Molecular Beacon Delivery Methods in 2D Cell Culture

Corey Holman

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Thesis Advisor

Dr. Eric M. Darling | Associate Professor of Medical Science, Engineering, and Orthopaedics, Department of Molecular Pharmacology, Physiology, and Biotechnology, Department of Orthopedics, School of Engineering

Second Reader

Dr. Edith Mathiowitz | Professor or Medical Science, Professor of Engineering, Department of Molecular Pharmacology, Physiology, and Biotechnology, Director of Biotechnology Graduate Program

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1. ABSTRACT

In recent years, molecular beacons have drawn a lot of attention from researchers across all fields due to their ability to image mRNA expression in any cell in real time. Molecular beacons are short hairpin oligonucleotides that fluoresce when bound to the mRNA of a gene of interest. Despite the utility of molecular beacons, current methods of delivering molecular beacons to cells lead to fluorescent debris and detrimental background signal that complicate analysis. This study investigates whether polymer-based delivery methods can improve upon current beacon delivery methods. We aimed to investigate whether two of these proposed methods, a diblock copolymer carrier system and a p(FA:SA) nanoencapsulation method, successfully deliver molecular beacons to multiple cell types while retaining functionality upon intracellular release. HEK293 and MG-63 cells were fixed and imaged after treatment with a polymer beacon complex for defined periods of time. A custom MATLAB script was used to quantify successful beacon delivery by associating DAPI-stained nuclei with positive beacon signal. Beacons were delivered using a polymer developed for siRNA delivery, diblock copolymer. The number of cells expressing beacon fluorescence increased over a 24 hour period for all beacon sequences delivered with diblock copolymer. At 24 hours, beacon conditions delivered with diblock copolymer all displayed fluorescent cells while beacon-only, diblock copolymer-only, and no treatment conditions exhibited none. Another polymer, p(FA:SA), originally developed for bioadhesion, drug delivery, and DNA transfection, was used to encapsulate beacons in preparation for functionality analysis and delivery. p(FA:SA) cellular treatment resulted in minimal cytotoxicity, while post-encapsulation analysis indicated an unquantifiable, low loading efficiency. Because of the low loading, microspheres could not be tested in cells. Though further research is required to verify the efficacy of these two delivery methods, if successful, these delivery methods can enable the study a myriad of cellular processes using molecular beacons.

2. INTRODUCTION

Nucleic acids are the backbone of all life and direct all cellular processes. Through novel experimentation, nucleic acids have revolutionized scientific research. The ability to assess the functionality of these important molecules has opened countless applications in numerous fields. Assessing nucleic acids in cells can be used for anything from basic science to tissue engineering, and even to cancer diagnostics. In the pharmaceutical industry, nucleic acids are used as an investigative tool, while polymerase chain reaction (PCR) and small interfering RNA (siRNA) is used to knock down gene expression, leading to improved treatment for diseases.[1] In the field of stem cell research, nucleic acids can be used to assess gene expression during differentiation.[2] Nucleic acids have even been used as the newest class of next-generation therapeutics, utilizing plasmid DNA along with viral and non-viral vectors in novel gene therapies.[3] Potential therapeutic applications motivate the use of nucleic acids for use as a tool to assess gene expression. All these revolutionary applications would not be possible without nucleic acid delivery, for all nucleic acid therapeutics are dependent on transport into the cell, making the delivery of these strands of genetic material the most important step of the process.

One application of nucleic acids delivery is the ability to assess *in vivo* cellular processes based on gene expression. A method for assessing real-time gene expression in live cells is using nucleic acid-based probes called molecular beacons. Molecular beacons are small labeled strands of DNA that fluoresce when bound to the mRNA of a gene of interest.[4] Beacons can detect mRNA in living cells, while commonly used techniques for gene expression analysis including PCR and micro arrays require lysed or fixed cells.[5] Molecular beacons are invaluable because they can fluorescently identify cellular gene expression in real time. Being negatively charged, beacons cannot pass through the cell membrane, and therefore need a carrier to get into the cell. The most

commonly used protocols for introducing molecular beacons to cells include electroporation and microinjection.[6] These methods have been used extensively, but can require long incubation times, be highly invasive, and potentially result in endosomal entrapment causing high levels of detrimental, brightly fluorescent debris.[5] The long incubation periods can prevent observation of some time-sensitive cellular behaviors, while fluorescent debris and heightened background noise levels make quantitative analysis of actual cellular signals difficult. As such, a faster and less noisy delivery protocol is needed.

The major benefit of molecular beacons is that they can be used in living cells to observe dynamic processes and allow for single cell analysis. Beacon fluorescence can be adapted to any imaging platform, and can be used to assess mRNA through fluorescence while not disturbing the cellular function.[6] Beacons are specifically designed to recognize only their exact target in the cell.[7] This makes beacons widely applicable in cellular analysis. Molecular beacons are a tool that allows isolation of a subpopulation of cells that can be used for a variety of therapies in many fields. Molecular beacons and fluorescence-activated cell sorting (FACS) have been used to sort adipose derived stem cells (ASCs) based on gene expression.[2] For example, to help treat cartilage degeneration, ASCs could be used as a source of chondrogenic cells,[8] eliminating the need for the highly invasive harvesting of cartilage cells. A small bit of fat could be obtained from minimally invasive liposuction as the source of ASCs, then molecular beacons could be designed to target the mRNA of chondrogenic cells and added to the pool of harvested ASCs. The chrondrogenic ASCs could be isolated by fluorescence activated cell sorting (FACS), and used directly for cartilage tissue regeneration. Molecular beacons open up a wide variety of applications, therapies, and cellular diagnostics because of their ability to assess gene expression in real time.

Before this specific application of nucleic acid delivery can be developed, functional beacons have to be successfully delivered to cells. Polymers have been shown to be effective nucleic acid carriers.[1] Polymer-based delivery using diblock copolymer[9] has been shown to be highly effective delivering siRNA, which is a similar molecule to molecular beacons. Diblock copolymer was developed to enhance cellular uptake by specifically protecting siRNA from nucleases and efficiently escape endosomal trafficking. Molecular beacons are shaped like a hairpin, while siRNA is double stranded and does not exhibit this hairpin structure. siRNA is used in gene knockdown to assess gene expression,[9] whereas beacons would bind to the mRNA for the same effect. Positively charged diblock copolymer complexes with negatively charged beacon or siRNA and allows cellular delivery, shown in mesenchymal stem cells (MSCs).[9] Diblock copolymer delivery has been shown to not adversely affect cellular survival or differentiation, which gives this delivery method promising potential for application in regenerative medicine.[9]

Besides diblock copolymer, p(FA:SA) microspheres have also shown promise with plasmid and drug delivery, and could potentially be used to deliver molecular beacons to cells. It has been shown that bioadhesive polymers, such as p(FA:SA) are excellent vehicles for delivery of pharmaceutical agents.[10] The microsphere facilitate a controlled release system that improves bioavailability by increasing absorption through direct contact and minimizing drug degradation before contact. This controlled system releases most of the drug in the first 12 hours,[11] which makes this system perfect to monitor beacon delivery at 24 hours. Because of the DNA transfection promise in vivo[12] facilitated by the bioadhesive properties of the microspheres and the optimal time course of release, p(FA:SA) has the potential to be an efficient delivery system for molecular beacons.

3. SPECIFIC AIMS

The overall aim of this study is to determine whether two different polymer-based delivery systems can deliver functional beacons to cells more effectively than current delivery approaches. First molecular beacons must be successfully complexed with diblock copolymer or encapsulated in p(FASA). Successful encapsulation will be quantified by performing functionality assays on released molecular beacons. Once beacon loading is confirmed, delivery effectiveness of beacon-polymer complexes must be confirmed. To quantify effectiveness of delivery methods, delivery efficiency and beacon functionality must be quantified. Beacon delivery efficiency is quantified through fluorescence microscopy and an image analysis script in MATLAB, while beacon functionality is confirmed through thermal denaturation assays. Beacon delivery methods with high delivery efficiency and beacon functionality metrics will be used to probe gene expression in live cells, in real time.

Aim 1: Diblock copolymer can be used to deliver functional molecular beacons to cells.

We hypothesize that the diblock copolymer-based delivery method, originally developed for siRNA delivery, can also be used to effectively deliver functional molecular beacons to cells, and will lead to more efficient transfection and less background noise than current methods. Since it has previously been proven to carry nucleic acids into cells, diblock copolymer would effectively deliver molecular beacons to cells. The uncertainty of this method is if the observed fluorescence means that the beacons are successfully delivered, binding to their mRNA target in the cell, or being degraded and unraveling, releasing the fluorophore and leading to false fluorescence. Further experiments are necessary to determine true beacon functionality.

Aim 2: The p(FA:SA) microsphere encapsulation process will maintain beacon functionality and can be used to deliver molecular beacons to cells.

We hypothesize that p(FA:SA) microspheres, previously used for drug encapsulation and delivery, can be used to effectively encapsulate and deliver molecular beacons to cells, while maintaining beacon functionality. Because of a harsh encapsulation process, many assays must be considered to determine microsphere loading and asses beacon functionality before delivering these microspheres to cells.

4. MATERIALS AND METHODS

Molecular Beacons

Beacons were diluted to 10 µM with 1x phosphate buffered saline (PBS) and used in diblock copolymer experiments. All beacons were purchased from Eurofins Genomics at HPLC purity. All sequences shown 5' to 3.'

Beacons Used with Diblock Copolymer:

Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**):

Function: Targets mRNA of a commonly used housekeeping gene[13]

Beacon: [6~FAM]*CGACGGAGTCCTTCCACGATACCACGTCG [BHQ1a~Q]**

MP: 73.1°C

*[6-FAM] = 6-carboxyfluorescein fluorophore

**[BHQ1a~Q] = Black Hole Quencher 1

Target: TGGTATCGTGGAAGGACTC

MP: 60.2°C

Random NP NoQ (**No QR**):

Function: No endogenous target, lacks quencher, should always exhibit fluorescence[6]

Beacon: [6~FAM] ACGACGCGACAAGCGCACCGATACGTCGT

MP: 73.1°C

Target: TATCGGTGCGCTTGTCG

MP: 59.6°C

Bao Random NP 6 FAM (**QR**):

Function: No endogenous target, has quencher, should not exhibit fluorescence[6]

Beacon: [Cy3] ACGACGCGACAAGCGCACCGATACGTCGT[BHQ2a~Q]

MP: 73.1°C

Target: TATCGGTGCGCTTGTCG

MP: 59.6°C

LONG STEM (**LS**):

Function: No endogenous target, the long stem results in more thermodynamic stability,[13] stem designed by Darling Lab.

Beacon: [6~FAM] CTCGCATGCGATATACGCATGCGAG [BHQ1a~Q]

MP: 67.9°C

Target: TATAT

MP: -35.1°C

Beacons Used with p(FA:SA) Microspheres:

Bao GAPDH NMR (**umGAPDH**):

Function: Control unmodified GAPDH sequence[13] without the 6~FAM fluorophore or BHQ1a~Q quencher, stem designed by Darling Lab.

Beacon: GGTCCGGAGTCCTTCCACGATACCAC GGACC

MP: 75.2°C

UBIQUITIN (**Ubiq**):

Function: Targets mRNA of a ubiquitin gene, sequence designed by Darling Lab.

Beacon: [6~FAM] CGACGTTCCAGCTGTTTTCCGCGTCG [BHQ1a~Q]

MP: 70.9°C

Target: CGGAAAACAGCTGGAA

MP: 52.4°C

Testing Beacon Functionality: Thermal Denaturation of Beacons

Melt buffer was made containing 3.5 mM magnesium chloride hexahydrate ($MgCl_2$) (Sigma) and 10 mM Tris Hydrochloride (Tris-HCl) (Fischer Scientific) in HyPure Molecular Biology Grade Water (HyClone) and titrated to pH 8 with 1M NaOH. Beacon was added to relevant conditions to a final concentration of 200 nM in an optically clear qPCR 96 well plate. Target was added to relevant conditions to a final concentration of 400 nM. Tested conditions included: beacon, beacon + target, and buffer-only. Work area was sprayed with RNAse away (Molecular Bio Products) to remove nucleases that might degrade beacons or their targets before samples were prepared in a qPCR plate. Conditions were run in triplicate and target was added just

before sealing the plate. The plate was sealed and spun at 1100 RPM for 2 mins to collect fluid and eliminate any bubbles. On a BioRad C1000 Touch Thermal Cycler (BioRad), experimental plates were heated to 80°C and cooled to 25°C in 1°C, 1 minute hold steps with FAM fluorescence measured at each step using the program CFX (Biorad).

Polymers

Diblock Copolymer

[pDMAEMA-*b*-p(DMAEMA-*co*-PAA-*co*-BMA)][9]

Diblock copolymer was generously provided by the Benoit Lab at the University of Rochester.[9] Freeze-dried stocks were rehydrated to a concentration of 1mg/mL using 1x PBS, sonicated for 20 mins to form a homogeneous solution, and stored at -20°C to be used as needed.

poly(FA:SA)

[Polyanhydride copolymers of fumaric and sebacic acid][14]

Poly(FA:SA) microspheres were synthesized through phase inversion nanoencapsulation (PIN).[14] 100 mg of p(FA:SA), co-polymers of fumaric acid and sebacic acid (molar ratios of 20:80),[10] was generously provided by the Mathiowitz Lab at Brown University. The whole procedure was completed under dark conditions to minimize photobleaching of the molecular beacons. The p(FA:SA) was added to 6.67 mL dichloromethane (DCM) vortexed and sonicated until dissolved. 100 µL of a 10 µM beacon stock solution was added to the DCM and was sonicated to re-form the emulsion. The DCM solution was added dropwise to a gently stirring beaker of 900 mL of petroleum ether. After all the DCM was added, the beaker was left stirring open on the bench for five minutes at room temperature. The solution was then vacuum filtered, allowing the sample to dry. The particles were then scraped off of the filter with a razor blade, collected in a pre-weighed 50 mL tube, and lyophilized to dry overnight. Alternatively, beacon-polymer complexes were dried at room temperature for two days in a kimwipe covered glass tube filled

partway with Drierite (Fisher). Once dry, the sample was weighed, sealed with parafilm, covered with tin foil to prevent light damage, and stored at -20°C Drierite filled bag. Encapsulations results shown in **Table 1.**

Microspheres	Amount Beacon Originally Added	Weight microspheres obtained (mg)	% Yield	% Loading	Amount Beacon Assuming Have in Particles (50%)	Amount particles for 35 nM beacon in 1 mL	Zeta Potential (mV)
Blank p(FA:SA)	0 ng, 0 nmol	297.1	67.5%	N/A	N/A	N/A	-15.9
umGAPDH p(FA:SA)	12,273 ng, 1.3 nmol	98.1	95.2%	0.012%	6136.5 ng, 0.65 nmol	5.303 mg	-0.292
Ubiq p(FA:SA)	8970 ng, 0.997 nmol	53.5	52.91%	0.0089%	4485 ng, 0.498 nmol	3.757 mg	-42.1

Table 1: Results of p(FA:SA) encapsulation efficiency, loading, and yield.

Cell Culture

HEK-293T human embryonic kidney cells, gifted to us from the Oancea Lab at Brown University, were used first as an easily transfectable cell line, and later MG-63 human osteosarcoma cells (purchased from ATCC) were used. Cell lines were cultured at 37°C and 5% CO_2 in growth medium containing 87% Modified Eagle's Medium (MEM) (Corning), 10% Fetal Bovine Serum (FBS) (ZenBio), 1% Penicillin/Streptomycin (HyClone), Cells were passaged at 80% confluence using 0.25% Trypsin-EDTA (ThermoFisher Scientific) and maintained in good health, changing medium every two days.

Diblock Copolymer Delivery

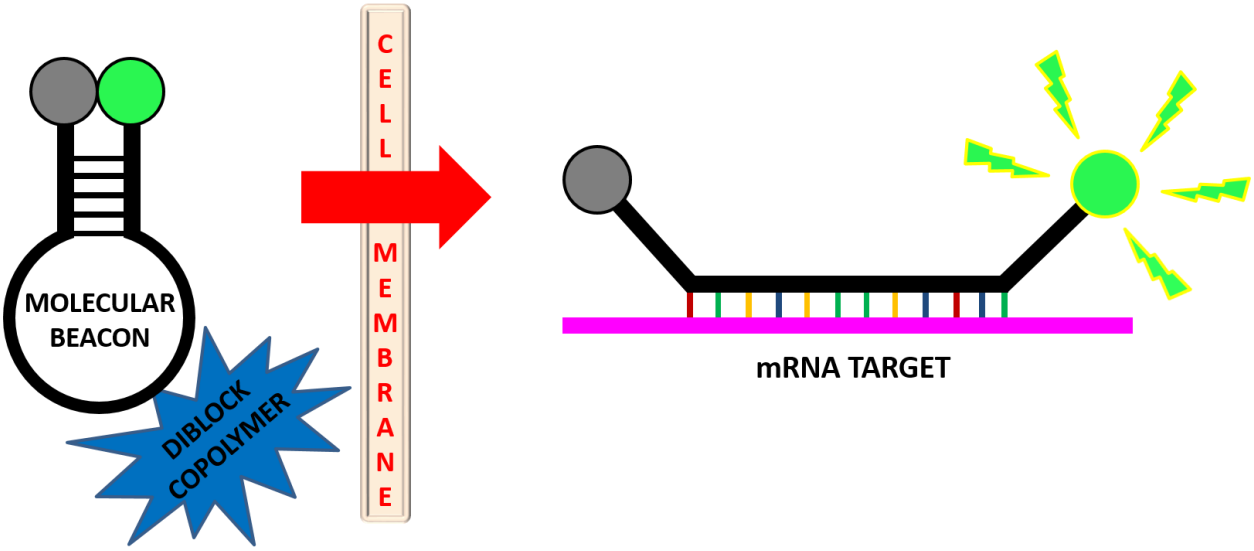


Figure 1: Schematic of diblock copolymer-mediated delivery of a molecular beacon.

Transfection

Cells were seeded at 16,000 cells per well, in a 96 well plate (Olympus) and allowed to attach overnight. Transfection solutions containing diblock copolymer and the molecular beacon were made at various polymer:beacon charge ratios as detailed in Table X, based off of calculations shown in **Appendix A**. 35 nM beacon was used for each condition with the respective amounts of diblock copolymer, PBS, and medium shown in **Table 2**.

Diblock Copolymer-mediated Delivery of Molecular Beacons: Experimental Conditions				
Condition	**Beacon (µL)**	**Diblock (µL)**	**PBS (µL)**	**Media (µL)**
1:1	0.750	0.128	19.12	180.0
2:1	0.750	0.256	18.99	180.0
3:1	0.750	0.384	18.86	180.0
4:1	0.750	0.512	18.73	180.0
5:1	0.750	0.641	18.60	180.0
6:1	0.750	0.769	18.48	180.0
7:1	0.750	0.897	18.35	180.0
8:1	0.750	1.025	18.22	180.0
beacon only	0.750	0.000	19.25	180.0
polymer only (1:1)	0.000	0.128	19.87	180.0
cells only	0.000	0.000	20.00	180.0

Table 2: Experimental Transfection Conditions for Diblock Copolymer Delivery. Final beacon concentration in well is 35 nM. Volumes shown for one well of a 96 well plate (200 µL well volume)

Each experimental condition was tested in triplicate. A stock transfection solution was made with PBS, beacon, and diblock copolymer in a 1.5 mL tube, the tube was covered with foil, and the solution was incubated at room temperature for 20 minutes to allow complexing of the diblock polymer and beacon. Cells were then treated with 20 µL of the stock transfection solution, and placed back in a 37°C humidified incubator with 5% CO_2 for either 1, 4, or 24 hours.

Cell Fixing and DAPI Staining

After incubation, wells were washed three times with Hank's Ballanced Salt Solution (HBSS) (HyClone). Wells were then treated with 200 µL 10% formalin (Fisher), wrapped in tin foil, and incubated at room temperature for 30 mins. During the incubation, a stock of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies) was made to stain the nuclei blue. 50 mL of PBS and 10 µL of DAPI stock was combined, vortexed, and covered in tin foil. After the incubation, wells were washed three times with 1x PBS and 200 µL of the freshly

made DAPI stock was added. The plate was covered in tin foil again and incubated for 30 mins at room temperature. After the incubation, plates were washed three times with PBS.

Imaging on a Biotek Cytation 3

Automated epifluorescence imaging was performed using a Biotek Cytation 3 imaging cytometer. An imaging protocol was created in Gen5 imaging software such that six bright field, blue fluorescent (DAPI excitation/emission: 377 nm/447 nm), and green fluorescent (GFP 469 nm/525 nm) images were taken for each experimental well in a 96 well plate. At each image location, an autofocusing algorithm using the blue fluorescence channel was used to establish the base focal plane and subsequent GFP and bright field images were taken at manually determined z focal plane offsets. Six DAPI, GFP, and bright field images were taken per well in a 3x2 grid with 1200 µm horizontal and vertical spacing, a representative majority of the well. Integration time, gain, and LED settings were all determined independently, but kept constant within each plate. A table showing these settings can be seen in **Appendix B**.

Image Analysis in ImageJ and MATLAB

Previously existing MATLAB code written by Nicholas Labriola in the Darling Lab used to relate beacon signal to corresponding DAPI-stained nuclei signal[15] was adapted to accept inverted, binary images created with a watershed ImageJ macro. The ImageJ macro, listed in Appendix C, makes the image binary and inverts the image such that nuclei become black and the background white. The MATLAB script then counts the nuclei, associates the nuclei with beacon signal over a certain size and brightness threshold and outputs an image showing the percentage of cells expressing beacon. The pixel intensity threshold was set to 75 by manually analyzing the

GFP fluorescence over cells in ImageJ verses the background values, while the size threshold was set to 50 μm^2 based off the area of a HEK293 cell. The process is illustrated below in **Figure 2**.

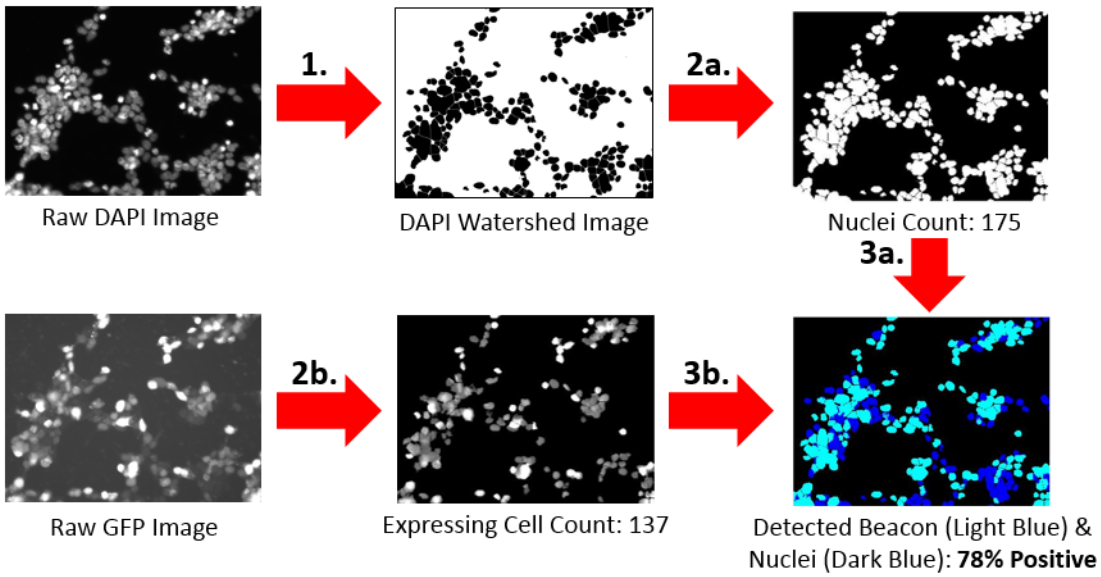


Figure 2: ImageJ and MATLAB Quantification of Beacon Delivery. 1. A custom ImageJ macro was written and used to make the raw DAPI images binary and apply a watershed function. 2. The ImageJ processed DAPI watershed image and raw GFP images were analyzed using an adapted custom MATLAB script to count nuclei (2a) and detect cells expressing beacon signal above a set threshold (2b). (Labriola et al 2015) 3. The custom MATLAB script quantifies percent expression by associating detected beacon signal (3b) with nuclei (3a).[15]

All other cytation images shown were made presentation ready using a custom ImageJ macro, outlined in **Appendix D**, which colors the cytation images, merges a DAPI (blue nuclei) and GFP image (green fluorescence), and adds a scale bar.

Flow Cytometry

Cells were seeded at 300,000 cells per well in a 6 well plate and treated with 200 µL of a 1:1 charge ratio of polymer and 35 nM beacon for 24 hours as outlined previously. Each well was washed with 1 mL MEM, treated with1 mL 0.25% trypsin and incubated at 37°C for 5 mins. After incubation the resultant cell suspension was added to a 15 mL tube with 1 mL of complete growth

medium to neutralize remaining trypsin. Cells were pelleted via centrifugation (5 min, 400 g), and resuspended in 1 mL HBSS. Resuspended cells were then analyzed on a BD FACSAria III (BD Biosciences) set to read 15,000 events with the 488 nm laser and FL1 green channel for detection. Data was analyzed using open-source analysis software Flowing (Perttu Terho, Turku Centre for Biotechnology).

Testing of p(FA:SA) Microspheres Encapsulated with Molecular Beacons

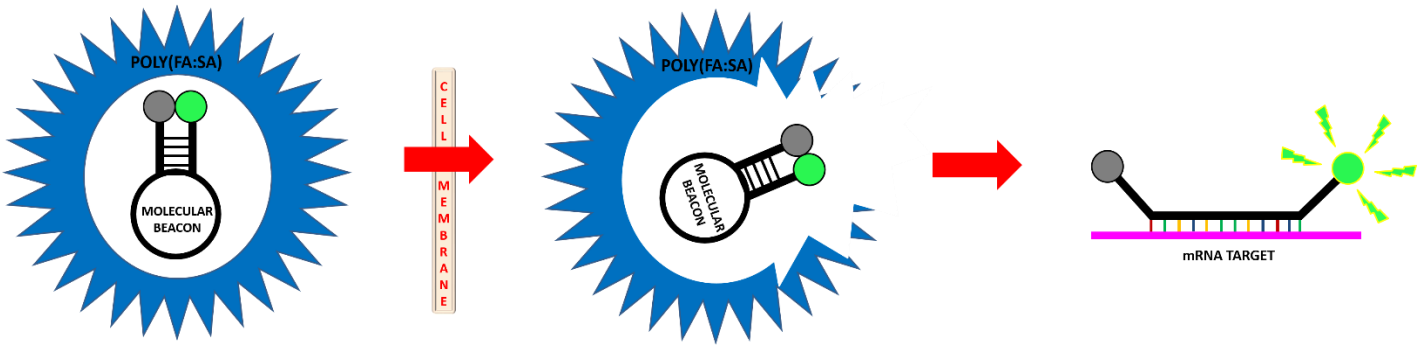


Figure 3: Schematic of proposed poly(FA:SA) microsphere delivery of a molecular beacon.

Morphological Characterization of Polymers: SEM

Samples of all p(FA:SA) microspheres, along with one sample of diblock copolymer, were mounted on aluminum SEM stubs using double sided SEM tape, then sputter coated with gold palladium at 3Amps for 3 Minutes with an Emitech K550 sputter coater (Emitech). Prepped samples were imaged using a Hitachi 2700 Scanning Elecron Microscope (Hitachi) equipped with Quartz PCI imaging software (Quartz Imaging Corporation) at 300x, 3000x, and 6000x. (Chickering, Jacob, and Mathiowitz 1996)

p(FA:SA) Cytotoxicity in Cells

HEK293T cells were seeded at a concentration of 47,000 cells per well in a 24 well plate and allowed to attach overnight. In the morning, media was changed, adding only 800 µL fresh media back to allow for 200 µL of polymer treatment. Amount empty p(FA:SA) polymer added was equivalent to ubiq p(FA:SA) particles with 70 nM encapsulated beacon. The concentration was calculated based off of a thin slice of the well (r=7.8 mm, h=20 µm, $V=3.88x10^{-3}$ mL), representing a layer of cells with microspheres sitting on top. Assuming that the umGAPDH p(FA:SA) microspheres have 0.65 nmol umGAPDH encapsulated within the particles, 0.0409 mg/well is necessary for [70 nM] in the $3.88x10^{-3}$ mL calculated well slice volume. Since this amount is so small, ten times the equivalent amount had to be measured to get an accurate weight for 15 wells (0.0409 mg/well x 15 wells x 10 = 6.135 mg empty p(FA:SA) weighed out). The 6.135 mg empty polymer was added to 1 mL HBSS and sonicated until dissolved, around 10 seconds. 100 µL of this solution was removed and added to 2.9 mL of HBSS and sonicated for an additional 10 seconds. Cells were then treated with their desired concentrations of microspheres (70 nM = 200 µL empty p(FA:SA) polymer solution + 0 HBSS, 35 nM = 100 µL polymer solution + 100 µL HBSS, 17.5 nM = 50 µL polymer solution + 150 µL HBSS, 8.75 nM = 25 µL polymer solution + 175 µL HBSS, 4.35 nM = 12.5 µL polymer solution + 187.5 µL HBSS). Plates were incubated for 24, 48, or 72 hrs. After incubation, three control wells were fixed with formalin to be used as a dead control. All conditions except for a cell-only control were then stained with Calcein and Eithidium homodimer-1 from a LIVE/DEAD ® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen). A stock of 2 mL PBS, 1 µL Calcein, and 4 µL of Ethidium homodimer-1 was made. The wells were washed 2x with 1x PBS, treated with 250 µL of the live/dead stain, and the plate was incubated for 20 mins at room temperature. Wells were washed

1x with PBS after the incubation and imaged on a Nikon Eclipse Ti Microscope (Nikon). Images were taken at 10x with a Nikon Plan Fluor 10x/.3 objective (Nikon) using GFP and Cy3/Texas Red filter sets (488 and 561 nm, respectively).

Testing Effects of Encapsulation Process on Beacons

Beacons were put through the most harmful stages of the encapsulation process to test the effects on the integrity of the beacons after encapsulation. In separate 3 mL glass vials, 3.83 nmol of umGAPDH was added to 495 µL of dichloromethane (DCM), and 5 nmol of Ubiq was added to 450 µL of DCM. The vials were sonicated three times for 10 seconds each to mimic the sonication step to dissolve the p(FA:SA) in the encapsulation process. After the sonication, a phase separation with molecular grade water was performed, adding 495 µL molecular grade water to the umGAPDH and DCM, and 450 µL water to Ubiq and DCM. An emulsion was created by vortexing the vials, and the bottom water layer was extracted with a syringe and used for analysis (40 µL collected for umGAPDH and 50 µL collected for Ubiq). The effect of this encapsulation process simulation on the DNA was tested in the following manners:

Nanodrop

DNA was tested on the Nanodrop 2000C Spectrophotometer (ThermoScientific) set to Nucleic Acid, measuring the absorbance at 260 nm and the 260/280 ratio. 1 µL of the phase extracted beacon was tested on the Nanodrop and amount retained was determined using the Beer-Lambert Law: A260 = ElC and the beacon's extinction coefficient, yielding data shown in Results, **Table 5.**

Acrylamide Gel Electrophoresis

A 15% acrylamide gel was made and pre-run at 50 V for 30 mins. Beacon samples were loaded to a final concentration of 1.25 µM in 14 µL 6x DNA loading buffer, and sonicated beacon samples were loaded with a final volume of 10 µL sample (unknown beacon concentration after sonication and phase extraction) in 6 µL loading buffer. Gel was run for 1 hr at 35 V and increased to 50 V for the last 2 hrs. Gel was submerged in 1x tris base, boric acid, and EDTA (1x TBE) made in lab, removed from plates with a razor blade and stained with ethidium bromide (EtBr) (Sigma) for 15 mins, then destained in 1x TBE for 15 mins. Gel was imaged on a UV lightbox (Fotophoresis).

Release Experiment

To determine concentration of umGAPDH beacon encapsulated in p(FA:SA) microspheres, we set up an experiment to release the beacon over fourdays and measure the supernatant for beacon. We removed umGAPDH p(FA:SA) and blank p(FA:SA) from storage in freezer, weighed out 15.9 mg of each type of microsphere, which is enough for 3 wells at 5.3 mg each for a concentration of 35 nM in 1 mL based on having 50% of the DNA left in particles (see microsphere data in Table X). The microspheres were then put in 1.5 mL tube, 600 µL of HBSS was added, and sonicated to form a homogeneous mixture. 200 µL of the microsphere solution was added to each insert of a 24 well transwell plate (Costar). 800 µL of HBSS was added to well the well, and the plate was stored in the 37°C incubator. 800 µL of HBSS was removed each day for 4 days, stored in the -20°C freezer, and replaced with 800 µL of fresh HBSS.

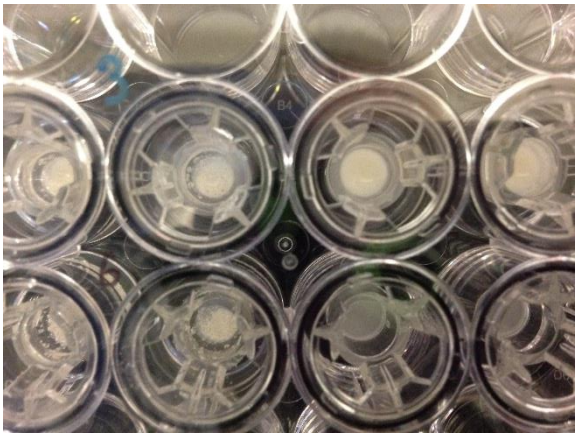


Figure 4: Image of release experiment transwell plates. Image shows plate with the transwell inserts containing white p(FA:SA) microspheres.

Once all four days of samples were collected, samples were spun at 10,000 g for 8 mins to pellet the polymer, while released DNA remained in the supernatant. The supernatant was moved to a different tube and these samples were evaporated in a Vacfuge (Eppendorf) at 60°C until dry (~4.25 hours). Samples were reconstituted in 20 µL molecular grade water, and spun at 10,000 g for 10 mins at 4°C to get polymer to pellet. The samples were measured by Nanodrop to confirm integrity and concentration of DNA. Days 1-4 of umGAPDH p(FA:SA) release (10 µL samples + 6 µL loading buffer) in addition to 33.3 µM umGAPDH beacon in 11.5 µL loading buffer, and 12 µL ladder were then run on a 15% acrylamide gel for 2.5 hrs at 35 V. The dye did not move, so voltage was increased slowly over the course of a half hour until 75 V was reached, then the bottom of the well was manually aggravated with a pipette tip to try to get sample to move in case polymer was coating bottom of well and preventing movement of the sample. Changing the voltage caused ladder to be warped, as seen in gel in Results, **Figure 18**.

Phase Extraction

5 mg empty p(FA:SA) microspheres was dissolved in 1 mL of ethyl acetate or butanol. Solution was vortexed and gently heated in a warm water bath for a few seconds to get polymer dissolved into the organic layer (ethyl acetate or butanol). 1 mL molecular grade water was added, and an emulsion was made by vortexing. The vial was left to sit on the bench for 1 hour until there were two distinct layers. The bottom H_2O layer was extracted with a syringe.

Ubiq p(FA:SA) Thermal Denaturation

A thermal denaturation was run as described previously, but p(FA:SA) encapsulated beacon conditions required additional prep than beacon conditions. Assuming that half the Ubiq is in the final particles, based on the 50% yield shown in the methods section, **Table 1**, 1.07 mg Ubiq p(FA:SA) is needed per well to be equivalent to 200 nM beacon required for the melt. Four times the necessary amount for one well of Ubiq p(FA:SA) was sonicated in 200 µL melt buffer (three times is necessary for completing wells in triplicate plus one extra). This was also completed for blank p(FA:SA) as a control. All conditions were incubated at 37°C for 24 hours to allow release of the beacon from the microspheres. Tubes were spun down for 20 mins at 4°C, and supernatant was taken off of polymer conditions and used in the melt.

Thermal Denaturation of Beacons with a Serial Dilution

A melt was run as described previously, but Ubiq was diluted 1:10 starting at the desired melt concentration of 200 nM and decreasing to 0.02 nM. The same amount of target (400 nM) was used in each condition.

pH Time Course of p(FA:SA) Degradation

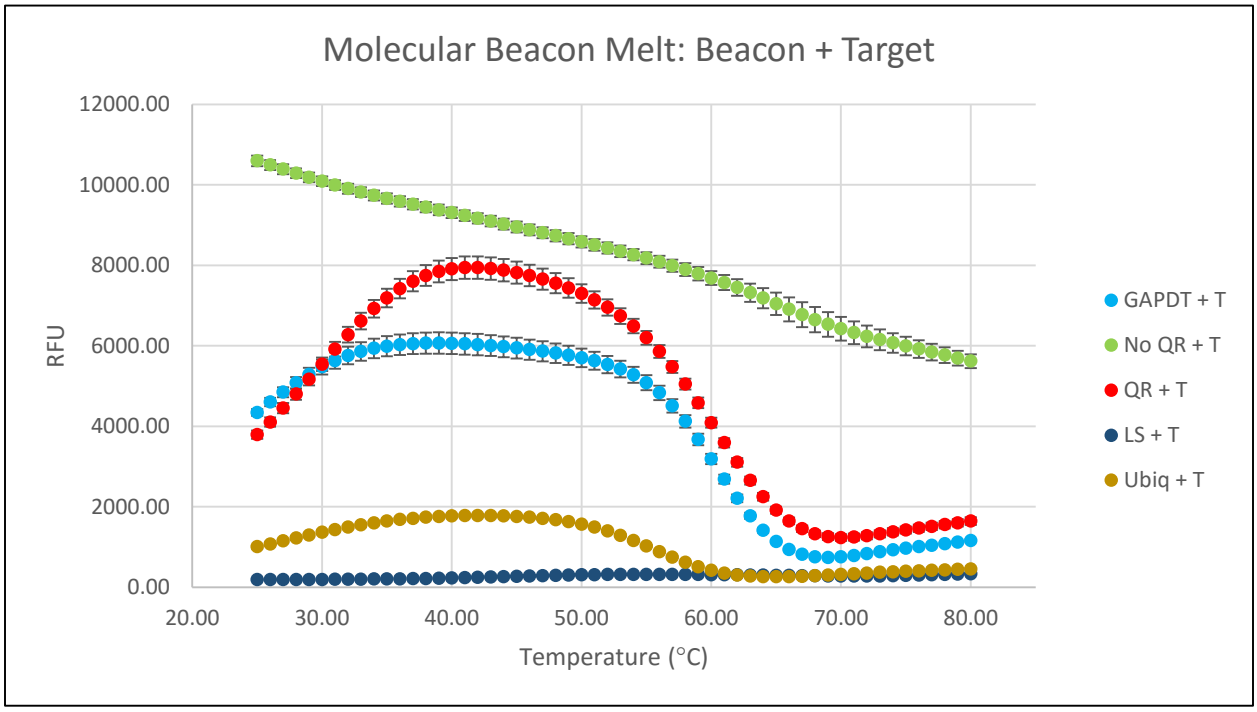
Stock p(FA:SA) 20:80 was graciously donated by the Mathiowitz lab at Brown University. 171.68 mg was added to 8 mL hybridization buffer (1 mM $MgCl_2$, 20 mM Tris-HCl in deionized water at pH 8) and alternately sonicated then vortexed until dissolved. This amount is equivalent of 200 nM beacon contained in p(FA:SA) scaled up so the pH could be measured. The tube was put in the 37°C incubator, and the pH was measured over the course of 5 days.

5. RESULTS

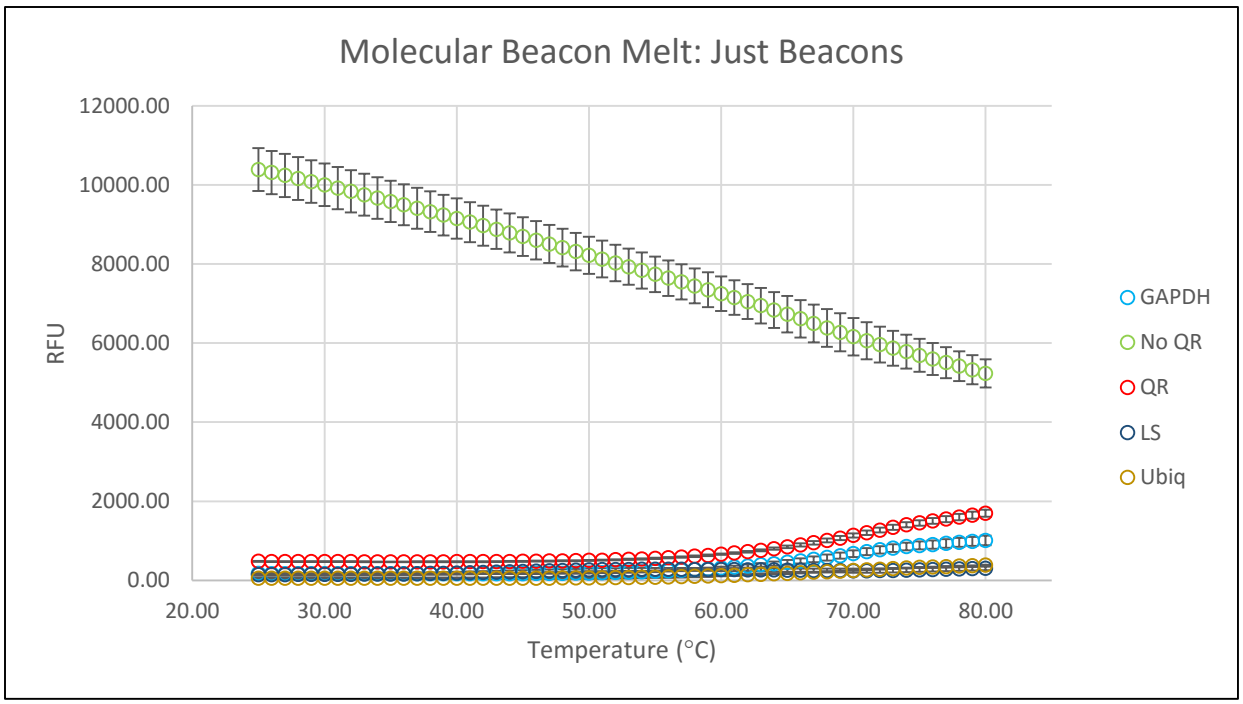
Characterization of Beacon and Polymers

Thermal Denaturation of Beacons

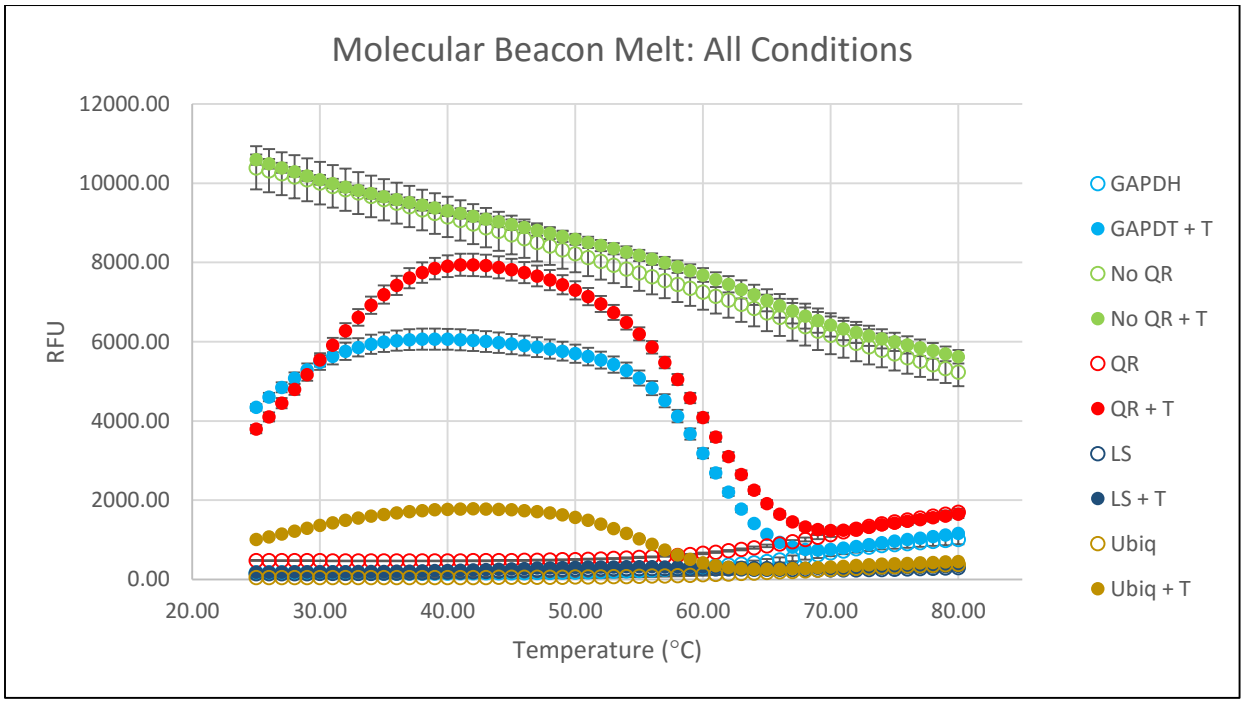
Beacon functionality testing was necessary before the beacons could be used for delivery. A beacon melt was run based on previously established protocols.[4] All beacons used for experiments were tested including GAPDH, No QR, QR, LS, and Ubiq. The melt provides information on the thermal stability of the beacon alone, and interactions between the beacon and target. Three wells of each condition were run, conditions were averaged, and error bars added to show the standard deviation. The background fluorescence of buffer-only conditions were used as blanks to subtract any fluorescent contributions from the buffer itself from all other conditions.



Graph 1: Molecular beacon melt showing thermal stability of beacons with their targets. Relative fluorescence for beacons is brightest in No QR, lowest in Ubiq, and not visible in LS.



Graph 2: Molecular beacon melt showing thermal stability of beacons without their targets. The increase in fluorescence corresponds to the beacon melting temperature, causing a physical separation of fluorophore and quencher and an increase in fluorescence.



Graph 3: Molecular beacon melt showing thermal stability of beacons with and without target. Beacon with and without target exhibits the same behavior at the melting temperature of the beacon, increasing in fluorescence due to denaturation of beacon causing the dissociation of the quencher from fluorophore. This fluorescence is significantly lower than the fluorescence of the beacon bound to its target.

Morphological Characterization of Polymers: SEM

Polymers used for beacon delivery were imaged to see morphology. SEM images were taken to compare p(FA:SA) microsphere morphology with that of the diblock copolymer. Images of p(FA:SA) microspheres were also taken to make sure the encapsulation process resulted in consistently spherical microspheres.

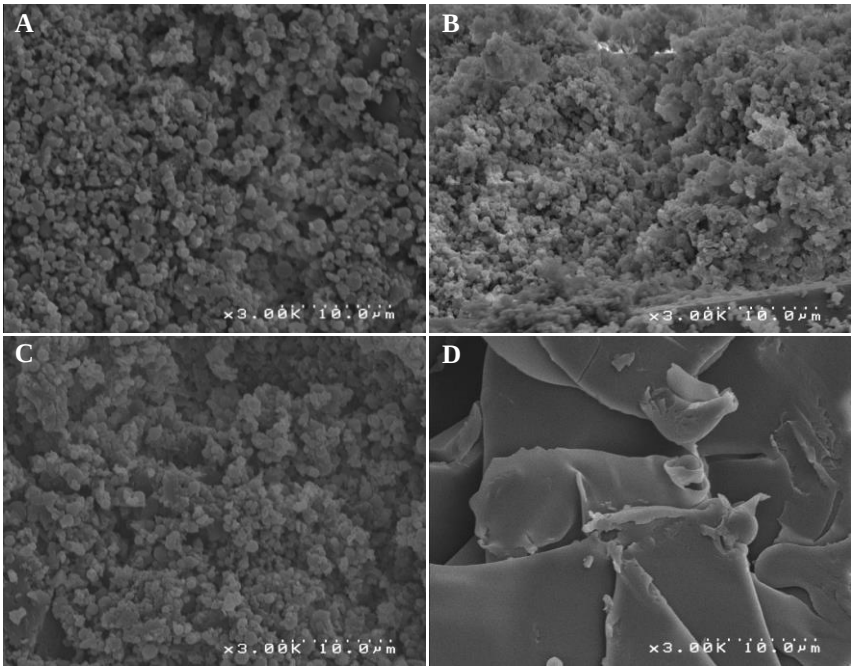


Figure 5: 3000x SEM images of dry polymers. A. Ubiquitin p(FA:SA), B. umGAPDH p(FA:SA), C. Empty p(FA:SA), D. Diblock copolymer. All p(FA:SA) images, although made in different batches with different loadings (A. Ubiq, B. umGAPDH, C. none) exhibit the morphology of small, intact microspheres as expected. The diblock copolymer has a more sheet-like morphology.

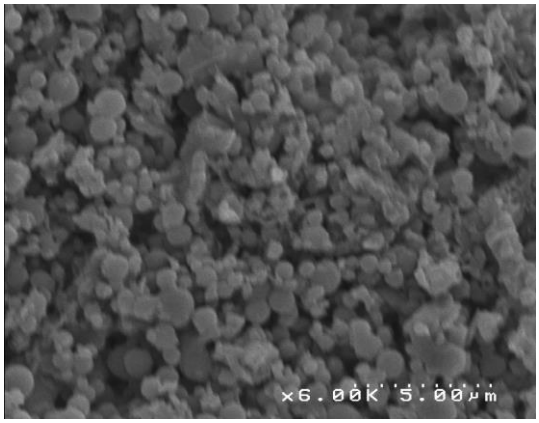


Figure 6: 6000x SEM image with 5 µm scale bar of dry Ubiq pFA:SA. Image shows a closer look at the microsphere morphology. Particles ~1 micron in diameter on average.

Diblock Copolymer Delivery

Effect of Serum on Transfection

Cellular transfection using various agents has been shown to work better in serum free media,[16] so transfection was tested in FBS-containing and FBS-free media. Results show that FBS-free media facilitates better transfection and was soley used for all future transfections.

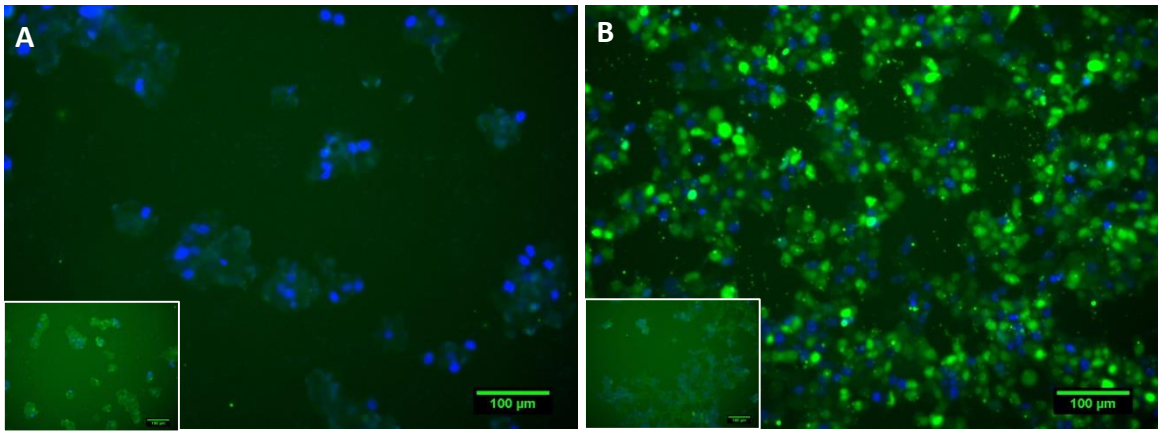


Figure 7: Effect of Serum on Transfection. 10x fluorescent images of diblock copolymer-mediated delivery of GAPDH beacon with a 1:1 charge ratio to HEK293 cells in A. FBS-containing and B. FBS-free media. Cells are fixed and DAPI stained. Control images of just GAPDH with no polymer in the respective media are shown in the inserts. Images suggest that serum-free medium facilitates better transfection.

Charge Ratio Optimization

In order to effectively deliver molecular beacons with diblock copolymer, the first few experiments consisted of optimizing a polymer to beacon ratio. Diblock copolymer delivery of siRNA was optimized to a 4:1 ratio, [9] so ratios surrounding that value were tested. To determine which ratio delivered beacons most effectively, ratios of 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1 were tested. Multiple experiments showed a charge ratio of 1:1 resulted in the most positive cells with the least fluorescent debris. A positive cell was determined by the amount of bright green GFP

fluorescence concentrated over the DAPI stained nucleus. Fluorescent debris appeared as bright particles not near cells.

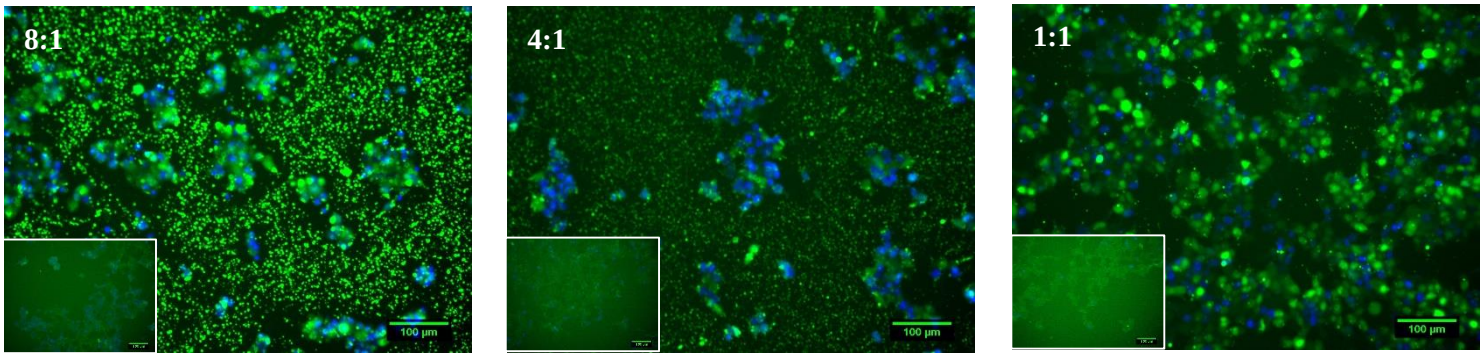


Figure 8: Charge Ratio Optimization for Transfection. 10x colored cytation images of GAPDH beacon delivery optimization conditions including 8:1, 4:1, and 1:1. Control images are included in the corner of images of just beacon without polymer shown in 8:1, just polymer in 4:1, and just cells in 1:1. Control images show that there is no fluorescence caused by the beacon alone, no fluorescence of the polymer, and no autoflorescence of the cells. 8:1 ratio of polymer to beacon shows lots of florescent debris, but not many positive cells (low cell count in general). 4:1 shows high florescence and more positive cells, and 1:1 shows less debris and some positive cells.

Conditions with higher polymer concentrations exhibited a marked increase in fluorescent debris, concentrated in the space between cells. To investigate this further, a condition was added to experiments of just beacon and polymer, without cells. A representative image of this condition is shown in **Figure 9** below. Because this fluorescent debris makes analyzing beacon delivery to cells challenging, a charge ratio of less polymer and less debris was used. In order to try and eliminate the sticking of the polymer and beacon complex to the bottom of the well, an experiment coating the plates with Poly-L-Lysine (Sigma-Aldrich) was completed. Results of this experiment did not show a marked difference between wells coated with PLL and wells without coating. Images show that the beacon and polymer condition without cells still displayed highly fluorescent debris in both coated wells and uncoated wells. To avoid this excessive debris, a 1:1 charge ratio was settled on, where in numerous experiments, of which an example was shown above in **Figure**

8, had the best transfection and the least fluorescent debris. A 1:1 charge ratio was used going forward.

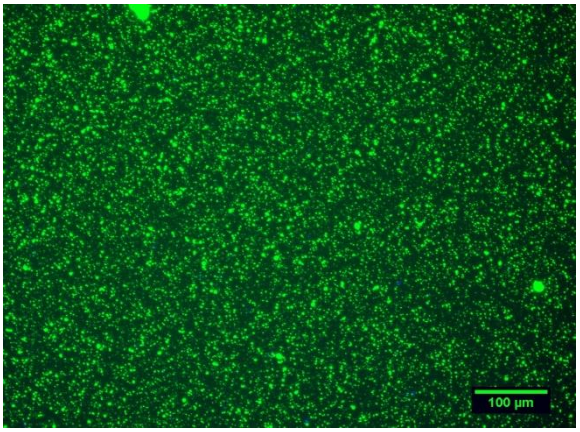


Figure 9: No QR + Polymer without cells control image. 10x fluorescent image showing complexation of beacon and polymer on the bottom of the well without cells. Image shows that polymer when complexed to beacon sticks to the bottom of the well, possible explanation for the fluorescent debris in the polymer dense conditions shown in **Figure 8**.

Time Course of Beacon Delivery

After transfection conditions including serum type and charge ratio were optimized, incubation time of the transfection was also tested. Cells were transfected with various beacons at a 1:1 charge ratio in serum free media then incubated for 1, 4, and 24 hours to assess the temporal delivery of molecular beacons. Diblock copolymer was effective at delivering siRNA at 24 hrs, [9] so these times were also tested for molecular beacon delivery. 20x cytation images were analyzed in MATLAB as described in the methods section. A positive cell is one that shows fluorescence over the DAPI stained nuclei.

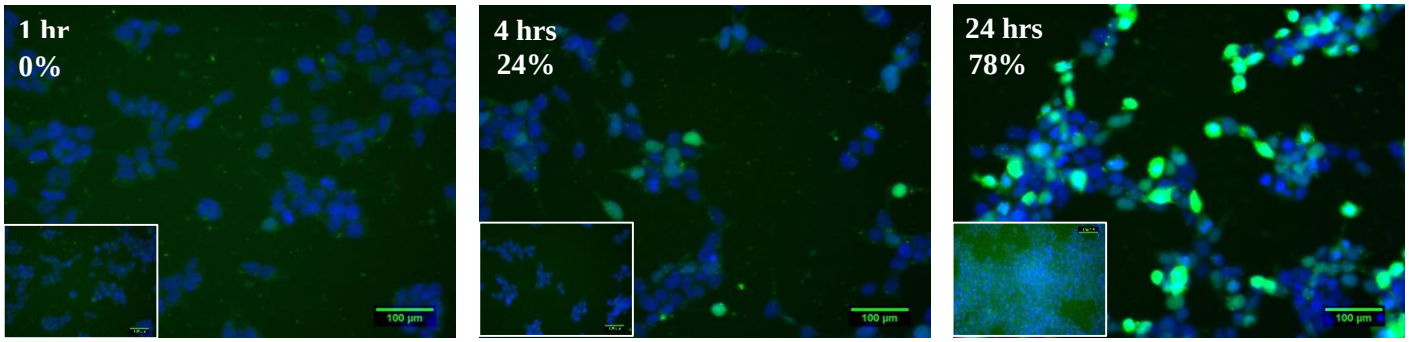


Figure 10: Quantified Temporal Long Stem Beacon Delivery. 20x colored cytation images showing temporal diblock copolymer-mediated delivery of the LS beacon. Cells were assessed at 1, 4, and 24 hours and displayed 0%, 24%, and 78% fluorescence, respectively. Fluorescence increases over a 24 hour time period. Control images of Just Cells shown in insert and all exhibit 0% fluorescence. DAPI-stained nuclei are blue while beacon fluorescence is green. Percent positive cells shown is from image analysis completed in ImageJ and MATLAB.

Diblock Copolymer-Mediated Delivery of Molecular Beacons Results: HEK293T

Based on the success of 24 hour delivery, GAPDH, No QR, QR, and LS were delivered with diblock copolymer to HEK293T cells with a 24 hour incubation time, and displayed 22%, 17%, 11%, 78% positive cells, respectively for the above images. The negative control conditions of just polymer and just cells both displayed 0% positive cells. The just beacon condition was omitted due to consistently negative results in previous experiments optimizing transfection conditions. All beacons were also tested at 1 and 4 hours for comparison, and results varied. For quantification graphs, 7-18 images were analyzed in MATLAB for each condition, some have more depending on quality of cytation images, all used a pixel intensity threshold of 75 and a area threshold of 50 μm^2.

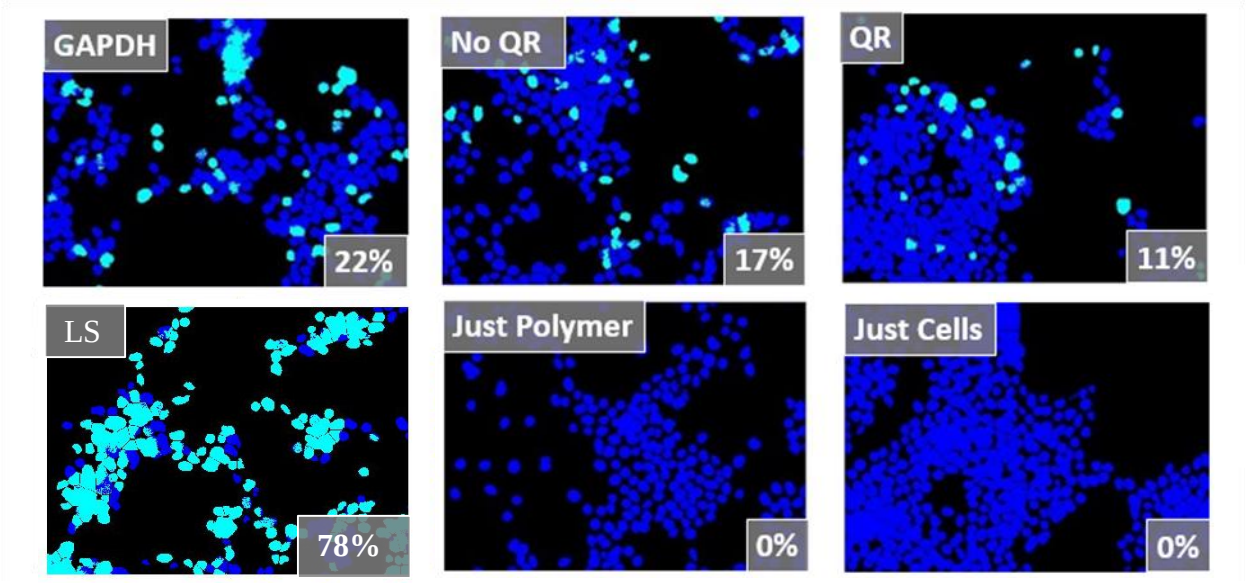
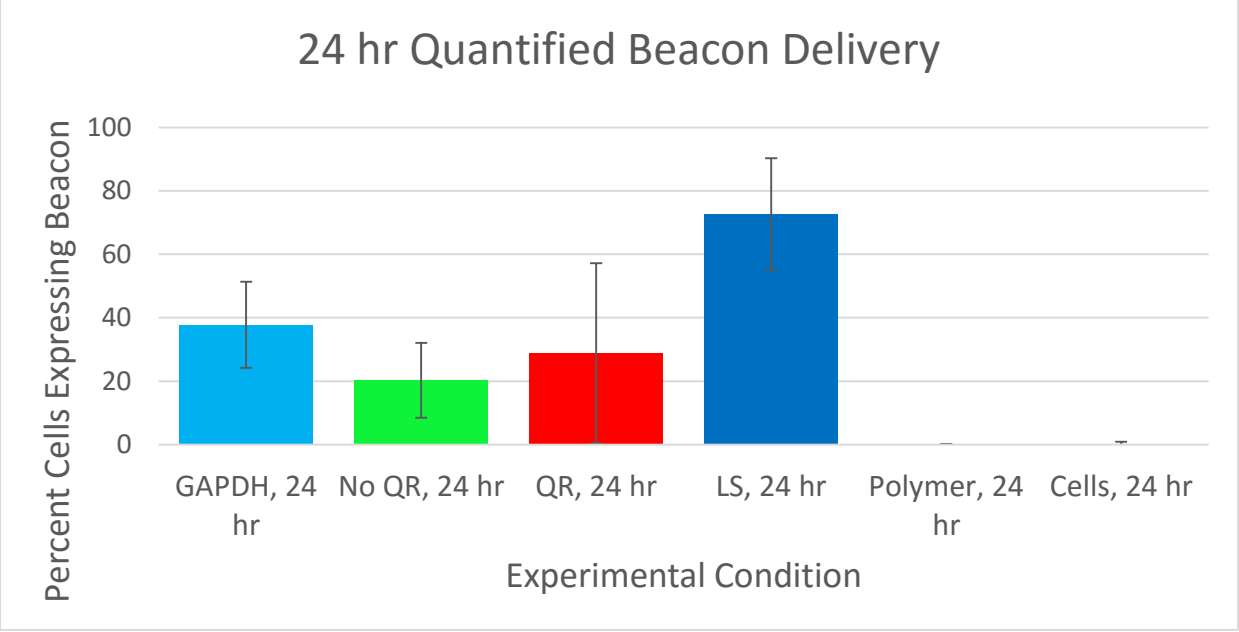
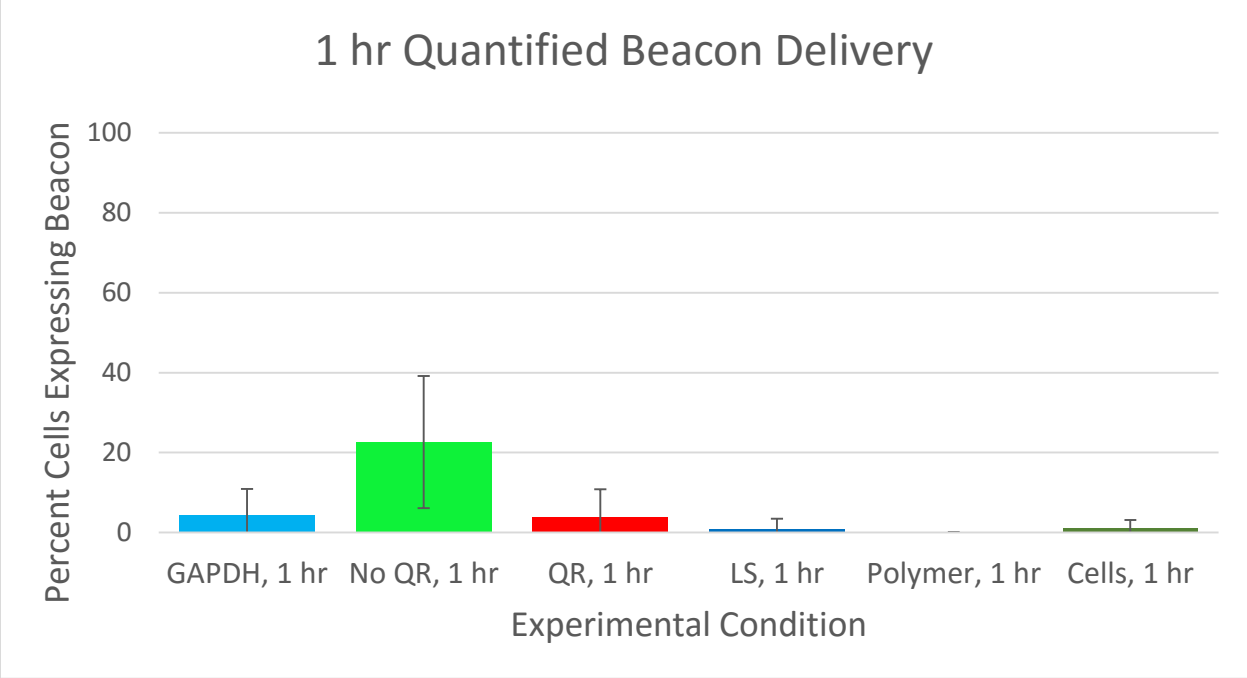


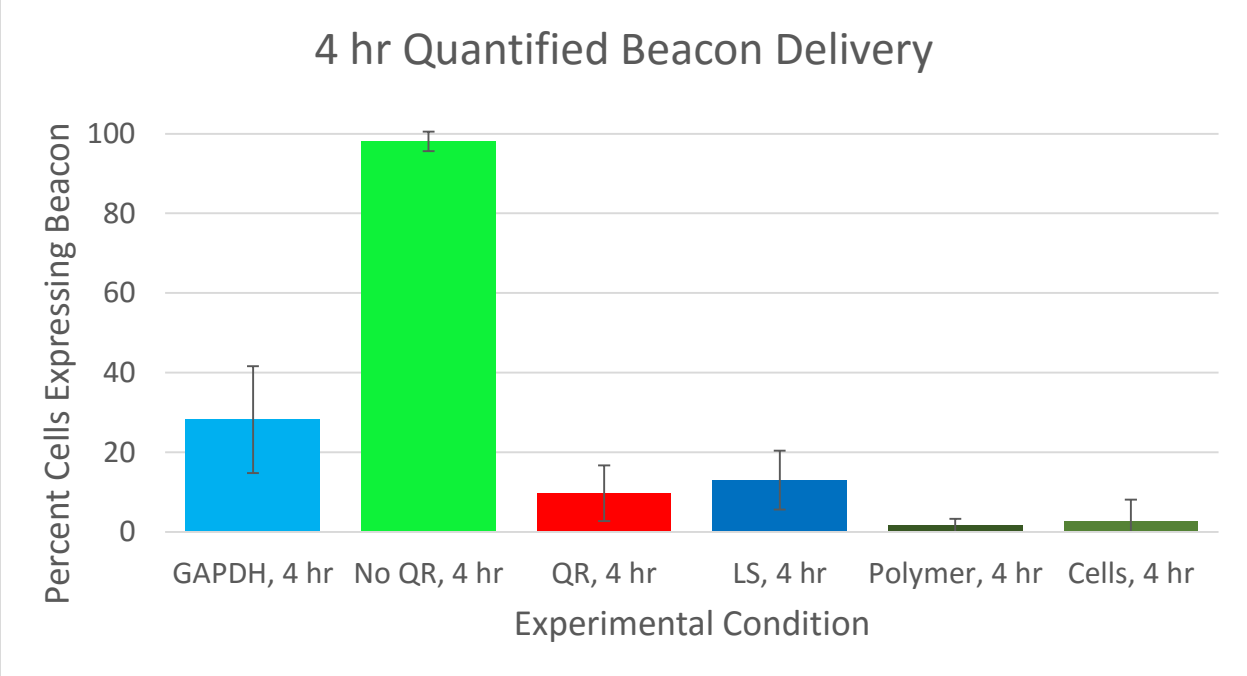
Figure 11: 24 Hour Quantified Beacon Delivery. MATLAB quantification images of 24 hour diblock copolymer-mediated delivery of various beacons to HEK293T cells. GAPDH show 22% positive, No QR shows 17% positive, QR shows 11% positive, and LS shows 78% positive. Negative controls of Just Polymer and Just Cells show 0% positive. LS quantification picture is the same picture from the above fluorescent picture for comparison. Figure shows representative images of each condition, demonstrating that fluorescence is high at 24 hours for beacon conditions compared to controls, with individual beacon fluorescence varying.



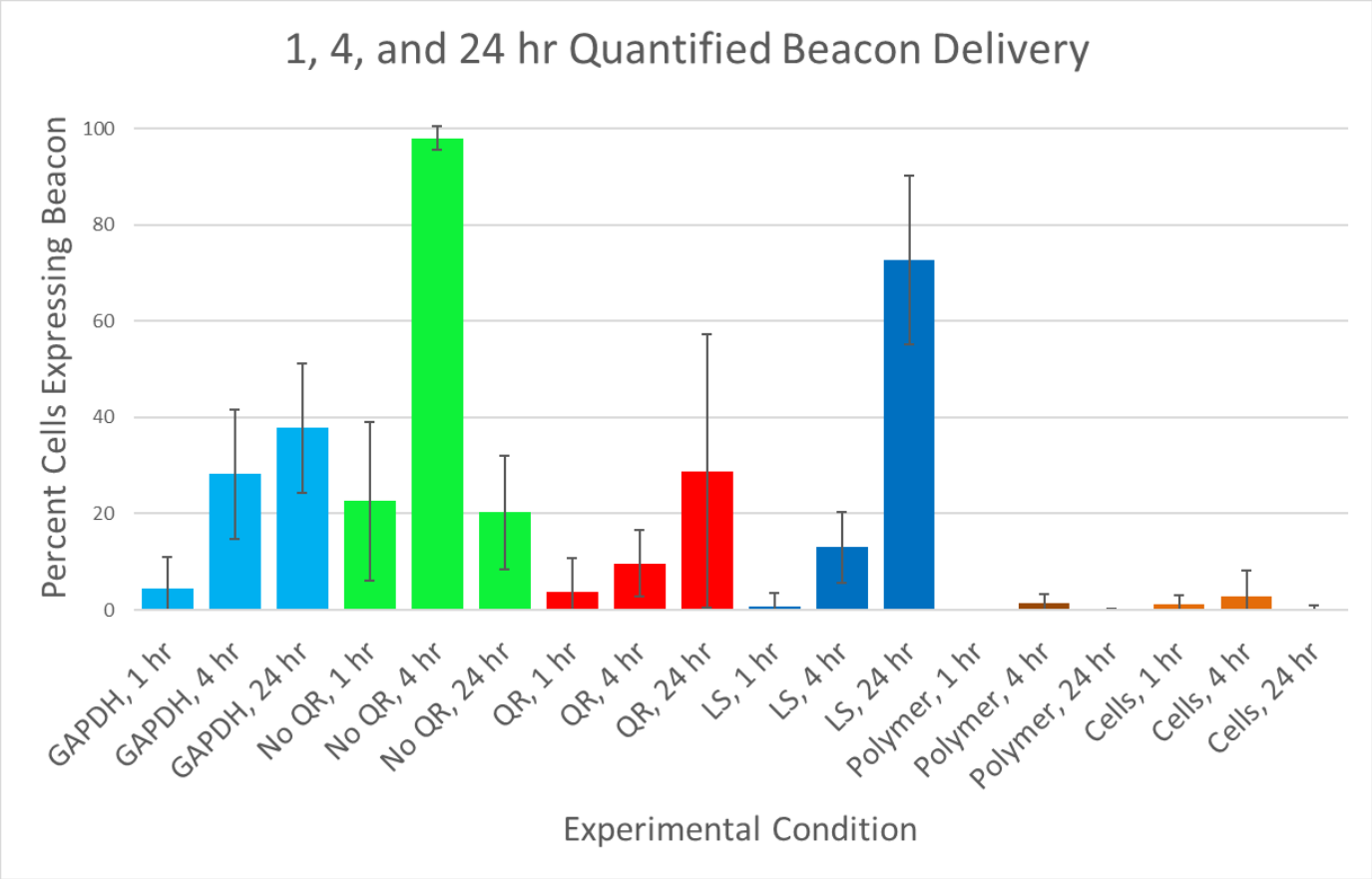
Graph 4: 24 Hour Quantified Beacon Delivery. MATLAB quantification data of 24 hour diblock copolymer-mediated delivery of various beacons to HEK293T cells of GAPDH, No QR, QR and LS beacons. Representative images of each condition showed above. Graph shows variability in beacon delivery at 24 hours. LS beacon had the highest fluorescence followed by GAPDH, QR, and No QR.



Graph 5: 1 Hour Quantified Beacon Delivery. MATLAB quantification data of 1 hour diblock copolymer-mediated delivery of various beacons to HEK293T cells of GAPDH, No QR, QR and LS beacons. Graph shows variability in beacon delivery at 1 hour.



Graph 6: 4 Hour Quantified Beacon Delivery. MATLAB quantification data of 4 hour diblock copolymer-mediated delivery of various beacons to HEK293T cells of GAPDH, No QR, QR and LS beacons. Graph shows variability in beacon delivery at 4 hours.



Graph 7: 1, 4, 24 Hour Quantified Beacon Delivery.* MATLAB quantification data of 4 hour diblock copolymer-mediated delivery of various beacons to HEK293T cells of GAPDH, No QR, QR and LS beacons. *Each incubation time (1, 4, 24 hrs) was completed on a different plate and thus imaged separately, but the cytation settings were kept consistent between runs.

Diblock Copolymer-Mediated Delivery of Molecular Beacons: MG-63

Because of the variability of beacon delivery, both another cell type and another method of analysis were used. All beacons were used in a 1:1 ratio with 35 nM beacon and delivered to MG-63 cells. Two runs were completed, one run was imaged on the cytation and the other run cells were uplifted and analyzed on a flow cytometer. The just cells condition was set as the negative population in the flow cytometer.

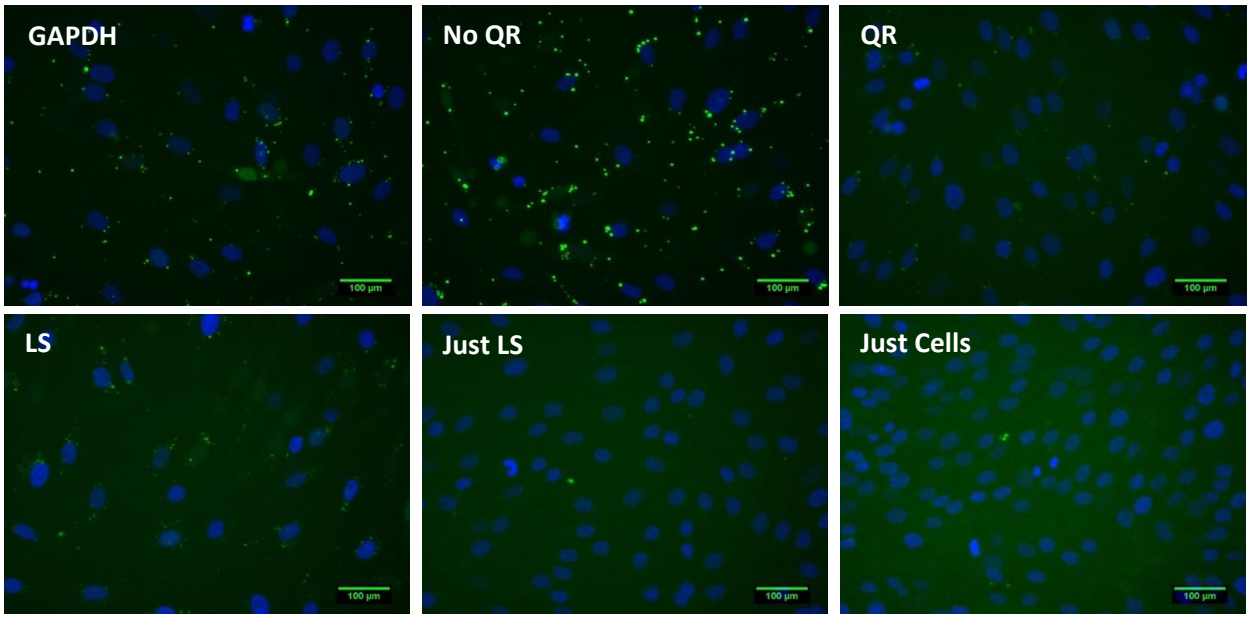


Figure 12: 24 hr colored cytation images of diblock copolymer mediated delivery of various molecular beacons to MG-63 cells, 35 nM beacon, 1:1 charge ratio. No QR shows most fluorescent cells, GAPDH shows some, QR and LS show no expressing cells, and controls of just LS and just cells are dark.

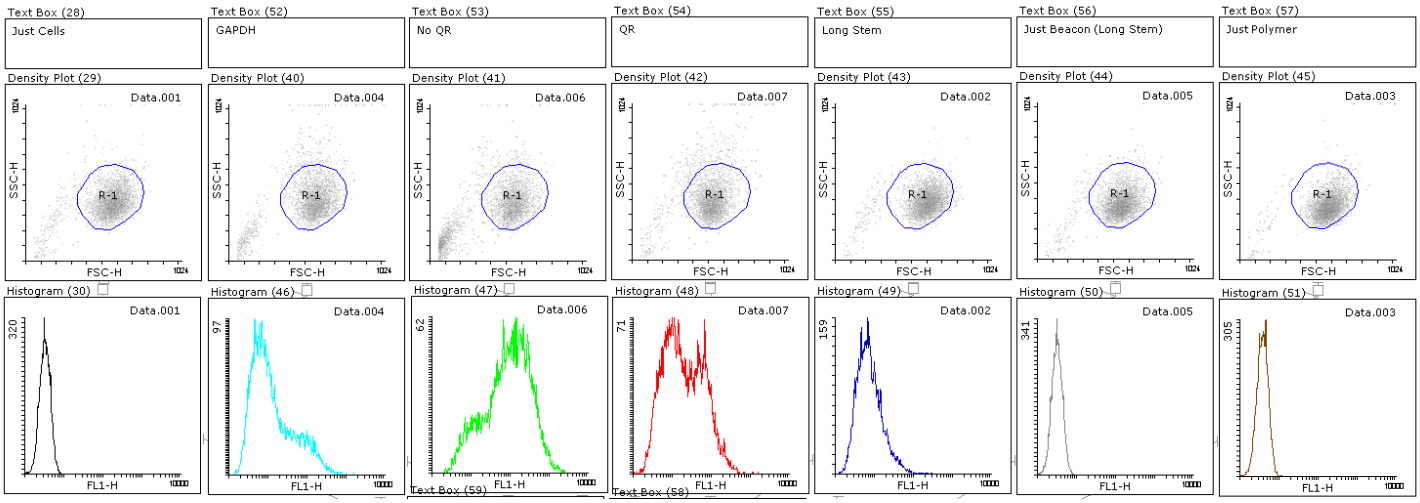


Figure 13: 24 hr Flow Cytometer data of diblock copolymer-mediated delivery of various molecular beacons to MG-63 cells, 35 nM beacon, 1:1 charge ratio. Top graphs show the R1 population of cells analyzed for each condition. Bottom graphs show the fluorescent data with X axis showing FL-1 florescence and Y axis presenting number of events. Conditions from left to right are Just Cells, GAPDH, No QR, QR, LS, Just LS Beacon, and Just Diblock Copolymer.

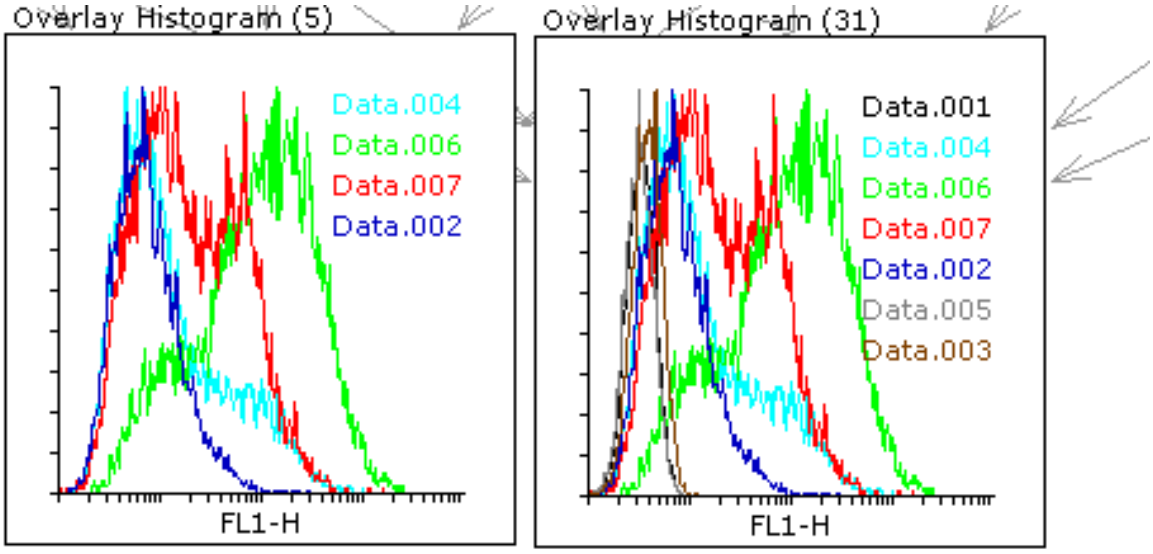


Figure 14: Flow Cytometer data of diblock copolymer mediated delivery of various molecular beacons to MG-63 cells, 35 nM beacon, 1:1 charge ratio polymer. Figure shows the individual graphs from Figure X combined for condition comparison. Black line .001 is Just Cells, light blue .004 is GAPDH, green .006 is No QR, red .007 is QR, dark blue .002 is LS, grey .005 is Just LS Beacon without polymer, and brown .003 is Just Polymer. All beacon conditions shown on the left graph, while controls and beacons are shown on the right. Histogram reveals that the LS beacon fluorescence is higher than controls, but lower than the positive peaks of other beacons. No QR exhibits largest number of highly fluorescent positive cells while GAPDH has the lowest number of fluorescent cells, and LS is slightly inconclusive.

Testing of pFA:SA Microspheres Encapsulated with Molecular Beacons

p(FA:SA) Cellular Cytotoxicity

Before pursuing delivery of molecular beacons through p(FA:SA) microspheres, the cytotoxicity of p(FA:SA) in cells had to be tested. Cells were treated with blank p(FA:SA) and incubated for 24 hours then stained with a live/dead cytotoxicity kit outlined in Methods. Images show that cells treated with p(FA:SA) look most like the alive control and have a few circular dead cells, with lots of red debris. Imaging the p(FA:SA) alone without live/dead stain reveals that in both the red and green channel the polymer is fluorescent, and the morphology looks similar to the red debris found in the polymer treated condition. This would reveal that the cells treated with are presumed to be alive.

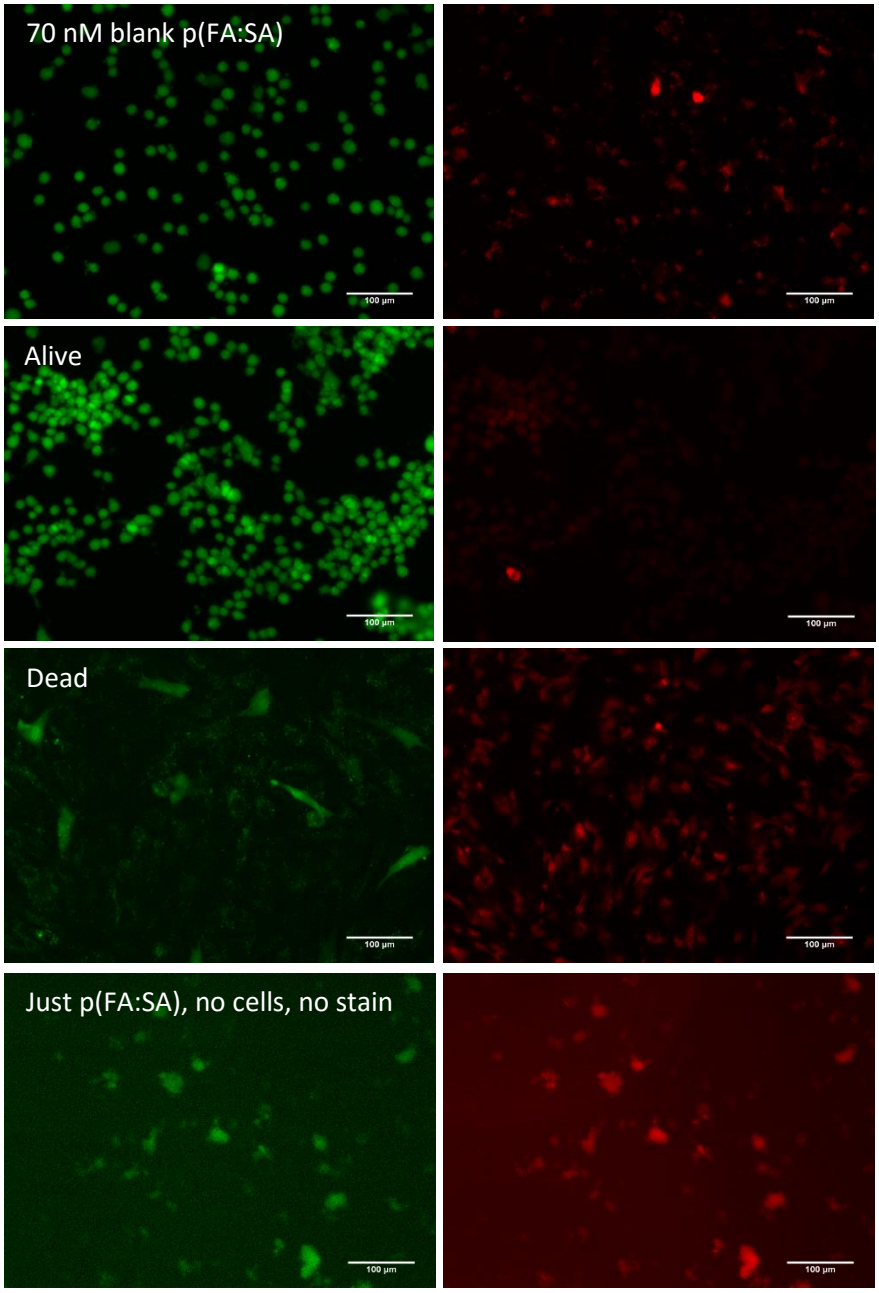


Figure 15: Cytotoxicity of blank p(FA:SA) microspheres in HEK293T cells. Green channel (right) shows Calcin live stain and red channel (left) shows Ethidium Homodimer-1 dead stain. Cells treated with 70 nM bank p(FA:SA) are mostly alive and the p(FA:SA) is autofluorescent in both channels.

Effect of Encapsulation Process on Molecular Beacons

After cellular cytotoxicity was determined, in order to test whether the beacon encapsulation process would damage the beacons, beacons were sonicated in DCM to simulate the harsh encapsulation process. To extract the DNA, a phase separation with water was then completed, and the resulting water/DNA solution was both tested in the Nanodrop and run out on a gel to assess the damage.

	UmGAPDH	Ubiq
Before sonication (nmol)	3.750	5.000
After sonication (nmol)	0.876	0.467
Total DNA lost	2.874 nmol, **76.64%**	4.533 nmol **90.66%**

Table 3: Nanodrop data showing loss of DNA to sonication in DCM. Both beacons, umGAPDH and Ubiq amount decreased after sonication in DCM to mimic the p(FA:SA) encapsulation process. umGAPDH lost 76.64% of the DNA, and Ubiq lost 90.66%.

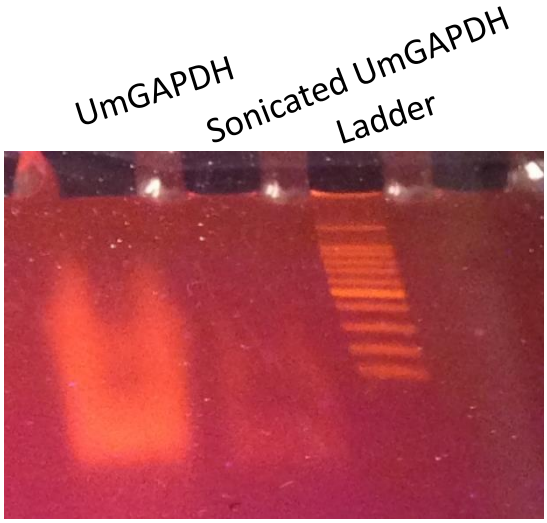
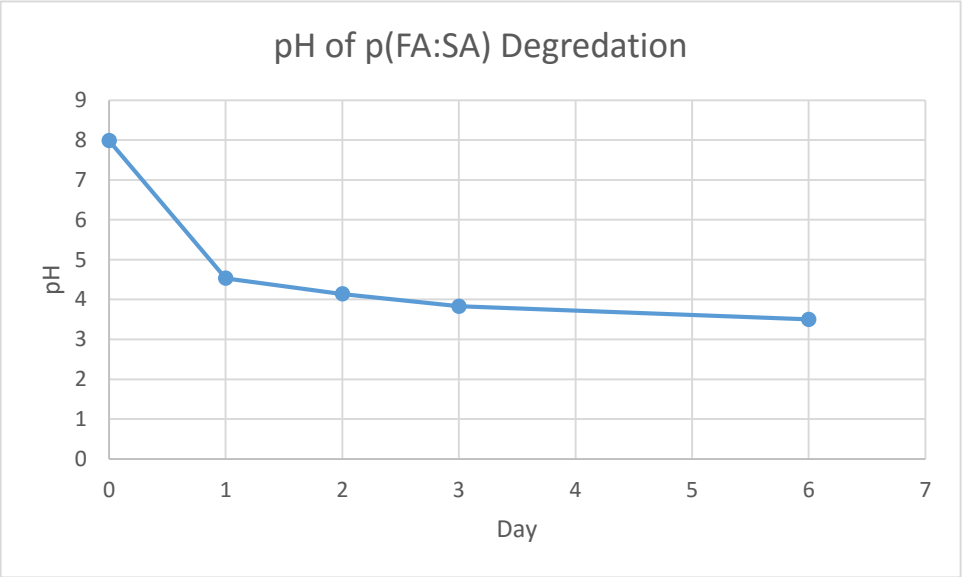


Figure 16: Gel showing loss of DNA to sonication in DCM. Amount of umGAPDH decreased after sonication in DCM tested to mimic the p(FA:SA) encapsulation process, shown on gel as a decrease in the prominence of the band. Ubiq and sonicated Ubiq (not shown) ran off the gel, as evident by the lack of visible dye.

To further test the encapsulation effect on beacons, an experiment was completed monitoring the pH over six days of p(FA:SA) in hybridization buffer. This reveals that the first day has the most drastic drop in pH, but it keeps getting more acidic every day as the p(FA:SA) degrades and dissolves in solution. Such an acidic pH may be troublesome for cells and beacon functionality.



Graph 8: pH time course of p(FA:SA) in Hybridization buffer. Graph shows that pH decreases drastically in the first 24 hours, and then decreases gradually down through day 6.

Determination of Molecular Beacon Loading of p(FA:SA) Microspheres

Phase Extraction

Phase extraction was explored to try and calculate the concentration of polymer from the Nanodrop. This was completed with the blank p(FA:SA) microspheres meaning any absorbance should be from the polymer only, which will show the concentration of polymer left in the aqueous phase. Ethyl acetate and butanol were tested for the phase extraction, and compared to the empty p(FA:SA) measured on the Nanodrop. Using a general extinction coefficient (E) of 100,000 (since

the extinction coefficient of the polymer has not been reported), the concentration of polymer was determined using Beer-Lambert Law: A260 = ElCm. Based on the comparison of concentrations, the phase extraction reduced the concentration of polymer in solution by about half in ethyl acetate. Other chemicals were tried including DCM and hexanes in water, but these did not work. Ethyl acetate gave the best results.

	A260	[polymer]
Ethyl Acetate + water	0.493	4.93 nmol
Butanol + water	0.731	7.31 nmol
Empty FASA in water	28.752	10 nmol

Table 4: Nanodrop results of phase extraction. Empty particles were used to see the concentration of polymer left after the phase extraction in either ethyl acetate and water or butanol and water, compared to just empty particles sitting in water.

Release Experiment

A release experiment was completed using transwell plates. umGAPDH p(FA:SA) was added to transwell insert and incubated at 37°C for four days in HBSS. Each day, the HBSS was removed from the well and stored. Samples were evaporated in the speed vac and reconstituted in PCR H_2O. Samples were then run out on a gel. The dye in the well did not run, but gel was still stained and is shown below.

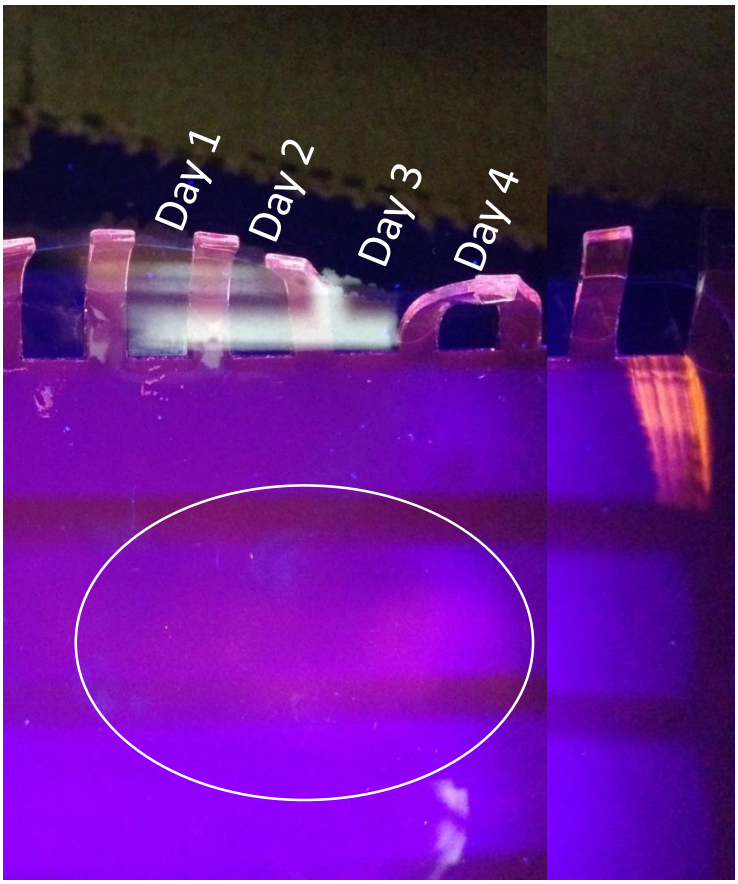


Figure 17: Gel showing release experiment results on gel. umGAPDH released from microspheres potentially shown in the white oval. Since there was no control umGAPDH beacon ran on the gel, it is hard to determine if the bright area in the white circle is DNA. *this procedure did not involve spinning at 10,000 RCF for 8, then 10 minutes as described in Methods section*

New samples from the release experiment were analyzed, and these were spun at 10,000 RCF for 8, then 10 minutes to try and get the polymer pelleted out of solution and leaving the DNA. This method still prevented the dye from running on the gel, as shown in the gel dock below. All conditions with p(FA:SA) did not run, but the ladder and just Ubiq beacon did run, pointing out that it is probably the p(FA:SA) that prevents the dye and sample from migrating. These samples were further analyzed in the Nanodrop and compared to the blank p(FA:SA), subtracting out that absorbance. However, day 1 obtained that there was 1 nmole of umGAPDH in the 5.3 mg sample, but in the whole batch of umGAPDH p(FA:SA) contained 1.3 nmol at the start of the

encapsulation process. Therefore, this method of attempting to find the loading by subtracting out the absorption from the polymer to isolate the absorption from the DNA is not accurate at all.

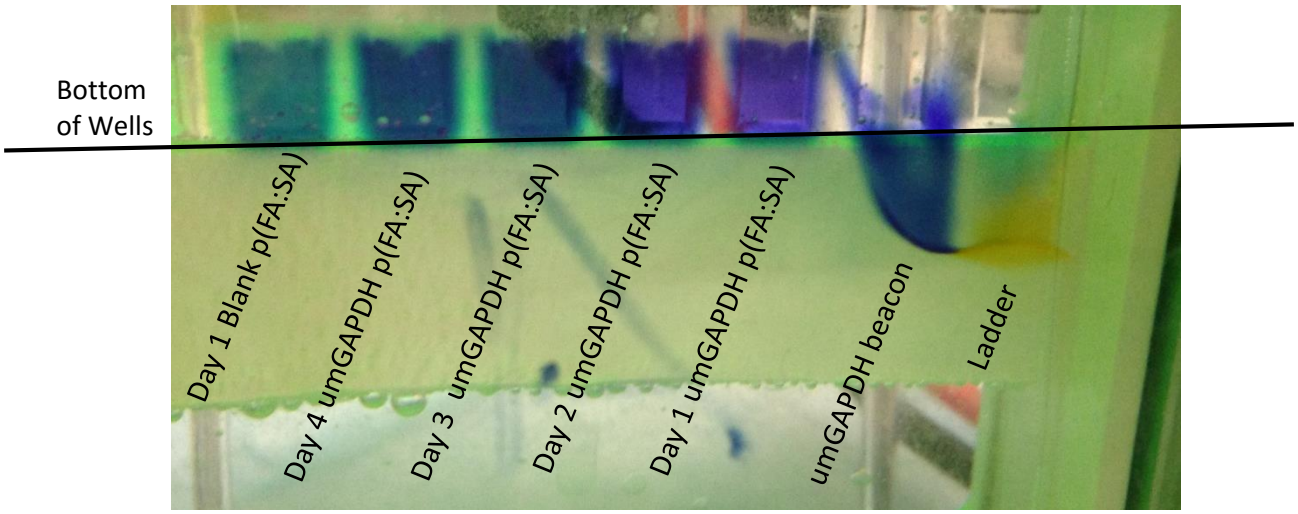


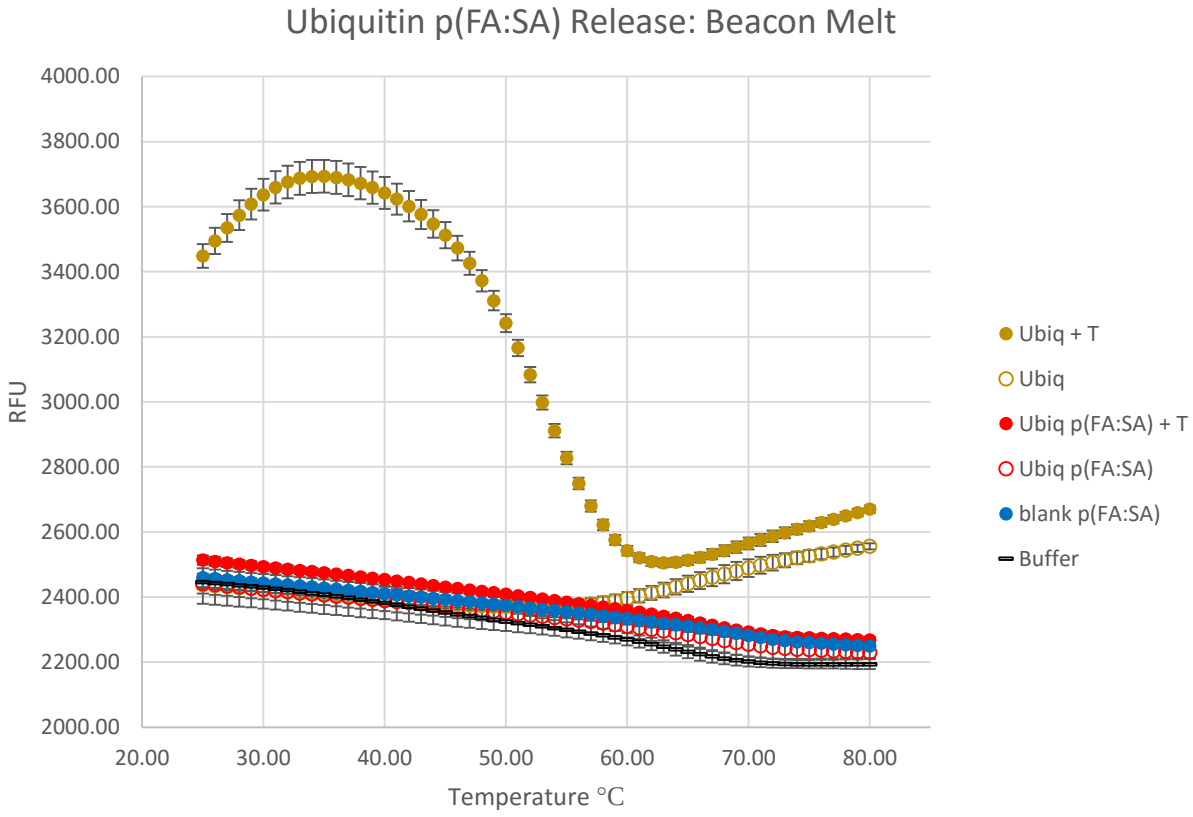
Figure 18: Gel dock showing release experiment results. This is a picture of the gel dock of running the release experiment on a gel that illustrates the impermeable well barrier of all conditions with p(FA:SA) in them. Ladder and beacon conditions are warped because of increasing the voltage to try and get other wells to run.

	A260 umGAPDH p(FA:SA)	A260 Empty p(FA:SA)	A260 umGAPDH p(FA:SA) – A260 Empty p(FA:SA)	Calculated concentration of umGAPDH in p(FA:SA) microspheres
Day 1	43.015	28.752	14.263	1 nmole
Day 2	23.786	23.081	0.705	49.6 pmol
Day 3	13.123	11.851	1.272	89.6 pmol
Day 4	7.183	7.111	0.072	5 pmol

Table 5: Nanodrop release experiment results. umGAPDH released from 5.3 mg microspheres, evaporated in the speed vac and reconstituted in PCR H_2O. Data shows the high absorbance of umGAPDH p(FA:SA), lowere empty p(FA:SA), the difference, and the calculated concentration based off Beer-Lambert law using the extinction coefficient of umGAPDH (284170), giving the theoretical concentration of umGAPDH in the p(FA:SA) particles.

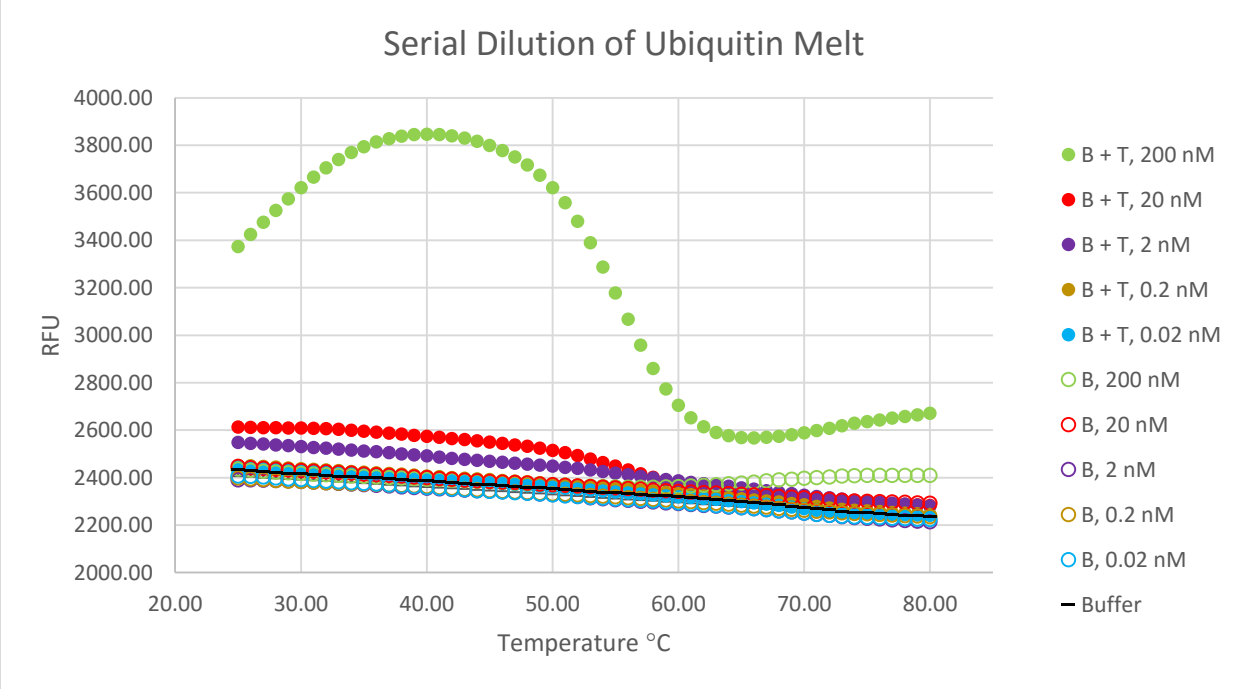
Ubiq p(FA:SA) Release Thermal Denaturation

Microspheres were then tested to see if functionality of encapsulated beacons was retained after the encapsulation process. A release experiment was completed, allowing beacons to be released from microspheres for 24 hours, then were analyzed with a beacon melt and compared to functional beacons.



Graph 9: Thermal denaturation showing results of Ubiquitin p(FA:SA) 24 hour release. Ubiq beacon functions as expected, increasing fluorescence when bound to target, decreasing as target degrades and then increasing again as beacon melts. Ubiq p(FA:SA) showed higher background than the buffer, but did not exhibit functional beacon fluorescence.

Because the Ubiq p(FA:SA) did not show beacon functionality, a study was completed to see the lower detection limit of the melt. A serial dilution of the ubiquitin beacon was made and melted. Results show that in the first step down from 200 nM to 20 nM, fluorescence decreases dramatically and by 0.02 nM there is no visible fluorescence, so 2 nM is the detection limit of the melt. It is necessary to note that Ubiq fluorescence at the normal melt concentration is much lower than expected, as determined in the previous melt with all the beacons.



Graph 10: Thermal denaturation showing serial dilution of Ubiq beacon. Graph shows that below 2 nM, no beacon fluorescence is detected. Error bars not shown because only one well of each condition was run.

6. DISCUSSION

The goal of this project was to investigate novel polymer-mediated delivery methods of molecular beacons. This was achieved by using diblock copolymer for delivery as well as encapsulating beacons in p(FA:SA) microspheres in preparation for delivery. We hypothesized that both diblock copolymer and p(FA:SA) microspheres would be effective delivery methods, delivering functional beacons to cells. Diblock copolymer was extensively tested in HEK293T and MG-63 cells and shows promise as an effective, versatile nucleic acid delivery method, both with molecular beacons and previously with siRNA.[9] Major findings include that transfection was optimized in serum free media and a 1:1 charge ratio and 35 nM beacon facilitated best delivery. Cellular fluorescence increased over a 24 hour period with beacon and diblock copolymer conditions only conditions exhibiting fluorescence while control conditions did not. In this study, both HEK239T cells and MG-63 cells exhibited fluorescence after beacon transfection with diblock copolymer. The variability of beacon functionality led to inconsistent results surrounding the cause of fluorescence. Our metric to determine delivery was the amount of cells expressing beacon fluorescence. With this metric, we can definitively say that the fluorophore and quencher of the beacon are unbinding to produce fluorescence. However, the cause of the fluorescence comes into question. It is either that the beacons are correctly binding to their target, they are getting degraded by cellular endonucleases, or some other process is causing beacons to degrade and exhibit fluorescence. Further experiments are necessary to figure out the mechanism of visible fluorescence. P(FA:SA) microspheres on the other hand, could not be tested with cellular delivery, because we could not detect functional molecular beacons after the encapsulation process. However, much was learned about the effect of the encapsulation process on the beacons, and the inability to detect beacons is either likely due to a loading lower than our detection limit, or degradation of the beacons in the encapsulation process. Furthermore, it was shown that p(FA:SA)

microspheres are not cytotoxic to the cells, even after three days of exposure, so if microsphere loading can be understood and corrected, cellular delivery work can proceed.

Characterization of Beacons and Polymers

Thermal Denaturation of Beacons

A thermal denaturation profile was completed as described previously[17] for all beacons. The initial increase and plateau of fluorescence shown in **Graphs 3** for GAPDH + T, QR + T, Ubiq + T occurring around 30°C follows the expected shape of beacon binding with target, and the decrease indicating a dissociation of beacon and target corresponds to the respective melting temperatures of the target,[7] as listed in the Materials and Methods section. Half way up the final increase in fluorescence between the baseline and plateau corresponds to the melting temperature of the denatured beacon,[4] causing coming apart of fluorophore and quencher and an increase in fluorescence. Relative fluorescence is brightest in No QR ~10500 RFU, then QR at 8000 RFU, GAPDH at 6000 RFU, Ubiq at 2000 RFU, and LS slightly above buffer. No QR is quencherless, so whether it is bound to target or not, the fluorophore exhibits high fluorescence,[6] as shown in the graph. LS beacon's fluorescence is only slightly higher than the buffer. This lack of fluorescence is likely due to the thermodynamic stability of the LS beacon, which was specifically designed to be highly stable with a very long stem and short loop. If the stem of a molecular beacon is too strong, as it is likely the case with the LS beacon, the beacon will remain closed, even when bound to target.[17] Overall the melt characterized the beacons, demonstrating that the beacon fluorescence is high when bound to target, low when closed in its hairpin shape, and increases again to a lower RFU value if the beacon alone is denatured.

Morphological Characterization of Polymers: SEM

The SEM images of the polymers shown in **Figure 5** reveal that all microspheres, although synthesized in different batches and all containing different loadings (none, umGAPDH, Ubiq) exhibit the morphology of small, intact spheres as expected.[18] The average size of the microspheres was ~1 micron which will allow for efficient cellular uptake. The diblock copolymer was observed to have a sheet-like morphology.

Diblock Copolymer Delivery

Effect of Serum on Transfection

FBS-free and FBS-containing media were tested to see the effect of serum on transfection efficiency. It was found that serum-free media led to higher percent fluorescing cells and least fluorescent debris. This is supported in the literature, as serum-free media is commonly used for transfections.[16] Serum containing media may prevent the polymer from being endocytosed, while the serum free media would allow complete polymer delivery.

Charge Ratio Optimization

After many trials based on the charge ratios previously used with diblock copolymer delivery of siRNA,[9] a 1:1 polymer to beacon ratio was found to facilitate the highest transfection efficiency with the lowest fluorescent debris. The diblock copolymer conditions optimized for siRNA found 4:1 to be the best ratio.[9] However, we found that a 1:1 ratio lead to the most efficient delivery of molecular beacons with the least fluorescent debris. Using No QR, at high polymer-to-beacon charge ratios, namely 8:1 and 4:1, as shown in **Figure 8**, there was excessive debris. This

was also observed in control wells containing the beacon and polymer complex without cells (**Figure 9**), even after the extensive washing used in the transfection protocol. Excessive debris was likely caused by the beacon and polymer complex sticking to the bottom of the well. To try and mitigate this problem, wells were treated with Poly-L-Lysine (PLL) (Sigma-Aldrich), process outlined in **Appendix E**. The polycationic nature of PLL and its common use of an adhesive solution was thought to prevent complexing of beacon and polymer complex to the bottom of well. There was no difference in wells treated with PLL and wells not treated with PLL, so a 1:1 ratio was settled on as the most efficient transfection condition.

Diblock Copolymer-Mediated Delivery of Molecular Beacons Results: HEK293T

Diblock copolymer as previously mentioned, is a chaperone polymer that facilitates delivery of nucleic acids to cells. The temporal delivery of LS beacon was assessed at 1, 4, and 24 hours. Cells expressing fluorescence increased over the 24 hour period from 0% to 24% to 78% in a representative MATLAB quantified image. This revealing that transfection is most efficient with a 24 hour incubation. At 24 hours, however, beacon functionality was variable as displayed in **Graph 4**. GAPDH, a positive control beacon that should fluoresce if bound to the mRNA of a reference gene exhibited an average of 37.8% positive cells. No QR beacon, another positive control beacon which has no endogenous target, lacks a quencher, and therefore should always exhibit fluorescence exhibited 20.3% positive cells. QR beacon which has no endogenous target, has a quencher, and should not exhibit fluorescence had 28.8% positive cells. Long Stem beacon which has no endogenous target and has a longer double stranded beacon stem resulting in more thermodynamic stability had 72.7% positive. The controls of just beacon, just polymer, and just cells, which should all be dark, displayed a fluorescence of less than 2%. There were substantial deviations in our data as evidence by the error bars within each condition, speaking to the fact that

the images were highly variable. There were cytation imaging difficulties focusing on fluorescing cells verses fluorescent debris, which caused variability in the images and made analysis in MATLAB difficult. One necessary note is that at 4 hrs, No QR exhibited fluorescence inconsistent with all other conditions, which is analyzed in **Appendix F**.

In all conditions, percent positive cells increased over the course of 24 hours. As previously mentioned, diblock copolymer was designed to protect siRNA from both nucleases and escape endosomal trafficking.[9] However, the control beacons did not behave as expected, and this behavior brings this function of diblock copolymer delivery of molecular beacons into question and will require further study. Our only assessment of beacon delivery was if the cells exhibited fluorescence. The specific mechanism of fluorescence once in the cell is not known. Beacons are designed to bind to their target inside the cell, opening up the hairpin and physically separating the fluorophore from quencher, resulting in a fluorescent signal upon excitation.[5] This is supported in the observed thermal denaturation profile, seen in **Graph 1**, showing fluorescence when the beacon is bound to its target. **Graph 2** demonstrates the other possibility of fluorescence, showing that when the beacon itself is denatured, all beacons including GAPDH, No QR, and QR exhibited fluorescence. From the melt, we can definitively say that beacons fluoresce both when bound to target and when denatured, physically separating the fluorophore from quencher and causing fluorescence. Our hypothesis is that either of these two mechanisms occurred in the cells. The beacons are either binding to their target, or the beacons are getting damaged by cellular processes, unquenching the fluorophore and causing fluorescence. There is also a possibility of probes binding to nontarget RNAs instead of the intended target, also resulting in an undistinguishable difference in fluorescence. This reveals a limitation in this study which is supported in the literature of molecular beacons indicating there is an inability to distinguish between fluorescence of probes

hybridized to target verses unbound, degraded probes.[5] This was potentially seen in QR, exhibiting fluorescence when it has a random target, indicating that it may be degrading or binding to a nonspecific target. A possible cellular degradation process to explain this is endosomal entrapment in the endosomal/lysosomal pathway, which results in rapid degradation by nucleases.[5] The LS beacon's behavior supports this cellular degradation hypothesis. LS has a long double stranded region, which makes it more susceptible to be destroyed by cellular endonucleases,[9] and would explain why it exhibited the highest fluorescence at 24 hours. Overall, in these experiments, beacon functionality was variable and may be attributed to either expected beacon functionality, beacon degradation from endonucleases, lysosomal uptake, or nonspecific binding to the cell membrane.

Diblock Copolymer-Mediated Delivery of Molecular Beacons Results: MG-63

MG-63 cells are larger and grow less plaque-like than HEK293T cells, making MATLAB image analysis more easily performed. Therefore, MG-63s were thought to allow further analysis of beacon delivery. In MG-63 qualitative imaging, the beacons delivered with a 1:1 polymer to beacon charge ratio and concentration of 35 nM exhibited expected functionality, shown by **Figure 12**. The positive control GAPDH beacon exhibited a few fluorescent cells, the highly positive No QR showed more positive cell fluorescence, while the negative controls of QR and LS did not show positive cells. Negative controls of just polymer, just beacon, and just cells display small amounts of fluorescent debris, but no fluorescent cells. This experiment was also analyzed on a flow cytometer. In **Figure 13**, graphs showing the green fluorescence verses the number of events reveal expected beacon functionality. Bimodal peaks indicate two major populations of cells. GAPDH exhibits a bimodal peak including a large peak of negative cells and a small peak of positive cells, while No QR establishes a small peak of negative cells and a large peak of positive cells. QR has a bimodal peak, half negative, and half positive, which indicates that this negative

control beacon did result in fluorescence, while LS acts as a true negative control and has a large, wide negative peak. When **Figure 14** is analyzed, the relative fluorescence units are visible in comparison to the other conditions and to the controls. This confirms the data shown in the aforementioned thermal denaturation profile. No QR shows the highest fluorescence, followed by GAPDH and QR both having similar fluorescence, with the number of positive cells lower in GAPDH. Because of the positive cells expressed in QR, the delivery of this beacon reveals that there is probably some endonuclease activity as was mentioned in the HEK293T delivery, or some other mechanism that would cause a negative control beacon that is not supposed to bind to a random target inside the cell to fluoresce. However, the LS beacon had a single negative peak, indicating that in this experiment it functioned as a negative control, with the long stem staying together as intended by its design. The controls of just beacon, just polymer, and just cells all show no fluorescence as expected. Because delivery to MG-63 cells analyzed with flow cytometry shows promise, more trials are necessary to confirm results, as is testing HEK293T cells on a flow cytometer both to compare results in different cell lines, and to compare results of quantified cytation imaging to flow cytometry data counting the number of fluorescent events.

Testing of p(FA:SA) Microspheres Encapsulated with Molecular Beacons

p(FA:SA) Cellular Cytotoxicity

Cells were treated with different amounts of p(FA:SA) and imaged after 24, 48 and 72 hours. Calculation of amount p(FA:SA) used was based on a slice of the well volume, as discussed in the methods section, because of the inability to image the preliminary cytotoxicity experiment (**Appendix G**) due to the full well volume requiring 31.8 mg microspheres 1 mL well volume.

Figure 15 clearly shows that p(FA:SA) is not cytotoxic to cells. To note, after 48 and 72 hours, p(FA:SA) treated cells were also alive, but images are not included in this analysis. Cells that stain green are alive and red are dead as mentioned in the methods section. The live control (just cells + stain) shows mostly green cells and a few red, the dead control (formalin fixed cells + stain) shows mostly red cells with a few green, possibly due to the formalin. Unstained just p(FA:SA) without cells shows that the polymer is autofluorescent in both channels. Looking at these control images compared to the p(FA:SA) treated cells reveals that most cells in the p(FA:SA) condition are living. The red fluorescence seen in the treated cells looks more similar to the autofluorescence of the polymer observed in the just p(FA:SA) condition than it does to whole dead cells. Because of this, we believe that the cells treated with p(FA:SA) contain autofluorescent debris from the polymer treatment but are living. The morphology of cells treated with live/dead stain appear balled up, but since they are green, the cells must be alive. Some unknown factor was making all cells treated with stain ball up, besides the cells previously fixed with formalin. This is a qualitative experiment that reveals that p(FA:SA) is not cytotoxic, as supported by the previous use of p(FA:SA) in vivo.[14]

Effect of Encapsulation Process on Molecular Beacons

Before the encapsulation of molecular beacons could occur, we decided to test the effects of the most harmful steps of the process on beacons. We first tested the concentration of molecular beacons before and after sonication in DCM with a phase extraction both on the Nanodrop (**Table 3**) and run out on a gel (**Figure 16**). The Nanodrop data revealed that there was a noteworthy loss of DNA in this process, either due to damage, or an inefficient phase extraction, leaving DNA in the DCM. The gel confirmed that there were less of the sonicated beacons in DCM, evident by its faint band, but this revealed that the DNA was not fragmented, or multiple bands would have been

visible. Since a phase extraction is not completed in the encapsulation process, DNA loss will not occur in this step.

The pH of p(FA:SA) was analyzed over 6 days to see if there was a drastic pH change that may affect the beacon functionality. The pH dropped from 8 to 4.53 in the first 24 hours, seen in **Graph 8**. It has been shown that pH plays an important role in fluorescence of a molecular beacon,[19] more specifically in the fluorescence of the fluorophore, so more experimentation must be completed to determine the effect of this highly acidic pH on the beacon functionality. Since this polymer is highly acidic, it was important to assess the extent of the effect of microsphere degradation on pH.

Determination of Molecular Beacon Loading of p(FA:SA) Microspheres:

Phase Extraction

Testing of a phase extraction's ability to determine loading of microspheres proved to not work. A phase extraction was completed with blank p(FA:SA) to see if all polymer could be removed from the aqueous phase. Blank microspheres were used in the phase extraction, and results were tested on the Nanodrop, meaning the only absorbance should be due to the polymer since no DNA was used. These data are shown in **Table 4**, where in ethyl acetate the concentration of polymer was reduced from 10 nmol in the blank particles down to 4.93 nmol in the phase extraction. This calculation was carried out as described previously. This indicates that p(FA:SA) is amphiphilic, therefore most goes into the organic phase but some goes into the aqueous phase too. The phase extraction with blank microspheres revealed that we cannot fully remove the polymer from the aqueous phase, making phase extraction an inaccurate way to assess loading.

Release Experiment

The release experiment intended to extract all DNA from microspheres using transwell plates in order to assess loading was completed over 4 days to ensure that all DNA was released from the microspheres.[14] **Figure 17** shows the results of the release on a gel. There was difficulty getting the sample to run, maybe because of the polymer. In the white circle in this figure, there is a possibility of bands indicating that there is DNA in the microspheres, but since no control DNA was run, the results are very uncertain. A second gel was run with a different protocol that used more spinning to try and pellet the polymer out of solution. **Figure 18** clearly demonstrates that this method similarly did not work, shown by the inability of conditions with polymer to migrate in the gel. We hypothesize that the polymer prevented running on the gel possibly by coating the bottom of the well. Since the gels were inconclusive, the release was tested on the Nanodrop and compared to just unloaded microspheres. The absorbance of the unloaded microspheres was subtracted from the loaded microspheres release Nanodrop data, in order to see just the absorbance from the loaded DNA. This was able to yield a calculation that 1 nmole DNA was in the day 1 release. However, since only 1.3 nmol were encapsulated in the umGAPDH particles to begin with, (which does not account for loss in the encapsulation process or the low loading) and the amount of polymer used was only 5.3 mg out of 98.1 mg (1/18.5th), the calculation of 1 nmol in the day 1 release is highly unlikely. For example, if all 1.3 nmol were encapsulated, there should only be 0.07 nmol in the 5.3 mg used for the release. These data indicate that this method of trying to determine the encapsulation efficiency is inaccurate, so the loading of the particles is still unknown.

A problem discovered with this experiment is both DNA and the polymer absorb at 260 nm. Therefore, p(FA:SA) is disrupting the signal from the DNA. To avoid this, similar experiment

must be completed, measuring the absorbance of DNA loaded microspheres at 260 nm and at a different wavelength that only the polymer absorbs, not the DNA. A possibility for this is at 345 nm, as was determined in the Mathiowitz Lab at Brown University as a wavelength at only the polymer will absorb. Underlying these issues, since there is so little DNA in the microspheres, due to the 0.012% calculated loading this means that out of the 1.3 nmol started with, there should be 1.56×10^{-4} nmol in the total batch of microspheres. Since the loading calculation was based on weighing particles, this method is inaccurate. Therefore, since around 50% yield in the encapsulation process, it was assumed that there was also a 50% loss of DNA, so 50% of the starting amount of DNA is encapsulated in the particles. This is very inaccurate, but since loading was unable to be determined experimentally, this is the assumption used. So, we assumed that since we lost half of the polymer, we lost half of the DNA.

Ubiq p(FA:SA) Release Thermal Denaturation

As demonstrated previously, the amount of DNA in solution can be measured in a homogenous mixture.[4] This was used to determine the lower detection limit of a beacon thermal denaturation in order to assess how much ubiq beacon is encapsulated in the p(FA:SA) particles. The standard curve showing ubiq beacon's thermal denaturation at different concentrations is shown in **Graph 10**. This serial dilution curve shows that below 2 nM, no ubiq beacon fluorescence is detected. From this, we can extrapolate that from the release experiment, since there was no detectable fluorescence in **Graph 9**, the amount of beacon encapsulated in the ubiq p(FA:SA) microspheres must be lower than 2 nM. Another possibility is that the beacon is not functional and the fluorophore damaged, because no measurable florescence was seen. Although they are different systems, the thermal denaturation using raw beacon and the ubiq p(FA:SA) thermal denaturation using encapsulated beacon that had to first be released and collected from the

particles, we infer that there is likely not enough beacon encapsulated in the microspheres. This means that our previous calculation saying that there was 1.0 nmol of ubiq in the microspheres determined by the Nanodrop was highly inaccurate. Conditions as we completed did not work, but our hypothesis is that if we add more beacon to surmount the lower detection limit of the thermal denaturation assay, there is a high probability that we would see signal.

7. CONCLUSIONS

This project looked to assess molecular beacon delivery methods using two different polymers, diblock copolymer and p(FA:SA) microspheres, in 2D cell culture. Diblock copolymer delivery was shown to be best at a 1:1 charge ratio using 35 nM beacon. Fluorescence in cells increased over a 24 hour period, and delivery was shown in both HEK293T and MG-63 cells. P(FA:SA) microspheres were shown to not be cytotoxic to cells, and more experimentation is necessary to test whether an increased concentration of beacons encapsulated would permit effective determination of loading and assess functionality of beacons after encapsulation, which must be completed before cellular delivery is possible. Both of these polymer delivery systems have the potential to be very beneficial in the delivery of molecular beacons, and more experiments will solidify this promise.

8. FUTURE DIRECTIONS

This exploration of polymer delivery systems with molecular beacons shows promise, and there are many options moving forward. In order to fully understand the percent positive cells, a way to distinguish between the fluorescence of beacons bound to the correct target, beacons bound nonspecifically, and beacons degraded must be determined. This is currently a limitation of molecular beacons and a definitive way to account for all these variables has not yet been developed. A potential way to determine if beacons are being degraded verses binding to their mRNA target would be to use GAPDH in cells without sequence homology to humans, such as in a mouse model cell line.[20] This would show that if there is fluorescence, beacons are probably being degraded. Although this is a cross-species control, it is a potential way to address these limitations. With the p(FA:SA) encapsulation, a way to determine loading must be discovered. All methods tried were failures, so moving forward looking at absorbance of DNA at 260 nm verses a different absorbance of the polymer at 345 nm could distinguish between the DNA and polymer and be used to determine loading. However, first and foremost, a higher concentration of DNA would be encapsulated in the particles to account for the dramatic loss in the encapsulation process and the miniscule loading. Once beacon can be successfully encapsulated in particles and measured, cellular work can move forward because it has already been determined in this project that p(FA:SA) is not cytotoxic to cells. Once these polymer-based delivery systems are optimized for molecular beacons, we will demonstrate the universal applicability in primary cells, opening up a myriad of therapeutic applications.

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  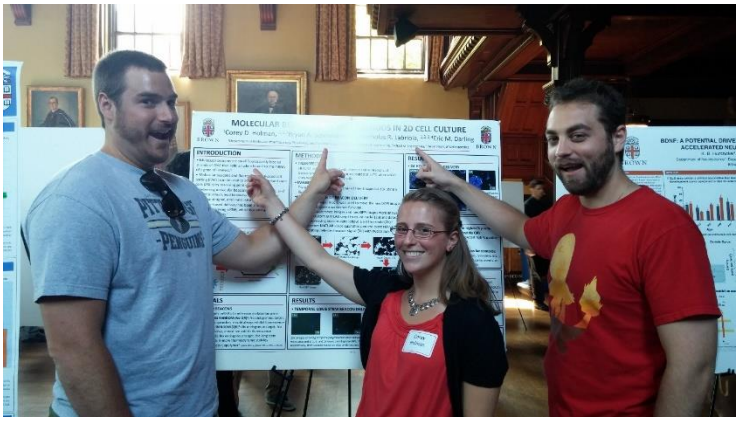
  L-R: Bryan, Corey, and Nick at the UTRA poster presentation, August 2015

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The Darling Lab, Summer 2015

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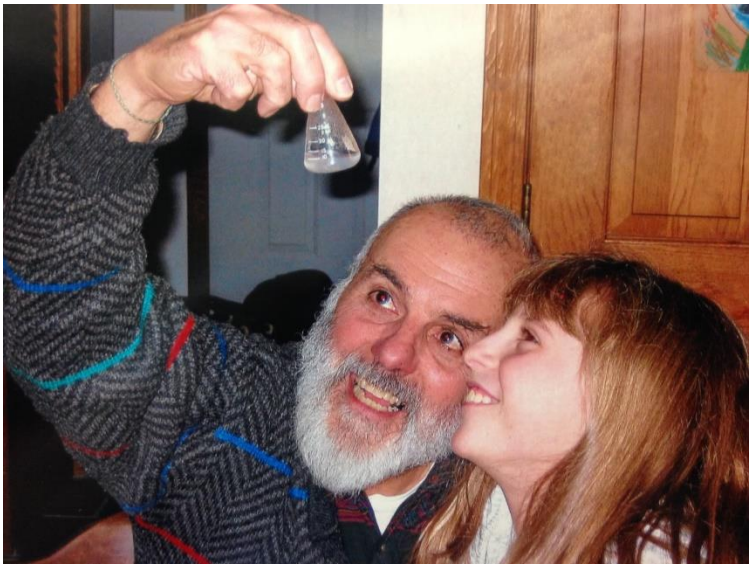

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11. APPENDICES

Appendix A: Calculation of Polymer:Beacon Charge Ratios

Necessary Concentrations	Values	Units
Desired beacon concentration per well	3.75E-08	mol/L
Cell culture volume per well (**96 well plate**)	0.0002	L
Concentration of beacon stock	1.00E-05	mol/L
Mol beacon per well	$7.5x10^{-12}$	Mol b/well
Mol (-) charges per mol beacon	29	Mol -/mol b
Mol (-) charges in beacon per well	$2.175x10^{-10}$	Mol – b/well
Diblock stock solution concentration	1	mg/mL
Molecular weight (MW) of diblock	45000	g/mol
Grams diblock per well	$1.23x10^{-7}$	g
Mol (+) charges per mol diblock	76.4	Mol +/mol d

Calculation of volume diblock needed per well for a 1:1 charge ratio of Polymer:Beacon:

(Desired beacon concentration per well)(cell culture volume per well) = Mol beacon per well

$(3.75 \times 10^{-8}$ mol beacon/L)(0.0002 L) = $7.5x10^{-12}$ mol beacon per well

(mol beacon per well)(mol (-) charges per mol beacon) = mol (-) charges in beacon per well

($7.5x10^{-12}$ mol beacon) (29 mol (-)) = $2.175x10^{-10}$ mol (-) charges in beacon per well

(mol (-) charges in beacon per well) / (mol (+) charges per mol diblock)(MW diblock)

= grams diblock per well

($2.175x10^{-10}$ mol (-) b/well) / (79.6 mol (+)/mol d)(45000g diblock/mol diblock)

= $1.23x10^{-7}$grams diblock per well

(grams diblock per well)(diblock stock solution concentration)(conversion)(conversion)

= amount diblock stock per well

($1.23x10^{-7}$ grams diblock per well)(1mL/1mg diblock)(1000 mg/1g)(1000 µL/1mL)

= **0.128 µL diblock stock per well, 1:1 ratio**

Calculation of volume diblock needed per well for a 2:1 charge ratio of Polymer:Beacon:

(amount diblock stock per well, 1:1 ratio)(2) = amount diblock stock per well, 2:1 ratio

(0.128)(2) = **0.256 µL diblock stock per well, 2:1 ratio**

Calculation of volume diblock needed per well for a X:1 charge ratio of Polymer:Beacon:

(amount diblock stock per well, 1:1 ratio)(X) = amount diblock stock per well, X:1 ratio

(0.128)(X) = **_____ µL diblock stock per well, X:1 ratio**

Calculation of volume beacon needed per well for a final concentration of 35 nM, all charge raios:

$C_1V_1 = C_2V_2$

$C_{1,\text{ concentration of beacon stock}} = 1x10^{-5}$ mol beacon/L

V_1=?

$C_{2,\text{ desired beacon concentration per well}} = 37.5 \times 10^{-9}$ mol beacon/L

V_2=200 µL

V_1= (37.5 x 10^{-9} mol beacon/L)(200 uL)/(1x10^{-5} mol beacon/L)

V_1= **0.75 µL beacon stock per well, 35 nM**

Appendix B: Cytation Imaging Settings

Setting	**LED**		**Integration Time**		**Gain**	
Image	*DAPI*	*GFP*	*DAPI*	*GFP*	*DAPI*	*GFP*
Figure 7	9	10	100	463	0.8	11
Figure 8	9	10	100	463	0.8	11
Figure 10*: 1 hr	5	10	65	180	0	15.6
4 hrs	5	10	60	180	0	15.6
24 hrs	5	10	64	180	0	15.6
Figure 11	5	10	64	180	0	15.6
Figure 12	9	10	100	390	0.5	15.6

*and Graphs 4-7

Appendix C: ImageJ Macro - Makes Watershed of DAPI images

Macro function:

1. Makes the DAPI image binary
2. Converts the DAPI image to a mask
3. Makes a watershed of the DAPI image

```
//CYTATION Watershed Prep for MatLab Analysis by Corey Holman
```

//*Cytation image directory must be sorted by name, i.e. have DAPI, GPF, and bright field images for the first well, then DAPI, GFP, and bright field images for the second well, etc. (As opposed to having all DAPI images in file, then all GFP, then all bright field.)*

```
inputDir = getDirectory("Choose your input directory!");

outputDir = getDirectory("Choose an output directory!");

fileList = getFileList(inputDir);

setBatchMode(true);

for(j =0; j<6; j++) {

        for (i = 0; i < fileList.length/18 ; i++) {

        showProgress(i+1, fileList.length);

open(inputDir+fileList[(i*18)+j]);

                dapi = getTitle();

setOption("BlackBackground", false);

run("Make Binary");

run("Convert to Mask");

run("Watershed");

saveAs("tiff", outputDir + "/" + dapi);

        close();

open(inputDir+ fileList[(18*i+6)+j]);

                gfp= getTitle();

saveAs("tiff", outputDir + "/" + gfp);

        close();

}

}

print("FINISHED!!")

//run("Close")
```

Appendix D: ImageJ Macro - Automates Presentation Image Processing

A macro was coded in ImageJ, to add scale bars to a batch of cytation images, merge the DAPI (blue) and GFP (green) channels from two images to create a merged DAPI and GFP image, and create individual false color images of each channel (DAPI, GFP, bright field). Images straight from the cytation are black and white, as with standard epifluorescence imaging, while images analyzed in ImageJ are colored.

Macro function:

1. adds scale bar to all images
2. overlays DAPI/GFP channels
3. colors all cytation images their respective colors
4. converts TIFF image to a JPG

For 10x cytation images:

```
//Cytation Image Processing by Corey Holman

//*Cytation image directory must be sorted by name, i.e. have DAPI, GPF, and bright field images for the first well, then DAPI, GFP, and bright field images for the second well, etc. (As opposed to having all DAPI images in file, then all GFP, then all bright field.)*

inputDir = getDirectory("Choose an INPUT directory!");

outputDir = getDirectory("Choose an output directory!");

fileList = getFileList(inputDir);

setBatchMode(true);

for(j =0; j<6; j++) {

for (i = 0; i < fileList.length/18 ; i++) {

    showProgress(i+1, fileList.length);

  //Merges the DAPI and GFP channels and add scale bar:

        open(inputDir+fileList[(i*18)+j]);

                dapi = getTitle();

        open(inputDir+ fileList[(18*i+6)+j]);

                gfp= getTitle();

        run("Merge Channels...", "c2=&gfp c3=&dapi c1=*None* create");

                //this merges the channels, setting image 2 to green (c2), image1 to blue (c3), and nothing in the red channel
```

```
	run("Set Scale...", "distance=1.55 known=1 pixel=1 unit=um");
		//this sets the scale bar...you can change the values for your specific image needs
	run("Scale Bar...", "width=100 height=8 font=28 color=White background=Black location=[Lower Right] bold");
		//this sets the scale bar look...you can change the color or background for your specific image needs
	saveAs("jpg", outputDir + "/" + dapi +"_merged");
	close();
  //Colors and creates scale bar on the DAPI image
	open(inputDir+fileList[(i*18)+j]);
		DAPI = getTitle();
	run("RGB Color");
	run("Merge Channels...", "c2=*None* c3=&DAPI c1=*None* create");
		//this merges the channels, setting image 2 to green (c2), image1 to blue (c3), and nothing in the red channel
	run("Set Scale...", "distance=1.55 known=1 pixel=1 unit=um");
		//this sets the scale bar...you can change the values for your specific image needs
	run("Scale Bar...", "width=100 height=8 font=28 color=White background=Black location=[Lower Right] bold");
		//this sets the scale bar look...you can change the color or background for your specific image needs
	saveAs("jpg", outputDir + "/" + DAPI);
	close();
  //Colors and creates scale bar on the GFP image
	open(inputDir+fileList[(18*i+6)+j]);
		GFP = getTitle();
	run("RGB Color");
	run("Merge Channels...", "c2=&GFP c3=*None* c1=*None* create");
		//this merges the channels, setting image 2 to green (c2), image1 to blue (c3), and nothing in the red channel
	run("Set Scale...", "distance=1.55 known=1 pixel=1 unit=um");
		//this sets the scale bar...you can change the values for your specific image needs
```

```
        run("Scale Bar...", "width=100 height=8 font=28 color=White background=Black location=[Lower Right] bold");
                //this sets the scale bar look...you can change the color or background for your specific image needs
        saveAs("jpg", outputDir + "/" + GFP);
        close();
   //Creates scale bar on the BRIGHT FIELD image
        open(inputDir+fileList[(18*i+12)+j]);
                brightfield = getTitle();
        run("Set Scale...", "distance=1.55 known=1 pixel=1 unit=um");
                //this sets the scale bar...you can change the values for your specific image needs
        run("Scale Bar...", "width=100 height=8 font=28 color=White background=Black location=[Lower Right] bold");
                //this sets the scale bar look...you can change the color or background for your specific image needs
        saveAs("jpg", outputDir + "/" + brightfield);
        close();
}
}
print("FINISHED!!")
```

For 20x cytation images: change line that says "distance=1.55" to "distance=3.1"

Appendix E: Protocol to coat wells with PLL

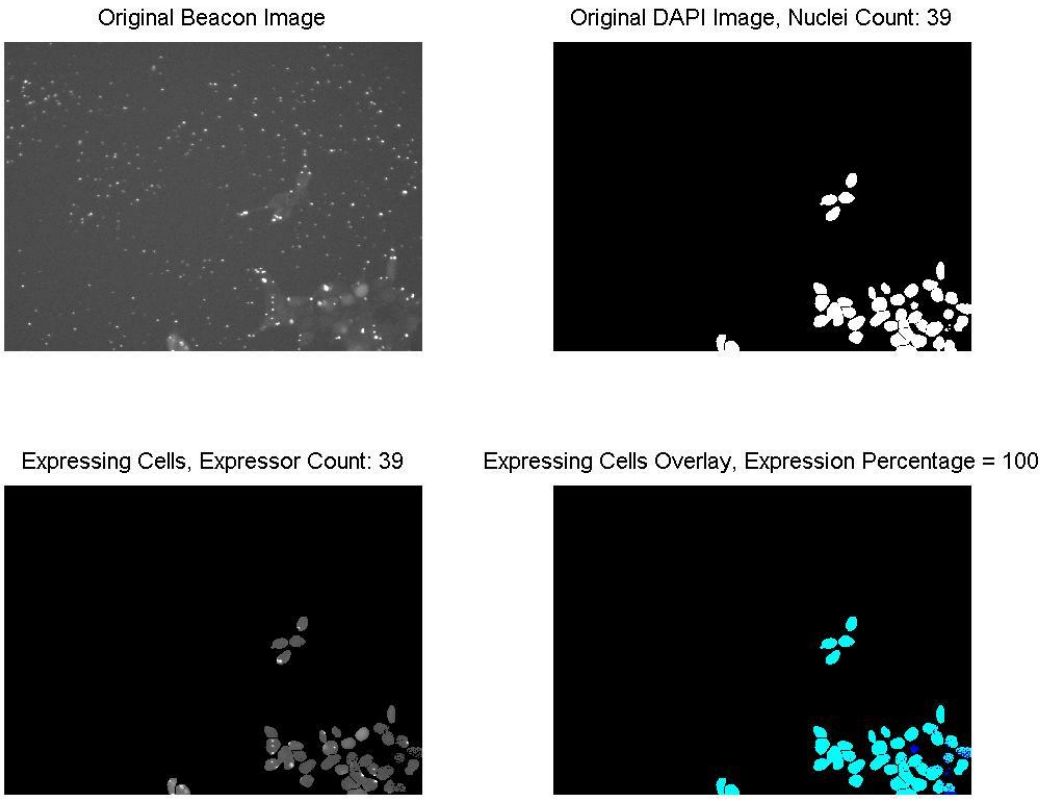
Coating Wells with Poly-L-Lysine

One beacon delivery experiment involved coating wells with Poly-L-Lysine, PLL, (Sigma-Aldrich) to asses PLL's ability to prevent the accumulation of fluorescent debris. The purchased stock PLL was diluted 1:10 with deionized water and filtered. 50 µL was then added to each desired well of a 96 well plate and left at room temperature for 5 minutes. Wells were then drained,

and left uncovered to dry in the hood at room temperature overnight. PLL-coated plates were then used for subsequent beacon delivery experiments.

Appendix F: Representative image of 4 HR, No QR Delivery Quantification

Original beacon image demonstrates that there was both a higher background fluorescence than seen in other conditions and less cells than other conditions. Because of this, 4 hr, No QR exhibited almost 100% fluorescence. But, since the background was unnaturally high for an unknown reason (same cytation image settings were used for all runs), these data are inconsistent with other runs.



Appendix G: Preliminary p(FA:SA) Cytotoxicity Experiment

The first attempt of this experiment used too much polymer, calculated based on the well volume. Going forward, the amount of polymer used was calculated on a well slice volume verses the entire well volume.

Conditions starting from top left in 3 well triplicates: Top row of conditions: 70 nM = 31.80 mg, 35 nM = 15.90 mg, Second row of conditions: 17.5 nM = 7.95 mg, 8.75 nM = 3.90 mg, Bottom row of conditions: 4.35 nM = 1.96 mg, one well of each of the controls: dead control of formalin fixed and stained cells, alive and stained control, just cells autofluorescent control.