Biodegradable Microparticles for Cystic Fibrosis Drug Delivery Applications

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Abstract

Cystic fibrosis (CF) is a genetic disease which affects about 70,000 patients worldwide, and is fatal by age 37 on average, usually due to severe lung infections. Thick mucus builds up in the digestive tracts and lungs of CF patients, which causes these respiratory infections as well as digestive problems and difficulty breathing.[1] Current treatment methods for CF are not optimal, as they do not effectively treat the disease and carry excessive risks and side effects. Therapies can be improved, however, by using biodegradable, drug-loaded, polymer microparticles as drug delivery vehicles.[2] These microparticles can be fabricated using electrohydrodynamic co-jetting.[3]

Electrohydrodynamic co-jetting of polymer solutions (jetting, for short) is a method developed in the Lahann Lab for fabricating biodegradable, drug-encapsulating, spherical microparticles. Jetting allows for control over size, shape, and internal architecture of particles, creating many possibilities for unique particle design. During jetting, polymer solutions are loaded into two syringes tipped with metal needles and "jetted" onto grounded metal plates. The jetting is caused by an electric charge applied to both the needles and the plate. After jetting, particles are using SEM (scanning electron microscope) to characterize size and shape distributions and confocal scanning laser microscopy (CSLM) to characterize internal compartmentalization.[3]

We have found that particles in the 1 micron range can be efficiently, consistently fabricated in high yields. This particle size will be optimal for CF treatment because it will allow for efficient lung deposition and thus targeted infection treatment.[2] These particles can be fabricated to allow for release of multiple antibiotics in a controlled, time-dependent manner, which will reduce dosing frequency as well as systemic and local toxicity with the aim of reducing side effects and improving patient compliance.[4] Further, dual antibiotic release will enable improved treatment of bacteria, which are often drug resistant. Moreover, particles can be surface modified to include targeting and stealth moieties, so as to target the infection while evading the immune system.[4]

It is believed that biodegradable, drug-loaded microparticles optimal for CF treatment will have a specific set of characteristics and will be widely reproducible. Here we have worked to determine this set of characteristics and produce particles accordingly. We have studied these particles' shapes, sizes, inner compartmentalization, degradation and drug release characteristics, which have proved to be appropriate for CF therapy. Our future work will include further experimentation with release studies, *in vitro* and *in vivo* works.

Introduction

1.1 Introduction to cystic fibrosis disease

Cystic fibrosis (CF) is an inherited, recessive and autosomal disease which affects about one in 2,000 newborns in Caucasian populations. The disease does, however, affect all ethnic groups. Approximately 70,000 patients worldwide are affected by CF, and the average lifespan of a CF patient is 37 years. CF primarily effects the respiratory system, and severe respiratory infections are the primary cause of death in CF patients. However, this multi-functional disease also effects the pancreas and liver, gastrointestinal tract and reproductive system, and the sweat glands. Symptoms of CF vary by person, but the main symptoms of the disease include difficulty breathing, continuous coughing (usually expelling mucus), wheezing, decreased ability to exercise, intestinal blockage, severe constipation, poor weight gain and growth, and severe, sometimes fatal, lung infections. [2] Here, we focus on understanding and treating bacterial infections in the respiratory tract, since these are the primary cause of death in CF patients and are seen in nearly all CF patients. [2]

CF is caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7. There are more than 800 known mutations that can cause the disease, the most common mutation being a deletion of phenylalanine in position 508 in the amino acid sequence. [2] Mutations in the CFTR gene lead to malfunction of the CFTR protein, which is responsible for regulating ion and water movement across the epithelium. The mutations result in defective chloride ion channels, leading to decreased chloride secretion into the airways. This then causes increased sodium absorption from the airways due to the disrupted ion concentration gradient. This causes the airway mucus to become dehydrated, and the volume of the airway surface liquid (ASL) decreases, leading to the accumulation of purulent secretions and impaired mucociliary clearance (MCC). Mucociliary clearance is the complex process by which the cilia lining the airways works to clear airway secretions so that they can be expectorated or swallowed. It is an important defense mechanism against infection. Without strong mucociliary clearance, CF patients are prone to frequent, severe lung infections, which can be fatal. Their inefficient MCC leads to buildup of thick mucus builds lungs which causes these respiratory infections in addition to difficulty breathing.[2]

We seek to fabricate drug delivery vehicles which can navigate this complex environment created by the reduction of ASL and of MCC function, in order to deliver antibiotics efficiently to the sites of infection in the lungs, thus reducing side effects and dosing frequency, and improving efficacy. We hypothesize that this can be done by electrohydrodynamic co-jetting of polyanhydride microparticles containing the commonly used antibiotic Azithromycin in one compartment, and the experimental antibiotic carboxy-TEMPO in the other compartment. The use of two antibiotics will improve the chances of killing drug resistant bacteria, which occur

frequently in respiratory infections and are the implicated severe, long-lasting, difficult-to-treat disease.

Both Azithromycin and carboxy-TEMPO target *Pseudomonas aeruginosa,* in addition to other bacteria. This makes them powerful for CF treatment, since *Pseudomonas* is the primary bacteria that infects the lungs of adult CF patients. It is found in 54.4% of CF patients overall, and it is found in 80% of the patients by age 18. While other pathogens contribute to morbidity and mortality as well, such as such as *Burkholderia spp., Achromobacter spp.* and *Stenotrophomonas maltophilia*, the high incidence of *P.aeruginosa* in adults makes it an important pathogen to note. [5, 6, 7]

We have chosen the drug Azithromycin because it is currently, commonly used to treat CF. It is a particularly useful drug for this disease due to its anti-inflammatory properties in addition to its antibiotic activity. Azithromycin is a 15-membered ring which is stable at acidic pH, important for treating infection in CF biofilms which usually have a pH of approximately 5.6. Azithromycin is a macrolide antibiotic in the azalide class. It inhibits protein synthesis in bacteria by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. This deactivates the ribosome by inhibiting peptidyl transferase activity and interfering with amino acid translocation during translation. Thus, the bacteria can no longer effectively make protein, and so it dies. Another benefit of using Azithromycin is that it has a long half life, which helps to reduce dosing frequency and administration lengths.[5]

We have chosen to complement with carboxy-TEMPO due to the promise shown by this drug and similar members of the nitroxide family as a drugs for CF. Nitroxide antibiotics have been shown to disperse biofilms, but have yet to gain complete FDA approval. The nitroxide drug was originally used, but later we decided to complement with Azithromycin due to the greater certainty in its effects in CF patients.[6]

1.2 Current treatment methods for Cystic Fibrosis

Many current treatment methods lack effectiveness and are accompanied by harmful side effects.[1] This is due in part to intravenous drug administration, which does not target infected areas and thus provides increased systemic exposure.[2] For example, antibiotics kill harmful bacteria in the lungs, but may also kill important bacteria in other areas of the body. Drugs administered via inhalation are likely to have fewer side effects and to be more effective because they are administered directly to the site of mucus buildup and infection in the lungs.[2]

Modern molecular drug delivery vehicles can also administer drugs more efficiently to infection sites with fewer side effects. Using these vehicles, and the inhalation route, better treatments can be achieved.[2] There are many molecular drug delivery vehicles currently being researched which could be taken via inhalation, including liposomes, polymeric micelles, and drug polymer conjugates.[2] These vehicles are also not optimal, however, because they do not have the multiple functionalities necessary to optimally treat CF, such as the ability to deliver multiple drugs simultaneously, or to chemically target the sites of infection. Additionally, they

are not optimized to evade the immune system, or to provide sustained and controlled drug release.[2] While the current treatment options for CF are not optimal, therapies can be improved using polymer microparticles as drug delivery vehicles.[2]

1.3 Drug Loaded Polymer Microparticles as Drug Delivery Vehicles

Biodegradable, drug-loaded, polymer microparticles are more advanced drug delivery vehicles, which can be optimized to chemically target infection sites, deliver multiple drugs simultaneously with different controlled release profiles, and evade the immune system.[3] Currently, the optimization of an efficient method for fabricating particles with these capabilities is an important goal in the field.[4] Electrohydrodynamic co-jetting of poly(lactide-co-glycolide) polymer solutions (jetting) is a method developed in the Lahann Lab for fabricating such microparticles.[8] Jetting allows for control over size, shape, and internal architecture of particles, creating many possibilities for unique particle design.[8] Particles with multiple compartments can be produced, allowing a single particle to deliver multiple drugs, with multiple, distinct release kinetics.[8] For CF treatment, this will allow antibiotics, mucus dispersal agents, and anti-inflammatory drugs to be delivered simultaneously over controlled periods of time, which will reduce dosing frequency as well as systemic and local toxicity with the aim of reducing side effects and improving patient compliance.[4] Further, particles can be surface modified to include targeting and stealth moieties, so as to target the infection while evading the immune system.[4]

Further, all of these goals can be better achieved by using the inhalation delivery route. Particles will be inhaled by patients as an aerosol. This method has been used before for pulmonary drug delivery, and has shown promise for many reasons. This is a simple, non-invasive delivery route, which improves patient comfort as well as compliance. This method also caters to the properties of the lung: the large epithelial surface area, the high vascularization, the thin alveolar epithelium, a relatively low enzymatic activity compared to the gastrointestinal (GI) tract and no first pass effect all make drug delivery via inhalation more effective. The first three characteristics provide improved conditions for drug absorption and thus efficient action. The lower enzymatic concentration prevents drugs from being degraded quickly, as they would be in the GI tract. And lastly, without the first pass effect, i.e. without having to circulate the body before finding its target, and thus risking degradation, toxicity, and/or removal from the system, the drug can be far more effective and less toxic to the body. There are, of course, caveats to pulmonary delivery as well, such as difficulty in determining an effective way to administer the drug as an aerosol, and concern of hindrance of drug transport into the lungs by metabolic enzymes or efflux pumps. However, these concerns are mitigated in part by delivering the drug in surface-modified, biodegradable polymeric microparticles, which will be more simply aerosolized, and can be optimized to evade some of the biochemical hindrances to lung entrance.

It is difficult to jet multi-compartmental particles with the correct size, shape, drug compartmentalization, and release profiles. For example, drug/polymer solubility in jetting solutions is an obstacle, as there are limited solvent and polymer options that meet

biocompatibility constraints while maintaining dielectric properties optimal for jetting. Addition of drug to jetting solutions can have severe effects on jetting, due to changes in dielectric constant and potential salt formation. Variable humidity alters jetting results by changing the dielectric constant of the air and thus the electric field between the plates and needles, making it difficult to produce optimal particles consistently. Importantly, production of CF drug delivery particles via jetting has not been studied before, so there is little base knowledge on expected outcomes. However, these challenges can be overcome with extensive optimization of the jetting process.

The polymer I am using is Polysebacic Acid, a biodegradable, FDA-approved polyanhydride. This polymer was chosen due to its biocompatibility, government approval, degradation characteristics, and ability to be jetted with. The solvent used includes both chloroform and dimethyl formamide (DMF), in concentration ratios ranging between 95:5 and 50:50 chloroform: DMF. These are common jetting solvents and ratios, which are optimized for each case to meet necessary solubility constraints while yielding particles of ideal size and shape.

Particle size and shape can be controlled by varying several jetting parameters, including flow rate (how fast solutions are pumped out of solutions; usually ranges from 0.1-0.4 mL for particle jetting), distance from needles to plate, polymer concentration, solvent ratio, drug concentration, additive concentration (liquid or solid chemical additives may be used), needle gage, and humidity. These parameters must thus be optimized in order to fabricate ideally size and shaped particles in high yields.

1.4 Overview: Hypothesis and Plan of Action

In order to develop a consistent protocol for fabricating particles optimal for CF drug delivery, I jetted particles with different drug and polymer concentrations, solvent ratios, and flow rates. Ideal particles will be 1-5 micron diameter spheres, as this will allow for efficient lung deposition and thus targeted infection treatment.[2] In the past, larger particles have been jetted using dimethylformamide (DMF) as the primary solvent, while for smaller particles, chloroform has been used. This is the case since DMF has a higher dielectric constant than chloroform. Therefore I hypothesized that by varying the ratio of chloroform: DMF as jetting solvent, I would be able to control particle size. I also hypothesized that by varying polymer concentration and solution flow rate I could control particle size and shape distributions, since it has been seen that higher polymer concentrations and slower flow rates yield larger particles, up to a limit at which fibers are produced.[4]

I characterized the particles I jetted in order to determine which formulation was optimal, using methods such as Scanning Electron Microscopy (SEM) to determine shape and size distribution and Confocal Laser Scanning Microscopy (CLSM) to determine internal compartmentalization.[3] Once an optimal formulation was determined, I performed degradation and release studies to quantify particle degradation characteristics and drug release profiles. Degradation can be qualitatively observed using SEM and quantified using ImageJ software (to

measure particle diameter change over time). Drug release (mg drug released per time) can be quantitatively monitored using UV-visible spectrophotometry and a calibration curve for strongly absorbent drugs, and High Performance Liquid Chromatography (HPLC) otherwise. The Lahann Lab, in Germany and in Ann Arbor, provided me with all of the resources necessary to complete these studies.

Materials and Methods

2.1 Materials

Chloroform, dimethylformamide (DMF), phosphate buffered saline (PBS), Tween 20, the polyanhydride Polysebacic Acid (PSA), carboxy-TEMPO, Azithromycin, the fluorescence dye poly[(m-phenyl-enevinylene)-*alt*-(2,5-dibutoxy-*p*-phenylenevinylene)] (MEHPPV) and the fluorescence dye poly[tris(2,5-bis(hexyloxy)-1,4-henylenevinylene)-alt-(1,3-phenylenevinylene)] (PTDPV) as the blue and green markers, respectively, for confocal imaging were used as purchased from Sigma Aldrich, USA. Polylactide-co-glycolide (PLGA) 5002A was purchased from Lakeshore Biomaterials.

2.2 Particle Fabrication

Microparticles were fabricated using the electrohydrodynamic cojetting procedure, a schematic of which is shown in Fig. 1. Briefly, polymer solutions were dissolved separately and loaded into separate 1 mL syringes which were held together by a syringe holder. The syringes were tipped with metal needles whose tips were held together in a thin plastic sleeve, forming a parallel dual capillary system. Using a syringe pump, the solutions were expelled at flow rates of 0.4 mL/hr. The cathode of a high voltage source was attached to the needles, and the anode was attached to the collecting substrate, a grounded metal plate. A large electric field was applied to the needles, causing formation of a Taylor cone and thus an electrified polymer jet. All jetting experiments were performed under ambient conditions.[8]
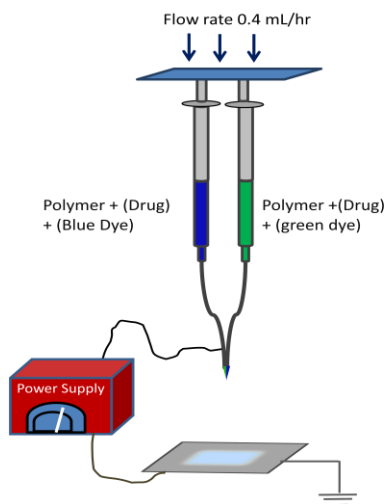
Fig. 1. Jetting Schematic. Solutions composed of polymer and (drug) are slowly pumped out of metal needles onto a grounded metal plate, forming a Taylor cone and yielding a thin layer of particles deposited on the plate.

A needle to-substrate distance of 30 cm was maintained throughout all experiments. A metal sheet acted as the counter electrode, onto which the microparticles were deposited and then collected for further processing. The jetted microparticles

were imaged via CLSM (Confocal Laser Scanning Microscopy) using an Olympus Confocal Microscope, and an Amray SEM (Scanning Electron Microscopy), at the Microscopy & Imaging Laboratory facilities at the University of Michigan in Ann Arbor, MI, USA and the imaging cores at the Karlsruhe Institute for Technology (KIT) in Karlsruhe, Germany. Microparticle and nanoparticle diameter measurements were done using Image-J software. 200 particles were counted per image.

2.2.1 Fabrication of non-drug-loaded PSA microparticles (control)

Particles were fabricated with both compartments containing Poly Sebacic Acid (PSA) 20% w/v, and one compartment containing green dye; the other, blue dye. Two solvent ratios were used: the first was 95:5 Chloroform: Dimethyl Formamide ($CHCl_3$: DMF) and the flow rate was 0.4 mL/hr. After analysis with ImageJ, the average diameter was 4.84 μm (100 particles measured), with a range of 2.8-7.1 μm. Images are included in Fig. 2, A. The second solvent ratio was 50:50 chloroform: DMF, seen in figure 15. ImageJ analysis will be performed as part of our future work. In order to dissolve jetting solutions with PSA, the polymer must be dissolved in DMF before adding chloroform, due to low PSA solubility in chloroform. First, solutions containing PSA and DMF must be bath sonicated for approximately 1 hour, then once chloroform is added, solutions must be bath sonicated for 1-3 hours, until solutions become clear.

2.2.2 Fabrication of carboxy-TEMPO encapsulating PSA microparticles

For particles containing solely carboxy-TEMPO in both compartments, all jetting solvents for the particles discussed in this section contain 2% v/v Triethyl amine (TEA) as additive in a 95:5 $CHCl_3$: DMF solvent ratio. All particles discussed in this section were jetted at a flow rate of 0.4 mL/hr, unless otherwise noted.

It is notable that jetting solutions containing both Carboxy-TEMPO and PSA are difficult to dissolve, and the following steps must be taken in order to successfully dissolve such solutions:

1. A stock solution of Carboxy-TEMPO must be dissolved in DMF+TEA, so that for each solution made, (half the amount DMF needed) + (20 uL TEA per 1 mL jetting solution) + (the full amount of Carboxy-TEMPO needed) can be pipetted into the complete jetting solution. For example, for a 1 mL jetting solution with 5% w/w loading of the drug, 25 uL DMF+20 uL TEA+0.01g Carboxy-TEMPO would be added to the complete jetting solution in order to achieve 50 uL of DMF total (5%).

This would use the drug loading calculation ([Drug] / [Polymer]) x 100=X% loading. Alternatively, the calculation (([D]/ ([D] + [P]))*100 = X% loading, could be used. P is the polymer, which is used at 20% w/v (or for a 1 ml solution, 0.2 g), and D is the drug.

This solution is then vortexed and then bath sonicated for 1 hour.

2. All of the PSA needed for the jetting solution (20% w/v) is then dissolved in half the amount of DMF needed for the solution (i.e. for 1 mL, 25 uL DMF is used). This solution is then vortexed and then bath sonicated for 30 min.

3. The Carboxy-TEMPO stock is at this point dissolved, and is mixed with the PSA solution, resulting in the full amount of DMF + TEA + Carboxy-TEMPO + PSA needed for the jetting solution. This solution is then vortexed and then bath sonicated for 25 min.

4. The full amount of $CHCl_3$, i.e. 950 uL per 1 mL solution (95% v/v), is then added to the solution, vortexed, then bath sonicated until clear, for approximately 2-3 hours.

5. Solutions are then ready to jet. Jetting is done at 0.4 mL/hr, and plates are changed every 20 min, thus depositing 0.133 mL of solution onto each plate.

Particles were fabricated with both sides containing Poly Sebacic Acid (PSA) 20% w/v and 5% carboxy-TEMPO w/w, and one side containing green dye, while the other side contained no dyes. The solvent was 95:5 $CHCl_3$: DMF and the flow rate was 0.4 mL/hr. After analysis with ImageJ, the average diameter was 1.96 μm, with a standard deviation of 0.642 and a range of 0.9-3.7 μm. Images are seen in Fig. 2, B.

Two additional sets of particles were jetted using the same parameters, with the exception that one set contained 2.5% carboxy-TEMPO w/w, and the other contained 10% carboxy-TEMPO w/w. The jetting for the 10% drug-loaded particles was ineffective, most likely due to the strong change in dielectric constant of the solution due to high drug concentration. The jetting of particles with 2.5% drug loading was effective, and particles yielded had an average diameter of 2.19 μm, and a standard deviation of 0.637, with a range of 1-3.5 μm. 2.5% drug-loaded particles are seen in Fig. 2, C.

Two additional sets of biphasic particles were jetted, with different compositions of each compartment. First, particles were jetted with one side containing only PSA 20% w/v, and the other side containing PSA 20% w/v and carboxy-TEMPO 5% w/w. After analysis with ImageJ, the average diameter was 2.06, with a standard deviation of 0.693 and a range of 0.9-3.5 μm. Images are included in Fig. 2, D. Second, particles were jetted with one side containing PLGA 5004A 10% w/v, and the other side containing PSA 9% w/v and carboxy-TEMPO 5% w/w. ImageJ analysis showed an average diameter of 3.37 μm, with a standard deviation of 1.19, and a range of 0.7-5.2 μm. Images are shown in Fig 2, E.

For particles containing carboxy-TEMPO in only one compartment, carboxy-TEMPO was simply dissolved in the green-dyed chloroform solution before adding to the PSA+DMF solution.

2.2.3. Fabrication of Azithromycin encapsulating PSA microparticles

Particles were fabricated with both compartments containing Poly Sebacic Acid (PSA) 20% w/v, and one compartment containing green dye; the other, blue dye. The solvent ratio used was 95:5 Chloroform: Dimethyl Formamide ($CHCl_3$: DMF) and the flow rate was 0.4 mL/hr. One compartment was loaded with 5% Azithromycin, which was simply dissolved in the chloroform solution, which also contained blue dye, before jetting. Images of Azithromycin-encapsulating particles are seen in figures 13, 14 and 16.

2.2.4 Fabrication of Azithromycin and carboxy-TEMPO encapsulating PSA microparticles

Particles were fabricated with both compartments containing Poly Sebacic Acid (PSA) 20% w/v, and one compartment containing green dye; the other, blue dye. The solvent ratio used was 50:50 Chloroform: Dimethyl Formamide ($CHCl_3$: DMF) and the flow rate was 0.4 mL/hr. One compartment was loaded with 5% Azithromycin, which was dissolved in the chloroform solution, which also contained blue dye, before jetting. The other side was loaded with 5% carboxy-TEMPO, which was dissolved in the chloroform solution containing green dye, before jetting. Images are seen in Figure 17.

2.3 Degradation Studies

In order to determine degradation characteristics of the microparticles containing 20% PSA w/v and 5% w/w carboxy-TEMPO, as well as microparticles containing only 20% PSA w/v, I used the following protocol.

Degradation study protocol

1. Collect 1 mg particles dry, using a plastic razor blade to scrape particles off of collecting substrates.
2. Place particles in a 2 mL centrifuge tube and add 1 mL Phosphate Buffered Saline solution (PBS) or pH 5 buffer +1% tween.
3. Leave this particle suspension on a shaker for various time periods of interest both at room temp and at 37C.
The time periods I chose were 15 min, 30 min, 60 min, 5 hr, 24 hr, 48 hr, and 5 days.
4. After the desired time period has elapsed, centrifuge the sample at 15000 rpm for 2 min.
5. Wash with deionized water 5 times.
6. Tip sonicate under the following parameters: 30 sec total, 1 sec on, 5 sec off, 20 amp. Sonicate with the solution on ice to avoid melting the particles.
7. Pipette 10 μL of the solution onto a silica wafer and image using SEM.

2.4 Carboxy-TEMPO Release Study Protocol

It is worth noting that the absorbance of carboxy-TEMPO is not very high (my calibration curve linear equation is 4.7x, while the Ann Arbor lab normally works with dyes they are about either 44 or 200x). This must be taken into account when determining the necessary concentration of drug loaded in my particles, and the amount of particles needed to do the release study.

The following release study protocol was developed:

(I) Calculating concentration of particles needed:

1. Set 0.001 as the lowest bar for 5%. This means at a minimum, it will be possible to get a reading for a 5% release of material. 0.001 is chosen because this is the lowest value at which the UV visible absorbance measurement can be consistently, accurately taken by a typical plate reader. Based on the specific plate reader in use, this amount may need to be varied.

Note: In this particular study, 0.001 absorbance is 0.00021 mg/ml concentration.

2. Based on this amount, calculate the 100% amount of the drug needed. In this case, that is 0.004197 mg/ml.

3. Use a total volume of 40 mL, and calculate the total amount of drug needed. In this case, that is 0.1678 mg.

4. For an X% loading of drug in the particles, calculate the mass of particles required. In this case, for 5% loading, 3.36 mg of particles are needed. It is possible to use a higher mass, but using a lower mass will not allow a detectable signal for 5% drug release, and this is suboptimal. Take release measurements in triplicate, but prepare four samples, in case one sample becomes damaged. In this release study, 5 samples were used, each with 7 mg of particles, as this was the maximum amount of particles available.

Release study:

1. Measure out the particles in small centrifuge tube and add a 2 mL solution of PBS and 1% tween. Disperse the particles gently. Do not use sonication or other harsh methods of dispersion, as this can cause the drug to release.

2. Rinse dialysis tubes with DI H_2O. Prepare one tube for each release sample. Fill the tubes with DI H_2O (inside and outside the tubing) and let stand for about 15-30 min before use.

3. Pour each 2 mL suspension of particles in PBS+1% tween and into a dialysis tube.

4. Rinse the small centrifuge tube again with 2 ml of PBS+1% tween and pour that into the dialysis tube again. Rinse again with 1 mL, in order to make sure that all of the particles are out of the small centrifuge tube and into the dialysis tube. Total volume will now be 5 mL.

It is important not to add the particles dry to the dialysis tube, because then it is possible for them to start off in aggregates, which will alter their release properties due to the blocking of the solvent from reaching all sides of each particle.

5. Fill 50 mL tubes with 35 mL of PBS + 1% tween and put the dialysis membranes in them. Put them on a shaker and incubate at 37 degrees Celsius. Take absorbance readings at 3 hr, a 5 hr, a 7 hr, and 24 hrs time periods, and if the release is still very rapid at 24 hrs, take a

reading at 30 hr as well. Then take readings every 24 hrs until a plateau is reached. It is also possible to take time points in between so long as they are consistent.

        6. Take absorbance readings for the fluid outside the membrane using a 96 well plate with 200 uL per well. If values are close to 0.005, take readings at longer time intervals (e.g. 24 hours vs. 12 hours).

For drug loading calculations:

$((D/(D+P))*100 = X\%$, where D is the Drug concentration and P is the Polymer concentration, which is 20% in this case.

Results and Discussion

3.1 Particle Fabrication

        Using the methodology described in prior sections, several sets of particles were jetted in order to test the effects of drug concentration on particle size and shape, and to determine the viability of jetting particles with various drug concentrations, in addition to the viability of jetting particles with different compartmentalization schemes and different polymers. Here I confirm the versatility of particles that the jetting method can produce, and I show that this versatility indeed applies to this specific project. The ability to fabricate particles with different properties opens up the utility of the technique developed here to apply to different drug delivery strategies for CF, and shows that other unique formulations needed for future CF and other antibiotic delivery applications are likely to be possible once optimized. Scanning Electron images of five different particle formulations are shown below.
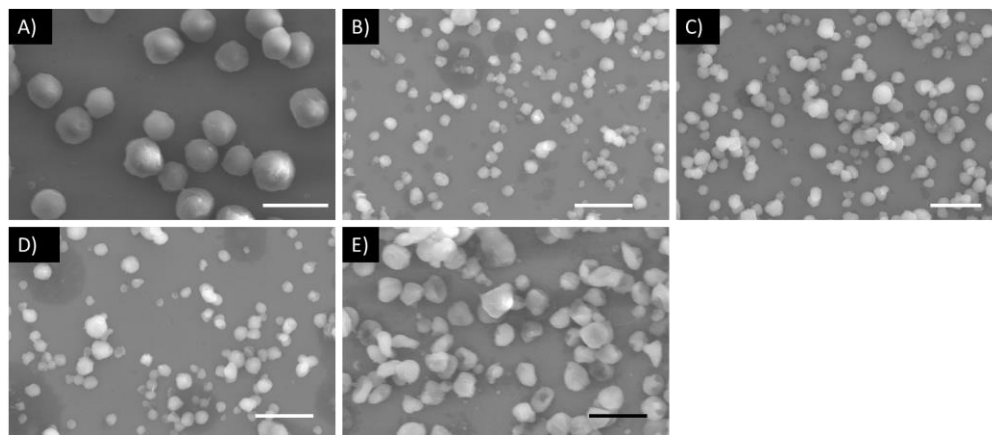
Fig. 2. Scanning Electron Microscope images showing particle size, shape, and surface morphology. A) Control- Poly Sebacic Acid 20% w/v. B) Poly Sebacic Acid 20% w/v, 2.5% drug loading, C) Poly Sebacic Acid 20% w/v, 5% drug loading, D) Poly Sebacic Acid 20% w/v, 5% drug loading in one compartment, no drug in the other, E) Poly Sebacic Acid 9% w/v in one side, PLGA 5004A 10% w/v in the other, 5% drug loading in both sides. All scale bars are 10 µm.

There is a significant change in particle size when jetting empty particles as compared to drug-loaded particles. In order to determine if this size change is due to the addition of the drug, or the addition of the TEA as additive to help dissolve the drug, I jetted empty PSA particles with TEA as additive for comparison: the particles contained PSA 20% w/v, 95:5 $CHCl_3$, and 2% v/v TEA as additive. Jetting did not yield particles, so a comparison could not be drawn. Future work could be done to determine effects on particle size with variable TEA concentration. However, to maintain jetting which yields particles (as opposed to amorphous, thin shapes), TEA concentration cannot be raised past about 2% of the jetting solution, so these experiments would be difficult to conduct, since varying the TEA concentration within the window of 0-2% would likely not yield a significant size difference.

3.2 Degradation Study Results for carboxy-TEMPO particles

Images of degradation studies are included in Figs. 3-7. Several different setups are shown to allow for different comparisons to be drawn.
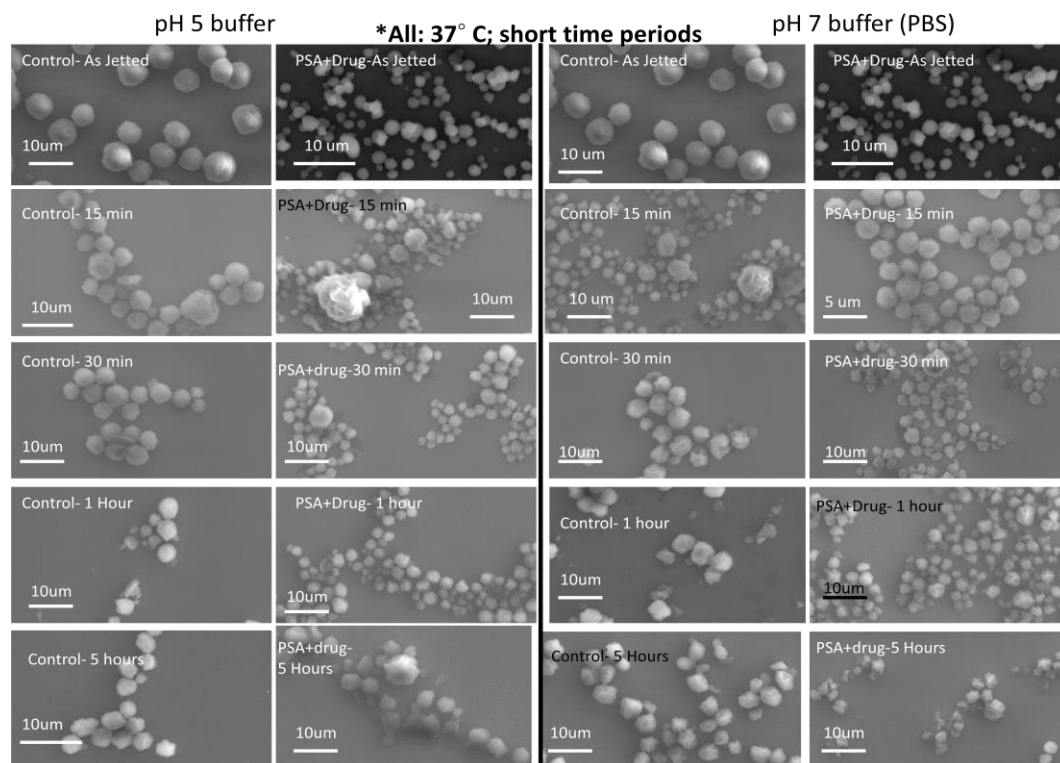
Fig. 3. Degradation studies at 37C for short time periods. Images taken using Scanning Electron Microscopy. Comparisons are shown between pH 5 and pH 7 degradation, for particles with and without drug loading.
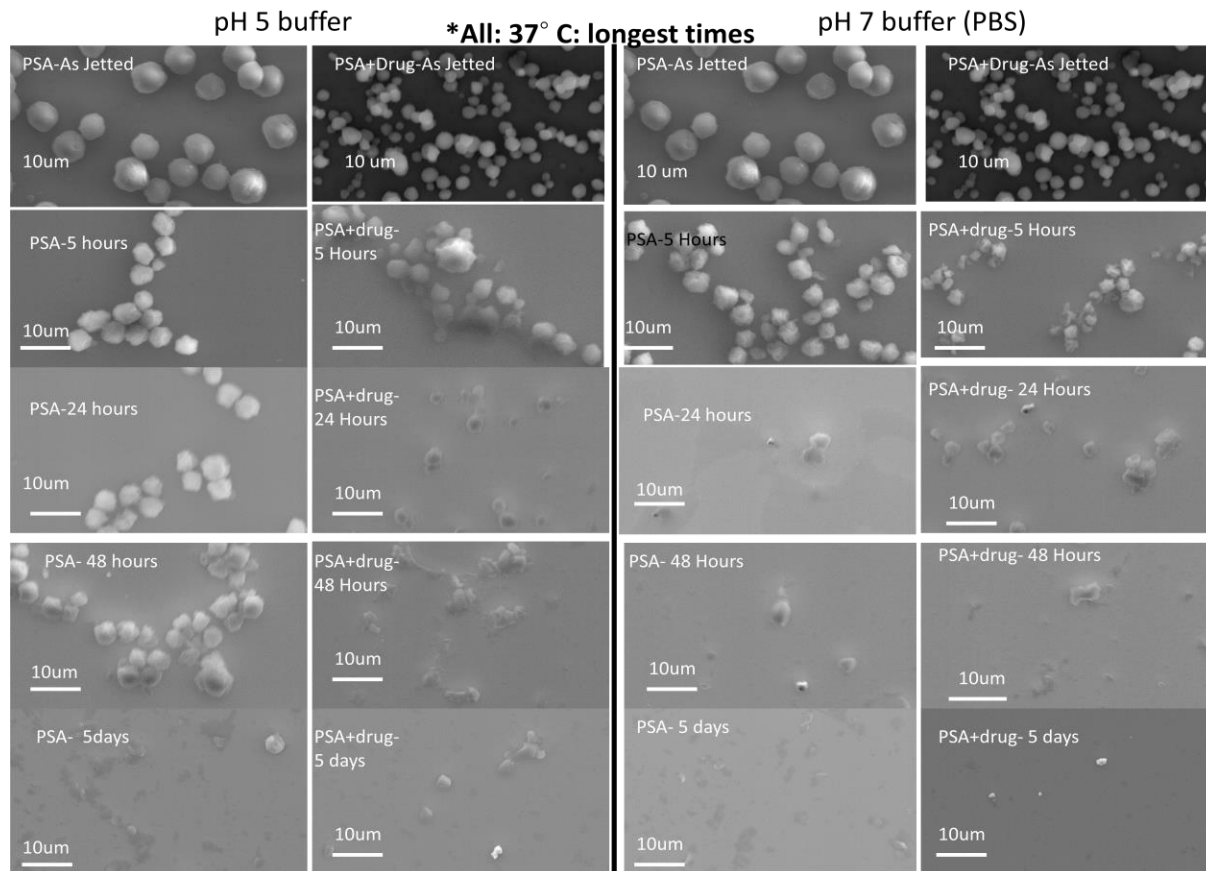
Fig. 4. Degradation studies at 37C for long time periods. Images taken using Scanning Electron Microscopy. Comparisons are shown between pH 5 and pH 7 degradation, for particles with and without drug loading.
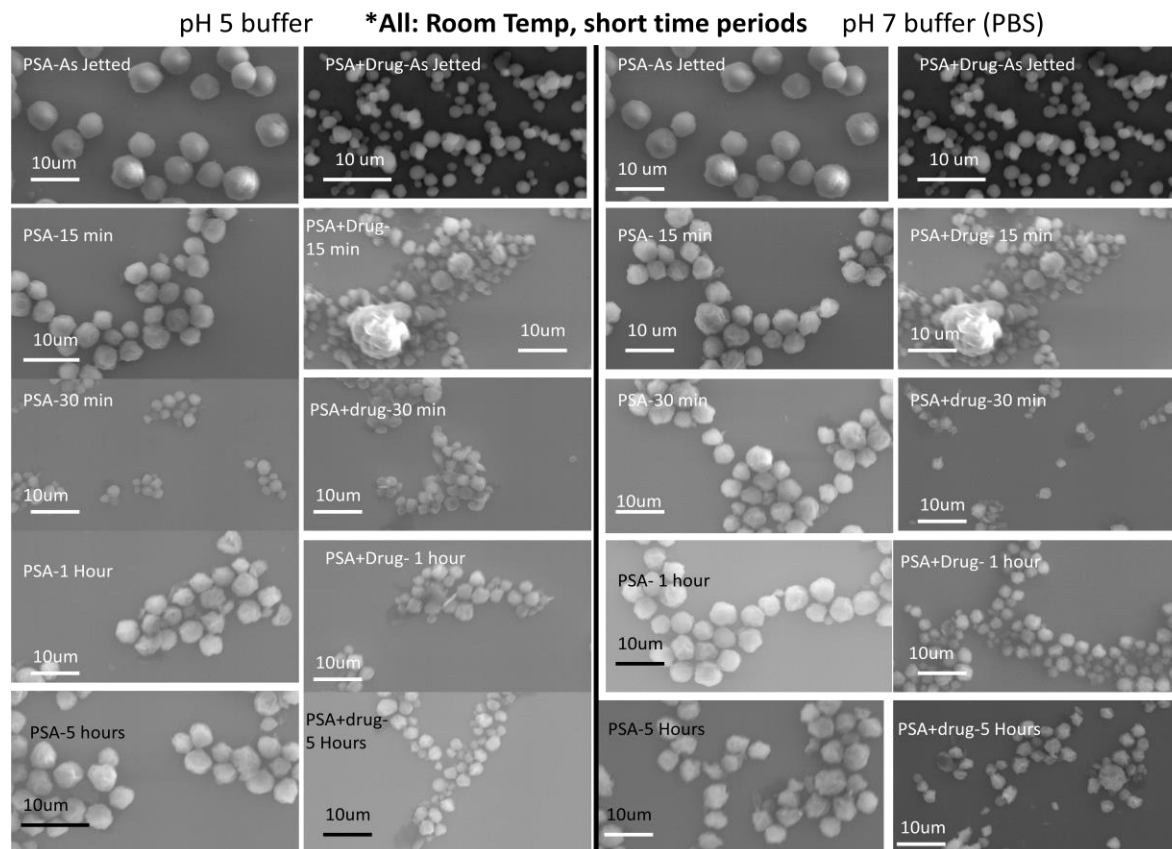
Fig. 5. Degradation studies at room temperature for short time periods. Images taken using Scanning Electron Microscopy. Comparisons are shown between pH 5 and pH 7 degradation, for particles with and without drug loading.
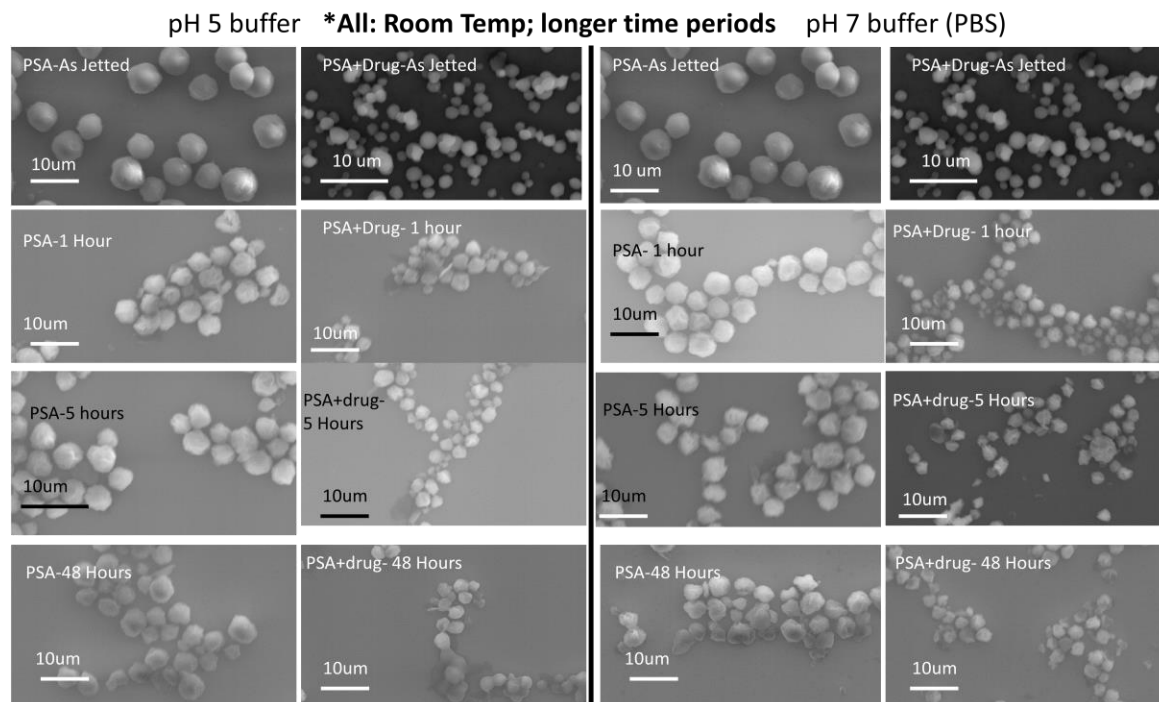
Fig. 6. Degradation studies at room temperature for long time periods. Images taken using Scanning Electron Microscopy. Comparisons are shown between pH 5 and pH 7 degradation, for particles with and without drug loading

The degradation of particles containing PLGA in one compartment was studied as well, using the same protocol with the exception of the use of different time periods. Results are shown in Fig. 7, as compared to empty PSA particles and drug-loaded PSA particles.
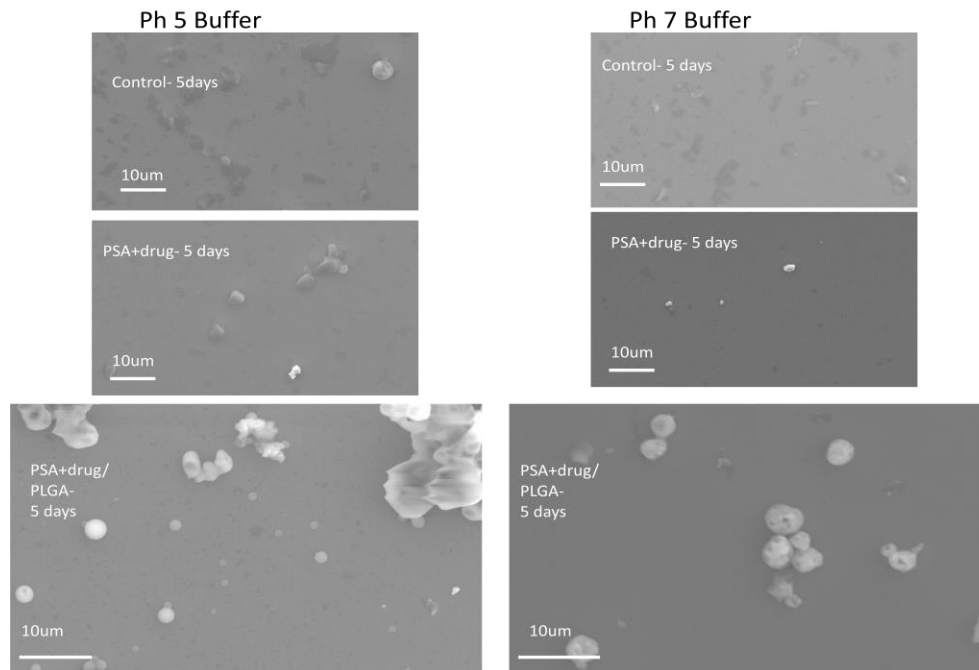
Fig. 7. Degradation of PSA particles with and without drug loading as compared to particles with different drug loading and different polymers in each compartment. All images taken after 5 days of incubation at 37C.

Correlation between degradation, pH, and temperature is somewhat unclear. Future experimentation studying drug release with additional monitoring experiments, such as High Performance Liquid Chromatography (HPLC), will be conducted in order to better elucidate if the particles do indeed degrade faster and release more drug sooner at higher pH, which would be optimal for CF drug delivery due to the lower pH biofilm microenvironment. This would allow for drug release at primarily the site of infection, reducing toxicity and improving efficacy.

3.3 Polymer degradation prior to jetting

In order to determine if the polymer was degraded prior to jetting, an IR spectrum was taken for a container of PSA which had been opened two days beforehand and stored under vacuum. The following spectrum in Fig. 8 was generated.
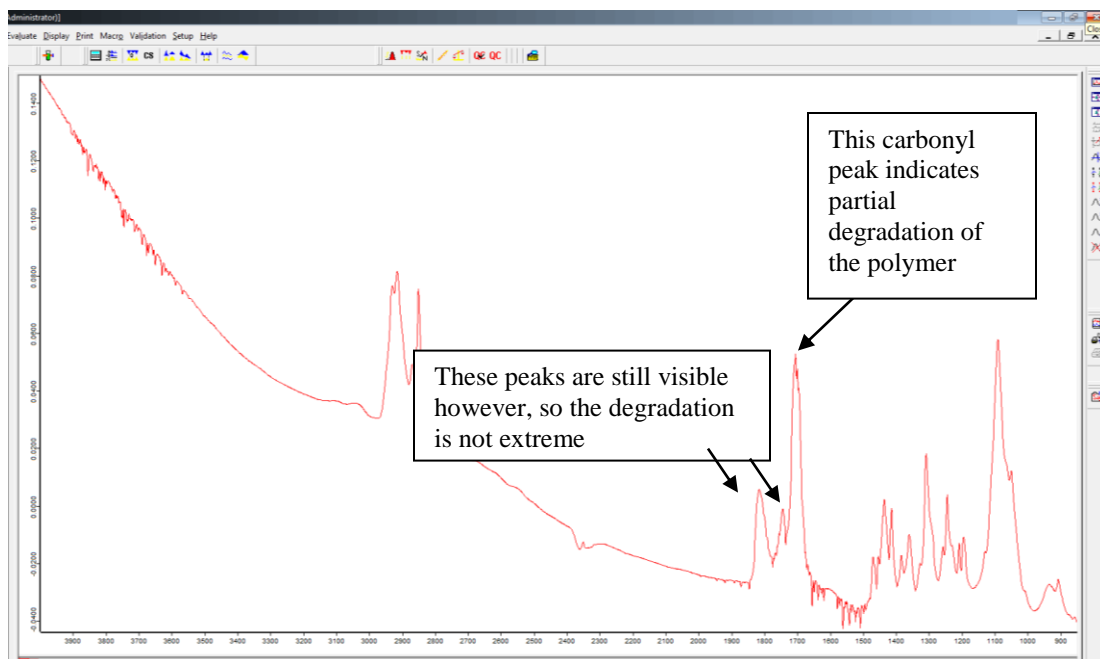
Fig. 8. IR spectrum of newly opened PSA, with annotated peaks. Degradation is visible due to a notable carbonyl peak near 1700 cm^{-1}. However, degradation is not extreme, since there are still peaks characteristic of non-degraded PSA visible. In completely degraded PSA, these peaks are overpowered by the carbonyl peak.

The Lahann Lab in Ann Arbor lab also took an IR spectrum with PSA used there. IR spectra of PSA stored in room conditions for greater than one month showed extreme degradation (carbonyl peak at 1700 entirely overpowered peaks near 1000 and 1600). IR spectra of newly opened PSA showed partial degradation. Sigma Aldrich was contacted regarding the issue, and they confirmed that the polymer is partially degraded upon packaging and sale. Resolution and receipt of non-degraded polymer has yet to occur.

3.4 Release studies

Despite partial polymer degradation prior to jetting, a preliminary release study was run in order to determine if the protocol methodology is effective.

The carboxy-TEMPO has a UV absorbance peak 245 nm, so a calibration curve was made in order to allow for UV absorbance to quantify drug release. The absorbance curve of the carboxy-TEMPO is shown in Fig. 9, and the calibration curve in Fig. 10.
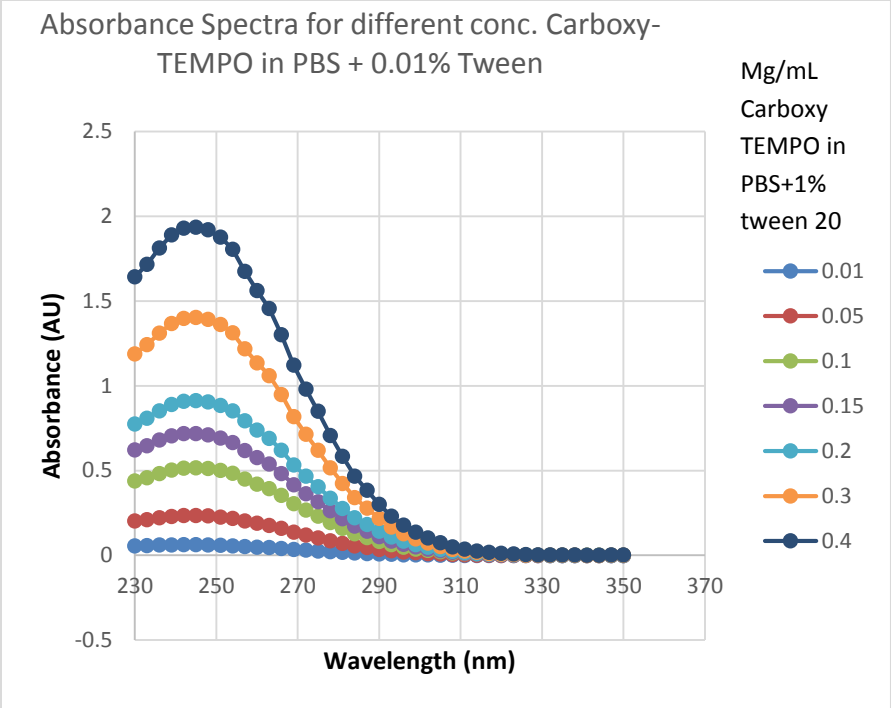
Fig. 9. Absorbance spectra for carboxy-TEMPO at different concentrations.
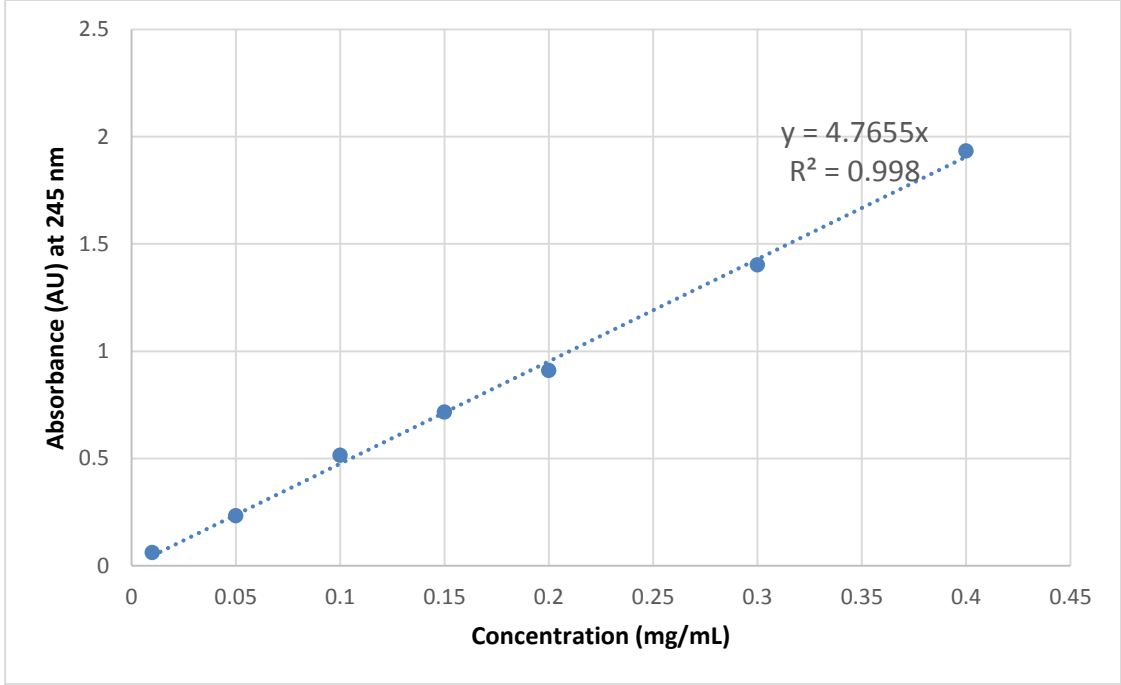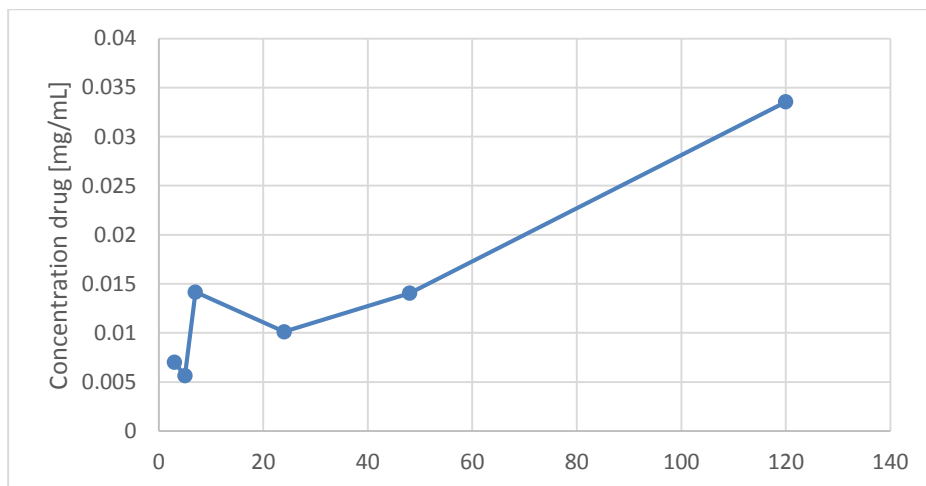

Fig. 10. Calibration curve for carboxy-TEMPO in PBS+0.01% Tween20.

In Fig. 11, a preliminary release curve is shown, which was generated using the protocol described previously. Concentrations of carboxy-TEMPO were calculated based on the calibration curve in Fig. 10 above.


Fig. 11. Carboxy-TEMPO Release from PSA Microparticles over 120 hours.

This release curve takes on an unexpected shape. Typically, with PSA microparticles fabricated using the jetting method, there is an initial burst release followed by a levelling off of the curve within 48 hours. It is important to note: between 0 and 3 hours, for part of this time, the incubation temperature was 60 degrees C, because someone changed the temperature setting of the machine where I had left my samples (even though I left a note saying not to do that). So this has probably effected the results.

Another important concern is that the absorbance values are much higher than expected: for example, the absorbance at 245 nm is 0.091 for the 48 hour sample, indicating a theoretical carboxy-TEMPO concentration of 0.19 mg/mL. The total amount of drug in the particles (7 mg particles with 4.76% loading) is 0.33 mg, in 40 mL. This means that for 100% drug release, there would be a concentration of 0.0083 mg/mL. Therefore, the data show absorbance values that indicate greater than 100% release. This may indicate the presence a contaminating substance with an absorbance in solution, a malfunction of the machine, or a problem of a different nature.

In order to resolve this issue, the shape of the spectra for the 48 hour sample was more closely analyzed. As seen in Fig. 12, the absorbance spectrum for the 48 hour release sample has a noticeable shoulder, which most likely indicates the presence of a contaminating substance with an absorbance in solution.
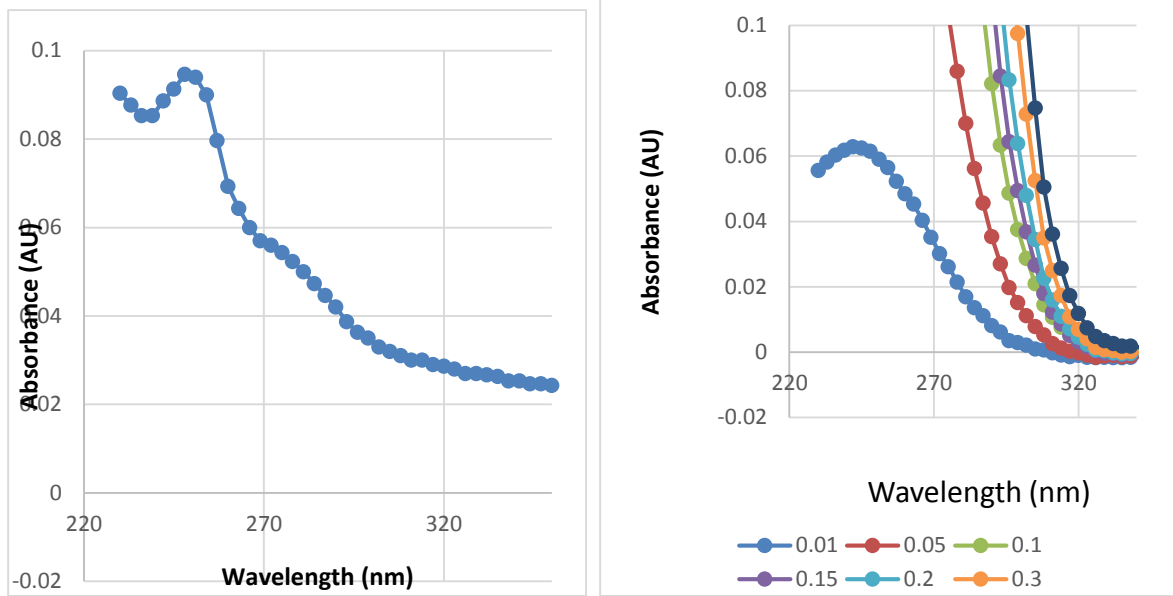
Fig. 12. Left: Spectra for the 48 hour release sample shows a significant shoulder as compared to Right: the pure carboxy-TEMPO calibration spectra, which show a smooth curves. In B, the colors of the lines correspond to amounts of carboxy-TEMPO in solution in mg/mL.

Several possible hypotheses have been considered to account for what could be occurring to cause these usual release results. It would have been possible that the release media (PBS + tween) could have been contaminated, but this hypothesis is refuted because the blank would have accounted for this. It is possible that release membranes were contaminated despite rinsing, but this is unlikely. Another possibility is that the dyes within the particles could have partially degraded at very high temperatures (e.g. the brief 60C stint) and yielded absorbent fragments which escaped the release membranes. The full length dyes would be too long to escape the membranes, as would the full length PSA, but it is also possible that the PSA degraded at 60C and the degraded polymer structure escaped the membrane. However, this structure would almost certainly not have an absorbance, so this is unlikely. It is possible that contamination occurred during collection of particles, though this would likely have become apparent before 48 hours of release experiments had elapsed.

It would be best to resolve this issue before the next release study is done, but as this has proved difficult, the best option seems to be to just try again. Of course there is always the chance of incorrect calculations, calibration, plate preparation, et cetera, and repeating the experiments carefully would ideally eliminate these types of error. Thus, future work will include another release study, performed with the greatest attention to detail.

3.5 Jetting in Germany as compared to jetting in Michigan

In order to determine if jetting done in the Lahann lab in Karlsruhe, Germany could be reproducible with significant accuracy in the Lahann Lab in Ann Arbor, MI, jetting of particles with 5% loading of carboxy-TEMPO, 20% w/v PSA, 95:5 CHCl$_3$: DMF solvent ratio, with 2% TEA as additive, and a flow rate of 0.4 mL/hr were jetted in the Ann Arbor lab. Particles jetted in Ann Arbor had an average diameter of 2.075 μm (200 measured), with a standard deviation of 0.6203 μm and a range of 0.64-5.39 μm. Particles jetted in Karlsruhe had an average diameter of 1.96 μm (100 measured) and a standard deviation of 0.642 μm with a range of 0.9-3.7 μm. Thus particle size is very similar. Images of both sets of particles are seen in Fig. 12. Shape and surface morphology are similar, although some difference is seen due to the lack of gold sputter coating on the particles from Karlsruhe. During my time in Germany, the gold sputter coater was not working, and its caretaker was on holiday. In all, particle size and shape can be fairly closely reproduced on different continents, which bodes well for the potential of future industrialization of the jetting method to make biodegradable drug delivery vehicles commercially.
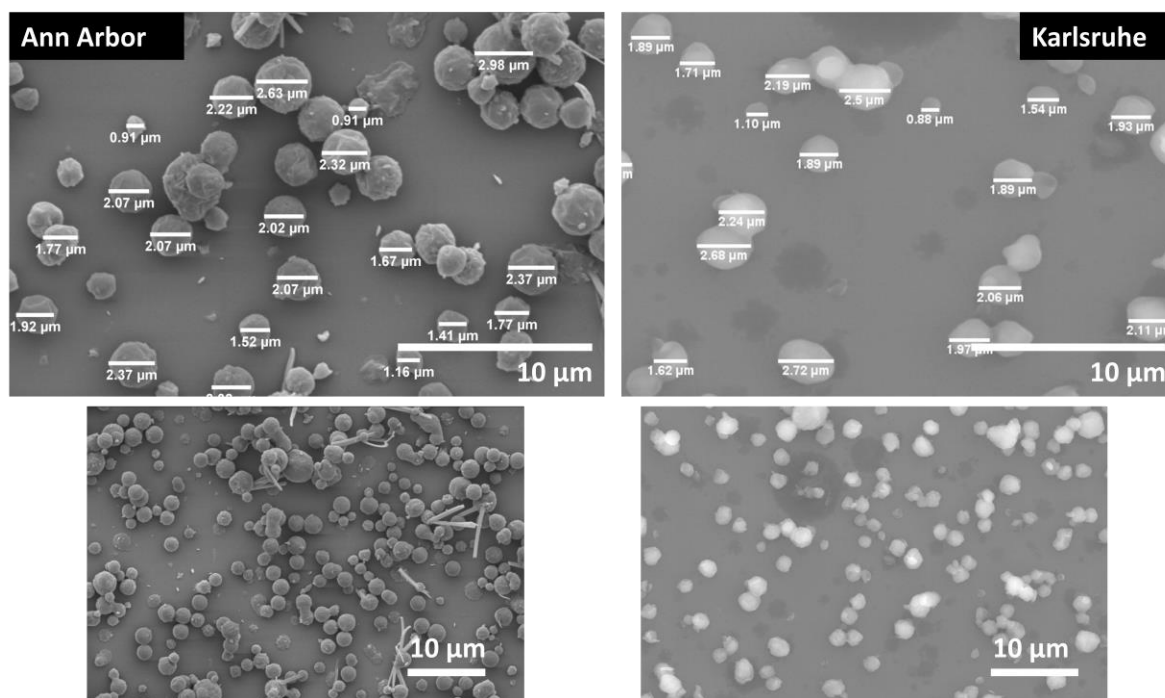

Fig. 12. Particles jetted in Ann Arbor as compared to particles with the same formulation jetted in Germany, showing comparable size and morphology. Note, however, that the images from Karlsruhe are not sputter coated because sputter coater was not working while I was there.

3.6 Jetting with Azithromycin

Azithromycin is an antibiotic more commonly used in the treatment of lung infections in CF patients. Particles containing Azithromycin were fabricated in order to supplement our data on carboxy-TEMPO, a nitroxide, which is part of a more experimental class of CF-related antibiotics. With jetting, optimization of jetting solution parameters such as polymer concentration, solvent ratio, and flow rate is always an important initial process.

Using a 95:5 solvent ratio with Azithromycin was attempted, since this was effective with carboxy-TEMPO. However, in Ann Arbor, there has been difficulty dissolving PSA in 95% $CHCl_3$. PSA is very soluble in DMF but only slightly soluble in $CHCl_3$, hence the complicated dissolution procedure necessary to produce PSA jetting solutions described previously. Dissolution of PSA in a 95:5 solvent ratio was possible in Germany, and thus the lack of solubility is currently a concern. Perhaps batch-to-batch differences occur in PSA purchased from Sigma, which is a concern. Of course, the polymer degrades over time, but even PSA from newly opened vacuum sealed bags has not proved soluble except in 70:30 solvents, or those with even higher DMF concentrations (e.g. 50:50).

Dissolving PSA has been possible in 95:5 solvents when TEA is used as an additive, but TEA causes instability in jetting unless coupled with carboxy-TEMPO. This causes jets to yield amorphous structures as opposed to spherical microparticles, as seen in Fig. 13. The exact chemical mechanism of TEA-carboxy-TEMPO co-stability is currently not known. It is probably that the two compounds form salts, neutralizing one another's charges and thus having a more limited effect on the dielectric constant of the solution. Since Azithromycin is largely non-polar and uncharged, it would not have the same interaction with the TEA.
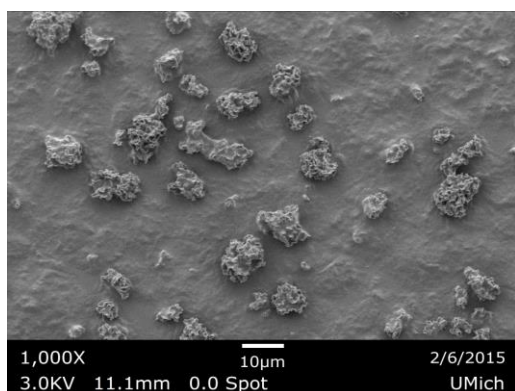
Fig. 13. Jetting with TEA in solutions that do not also contain carboxy-TEMPO yields amorphous structures. TEA can thus not be used as an additive to improve PSA solubility in particles which do not also encapsulate carboxy-TEMPO.

Thus, a 50:50 chloroform: DMF solvent ratio was chosen. A 70:30 solvent ratio was also tested, but particles did not take on as smooth of a spherical shape. It is seen that encapsulating drug

does not adversely affect particle size or shape, and that the 50:50 solvent ratio yields adequate particles for further analysis, as presented in Fig. 14.
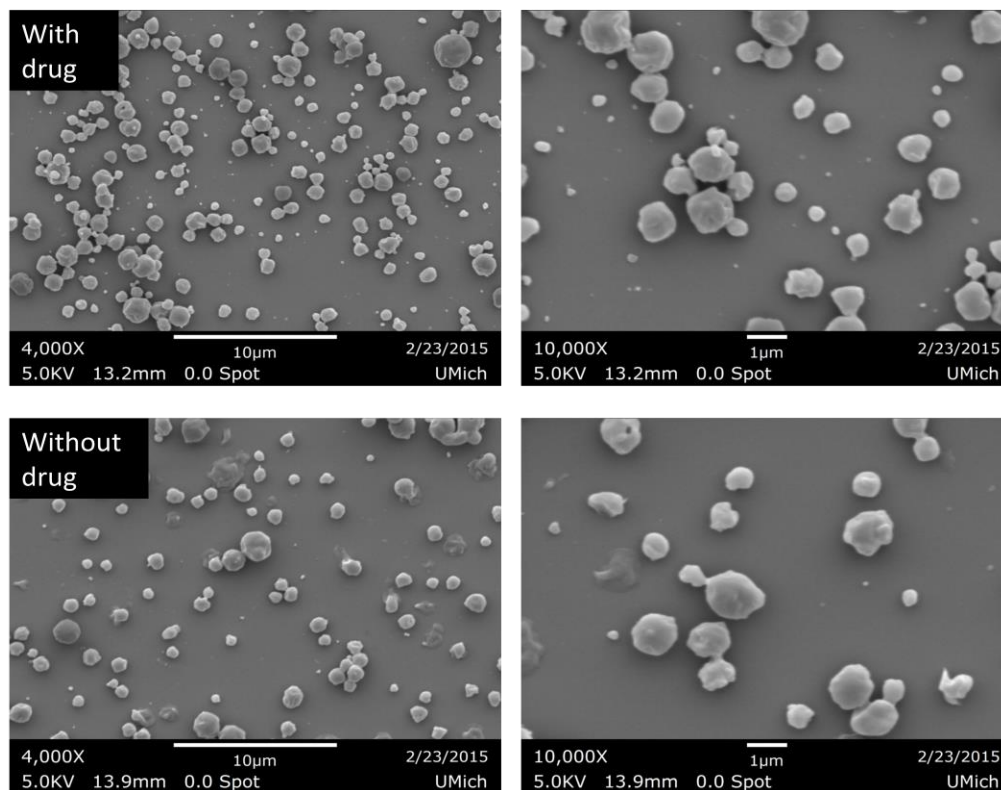

Fig. 14. Scanning electron images of PSA microparticles with and without Azithromycin, using a 50:50 solvent ratio for the particles in both images.

3.7 Dual Drug Loading

Particles were jetted which contain carboxy-TEMPO in one side only, Azithromycin in one side only, and both carboxy-TEMPO and Azithromycin in separate compartments. Confocal imaging was performed in order to confirm the bi-compartmental nature of the particles. Images are shown in Fig. 15-17: biphasicity is clearly visible. All particles were fabricated using 20% PSA weight/volume, 5% drug weight/weight, a solvent ratio 50:50 $CHCl_3$: DMF, and a flow rate of 0.4 mL/hour. Blue dyes and green dyes were included in compartments containing Azithromycin and carboxy-TEMPO, respectively, and for particles with blank compartments, the dye for that compartment was chosen to be the opposite color (blue if the other side was green, and vice versa) of the other compartment.
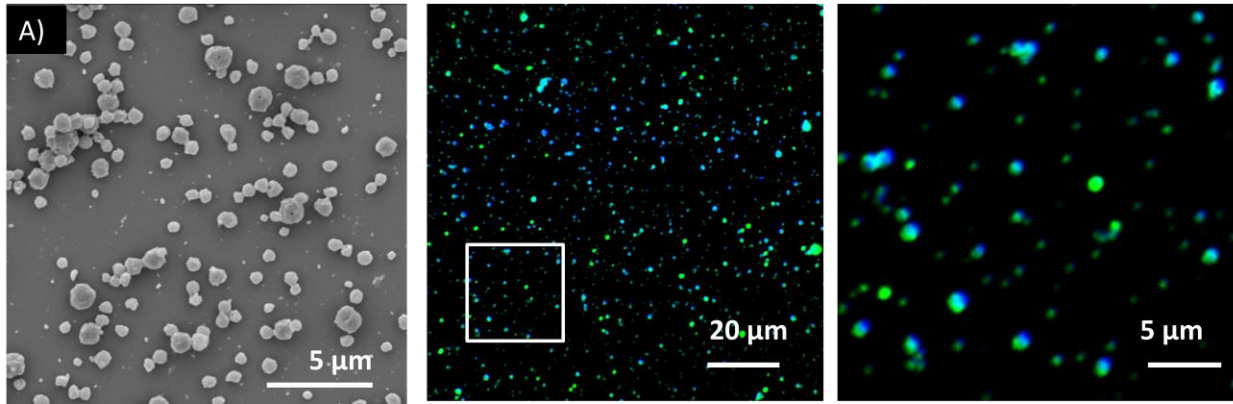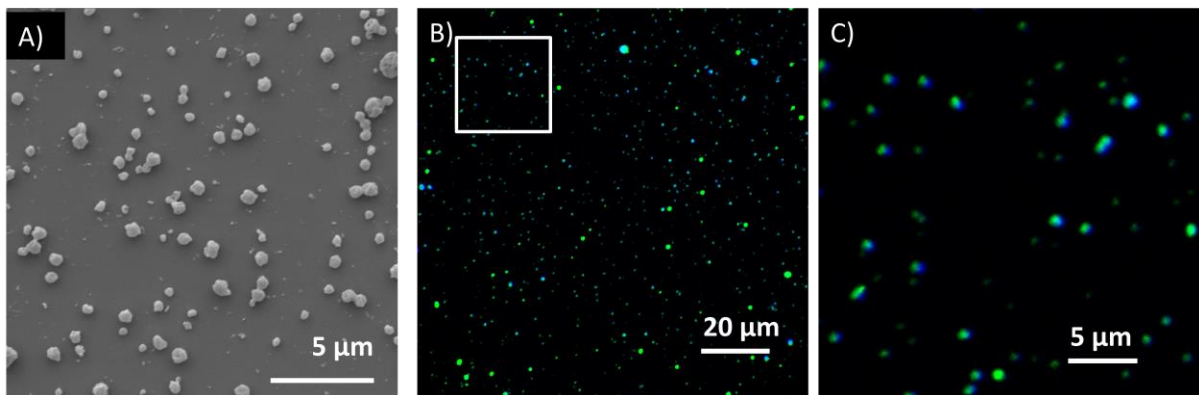
Fig. 15. Scanning Electron Microscope and Confocal Laser Scanning Microscope images of Particles containing carboxy-TEMPO in one side and nothing in the other.
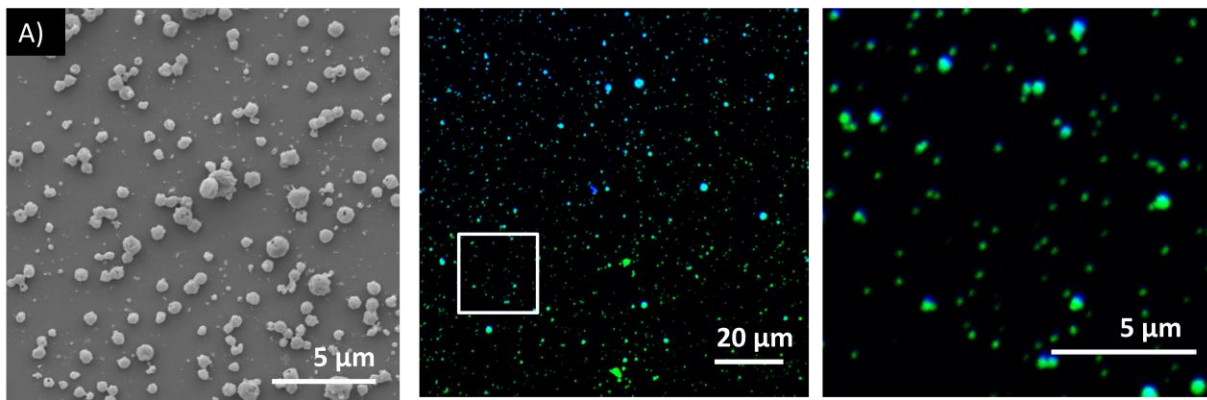
Fig. 16. Scanning Electron Microscope and Confocal Laser Scanning Microscope images of Particles containing Azithromycin in one side and nothing in the other.

Fig. 17. Scanning Electron Microscope and Confocal Laser Scanning Microscope images of Particles containing carboxy-TEMPO in one side and Azithromycin in the other.

These dual drug delivery particles will enable enhanced treatment for drug resistant bacteria, since it is far less likely for bacteria to evolve resistance to both drugs than it is for them to evolve resistance to one drug only.

3.8 Future Work

Future work will include solid state IR with PSA particles, both shortly after jetting and after being stored for different lengths of time, to determine if the polymer is degrading during jetting and/or storage. Other ideas will be brainstormed as well to determine whether the variable degradation of the polymer prior to release studies is effecting the release rate of drug from the particles in an uncontrollable way. Release studies will be performed with particles containing only carboxy-TEMPO, only Azithromycin, and both carboxy-TEMPO and Azithromycin, in order to compare release profiles and to determine if the bi-compartmental particles yield different release rates for the two drugs. Dynamic Light Scattering and Nanosight nanoparticle tracking analysis will be used to further quantify particle size distributions.

Future work with successfully fabricated particles, whose characteristics are seen as appropriate for CF drug delivery, will include cell toxicity testing and animal testing. A cell line has been identified as a promising candidate for cell studies. MucilAir is a commercially available in vitro, 3D model of the human epithelium sold by Epithelix in Switzerland. It is fully differentiated and has a shelf life of up to one year, but requires continuous surveillance because its media must be changed every two to three days. MucilAir mimics the *in vivo* tissue of the human respiratory epithelium by including features such as basal, goblet, ciliated cells, and mucus; cilia beating; metabolic activity; tight junctions; and active ion transport. MucilAir promises very high batch to batch reproducibility, so that multiple experiments can be performed without concern of error introduced by batch to batch inconsistencies. MucilAir sends cells in 24-well plate inserts, which can easily be transferred between well plates if needed. Each insert is about $100, depending on the pathology, and MucilAir will send three inserts for free as a sample. Available for purchase are cells taken from healthy patients or patients with a variety of diseases, including CF, COPD, Asthma, Allergic Rhinitis, and Smoker. Epithelix ships worldwide, and constantly has cells ready so that they can ship within days.

A caveat of using MucilAir is that one must purchase their media directly from Switzerland, because they refuse to share exactly what makes up their media for commercial reasons. I personally emailed them and they said they could not disclose this information, which is frustrating as a scientist who wishes to know exactly what they are feeding to their cells. In the end, the pros of using MucilAir seem to outweigh the cons, and I would advise that future in vitro studies be done using this model. Interfacing with Epithelix via email tends to yield a response within 3 days, so samples can be sent quickly and experiments may be done in an efficient manner.

After cell toxicity testing, in vivo studies will be performed. CF in vivo work is generally done using either mice or pigs as models. The eventual goal is to put these particles into clinical trials, and to eventually use them to better treat CF disease.

Overall, it is important to develop CF-optimized microparticles with highly specific drug delivery benefits in order to heal tens of thousands of patients worldwide. Moreover, the methodology developed from my research also will have potential uses for applications to other disease treatments.

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