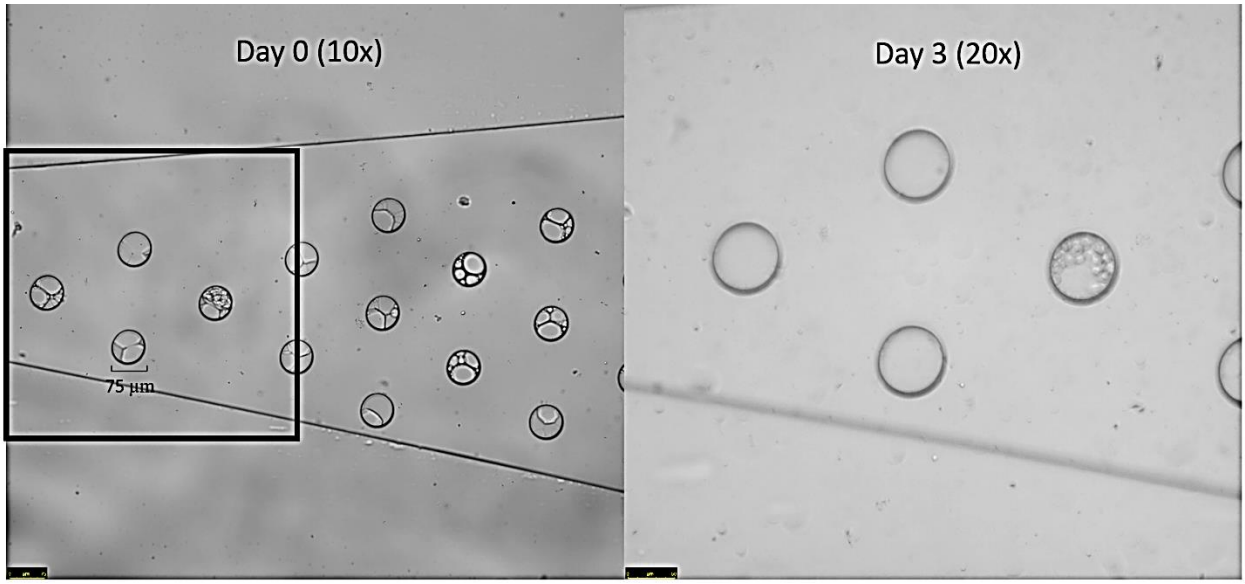


Figure 2.7. Microfluidic droplet images with cell density 3×10^6 cells/mL

2.7. Conclusion and Future Work

The thiol-acrylate hydrogel was evaluated for use with MDA-MB-231 and MCF-7 breast cancer cells with confocal microscopy and droplet-based microfluidics. The confocal microscopy images confirmed that the hydrogel was able to sustain and possibly promote growth of the breast cancer cells. This growth could not be confirmed until the droplet-based microfluidics was tested. The microfluidic images, however, are less conclusive. The 10.5% hydrogel does not kill

the cancer cells, but this particular hydrogel prevents division of cells. The 9.5% hydrogel appears to show growth, but because of the lack of single-celled droplets, it was hard to quantify growth from day 0 to day 3. The gel stiffness does not appear to be a problem with the 9.5% hydrogel because cells were either able to divide or they were at least able to spread out across the trapped droplet. This was different from the 10.5% hydrogel where the cells, did not move over the 5-7 days of imaging across multiple microfluidic devices.

Future work on this project can be directed into changing the molecular weight of the specific thiol and acrylate. Raising the molecular weight of either the thiol or acrylate will allow for a less crosslinked polymer, which can lower the stiffness and keep the same gel life time. Another aspect that should be considered is adding lipids, proteins, and carbohydrates to better simulate an *in vivo* environment. The droplet generation needs to provide a higher percentage of single cell encapsulation as well, so that future drug testing can be done to see if the cancer cells can then be killed, while trapped inside the hydrogel. Hopefully, this work has laid the foundation to show that thiol-Michael addition hydrogels can be used to provide an extracellular environment that can support tumor spheroid culturing.

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