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A Fabrication System for Chondroitin Sulfate Methacrylate Particles for Drug Release

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Abstract

Neuroblastoma is a rare cancer that develops in the sympathetic nervous system of infants and young children. Neuroblastoma has relatively high pediatric mortality rates and is currently treated with systemic chemotherapy. Systemic chemotherapy causes adverse side effects that impede the growth and development of pediatric patients. The localized, sustained release of chemotherapy drugs is a desirable alternative to systemic chemotherapy since its location makes it more adaptive and targetable compared to systemic chemotherapy. There is large need for the development of a drug delivery system (DDS) to facilitate the local delivery of chemotherapy drugs as well as the sustained release of the medication. To address this need, this project aims to design, fabricate, and validate a particle fabrication device and process for producing drug carrier particles for the delivery of chemotherapeutic drugs. The final product of this project targets neuroblastoma cells in culture, which serve as a primitive 2D disease model to 3D tumors that are most commonly found in infants. First, the project identifies current chemotherapy treatment options. Next, the project identifies a chemotherapeutic drug to be delivered and the most effective administration technique. The final goals of the project are to manufacture a sustained-release DDS and to characterize the results. This project concludes by identifying future work to continue the progress of this research.

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I. Introduction

Neuroblastoma is a cancer that stems from the neural crest, which is part of the sympathetic nervous system and can be found throughout the body. Neuroblastoma most commonly occurs in children under five years of age, an especially vulnerable risk group (Maris, Hogarty, Bagatell, & Cohn, 2007). Children are most negatively affected by cancer, because many systemic treatment and administration options for pediatric neuroblastoma impede the growth and development of pediatric patients. Since systemic chemotherapy is not cell-specific, it can harm healthy tissue as well as cancerous tissue. Prolonged systemic chemotherapy can lower blood cell counts, weaken the immune system, cause improper circulation and hair loss, and increase the risk for leukemia. Localized drug delivery can bypass these issues and side effects associated with systemic delivery in treating pediatric neuroblastoma.

According to Wolinsky et al, hydrogels, specifically, drug-loaded hydrogel microparticles, are being developed as potential alternatives to systemic chemotherapies such as intravenous chemotherapy treatments. Hydrogels are networks of dispersed organic and/or inorganic molecules suspended in an aqueous phase that solidifies into gel (Wan, Stylios, Giannoudi, & Giannoudis, 2015). Since various materials can compose hydrogels, they can be altered to have desired properties and can be designed for multiple purposes, including drug delivery. Hydrogel drug delivery is achieved by designing hydrogels with materials that are compatible to the desired drug along with designing a structure that will permit the sustained-release of the desired drug. In the case of pediatric neuroblastoma, the most desirable type of hydrogel drug delivery system (DDS) would be a microparticle made of a biocompatible polymer that can load chemotherapeutic drugs and slowly release them intratumorally over time. Microparticles are the most desirable hydrogel shape, because they can be repeatedly produced via microfluidics and they have predictable sustained drug release kinetics. Microparticles are also great at higher drug loading as well as a long duration of release to achieve sustained release (Wolinsky, Colson, & Grinstaff, 2012). In order to

produce a replicable and repeatable hydrogel DDS, the microparticle fabrication techniques and the drug-loaded microparticles must be researched, designed, and tested appropriately.

1.1 Project Scope and Goals

The end goal of this project is to design a device that fabricates hydrogel microparticle DDS via microfluidics and to create a DDS that can load and release doxorubicin (DOX) chemotherapy drugs for inducing apoptosis in neuroblastoma cells. Thus, this project will contribute to a long-term goal of research in DDS, which is to develop alternative cancer treatment options that decrease the need for adversely effective systemic chemotherapy treatments. The team's vision is to improve chemotherapy treatment by reducing the side effects associated with systemic chemotherapy and enhancing the potential to treat neuroblastoma efficiently and effectively via locally-administered DDS.

There is a need for a delivery vehicle for the local sustained release of chemotherapeutic drugs on cancerous tissue within the body. The overarching goal of this project is to develop and validate a fabrication device and standard operating procedure (SOP) for creating a precursor to an effective chemotherapy drug delivery method for inducing apoptosis in neuroblastoma cells that can be further developed in the future for *in vivo* studies. The team will achieve this goal by meeting the following specific goals:

- Engineer a system for the fabrication of the unloaded drug carrier
- Design and produce a drug carrier for the sustained release of chemotherapy drugs
- Load chemotherapeutic drug into the unloaded drug carrier
- Quantify the drug release profiles from the chemotherapeutic-loaded drug carrier in order to validate the fabrication device and process.

To prepare the project for enhancement of treatment of neuroblastoma, the team must be knowledgeable in neuroblastoma tumor characteristics, current treatment options, and current clinical

need. Additionally, the team must have a broad understanding of which polymeric materials and chemotherapeutic drugs can be used to create drug carriers and how microfluidics can be implemented in order to create a fabrication device to produce these drug carriers. Background on these topics are provided in the following section.

II. Literature Review

2.1 Neuroblastoma

Neuroblastoma tumor characteristics are necessary to understand since knowledge of the targeted tissue cell type, location, and properties are necessary for designing an effective neuroblastoma treatment enhancement. Further investigation into the irregular angiogenesis of tumors is also critical since it determines variations in clearance mechanisms, drug targeting and drug-carrier-to-tumor relationship. As the enhancement of any treatment relies on current treatment options, the team will also have to know about the details, advantages, and disadvantages of the current technology available to treat neuroblastoma in order to make appropriate modifications and/or improvements. The team's goals should ultimately align with the most beneficial option that has the most impact on current neuroblastoma treatments, and thus the team will also investigate what the biggest clinical need out of all the current options is by evaluating which options are used the most as well as what gap in technology is present for the clinically relevant treatment options.

2.1.1 Tumor Characteristics

Neuroblastoma tumors develop in diseased sympathoadrenal lineage of the neural crest and spread along the sympathetic nervous system (Davidoff, 2012). Since the sympathoadrenal system is connected to the sympathetic nervous system and the adrenal medulla, neuroblastoma cells can metastasize anywhere in the body where there are cells that are a part of either system (Marieb & Hoehn, 2016). Additionally, since neural crests are located in various parts of the body where stem cells differentiate into sympathoadrenal lineages, neuroblastoma can appear in different locations on the body. While 65% of neuroblastoma tumors initially appear within the abdomen, tumors may also appear in the neck, chest, and pelvic regions (Martin, Gaffney, Gatenby, & Maini, 2010).

Although neuroblastoma can develop in different locations across the body, it can be targeted by finding a biological similarity between all of the cancerous masses. When histologically analyzed, neuroblastoma cells appear to develop around stromal cells, which compose the epithelial layer of connective tissue and blood vessels surrounding the tumor. One way to overcome the complex structure of a neuroblastoma tumor is to target the highly acidic pH of tumor masses and the irregular blood vessels of a tumor mass (Martin et al., 2010). Tumors metabolize through aerobic glycolysis; as a result, tumors undergo a process called the Warburg effect, wherein the cancer cells produce acidic metabolic byproducts, such as lactic acid, and acidify the environment in the interior of the tumor (Damiani et al., 2016; Guy Makin, 2018; Renu, V.G, Picchia P.B, & Arunachalam, 2018; Schläger & Dräger, 2016; SM Ong et al., 2017). This drop in pH is a common identifying trait of neuroblastoma tumor masses. Additionally, tumor masses have been identified to have irregular, "leaky" vasculature with enlarged fenestrations that allow larger molecules to permeate through the capillary walls in comparison to healthy capillary walls that do not permit large molecules to permeate through (Robert S Kerbel & Yuval Shaked, 2017; Yu et al., 2016). The acidity and the leaky vasculature of tumor masses are targeted by future treatments pH-specific and/or large enough to permeate through leaky vasculature. However, in order to develop alternative methods for cancer treatment, the team must understand the current treatments available on the market.

2.1.2 Current Treatments

The four categories of the Gold Standard treatments available for the treatment of neuroblastoma are: surgery, chemotherapy, immunotherapy, and radiation therapy (Coughlan, Lynch, Gianferante, Stevens, & Harlan, 2017). Surgical treatment encompasses the full or partial removal of the tumor via resection. An advantage of this treatment is that surgical tumor removal does not introduce the body to systematically-delivered foreign objects or foreign drugs. In fact, surgery can be the only treatment necessary to eliminate the tumor mass. However, in most cases, the tumor is too large for the surgeon's

comfort or its growth surrounds critical vasculature. In these cases, surgery is not an ideal first-choice option. Surgery could also complicate the child's health overall due to the location of the tumor in their developing bodies (Mullassery & Losty, 2016). Other drawbacks of surgery are that there is no immediate assurance of full removal of the tumor, even if the surgeon can no longer detect it. It is most common to couple surgery with other treatments to ensure full removal of the cancerous cells from the body due to risk of metastasis and hidden portions surrounding critical body systems. The common complications in surgery include excessive bleeding, infections, and damage to nearby organs and vascular systems. These issues come in addition to complications related to anesthesia administered during the surgery (Coughlan, Lynch, Gianferante, Stevens, & Harlan, 2017; Davidoff, 2012; Mullassery & Losty, 2016).

Chemotherapeutic treatments involve administering a combination of anti-cancer drugs suitable for a patient's specific risk group, or people that share a common negative event like tumor histology, and other personal factors including age and weight. This treatment can be given pre- or post-surgery. Chemotherapeutics are typically administered intravenously or orally so that the drugs are able to travel through the bloodstream to reach the target site. The common drugs used for neuroblastoma treatment are carboplatin, cyclophosphamide, doxorubicin, etoposide, vincristine, topotecan, and busulfan (Coughlan, 2017).

The drawbacks of chemotherapy include the frequent, consecutive hospital visits to treat the patient with the appropriate chemotherapeutic medication (Davidoff, 2012; Mullassery & Losty, 2016). Since high concentrations of chemotherapeutics are extremely toxic, chemotherapy is often distributed as metronomic chemotherapy, or in lower doses and shorter intervals without extended resting periods (Robert S Kerbel & Yuval Shaked, 2017). Some of the main drawbacks to administering chemotherapy drugs systemically over time are mouth sores, bladder irritation, peripheral neuropathy, loss of fertility, decreased counts in leukocytes, red blood cells, and blood platelets, as well as hair loss and loss of appetite (Mullassery & Losty, 2016). While these side effects are not permanent and typically subside

once the treatment is concluded, they are still considered to decrease the patient's quality of life during the time of metronomic treatment due to organ damage especially on children.

Immunotherapy is another option of treatment for neuroblastoma. Immunotherapy is a technique to help the body's own immune system recognize the cancer cells in order to target and eliminate malignant cells. Currently, the gold standard for neuroblastoma-targeting immunotherapy is delivering a monoclonal antibody, unituxin, to the tumor site to attach to a molecule commonly found on the surfaces of neuroblastoma cells, GD2 (American Cancer Society, 2016).

The U.S Food and Drug Administration (FDA) has recently approved unituxin for use as part of a multimodality regimen, including surgery, chemotherapy and radiation therapy for patients who achieved at least a partial response to prior first-line multiagent, multimodality therapy (National Cancer Institute). Since this technique relies on the patient's own immune system, it is not always a feasible first-option for treatment, since most of the patient population is under five years of age with an underdeveloped immune system. Oftentimes, immunotherapy is administered in combination with additional drugs to further help the immune system attack the proper cells (American Cancer Society, 2016).

Another common treatment for neuroblastoma is radiation therapy. Radiation therapy involves the exposure to high energy rays that are meant to have a shrinking effect on the size of the tumor. There are many forms of administration: one being localized administration and another being full-body administration. Localized administration is given when the tumor develops in a condense, identifiable area and when it is visible and not blocking other organs (Davidoff, 2012; Mullassery & Losty, 2016) Full-body administration is given when there is a high risk of tumor metastasis as well as undiscoverable masses. The latter option is not as popular as the former option due to the long-term side effects that have been tied back to full-body administration. Some of these side effects include, DNA destruction, secondary cancer, impeded growth, and other internal organ problems.

While chemotherapy is commonly used in treating neuroblastoma, chemotherapy causes many secondary side effects, such as the destruction of bone marrow reserves (Davidoff, 2012; Mullassery &

Losty, 2016). Additional drawbacks to the chemotherapeutic treatment of neuroblastoma can be identified when considering the common age among the specific patient pool. The majority of the patient pool are infants. Children, especially those with high-risk neuroblastoma, frequently develop anemia, bone marrow metastasis, malnutrition, and hemorrhage as a result of chemotherapy. Most of these children will require red blood cell transfusions during and/or after systemic chemotherapeutic treatment (Vazquez-Mellado, 2015).

2.1.3 Clinical Need

The different treatments prescribed for each patient depend on a multitude of variables, including the patient's risk group, tumor histology and age. Patients can be loosely characterized into three risk groups: low-risk, intermediate-risk, and high-risk, according to the Children's Oncology Group (COG) classification (Davidoff, 2012). It is common for physicians to choose to pursue no treatment with lower risk groups. Due to the spontaneity of pediatric neuroblastoma, patients at low-risk level may experience unfacilitated tumor regression. Low-risk group treatments usually start with surgery. Surgery offers an effective way of eliminating the bulk of tumor masses. The disadvantage with surgery is that it is not always possible to achieve full elimination of residual tumor tissue, which is why chemotherapy is crucial to the success of full tumor elimination, whether it is administered as the primary treatment or as a supplementary mode of treatment. Because of this, there is a need for a locally-delivered treatment that supplements tumor-removal surgery to reduce the immunogenic effects and side effects of systemic treatments.

2.2 Chemotherapy Drugs

Doctors administer chemotherapy in cycles, which consists of treatment on a few days in a row, followed by time off to allow the body time to recover. The cycles are typically repeated every three or

four weeks. The total length of treatment depends on which risk group the child is in – higher risk groups usually require longer treatment. The length of treatment also varies depending on the drugs that are being administered, the type of cells that the treatment is targeting, the rate at which the cells proliferate, and the point in cancer cell proliferation at which a given drug is most likely to be effective (American Cancer Society, 2016; Davidoff, 2012).

The most common combination of chemotherapy drugs includes carboplatin (or cisplatin), cyclophosphamide, doxorubicin, and etoposide, which are considered the gold standard for chemotherapeutic drugs (Davidoff, 2012; Vazquez-Mellado, Aguilar, & Rocha-Zavaleta, 2015). It is important to note that other drugs are also being used i.e. topotecan, vincristine and sunitinib. The drug name, methods of delivery, and side effects of each drug are discussed in Table 2.1 (Cancer.Net Editorial Board, 2017; Davidoff, 2012). Table 2.1 lists common chemotherapeutic drugs along with their mechanism of action, common cancers treated, method of delivery and potential side effects.

Table 2.1: Drugs used for chemotherapy

Drugs	Mechanism of Action	What types of cancer do they commonly treat	Method of Delivery	Potential Side Effects
Carboplatin	Alkylating agent[1]	Ovarian, Lung, Head, Neck and Endometrial	Infusion into a vein, Intravenous (IV) and Intra-peritoneal (Peritoneal cavity in Abdomen)	Taste Changes, Nephrotoxicity, Ototoxicity, Abnormal Magnesium Levels, Diarrhea, Peripheral neuropathy and Central Neurotoxicity
Cyclophosphamide	Alkylating agent	Breast, Endometrial and Lung	Infusion, Orally (tablet), and Injection into a muscle or the lining of the lung	Blatter Irritation, Bleeding, Diarrhea, Leukemia and Myelodysplasia
Doxorubicin	Anthracycline[2]	Breast, Gastric, Liver, Kidney, Neuroblastoma and Thyroid	Intravenous or Infusion	Pain, Nausea or Vomiting, Low Blood Counts, Mouth sores, Cardiotoxicity and Problems with Fertility
Etoposide	Plant Alkaloid[3]	Testicular, Bladder, Prostate, Lung and Stomach	Oral (tablet) and Intravenous (IV)	Menopause, Loss of Fertility, Low Blood Pressure, Radiation Recall and Poor Appetite
Sunitinib	Antiangiogenic Agent[4]	Gastrointestinal Stromal Tumor, Advanced Renal Cell, Advanced Pancreatic Neuroendocrine Tumor	Orally	Fatigue, Heartburn, Low Blood Counts, Shortness of Breath and Increased Liver Enzymes
Vincristine	Antimicrotubule Agent[5]	Acute Leukemia, Hodgkin's and non-Hodgkin's Lymphoma, Neuroblastoma, Rhabdomyosarcoma and Brain Tumors	Intravenous (IV) and Infusion	Hair Loss, Constipation, Low Blood Counts, Loss of Appetite and Peripheral Neuropathy

Referenced and adapted from ((Davidoff, 2012; Mullassery & Losty, 2016) Davidoff, 2012; Guy Makin, 2018; National Cancer Institute, 2011; Renu et al., 2018)

(1) An alkylating agent refers to drugs that are most active in the resting phase of the cell. Alkylating agents could also be known as cell-cycle nonspecific.
(2) An anthracycline drug is a drug derived from certain types of Streptomyces bacteria. Anthracycline drug combats tumors by blocking an enzyme called the topoisomerase 2, thus halting the growth of cancer cells. DNA topoisomerases are enzymes that catalyze the passage of individual DNA strands or double helices through one another. The cancer cells need the topoisomerase 2 to be able to replicate DNA, proliferate, and develop into a tumor mass.
(3) Plant alkaloids are secondary compounds that have been derived predominantly from plants.
(4) An antiangiogenic agent prevents the formation of new blood vessels
(5) Antimicrotubule agents inhibit microtubule structures within the cell, which will ultimately result in cancer cell apoptosis and cancer cell death. Microtubules are founded in the cell's apparatus for division and replication.

Out of the drugs mentioned in this section, DOX serves as a model drug, is available, is one of the gold standard chemotherapeutic drugs and is one of the leading drugs for neuroblastoma research (Chemotherapy for neuroblastoma; Davidoff, 2012; Mullassery & Losty, 2016). Because of this, the team will be focusing primarily on DOX.

Chemotherapeutic drugs have a common disadvantage. Even though they are able to eliminate cancer cells, the chemotherapy drugs do not detect the difference between healthy cells and cancer cells. Since healthy cells are also affected by chemotherapeutics, some side effects will occur due to the healthy cell apoptosis and tissue damage. Some common side effects include: hair loss, mouth sores, bladder irritation, shortness of breath, back pain, tightness in the chest, infection, abdominal pain, diarrhea and low platelet count. The low platelet count is usually a common side but it can be severe since the patient is more susceptible to leukemia or myelodysplasia. Other more severe side effects include problems with fertility, decrease in the heart's pumping capability, peripheral neuropathy, central neurotoxicity, nephrotoxicity and hearing loss in pediatric patients (Damiani et al., 2016). Therefore, optimization of the administration of such drugs is important to reduce the effect of the chemotherapy on patients.

2.2.1 Methods of Administration

Methods for administering chemotherapy and for controlling dosage vary. The method of delivery can greatly impact the efficacy of the chemotherapeutic drugs. For example, oral delivery of pH-sensitive chemotherapeutics exposes drugs to highly acidic stomach acid. The acidic environment can hydrolyze drugs into inactive metabolites, thus lowering drug efficacy. However, administering high concentrations of the drug in order to combat the declining drug efficacy may cause adverse effects. Administration methods, such as hydrogel DDS, can prove to be superior to current drug delivery methods. Common methods of administering chemotherapeutic drugs are described below:

i. Oral Delivery

Oral chemotherapy medications are ones that can be swallowed and are, thus, non-

invasive. This method of chemotherapy is also known as the enteral method. Oral chemotherapeutics come in a variety of forms such as pills, tablets, capsules, and liquids. The drugs are enclosed in a protective coating, which is degraded in the stomach by digestive acids (Damiani et al., 2016). The drugs are then absorbed through the stomach lining. Depending on the coating, the medication can be released throughout the day allowing for an extended release. This form of delivery does not suit all types of chemotherapy drugs. Certain medications can be destroyed by stomach acids or may cause adverse effects in the stomach. Additionally, it is easy to overdose with oral medication and cause unintended interactions between the drug and food (Sandeep D. Parsad & Mark J. Ratain, 2007).

ii. Subcutaneous Injection

Subcutaneous injections utilize a short needle and apply the drug between the skin and the muscle directly or in an encapsulated form. This form of treatment is often used in order to prevent bleeding when the patient's platelet count is low.

iii. Intravenous Treatment

IV treatment allows for rapid entry of the drug into the body's systemic circulation (Guy Makin, 2018). This is the most common form of chemotherapy treatment and the drugs are readily absorbed through the bloodstream. Intravenous treatment allows for more flexibility with drug dosing. Treatment dosages can be tailored to the patient's needs over time. Infusion of the drug can be given over the course of a few days or weeks. One of the disadvantages of this method is the extravasation in which the medicine can leak out of the veins causing adverse effects on the skin which can lead to patient discomfort and unnecessary complications (Guy Makin, 2018).

iv. Intraperitoneal Treatment

Chemotherapy drugs can be given directly into the abdominal cavity using a catheter placed through the abdominal wall and emptying into the cavity (Guy Makin, 2018). To facilitate

treatment, the patient is advised to move side to side and lying on the back right after administration. The idea of this treatment is to concentrate the drug around tumors in the abdomen, thereby reducing systemic exposure. After treatment, the drugs can be drained out or allowed to remain in the cavity to be slowly absorbed by the tumor. This treatment is controversial since cancer reduction has not been demonstrated well in clinical studies.

v. Intra-Arterial Treatment

After locating the blood vessels supplying blood to the tumor using angiography or special X-rays, chemotherapy drugs can be supplied intra-arterially using an external catheter or an implanted pump (Guy Makin, 2018). The idea behind this administrative method is to reduce overall toxicity of the drug in the body and to concentrate it at the tumor site.

vi. Topical treatment

Some forms of chemotherapy drugs can be applied as creams directly to the surface of the skin usually to treat skin cancer. The drug is absorbed through the skin into the cancerous lesion (Guy Makin, 2018). The number of applications of this method is limited. Its primary advantage is that it is easy to administer but can result in burns and rashes on the skin.

Each of these methods of chemotherapy administration have their drawbacks. Most notably, the majority of these methods expose large areas of the body to toxic chemotherapy. In order to reduce the cytotoxic effect of chemotherapy, a vehicle containing a concentrated amount of drug must be directly applied to the cancerous tissue. This vehicle, most of the administration methods listed above, would perform local drug delivery by slowly eluting fractions of the drug into the surrounding cancerous tissue. The DDS would have to be designed to obtain a predictable drug release rate at judicious drug concentrations that are effective on cancer cells, but do not reach healthy tissue. The efficacy of a DDS for local drug delivery is dependent upon the DDS's ability to achieve a sustained drug-release rate.

2.2.2 Sustained Release

Sustained release, in this context, will refer to a controlled delivery of a therapeutic reagent comprised of a polymer or material that can be engineered to control the rate of drug release and/or desorption over an extended period of time (Saltzman, 2014). The rate of release can depend on multiple mathematical factors including drug diffusion rate, polymer degradation rate, and overall polymer-drug desorption rate. One equation used to calculate the diffusion rate is:

$$\frac{\partial c_p}{\partial t} = D_{i:p} \frac{\partial^2 c_p}{\partial x^2}$$

Equation 1: Diffusion rate for zero-order release for slab geometry

where $D_{i:p}$ is the diffusion coefficient for the drug within the polymer and c_p is the drug concentration (mg/mL) (Liechty, Kryscio, Slaughter, & Peppas, 2010; Saltzman, 2014). This equation can calculate total diffusion rate of drug through the polymer matrix for an ideal zero-order release system.

Researchers are pursuing the achievement of zero-order release systems because of their advantages. Zero-order release systems present a desirable theoretical ideal of maintenance of the drug concentration within the body at an optimal level over time (Tang et al., 2015). Figure 2.1 shows an example of various types of drug release including, zero-order, sustained, and immediate release.

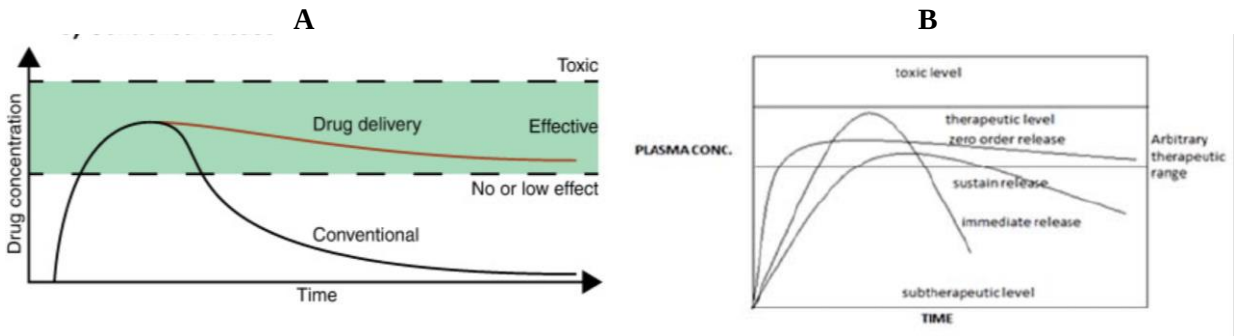


Figure 2.1: Drug release curves **(A)** drug concentration over time of a generic drug delivery method, **(B)** specific drug release profiles over time (Garg, Panday, & Patel, 2016)

Within Figure 2.1, both graphs contain two horizontal lines indicating an arbitrary therapeutic range, where the lower line is considered the threshold for subtherapeutic drug levels, and the top line is the threshold for the toxic level. DDS aim to attain a drug release as shown in Figure 2.1A, however not all DDS achieve this, as shown in Figure 2.1B.

Sustained drug release is an imperative function of a competitive DDS. There are multiple types of DDS that have been proven to achieve sustained drug release. These DDS are composed of different material and come in a variety of shapes and sizes.

2.3 Alternative DDS

Many types of DDS have been studied for their ability to load and release chemotherapy drugs. The most common DDS are hydrogel matrices, particles, and self-assembled particles (Norouzi, Nazari, & Miller, 2016); Yu et al., 2013).

Hydrogels are customizable matrices of polymeric materials that gel in an aqueous solvent (Wan et al., 2015). Hydrogel matrices have customizable mesh sizes and material properties that make it possible to control their functions. Hydrogels are desirable excipients for drug loading, because they are capable of absorbing drugs at controlled concentrations and releasing them at controlled rates. Additionally, hydrogels have been extensively studied for the purpose of intratumoral delivery (Norouzi et al., 2016). This application of hydrogels is specifically favorable, since intratumoral delivery of drugs is the ideal method to ensure a more localized delivery of chemotherapy. A hydrogel's capability to achieve sustained drug release of a drug depends on its shape, size, material properties, and interactions with the drug.

DDS can take on many shapes and sizes such as particles. Particles can be composed of a variety of materials. The two main materials that have been studied are metals (e.g. gold nanoparticles) and polymers (e.g. polyvinyl alcohol). With the move towards biodegradable DDS to avoid the need of a

secondary surgery for the removal of systems, metallic materials have been less favorable to use. These materials may not only require second surgeries but can also cause chronic foreign-body immune response (Wolinsky et al., 2012). Polymers have proven to be the most versatile material to create DDS due to their controllable characteristics, availability on the market, versatility of material selection (e.g. natural and synthetic options), and ease of production (Garavand, Rouhi, Razavi, Cacciotti, & Mohammadi, 2017). Particles can also be produced to create different shapes such as rods and spheres. While both rods and spheres have proven to be useful, spherical particles have been incorporated into more delivery methods (e.g. films, wafers, and gels) making them more attractive options for DDS (Wolinsky et al., 2012).

Another alternative for DDS are self-assembled particles. These particles include structures such as liposomes and polymersomes. Liposomes are structures that organize themselves similarly to the phospholipid bilayer of a cell. The inside of a liposome forms a capsule, with the amphiphilic material aligning itself so that the hydrophilic ends point towards the inside of the bilayer, while the hydrophobic ends form the inner and outer sides of the membrane. These structures are formed by using natural biomaterials such as phospholipids, whereas polymersomes are the synthetic polymer equivalent of liposomes. Polymersomes are produced with synthetic amphiphilic block copolymers and are known to be easier to design for DDS and more mechanically stable than liposomes (Cui, van Koeverden, Müllner, Kempe, & Caruso, 2014). The biggest drawback of these systems are their reversibility. To process liposomes and polymersomes, they must be submerged in a non-solvent that induces the self-assembly. When in contact with a non-solvent that no longer induces this assembly, the molecules can return to a non-assembled form and burst-release the encapsulated drug, which is not a form of sustained release. Another drawback of this system is the broad particle size distribution due to the non-tunable, self-assembling characteristics (Cui et al., 2014).

Although many systems exist to deliver drugs in order to facilitate chemotherapy, only some are useful to obtain a biodegradable, sustained release system that is easily produced. The most promising makeup of such as a system must be made of polymers that can be processed as hydrogels to form into spherical particles. Two important factors that must be considered when engineering drug delivery particles are the materials and methods used for the fabrication.

2.3.1 Achieving Sustained Drug Release Using Polymeric Hydrogels

DDS incorporate polymers to encapsulate, embed, or otherwise carry drugs. Polymers can be engineered to release the drug over a specified and desired time, creating a release profile for controlled or sustained drug release from the polymeric carrier. When incorporated into a hydrogel DDS, polymers are capable of achieving sustained drug release along with biocompatibility and biodegradability.

The pursuit of a DDS that achieves sustained drug release has incentivized researchers to alter the geometry of loaded hydrogel matrices in order to modify the release surface and the controlling relaxation (Chidambaram, Porter, Flood, & Qiu, 1998). The entry and exit of water from a hydrogel matrix controls the rate of drug release and/or drug dissolution. The entry of water causes the polymeric hydrogel matrix to swell, particularly if the matrix is composed of a hydrophilic polymer (Bastiancich, Danhier, Préat, & Danhier, 2016). There are two types of hydrophilic matrices (Maderuelo, Zarzuelo, & Lanao, 2011):

Matrix 1: Release is controlled by swelling. The drug diffuses through the hydrogel matrix formed by the swelling of the polymer chains when water enters the matrix. This includes the dissolution and the diffusion of the drug towards the outside of the matrix.

Matrix 2: Release is controlled by dissolution. The water enters the system and gels the polymer by crosslinking the polymeric molecules. The process of swelling and dissolution/erosion of the polymer is what controls the release of the drug.

The phenomena of diffusion, swelling and erosion related of the drug will vary with the square root of time which is shown in Equation 2 below:

$$M_t = A\sqrt{DC_s(2C_o - C_s)t}$$

Equation 2: Total mass released function for a slab system

In this equation, M_t is the total mass released up to time t; A is the total area of a two-sided slab; C_0 is the initial concentration and t is the time (Maderuelo, Zarzuelo, & Lanao, 2011).

The total rate of sustained release also depends on the type of polymer that a hydrogel is comprised of. Mathematical models can produce theoretical release rates; however, the polymer-drug interactions within a loaded hydrogel system produce the imperfections in release kinetics that cannot be easily calculated. In order to produce the most effective drug carrier system, it is important to select the polymer material with the most compatible chemical characteristics.

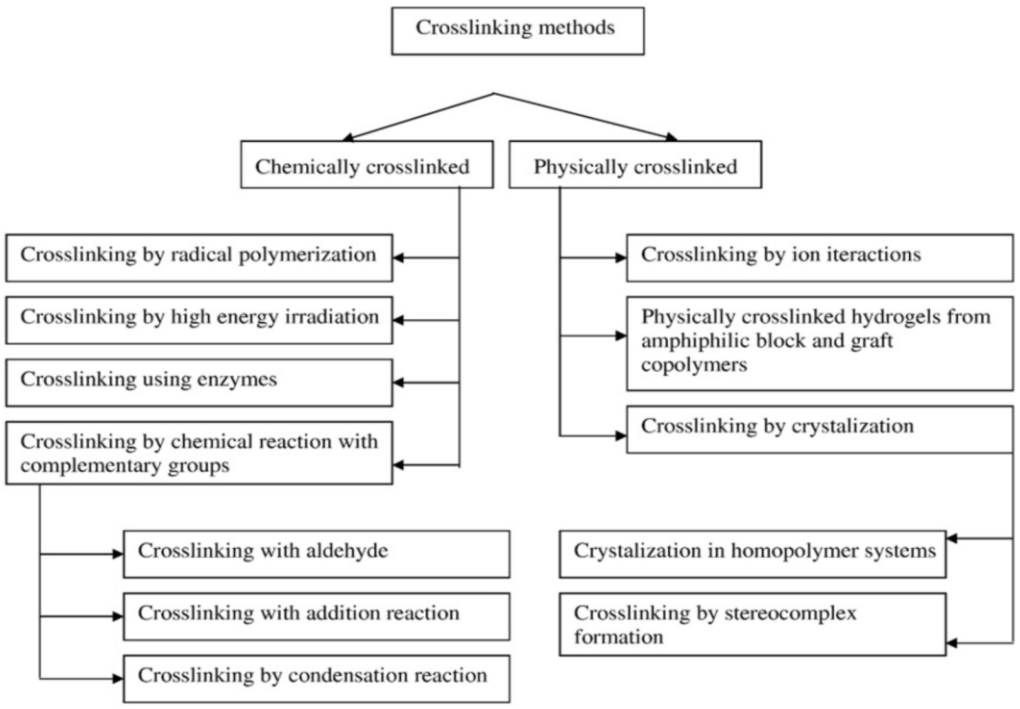
2.3.2 Polymer Selection for Drug Delivery

Polymers can be used to provide sustained release of therapeutic agents, controlled dosage, and the tunable release of both hydrophilic and hydrophobic drugs (Liechty, Kryscio, Slaughter, & Peppas, 2010). Polymeric drug carriers can be either degradable or non-degradable depending upon their material properties (Sawyer, Piepmeier, & Saltzman, 2006). Non-degradable polymers are usually used when there is a need for a permanent implant, such as a polymeric coating for a hip implant. Biodegradable polymers are preferred for drug-release purposes. They have the advantage of being completely cleared from the body without the need for a procedure to remove them. Many degradable systems are designed to erode via hydrolysis. This is beneficial since most of the body consists of water and enzymes to facilitate this process. However, this degradability must be controlled to ensure that the hydrogel is stable in aqueous environments and that the drug does not undergo bulk release.

2.3.3 Modification of Polymers to Create Hydrogels

Oftentimes, polymers need modifications for use in controlled sustained release DDS. Modifications include incorporating functional groups and/or crosslinking to achieve the desired characteristics. These modifications convert the polymeric raw material to a polymeric hydrogel that can absorb and release the desired drug. The chosen crosslinking method depends upon the existing groups on the polymer chains. The three basic crosslinking methods are physical crosslinking, chemical crosslinking, and mixed mode (Wan et al., 2015). Radical chain polymerization, a type of chemical crosslinking, is the most common crosslinking method (Wan et al., 2015).

The crosslinking method ultimately depends on the materials being used. Before material selection, it is important to fully understand the efficacy and user-friendliness of different crosslinking strategies. Figure 2.2 shows an overall overview of the different crosslinking methods.



Licensed to use through RightsLink (Hamidi, Azadi, & Rafiei, 2008) Appendix A; Hamidi, Azadi, & Rafiei, 2008)

Figure 2.2: Crosslinking Strategies

2.3.3.1 Chemical Crosslinking

Chemical crosslinking is a common crosslinking method for polymeric hydrogels. This method is specifically useful for water-soluble hydrogels containing -OH, -COOH, and -NH2 groups (Shivakumar, Satish, & Satish, 2006). Some forms of chemical crosslinking require crosslinking agents. Some of these agents have proven to be highly toxic, therefore a precaution that must be taken when using this method is ensuring the proper removal of all unreacted agents.

A common subset of this method is radical chain polymerization. Radical chain polymerization is a crosslinking method that is applicable to both natural and synthetic polymers (Garret D Nicodemus & Stephanie J Bryant, 2008). Prior to crosslinking, it often involves a functionalizing step to add vinyl groups that play an important role in the later crosslinking steps. This functionalizing step allows the polymer monomers or dimers to become multifunctional macromers. Once the polymer is functionalized, an initiator is introduced to create free radicals within the system. The free radicals are propagated through carbon-carbon double bonds by a signal in the form of heat or light of a certain wavelength. The free radicals form kinetic chains on the functionalized group of the polymer in order to produce the covalently crosslinked network with the polymer. A large advantage of this mechanism is that it often occurs in a length of time on the order of minutes, and in some cases, seconds. There are polymers, such as hyaluronic acid (HA) or chondroitin sulfate, that can be modified with side groups to create chemically produced crosslinks (Garret D Nicodemus & Stephanie J Bryant, 2008). Although polymers such as chondroitin sulfate and HA can be crosslinked using this method, there are typically various methods of crosslinking that work on the same chemistry type (e.g. HA can be crosslinked without the need of an initiator is modifying it with thiol side groups and to form a disulfide crosslink with the simple exposure to air) (Garret D Nicodemus & Stephanie J Bryant, 2008).

Crosslinking through irradiation is another type of chemical crosslinking mechanism (Shivakumar et al., 2006). This mechanism works by using high energy radiation (e.g. gamma rays or

electron beam). Radicals form on the polymer chains directly through irradiation of the aqueous polymer solution. If in contact in water, the radiation may also cause radiolysis so that hydroxyl radicals form from the water molecules which results in the formation of macroradicals when the radicals attack the polymer chains. A large disadvantage to this technique is the necessity to control the environment so that it occurs in an inert atmosphere (e.g. nitrogen or argon gas instead of oxygen). A large advantage to this method are the creation of almost pure, residue-free hydrogels. Polymers that can be crosslinked using this method include polyvinyl alcohol (PVA), polyethylene glycol (PEG), and polyacrylic acid (PAA).

Another common subset of the chemical crosslinking mechanism is a michael-type addition reaction (Shu, Liu, Luo, Roberts, & Prestwich, 2002; Zheng Shu, Liu, Palumbo, Luo, & Prestwich, 2004). This reaction involves a nucleophilic thiolate and an electrophile to create a thioether linkage. An advantage of the chemical crosslinking methods is that no additional agents are needed, and therefore no additional degradation by-products are formed. However, the disadvantages of chemical crosslinking include the length of time for gelation being on the order of hours.

2.3.3.2 Physical Crosslinking

Physical crosslinking is a method to use when avoiding a purification step (Shivakumar et al., 2006). Due to the need of crosslinking agents employed for chemical crosslinking, studies with sensitive systems have employed this method instead.

One type of physical crosslinking occurs through reversible ionic crosslinking where ionic bridges form between polymeric chains (Shivakumar et al., 2006). Another type is through hydrogen bonding. This bonding will only occur if the polymers' carboxyl groups have been protonated (Akhtar, Hanif, & Ranjha, 2016). An example of polymers that can be crosslinked using this method include PEG and poly(methyl methacrylate) (PMMA). Amphiphilic graft or block co-polymers may also undergo physical crosslinking through self-assembly. When induced by their surroundings, the polymers can form into micelles. Poly(lactic-co-glycolic acid) (PLGA) and PEG are examples of polymers that this type of

crosslinking may apply to (Akhtar et al., 2016). Crystallization is another method of physical crosslinking (Akhtar et al., 2016). This method refers to the crystallization of homopolymers using a freeze-thaw process cycle. Another example which employs this method is stereocomplex formation which applicable to stereoisomers (e.g. polylactic acid or PLLA and poly-d-lactide or PDLA). Due to the availability of complementary sites that stereoisomers have, intermolecular interactions drive the physical crosslinking.

2.3.3.3 Mixed-Mode Crosslinking

A less explored mechanism for crosslinking is mixed-mode polymerization. This mechanism involves properties from both radical chain polymerization and chemical crosslinking. The mechanism for this type of reaction requires the macromolecules involved in the reaction to contain thiol and acrylate groups to form thioether linkages while the kinetic chains form with the homopolymerization of the acrylate groups (Shu, Liu, Luo, Roberts, & Prestwich, 2002). The mixed-mode polymerization mechanism has been studied to have a faster gelation period than chemical crosslinking and smaller kinetic chain formation that is relevant when focusing on adjusting degradation characteristics. However, this method is very specific to polymers that contain specific side groups as mentioned above.

2.3.4 Examples of Polymeric Hydrogel Materials

2.3.4.1 PLGA Hydrogel Carriers

Poly(lactic-co-glycolic acid) or PLGA is a biodegradable synthetic polymer that has been incorporated into hydrophobic polymeric hydrogel DDS (Liechty, Kryscio, Slaughter, & Peppas, 2010). Due to the hydrophobicity of PLGA, it has limited water absorption. This can be modified by the amount of lactic acid to glycolic acid on the polymer. The higher the percentage of lactide acid units, the longer the polymer would last before degrading in the presence of water. Hydrogels made of PLGA are minimally toxic to the body, because they break down into lactic acid and glycolic acid, which are both metabolized by the Krebs cycle (Garret D Nicodemus & Stephanie J Bryant, 2008; Rydholm, Bowman, &

Anseth, 2005). Additionally, PLGA is a component of commercially available controlled release drug delivery products such as OncoGel™. OncoGel™ is a thermosensitive gel designed for the local delivery of Paclitaxel chemotherapy drug into solid brain tumors via intralesional injection or direct intertumoral placement (Bastiancich, Danhier, Préat, & Danhier, 2016b). OncoGel™ is a hydrogel that incorporates ReGel™, a copolymer of PEG and PLGA.

Another application of PLGA in a hydrogel carrier is through a thermosensitive PEGylated paste developed by Wolinsky et al. This paste contains PLGA/PEG microparticles working together with active agents such as Trichostatin A, anti-inflammatory agent methotrexate, and chemotherapy drug etoposide (Bastiancich et al., 2016). This paste has been tested in vitro and is being developed for intertumoral applications.

2.3.4.2 Polyvinyl alcohol and Polyvinyl Alcohol Methacrylate

Polyvinyl alcohol (PVA) is a synthetic hydrophilic polymer that is commonly used in biomedical applications due to its good mechanical properties, biocompatibility, and non-cytotoxicity (Zhou et al). In one study, PVA is used in a polymer blend with chitosan to increase the mechanical integrity of fragile pure chitosan. Given its reliable mechanical properties, PVA is a useful polymer for hydrogel applications. However, PVA alone is soluble in water (Zhou et al). PVA can be stabilized for use in aqueous environments via methacrylation (Zhou et al).

Polyvinyl alcohol methacrylate (PVA-MA), is a less soluble derivative of PVA due to the intermolecular photo-crosslinking of PVA macromers which makes the hydrolytic degradation. This is because methacrylation adds methyl groups to various parts of the PVA structure. PVA cross-links its molecules together when UV light irradiates at 365 nm in the presence of a photoinitiator to form a stronger bond between PVA-MA groups (Martens, Holland, & Anseth, 2002).

2.3.4.3 PCL Hydrogel Carriers

Poly(ε-caprolactone) or PCL is a biopolymer currently being developed as a component of DDS (Wolinsky et al., 2012). PCL is a biocompatible polyester with good drug permeability and a slow rate of biodegradability compared to other polymers mentioned in this review (Akhtar et al., 2016). These properties make PCL a desirable polymer to use in DDS with long-term drug release. Due to its slower biodegradation rate and chemical composition, PCL byproducts do not increase the acidity of the environment as they degrade over time, which further emphasizes the biocompatibility and non-toxicity of PCL. Additionally, PCL can be crosslinked via ionizing radiation. PCL has been used as a biomaterial for producing DDS in by being blended with water-soluble polymers such as PVA.

2.3.3.4 Chondroitin Sulfate (CS) and Chondroitin Sulfate Methacrylate (CS-MA)

CS is a glycosaminoglycan (GAG) composed of repeating units of the disaccharide b-1,3-linked N-acetylgalactosamine (GaINAc) and b-1,4-linked d-glucuronic acid (GlcA) with sulfate groups (Zhao, Liu, Wang, & Zhai, 2015). The extracellular matrix (ECM) is rich in polysaccharides such as CS (Zhao, Liu, Wang, & Zhai, 2015). CS can be extracted from the bone, cartilage, skin, extracellular matrix, nerve tissue, and blood vessels. The average molecular weight of CS derived from animal tissues is approximately 20 kDa (Zhao et al., 2015). The variation of CS derived depends on the natural variation in sulfation from molecule to molecule of CS. Three variations of CS include the A-type, C-type, and E-type.

Currently, CS is used as an FDA-approved orally administered osteoarthritis therapeutic. Since CS has an anti-inflammatory property and since it is naturally found in cartilage and bone, it is a promising GAG for the treatment of hard tissue inflammation. This anti-inflammatory property stems from CS's ability to diminish the nuclear factor NF-kB activation along the membrane receptors of cells (Vallières & du Souich, 2010; (Wan et al., 2015). NF-kB has been linked to contributing towards beginnings of pathogenic diseases such as gastritis, rheumatoid arthritis, psoriasis, and even cancer. The

anti-inflammatory properties of CS make it a desirable material for facilitating drug delivery to tumor sites.

Using a CS derivative as the polymer to create a DDS is advantageous since it is biocompatible and naturally derived. Naturally occurring polysaccharides have reactive side groups, which can be adapted to suit specific needs (Zhao et al., 2015). CS's reactive side groups are hydroxyl groups. Moreover, CS is biocompatible and biodegradable, which eliminates the risk of long-term adverse effects within the body (Zhao et al., 2015). However, CS is inherently water soluble, which limits its use as a candidate for the material for a drug delivery vehicle. There are various techniques that have been developed to modify CS so that it can successfully be used as a drug carrier. A widely explored technique includes a two-step process, methacrylation and crosslinking. The crosslinking occurs by the aforementioned radical chain polymerization mechanism. However, methacrylation is necessary to make GAGs like CS photo-crosslinkable. This is accomplished by functionalizing the hydroxyl groups with vinyl groups. The vinyl groups that are added on are involved in the kinetic chain growth during the process of free radical production from the initiator (Martens, Holland, & Anseth, 2002). The methacrylation of CS can be accomplished by utilizing various reagents such as methacrylic anhydride, methacryloyl chloride, and glycidyl methacrylate. Glycidyl methacrylate is the ideal option to choose from since the reaction is more efficient and is less cytotoxic in comparison to the other options (Anahita Khanlari, Michael S. Detamore, & Stevin H. Gehrke, 2013). An additional disadvantage of using methacrylic anhydride is the necessity to use more product since it is prone to hydrolysis (Li et al., 2004). This can be avoided by increasing the pH of the reaction, but this increases the risk of CS chain-scission as well as hydrolysis of the ester bonds necessary for the crosslinking reaction.

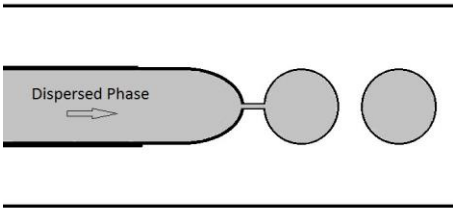
Additionally, CS-MA binds electrostatically to certain chemotherapy drugs, such as doxorubicin. Once it is loaded into CS-MA, DOX molecules begin to interact with each other and stabilize the drug release rate from the CS-MA. Drug-DDS interactions are important considerations when deciding on the material properties of the DDS.

2.4 Methods for Fabricating Microparticles

For the purpose of drug delivery, polymeric carriers are fabricated into micro-scale or nanoscale carriers for improved drug delivery with higher loading and faster and consistent diffusion kinetics. There are several methods that can be utilized such as the emulsion method or spray drying. However, microfluidic droplet generators are the most common method of fabricating micro or nano spheres of the desired polymer (Seemann, 2012). Droplet-based microfluidics is based on the high frequency production of droplets with consistent and controllable sizes. The microfluidics system involves flowing two or more immiscible liquids through very small channels and producing spherical particles. Droplet-based systems incorporate different architectures or geometries that assist in particle fabrication. The three main microfluidic geometries for droplet formation are co-flow, flow focusing, and cross-flow droplet generators and are discussed below (Christopher, 2007).

2.4.1 Co-Flow Droplet Generators

In a co-flow device, two to three phases of liquids are utilized to draw out spherical shaped products. The underlying idea is that two liquid streams are flowing together, where the continuous phase (the outer liquid stream) surrounds the dispersed phase (inner liquid that will form the droplets), until the interfacial instabilities it breaks into spheres (Christopher, 2007). Figure 2.3 is a schematic diagram exemplifying a co-flow geometry.



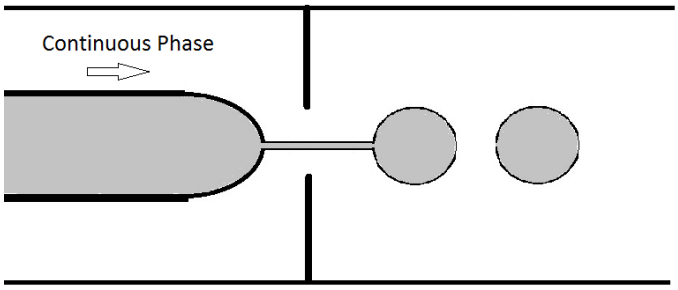
Adapted from (Seemann, Brinkmann, Pfohl, & Herminghaus, 2012)

Figure 2.3: Co-flow Schematic

Particle size of the pinched off dispersed phase can be controlled by either altering the flow rates, thereby changing shearing forces between the liquid phases, or by varying the interstitial surface tension. Surface tension depends on the selection of which liquid phase flows through the channels and their viscosities.

2.4.2 Flow Focusing Droplet Generators

Flow focusing involves the co-flowing liquids to flow through an additional orifice. The two immiscible liquids are forced to flow through a narrower channel and this also results in an elongation of the two phases. This also causes instability in the interfacial surface tension that results in the dispersed phase pinching off and forming droplets or spheres. The complexity in the fabrication and operation in flow focusing devices is higher compared to other geometries but it allows the production of even smaller particles by only controlling flow rate. Having a small orifice or flow focusing aperture in the flow focusing channel enables generation of very small droplets. The two liquid phases are forced to flow through a smaller channel increasing flow rate and shear causing the particles to pinch off faster from the dispersed phase. Flow focusing is a commonly used method in microfluidics as a result that is used in generating monodispersed microdroplets. Figure 2.4 below shows a typical schematic for a flow focusing device.

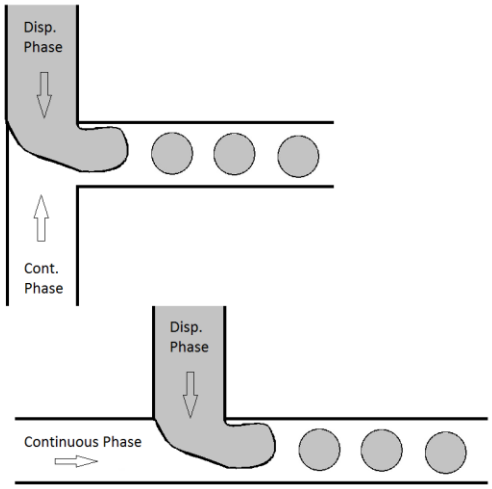


Adapted from (Seemann, Brinkmann, Pfohl, & Herminghaus, 2012)

Figure 2.4: Typical Flow Focusing Schematic

2.4.3 Cross-flow Droplet Generators

Cross-flow geometries or T-junctions allow for the easy and controllable production of particles. Unlike co-flow where the dispersed and continuous phase flows either in parallel or less than 90° to each other, cross-flow geometry forces liquids to flow at right angles to each other or directly head-on in opposite directions. In cross-flow the dispersed phase in pushed against the channel walls by the continuous phase causing it to shear off and produce particle. Typically, the flow rate of the continuous phase is higher than the dispersed phase. There are many papers that have identified similar variables that affect droplet generations such as flow rates and viscosities (Guillot, 2005), channel wall wetting properties (Xu, 2006), interfacial tension (Wang, 2009), channel dimensions (Abate, 2009) and crossing angles (Zhao, 2006) which are more well known for cross-flow geometries than co-flow. Variations of the T-junction have also been developed to allow for increased droplet production or to tune droplet size. The schematic for the standard T-junction and one with a 'head-on' geometry is shown below in Figure 2.5.



Adapted from (Seemann, Brinkmann, Pfohl, & Herminghaus, 2012)

Figure 2.5: T-junctions with **(bottom)** regular geometry and **(top)** head-on geometry

2.4.4 Device Fabrication Methods

Microfluidic droplet generators can be fabricated using a variety of materials and/or techniques. The main criteria governing the type of fabrication method utilized is the flow geometry and the wetting properties that is desired on the channel wall, which are needed to create particles (cross-flow). Common co-flow devices utilize glass microcapillaries that are usually narrowed using a pipette puller (Utada, 2005). Flow focusing features can also be added to the glass capillaries using micro forging techniques which can also be used to control the width of the capillary channels. Photolithography and soft lithography is another method that can be used to fabricate fluidic devices in micro-scale. Photolithography is the common term used to describe the process of deposition, exposure to specific energy emitting light, and developing (Koch, Rubino, Quan, Yoo, & Choi, 2016) & Zaouk, Park, & Madou, 2006). Typically, the process is initiated on a silicon wafer that acts as the foundation for the master template required for molding the actual device. Height of the mold determines how deep the channels will be in the device, thereby affecting cross sectional area and flow kinetics needed for droplet generation. A photomask with the desired channels imprinted upon is used to selectively control UV transmission. A negative photoresist is one that is initially soluble and after crosslinking the features of the device that are desired, the remaining uncrosslinked photoresist can be washed away. A positive photoresist is solid from the beginning and UV light is used to solubilize the unwanted regions on top of the wafer leaving behind the channels needed for imprinting. The photoresist layers are developed, and the remaining cross-linked material is left behind on the silicon wafer to form the master template. Polydimethylsiloxane (PDMS) can be poured over the template and later peeled off to create the microfluidics device. Once the device is fabricated, it can be bonded on glass with the side with channels. This causes the channels to be fully enclosed albeit the entries for the input and output which are made prior to bonding. Figure 2.6 shows a summary of the general photolithography and soft lithography process:

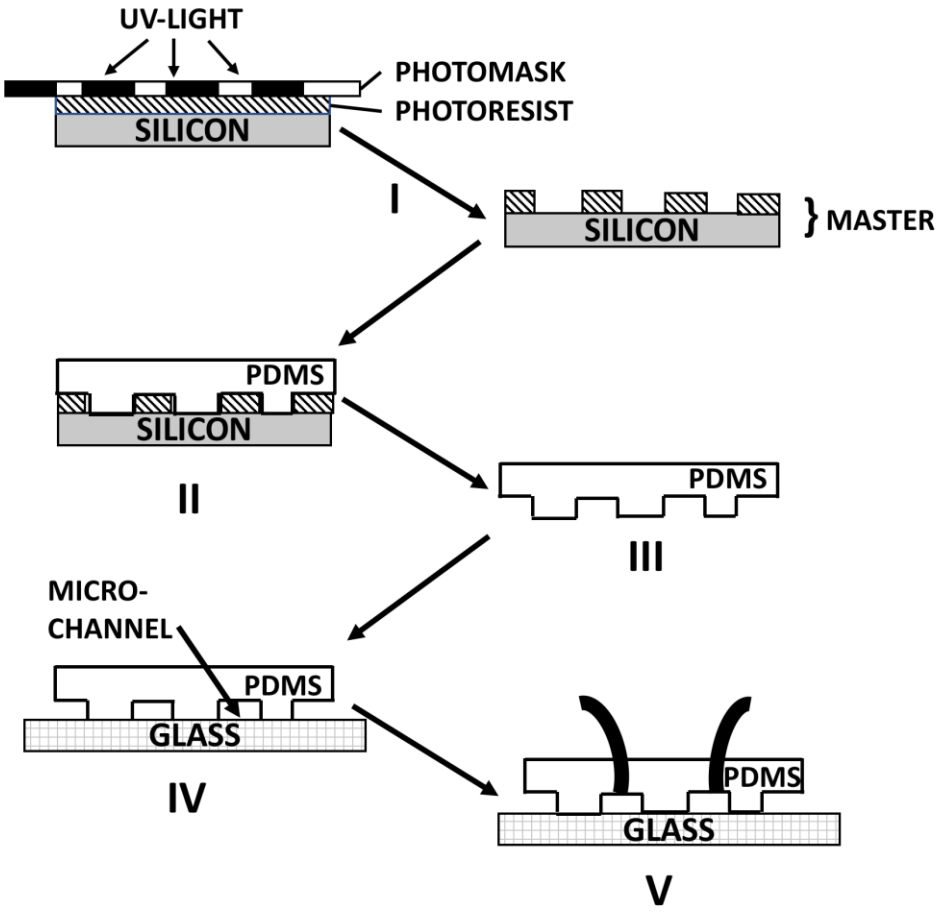


Figure 2.6: Photolithography and Soft-Lithography Process: **(I)** Photolithography process. **(II)** Poured PDMS on the master and cured. **(III)** Peeled PDMS from the master. **(IV)** Sealed against flat surface (e.g. glass). **(V)** Connected tubing for *use*. (Adapted from Seemann, 2012)

2.5 Experimental Cell Lines

In order to assess the efficacy of loaded drug carriers on neuroblastoma, several cell lines can be used as experimental 2D models; among those cell lines are KELLY human neuroblastoma cells, SKNAS cells, and SH-SY5Y human-derived cells. KELLY cells have been studied for their receptors and released hormones, which are indicative of neuroblastoma cells *in vivo* (Schlumberger, Jäggin, Tanner, & Eberle, 2002). SKNAS neuroblastoma cells have been utilized in animal studies for inducing the expression of neural factor kB (NF-kB) in mice (Tsutsumimoto, Williams, & Yoneda, 2014). Adrenergic SH-SY5Y

human-derived neuroblastoma cells are frequently studied for changes in the neuron-like cells' behavior based on environmental changes, such as temperature change (Tsutsumimoto et al., 2014).

KELLY cells are used as a great experimental model for neuroblastoma. The expression of melanin-concentrating hormone (MCH) receptors is important since it is commonly found in human neurons. This cell line also represents models for analysis of complement biosynthesis by human neurons. Kelly cells express the mRNA for MCH similar to that of the human brain. It is important to note that the major site of MCH expression is the central nervous system (Schlumberger, Jäggin, Tanner, & Eberle, 2002).

III. Project Strategy

The team has developed a project strategy in order to assure project success. This section addresses the client's needs and wants. First, the initial client statement is introduced. Second, the technical design requirements are identified. Third, design requirements based on engineering standards will be addressed. Then, a revised client statement will be established. Finally, taking all aspects of the project into consideration, a project approach is explained in order to ensure proper time management.

3.1 Initial Client Statement

The initial client statement as given by the team's advisor, Professor Jeannine M. Coburn, PhD is:

"Develop a fabrication technique to obtain water-insoluble, spherical particles composed of chondroitin sulfate for sustained release of chemotherapy for neuroblastoma treatment."

As more information is discovered by the team, as well as meeting with the advisor and receiving feedback, a revised client statement has been crafted and is presented in a later section.

3.2 Design Requirements

In this section, the design requirements of the project will be outlined. The requirements are grouped into project objectives, constraints, functions, and specifications. Some of these requirements are related to each other, such as defining a specification in relation to an objective. In contrast, not all requirements can be quantified and a qualitative explanation will be given instead.

3.2.1 Objectives

The objectives of the project are focused on creating a DDS that facilitates chemotherapeutic treatment for neuroblastoma. This focus was determined based on the needs of the client and discussions during project team meetings. The objectives are split into two categories and are formulated based on the initial client statement, requirements to develop a chemotherapy drug carrier to treat neuroblastoma, as well as the limitations of current solutions. The two categories are objectives for the particle fabrication system (referred to as "Fabrication System") and the resulting DDS (the loaded particles; "DDS"). The two categories and their respective objectives appear in Figure 3.1 below:

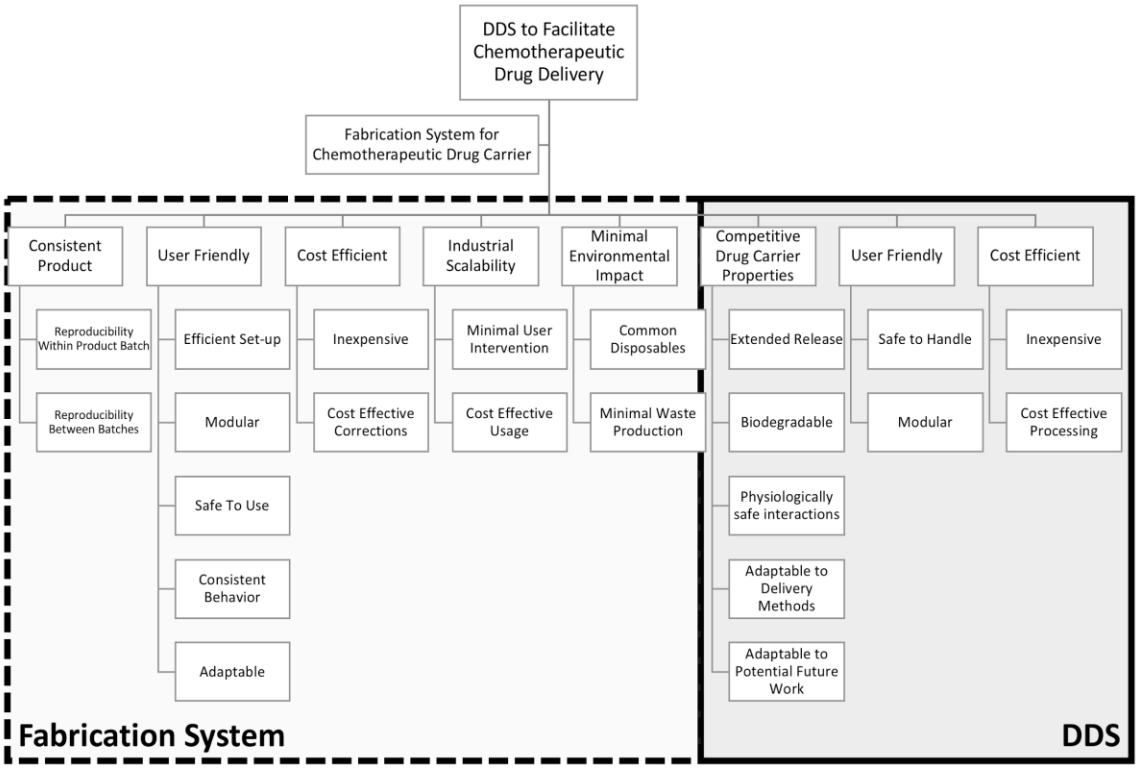


Figure 3.1: Objective and Sub Objective Tree

Table 3.1 through Table 3.8 explains the definition for each objective listed in Figure 3.1.

Table 3.1: Consistent Product and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Consistent Product	The immediate output of the fabrication method must be produced with consistent and replicable qualities
Reproducibility within the product batch	The qualities of the immediate output of the fabrication method within one produced batch must not be statistically different
Reproducibility between product batches	The qualities of the immediate outputs of the fabrication method between two separately produced batches using the same conditions must not be statistically different

Table 3.2: User Friendly and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
User Friendly	The users of the fabrication system must be able to conduct the fabrication process with ease
Efficient set-up	The users must be able to follow a protocol to initiate the fabrication process without outside help or large amounts of human labor
Modular	The fabrication system must include modular resources so that equipment can be easily stored, reused, and/or replaced
Safe to use	The fabrication system should not cause any harm to the user conducting the fabrication process
Consistent Behavior	The fabrication system settings (condition variables) and corresponding behavior are replicable
Adaptable	The fabrication system must easily allow for improvement, further research, experimentation on alternative settings and corresponding outputs, and optimization

Table 3.3: Cost Efficient and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Cost Efficient	The aggregate value of the materials does not use a significant portion of the project budget
Inexpensive	The individual materials must be of a reasonable cost or available from the lab stock
Cost effective Corrections	Any corrections that may potentially arise must be able to be dealt with at a reasonable price (using reusable materials or inexpensive disposables)

Table 3.4: Minimize Environmental Impact and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Minimize Environmental Impact	The materials used as part of the fabrication system do not cause substantial harm to the environment
Common Disposables	Any disposables used must be able to be disposed of properly
Minimal Waste Production	The fabrication system should aim to reduce any gaseous, liquid, or solid waste

Table 3.5: Industrial Scalability and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Industrial Scalability	The fabrication system should be able to be adapted to an industrial production scale
Minimal User Intervention	The fabrication system should be able to operate with automated systems or with minimal human labor or intervention
Cost Effective Usage	Should the fabrication system have parts that must be replaced, these should not affect the overall market competitiveness of the system

Table 3.6: User Friendly and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
User Friendly	The final output must be easy to handle for users
Safe to Handle	The final output must not cause any bodily harm or injury to the users
Modular	The final output must be capable of being stored for later use

Table 3.7: Competitive Drug Carrier Properties and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Competitive Drug Carrier Properties	The drug carrier material properties must be competitive to the current gold standard by providing sustained release and inactive by-products
Sustained release	The release profile must occur during a clinically relevant time period
Physiologically Safe Degradation	Any by-products present due to the drug carrier material degradation must not create any adverse effects to the body
Physiologically Safe Interactions	The drug carrier material must not worsen the condition being treated
Adaptable to Delivery Methods	The drug carrier properties should lend themselves to different potential forms of delivery
Adaptable to Potential Future Work	The drug carrier properties must easily lend themselves to improvement, further research, experimentation, and optimization

Table 3.8: Consistent Product and Sub-Objectives Definitions

Objective & Sub-objectives	**Definition**
Cost Efficient	The aggregate value of the materials and any additional processing to acquire the final product does not uses a reasonable portion of the project budget
Inexpensive	The individual materials must be of a reasonable cost or available from the lab stock
Cost effective Processing	Any processing that may be needed must be able to be dealt with at a reasonable price (using common methods and/or available supplies)

The project team has been able to identify various objectives that suit the needs of both the client and the project team. However, for the design process the project team also had to use a system to identify what objectives could be categorized as wants and which ones are needs. With this categorization the project team can then identify specific design functions that must be met to satisfy the chosen needs.

Due to the need of prioritizing objective, the project team completed a series of pairwise-comparison charts (PWC) for the main objectives and individual sub objectives under each main objective as listed in the above tables (Table 3.1 through Table 3.8). The comparisons were done using a scale of 0, .5, and 1. The objectives were placed in the matrix row and column-wise. Starting with objectives in each row, a score was given to each comparison to the objective on the respective column, left to right. A score of 0 was given when the row objective was less important than the column objective. A score of .5 was given if they had similar importance, and a 1 was given if the row objective was more important than the column objective. The PWC used to identify the scores for each objective can be found in Appendix B. The following is a summary table (Table 3.9) for all the objectives and their scores.

Table 3.9: Main Objective Rankings

Project Category	Main Objectives	Score
Fabrication System	User Friendly	5.5
	Cost Efficient	4
	Consistent Product	3
	Industrial Scalability	0.5
	Minimal Environmental Impact	0.5
DDS	Competitive Drug Carrier Properties	6.5
	Cost Efficient	6
	User Friendly	3

The team identified that objectives scoring 3 or above are identified as needs and all others are considered wants. Therefore, the fabrication system must: be user friendly, be cost efficient, and have

consistent product. The DDS must: have competitive drug carrier properties, be cost efficient, and be user friendly. To further analyze how to identify functions to satisfy the needs of the project, the sub-objectives for each identified need were also scored in order to identify what sub-objectives are not necessary for the overall completion of the main objective. The PWC can also be found in Appendix D. Table 3.10 shows the summary scores for the ranked sub-objectives.

Table 3.10: Sub-objective Ranking

Project Category	Main Objective	Sub-Objective	Score
Fabrication System	User Friendly	Safe to Use	3.5
		Adaptable	3
		Modular	1.5
		Consistent Behavior	1.5
		Efficient Set-up	.5
	Cost Efficient	Inexpensive	.5
		Cost Effective Corrections	.5
	Consistent Product	Reproducibility Between Batches	1
		Reproducibility Within the Batch	0
DDS	Competitive Drug Carrier Properties	Sustained release	3.5
		Physiologically Safe Interactions	3.5
		Adaptable to Potential Future Work	1.5
		Adaptable to Delivery Methods	1
		Biodegradable	.5
	Cost Efficient	Inexpensive	.5
		Cost effective Processing	.5
	User Friendly	Safe to Handle	.5
		Modular	.5

Using the scores from Table 3.10 the team could identify what sub-objectives could be considered less important to achieving the corresponding main objective. If the sub-objectives scored the

same as another sub-objective of the same category that only had 2 objectives, both were considered as needs to achieve the main objective. To create user friendly fabrication system the project team will consider the following sub-objectives: safe to use, adaptable, modular, consistent behavior, and efficient set-up. To create a cost-efficient fabrication system the project team will consider the following sub-objectives: inexpensive and cost-effective corrections. To create a consistent product the project team will consider the following sub-objective: reproducibility between batches. To create a DDs with competitive properties the project team will consider the following sub-objectives: sustained release, physiologically safe interactions, adaptable to potential future work, and adaptable to potential future work. To create a cost-efficient DDS the project team will consider the following sub-objectives: Inexpensive and cost-effective processing. To create a user-friendly DDS the project team will consider the following sub-objectives: safe to handle and modular. The objectives and sub-objectives chart can now be seen color coded in Figure 3.2 to illustrate the needs and wants.

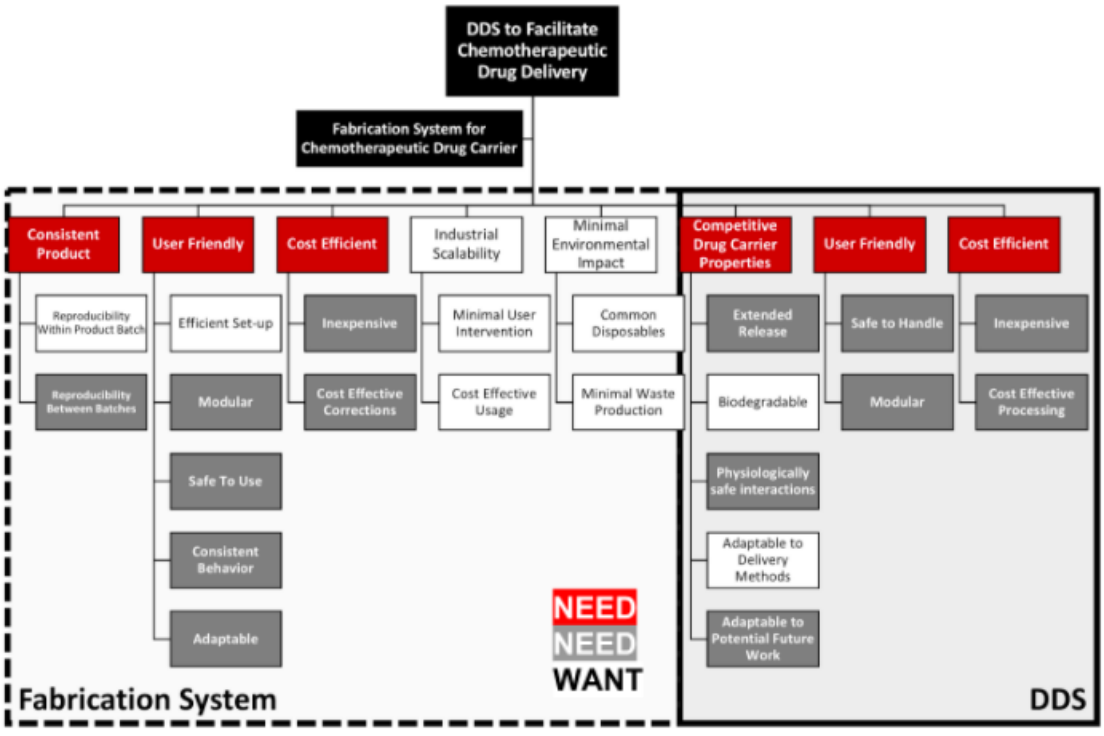


Figure 3.2: Color-Coded Objectives and Sub-Objectives Tree

3.2.2 Constraints

The general project constraints are an important part of the design requirements since they are the factors that would limit the success of the project outcomes. Therefore, the team made identifying the constraints a priority. The following table (Table 3.11) shows a list of the general project constraints along with their definition.

Table 3.11: General Project Constraints

Constraint	Definition
Time	All project work must be completed by April 20, 2018. Testing timeline is limited to (~6 months; Nov-April 2018).
Money	The team has a total budget of $1000 ($250/member).
Material Availability	The materials to be used in the project must be either purchasable by the project team or available from the WPI Coburn Laboratory.
Sterility	The materials chosen must be capable of being sterilized with available sterilizing techniques.
User Safety	Materials must be able to be handled by users without the need of special PPE.

The constraints that the project team could identify are time, money, material availability, sterility, and user safety. First, time is a constraint since the Major Qualifying Project (MQP) timeline is limited to the school year (September- May 2018). Due to this constraint, the project team must complete all work by April 20, 2018 (project presentation day) as the client must receive ample time to review the final report and the team must present the project on project presentation day. The months of September and October were utilized to obtain a theoretical understanding of the project and possible solutions. The experimentation phase of the project design was limited to November - April 2018 (~6 months). Secondly, money is a constraint since MQP team members are typically allowed a budget of $250 per team member. The project team contains a total of 4 students; therefore, the budget is $1000. Third, the materials that are chosen to complete the project must be easy to procure by the project team. Due to the

team budget, the team must be able to obtain and/or process said materials through the purchasing system WPI has in place or the WPI Coburn Lab. The fourth constraint is sterility. The final design must incorporate sterilization of the final product due to its expected use in the human body. The sterilization technique must be easily achievable with the resources the project team has or can procure. Lastly, user safety is the final constraint since the project team will be working hands on during the experimentation phase. The project team has common PPE and lab supplies to handle and synthesize the resources for the project. Therefore, the chosen resources must not need professional level equipment to handling and processing purposes.

3.2.3 Functions and Specifications

The project team identified functions and specifications that should be met. The chosen functions and specifications serve as part of the design criteria for our project to successfully meet the main objectives. The objectives that were identified as needs will serve as a guide to drive the fabrication device and DDS criteria.

According to the objective analysis, the following must be met for the fabrication device:

1. consistent product - reproducibility between batches
2. user friendly - modular set-up
3. user friendly - safe to use system
4. user friendly - consistent behavior
5. user friendly - adaptable to future needs
6. cost efficient - inexpensive base materials
7. cost efficient - cost effective corrections

According to the objective analysis, the following must be met for the DDS:

8. competitive drug carrier properties - sustained release

9. competitive drug carrier properties - physiologically safe interactions
10. competitive drug carrier properties - adaptable to potential future work
11. user friendly - safe to handle
12. user friendly - modular
13. cost efficient - inexpensive
14. cost efficient - cost effective processing

Using this objective breakdown will aid in communicating the exact objectives that each individual criterion satisfies. Once the criteria are introduced, the objective that it satisfies will be identified by the corresponding number in parentheses. Criteria 1- 4 refers to the fabrication device while criteria 5-9 refers to the DDS.

3.2.3.1 Criterion 1: Produce DDS with no Significant Difference Between/Within Batches

The first criterion for the fabrication system is to produce a DDS with no significant difference between or within batches. This criterion satisfies (1) and (4). These objectives will allow the team to operate a fabrication system with confidence that the system itself should not contribute to the variances in the batches. Therefore, when specific parameters for the system are set, the outcome should be statistically similar both within one run or batch, as well as between batches. To verify the success of this criterion the team must run more than 1 batch (at least 3) with similar parameters so that all variables that may alter the output are eliminated. Then the team will have to run statistical analysis using t-test and ANOVA to obtain a p-value that is > 0.05 to prove that the batches are statistically similar.

3.2.3.2 Criterion 2: Consists of Detachable Parts and Works with Tunable Characteristics

The second criterion is for the fabrication system to consist of detachable parts as well as being operable with tunable characteristics. This criterion satisfies (2) and (5). The fabrication system must consist of individual components that can be removed and interchanged as experimentation continues for either design considerations, improvements, or troubleshooting. Additionally, the fabrication system

should allow itself to either be easily optimized by using different parameters or by allowing easy switching between components. To verify this criterion, the team will have to design a system that does not have permanency. The system must contain options for operable ranges so that a broad range of experimentation is permitted. Creating a fabrication system that is modular is advantageous since modularity allows for easy replacements and does not halt experimentation progress.

3.2.3.3 Criterion 3: Must Be Operable with Common Personal Protective Equipment (PPE) and Lab Resources

The third criterion of the fabrication system is to be operable with common labwear such as safety glasses, gloves, lab coat, etc. This criterion satisfies (3). The fabrication system must not inherently require industrial-grade equipment to operate constantly. The operators of the fabrication system must not require extensive training for proper operation of the system. To verify this criterion, the team will need to participate in research that involves exploring fabrication methods that can easily be achievable in the lab as a benchtop model. The success rating of this criterion is of a binary value where the chosen fabrication system will either be or not be operable with common PPE and lab resources.

3.2.3.4 Criterion 4: Purchased Components must not exceed 50% of the Project Budget

The fourth criterion is to utilize components that will not require the team to use more than 50% ($500) of the allotted project budget. This criterion satisfies (6) and (7). When selecting what resources or components to use within the fabrication system, the project team must take into account the usability and durability of each component. In doing so, the team assures that both the base level costs as well as replacement parts do not exceed $500. An example of a non-ideal component would be something that initially requires a substantial fraction of the budget but is prone to breaking and has costly reparations. An example of a suitable component is one that initially is within budget and does not require costly reparations.

3.2.3.5 Criterion 5: Load and Release DOX

The fifth criterion is to load and release DOX. This criterion satisfies (8). The leading drug for treatment and testing new treatment options for neuroblastoma is DOX. The project is making use of this drug in order to be consistent with published literature and research. Therefore, this criterion is important since the loading and releasing of this model drug is imperative to obtain a DDS with competitive drug carrier properties. The sustained drug release is also crucial to keeping the dosage safe and efficient. To verify this, the project team must develop an SOP for measuring the loaded and released drug from the DDS. The loading of the drug must be quantifiable in the chosen DDS. Additionally, the release of the drug must be quantifiable and observable over a substantial period of time. Finally, the DOX activity must be confirmed as it is being released over time. One technique that can be used is mass spectrometry and/or spectrophotometry.

3.2.3.6 Criterion 6: Low Cell Toxicity

The sixth criterion is that the unloaded DDS must have low cell toxicity. This criterion satisfies (9). Although the delivered drug is meant to induce apoptosis in cells, the unloaded DDS must not be the cause of cell death since its purpose is to reduce the untargeted activity of the drug. This purpose would only be satisfied by a DDS that would inherently not kill surrounding cells unless the drug was being actively released. To verify this, the project team can perform experiments using positive and negative controls for cell viability and compare it to the test results of cell viability with the resulting DDS in the absence of the chemotherapeutic drug.

3.2.3.7 Criterion 7: Tunable Material Characteristics

The seventh criterion is for the DDS material to have tunable characteristics. This criterion satisfies (10). As this project is in an exploratory phase, it is highly important to consider options for materials that are tunable to suit various needs and potential future experimentation. DDS are typically difficult to optimize since there are so many variables to consider when creating one. However, if the

materials used for the DDS have tunable characteristics, it allows for a more robust system that is customizable for more applications. Such characteristics that would be ideal to experiment with and optimize are size and concentration. The project team can satisfy this criterion by choosing a material that has a shape that can easily be characterized at different sizes as well as customizability that can be performed at the benchtop.

3.2.3.8 Criterion 8: Handled with Common PPE and Lab Resources

The eight criteria of the project are to create a DDS that can be easily handled with common PPE and lab resources. This criterion satisfies (11) and (12). The DDS must not inherently require industrial-grade equipment and personal protection to handle both during and after production. The handling of the DDS material must not require extensive training to maintain safety standards within the lab. To verify this criterion, the team will need to participate in research that involves exploring DDS materials that do not require uncommon handling procedures. The success rating of this criterion is of a binary value where the chosen DDS material will either be or not be safe to handle with common PPE and lab resources.

3.2.3.9 Criterion 9: Purchased Materials must not exceed 50% of the Project Budget

The ninth criterion is to utilize materials that will not require the team to use more than 50% ($500) of the allotted project budget. This criterion satisfies (13) and (14). When selecting what materials and resources to use within the DDS, the project team must take into account the usability and durability of each component. In doing so, the team assures that both the base level costs as well as processing methods do not exceed $500. An example of a non-ideal material is one that initially requires a substantial fraction of the budget and has expensive processing methods. An example of a suitable material is one that is initially within budget and has cost efficient processing methods.

3.3 Design Requirements (Standards)

3.3.1 Specifications

This section will cover the industry, regulatory, engineering standards as per the BME MQP Project Guide. It will cover topics and standards such as sterility, cytotoxicity, and biocompatibility. The team will also incorporate other industry standards and guidelines as per FDA, USP, ISO, UL, ASTM, and others.

The team will follow standards and regulations with the final product of the project. The U.S. Food and Drug Administration (FDA) has created a term called a combination product. A combination product is defined as:

"A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, e.g., to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose" (Combination product definition.2017).

The regulations of combination products are mentioned in Title 21: Foods and Drugs from the Code of Federal Regulations (CFR) in which Parts 3 and 4 from subchapter A in Chapter I apply. Part 3 is about the Product Jurisdiction. The FDA describes it as "the regulations pertaining to current good manufacturing practices and postmarket safety reporting requirements for combination products" (FDA, 2017). Part 4 of the 21 CFR talks about the regulations of combination products in which the FDA describes it as "the regulations pertaining to current good manufacturing practices and postmarket safety reporting requirements for combination products" (FDA, 2017). Apart from the FDA, ISO 10993 entitled "Biological Evaluation of Medical Devices" includes considerations for products that could have a

cytotoxic and other effects that could potentially harm the human body (Yu et al., 2016). Under this ISO number, there are titles in which explains the guidelines that the product has to go through to be approved as safe. Title 3 of the ISO 10993 is used to test for genotoxicity, carcinogenicity, and reproductive toxicity. Title 11 of the ISO 10993 is used to test for any systemic toxicity.

The FDA has published various guidelines about how to approach the design of a product or device so it falls under the regulations and be able to be accepted and approved. One of the guides is the Guidance for Industry and FDA Staff - Early Development Considerations for Innovative Combination Products (2006) (FDA, .2017). This guide is intended to provide a context for initial discussion in the type of scientific and technical information that might be necessary for the investigation and marketing applications of combination products. Another important guide is the FDA Guidance of Industry: Container Closure Systems for Packaging Human Drugs and Biologics. This guide has the information about the quantification and qualification of control of the packing components (FDA, 2017).

The ISO 10993-5 states the tests for in vitro cytotoxicity. In here, the FDA mentions the extract, direct contact and indirect contact tests as well as the agar overlay assay. The agar overlay assay is used as a standard procedure for the evaluation of the cytotoxicity of biomaterials by using a monolayer of cells that are grown on the bottom of a petri dish and are later stained with neutral red while the test material is placed on top of the agar. If none of these tests work, the MTT Assay and MEM Elution, which reveals cytotoxic effects of potential leachable from a device/material. is recommended instead. Other studies that are suggested include a stability study, Alamar Blue Assay for a cell cytotoxicity study and hemocompatibility study such as the ASTM F756: standard practice for assessment of hemolytic properties of materials.

Dose delivery testing is also applicable for our DDS. The ISO 11608 specifies how needle-based injections are handled for medical use, given that the system is administered through a needle. The USP

also addresses some chapters about dose delivery testing including chapter 698: Deliverable Volume, chapter 755: Minimum Fill and chapter 905: Uniformity of Dosage Units.

The USP General Chapter 800 Hazardous Drugs tells the team on how to handle hazardous drugs. Their criteria for a hazardous drug is any of the following:

- Is it carcinogenic?
- Is it teratogenic or develop toxicity?
- Does it cause reproductive toxicity in humans?
- Does it cause organ toxicity at low doses in humans or animals?
- Is it genotoxic?
- Does it mimic existing hazardous drugs in structure or toxicity?

Following these criteria, doxorubicin falls under the category of hazardous drugs and therefore should be handled with care according to the guidance and standards along this chapter. The USP has a "Drug Release" guide which is found in chapter 724. This chapter dictates a test to determine compliance with drug-release requirements.

3.4 Revised Client Statement

After considering the requirements of the project, the revised client statement is to:

"*Develop a microfluidics device that fabricates methacrylated chondroitin sulfate microparticles. Validate microparticle loading of doxorubicin for the sustained release of the drug to induce apoptosis in neuroblastoma cells*."

3.5 Management Approach

List of Major Milestones - Here the team outlines the project's major milestones along with their estimated dates of completion. The major milestones were divided according to how the team decided to approach the problem and also divided into terms for a more equally distributed workload throughout the 2017-2018 academic year. These dates can also be found in Appendix C.

3.5.1 Work Completed in A-Term (Sep-Oct 2017)

In A-term, the team started working on the project by defining the problem and the project goals. The continually augmented literature review provides the background and the significance of the problem along with how the project and specific aims will advance the field. The literature review was completed by April 15, 2018 since it passively added more information due to the nature of the project. The team created a project strategy in which includes initial client statement, objectives, constraints, a revised client statement and the project approach. The project strategy was completed by October 8, 2017. After creating the project strategy, the team developed a Gantt chart to have a timeline with important deadlines and developed the breakdown of the project.

3.5.2 Work Completed in B-Term (Oct-Dec 2017)

In B-term, the team continued developing alternative designs of the project. Along with this, the team made a needs analysis, functions and alternative design concepts. Along with the alternative designs, the team has conducted a design verification and validation process, including experiments and preliminary data.

3.5.3 Work Completed in C-Term (Jan-Mar 2018)

During C-term, the team completed validating the parameters to run a successful particle fabrication procedure. This involves the validation of the set-up and protocols to follow to allow for

reproducibility and replicability of the produced DDS. The team started with surrogate solutions in order to conserve expensive resources, and gradually move on to the final polymer that will be in use for the final product. Additionally, this time-frame also served to begin exploring the post-processing procedures for the DDS once produced by the particle fabrication procedure.

3.5.4 Work Completed in D-Term (Mar-Apr 2018)

During D-term the team had an *in vitro* characterization of the DDS on neuroblastoma cells. The team followed the required protocols for cell culturing and cytotoxicity assays. Along with the required cytotoxicity assays, the team wrote conclusions about the project. The conclusions talk about the analysis of limitations of the project and the recommendations that the team want to address for future work.

Finally, the team finalized the final report and prepared for the final presentation at the end of the academic year. The report was expected to be completed and submitted by April 25, 2018 while the final presentation was finished by April 20, 2018. Note that the date for the final presentation is different than the one the team has written down in the Gantt chart, which will be shown in the next section.

IV. Design Process

The design process will be what follows the project strategy. In the project strategy, the design requirements were identified and ranked to gain a better understanding of the project's goals. To better understand the project in terms of needs and feasibility as well as being contrasted against competing designs, any and all necessary criteria that was deemed important by the team was assessed and quantified in this chapter.

4.1 Needs Analysis

To begin the quantitative analysis of the means to each function, the objectives that were defined as needs in the project are organized in percentage form. Table 4.1 lists the projects needs in percentage from most important to least important.

Table 4.1: Ranked Objectives by Percentage

Project Category	Main Objectives	Percentage
Fabrication System	User Friendly	41
	Cost Efficient	30
	Consistent Product	21
	Industrial Scalability	4
	Minimal Environmental Impact	4
DDS	Competitive Drug Carrier Properties	42
	Cost Efficient	39
	User Friendly	19

By prioritizing all the objectives, the needs and wants became clear. A need for this project is a component of feature that is crucial to the success of the project outcomes. According to the PWC

comparison chart (Appendix 2), the needs for the project fabrication device are user friendliness, cost efficient, and consistent product. The needs for the project DDS are competitive drug carrier properties, cost efficiency, and user friendliness.

A want for this project is component or feature that would be nice to have but is not crucial to the successful completion of the project outcomes. The wants that were identified are for the fabrication system and include industrial scalability and minimal environmental impact. The final project goal could nonetheless be achieved without these specific traits but they could still help in improving the device itself and open up future applications for it.

4.2 Concept Map

To make an initial conceptual design for the drug delivery vehicle for the treatment of neuroblastoma, it can be broken down it several simple parts. First is the selection of the polymer material for the drug carrier. The client wants the team to utilize CS which is a soluble biopolymer in the DDS. First would be to decide if this is the right polymer to be used. There needs to be alternative materials that can be used for the DDS which should then be contrasted against each other. For CS to be a contender it needs to be made insoluble first. To make polymeric hydrogel drug carriers, and to allow a mode of crosslinking to prevent particles from coalescing in the DDS fabrication system, there would be a need for a component to facilitate crosslinking of the polymer. A device to help fabricate the polymer or material into a spherical microparticle which would be beneficial for the treatment of neuroblastoma would then be needed. Lastly once the final design is completed and the drug delivery vehicle is fabricated, it needs to be loaded with the chemotherapeutic drug. To simplify the steps needed to make the final design, it is shown schematically below in Figure 4.1.

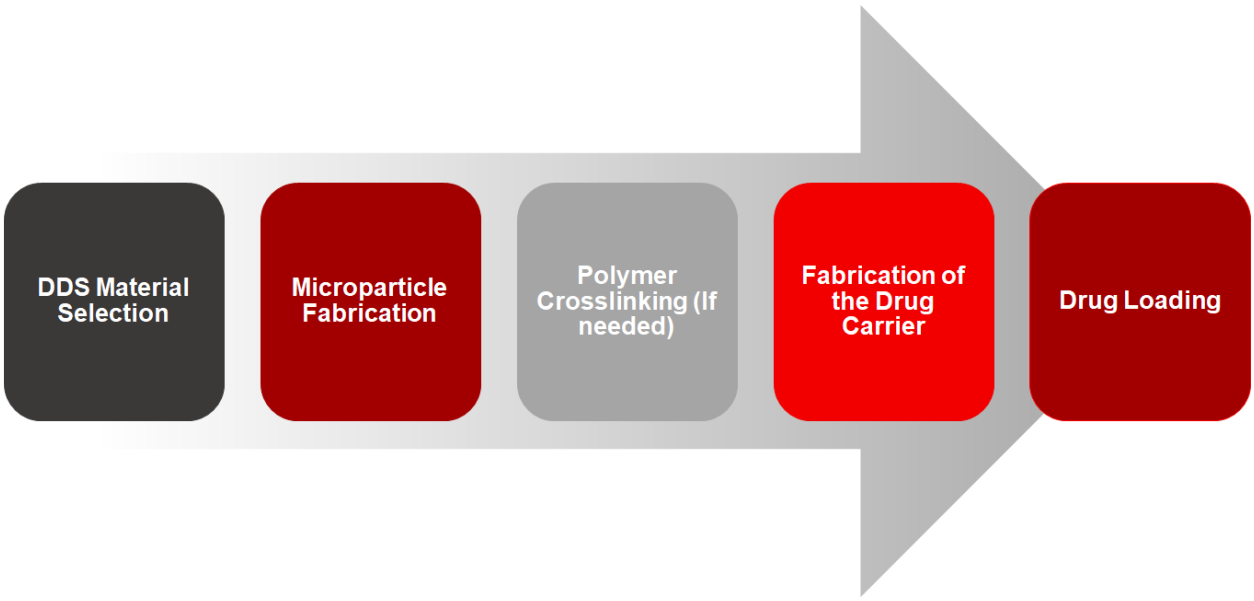


Figure 4.1: Conceptual Flow Diagram for the Fabrication of a DDS for Neuroblastoma Treatment

4.3 Alternative Design Concepts

Functions were previously identified in order to ensure proper completion of the project objectives. Design alternatives for the fabrication system and DDS of the project were identified to ensure a robust design process. Each mean was identified by taking into consideration the project constraints and functions. Then, the identified means were compared against the identified needs. This evaluation can be found in Appendix D.

4.3.1 Alternative Design Concepts for the Drug Carrier

The preferred characteristics of drug delivery materials as mentioned in the literature review are polymeric hydrogel spherical particles. Hydrogels can be composed of many types of polymers but the project has identified two main types: synthetic and natural polymers. Two synthetic polymers that were identified as options for materials to use in this application were PVA-MA and PLGA-PEG. Two natural

polymers (or semi-natural) that were identified for materials to use in this application were PCL-PVA and CS-MA. The identification of the best materials can be found in Appendix D.

Out of the 4 options, PVA-MA and CS-MA were identified to meet the project needs the best. However, CS-MA has inherent properties that would make it a better carrier material of the specific drug the project is testing against. Due to the electrostatic interactions between CS-MA and DOX, CS-MA has a better advantage on competitive drug carrier properties than PVA-MA.

4.3.2 Alternative Design Concepts for the Particle Fabrication Device

The preferred method for making spherical microparticles is the use of co-flow systems as mentioned in Section 2.4 in the Literature Review. Particle size can be controlled by varying the flow rates of the liquid phases or by altering the dimensions of the flow focusing channels. The particle fabrication device could be made using one of three geometries: co-flow, flow focusing or cross flow. Various designs and models for the alternate designs which utilize at least one of the three geometries and not only limited to microfluidics devices are listed below along with their pros and cons.

4.3.2.1 Coaxial Co-Flow Needle

The coaxial co-flow needle allows for a robust droplet generator that can be made using precision machinery or outsourced and purchased as a readymade electrospinning needle from companies such as Rame-Hart Instrument Co. Though the company specializes in making coaxial needles for electrospinning functions, they can be custom made to be used specifically for generating microparticles using a co-flow system. Though it can be easily obtained, each piece is expensive, and it would be difficult to calibrate it and clean. If the device were to clog and malfunction, replacement devices would make it expensive to keep using. Also, fluid flow cannot be visualized and quantified under a microscope. A schematic for the device is shown below in Figure 4.2. Dimensions for custom needles are limited to the standard gauge diameters that they are manufactured at and would be difficult to obtain smaller flow channels without

having to modify the device either by narrowing the needle by heat treating and expanding the metal or by addition of a component.

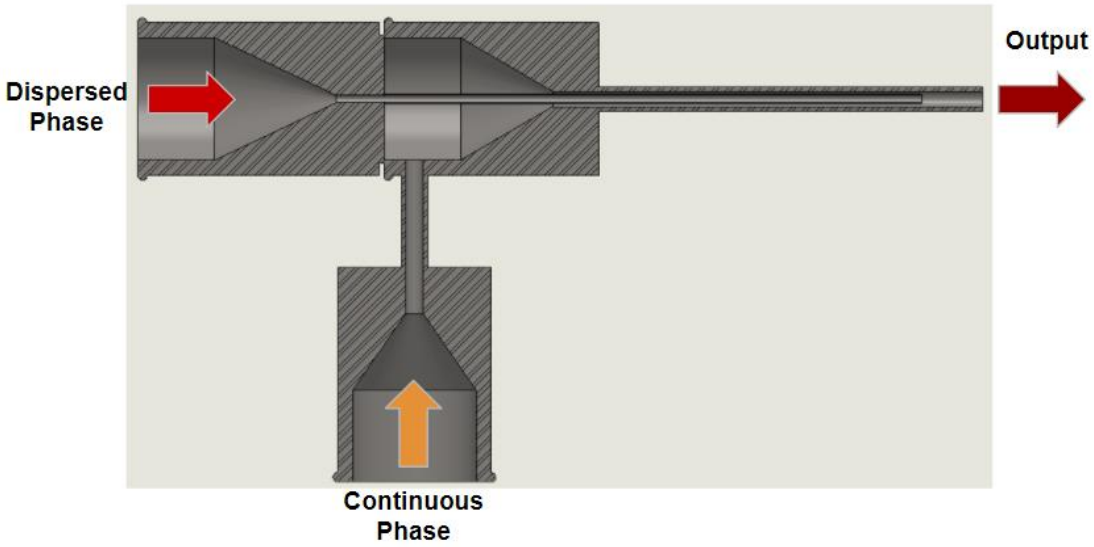


Figure 4.2: Cross-Section of a Coaxial Co-Flow Needle

4.3.2.2 Coaxial Glass Drawn Capillaries

This design is for a two-phase coaxial glass capillary co-flow droplet generator (Figure 4.3 and 4.4). It is made using glass capillaries which are readily available in the lab and can be stretched and pulled either manually or using an automated glass puller to get the desired channel dimensions. Variability between devices can be eliminated using specialized tools such as the Microforge MF-900 automated glass puller. The end of the glass capillary can be narrowed to form a narrow channel which can act as a flow focusing aperture. This will allow the dispersed phase to be easily sheared off to make microparticles. Since the device consists of very thin glass, it is susceptible to fracture. Fabricating the device to allow for concentric symmetrical alignment would be another challenge. To allow production of the particles in the microscale and potentially in the nanoscale, the inner capillary will have to have an inner diameter of at most 100 μm (Seemann, Brinkmann, Pfohl, & Herminghaus, 2012).

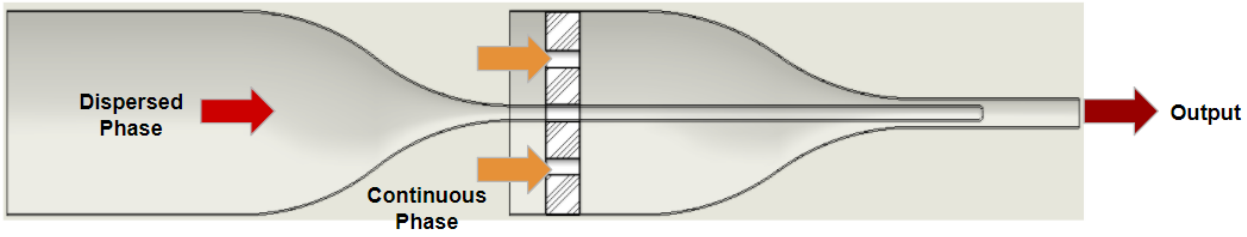


Figure 4.3: Schematic for a Coaxial Glass Drawn Capillary Co-Flow Device

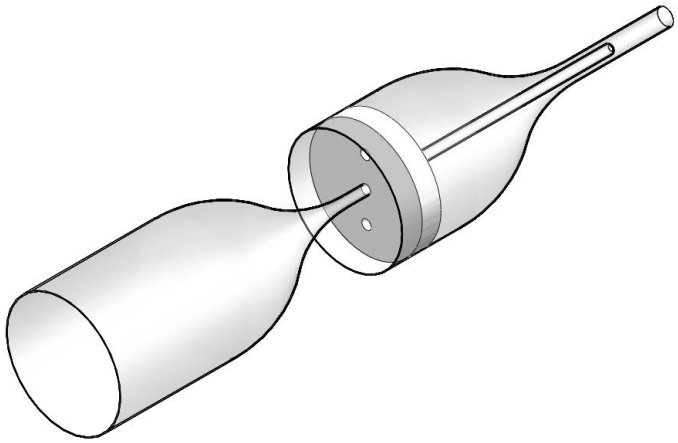


Figure 4.4: CAD Model for a Coaxial Glass Drawn Capillary Co-Flow Device

4.3.2.3 PDMS Based Co-Flow Microfluidics Devices

Microfluidics devices fabricated using soft lithography can be made with any desired flow geometry with modifiable channel dimensions. They are easy and cheap to manufacture and as multiple devices can be made using the same master template, it reduces variability between devices which is usually problem with systems incorporating glass drawn capillaries. The smallest channel size possible depends on the resolution of the photolithography process used to make the device. PDMS is clear and allows for easy monitoring. As of writing this paper, PDMS based co-flow microfluidics devices are utilized in the Coburn Lab by Natalia Vargas Montoya to make silk nanoparticles. Microfluidics device master templates currently available all have co-flow geometries with flow focusing channels ranging from 40 to 100 μm. In co-flow microfluidics the particle size is controlled by changing the flow rates and

it is easier to get smaller particles with co-flow geometries compared to other flow geometries. A CAD drawing of the device being used by Natalia is shown below in Figure 4.5.

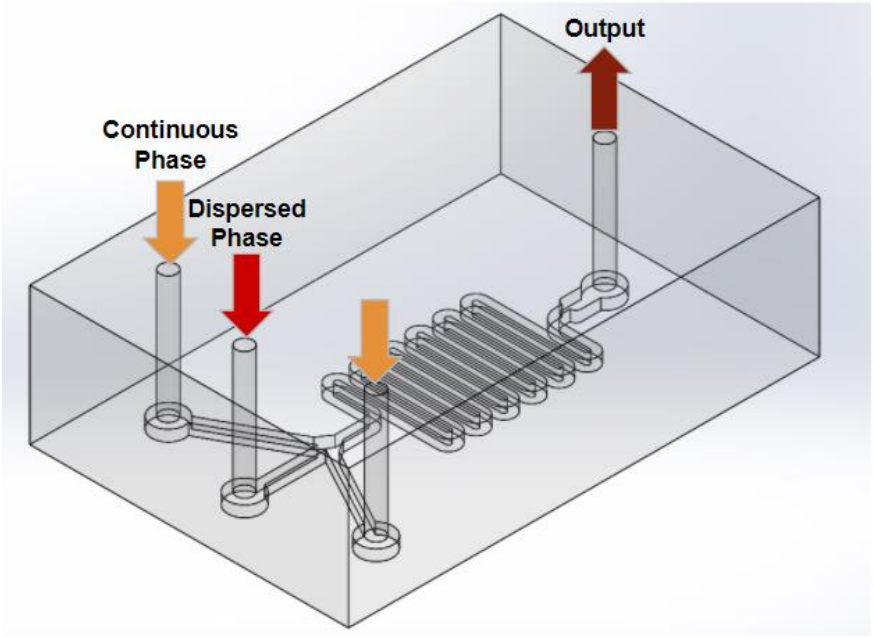


Figure 4.5: CAD Model for 3 Input Co-Flow/Flow Focusing Device

The design has a serpentine channel prevent silk particles from coalescing in the channels. The team theorizes that the serpentine channel could also serve to increase residence time of particles in the device for pre-curing purposes and to aid in the breakdown of the dispersed phase and formation of droplets by serving as an extended flow focusing channel (Zhou & et al.). Figure 4.6 shows a top-down image of the co-flow devices made using SolidWorks.

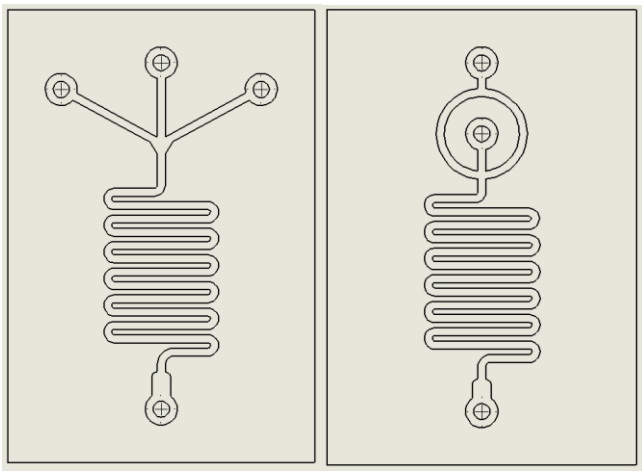
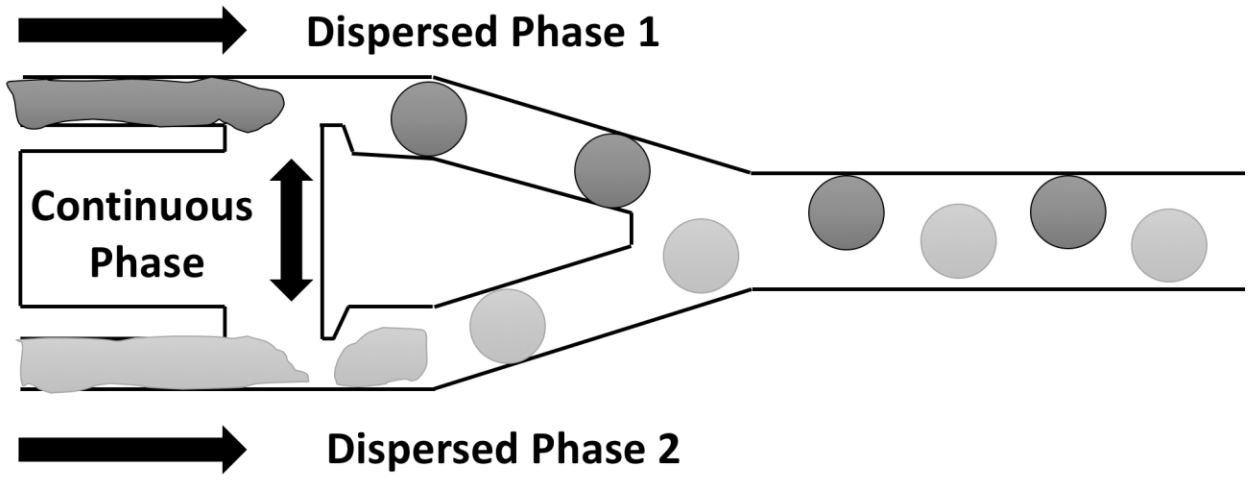


Figure 4.6: Schematic for the PDMS Based Co-flow/Flow Focusing Devices. (left) 3 input device, (right) 2 input variant

4.3.2.4 PDMS Based Cross-flow Microfluidics Devices

Cross-flow or T-junction microfluidics utilizes a variant of the flow focusing geometries where the dispersed phase or particle is pinched off by the continuous phase as it forces the fluid to flow against the channel walls. The width of the flow focusing aperture plays a role in particle size, but the primary deciding factors lie in varying flow rates and the wetting properties of the channel surface which affects interfacial surface tension. Figure 4.7 shows a variation of the regular T-junction that allows for the production of particles in two dispersed phases to allow for higher droplet frequency. Instead of having one channel for the dispersed phase, it can be flowed in parallel side by side, thereby, increasing production of particles two-fold. The primary disadvantage with cross-flow systems when compared to co-flow is that is that it more difficult to obtain smaller sized particles when using the same channel width and flow rates.



Adapted from (Seemann, Brinkmann, Pfohl, & Herminghaus, 2012)

Figure 4.7: Schematic of a Double T-Junction

Based off of the PWC charts in Appendix 2, the PDMS based microfluidics devices utilizing both co-flow and cross-flow were considered the best options. The final design that the team opted for was the PDMS based co-flow device due to the immediate availability of the master template required for device fabrication and its advantage over the crossflow geometry of T-junctions in the making of smaller sized particles.

4.3.4 Alternative Materials to Facilitate UV Permeability and Crosslinking

For the particles being produced in the particle fabrication device (droplet generator), there is a need for proper UV curing or crosslinking before collecting in the final output to ensure that the individual droplets do not mix and end up coalescing. The droplets, thus, should be properly cured while still flowing through the output tubing so as to guarantee that the individual particles retain their shape and form. This can be achieved using physical or chemical crosslinking, and in the case UV polymerization is selected, there would also be a need for a UV light source.

To facilitate UV-polymerization, the generated droplets must be flowed under UV light for a minimum amount of time to allow for proper crosslinking to occur. There is also a need for an initial dose of UV light treatment, or "pre-curing treatment", to ensure that the droplets do not collide and coalesce in the tubings themselves while still flowing. The droplets can be pre-cured in the PDMS device itself, provided the microfluidics channel has sufficient residency time for the particles to be UV treated or in the immediate output tubing. Efficiency of UV light penetrating through the PDMS device and the tubing is another factor that must also be taken into consideration when choosing a proper UV crosslinking device. The different crosslinking strategies as well as methods to enable UV crosslinking in the system are discussed below.

4.4.3.1 Tygon™ Output Tubing

The Tygon™ ND 100-80 Tubing from Saint-Gobain™ is a transparent flexible tubing of medical grade that can be used in the transfer of fluids into and out of the microfluidics device. It has an outer diameter of 0.06 in (1.524 mm) and an inner diameter of 0.02 in (0.508 mm). UV penetration and absorbance in the Tygon™ will vary depending on the type of tubing as well its wall thickness. There is no readily available information regarding UV light penetration in Tygon™ tubings and thus must be tested to check efficiency. With the aforementioned flow rates, particle velocity in this tubing will lie between 9.87 and 24.67 m/hr. The minimum length of tubing required to ensure a residence time of 20 minutes is approximately 8.22 m. That is a large amount of tubing that may be difficult to maintain and has a higher probability of getting clogged during operation. This may suggest that using a smaller length of tubing could facilitate pre-curing of the particles instead.

4.4.3.2 Polyvinyl Chloride (PVC) Output Tubing

VWR® Clear, Flexible PVC Tubing from VWR Scientific was chosen as a potential candidate for UV curing particles, due to the high penetrance of UV light through PVC material (Measurement of Optical Characteristic of Plastic by UH4150 Spectrophotometer) The tubing chosen has an outer diameter

of 0.125 in (3.175 mm) and an inner diameter of 0.0625 in (1.5875 mm). With the same flow rates, average particle velocity will lie between 1.01 to 2.53 m/hr. PVC tubing measuring at least 0.85 m will be required to ensure a residence time of 20 minutes for the particles. Due to the comparatively shorter length of tubing required, UV crosslinking of particles can be carried out with less material.

4.4.3.3 Polymethyl Methacrylate (PMMA) Output Tubing

PMMA has a very high UV transparency making it another candidate for the material in the tubing for UV curing (Measurement of Optical Characteristic of Plastic by UH4150 Spectrophotometer) However, it lacks the flexibility that is required in the setup for the microfluidics device which may become a major flaw in the design. As such, PMMA tubing was not obtained for testing or for future designs as the team decided that its one con far outweighed any advantage that it may offer in the UV crosslinking apparatus.

4.4.3.4 Microfluidics Channels in the PDMS Device

The team wanted to determine if it is possible to crosslink the particles in the PDMS based microfluidics device during particle formation. This would allow for a very simplified fabrication system utilizing mainly the device and would not require as much components. The rationale for this is that since PDMS is a clear material it should potentially allow the passing of UV light. Figure 4.8 below shows a to-scale drawing of the co-flow/flow focusing channels used in the microfluidics device for droplet generation. For pre-curing purposes, particles would be UV treated between the flow focusing channel, point A, and the end of the serpentine channel at point B. The channel consists of a rectangular cross-sectional area 100 μm x 100 μm ideally with a total length of roughly 27.94 ± 0.53 mm.

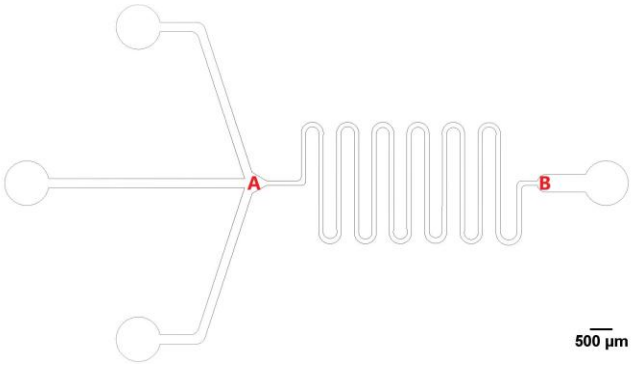


Figure 4.8: Scaled Image of the Schematic of the Microfluidics Device with Serpentine Channel

With volumetric flow rate lying between 2-5 ml/hr, generated particles flowing between points A to B with an average velocity 200-500 m/hr. Thus, the approximate residence time of generated particles lie between 5.59×10^{-5} s and 8.38×10^{-3} s. Minimum standard residence time for UV curing material is defined as 20 minutes by the client. This means that the particles do not reside long enough in the microfluidics device for sufficient UV treatment. If the cross section of the channels and flow rates remain the same, to allow for a residence time of 20 minutes would require a maximum length of approximately 167 m. That would require a lot of PDMS to construct the required channels. Also depending on the ratio of curing agent to PDMS monomers used to fabricate the device using soft lithography, as well as the thickness of the device, amount of UV light penetration can vary.

4.4.3.5 Alternative Designs for the Facilitation of Crosslinking Microparticles

As there were many uncertainties regarding the different types of tubing for UV penetrability, a PWC chart was not utilized and instead material selection was based off of UV penetration tests in Section 5.2.2. Using the UV data, the team came up with 2 primary designs concepts. All design concepts involved UV treating particles in the final collection vial for 20 minutes to ensure that all particles are completely methacrylated and crosslinked. The concepts are listed in Table 4.2.

Design 1:

Table 4.2: Concept Design Table for Design 1

Design Step	UV Curing Channel/Tubing	Length (m)	Residence Time (min)	UV Source
Pre-Curing	Microfluidics Device (Serpentine Channel)	~0.03	$<10^{-5}$	Commercially Available UV pen
Primary Curing	PVC Tubing	1	24 ~ 60	Broad Spectrum UV Bulb
Post-Curing	Glass Vial (Collection Container)	N/A	20	Broad Spectrum UV Bulb

In this design, the pre-curing system was setup by using a commercially available UV Glue Pen (5 Second FIX) facing down on the serpentine channel of the microfluidics device during droplet generation. Even with the negligible residence time, the idea was that there would be sufficient crosslinking of the particle to prevent coalescing inside the outlet tubing. The inlet tubing was protected using aluminum foil to prevent crosslinking of the particles before reaching the flow focusing channel. The generated particles would leave the device using the smaller Tygon™ tubing and then enter the larger PVC tubing where primary curing of the particles will commence. The PVC tubing was placed at a lower elevation compared to the device to assist the particles to flow through using gravity (the particles are heavy and tend to settle down in the tube if the flow rate is not high enough) and was also run directly under a broad-spectrum UV bulb to facilitate UV curing. The two-different tubing were joined together using Luer-Lok style connectors. A three-way stopcock was utilized to assist in removing air bubbles that may have resided in the microfluidics device before commencing the experiment. Particles were collected at the end of the PVC tubing in a glass vial. At the end of each particle fabrication run, fluid still residing in the tubing is pushed out using air into the collecting vial.

With this design, sufficient pre-curing was not achieved since particles were seen to coalesce and build up in the tubing. Sufficient UV light transmission through the PVC tubing might have also been another issue since collected particles were seen to larger than the ones being generated by the microfluidics device. This was seen in an exaggerated form in the stopcock since it had a had a much wider channel and slowed down flow significantly causing particles that were not sufficiently crosslinked to merge together. As such, a second design was proposed (Table 4.3).

Design 2:

Table 4.3: Concept Design Table for Design 2

Design Step	**UV Curing Channel/Tubing**	**Length (m)**	**Residence Time (min)**	**UV Source**
Pre-Curing	Tygon™ Tubing	0.6	1.5 ~ 4	Broad Spectrum UV Bulb
Primary Curing	PVC Tubing	1	24 ~ 60	Broad Spectrum UV Bulb
Post-Curing	Glass Vial (Collection Container)	-	20	Broad Spectrum UV Bulb

For the second design, pre-curing the particles in the microfluidics device was omitted. This modification was made, because the pre-curing process was ineffective at preventing particle coalescing. Instead particles were pre-cured within the Tygon™ tubing under a UV bulb for a longer amount of time (1.5~4 minutes which is significantly longer than the pre-curing step in Design 1) to prevent particle coalescing. The primary curing step remained unchanged except for modifications which were made to the setup over time according to observations made during fluid flow from experimentation.

Span 80 surfactant, also known as sorbitan monooleate, (Alfa Aesar) was used in the continuous phase to prevent particles from the dispersed phase from sticking together. Luer-Lock style connectors as well as the stopcock were removed between the two different tubing. The connectors introduced wider flow channels which caused sudden pressure changes to the fluid flow resulting in the formation of dead zones (the flow rate is zero or negligible). These pressure drops resulted in deposition of particles in the connectors which ended building up and clogging the channels. The tubing was modified by slightly widening the Tygon™ tubing so that it could be press fitted directly into the PVC tubing without the need for extra parts. This removed unnecessary pressure drops in the system and ensured a gradual change of velocity profile for the particles. For improving the efficiency of crosslinking particles in both the Tygon™ and PVC tubing, a V-shaped platform covered in aluminum foil was created. The tubing was run along the V-shaped platform and back. This system would allow the UV light too be reflected back and hit the tubing from all sides. After fluid flow is stopped at the end of the experiment, as in the previous design, any residing particles in the tubing are pushed out with air into the collecting vial.

4.4 Final Design Selection

After comparing the researched particles for hydrogel DDS, CS-MA was chosen for producing spherical microparticles for sustained drug delivery. CS-MA was chosen for its ability to release drug at a sustained rate, biocompatibility, cost-effectiveness, safety as a material, and adaptability to work in the future. PVA-MA was also ranked high for these same characteristics; therefore, it was used as a preliminary material for the first iterations of experiments.

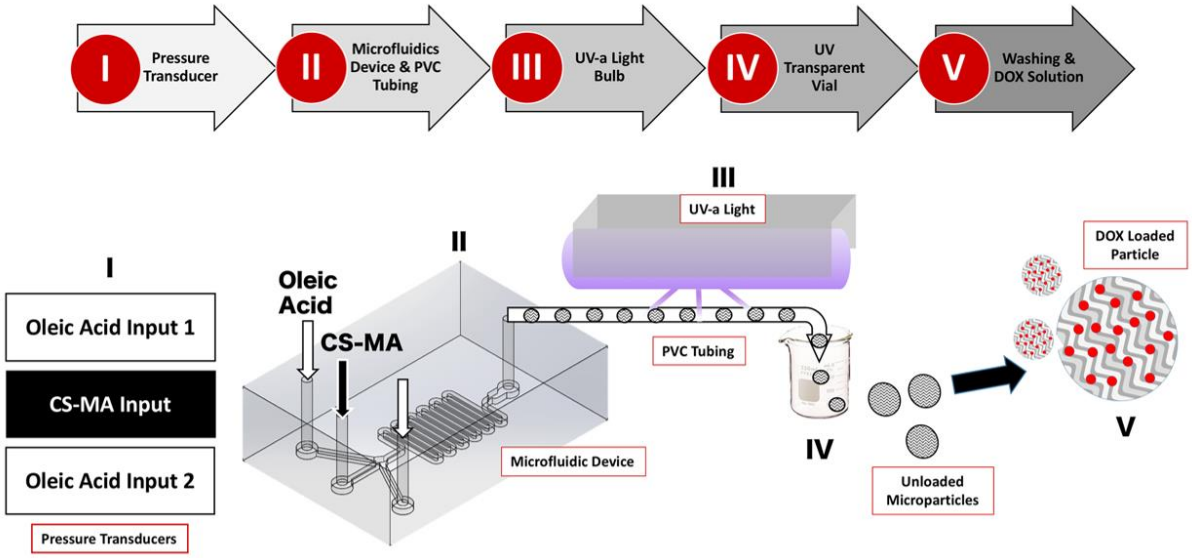


Figure 4.9: Final Design Setup

The final design (Figure 4.9) includes two pressure transducers, one of them that has two syringes loaded with oleic acid with span 80 while the other pressure transducer that has one syringe loaded with CS-MA. The syringes are then connected with a small Tygon™ tube that has a metal pin at the end, which connects to the input channels of the microfluidics device. The oleic acid with span 80 is the continuous phase, hence the two tubing are connected on the top and bottom channels, leaving the middle channel for the disperse phase which is CS-MA. The CS-MA is being pinched by the oleic acid with span 80 and form droplets, which are transported through the serpentine channel. The droplets are then passed through a PVC tubing that has a UV-a light bulb that shines on the tube for the crosslinking of CS-MA. The droplets, now theoretically crosslinked hence referred to as particles after this point, are collected on a UV transparent vial which is blasted with UV-a light at the end of collection. The collected particles are then washed with 100% ethanol and sterile deionized water to remove the oleic acid/span 80. Then, the particles are loaded with doxorubicin for further testing of cell viability and drug release profile.

V. Design Verification - Results

5.1 Experimentation Summary

The final design chosen was a soft lithography-based co-flow microfluidics device with a flow-focusing inlet aperture and serpentine channels based off of the designs by Natalia Vargas Montoya which was to make silk micro/nano particles (Coburn Lab). To make micro particles of CS-MA which will act as the drug delivery vehicle, various modifications were made to the original design setup to facilitate the fabrication of a new product. Before making the actual product, the team performed a set of low-cost preliminary experiments to verify that the device was conducive to different materials for continuous and dispersed phases, and to check and see how flow rates affected particle size distribution. Tests were also done in checking the efficacy of UV light penetration of various materials/tubing in the UV crosslinking apparatus to facilitate crosslinking of the polymers. Since the need of the project is to design a device to produce microparticles, this initial design verification focuses solely on the goal of producing particles in the micro-scale with the particle-fabrication device. To conduct preliminary experiments and collect preliminary data, the project utilized inexpensive materials including olive oil, oleic acid, 5-20% PVA solutions, and water to test the device without using the more expensive materials needed for the final design verification.

To quantify the size distributions of the microparticle products, bright field and phase microscopy were used to measure the dimensions of the microparticles. Statistical analysis was conducted to verify if the microparticles being fabricated are both reproducible. Scanning electron microscope (SEM) images of one batch of microparticles was also taken to obtain a qualitative observation of the particles.

Cytotoxicity assays can be performed using methylthiazol tetrazolium (MTT) assay or an Alamar blue assay to meet the engineering standards. These assays can be performed on KELLY cells which were

provided by the project advisor, Professor Coburn. The same cytotoxicity assays can also be used to check for polymer and drug compatibility to see if encapsulation had altered the drug chemistry. This can be done by checking and comparing drug efficacy while encapsulated and in its pure form.

Degradability can be quantified *in vitro* using a combination of microscopy and spectrophotometry and measuring the time it takes for the polymer carrier alone to lose both 3-D structure and molecular weight when placed in plates containing the chosen neuroblastoma cell line. The sustained release of the drug as well as its reproducibility and replicability can be quantified using spectrophotometry to measure the initial loading and then subsequent release of the drug *in vitro*. Using different samples and compiling their respective loading and release profiles, sustained release can be quantified.

Along with verification of the particle-fabrication device, a bulk CS-MA hydrogel was tested for its ability to load and release doxorubicin (DOX) over time. Since the particles had not yet been fabricated, the CS-MA was prepared into a bulk hydrogel. This preliminary data will guide future decisions on how much DOX to load onto CS-MA in its final micro-to-nanoparticle form.

5.2 Final Design Verification for the Fabrication System

In this section, the team has verified the final design for the fabrication system that was selected back in section 4.3. The final design was divided into different parts including: the PDMS microfluidics device, the droplets generation in the device and tubing verification for UV permeability.

5.2.1 Verification of PDMS Microfluidics Device

In order to verify the replicability and reproducibility of the PDMS microfluidics devices, the PDMS was validated for consistent size and shape before use. The PDMS microfluidics devices were made according to the protocols in Appendices E and F. PDMS devices underwent burst pressure tests by

pumping water through the channels after plasma bonding in order to check if PDMS devices "burst" and leaked water or not. Additionally, PDMS devices were manually sliced at the site of the three inlet channels and all three inlet channels were to be measured using simple microscopy and ImageJ.

5.2.2 UV Permeability of the Device and Tubing

The devices were tested for their UV penetrability. The following experiment was used to help determine if sufficient UV light can penetrate the device so that the particles can be pre-crosslinked in the device and do not coalesce. Six PDMS pieces of varying thicknesses (9:1 silicone to elastomer) were cleaned with DI water and ethanol. The devices were treated with tape to remove dust. Two different sources of UV light were used: a commercially available UV pen (5 Second Fix), and a broad-spectrum UV bulb. The experiment was set up such that the UV light source was fixed a set distance above the UV sensor (SPER Scientific UVA/B Light Meter 850009). The PDMS pieces were placed on top covering the sensor and the UV was passed through it. Readings were collected with and without the PDMS. The data was then plotted for a better visualization. Figure 5.1A shows the plot of relative UV light penetration through PDMS. A line of best fit is also plotted to better represent the trend of both UV pen and broad-spectrum UV bulb. The shaded area represents the average thickness of the PDMS devices used for generating particles.

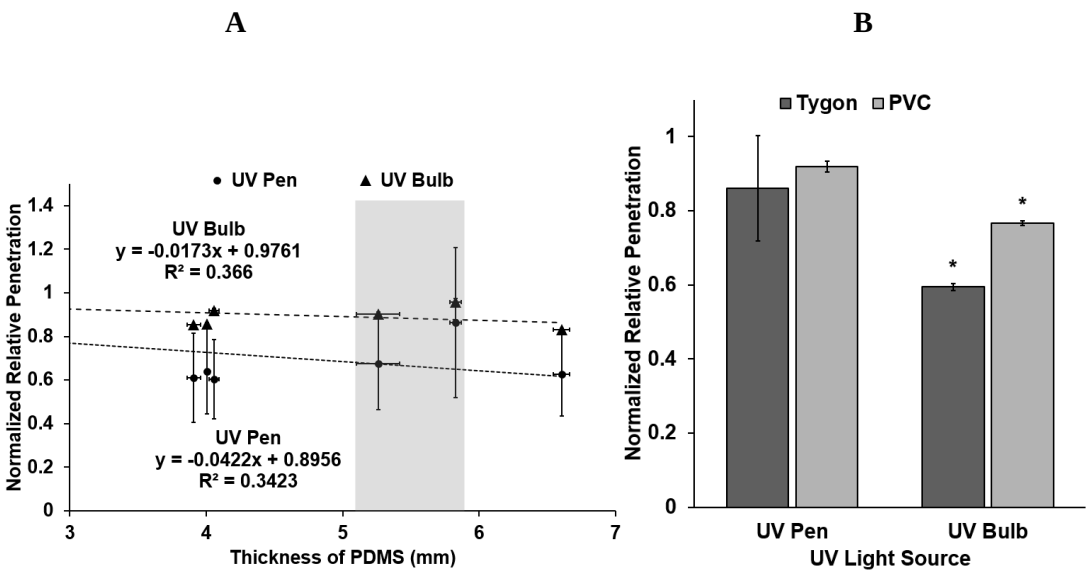


Figure 5.1: Normalized relative UV light penetration through **(A)** PDMS at different thicknesses and **(B)** Tygon™ and PVC tubing using a commercially available UV Pen and a higher-powered UV Bulb. Each reading was taken with 3 replicates to check for reproducibility and variability. Average UV penetration for the PDMS based on operating thickness as highlighted by the shaded area was 77% and 93% for the UV Pen and Bulb respectively.

The plot shows that with increasing thickness, there is a decrease in penetrance of UV light. The device operating thickness lies between 5.2 and 6 mm and still allows for ~90% penetration which is sufficient for pre-curing. The data for the UV pen shows a high deviation and variability. This may be due to it being a cheap commercially available product that utilizes an internal battery which does not provide the light source with a constant voltage. For the UV pen, UV intensity is seen to decrease with time making the data not reliable. For the broad-spectrum UV bulb, the data is more consistent and at a thickness of 6.6 mm for PDMS, over 80% of the UV light manages to penetrate it.

The next experiment was to test how much UV penetrates through Tygon™ tubing and PVC tubing. The same experimental setup in the PDMS experiment was used. To ensure that the only UV light being detected on the sensor came through the tubing, black tape was used to cover up any part of the

sensor that was open on either side of the tubing. Graphical representation for UV penetration for both types of tubing is shown above in Figure 5.1B. The experiment showed that UV absorbance was comparatively higher for the Tygon™ tubing than the PVC tubing. Within the same tubing, the decrease in UV penetration being significant for the broad-spectrum UV bulb may be accounted for the vertical alignment of the light source above the tubing. As it was easier to align the UV pen directly over the tubing and light source, it ensured that more light could penetrate. Using the UV Bulb data, it can be seen that the PVC tubing allowed more of the UV light to penetrate than the Tygon™ tubing.

5.2.3 Verification of Microfluidics Droplet Generation

To begin experimentation on the device, the team was introduced to the particle fabrication device setup, imaging, and troubleshooting the flow rates of the phases within the device. To do this, the team used olive oil and water as the continuous phase and the dispersed phase, respectively, and used similar flow rates as found in literature (0.025 mL/hr to 2 mL/hr). From these experiments, the team learned various factors including device maximum parameters to limit syringe pump "popping". This is the phenomenon where the actuator or moving head of the pump is not properly aligned and gets dislodged creating a significant noise. As the syringe gets dislodged the flow rate of the fluid within the respective tubing is not consistent and affects particle fabrication.

The tubes were adjusted by altering the maximum height as well as its tension to keep head pressure consistent. More information about the setup of the pumps, the microfluidics device, computer and UV crosslinking apparatus can be found on Appendix G. To work with a material with a viscosity similar to that of CS-MA and able to be modified with methacrylate groups, the team conducted additional experiments with a 5% mixture of PVA (Appendix M) as the dispersed phase and olive oil as the continuous phase. The goal was to test 20% PVA solutions with olive oil to approach the viscosity of CS-MA; therefore, the subsequent experiment was conducted with an increased concentration dispersed phase of 10% PVA. Using this material with much higher viscosity, the team attempted to troubleshoot

by altering the tubing but this experiment allowed us to see that the tubing itself must not be changed in order to troubleshoot issues such as backflow or multi-breakdown. Continuing the backflow and multi-breakdown outcomes were avoided by changing the flow rates and keeping the tubing set-up as the experiment had it to begin with.

After further consideration of how to continue with the design verification, the project changed courses to experiment with the actual material that would be used in the continuous phase: oleic acid. To have a set-point and direct comparison, we continued testing with 10% PVA solution. With this experimentation, the project concluded that paying closer attention to the continuous to dispersed flow rate ratios was more efficient than the singular ratios, as the team saw an inverse relationship of ratio to particle size (with some outliers) similarly to what literature suggested. The variables that were changed at any given time were also decreased. After these experiments were concluded the project continued with the final conditions of oleic acid and 20% PVA solution. The conclusions made for this experiment thus far are to test specific ratios to be able to make direct comparisons, and note down all aspects of the experiments, including device used, environmental temperature, accidental changes in tubing or other set-up aspects, and time at which images were taken. The experimentation summary can be found in Table 5.1 for a quick snapshot of the experiments done prior to final testing.

Table 5.1: Preliminary Experimentation Summary

Oil	Reasoning	Conclusion(s)
Water	• Understand Microfluidics device • Backflow • Imaging • Multi-breakdown • Troubleshooting • Set-up • Clean-up • Low cost	• Head pressure • Flow rate ranges • Particle fabrication device limits • Set-up must be consistent
5%PVA	• More viscous material • Different interactions to oil than just water	
10% PVA	• Higher viscosity with small steps	• Changing tubing to alter products was a bad idea • Decided to keep tubing consistent and fix backflow by only changing flow rates temporarily
Switched to Oleic Acid; can be filtered out from particles (it dissolves in ethanol) & used in literature		
Oleic Acid	**Reasoning**	**Conclusion(s)**
10% PVA	• Direct comparison with oleic acid • Had much more data with 10% PVA	• Flow rates did not apply • More efficient to work with more realistic conditions • Don't vary all variables at once • Don't use or take pictures of device with dust particles
20% PVA	• Time constrictions • Closer to CS viscosity	• Stabilizing time • Testing based on ratio • Have set ratios to test • Careful with image quality • Slight changes in environmental temperature may change behavior of liquid (viscosity)

Table 5.1 only includes the following configurations: Olive oil with water, 5% and 10% PVA, oleic acid with 10% and 20% PVA. The experiments done with 5%, 7.5%, and 15% PVA were not included due to the insufficient amount of data. The raw data for the approximations of the particle sizes can be found in Appendix N.

Table 5.2 discusses the experimentation summary that includes a brief overview of the experiments performed from September 2017 up until April 2018.

Table 5.2: Experimentation Summary

Disp. Phase	Cont. Phase	Parameters Studied	Conclusions
Water	**Olive Oil**	• Flow rate min/max for device	• Do not surpass 5 ml/hr total
PVA (5, 10, & 20%)	**Olive Oil** **Oleic Acid** (w/o Span 80)	• Behavior based on viscosity • Coalescing (inside the device)	• Flow rate ratios do not translate linearly • Olive oil is not ideal as cont. phase
PVA-MA	**Oleic Acid** (w/o Span 80) **Oleic Acid** (w/ Span 80)	• UV Crosslinking set-up alternatives • Particle separation (purification) • Coalescing (collected output)	• Completed cross linking set-up • Do not separate using conventional centrifuges • Coalescing experiment inconclusive
CS-MA	**Oleic Acid** (w/ Span 80)	• Particle separation • Coalescing (collected output)	• Do not separate using conventional centrifuges

The following figures represent the particles that the team has been able to make. The device was setup by the team as per Appendix G. Figure 5.2 below have the flow rates of both the dispersed and continuous phase along with the measured diameters of the particles.

The team also considered testing alternative flow focusing dimensions of the device including the main design that has a 100 µm width, as well as the 80 µm, 60 µm and 40 µm flow focusing channel alternatives. This experiment was designed to test the resulting changes in diameters of the formed PVA particles based on varying the flow focusing channel while using one optimized flow rate pair. Figure 5.3 below shows some images while experimenting with differently-sized flow focusing channels.

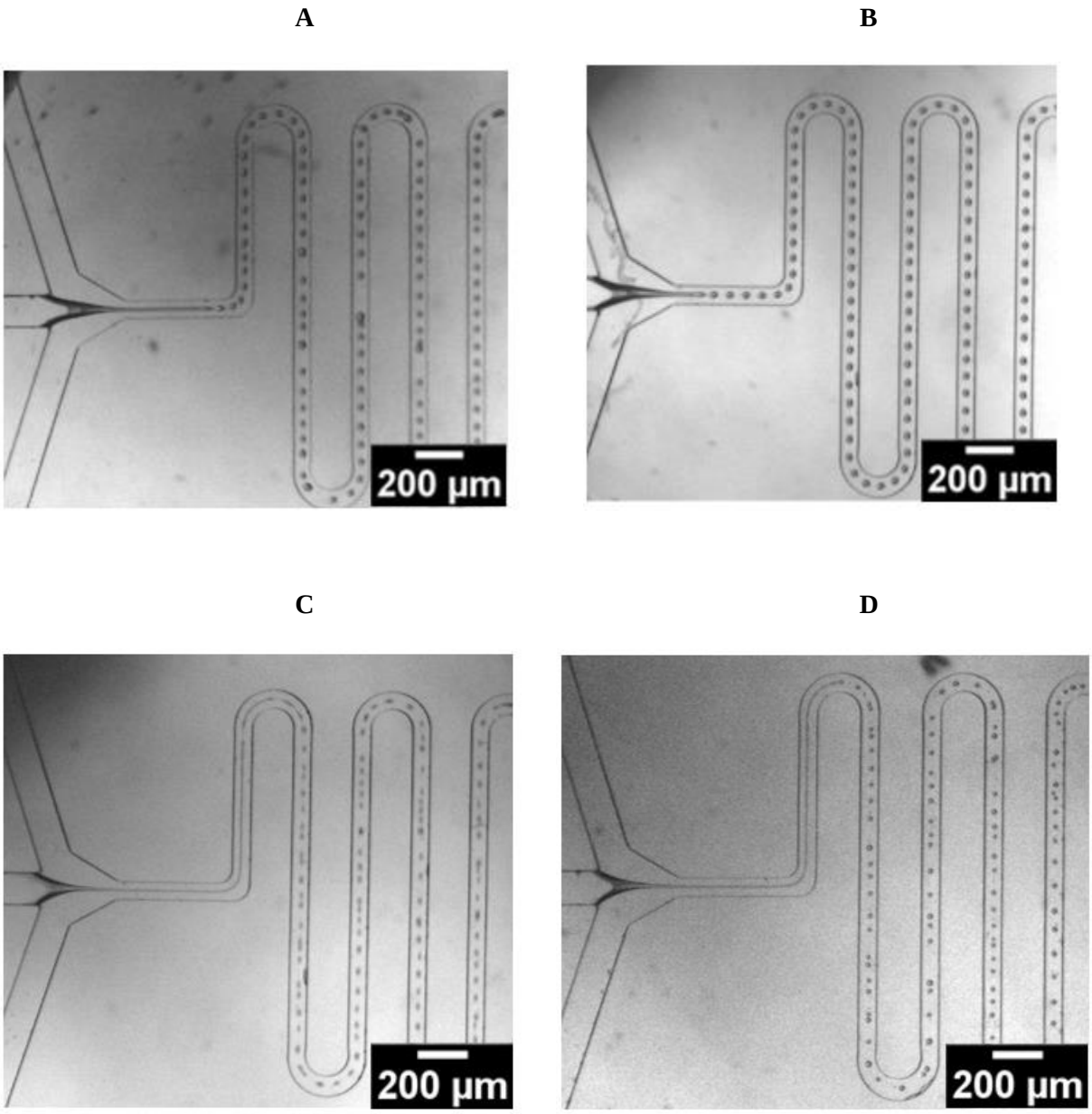


Figure 5.2: 10% (w/v) and 20% (w/v) PVA Particles. A) Particles of 64.84 µm in diameter using oleic acid at 1.45 mL/hr and 10% PVA at 0.185 mL/hr, B) Particles of 57.17 µm in diameter using oleic acid at 1.5 mL/hr and 10% PVA at 0.25 mL/hr, C) Particles of 36.90 µm in diameter using oleic acid at 2 mL/hr and 20% PVA at 0.06 mL/hr, and D)Particles of 36.04 µm in diameter using oleic acid at 2 mL/hr and 20% PVA at 0.08 mL/hr.

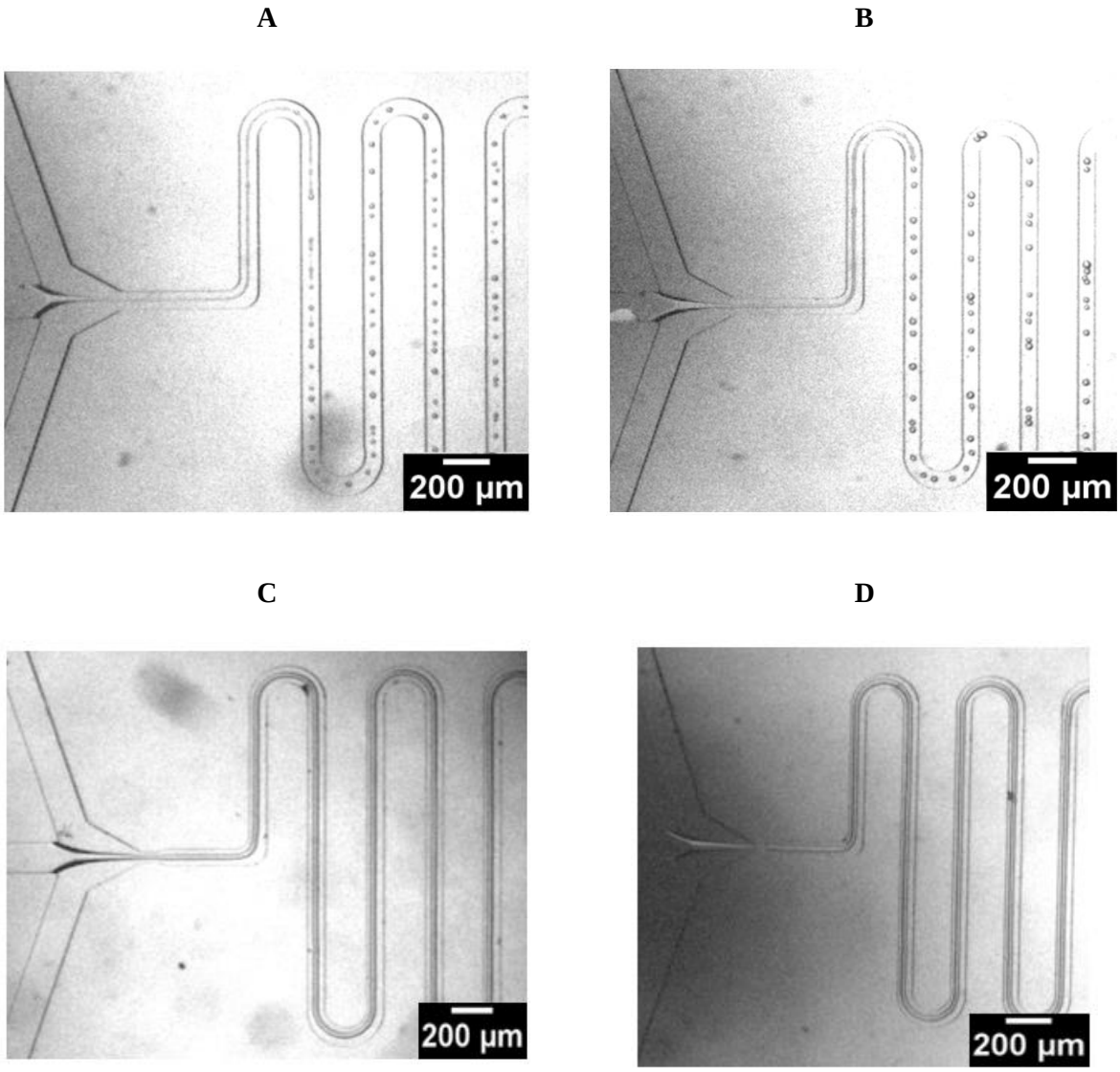


Figure 5.3: Testing of Different Sized Flow Focusing Devices All the experiments used the same flow rate of 1 mL/hr for oleic acid and 0.07 mL/hr for 20% PVA. A) 100 µm device B) 80 µm device C) 60 µm device D) 40 µm device.

Data was collected for 20% PVA-MA. Experiments were conducted using various flow rates to see what type of behavior was occurring in a 100 µm device. Figure 5.4 shows these results.

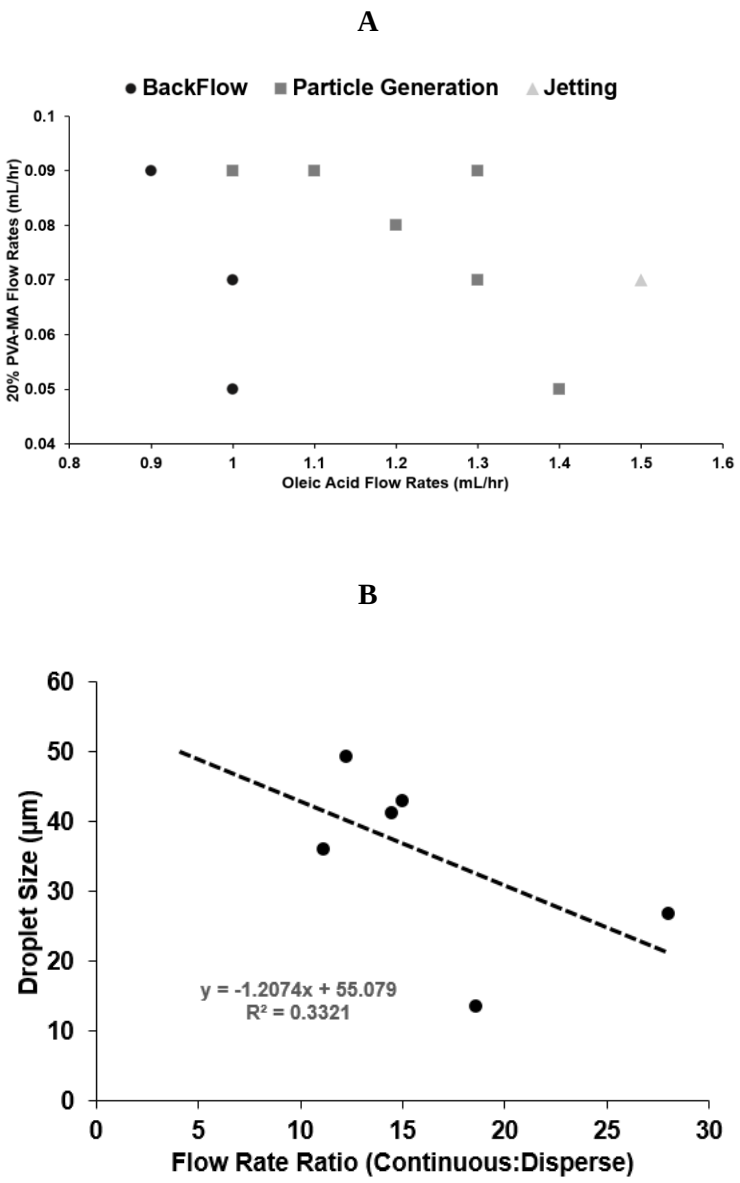


Figure 5.4: Flow Rate Comparison Between the Continuous Phase, Dispersed Phase and Droplet Size. **(A)** Different oleic acid and 20% PVA-MA flow rates were tested on a 100 µm device. For each combination of flow rates, the behavior of the dispersed phase was observed and noted as backflow (circle), particle generation (square) or jetting (triangle). **(B)** Regression analysis used for comparing flow rate ratio of continuous to dispersed phase in comparison with droplet size. The regression line equation and its R^2 value are shown.

Additionally, throughout the experimentation process, the team has also learned lab techniques to allow longer and improved usability of the microparticle fabrication device. To begin, the flow rate ratios did not exceed 10:1 continuous to dispersed phase, respectively. During the testing that used this flow rate ratio, the device was treated with ethanol by flushing the microparticle fabrication device with 100% ethanol and placing it in the oven overnight at 60°C. Ethanol treatment was meant to make the inner surfaces of the microfluidics channels hydrophobic to prevent the hydrophilic dispersed phase from sticking to the sides. As the flow rate ratio increased (the highest being 50:1), the device no longer worked to the optimal condition and the flow of the two liquids were stuck at the sides of the channels as they should (and were) under the previous conditions. To continue with experiments, the device was treated with pluronic instead. This was done by flushing the microchannels with pluronic F-127 (Sigma-Aldrich) (a triblock copolymer with hydrophilic ends and a hydrophobic center) for 5 minutes as indicated by Coburn lab protocol. This is to help prevent the dispersed phase from sticking to the sides and to improve flow rate and droplet generation.

CS-MA was prepared and tested for particle generation on the final fabrication system. First, CS-MA was prepared as stated in Appendices H and I. Figure 5.5 shows how the CS-MA droplets looked like inside the microfluidics device while having oleic acid with span 80 as the continuous phase.

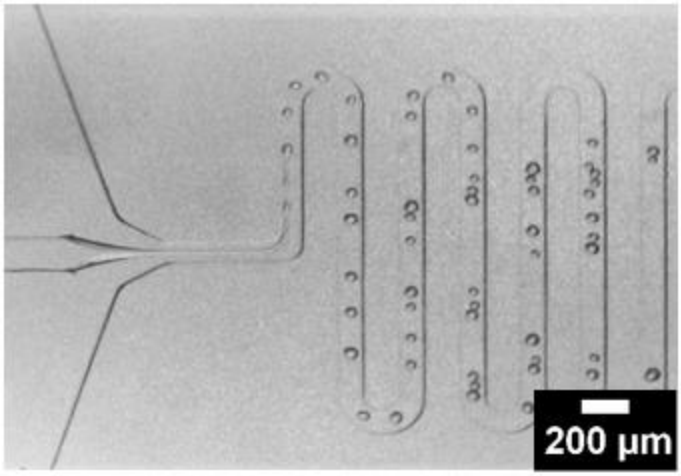
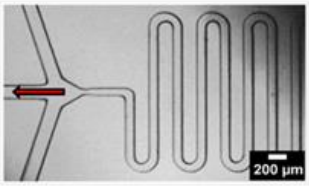
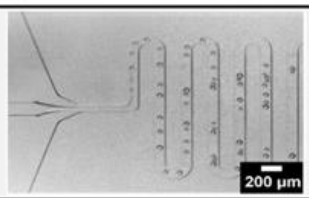
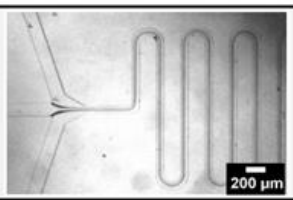


Figure 5.5: CS-MA Droplets (using oleic acid with span 80 flow rate of 1 mL/hr and CS-MA flow rate of 0.1 mL/hr.)

The flow rate of CS-MA inside the microfluidics device was optimized by using different batches of CS-MA denoted as Batches 1, 2, and 3 respectively. Table 5.3 shows the flow rate of CS-MA ranges as well as their observed behavior, an example image and if it was desired or not for the team. It is worth noting that the oleic acid with span 80 was kept at a constant rate of 1 mL/hr for the optimization process due to the easiness of controlling the CS-MA generated droplets.

Table 5.3: Flow Rate Optimization of CS-MA

CS-MA Flow Rate (mL/hr)	< 0.08	**0.08 - 0.3**	> 0.3
Observed Behavior	Backflow	Particle Generation	Jetted Flow
Image			
Desired?	X	✓	X

5.3 CS-MA Microsphere Particle Generation

After verifying the fabrication system as well as optimizing flow rates for producing microsphere particles, CS-MA particles were generated in multiple batches and quantified for size distribution. Batches 1, 2 and 3 were used previously in flow optimization and experiments. Batches 4 through 7 were collected to run drug loading and release assays. For simplicity, these batches will be labeled Batch A, B, C and D respectively from now on. Batches A and B were collected on one day and Batches C and D were collected on another. After collection of the first batch on each day, the microfluidics device and tubing were disconnected and cleaned before setting up the subsequent experiment. This was done to ensure that each experimental batch collected could be classified as a separate sample group.

5.3.1 CS-MA Particle Collection and Isolation

CS-MA (3 mL, 24% methacrylated) was prepared immediately before each experimental setup following protocol (Appendices H and I). After setting up all the components of the microfluidics

fabrication system (Appendix G), oleic acid (7 mL) with 5% span 80 at 2% (w/v) was filtered and pipetted into each continuous phase syringe. The CS-MA was pipetted into the dispersed phase syringe. These volumes were selected to ensure there was sufficient material to conduct two separate experiments while at the same time not wasting them. The phases were pushed using the syringe pumps at 1 mL/hr (continuous phase) and 0.1 mL/hr (dispersed phase). The UV crosslinking apparatus ensured that generated particles were crosslinked and would not coalesce. Particles from each batch (run for ~3 hours each) was collected in glass vials. During the cleanup process between and at the end of each experiment, the collected particles in oleic acid was then placed under the UV light source for 20 minutes to allow for crosslinking saturation.

Collected particles were centrifuged at 4,400 RPM for 5 minutes (Centrifuge Eppendorf 5702). The oleic acid was aspirated off and the particles were then resuspended in 3 mL of 100% ethanol. The particles were then centrifuged again. The supernatant was then aspirated off and the particles were washed again with 100% ethanol. This process was repeated until the particles were washed a total of four times using ethanol (also found in Appendix G). The ethanol helps dissolve the oleic acid which can then be removed. It also helps in disinfecting the particles prior to drug loading and cell viability testing.

5.3.2 CS-MA Particle Size Characterization

The washed batches of CS-MA particles were dried overnight and weighed using a digital mass balance. After allocating batches for doxorubicin loading, the remaining samples were left in deionized water for 1 week. 20 μL of each batch left out after allocating for doxorubicin loading was pipetted onto glass slides and held in place using cover slips and nail polish to prevent unnecessary motion of the particles. The particles were viewed and imaged under bright-field and phase contrast microscopy at 10X and 32X objective. The particles were scaled against a measured length using ImageJ. During particle fabrication previously, particle diameter in the device was also imaged and recorded. These two measurements were compared as shown in Figure 5.6.

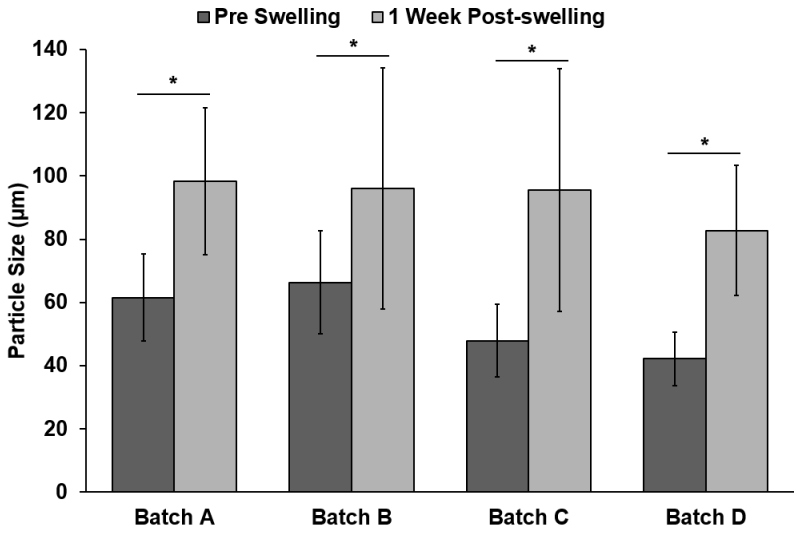


Figure 5.6: Size comparison of different batches of CS-MA particles during fabrication (pre-swelling) and after sitting in deionized water for 1 week (post-swelling).

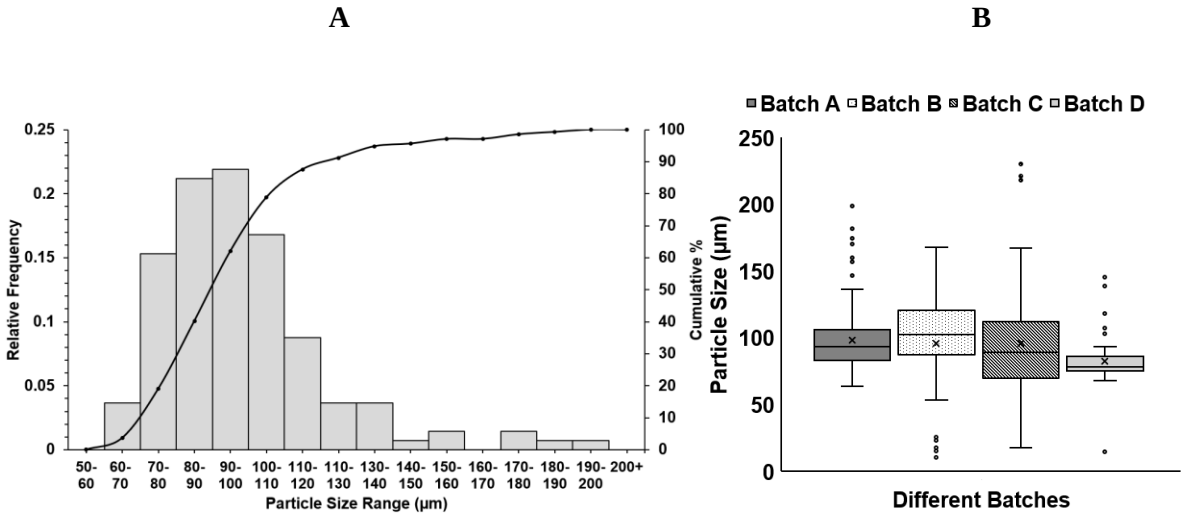


Figure 5.7: Size distribution of swollen particles **(A)** within Batch A as a histogram and **(B)** between batches as a box and whisker plot. The average particle size of the CS-MA particles for this Batches A, B, C and D were 98.44±23.26 µm, 96.15±38.11 µm, 95.59±38.48 µm and 82.79±20.6 µm. The number of particles tallied in the batches were 137, 62, 91 and 38.

5.4 Doxorubicin Loading and Release Experiments for CS-MA Microparticles

5.4.1 Loading CS-MA Bulk Gels with Doxorubicin for Proof of Concept

The team conducted preliminary tests on loading DOX into CS-MA (24% methacrylated) in bulk hydrogel form. This experiment was conducted as a proof of concept to ensure that doxorubicin would bind to our modified CS-MA material even after methacrylation. To do this, the team followed the CS-MA DOX Bulk Loading protocol in Appendix O. After incubating the CS-MA bulk hydrogels (2 mg) in DOX solution (1 mg/mL) over 3 days, the amount of DOX loaded onto each 20% CS-MA hydrogel (crosslinked for 20 minutes) was determined by reading the light absorbance values of the leftover supernatant on a spectrophotometer and comparing the values to a standard curve. By using a serial dilution of the doxorubicin solution (LC Laboratories, 1 mg/mL), a standard curve was created to quantitatively associate the amount of light absorbance per sample to the different concentrations of DOX (1.56-200 µg/mL, Figure 5.8). The amount of total loading stock DOX before loading was determined to be 1047.6 µg, or approximately 1 mg of doxorubicin. The raw data from this experiment can be found in Appendix P.

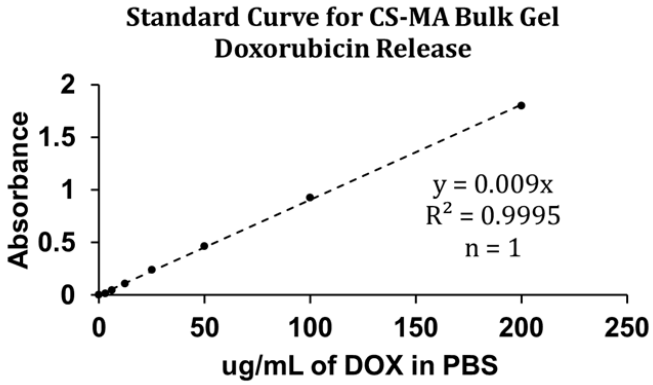


Figure 5.8: Standard Curve of the Light Absorbance Per DOX Concentration

The concentration of doxorubicin (ug/mL) in the samples of doxorubicin were quantified as follows:

$$Concentration\ of\ Doxorubicin\ (ug/mL) = \frac{Light\ Absorbance\ Value}{Absorbance/\ ug*mL\ (slope\ of\ Standard\ Curve)}$$

The CS-MA bulk gels were incubated at 25°C for three days to absorb the doxorubicin in the surrounding solution. After this incubation period, the absorbance value of the remaining doxorubicin in the supernatant was divided by the slope of the standard curve (n=1, 3 technical replicates). The average mass of DOX loaded into each 2 mg gel is approximately 509.5 µg. From here, the team calculated the background noise in any given well containing a DOX-loaded CS-MA gel in PBS (1 mL). The progress of total drug release on Days 1, 2, 4, 7, 9, 11, and 14 can be seen in Figure 5.9 below by mass released and percentage of total drug released.

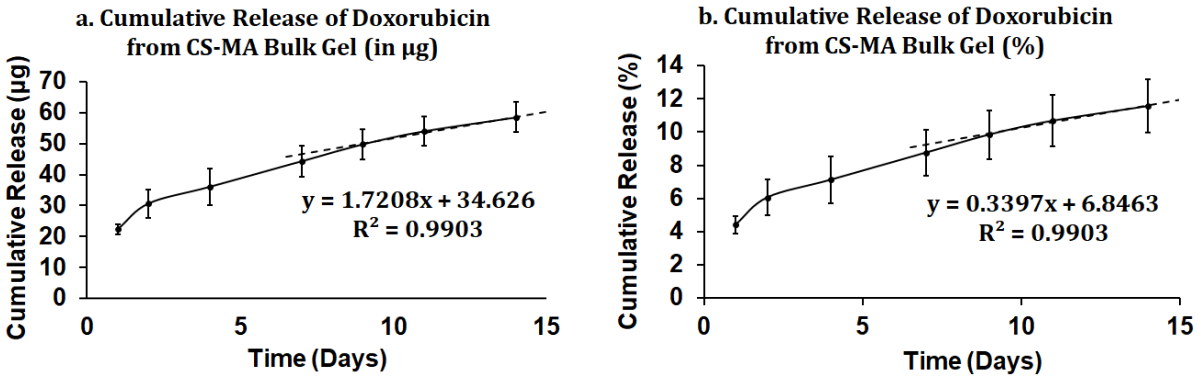


Figure 5.9: DOX Release. a) mass (ug) of DOX and b) the percentage of DOX released over 14 days.

Figure 5.9 shows an upward trend in cumulative DOX release, which is comparable to the data in previous CS-MA bulk hydrogel drug-release experiments. Additionally, the data shows a linear trend in doxorubicin release after Day 9 of incubation. From this experiment, the team proceeded to load the CS-MA microparticles with doxorubicin solution at the same concentration.

5.4.2 Loading CS-MA Microparticles with Doxorubicin

Similar to the CS-MA bulk loading experiment, DOX (LC Laboratories, 1 mg/mL) was added to CS-MA microparticles (2 mg) in an aqueous suspension (1 mL, n=4). A standard curve of absorbance values was produced via serial dilution of stock DOX (1.56-200 µg/mL). DOX concentration was determined by dividing absorbance values by the slope of the standard curve and converted to mass by multiplying by the volume. DOX-loaded particles were incubated in 1 mL phosphate buffered saline at 37°C for 30 days. As previously stated, a total of 4 batches of CS-MA particles were produced for drug loading and release studies (Batches A, B, C, and D). More details on loading DOX onto the CS-MA microparticles are found in Appendix Q and the protocol for reading absorbance values can be found in Appendix R. The DOX loading data can be found in Table 5.4.

Table 5.4: DOX Loading Data

CSMA Batch	Percentage Loaded (%)	Concentration (ug/mL)	Standard Deviation
A	76.2	767.1	29.3
B	88.8	890.1	82.7
C	8.9	889.1	6.5
D	53.1	530.7	44.1
AVG	56.7	769.3	32.0
mg/mg ratio		384.7	16.0

Released DOX solutions (Figure 5.10) were collected for thirty days and stored at 4°C for later cytotoxicity studies.

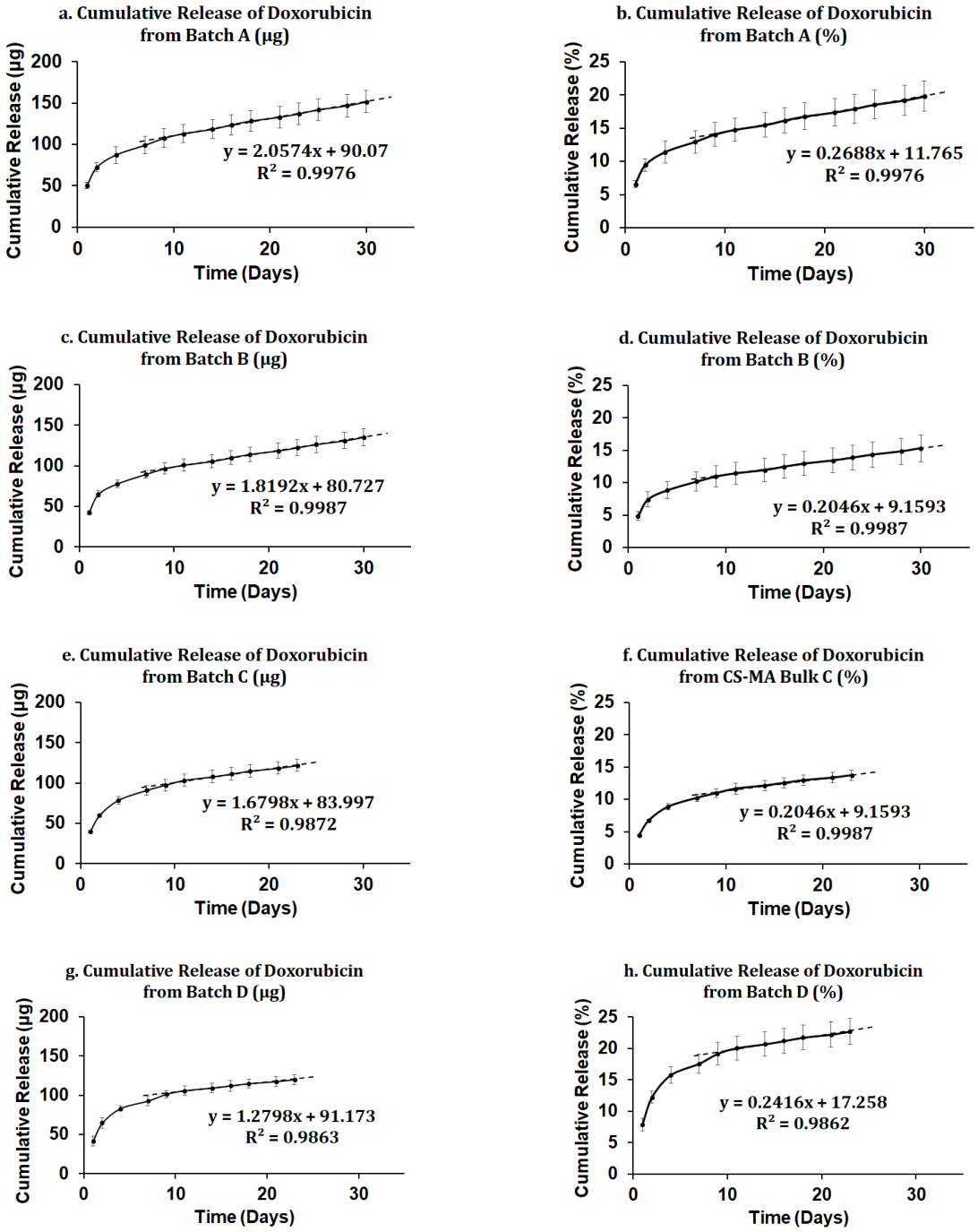


Figure 5.10: The mass and percentage of doxorubicin released for Batch A (a, b), Batch B (c, d), Batch C (e, f), and Batch D (g, h). Doxorubicin release was collected for 30 days from Batches A and B. Doxorubicin release was collected for 23 days from Batches C and D. After Day 9, the drug release rates of all four batches approached linear drug release rates

. An average release profile was created using all four CS-MA batches. Figure 5.11 depicts the release of doxorubicin from CS-MA microparticles.

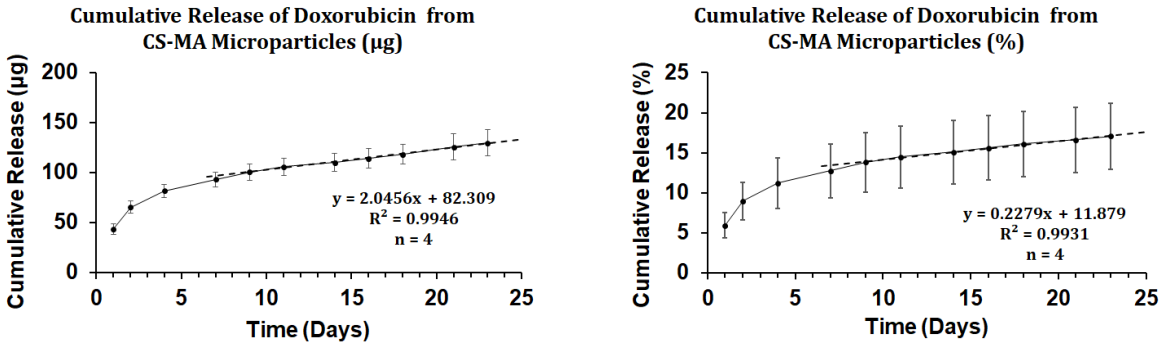


Figure 5.11: The Cumulative Release of all CS-MA Batches (n=4). The data up until Day 23 are graphed above. Linear drug release is achieved after Day 9.

5.5 Cytotoxicity Assay of DOX-Release Supernatant and of Unloaded CS-MA Microparticles

KELLY neuroblastoma cells (Sigma Aldrich) were plated at a density of 10,000 cells/well in a 96 well plate using Appendix S and incubated at 37°C overnight. Cell culture media was prepared with RPMI media (Sigma Aldrich), 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum. The following day, cells were treated with doxorubicin release supernatant diluted in media at ratio of 1:10, 1:100, 1:1000 (n=3), unloaded CS-MA particles (material control), and untreated (negative control). Cells were incubated in media (final volume: 250 mL) for 3 days at 37°C.

A resazurin metabolic assay was performed to assess the metabolic activity in each well, which is used to determine cell viability (resazurin solution: 0.15 mg/mL, six-fold dilution in media, Sigma Aldrich). Resazurin is a blue redox-driven pH indicator that turns pink and increases fluorescence upon reduction by metabolic activity in cells. Higher fluorescence values indicate the presence of living cells

while lower fluorescence values suggest that there are less living cells in a sample. The KELLY cells were incubated in resazurin solution (250 µL) at 37°C for three hours and the fluorescence values of the cell media were assessed on a fluorescence microplate reader (Figure 5.12).

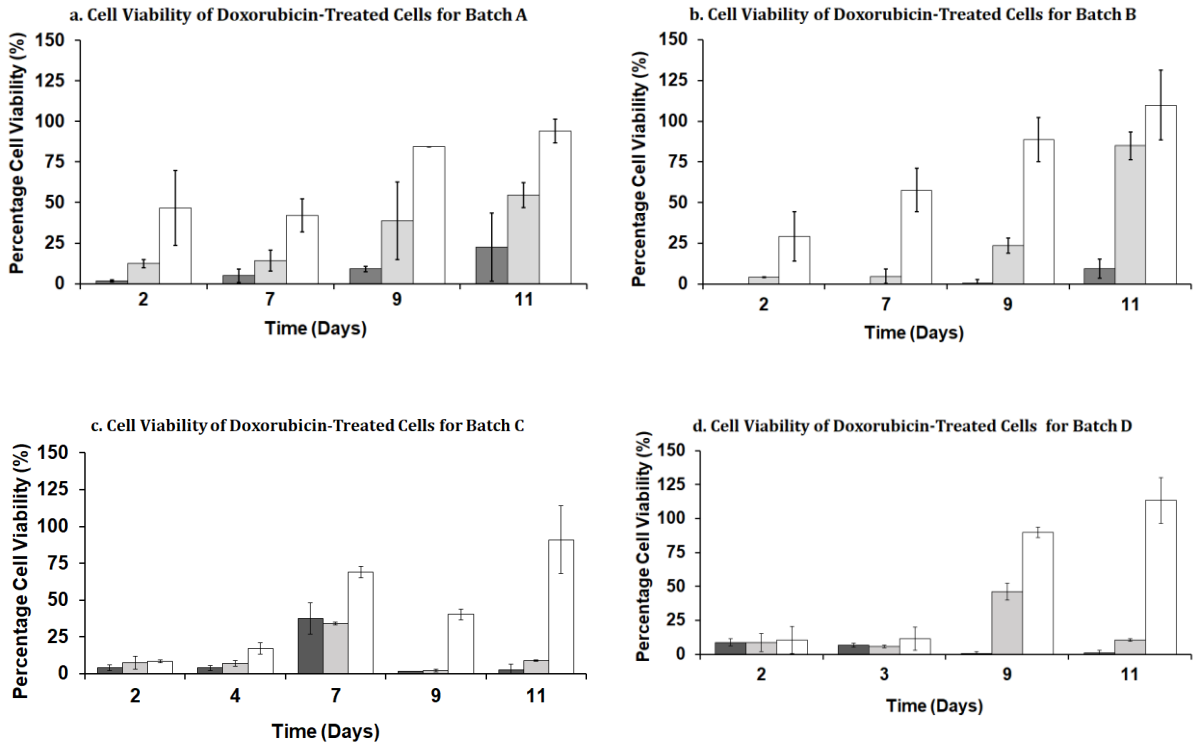


Figure 5.12: The Cell Viability (%) of the DOX Released From the Four CS-MA Batches. The dark gray bars are 1:10 dilutions of DOX in cell media, the light gray bars are 1:100 dilutions of DOX in cell media, and the white bars are 1:1000 dilutions of DOX in cell media.

The concentrations of each dilution of doxorubicin per day per batch is summarized in the Table 5.5 below.

Table 5.5: Concentrations of Doxorubicin in CS-MA Batches (ng/mL)

Concentration of DOX in CS-MA Batch A (ng/mL)			
Day	**1:10 Dilution**	**1:100 Dilution**	**1:1000 Dilution**
2	2193.4	219.3	21.9
7	1178.2	117.8	11.8
9	844.7	84.5	8.4
11	554.2	55.4	5.5
Concentration of DOX in CS-MA Batch B (ng/mL)			
Day	**1:10 Dilution**	**1:100 Dilution**	**1:1000 Dilution**
2	2268.7	226.9	22.7
7	1168.6	116.9	11.7
9	696.9	69.7	7.0
11	438.4	43.8	4.4
Concentration of DOX in CS-MA Batch C (ng/mL)			
Day	**1:10 Dilution**	**1:100 Dilution**	**1:1000 Dilution**
2	2020.3	202.0	20.2
7	1225.9	122.6	12.3
9	648.8	64.9	6.5
11	605.8	60.6	6.1
Concentration of DOX in CS-MA Batch D (ng/mL)			
Day	**1:10 Dilution**	**1:100 Dilution**	**1:1000 Dilution**
2	2295.0	229.5	23.0
7	968.8	96.9	9.7
9	845.2	84.5	8.5
11	462.3	46.2	4.6
Average Concentration of DOX in all CS-MA Batches (ng/mL)			
Day	**1:10 Dilution**	**1:100 Dilution**	**1:1000 Dilution**
2	2194.3	219.4	21.9
7	1135.4	113.5	11.4
9	758.9	75.9	7.6
11	515.2	51.5	5.2

To quantify the cell viability across all of the batches, the averages of all of the cell viability assays were taken for each day and for each dilution of DOX. These averaged values were plotted in Figure 5.13 below and the standard deviations across each respective group were recorded.

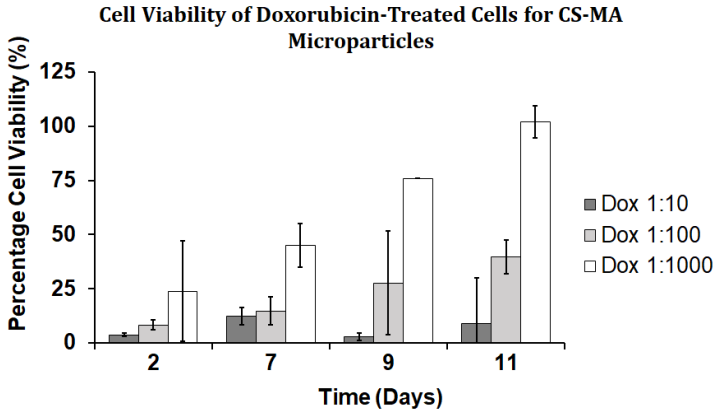


Figure 5.13: The Cell Viability Percentages Across all Four Batches (n = 4)

To test the biocompatibility of the unloaded CS-MA particles, a resazurin assay was also carried out for cells treated with microparticle suspension in sterile water (50 µL, Figure 5.14). A Student's t-test was performed to assess the difference in viability between particle-treated cells and control cells.

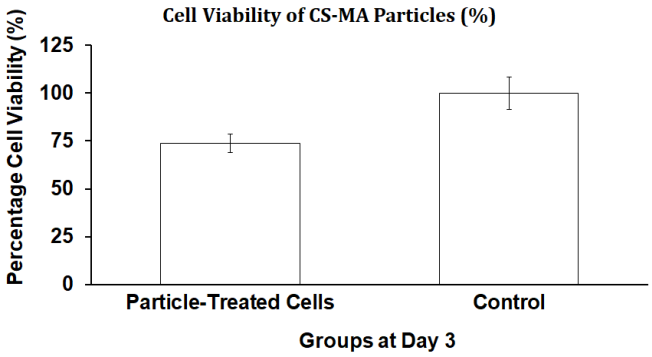


Figure 5.14: Cell Viability of Particle-Treated Cells. The null hypothesis (H_0) was that there was no statistically significant difference between the two groups. A Student's t-test was performed ($p = 0.051$), which supports the H_0 to be true. Therefore, there is not statistical difference between the cell viability percentage of the particle-treated cells versus that of the control group.

VI. Final Design and Validation

The project has developed and executed various tests and tasks in order to achieve a successful final design. however, to complete the design it must be validated. This section will have a detailed overview of how the initial goals were met with the final design since that is the ultimate objective of the design process. Additionally, this section will also briefly discuss what role industry standards play in the design process for the current phase of our product.

6.1 Final Design Overview

The following section will outline the fabrication of CS-MA microparticles as well as the loading of doxorubicin onto the particles.

The first component necessary for the fabrication of CS-MA microparticles was the creation of the microfluidics device. The device was fabricated using soft lithography. The protocol for soft lithography as well as the photolithography fabrication of the final master template provided by Natalia are provided in Appendices E and F. The processing techniques which involve the methacrylation and crosslinking utilizing both glycidyl methacrylate and Irgacure 2959 can be found in Appendices H, I and T. The CS-MA and oleic acid with span 80 are then filtered using a 40-μm filter in separate 50-mL conical tubes. Three 10-mL syringes were then prepared by connecting to separate three-way stopcocks. All of the stopcocks had a 5-mL syringe connected at the top side and the Tygon™ tube connected to the opposite side of the syringe using a Luer lock and blunt needle as shown in Figure 6.1.

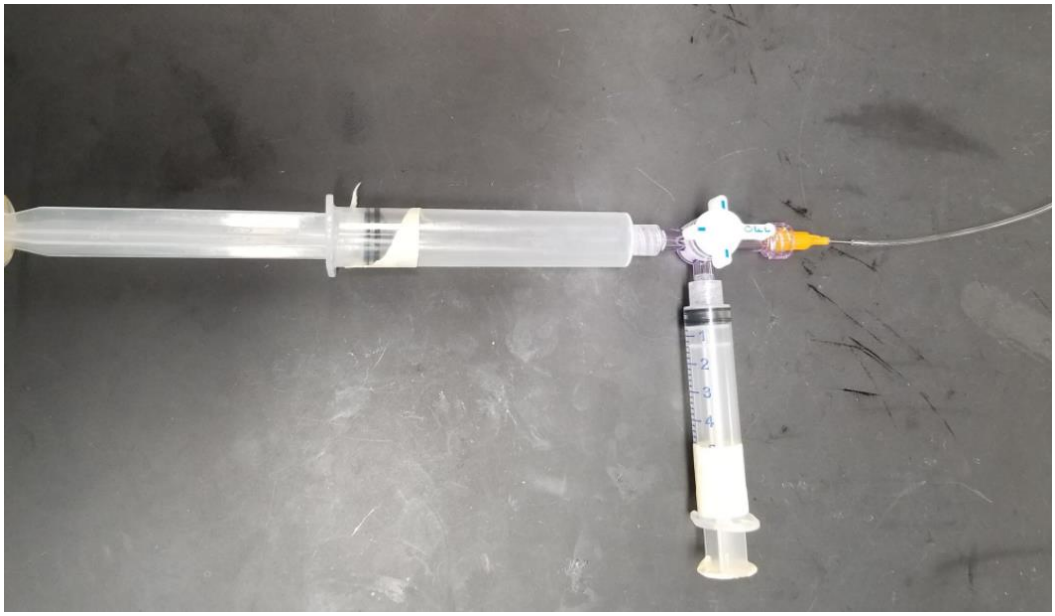


Figure 6.1: Syringe Stopcock Complex. The Three-way stopcock connected to the 10-mL syringe (left), the 5-mL syringe (bottom) and the Tygon™ tubing via the Luer Lock and hypotube needle (right)

3 mL of filtered CS-MA was pipetted into the 10-mL syringe meant to be the dispersed phase. After pushing out most of the air in the 10-mL syringe into the 5-mL one, the stopcock was turned allowing fluid flow for the larger syringe in the tubing. Similarly, 7 mL of filtered oleic acid with span 80 was pipetted into the other two syringes separately and prepped.

The liquids were then pushed through the Tygon™ tubing to remove any air inside the tubing. Both oleic acid with span 80 syringes and the CS-MA syringe were then mounted onto the syringe pumps and placed securely.

The microfluidics device was placed under a microscope (AmScope FMA050). The ends of the tubes from the syringes were then connected to their respective inlets using blunt hypotube. The computer was then setup as explained in Appendix L. The needle end of the output tube was placed in the output channel and the other end was placed inside a beaker for collection. The pumps were set to 1 mL/hr flow rate for the oleic acid with span 80 and 0.1 mL/hr flow rate for the CS-MA pump. Both pumps were

started simultaneously. The UV curing system was setup as detailed in Appendix G. Adjustments were made depending if the system started back flowing or jetting. When the first generated particle started to reach the end of the output Tygon™ tubing, the connecting blunt needle at the end of the output was removed and the end of the Tygon™ was inserted directly into the end of the PVC tubing. The broad-spectrum UV bulb was then turned on. With the given volume the system was run for 3 hours for the first run and another 3 hours for the second. When the experiment was done, the cleanup procedure was followed as mentioned in the previous protocol. The final output which was collected in a glass vial consisted of oleic acid with span 80 and generated particles of crosslinked CS-MA. The collected particles were isolated and washed as mentioned in section 5.3.1.

Washed particles of CS-MA were loaded with DOX dissolved in water as described in section 5.4.2. The DOX diffuses into the CS-MA hydrogel matrix and binds electrostatically. Once loaded, the extra unloaded DOX in solution was aspirated off leaving only the loaded particles. The final product is the complete DDS.

6.2 Final Design impact

It is of high importance to analyze the impact the final design has various aspects of the world. Although our design may not have direct contact with a patient, it may have overseen aspects in economics, the environment, society, ethics, health and safety, and manufacturability. This section will briefly discuss the latter with respect to the project's work.

6.2.1 Economics

The project's work aims at improving the current DDS that is incorporated into chemotherapeutic treatments. The current gold standard is the classic IV drip that systemically delivers lowers doses of drugs in burst releases. With the system this project has done work on, classic chemotherapy with an IV drip is no longer necessary after surgery since the product can be delivered intratumorally. Currently the

product has been manufactured successfully in the presence of an academic lab, students, and academic resources.

Since the developed system follows current neuroblastoma treatment quite closely the economic impact is not great. There is an added cost of the new delivery method and materials used but they may be outweighed by the decrease need of IV treatment, lower systemic side effects, as well as more precise drug dosages. The economic standpoint from a manufacturability point of view is the rigor of the tests such a medical device must endure. The device and manufacturing method will have to undergo testing according to all the regulations deemed by the FDA. The approval process for medical devices is known to take years and be very costly. Additionally, the finally produced product must also be used in humans and that will require human trials which even further rise the expenses and time in approving such a method and product. Although there are many economic challenges to transfer this product from the benchtop to the bedside, positive economic impacts are also a possibility in the long run.

6.2.2 Environmental impact

The environmental impact of products is important to analyze to assure that we are not harming our surroundings. As the project's work remains in the exploratory phase, the large-scale environmental impacts aren't a concern. However, the possible future environmental impacts can be discussed. The environmental impact for this project can be divided into benchtop and bedside materials. The materials from the benchtop include any devices, materials, and tools that are used in the DDS fabrication. The fabrication involves the use of various glass and disposable labware. Another solid material used in quantity is PDMS, along with aluminum foil, PVC, and Tygon™ tubing. All these materials are small and discarded rarely. These materials can be of concern only if the project's work is expanded to a much bigger scale. Future considerations may include alternative discarding and sorting of discarded materials to lessen the environmental impact. Major chemicals that are used are diluted ethanol, pluronic, oleic acid, and CS-MA and doxorubicin waste. These chemicals are either used in too small of quantities,

diluted to an extent that is not of environmental concern when disposed, or disposed of according to local OSHA and/or lab standards.

The second set of materials are the bedside materials; these include the CS-MA DOX loaded particle. While the DDS remains active within a patient's body (this component will be completely inside a patient during this period), the environmental impact should be of no concern. This project timeline does not allow sufficient time to characterize the behavior of the degraded materials and how it is excreted by the patient. Future work before animal testing or clinical trials would have to include *in vitro* testing of the behavior of the DDS over long periods of time as it degrades in a physiological and enzymatic environment in order to assure a safe environmental impact.

6.2.3 Societal influence

The current societal influence for this project's work is minimal. The project focused on designing a fabrication device and validating the final product. The final product is not capable of being tested on human subjects, so there is very little influence on that front. However, if the fabrication design is further developed and a proper DDS is produced to better help the current treatment options for neuroblastoma then the societal influence will be high. First the influence will be high due to the rigorous testing necessary to bring the product to the patient's bedside. When proven successful and indeed superior to current drug delivery methods, the societal influence will be high. The reach for the potential of this product is high and can possibly be applicable to future improvements for treatments in other cancer research. The societal influence will ultimately depend on the success of this project's work and who and what it inspires.

6.2.4 Political ramifications

This project has no political ramifications due to the nature of this project's work. Both its project phase and potential effect do not allow for any significant political impacts. The final objectives of the

project can only potentially enhance known treatments. The only consideration to take for political ramifications of this project are that a better alternative to neuroblastoma treatment would have originated in the United States. However, this consideration is only applicable once the product advances from its exploratory phase to a more developed product closer to market.

6.2.5 Ethical concern

With advancements in biomedical engineering, drug delivery research has seen much growth, especially in recent years. With increasing competition in publishing data as quickly as possible, certain factors pertaining to one's research or practice tend to be overlooked, particularly ethical considerations. Unlike practitioners of medicine, biomedical engineers are not bound by the hippocratic oath and as such ethics in medical research can vary. This can result in haphazard animal testing and rushed clinical trials to get results or place a new drug on the market. The team's objective is to design a system for the fabrication of a DDS and characterize drug loading and release *in vitro*. None of the tests involve human trials or animal experimentation. The sole purpose of the project is to serve as a foundation for a new type of polymeric drug carrier to see if there is a future potential for this product or a modified version of it for further research. Regardless, as researchers, care should be taken while conducting tests and collecting data that they are not falsified intentionally show the efficacy of the drug carrier or to cut corners in research to save time. the data and procedures should be sound and not altered. There is no conflict of interest. The research team is an individual entity, completing their senior year project, with no ties to third parties or companies that might have a stake on the final results.

6.2.6 Health and safety issue

The health and safety issues for this project are applicable to the people that come into contact with it: the fabricators/administrators and the patients of this kind of product. The fabrication process does not pose any danger to the operator. However, the point where the DOX must be loaded onto the particles poses risks. However, this risk is not new since DOX is a typical chemotherapeutic drug that is handled

for cancer patients regardless so the necessary protocols and safety precautions would adequately be followed. Secondly, the health and safety issue of the patient would potentially be assured. Although the current product phase is distant from seeing a patient, future health and safety issues should be minimal due to strict regulations by the government on making sure a product is safe prior to introducing it commercially.

6.2.7 Manufacturability

The project does have some manufacturability concerns. The final procedure and product have proved to be relatively low cost and requires low cost maintenance as well. However, the current manufacturing world typically leans towards automation and the procedure still involves frequent monitoring. Since the project is still in an exploratory phase, this isn't a high priority item to improve but it can be a future task to be considered. Additionally, other considerations than can be made is making sure that the integrity of molds and reused equipment yield reproducible and replicable results.

6.2.8 Sustainability

The project has a low sustainability impact. The fabrication process still has room for improvement but insignificant amounts of materials are being lost in the process. Additionally, the main materials that may affect the biological world are oleic acid and CS. CS is a GAG that is naturally derived and abundant in the ECM. Currently, the benefits of using CS biologically are worth the slight disadvantage of having a project that relies on a naturally derived source. However, this material is not rare and is easy to obtain. However, if the project should be continued to a further degree, a higher consideration of industrial scalability must be taken when identifying the most suitable materials to use in this application at a larger scale.

VII. Discussion

7.1 Microfluidics Particle Generation Experiments

Future studies and considerations will be made in order to find the proper amount of time that must be waited in order to consider the produced particles usable as it takes the system a couple of minutes to adjust. The different configurations in Tables 5.1 and 5.2 shows the different continuous and dispersed phases the team has used for the experiments. Oil was first used along with water as an introduction for microfluidics and the microfluidic devices. The team then changed the water to PVA on different percentages to learn how to deal with more viscous non-Newtonian fluids on the same microfluidic device. The oil was then changed to oleic acid since it can be filtered out from particles easier since it dissolves in ethanol. The PVA percent went up to 20% since it is closer to CS. Figure 5.2 depicts images of both 10% and 20% PVA along with oleic acid with their respective flow rates. The particle size varied between 36.04 and 64.84 µm due to different temperatures in the atmosphere at the times of the experiment and other miscellaneous reasons that are outside of the team's control. Figure 5.3 shows the testing of different sized flow focusing devices. The team decided that using the 100 µm device was the best choice since it is easy to handle and it yields particles with diameters smaller than the channel width (<100 µm). The smaller diameter particles were due to the pressures inside the device being more stable as well as the flow rates being more optimized compared to earlier flow rates. The smaller size flow focusing devices were harder to handle and some of them did not generate particles. More specifically, the 40 µm and 60 µm device were not able to generate particles. The smaller sized flow focusing devices undergo more pressure, due to the smaller area, than the bigger sized flow focusing fittings and jetting is more likely to occur. Figure 5.4A plots the flow rates collected during the experiment. The x-axis represents the oleic acid flow rates while the y-axis represents the 20% PVA-MA

flow rates. The plotted points are represented by a shape indicating what type of behavior was recorded during the experiment. Backflow is when there is too much pressure on the continuous phase compared to the dispersed phase and thus makes it backflow on the dispersed phase. Dripping is when the droplets are generating, whether it is on the flow focusing part of the device or even into the serpentine channel. Jetting is when there is a single stream going throughout the entire device and this does not generate particles. Figure 5.4B shows the regression analysis of said flow rates in part A and they are compared to the size of the droplet that was generated. The linear equation is $y = -1.2074x + 55.079$ and its R^2 value is 0.3321. The line is going downwards suggesting it has a negative relationship between the flow rate ratio and the droplet size generated. However, the R^2 value suggests that the line is not a good representation of said relationship and therefore there isn't a direct negative correlation. Further data points should be obtained to have stronger data that could suggest a correlation.

The CS-MA flow rates used during the CS-MA experiments were taken from the preliminary data from 20% PVA-MA. Since 20% PVA-MA has a similar viscosity to CS-MA, the flow optimized flow rates were transposed with little corrections. The optimized flow rate for PVA-MA was 1 mL/hr for the oleic acid with span 80 and 0.13 for PVA-MA. The newly optimized flow rate for CS-MA was 1 mL/hr for the oleic acid with span 80 and 0.1 mL/hr for CS-MA. The small modification of the flow rate was due to the difference of properties in the polymers like similar viscosity but not equal to each other, how each polymer reacts to differences in pressure and also the degree of methacrylation of both polymers.

After collection of the particles from batches A, B, C and D, particle size distribution was measured and compared both before swelling and one-week post swelling in water. Figure 5.7A shows the size distribution of particles within Batch A. The particles were distributed in a bell curve with the peak lying at the mean data. The mean and median are close but do not intersect as shown in the box and whisker plot in Figure 5.7B. This means that the particles are not normally distributed diameter wise. This may be due to having a low sample size and if more images were taken of the particles and sized, it could

show Gaussian distribution. The particle sizes for batches A, B, C and are 98.44±23.26 μm, 96.15±38.11 μm, 95.59±38.48 μm and 82.79±20.6 μm. ANOVA showed no significant difference in particle diameters between batches. This shows that with the final design for the fabrication system it was possible to get reproducible sized particles between batches. Batch D had the smallest mean particle size but this may account to its small sample size of 38. The CS-MA particles are hydrogels and swell when in contact with an aqueous solution. Figure 5.6 shows the diameter change of particles pre and post swelling. When compared within batches using a student's t-test, the increase in diameter was significant which was predicted. This means that particles produced in the microfluidics device which were initially much less than 100 μm could swell up to more than that. This issue, however, should not pose as a problem since the drug delivery vehicle is still within the microscale.

7.2 Doxorubicin Loading and Release Experiments for CS-MA Microparticles

An average of 385 μg of doxorubicin was loaded per mg of particles. According to the data, the loading was repeatable with approximately the same amount of doxorubicin being absorbed into the microparticles during the incubation time. On Day 1, there was an initial burst release of 44 μg of doxorubicin, which is 6% of the total doxorubicin loaded onto the 2 mg of particles. By Day 9, the release profile becomes linear with a release rate of 2.04 μg/day. These data validate that the CS-MA microparticles achieved sustained release of a chemotherapy drug at a linear release rate.

Currently, systemic chemotherapy is administered over the course of a month-long cycle. During the first week, the patient must be treated with intravenous (IV) chemotherapy for a few hours each day. The next three weeks are spent as a resting period between IV treatments. Based on this schedule, an ideal DDS would be able to release 100% of the desired drug amount over the course of one week. At one

week, the CS-MA microparticles released an average of 12.7±3.37 % of the total loaded drug. To increase the rate of drug release, the properties of the CS-MA microparticles must be modified.

7.3 Cytotoxicity Assay of Unloaded and DOX-Loaded Particles

The drug activity of doxorubicin after binding to and desorbing from the CS-MA particles was validated by running a biocompatibility test. The data shows that the doxorubicin release was more cytotoxic at higher concentrations and at earlier time points, as predicted. This is reflective of the initial burst release of drug from the particles and the decreasing amount of doxorubicin released per day. The particle-treated cells had a lower cell viability percentage, however, this may be due to residual solvents on the particles such as ethanol or the mechanical shearing of the particles against the KELLY cells. Overall, these data verify that the doxorubicin released from the CS-MA particles remains active over time, which is necessary for a sustained drug release system.

VIII. Conclusions and Recommendations

In conclusion, our project team developed and validated a successful fabrication system. The fabrication system creates consistent particles. This completed objective was validated by characterizing the size of CS-MA microparticles. The system also proved to be user friendly and cost efficient. These completed objectives were validated by providing easy to use operating protocols and by using the allotted budget efficiently for component selection. The resulting DDS has competitive drug carrier properties. This objective was validated by loading the microparticles with doxorubicin, characterizing the release profile, and confirming doxorubicin activity. The DDS also proved to be user friendly and cost efficient by easy handling and efficient use of the budget for material selection. Future directions include experimentation with the microfluidic device by altering the channel size to explore how to decrease particle size; long-term drug release studies; different CS-MA concentrations and how it affects the particle production; as well as experimenting with other chemotherapeutic drugs and sterilization techniques. More long-term recommendations include exploring how to incorporate the DDS into delivery methods such as gel injections, sprays (using aerosol science to create monodisperse nanoparticles), and wafers.

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Zhou, Y., & et al.Photopolymerized water-soluble maleilated chitosan/methacrylated poly (vinyl alcohol) hydrogels as potential tissue engineering scaffolds.

X. Appendices

Appendix A: Licensed use of Figure 2

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Title: Injectable hydrogel-based drug delivery systems for local cancer therapy
Author: Mohammad Norouzi,Bahareh Nazari,Donald W. Miller
Publication: Drug Discovery Today
Publisher: Elsevier
Date: November 2016

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Appendix B: Objectives and Sub-objectives PWC

Main Objectives PWC

		Device					DDS			
		Consistent Product	User Friendly	Cost Efficient	Industrial Scalability	Minimal Environmental Impact	Competitive Properties	User Friendly	Cost Efficient	Sum
Device	Consistent Product		0	0.5	1	1	0	0.5	0	3
	User Friendly	1		1	1	1	0	1	0.5	5.5
	Cost Efficient	1	0.5		1	1	0	0.5	0	4
	Industrial Scalability	0	0	0		0.5	0	0	0	0.5
	Minimal Environmental Impact	0	0	0	0.5		0	0	0	0.5
DDS	**Competitve Properties**	1	1	1	1	1		1	0.5	6.5
	User Friendly	0.5	0	0.5	1	1	0		0	3
	Cost Efficient	1	0.5	1	1	1	0.5	1		6

Consistent Product PWC			
	Reproducibility Within Product Batch	Reproducibility Between Batches	Sum
Reproducibility Within Product Batch	-	0	0
Reproducibility Between Batches	1	-	1

Cost Efficient PWC			
	Inexpensive	Cost Effective Corrections	Sum
Inexpensive	-	0.5	0.5
Cost Effective Corrections	0.5	-	0.5

User Friendly PWC						
	Efficient Set-up	Modular	Safe To Use	Consistent Behavior	Adaptable	Sum
Efficient Set-up	-	0	0	0.5	0	0.5
Modular	1	-	0	0.5	0	1.5
Safe To Use	1	1	-	1	0.5	3.5
Consistent Behavior	0.5	0.5	0	-	0.5	1.5
Adaptable	1	1	0.5	0.5	-	3

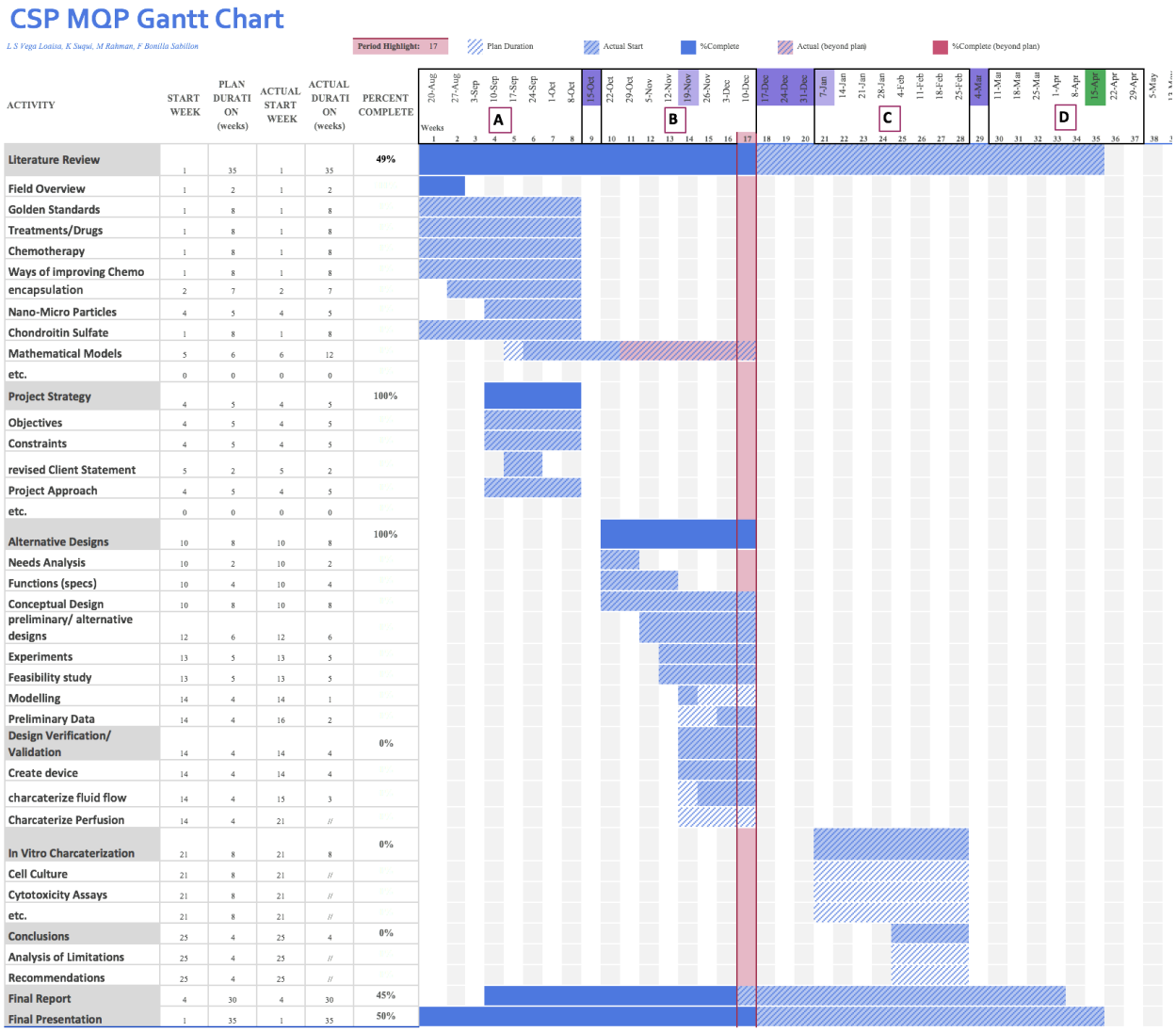
Competitive Drug Carrier Properties PWC						
	Extended Release	Biodegradable	Physiologically Safe Interactions	Adaptable to Delivery Methods	Adaptable to Potential Future Work	Sum
Extended Release	-	1	0.5	1	1	3.5
Biodegradable	0	-	0	0	0.5	0.5
Physiologically Safe Interactions	0.5	1	-	1	1	3.5
Adaptable to Delivery Methods	0	1	0	-	0	1
Adaptable to Potential Future Work	0	0.5	0	1	-	1.5

User Friendly PWC			
	Safe to Handle	**Modular**	Sum
Safe to Handle	-	0.5	0.5
Modular	0.5	-	0.5

Cost Efficient PWC			
	Inexpensive	Cost Effective Processing	Sum
Inexpensive	-	0.5	0.5
Cost Effective Processing	0.5	-	0.5

Appendix C: Gantt Chart

The following page (Figure 8) illustrates the team's projected schedule in the form of a Gantt chart.



Appendix D: PWC for alternative designs

		Objectives			
		Competitive Drug Carrier Properties	**Cost Efficient**	**User Friendly**	
		1-extended release	1-inexpensive	1-safe to handle	
		1-physiologically safe interactions	1-cost effective processing	1-modular	
		1-adaptable to potential future work			**Total**
DDS Material	**PVA-MA**	**2**	**2**	**2**	**6**
	PLGA-PEG	3	0	2	5
	CS-MA	**3**	**1**	**2**	**6**
	PCL-PVA	2	0	1	3

		Objective			
		User Friendly	**Cost Efficient**	**Consistent Product**	
		2-Modular	1-inexpensive	1-Reproducibility between batches	
		1-safe to use	1-cost effective correction		
		2-consistent behavior			
		1-adaptable			**Total**
Fabbrication System	**Coaxial Co-flow**	3	0	1	4
	Coaxial Glass D	1	2	0	3
	PDMS Co-flow	**6**	**2**	**1**	**9**
	PDMS Cross-flo	**6**	**2**	**1**	**9**

Appendix E: Making the Master Template for the PDMS Device via Photolithography

Adapted from Albrecht Lab (WPI) and Natalia Vargas-Montoya (WPI)

Materials:

4 ml SU-8 2035 photoresist

4-inch silicon wafer

Spin coater

Hot plate

UV photomask

UV mask aligner

Isopropyl alcohol

Nitrogen gas

Fabrication of the Master:

1. Dispense around 2 ml of SU-8 2035 photoresist onto a clean and dry 4-inch silicon wafer
2. Coat the dispensed photoresist evenly using a spinner at 1875 RPM to create 50 μm thickness
3. Soft (Pre)-bake the wafer for 1 min 40 s at 65°C and for 8 min at 95°C and then again at 65°C for 2 min on a hot plate (Keep the wafer level at all times)
4. Dispense another 2 ml of SU-8 2035 on top of the baked photoresist and repeat steps 2 and 3 to create a final thickness of 100 μm
5. Use a transparency photomask to produce the master by exposing the wafer to 12 s of UV light at a dosage of 230 mJ/cm^2
6. After exposure bake the wafer at 65°C for 5 min and then at 95°C for 10 min and again at 65°C for 2 min
7. Develop for 5-6 min in photoresist developer

8. Rinse the substrate with isopropyl alcohol to remove uncrosslinked photoresist
9. Hard bake for 45 min at 150^{o}C
10. Dry gently with pressurized nitrogen gas and store the master under a cover to prevent dust exposure

Appendix F: Making PDMS Device from Master Template (Soft Lithography)

Natalia Vargas-Montoya provided the team with the master template to pour PDMS into.

Materials:

PDMS

Elastomer reagent

Weight boat

Disposable Stirrer

Vacuum

Master Template in Petri dish

Mass balance

Oven

PDMS Preparation

1. Ratio of 9:1 PDMS to elastomer
2. Well mixed into a total PDMS solution (100g) for even consistency (or 40g if ring of PDMS is present)
3. Place PDMS solution in a vacuum in order to remove bubbles
4. Carefully, pour PDMS into the silicon master template in a petri dish (15cm)
    a. *take caution to avoid trapping air bubbles in the PDMS while pouring
    b. *possibly pour PDMS with slight excess (making fence around with lab tape
5. Leave the dish of PDMS in a 60^{o}C oven overnight

Tubing Assembly

Materials:

"Needle ends" (stiff hypotubes) estimated to be about 1.5cm long

Hypotubes with Luer-Lok ends

Plastic tubing (Tygon™ Box)

Razor blade

1. Pull Tubing out to 50 cm length and cut
2. Use razor blade to cut
3. Hypotubes were inserted 1/3 of the way into the Tygon™ plastic tubing
    a. *used gloves and ethanol to assist with inserting the tubing
4. Lone hypotubes go in on one end and hypotubes with the luer lock go into the other end of the plastic Tygon™ tubing

Preparing the devices

1. Cut PDMS mold out making sure not to crack the plate underneath and to avoid air bubbles
    a. The cut is made along the wafer's line markings
2. Gently slide a razor blade horizontal to the mold and cut straight down in order to cut out the individual devices, do not slide the razor or else It will cut the device at an angle
3. A small piece of tape is placed on the side of the device with the holes and channels imprinted on them
4. Use a sharpie to mark each input and output hole to be punched out
5. Using a 1mm biopsy needle, punch holes where indicated by the sharpie
    a. *the biopsy needle will get shorter if this is done at the bench due to the force; to prevent: punch in the air; to fix: stick a paperclip inside the needle and firmly pull out to "lengthen" the hollow punching needle
6. During punching, if the polymer tube is not visible outside of the device, use a thinner instrument to ensure the polymer tubule is no longer present in the punched hole
7. Flow 1) deionoized water 2) 100% ethanol 3) deionized water through each hole
8. Dry devices and remove dust by taping

9. Leave a piece of tape on the microchannel side of the devices in order to prevent dust from making contact with the surface
10. Repeat steps 6-9 for glass slides and only on the side that is labeled to later plasma bond

Plasma Bonding

This step is done to covalently bind the PDMS devices to the glass slides. It is done by oxygen plasma treatment of both clean surfaces.

Materials:

Glass tray

Slides and test slide

Scrap PDMS device

Tape

Plasma cleaner

Vacuum pump

Desired PDMS devices

The set-up was done as outlined by the Albrecht Lab (WPI Gateway BME department) Protocol

PDMS Bonding

*These steps can be followed first with a scrap piece to practice

1. Remove the pieces of tape of the device and slide and place next to each other on glass tray facing up (the side to be treated)
2. Insert tray to the chamber, close door valves, turn on the vacuum pump, and make sure the door is held in place
3. The machinery and set-up to plasma treat the surfaces was followed as stated by the Albrecht Lab (WPI Gateway BME department) Protocol
4. Wait 30 seconds to complete the plasma treatment

5. Turn the power switch off and the vent on before carefully removing the treated objects
6. Invert the PDMS device onto the surface of the glass slide with desired alignment carefully as it is bonded instantly
7. Apply light pressure to remove air bubbles trapped at the interface once PDMS device is sealed
8. Wait 30 seconds to test an edge for bonding by gently tugging at a corner.

Appendix G: Final Fabrication System Setup

By: Mohammed Masrur Rahman,

Materials:
Tygon™ Tubing
PVC Tubing
Razor Blade
Hypotubes, various Luer Locks
Three Way Stopcocks
Pre-prepared microfluidic device
Dispersed phase (CS-MA)
Irgacure 2959
Continuous phase (Oleic Acid with Span 80)
Oleic Acid
5% Pluronic
milliQ H_2O
(3) 5 mL
(3) 10 mL
(2) 40 μm filters (one for each phase)
1 single syringe pump
1 double syringe pump

Pre-Set-Up

1. Prepare tubes (using razor blade)
    a. 3 Tygon™ tubes inputs cut to 50 cm lengths
        i. Place hypotubes on one end, hypotubes with luer lock on the other end (⅓ of the way in)
    b. 1 Tygon™ tube output cut to 100 cm length
        i. Place hypotube in one side
    c. 1 PVC tubing cut to 100 cm
        i. Place male luer lock one end (attach a three-way stopcock to that end)

A **B**

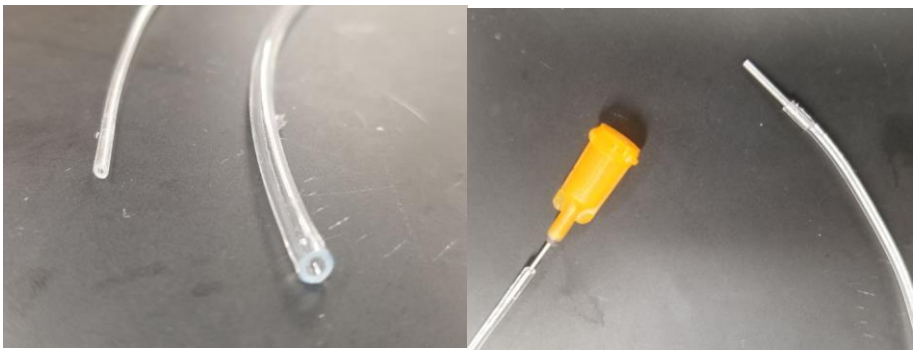


Figure 1: (A) Tygon™ Tubing (left) and PVC Tubing (right) and **(B)** Tygon™ Tubing with the hypotube and Luer Lock (left) and just hypotube (right)

Set-Up

2. Obtain pre-prepared device (protocol in Appendices E and F)
3. Turn off the lights in the experimentation room
4. Prepare 20% CS-MA with Irgacure 2959 (protocol in Appendices H and I)
    a. Filter using 40 μm filter into a 50-mL conical tube
    b. Cover conical tube with aluminum foil to prevent unwanted premature crosslinking of CS-MA
5. Prepare Oleic Acid with Span 80 (protocol in Appendix J)
    a. Filter using 40 μm filter into a 50-mL conical tube
6. Run 5% Pluronic (protocol on Appendix K) through all tubing and the microfluidics device
    a. Let it sit inside the device or tubing for 5 minutes
    b. Rinse out the Pluronic using milliQ H_2O
7. Prepare the PVC outlet tubing by running oleic acid through the connected stopcock and closing it until the experiment starts
    a. If white flakes of CS-MA (from a previous experiment) are noted inside the PVC tubing, push it out using air and then running step 6 on the tubing
    b. Repeat 7 until no white flakes are observed
8. Prepare syringes and inlet tubing
    a. Connect the 10 mL syringe and a 5 mL syringes onto a 3 way stop cock. Repeat this process twice more
    b. Connect the respective inlet tubing to the stopcocks by attaching the luer lock hypotube
    c. In one syringe place 3 mL of the dispersed phase (CS-MA)
        i. Cover around syringe with aluminum foil
    d. In the other two, place 7 mL of the Oleic Acid

    These volumes are sufficient for two 3 hour runs. Using less than 2 mL for any of the phases is ***not advised*** *as the amount of liquid left in the syringes after pushing it through the stopcock and tubing in a later step, is not enough, making it* ***NOT*** *cost effective.*
    e. After rotating the syringe-stopcock complex such that that the end connecting the tubing is facing upwards, get rid of air bubbles in the 10mL syringe by using the 5mL syringe as air storage

i. In case the 5mL syringe is fully extended, close the larger syringe using the stopcock and disconnect the smaller syringe.
ii. Reconnect the smaller syringe after pushing the plunger back inside
iii. After all the air is expelled in the syringes, reconnect the smaller syringe with the plunger fully extended outwards

Figure 2: Syringe-stopcock complex in the upward direction to push air out of the 10mL syringe into the 5mL syringe. The stopcock is closed towards the tubing direction to allow air flow in between syringes.

f. Keep the outlets of the input tubing in a waste beaker and push the fluids through the tubing using the syringe expelling the excess air
    i. Once the air is expelled and the fluid reaches the ends, close off the stopcocks
g. Connect the two syringe pumps and place them in their designated positions

[Prepare syringes Picture]

h. Place syringes so that the 10mL syringes are parallel to the pump actuators and the 5mL one is perpendicular and vertical to the pump surface.
    i. Put the syringes in place in the syringe pumps
    ii. Move the actuators such that they are touching the ends of the plungers and lock the actuators
    iii. Ensure the actuator is fixed in place by manually moving it while the system is locked in place
        1. If the pump makes noise during running, it means that the actuators were not properly secured and got displaced
i. Ensure that there are no air bubbles in the syringe system prior to starting the pumps and after

i. Extra air bubbles in the tubing can be pushed out using the syringe pumps set to 3mL/hr.
ii. Turn off the syringe pump once the air bubbles are not visually observed in the tubing

Figure 3: The syringe pumps placed on the aligned to the marked red tape. The single syringe pump (red) is meant for the dispersed phase and the double syringe pump (black) is meant for the continuous phase

9. Connect the microscope and camera
10. Set up computer (protocol in Appendix L)
11. Set up pumps and connect inlets
    a. Make sure the pumps are set to the right syringe dimensions (14.5mm for 10 mL syringes) and flow rate (1mL/hr for both continuous phase and dispersed phase initially)
    b. Ensure the inputs are flowing properly into a collection beaker prior to connecting to the device
    c. Wrap excess inlet tubing around the perpendicular 5 mL syringes and up to the top of the extended plunger to ensure consistent heights
    d. Connect the inlet tubing to their respective holes in the microfluidic device
    e. Place the microfluidics device under the microscope and clip it in place
    f. Ensure that the flow focusing channel of the device is focused on using the microscope

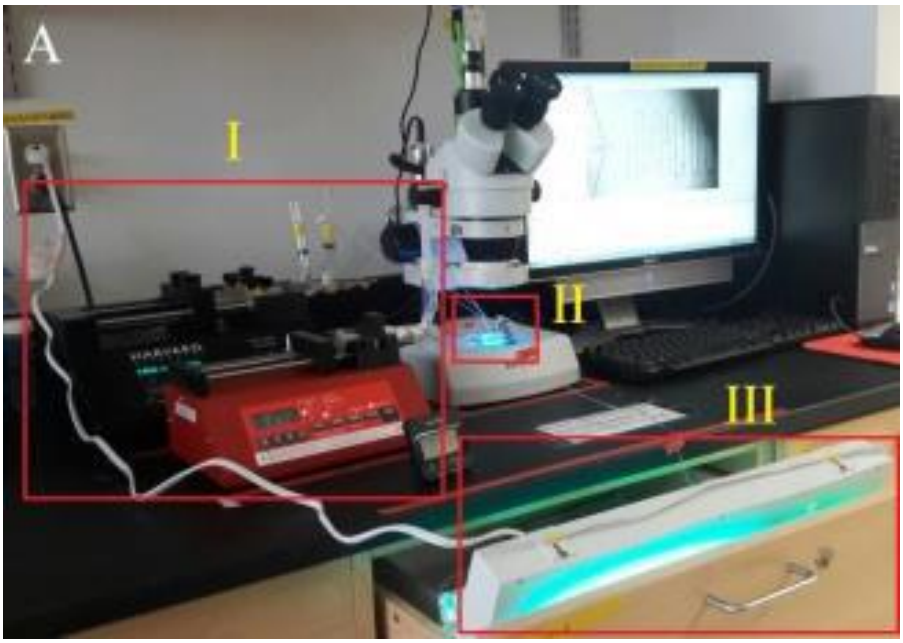


Figure 4: Final experimental setup as (I) Syringe pumps, (II) Droplet generator and (III) UV treating setup

12. Set-up the UV crosslinker facilitating V-boat lined with aluminum foil and collecting vial
    a. Open drawer underneath the benchtop and place the V-boat and vial securely.
    b. Use aluminum foil generously to prevent UV light from escaping from the drawer
    c. Place the oleic acid filled PVC tubing in the valley of the V-boat with the stopcock end inside the collecting vial
13. Connect output Tygon™ and place the other end into the waste beaker and begin running the pressure transducers
    a. Run both the syringe pumps simultaneously
    b. Once the dispersed phase in observed to be flowing through the channels in the device, decrease the flow rate of the dispersed phase gradually to 0.1mL/hr (optimized flow rate)
    c. While particles are initially generated use the UV bulb to help pre-crosslink particles until all the air is pushed out of the output Tygon™ tubing
14. Once all the air in Tygon™ output tubing is expelled, connect it immediately to the open end of the PVC tubing by inserting it inside directly
    a. Place the excess Tygon™ output tubing inside the valley of the V-boat so that particles can be pre-crosslinked
    b. Remove the three-way stopcock and male Luer Lock from the end of the PVC tubing and place the newly open end into the collecting vial
    c. Place the UV Bulb on top of the drawer directly over the V-boat

Figure 5: Setting up of the (I) V-boat and (II) collecting vial in the drawer with the UV light bulb above it. An extra sheet of aluminum foil is on the top which can be moved in placed to protect the contents of the drawer other than the setup from UV exposure

During Fabrication

1. Monitor particle fabrication through the computer monitor

[Monitor Picture]

2. Troubleshooting
    a. **If Jetting** - decrease dispersed phase flow rate
    b. **If backflowing** - increase dispersed phase
3. Once enough particles are collected or the syringes have run out of liquid, stop the pumps
    a. Disconnect all tubing from the device
4. Push the remainder of the fluid inside the output Tygon™ tubing and PVC tubing into the collecting vial using syringes filled with air (empty dry syringes that are fully extended)
    a. Shine UV light into the open collecting vial for 20 minutes to ensure crosslinking saturation

Clean Up

5. Empty the contents of the syringes into their respective containers if they are still useable.
    a. Empty the contents of the inlet tubing into the waste beaker
    b. Push out the fluid in the tubing using air
6. Clean all parts used in the fabrication system including device, syringes, stopcocks and all tubing
    a. Rinse with deionized water
    b. Rinse with soap and water
    c. Rinse with deionized water
    d. Rinse with 70% ethanol
    e. Rinse with deionized water
    f. Hang the tubing to dry
    g. Place the microfluidics device in the 60^{o}C oven overnight to dry
    h. Leave the syringes with the plungers disconnected, outside and covered to dry
7. Disconnect the computer program and the microscope/camera
8. Store away the pumps and other devices used

Clean Up

9. Collect the contents of the collecting vial in a 15mL conical and label correctly
    a. Wash the particles in 100% ethanol to remove wash the oleic acid as mentioned in Section 5.3.1 (The protocol is mentioned again below for ease of reading)
    b. Centrifuge collected particles at 4,400 RPM for 5 minutes (Centrifuge Eppendorf 5702).
    c. Pipette out the supernatant (oleic acid) and resuspend the particles in 3 mL of 100% ethanol
    d. Repeat steps 9b and 9c three more times
    e. Store the particles in 100% ethanol at room temperature
        i. This keeps the particle disinfected for further use or testing

Appendix H: Making CS-MA

Materials:

CS
HCL
GMA
Acetone

Protocol

1. 5 g CS (87-90% hydrolyzed avg MW 30,000-70,000 Sigma P8136) dissolved into 45 mL water
2. Add 32.5 mL GMA (97%, 100 ppm MMEQ inhibitor, Sigma 151238)
3. Adjust pH to 1.5 with concentrated HCl
4. React at 25C w/ stirring for 3 hrs, light protected
5. Precipitate immediately with acetone
6. Wash once with acetone
7. Let dry at RT overnight
8. Resuspend in 50 mL H2O
9. Dialyze minimum 48 hrs
10. Freeze at -80C overnight (max 30 mL in 50 mL tube)
11. Lyophililze minimum 72 hrs

Appendix I: Preparing Irgacure

Preparing Irgacure Solution for Crosslinking PVAMA and CS-MA Particles
By: Leonela Vega

Materials:
Irgacure Powder (Add Manufacturer)
70% Ethanol
PVAMA Solution at desired concentration
Pipettes and pipette tips

Procedure:

1. Weigh out Irgacure powder to form a 10% concentration in 70% ethanol
    a) 10% (w/v) of Irgacure is 100 ug of Irgacure/ 1 mL of ethanol
2. Pipette up and down gently to mix a uniform solution of 10% Irgacure in ethanol
3. Add the 10% Irgacure into PVAMA or CS-MA solution
    a) The final concentration of Irgacure in the polymeric solution (PVAMA or CS-MA) should be 0.1%

Note: Make a small enough volume to be used that day. CANNOT BE STORED.

Appendix J: Preparing Oleic Acid with Span 80

Producing span 80 Surfactant
By: Natalia Vargas-Montoya, Fabian Bonilla

Materials:
Oleic acid
Sorbitan monooleate (Span 80 surfactant)

Procedure:
For 40 ml of total volume (oleic acid + span 80):

1. Get 34 ml of oleic acid in a conical tube
2. 6 mg of span 80 should be added
3. This will yield 2% wt span 80 in oleic acid
4. Shake manually

Note: span 80 is very viscous! Handle with care.

Appendix K: Preparing 5% Pluronic

Producing Pluronic F-127 for Flow-Focusing Microfluidics Devices and Fabrication of Polymeric Microparticles
By AAT BioQuest, modified 1/31 by Kathy Suqui

Materials:
Pluronic F-127 solid powder
Distilled water or anhydrous DMSO
Conical Tube (50 mL)
Metal or Plastic Spatula
Micropipettor
Pipette tips (appropriately sized)
Personal Protection Equipment

If Performing Fluorescence Work on Cells, also prepare:
DMSO AM ester

Procedure:

Preparing a stock solution of Pluronic F-127

1. Weigh out the required amount of solid powder Pluronic for the appropriate concentration in liquid.

ex: *1 g of Pluronic F-127 powder for 10 mL of distilled water for 10% (w/v) of Pluronic, or 2g of Pluronic F-127 for 20 mL of anhydrous DMSO organic solvent for 20% (w/v) of Pluronic*

2. Dissolve Pluronic in the amount of water or DMSO as determined in Step 1.
3. Heat solution in water bath (40°C) for 20-30 min until solution is well-mixed and Pluronic is completely dissolved.
4. Store at room temperature for future use.

Preparing a working solution of Pluronic F-127

1. Dilute Pluronic to 5% (w/v) by adding solvent from Step 2 above until proper concentration of Pluronic is reached.
2. To load Pluronic onto cells, add Hanks or Hepes buffers to the working solution to a 1:1000 to 1:500 dilution for a 0.02% to 0.04% working solution

Appendix L: Setting up the Computer

This is the simplified setup procedure. The complete procedure is available in Coburn Lab in the drawer underneath the computer

Procedure:

1. Connect camera and scope to outlet
2. Connect the camera ethernet port to the computer
3. Open “IP Configurator” on the computer
    a. If the camera name is shown, all parts were connected correctly.
    b. If it does not show, wait a bit and refresh the screen. If problem persists ensure all connections are correct and try again
4. Open “Viewer”
    a. Click on “devices” at the bottom left. The camera should appear under GigE. If it does not appear check IP Configuration
    b. Click on “feature”
    c. Load features (there should only be one mapped, otherwise check the main hard copy of the protocol)

Appendix M: Preparing 5%* PVA Solution (Adapted from Prof. Coburn's Lab)

Materials:

200 mL MilliQ H_2O

10 g PVA powder

Procedure:

1. Weigh 10 grams of PVA
2. Using a graduated cylinder, obtain 150 mL of MilliQ H_2O
3. Add 10 grams of PVA and 150 mL of MilliQ H_2O to the beaker
4. Place the beaker on a hot plate with a set temperature of 70°C. Monitor the temperature until it reaches the constant 70°C (takes approximately 2 hours checking on it every ~20 minutes).
5. Once the temperature is consistently at 70°C, cover the beaker with aluminum foil and let the PVA stir and dissolve in the water overnight.
6. The next day, transfer contents to a graduated cylinder and add MilliQ H_2O to a final volume of 200 mL making sure that the finals contents are also fully mixed by pipetting technique or using magnetic stir bars in a beaker.

*To make alternative percentages of PVA, the ratios of water to PVA content must be changed by using the following equation:

$$m_1v_1=m_2v_2$$

Appendix N: Raw Data of the Estimated Size of the Obtained Particles Under Various Conditions

L	M	N	O	P	Q	R	S	T	U	V	W	X
	Date	Oleic Acid	10% PVA	Ratio	Diameter (µm)			AVG (µm)	std dev	comments		
	1-Dec	1	0.25	4.00						multibreakdown		
	1-Dec	1.3	0.25	5.20						multibreakdown		
	1-Dec	1.5	0.25	6.00								
	1-Dec	1.5	0.25	6.00	81.9	68.17	77.71	75.93	7.037			
	1-Dec	1	0.15	6.67						multibreakdown		
	1-Dec	1.45	0.185	7.84	67.4	71.25	55.86	64.84	8.009			
	1-Dec	1.6	0.15	10.67	84.14	65.51	72.26	73.97	9.432			
	1-Dec	1.7	0.15	11.33	72.7	68.65	65.83	69.06	3.453			
	1-Dec	1.7	0.145	11.72	70.51	75.08	65.67	70.42	4.706			
	Date	Oleic Acid	20% PVA	Ratio	Diameter (µm)			AVG (µm)	std dev	comments		
	6-Dec	1.9	0.28	6.79	52.69	47.67	40.84	47.07	5.948			
	6-Dec	1.9	0.12	15.83	47.83	46.45	42.39	45.56	2.827885	some 5microns		
	6-Dec	2	0.12	16.67	85.5	81.06	75.57	80.71	4.974			
	6-Dec	2	0.12	16.67	51.53	46.22	51.92	49.89	3.184			
	6-Dec	2	0.1	20.00						unclear image;		
	6-Dec	1.9	0.08	23.75	42.58	28.65	36.88	36.04	7.003			
	6-Dec	2	0.08	25.00						unclear image;		
	6-Dec	1.2	0.04	30.00						multibreakdown		
Stabalizing Time Study	Date	Oleic Acid	20% PVA	Ratio	Diameter (µm)			AVG (µm)	std dev	Comments	Time (minutes)	Device Name
	8-Dec	2	0.1	20.00	50.44	60.08	60.08	56.87	5.565657	blurry image	5	6-28-27 100µm
	8-Dec	2	0.1	20.00	73.3	80.37	73.06	75.58	4.152883	blurry image	10	6-28-27 100µm
	8-Dec	2	0.06	33.33	49.34	37.805	23.7	36.95	12.84145	blurry image	5	6-28-27 100µm
	8-Dec	2	0.06	33.33	57.37	40.57	29.59	42.51	13.99124	blurry image	10	6-28-27 100µm
	8-Dec	2	0.08	25.00	47.6	56.4	69.1	57.70	10.80879	Very Blurry	5	6-28-27 100µm
	8-Dec	2	0.08	25.00	67.96	59.78	45.16	57.63	11.55059	very blurry	10	6-28-27 100µm
	8-Dec	2	0.12	16.67	86.07	60.21	75.26	73.85	12.9878	blurry image	5	6-28-27 100µm
	8-Dec	2	0.12	16.67	55.14	31.55	66.01	50.90	17.61693	blurry image	10	6-28-27 100µm
	8-Dec	1	0.04	25.00	82.02	55.25	45.3	60.86	18.9912	clear image	5	6-28-27 100µm
	8-Dec	1	0.04	25.00	69.98	60.85	111.13	80.65	26.78544	clear image	10	6-28-27 100µm
	8-Dec	1	0.06	16.67	61.8	38	50.18	49.99	11.9011	clear image	5	6-28-27 100µm
Flow Focusing Dimension	Date	Oleic Acid	20% PVA	Ratio	Diameter (µm)			AVG (µm)	std dev	Comments	Time (minutes)	Device Name
	13-Dec	2	0.06	33.33	38.82	28.66	47.24	38.24	9.303569	"later time"	10	80µm B
	13-Dec	2	0.06	33.33	38.8	22.93	34.4	32.04333333	8.193268		10	80µm B
	13-Dec	2	0.08	25	41.65	33.76	45.65	40.35333333	6.050127		10	80µm B
	13-Dec	2	0.12	16.66666667	63.5	22.99	47.61	44.7	20.41118		10	80µm B
	13-Dec	2	0.06	33.33333333								60µm B
	13-Dec	2	0.08	25								60µm B
	13-Dec	2	0.12	16.66666667								60µm B
	13-Dec											

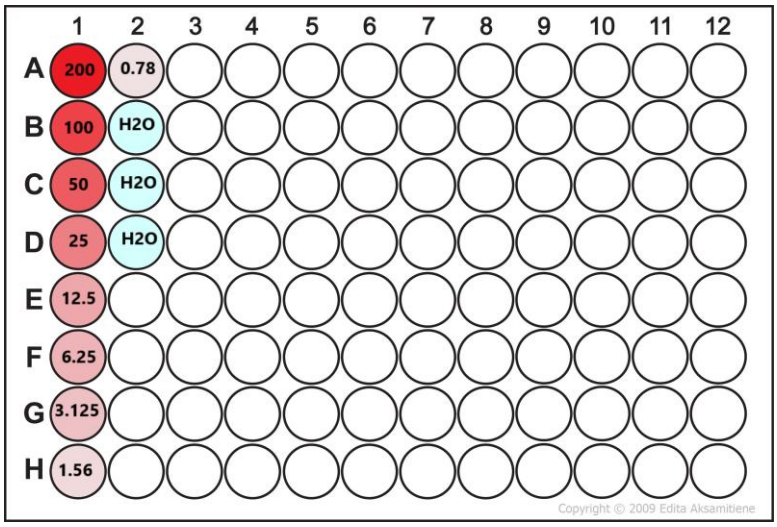
Appendix O: Chondroitin Sulfate Methacrylate (CS-MA) Doxorubicin (DOX) Bulk Loading SOP

Objectives:

- To create a standard curve from working concentration of doxorubicin (1 mg/mL) in phosphate buffered saline (PBS) (0.781 - 200 μg/mL; not sterile)
- To quantify how much DOX (μg) was loaded onto each CS-MA gel by using the standard curve
- To incubate each CS-MA gel in PBS (1 mL) at 25℃ and to assess DOX release at Days 1, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, and 30.

Preparing the DOX Standard Curve:

1. Perform a sixfold dilution of DOX stock concentration (5 mg/mL) in order to obtain the working concentration of DOX (1 mg/mL).
2. Dilute the working concentration of DOX (200 μL) in milliQ water (800 μL) to achieve a 200 μg/mL DOX solution (1 mL) in a microcentrifuge tube. Mix well.
3. Perform a two-fold serial dilution to achieve DOX solutions at 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.781 μg/mL in individual microcentrifuge tubes. Label the tubes according to the respective concentration of the DOX solution that they contain.
4. Pipette 200 μL of each DOX solution onto a UV/vis plate. Next pipette three samples of milliQ water (200 μL) into the UV/vis plate as shown below:



Read the light absorbance values of the plated samples on a spectrophotometer. The average light absorbance value of the water is the background of the DOX solution absorbance values. Subtract this background value from all of the DOX solution absorbance values. Save data on an Excel Sheet.

Preparing the CS-MA Bulk Gels:

1. Label three microcentrifuge tubes with "DOX." Label one microcentrifuge tube with "H_2O"
2. Place CS-MA gels (n=4) into the four labeled microcentrifuge tubes.
5. Pipette the working concentration of DOX (1 mg/mL; 1 mL) into the tubes labelled "DOX." Pipette milliQ water (1 mL) into the fourth microcentrifuge tube.
6. Incubate (25°C, CO_2 5%-10%) for 24 hours to allow CS-MA bulk gel to absorb the DOX.
7. Pipette 200 μL of supernatant (3 times) from each of the microcentrifuge tubes into a plate reader. Record the light absorbance values of the samples and quantify the mass of unloaded DOX by dividing the average absorbance value by the slope of the standard curve. Subtract this value from the total DOX (mg) to calculate how much DOX was absorbed.
8. Aspirate the supernatant off of the particles. Add PBS (1 mL). Allow to incubate over thirty days and read absorbance values on Days 1, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, and 30.

Appendix P: Raw and Preliminary Data from DOX Bulk Loading and Release

Raw Data

	D1	D2	D4	D7	D9	D11	D14
1	0.321	0.149	0.132				
2	0.301	0.094	0.098				
3	0.25	0.101	0.112				
4	0.028	0.03	0.032				
PBS	0.027	0.024	0.033				
PBS	0.024	0.024	0.027				
PBS	0.024	0.023	0.029				
Empty	0.031	0.032	0.03				

Subtract Background

Background	0.025	0.023667	0.029667	#DIV/0!	0.029667	#DIV/0!	#DIV/0!
D1	0.296	0.125333	0.102333	#DIV/0!	-0.02967	#DIV/0!	#DIV/0!
D2	0.276	0.070333	0.068333	#DIV/0!	0.068333	#DIV/0!	#DIV/0!
D3	0.225	0.077333	0.082333	#DIV/0!	0.082333	#DIV/0!	#DIV/0!

Converto ug using Standard

D1	46.84201	19.834	16.19425	#DIV/0!	-4.69475	#DIV/0!	#DIV/0!
D2	43.67701	11.13025	10.81375	#DIV/0!	10.81375	#DIV/0!	#DIV/0!
D3	35.60626	12.238	13.02925	#DIV/0!	13.02925	#DIV/0!	#DIV/0!

	Starting Amount	Percent Released						
D1	256.0485403	18.29419	7.746189	6.324681	#DIV/0!	-1.83354	#DIV/0!	#DIV/0!
D2	249.4020392	17.51269	4.462775	4.335871	#DIV/0!	4.335871	#DIV/0!	#DIV/0!
D3	203.351282	17.50973	6.018158	6.407263	#DIV/0!	6.407263	#DIV/0!	#DIV/0!
Average	236.2672871							
Stdev	28.69915576							

Cumulative Amount Released (ug)

		1	2	4	7	9	11	14
	Well #1	46.84201	66.67601	82.87026	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	Well #2	43.67701	54.80726	65.62101	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	Well #3	35.60626	47.84426	60.87351	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Average		42.04176	56.44251	69.78826	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Stdev		5.793623	9.521778	11.57535	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Cumulative % Released

	D1	18.29419	26.04038	32.36506	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	D2	17.51269	21.97547	26.31134	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	D3	17.50973	23.52789	29.93515	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Average		17.7722	23.84791	29.53718	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Stdev		0.452057	2.051266	3.04642	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

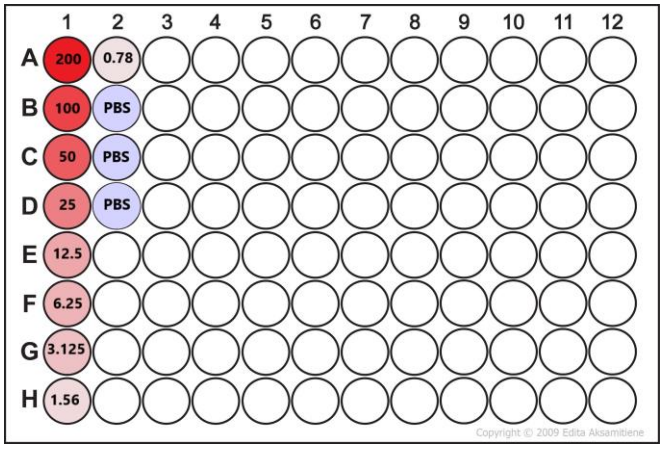
Appendix Q: Sterile DOX Loading onto CS-MA Microparticles

Day 1: Washing and Weighing Particles

1. Purify CS-MA particles until they sit in a known volume (mL) of sterile, deionized water
    a. *Wash particles four times in 100% ethanol and then four times in sterile deionized water.*
    b. *Note: all ethanol must be washed off through purification process as ethanol reacts poorly with the pi stacking interactions in doxorubicin*
2. Vortex the solution for uniform particle distribution
3. Aliquot 500 μL of the solution onto a pre-weighed weight boat.
    a. *Note: this aliquot does not need to be kept sterile since it will be discarded after taking the mass.*
4. Record the mass of the weight boat and the weight boat with the particle liquid suspension. Allow to dry overnight, partially covered with a lid or Kimwipe.

Day 2: Treating Particles to DOX and Preparing a Standard Curve

5. After sample is dry, weigh the dry mass
    a. *Note: if dry mass is giving a negative reading, treat the weight boat with an anti-electrostatic gun to allow the particles to settle onto the bottom of the weight boat*
6. Calculate the concentration of particle mass/volume of particle suspension:
    Dry Mass of Particles/ 500 μL = Concentration of particles in suspension
7. Calculate how much volume of the particle suspension is needed in order to pipette 2 mg of particles in a microcentrifuge tube
    a. If more than 0.5 mL of particle suspension is needed, then spin down the particles, remove some of the water, and recalculate what concentration of particles you have and how much volume you will have 2 mg of particles in.
    b. This step ensures that the particle suspension (calculated volume from this step) and the doxorubicin (1 mL) will fit in one microcentrifuge tube.
8. Pipette particle suspension (2 mg, volume calculated in Step 7) into three microcentrifuge tubes
9. Add 1 mL of 1 mg/mL DOX solution to each microcentrifuge and allow the particles to incubate at 37°C and absorb DOX over the weekend (3 days)
10. Perform a two fold serial dilution eight times with working concentration of DOX (200 μg/mL) in PBS to create a standard curve. Place these DOX samples (200 μL each) and three samples of the PBS used to dilute the DOX into the UV/vis plate as shown below:



11. Read samples on a spectrophotometer and record the absorbance values on an Excel sheet. Subtract the average absorbance value of the PBS samples from every absorbance value reading.

Reading and Quantifying DOX Loading and Release

12. After incubation period, centrifuge particles at 14,000 rpm for 5 min. Pipette 200 µL samples (3 samples) onto a 96 well plate.
13. Once light absorbance values are collected and checked (i.e, the amount released is not more than the amount loaded), aspirate off left over supernatant from each microcentrifuge tube.
    a. Measure how much DOX was left by dividing the absorbance value of Day 0 by the slope of the DOX standard curve and subtracting it from the total amount of DOX that was introduced to the particles.
14. Add 1 mL of PBS to the particles and incubate at 37°C. Allow absorbed DOX to release into the PBS over time.
15. Collect DOX release data at Days 1, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, and 30.
    a. Note: read PBS as a background control each time
        i. If reading matches PBS, let particles sit longer
16. Enter data into an Excel Sheet for calculating cumulative mass released and cumulative percentage released.

Appendix R: Plate Reading for Release/Absorbance Studies

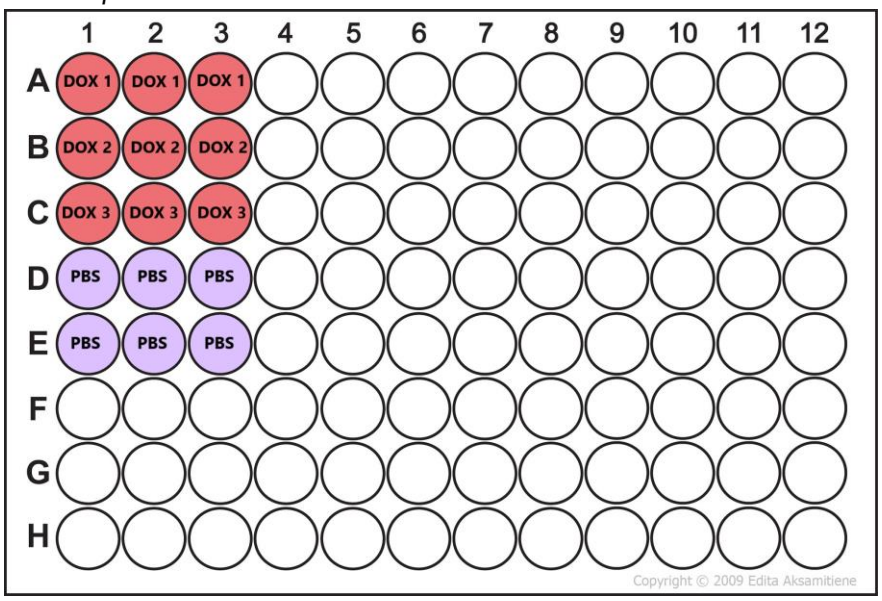
By Leonela Vega and Kathy Suqui

1. Obtain samples that will be read from the MQP drawer or incubator
    - Read groups: Dox 1, Dox 2, Dox 3 vials only
    - Read PBS from the bottle on the benchtop or from the sterile PBS

2. Centrifuge the samples at 14,000 rpm for 5 minutes.

2. Aliquot 3 samples of 200 μL of DOX release supernatant from each group to be read on a spectrophotometer
    - The sample volumes will be placed on a 96-well plate found in the oven

Note: For each of the tubes that are being read, 3 samples must be taken, therefore if given 5 samples, there would be 15 wells filled.



3. Bring the plate to the spectrophotometer to read.
4. Turn the machine on and carefully place the plate in the plate slot. Do not try to push it shut as this will break the machine. The plate slot will shut automatically when you read the plate using the computer software as described below:

Program:

5. Sign in with the given credentials and open the plate reading program.
    a. Click on “Set-up plate.”
    b. Change wavelength to “485 nm” using the number pad.
    c. Select the columns that you want to read.
    d. “Read” plate by pressing “read” button on the top of the window.
    e. Press “NORMAL”, not pre-read.
    f. Press “OK”. Program will start running and plate slot will shut automatically.
6. Obtain the results and save them in the native file and export as a text file into a USB.

    a. Naming convention for this project is 03192018_DoxReleaseD7_CS-MA2.
7. BEFORE DISPOSING OF SAMPLES, make sure that the data collected makes sense with the previously collected data:
    a. The CS-MA particles have an initial burst release on the first day of release, and then a very gradual release over time.
    b. Each day, the particles should be releasing less DOX. Less DOXin the supernatant indicates less light absorbed by the color of the liquid. Less color and less light absorption means a lower light absorbance value (given by the software in the .txt file
    c. Individually enter in the mean of each group's release light absorbance values into the excel sheet on the Coburn lab drive
    d. Once the data is checked, you can begin the clean-up protocol. If the data does not makes sense, try to run your plate again. If it still looks wrong, run it a third time. If it still looks wrong, re-plate your samples with the supernatant left in the old vials
8. Delete the files from the computer in order to avoid slowing down the desktop. Log off the computer.
9. CLOSE the drawer by pressing "drawer" button on the plate reader. Then switch the machine to "off".
10. Commence clean-up procedure:

Clean-up Procedure:

1. Aspirate the rest of the supernatant from the microcentrifuge tubbes containing particles.
    a. Pipette all DOX waste into the appropriate biohazard/chemical waste container.
    b. Rinse the plate with milliQ water 3 times
2. Replenish supernatant liquid by adding 1 ml of PBS to each microcentrifuge tube.
3. INCubate microcentrifuge tubes until next reading point.

Appendix S: Seeding KELLY Cells into a 96-well plate

By: Kathy Suqui
March 27, 2018

MATERIALS

KELLY cells (frozen or from cell culture flask)
KELLY cell media (RPMI medium, 10% FBS, 1% Penn-Strep, 1% L-glutamine)
15 mL conical tube
Sterile pipette tips (10 µl*, 20 µl, 1000 µl)
Pipettes (20 µl*, 200 µl, 1000 µl)
96-well plate (sterile)

*Ideal material if handling small quantities, but not necessary

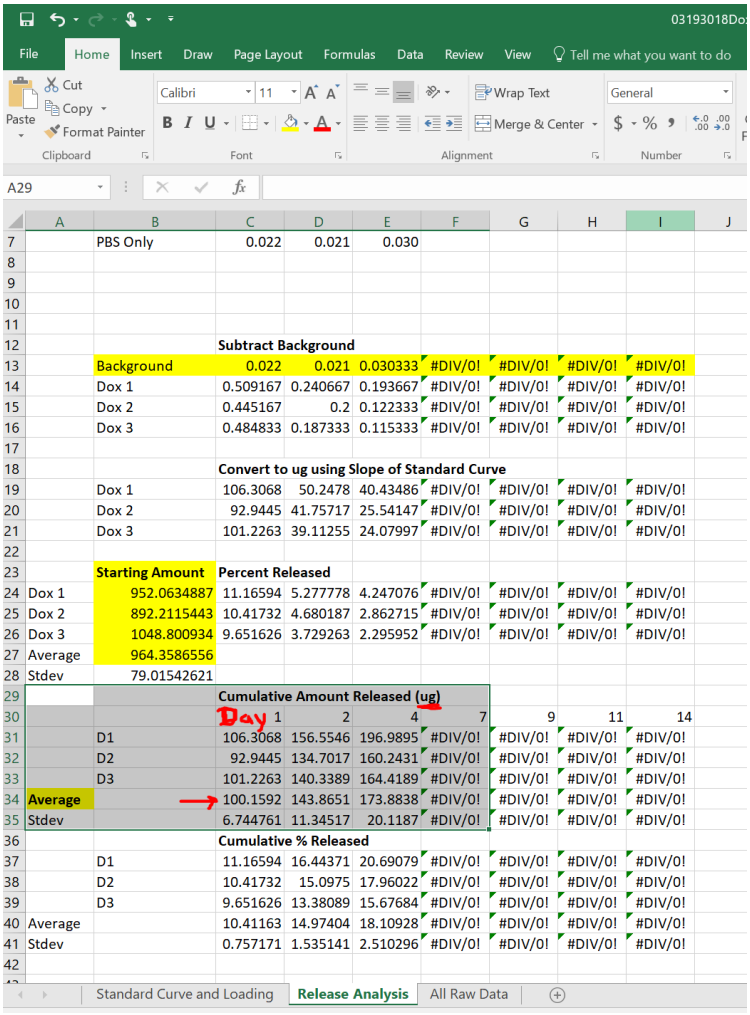
PRE-LAB CALCULATIONS AND PREPARATIONS

- Before coming into lab, calculate how many cells, how much media, how much DOX, and how to dilute your doxorubicin to the cytotoxic range.

Cytotoxic range: the concentration of doxorubicin at which it will kill cells effectively. Cytotoxic range for doxorubicin to KELLY cells is 50-100 ng/mL. On Day 1 of release, CS-MA particles release an average of 80-100 µg/mL, also known as 80,000 - 100,000 ng/mL. This must be diluted down to 10,000 ng in 250 µL of total volume, 1,000 ng in 250 µL of total volume, and 100 ng in in 250 µL of total volume. Total volume is the volume of the doxorubicin solution added to the volume of cell suspension and added to any cell media added at the end.

$$\boldsymbol{Total\ Volume\ of\ a\ Well\ in\ a\ 96\ Well\ Plate} = \mathbf{250}\ \boldsymbol{\mu L}$$
$$= \boldsymbol{V_{doxorubicin\ in\ PBS}} + \boldsymbol{V_{cell\ suspension}} + \boldsymbol{V_{cell\ media}}$$

Cell Seeding Density: the concentration of cell suspension that is pipetted into a well. The cells in the suspension settle to the bottom of the well and attach to the bottom of the plate. These cells will not come off from aspirating media if done properly. The cell seeding density for one well is 10,000 cells per well. This means that if you have a cell suspension at 0.5 million cells/mL, you will need to calculate how much volume it takes to get 10,000 cells. In this example, 10,000 cells is present in 50 µL of cell suspension. This volume must be accounted for during the calculation of the total volume (must be at 250 µL total volume)



	A	B	C	D	E	F	G	H	I	J
7		PBS Only	0.022	0.021	0.030					
8										
9										
10										
11										
12			**Subtract Background**							
13		Background	0.022	0.021	0.030333	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
14		Dox 1	0.509167	0.240667	0.193667	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
15		Dox 2	0.445167	0.2	0.122333	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
16		Dox 3	0.484833	0.187333	0.115333	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
17										
18			**Convert to ug using Slope of Standard Curve**							
19		Dox 1	106.3068	50.2478	40.43486	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
20		Dox 2	92.9445	41.75717	25.54147	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
21		Dox 3	101.2263	39.11255	24.07997	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
22										
23		**Starting Amount**	**Percent Released**							
24	Dox 1	952.0634887	11.16594	5.277778	4.247076	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
25	Dox 2	892.2115443	10.41732	4.680187	2.862715	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
26	Dox 3	1048.800934	9.651626	3.729263	2.295952	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
27	Average	964.3586556								
28	Stdev	79.01542621								
29			**Cumulative Amount Released (ug)**							
30			Day 1	2	4	7	9	11	14	
31		D1	106.3068	156.5546	196.9895	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
32		D2	92.9445	134.7017	160.2431	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
33		D3	101.2263	140.3389	164.4189	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
34	**Average**		100.1592	143.8651	173.8838	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
35	Stdev		6.744761	11.34517	20.1187	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
36			**Cumulative % Released**							
37		D1	11.16594	16.44371	20.69079	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
38		D2	10.41732	15.0975	17.96022	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
39		D3	9.651626	13.38089	15.67684	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
40	Average		10.41163	14.97404	18.10928	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
41	Stdev		0.757171	1.535141	2.510296	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	
42										

Diluting Doxorubicin: the doxorubicin release samples must be diluted with sterile PBS before treating cells. This is because the concentration of doxorubicin released is too high for the number of cells (only 10,000 cells) that fit in the well of a 96-well plate. If using a larger sized plate with a larger cell seeding density, this step may be modified or omitted. The amount of doxorubicin released (in µg) is located in the Dox Release Analysis excel sheet for the respective batch you made.

Appendix T: Methacrylation of PVA or CS

Materials:

PVA or CS
GMA
HCL
Acetone

Protocol:

1. 5 g PVA (or CS) (87-90% hydrolyzed avg MW 30,000-70,000 Sigma P8136) dissolved into 45 mL water
2. Add 32.5 mL GMA (97%, 100 ppm MMEQ inhibitor, Sigma 151238)
3. Adjust pH to 1.5 with concentrated HCl
4. React at 60C w/ stirring for 24 hrs, light protected
5. Precipitate immediately with acetone
6. Wash once with acetone
7. Let dry at RT overnight
8. Resuspend in 50 mL H2O
9. Dialyze minimum 48 hrs
10. Freeze at -80C overnight (max 30 mL in 50 mL tube)
11. Lyophililze minimum 72 hrs