Molecularly Imprinted Solid Phase Extraction of Taxanes from Plant Cell Culture

A Major Qualifying Project
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Submitted by:

Clydeblaise Niba
Matthew Portugal
Benjamin Small
Shawn Wile

Submitted to: Professor Susan Roberts, Project Advisor

Abstract

There are many drugs approved by the Food and Drug Administration that help to combat various forms of cancer. One such drug is paclitaxel, also known as Taxol®, which is a chemical that is in a class of compounds known as taxanes. Formed in suspension cultures with other taxanes, purifying paclitaxel is a difficult procedure because most taxanes have a chemically similar structure. A process called molecular imprinting has the potential to alleviate this problem. This technique involves creating artificial receptors that have the capacity to selectively and specifically bind to target molecules. Molecularly imprinted polymers, or MIPs, are engineered for many different compounds, including drugs such as paclitaxel. The goals of this project were to determine the optimum conditions for producing paclitaxel-specific MIPs and test their overall effectiveness in separating paclitaxel from a mixture of other taxanes. So far, successful demonstrations of forming MIPs have been found in many sources of literature. One of which includes the small-scale formation of MIPs as performed by Lanza and Sellergren, where the molar ratio of template:functional monomer:cross-linker was found to be 1:4:20 for favorable conditions. From the various trials that were performed in this study, we report that the method of bulk polymerization using a ratio of 2:4:20 with paclitaxel as template, methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker, and chloroform as solvent to make the MIP for paclitaxel had the best rebinding capacity, with 70 percent of the paclitaxel in solution binding to the MIP. While the binding capacity was high, it was not highly specific as the control polymers rebounded 57 percent of the paclitaxel in the same concentration of solution. What was determined from these results is that a larger sample pool of successful trials and polymerization methods, as well as variations in functional monomer, cross-linker, and solvent could be used to yield more favorable results in the future.

Acknowledgements

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List of Experimental Chemicals

Templates:

- Paclitaxel (Taxol®)

Functional Monomers:

- Methacrylic Acid (MAA)

Cross-linkers:

- Ethylene Glycol Dimethacrylate (EGDMA)
- Trimethylolpropane Trimethacrylate (TRIM)

Solvents:

- Dichloromethane (DCM)
- Toluene
- Chloroform

Initiator:

- Azo-N,N’-bisisobutyronitrile (AIBN)

Chapter 1: Introduction

There are several drugs approved by the Food and Drug Administration (FDA) that help fight against cancer. In 2016 alone, the FDA approved various new drug treatments to treat different types of cancer, including Tecentriq (bladder cancer), Xalkori (lung cancer), and Opdivo (Hodgkin Lymphoma) ("New Cancer Drug Approvals From 2016" 2016). One of the most notable FDA-approved drugs is paclitaxel, known commercially as Taxol®, which is used in the treatment process of breast, lung, ovarian, and other types of solid tumor cancer (Taxol, n.d.). Isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) in 1964, paclitaxel is one of over 400 taxanes, which are a class of diterpenoid compounds that possess the taxadiene core (Team, E.W., n.d.). Paclitaxel is formed in *Taxus* suspension cultures that also produce a wide range of other taxanes. Due to the structural similarities paclitaxel has with other taxanes, downstream processing and purifying paclitaxel becomes difficult.

A process called molecular imprinting could potentially be utilized to address this issue. Molecular imprinting is a technique to produce artificial receptors that are designed to specifically and selectively bind to a given molecule (Vasapollo et al., 2011). Molecular imprinting proves to be less expensive and better for the environment. In addition, the technological process of molecular imprinting provides a way to prepare new polymer materials containing recognition sites for target molecules that can be employed in a variety of applications, such as separation, sensors, catalysis, organic synthesis, enantiomeric separation and drug delivery systems (Wulff, 1995). Molecularly imprinted polymers (MIPs) can be engineered for a variety of molecules, including as drugs such as paclitaxel.

MIPs are created by the reaction of a functional monomer and cross-linking monomer in the presence of a template molecule. The functional monomer and cross-linking monomer form the MIP around the template, which can then be washed away leaving imprinted cavities that specifically bind to the template. Numerous approaches have been used to obtain MIPs with a controlled size and shape distribution, such as bulk polymerization, precipitation polymerization, suspension polymerization, swelling polymerization and emulsion polymerization (Ye, Weiss, & Mosbach, 2000).

The goals of this project were to produce paclitaxel-specific MIPs and test their effectiveness in separating paclitaxel from a mixture of taxanes. Due to time constraints, only the first objective (producing paclitaxel-specific MIPs) was investigated.

I. Screen Conditions for MIP formation: Some of the important factors we varied when forming our MIPs were the molar ratios between the template molecule, functional monomer, and cross-linker, the types of cross-linkers, the type and volume of solvents the MIPs were formed in, and the method of polymerization. Other factors such as heating temperature, type of functional monomer, and polymerization initiators could have also been varied, but time constraints prevented us from doing so. By comparing the rebinding capacity of the MIPs to non-imprinted control polymers, which are formed in the same manner as the MIPs with the absence of the template molecule, the overall effectiveness

of the MIP can be determined. For this project, screening of all conditions were performed on a small-scale as demonstrated in the MIPs formed by Lanza and Sellergren (1999).

II. Separation of Taxane Mixture: With more time, we would have tested the most effective MIPs on larger scale in a mixture of taxanes. This would have tested the selectivity of the MIPs for paclitaxel against molecules with a similar structure. The most ideal MIPs would have a strong affinity for paclitaxel and a weak affinity for other taxanes. This is a rich area for future work.

Chapter 2: Background

This section covers information necessary for understanding the material presented in this report as well as how it applies to the broader research project at hand. As mentioned previously in the introduction, the goal of this project is to produce a paclitaxel-specific molecularly imprinted polymer (MIP) that will be effective in separating paclitaxel from a mixture of taxanes. The importance of paclitaxel as well as the theory behind the construction and effectiveness of MIPs will be explained in this section.

2.1 History of Paclitaxel

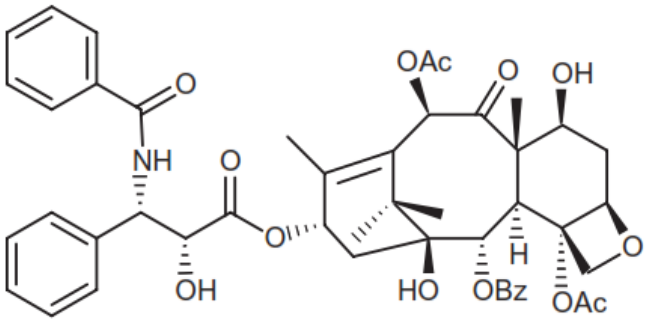

Figure 1: Chemical structure of paclitaxel (Dunn, Wells, & Williams, 2010)

According to the Nationals Cancer Institute, paclitaxel (seen in Figure 1) is the most well-known natural-source cancer drug in the United States. The drug was discovered in bark and other samples collected from the Pacific yew tree by a USDA botanist named Arthur Barclay in the state of Washington ("A Story of Discovery," n.d.). The bark is depicted in Figure 2.

Figure 2: Bark of the Pacific yew tree ("Taxus brevifolia," n.d.)

From these samples, an extract of the bark showed cytotoxicity against a KB (oral epidermoid carcinoma) cell culture, which is a cell line from a human cancer ("Discovery of Camptothecin and Taxol," 2003). This work was performed by Dr. Monroe Wall and Dr. Mansukh Wall of the Research Triangle Institute (RTI), both of whom worked together to discover the structural formula and came up with the name for paclitaxel as well as its commercial name, Taxol®. Dr. Wall derived this name from the scientific name of the Pacific yew tree (*Taxus brevifolia*) and the fact that paclitaxel has hydroxyl groups in its structure, making it an alcohol ("Discovery of Camptothecin and Taxol," 2003).

In 1992, after years of research and testing, the United States Food and Drug Administration approved paclitaxel for treating ovarian cancer. Two years later in 1994, paclitaxel was approved for treating breast cancer treatment ("Success Story: Taxol," n.d.). Paclitaxel has since been used against several other forms of cancer ("Taxol," n.d.). With such a wide variety of use against cancer, paclitaxel has become the best-selling cancer drug ever produced, with annual sales peaking in 2000 with $1.6 billion in sales ("Success Story: Taxol," n.d.).

2.1.1 Use of Paclitaxel Against Cancer

Paclitaxel is a chemotherapy medication. It interferes with the growth of cancer cells and slows their growth and spread in the body (Cunha, 2016). Currently, paclitaxel is used to fight the following types of cancer ("Taxol," n.d.):

- Breast cancer
- Ovarian cancer
- Lung cancer
- Bladder cancer
- Prostate cancer
- Melanoma cancer
- Esophageal cancer
- Other solid tumor cancers

Paclitaxel is delivered to the body via injection or infusion into the vein. It belongs to a class of chemotherapy drugs broadly called plant alkaloids. Plant alkaloids are cell-cycle specific, meaning they attack cells during cell division. Paclitaxel specifically acts as an antimicrotubule agent by stabilizing a cell's microtubules, which is a part of the cell responsible for dividing and replicating itself. By stabilizing the microtubules of cancerous cells, cells cannot divide normally and undergo programmed cell death ("Taxol," n.d.).

2.1.2 Paclitaxel Production Methods

The amount of natural paclitaxel that can be acquired is very limited. The bark of the Pacific yew tree consists of only 0.0004% paclitaxel, which is not concentrated enough to be a sustainable source. Bark-stripping to obtain paclitaxel is fatal for yew trees and poses significant environmental problems. Yew trees can take 200 years to mature, and they are also the main habitat the endangered northern spotted owl. To address this issue, two methods of producing paclitaxel, a semi-synthetic process and a plant cell fermentation process, have been actively developed. These methods are described in detail in "Green Chemistry in the Pharmaceutical Industry" by Peter Dunn, Andrew Wells, and Michael Williams.

2.1.2.1 Semi-Synthetic Paclitaxel

The semi-synthetic process was first developed by Pierre Potier and Andrew Greene. Potier and Greene discovered that a compound known as 10-deacetylbaccatin III, or 10-DAB (Figure 3), closely resembles the tetracyclic ring of paclitaxel and contained the proper stereochemistry found in paclitaxel. Therefore, they hypothesized that 10-DAB can be used as an initial start for the semi-synthesis of paclitaxel.

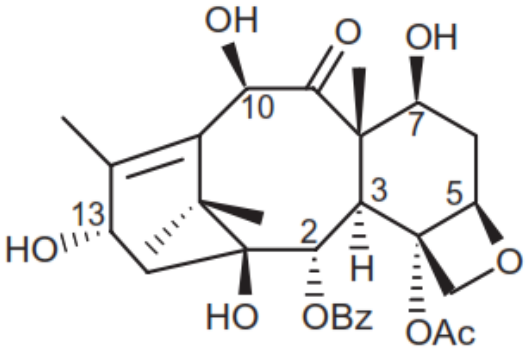

Figure 3: Chemical structure of 10-deacetylbaccatin III (Dunn et al., 2010)

The advantage of this approach was that 10-DAB could be found in leaves and twigs of the European yew tree, a relative of the Pacific yew tree. The leaves and twigs were found to be made up of 0.1% 10-DAB, a far greater concentration than the 0.0004% of paclitaxel in Pacific yew tree bark. Additionally, harvesting the leaves and twigs of the European yew tree does not harm the tree, making the source of 10-DAB far more sustainable than the source of natural paclitaxel.

There are two main structural differences between 10-DAB and paclitaxel. The first main difference is the hydroxyl group located on the 10-position carbon of 10-DAB instead of the acetyl group found in paclitaxel (highlighted in blue in Figure 4). The other main difference is the hydroxyl group on the 13-position carbon on 10-DAB instead of the complex side chain found in paclitaxel (highlighted in orange in Figure 4).

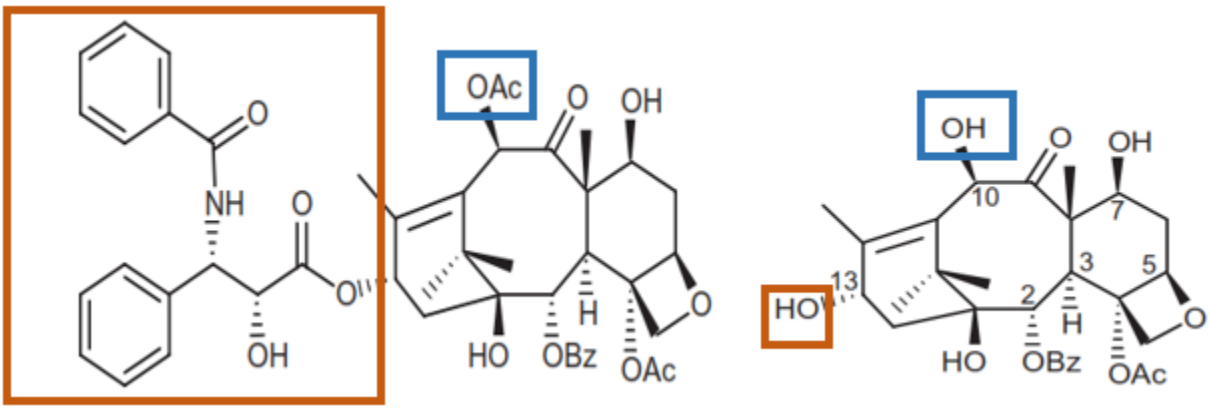

Figure 4: Comparison of paclitaxel (left) with 10-DAB (right) (Dunn et al., 2010)

Acetylation of the hydroxyl group on the 10-position carbon turned out to be the most complicated part of the procedure due to the presence of three hydroxyl groups with different reactivities. Dealing with this required protection steps for the 7-position and 13-position carbons, low temperatures, and hazardous reagents and solvents. This process is detailed in Figure 5.

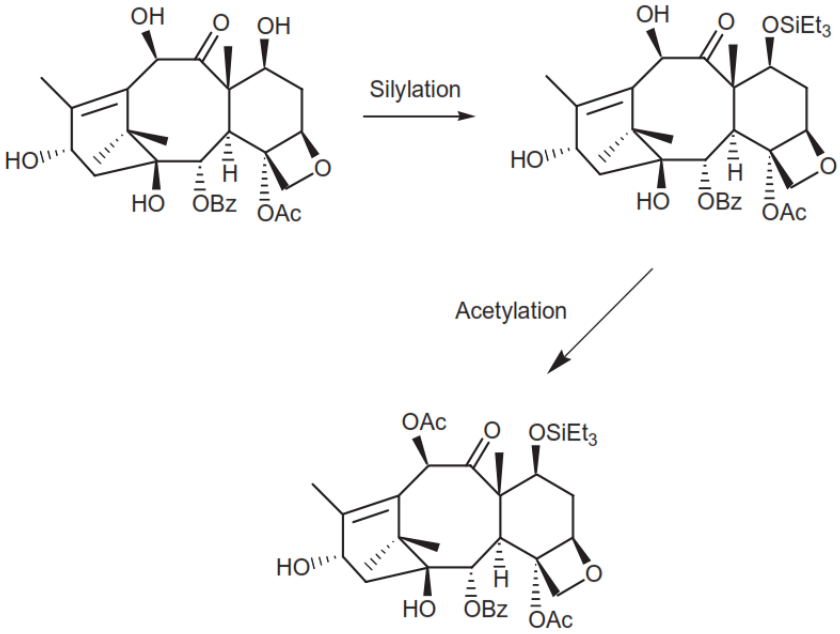

Figure 5: Acetylation of 10-DAB (Dunn et al., 2010)

The other main challenge was producing and attaching the side chain to form paclitaxel. Potier and Greene were able to successfully carry out the synthesis at low yields, but scientists at Bristol-Myers Squibb (BMS) were able to produce the side chain with the process detailed in Figure 6 and attach the side chain to the 10-DAB with the process detailed in Figure 7.

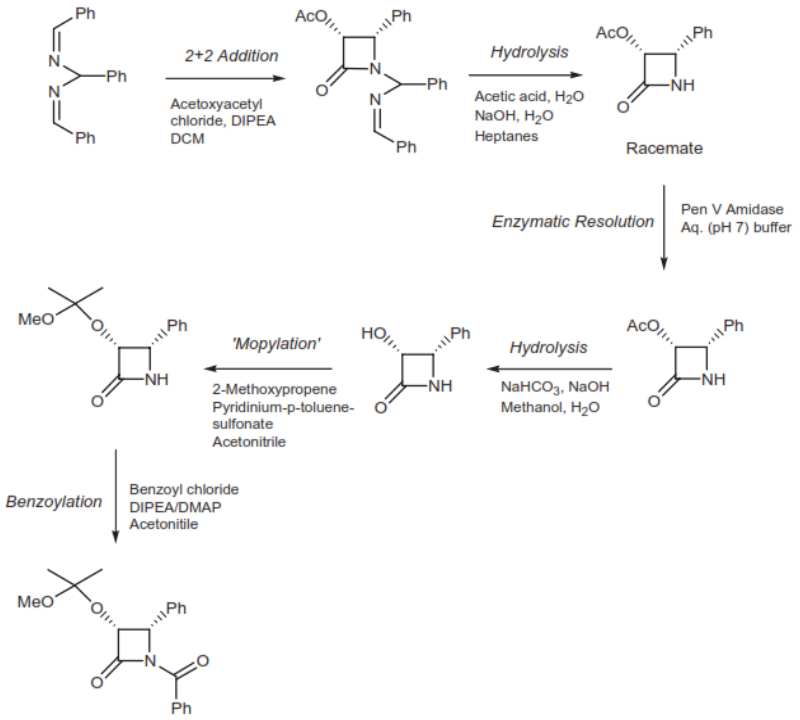

Figure 6: Production of the paclitaxel side chain (Dunn et al., 2010)

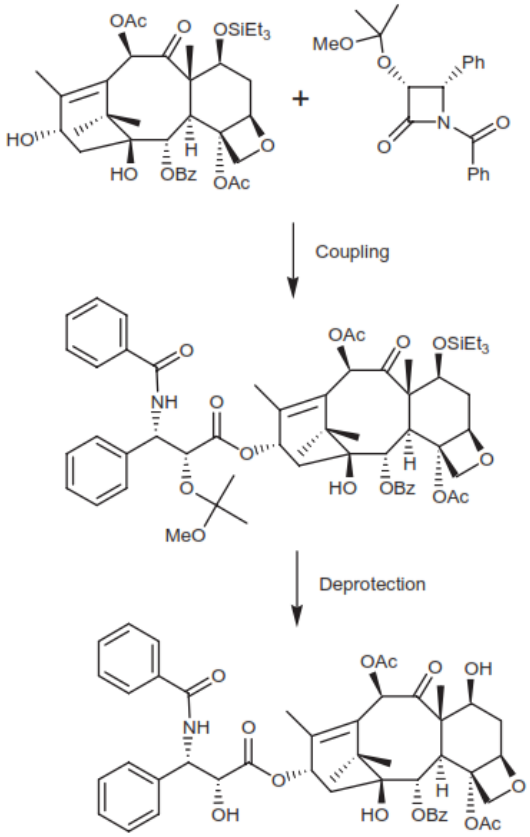

Figure 7: Formation of paclitaxel from 10-DAB and side chain (Dunn et al., 2010)

The main issues with this process is that, while renewable, there are still environmental problems. The semi-synthesis procedure requires the use of some hazardous solvents, toxic reagents, waste streams, and high-energy steps. Despite these issues, the semi-synthesis production of paclitaxel became economically viable in 1993.

2.1.2.2 Plant Cell Fermentation

To address the issues with the semi-synthesis of paclitaxel, BMS scientists using Phyton Biotech GmbH technology developed a plant cell fermentation (PCF) process using culture cells from Chinese yew tree needles. With this process, Phyton became and still is the largest commercial application of plant cell fermentation.

At Phyton, the PCF process starts by growing approximately 1 g of cells on a solid agar medium plate to form cell calli. These calli are then transferred to a liquid growth medium. These calli are grown and maintained over time by replacing the growth medium weekly. At a certain point, the cells in the calli are fed with a special production medium to produce paclitaxel and other specialized metabolites. The cell broth is subsequently extracted to recover crude paclitaxel and is then purified by chromatography and crystallization. Figure 8 lists the specific steps of PCF.

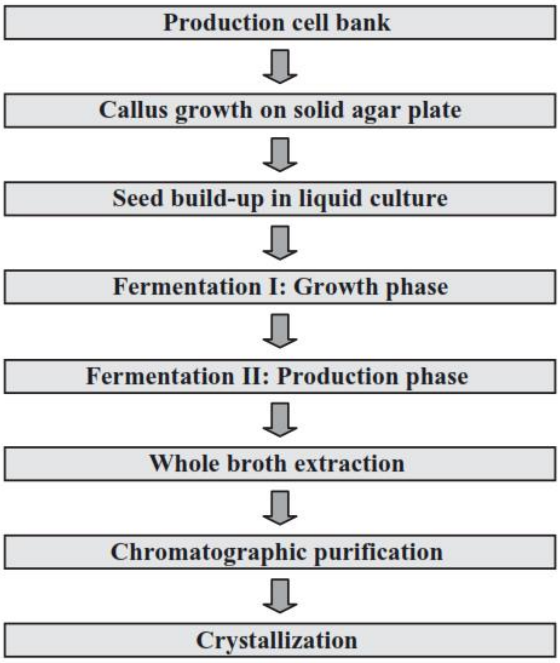

Figure 8: Plant cell fermentation process (Dunn et al., 2010)

The advantages of PCF is are as follows:

- Solid waste is negligible.
- No chemical reactions occur, so there is no need for reagents.
- There is only one drying step.

The steps of PCF being addressed by our project are the whole broth extraction, chromatographic purification, and crystallization of paclitaxel. Currently, these steps require the use of several solvents and chemicals, including isobutyl acetate (IBA), isopropanol (IPA), sodium hydroxide, dichloromethane (DCM), dimethylformamide (DMF), formamide, and a mixture of acetonitrile (ACN) and water. A direct extraction using MIPs could potentially reduce the amount of steps or solvents used in the purification steps.

2.2 Molecularly Imprinted Polymer Theory

Figure 9 describes visually how MIPs are made. The process begins with a reaction mixture made up of a template, a functional monomer, a cross-linking monomer, and a polymerization initiator. This reaction forms a complex between the template and the functional monomer, which is then surrounded by the cross-linking monomer. The cross-linking monomer polymerizes around the complex, yielding a three dimensional polymer network (the MIP) where the template molecules are trapped after the polymerization is complete. By thoroughly washing the MIP with appropriate solvents, the template molecule can be extracted from the MIP, leaving specific binding sites, or cavities, complementary to the template in size, shape, and molecular interactions (Cheong, Yang, & Ali, 2013).

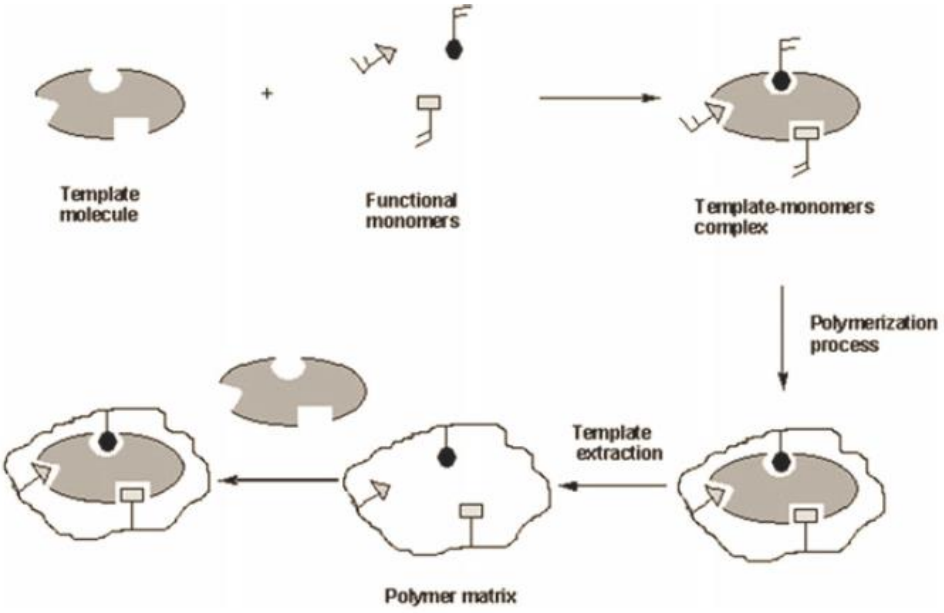

Figure 9: An illustration on the preparation of MIP (Cheong et al., 2013)

There are several types of polymerization methods used to synthesize MIPs. Some common polymerization methods include bulk polymerization, precipitation polymerization, and emulsion polymerization. The most used and simple polymerization method is bulk polymerization, which uses a porogenic solvent to create a block of polymer (Mayes & Mosbach, 1996). The advantages of this method include its simplicity, which makes it possible to be performed in any laboratory, and that no particular skills and difficult instruments are required (Pardeshi & Singh, 2016). However, its disadvantages include tedious procedures of grinding and sieving the polymers, which produces irregular particle shapes and can destroy the imprinted sites of the polymers (Pardeshi & Singh).

Another method of polymerization is known as precipitation polymerization. A precipitation polymerization is essentially a heterogeneous polymerization process that is initially a homogenous solution that is in the continuous phase. At first, the initiator and monomer are completely miscible in each other, but once initiator is added the polymer becomes insoluble and finally precipitates out of reaction. After precipitation occurs, the polymerization is commenced by the absorption of both the initiator and the monomer by the polymer particles (Pearce, 1992). Advantages of this method of polymerization include the lack of need for stabilizers and the production of suitable polymer beads in high yield, but disadvantages include a potential for this method to not suit the template molecule, as literature exists describing the obtainment of agglomerates instead of independent beads (Tamayo, Turiel, & Martín-Esteban 2007).

One final method of polymerization is called emulsion polymerization. This method makes it possible to predetermine the polymer particle and involves the formation of spherical particles. It is difficult to perform because the use of surfactants and stabilizers are hard to separate from the resulting MIPs that are made. Emulsion polymerization is a certain kind of radical polymerization that begins with the emulsion of monomer and surfactant in water. The most common type of emulsion polymerization is an oil-in-water emulsion. During this kind of emulsion, the monomer is oil and droplets of oil are emulsified with the surfactant in water that is in a continuous phase. The actual polymerization step takes place in particles that rapidly form within the first five minutes of the process. The particles are roughly 100 nm and are made up of a variety of individual polymer chains. The surfactant used for this method is soap and the particles are stopped from forming solids when the surfactant surrounds them (Whitby & Katz, 1933). This method helps predetermine the size and shape of the MIPs formed, but the complex nature of the polymerization makes it less convenient than bulk or precipitation polymerization (Pradeshi & Singh, 2006).

There are several other polymerization methods used to form MIPs. Figure 10 shows the most popular polymerization methods with advantages and disadvantages of each method (Yan & Row, 2006).

MIP format	Benefits	Limitations
Bulk polymerization	Polymerization simplicity and universality, No require particular skills or sophisticated instrumentation	Tedious procedures of grinding, sieving, and column packing, Irregular particle in size and shape, low performance.
Suspension polymerization	spherical particles, Highly reproducible results, Large scale possible	Phase partitioning of complicates system, Water is incompatible with most imprinted procedures, Specialist surfactant polymers required
Multi-step swelling polymerization	Monodisperse beads of controlled diameter, Excellent particle for HPLC	Complicated procedures and reaction conditions, Need for aqueous emulsions,
Precipitation polymerization	Imprinted microspheres, Uniform size and high yields	Large amount of template High dilution factor
Surface polymerization	Monodisperse product, Thin imprinted layers	Complicated system, Time consuming
In-situ polymerization	One-step, in-situ preparation, Cost-efficient, good porosity	Extensive optimization required for each new template system

Figure 10: List of common polymerization methods (Yan & Row, 2006)

2.3 Ultra Performance Liquid Chromatography

In Greek, Chromo means color, and graphic means writing. This color writing, or chromatography, is a useful analytical technique that chemists utilize. Chromatography is used to identify biological materials in the liquid or gas phase. A simple way of understanding what chromatography is by using the example of ink on wet paper. The ink is a liquid which dissolves in the water and moves across the surface of the paper. In a lab chromatography separates a mixture of chemicals in the gas or liquid phase by allowing them to move past another substance which is either a liquid or solid.

For chromatography to work we must have a mixture in one state, either gas or liquid, move over the surface of something else in another state, the liquid or solid that stays stationary. The mixture that is moving is known as the mobile phase and the other substance is known as the stationary phase. When the mobile phase is moving across the stationary phase, its different components are separated out on the stationary phase. This then allows us to analyze those different components individually.

This laboratory utilized an Ultra Performance Liquid Chromatography device (UPLC). A UPLC is an improvement on previous chromatography equipment in its chromatographic resolution, speed, and sensitivity whose packing materials have smaller particle size than 1.7-1.8 µm. Figure 11 shows the UPLC used for our experiments.

Figure 11: UPLC Device

Our UPLC is connected to a computer running the columns software, and it can be used to test many different samples with its autosampler. The user creates all of the samples they would like to test with a set of standards placed at the beginning and end of the set of samples. The UPLC then uses a needle to take the first sample and inject it into the column. The sample then begins to form a band of colors that can be read. The sample takes about 5 minutes in total to flow through the columns packing material. The different bands move separately at different speeds through the column. This occurs because the bands are being attracted to the mobile phase and stationary phase. The faster the band exits the column, the more attracted to the mobile phase it is versus the stationary phase. The slowest moving compound is the most retained compound in a sample.

The detector in the column helps to create the chromatogram on the computer, which is how the sample is identified. Different peaks can be seen on the computer, which represent each of the different components in the sample. The peaks each have a characteristic shape, which is how the components the peak represents can be identified. The concentration peak of paclitaxel and characteristic shape of paclitaxel on the UPLC can be seen in Figure 12.

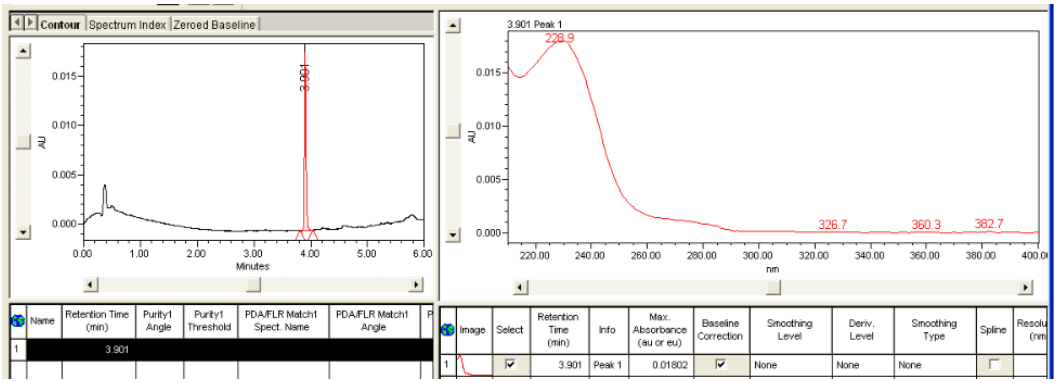

Figure 12: Concentration peak of paclitaxel (chromatograph on left) with characteristic paclitaxel shape (chromatograph on right)

The area of the peaks produced by the UPLC can then be used to determine the concentrations in the samples. By comparing the area of the standards peaks to the area of the samples peaks, the specific concentration of the paclitaxel in a sample can be determined. An example standard curve can be seen in Figure 13.

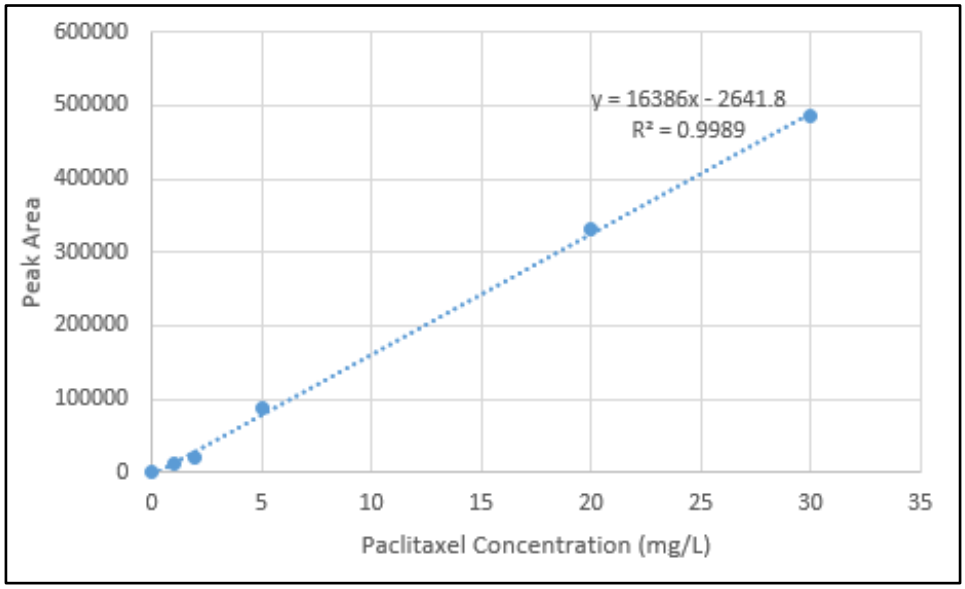

Figure 13: Standard curve for paclitaxel concentration generated by the UPLC

Chapter 3: Methodology

As mentioned previously, one of the main goals of this project was to determine the optimum conditions to form a paclitaxel-specific MIP. To accomplish this goal we completed the following objectives:

1. Synthesized MIPs with a variety of solvents, solvent volume:paclitaxel mass ratio, cross-linkers, template mole:functional monomer mole:cross-linker mole ratios, and polymerization methods. Control polymers were synthesized in the same manner as the MIPs with the absence of paclitaxel.
2. Removed the paclitaxel from the MIPs by sonicating them in acidified methanol. The UPLC was used to measure the concentration of paclitaxel in solution to determine if it had been removed from the MIPs.
3. Rebounded paclitaxel to the MIPs and control polymers to determine rebinding capacity and selectivity of the MIPs. The UPLC was used to measure the concentration of paclitaxel in solution to determine if it had bound to the MIPs and control polymers.

The common conditions that we varied for each MIP synthesis are listed as follows:

- Template
    - Paclitaxel, only template used
- Functional Monomer
    - Methacrylic Acid (MAA), only functional monomer used
- Cross-linker
    - Ethylene Glycol Dimethacrylate (EGDMA)
    - Trimethylolpropane Trimethacrylate (TRIM)
- Porogenic Solvent
    - Dichloromethane (DCM)
    - Toluene
    - Chloroform
- Molar Ratio of Template:Functional Monomer:Cross-linker
    - 1:4:20
    - 0.25:3:8
    - 2:4:20
    - 1:4:16
- Ratio of Solvent Volume:Template Mass
    - 25 μL/mg
    - 40 μL/mg
    - 50 μL/mg
- Initiator
    - Azo-N,N’-bisisobutyronitrile (AIBN), only initiator used

3.1 Molecularly Imprinted Polymer Synthesis

The two types of polymerization that were performed in our experiments to synthesize MIPs were bulk polymerization and precipitation polymerization. These procedures are detailed in the following sections.

3.1.1 Bulk Polymerization Procedure

The bulk polymerization procedure was based off the procedure by Tsermentseli, Manesiotis, Assimopoulou, and Papageorgiou (2013), which formed MIPs targeting the drug shikonin. A typical procedure was as follows: 20 mg of paclitaxel (template) were dissolved in 800 μL of chloroform (porogenic solvent) in an Eppendorf tube. This solution was split into two UPLC vials, each containing 400 μL of the initial solution (each solution now contains 10 mg, or 11.7 μmol, of paclitaxel). 1.99 μL, or 23.4 μmol, of MAA (functional monomer) and 22.1 μL, or 117.1 μmol, of EGDMA (cross-linker) were then added to each solution. Finally, the last chemical that was added to each vial was 2 μL of the initiator, AIBN, to allow the reaction to proceed and form the polymers. The initiator does not perform in the presence of oxygen, so the vials were capped and purged with nitrogen gas for 10 minutes to remove the oxygen. The solutions were then added to a heating block at 70 ^{o}C for 24 hours. Corresponding control polymers were prepared in the same manner without the presence of the template molecule, paclitaxel.

3.1.2 Precipitation Polymerization Procedure

The polymerization procedure was based off the miniemulsion experiments by Ishuh et al. (2014), which formed MIPs specific to paclitaxel, our target drug. A typical procedure was as follows: 22.1 μL, or 117.1 μmol, of EGDMA (cross-linker), 1.99 μL, or 23.4 μmol, of MAA (functional monomer), and 10 mg, or 11.7 μmol, of paclitaxel (template) were added to Eppendorf tubes, which were then sonicated for 18 minutes. After sonication, 400 μL of chloroform (porogenic solvent) and 0.5 μL of AIBN (initiator) were added to the Eppendorf tubes, which were then continuously stirred for 3 hours. After 3 hours, the contents of the Eppendorf tubes were transferred to UPLC vials and purged with nitrogen gas for 5 minutes. The solutions were then added to a heating block at 70 ^{o}C for 16 hours. Corresponding control polymers were prepared in the same manner without the presence of the template molecule, paclitaxel.

3.2 Standard Curves and UPLC Analysis

To interpret the values of the peaks given by the UPLC for the template extraction and template rebinding steps, a set of known standards were then prepared. Seven standards were prepared to contained between 0-30 mg/L of paclitaxel in water/acetonitrile (H_2O/ACN) solution (70:30, v:v). Based on the paclitaxel peak measurements of the standards, we were able to determine the paclitaxel concentration of the samples we were analyzing. This process was set to run overnight with results available for analysis the following day.

3.3 Template Extraction

If the polymers successfully formed, then both the MIP and control vials were safely cracked open using a hammer in a paper towel and added to Eppendorf tubes. The polymers formed by bulk polymerization, except for those using chloroform as the solvent, were also grinded in a mortar and pestle to increase the surface area of the particles. The polymers made in chloroform stuck to the walls of the mortar, so grinding was abandoned for these polymers. Once the polymers were transferred to the Eppendorf tubes, they were sonicated in 1 mL of methanol/acetic acid solution (90:10, v:v), or acidified methanol, for 20 minutes to remove the paclitaxel (template). This step was repeated between 5-8 times, depending on if the UPLC readings determined that the paclitaxel was removed from the polymers. At first, readings were taken after every acidified methanol wash to determine how much paclitaxel was removed after each sonication. However, to save time only the final wash sample was analyzed by the UPLC in future experiments. These samples were analyzed by diluting the liquid solution around the MIPs and control polymers in water/acetonitrile (H_2O/ACN) solution (70:30, v:v) to prevent the UPLC unit from becoming oversaturated. If the UPLC did not read any paclitaxel peaks, it was assumed that all of the paclitaxel in the MIPs was removed by the acidified methanol.

3.4 Template Rebinding

After the chromatographic analysis of the samples, if it was shown that all the paclitaxel had been washed away, then the rebinding procedure was attempted to evaluate how well paclitaxel rebounded to the MIPs and control polymers. To test this, 1 mL of a 10 mg/L solution of paclitaxel and porogenic solvent was prepared around each MIP and control polymer. This concentration was chosen because the PCF process by Phyton Biotech GmbH produces paclitaxel at 10 mg/L. The solutions in the tubes were sonicated for 20 minutes, and then 500 µL of the initial 1 mL of the solutions were transferred to fresh Eppendorf tubes. The tubes were then added to an evaporative centrifuge to remove the liquid and leave behind any paclitaxel that was left in solution. Following evaporation, 500 µL of water/acetonitrile (H_2O/ACN) solution (70:30, v:v) were added to each Eppendorf tube and sonicated for 6 minutes. The contents of the

Eppendorf tubes were then added to UPLC vials so that the concentration of paclitaxel could be monitored using the UPLC to see if the template bound to the polymer. Readings of less than 10 mg/L of paclitaxel in solution would imply that the missing paclitaxel successfully rebounded to the MIPs and control polymers. Ideally, more paclitaxel would rebind to the MIPs than control polymers, which would indicate a higher paclitaxel-specificity for the MIPs than control polymers.

Chapter 4: Results and Discussion

This section covers the observations and results of the experiments. The experimental results are summarized in Table 1. Table 1 lists the conditions of the MIP formation including:

- Amount of template used
- Amount and type of functional monomer used
- Amount and type of cross-linker used
- Amount and type of solvent used
- Molar ratio between template, functional monomer, and cross-linker
- Method of polymerization

Table 1 also lists the steps taken after the polymer formation (if applicable) and the results of those steps, including:

- If the polymers were grinded
- How often the polymers were washed in 90/10 methanol/acetic acid
- If the paclitaxel was successfully removed from the polymer
- The amount of paclitaxel successfully rebounded to the MIPs and controls

Table 1: MIP Results Summary

	Date of Procedure	Template	Functional Monomer	Cross-Linker	Solvent	Molar Ratios	Method of Polymerization	Polymers Formed?	Polymer Grinding?	Amount of Washes in 90/10 MeOH/AcOH	Taxol Removed?	Taxol Rebounded?	Percent Rebounded in MIP	Amount Rebounded in CTL
1	10/28/2016	20 mg Taxol	7.95 µL MAA	88.3 µL EDMA	500 µL DCM	1:4:20	Bulk	Yes	No	1	No	n/a	n/a	n/a
2	11/15/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL DCM	1:4:20	Bulk	No	n/a	n/a	n/a	n/a	n/a	n/a
3	11/15/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL Toluene	1:4:20	Bulk	No	n/a	n/a	n/a	n/a	n/a	n/a
4	11/17/2016	10 mg Taxol	3.97 µL MAA	44.2 µL EDMA	250 µL DCM	1:4:20	Bulk	Yes	No	1	No	n/a	n/a	n/a
5	11/17/2016	10 mg Taxol	3.97 µL MAA	44.2 µLEDMA	250 µL Toluene	1:4:20	Bulk	Yes	No	1	No	n/a	n/a	n/a
6	12/1/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL DCM	1:4:20	Bulk	Yes	Yes	1	No	n/a	n/a	n/a
7	12/1/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	250 µL DCM	1:4:20	Bulk	Yes	Yes	1	No	n/a	n/a	n/a
8	12/1/2016	5 mg Taxol	5.96 µL MAA	59.8 µL TRIM	125 µL DCM	0.25:3:8	Bulk	Yes	Yes	1	No	n/a	n/a	n/a
9	12/1/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL Toluene	1:4:20	Bulk	Yes	Yes	1	No	n/a	n/a	n/a
10	12/1/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	250 µL Toluene	1:4:20	Bulk	Yes	Yes	1	No	n/a	n/a	n/a
11	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL DCM	1:4:20	Bulk	Yes	Yes	5	Yes	Yes	27% ± 3%	28% ± 2%
12	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	250 µL DCM	1:4:20	Bulk	Yes	Yes	5	Yes	Yes	37% ± 20%	41% ± 6%
13	12/5/2016	5 mg Taxol	5.96 µL MAA	59.8 µL TRIM	125 µL DCM	0.25:3:8	Bulk	Yes	Yes	5	Yes	Yes	31% ± 9%	23% ± 2%
14	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	125 µL Toluene	1:4:20	Bulk	Yes	Yes	5	Yes	Yes	20% ± 13%	27% ± 6%

	Date of Procedure	Template	Functional Monomer	Cross-Linker	Solvent	Molar Ratios	Method of Polymerization	Polymers Formed?	Polymer Grinding?	Amount of Washes in 90/10 MeOH/AcOH	Taxol Removed?	Taxol Rebounded?	Amount Rebounded MIP	Amount Rebounded CTL
15	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	250 µL Toluene	1:4:20	Bulk	Yes	Yes	5	Yes	Yes	23% ± 2%	25% ± 2%
16	1/23/2017	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	200 µL Chloroform	1:4:20	Bulk	Yes	No	5	Yes	No	n/a	n/a
17	1/23/2017	10 mg Taxol	1.99 µL MAA	22.1 µL EDMA	400 µL Chloroform	2:4:20	Bulk	Yes	No	8	Yes	Yes	70% ± 1%	57% ± 3%
18	1/23/2017	5 mg Taxol	1.99 µL MAA	17.7 µL EDMA	200 µL Chloroform	1:4:16	Precipitation	Yes	No	5	Yes	No	n/a	n/a
19	1/23/2017	5 mg Taxol	5.96 µL MAA	60.3 µL TRIM	200 µL Chloroform	0.25:3:8	Precipitation	Yes	No	8	Yes	No	n/a	n/a
20	1/31/2017	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	200 µL Chloroform	1:4:20	Bulk	Yes	No	5	Yes	No	n/a	n/a
21	1/31/2017	5 mg Taxol	1.99 µL MAA	22.1 µL EDMA	400 µL Chloroform	1:4:20	Bulk	Yes	No	5	Yes	No	n/a	n/a
22	1/31/2017	5 mg Taxol	1.99 µL MAA	17.7 µL EDMA	400 µL Chloroform	1:4:16	Precipitation	Yes	No	5	Yes	No	n/a	n/a
23	1/31/2017	5 mg Taxol	1.99 µL MAA	60.3 µL TRIM	400 µL Chloroform	0.25:3:8	Precipitation	No	n/a	n/a	n/a	n/a	n/a	n/a
24	2/21/2017	10 mg Taxol	1.99 µL MAA	22.1 µL EDMA	400 µL Chloroform	2:4:20	Bulk	Yes	No	7	Yes	?	?	?
25	2/21/2017	10 mg Taxol	1.99 µL MAA	37.7 µL TRIM	400 µL Chloroform	2:4:20	Bulk	Yes	No	7	Yes	?	?	?
26	2/21/2017	10 mg Taxol	1.99 µL MAA	22.1 µL EDMA	400 µL Chloroform	2:4:20	Precipitation	No	n/a	n/a	n/a	n/a	n/a	n/a
27	2/21/2017	10 mg Taxol	1.99 µL MAA	37.7 µL TRIM	400 µL Chloroform	2:4:20	Precipitation	No	n/a	n/a	n/a	n/a	n/a	n/a

4.1 Experimental Results

From the results summarized in Table 1, the following observations can be made:

1. **The functioning MIPs formed in chloroform were able to rebind to paclitaxel well, but were not highly paclitaxel specific.**

The MIPs made in row 17 of Table 1 using a molar ratio of 2:4:20 between paclitaxel, MAA, and EGDMA were the most effective functioning MIPs at rebinding to paclitaxel, rebinding 70% ± 1% of paclitaxel in a 10 mg/L solution. No other functioning MIPs were able to rebind more than 37% ± 20% of paclitaxel in a 10 mg/L solution.

However, the control polymers for these conditions rebounded 57% ± 3% of paclitaxel in the same concentration of solution. This 13% ± 4% difference between the MIPs and control polymers is higher than those of the other MIPs and control polymers, but greater differences have been observed, such as those in the miniemulsion experiments by Ishkuh, Javanbakht, Esfandyari-Manesh, Dinarvand, and Atyabi (2014). In those experiments, differences between 15.7% and 38.4% were observed. In addition, those experiments resulted in rebinding for control polymers between 2.2 and 11.8%, far less than the 57% ± 3% observed in our experiments.

The main advantage of our most successful MIP is the high rebinding capacity of 70% ± 1%. The rebinding capacities observed in Ishkuh et al. (2014) were only between 18.4% and 48.4%. However, with the low difference in rebinding percentage between the MIPs and control polymers, this advantage is less meaningful.

2. **The functioning MIPs formed in dichloromethane and toluene neither rebounded to paclitaxel well nor were paclitaxel specific.**

The MIPs and control polymers described from rows 11 to 15 in Table 1, were able to successfully rebind to paclitaxel, but were not able to do so as well as the successful one from row 17. The MIP in these rows with the highest rebinding capacity was made with the conditions in row 12. This resulted in a rebinding capacity of 37% ± 20%, far below the 70% ± 1% in row 17.

These MIPs were also far less selective than the MIPs in row 17. The control polymers in rows 11, 12, 14, and 15 actually rebounded a higher percentage of paclitaxel than the MIPs. For example, in row 12, the control polymers rebounded 41% ± 6% of the paclitaxel in solution, a higher percentage than the 37% ± 20% that the MIPs rebounded to. The most selective MIP in these rows was row 13, which used TRIM as the cross-linker and template:monomer:cross-linker ratio of 0.25:3:8. The difference in selectivity between the MIPs and control polymers of 8% ± 11%. However, because of the potential error involved in this calculation even this MIP could still be less selective to paclitaxel than the control polymers.

3. **Increasing amount of dichloromethane solvent increased average rebinding capacity for paclitaxel, but did not increase paclitaxel selectivity.**

The main difference between rows 11 and 12 was the amount of dichloromethane used as solvent in the MIP formulation. Row 11 used 125 μL of dichloromethane while row 12 used 250 μL of dichloromethane. All other conditions between the two rows were kept constant. In row 11, these conditions resulted in a rebinding capacity of 27% ± 3% in the MIPs and 28% ± 2% in the control polymers. In row 12 with the extra solvent, these numbers jumped to 37% ± 20% in the MIPs and 41% ± 6% in the control polymers. These numbers indicate that while increasing the amount of dichloromethane increased the rebinding capacity, the selectivity for paclitaxel did not improve as well.

4. **Paclitaxel does not completely dissolve in toluene.**

The MIPs and control polymers formed in rows 14 and 15 were set up in the same manner as those in rows 11 and 12, with the exception of toluene used as solvent rather than dichloromethane. Row 14 used 125 μL of toluene while row 15 used 250 μL of toluene, with all other conditions kept constant.

It was discovered during the experiment that paclitaxel does not dissolve well in toluene. Figure 14 illustrates this observation.

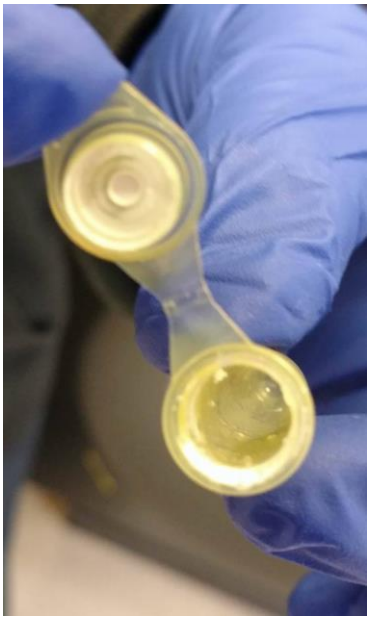

Figure 14: Paclitaxel (white particles) in toluene

We hypothesized that this occured because of the polarity of paclitaxel and the non-polarity of toluene, since polar molecules tend to dissolve better in polar solvents than non-polar

solvents. Figure 15 shows the potential sites for hydrogen bonding in paclitaxel compared to the lack of such sites for toluene.

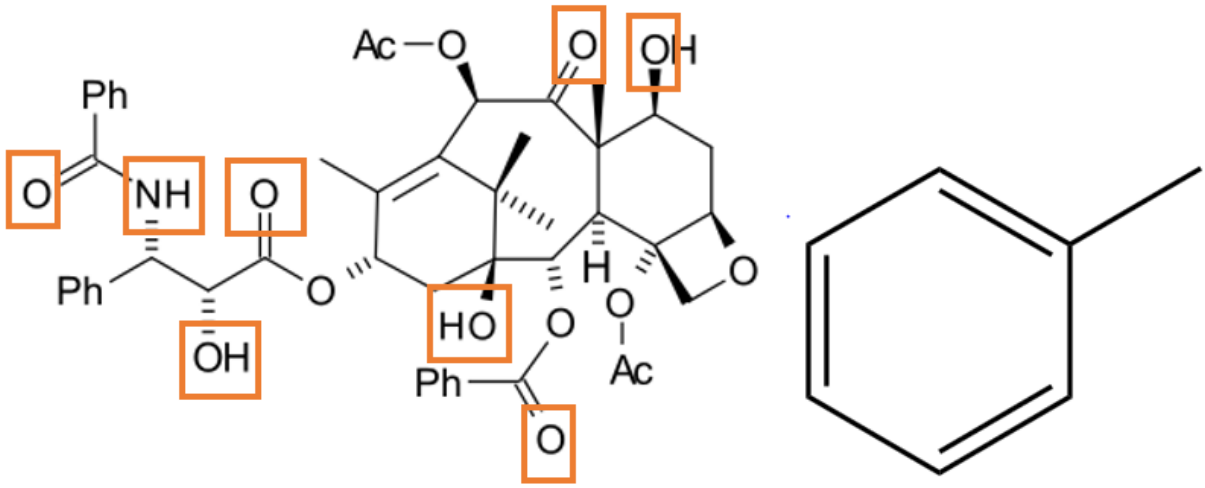

Figure 15: Paclitaxel (left, with polar sites highlighted in orange) and toluene (right, with no polar sites)

5. **Increasing the amount of toluene solvent did not significantly increase rebinding capacity or paclitaxel selectivity.**

The lack of solubility for paclitaxel in toluene seems to be a roadblock for effective MIP formation in toluene. In row 14, the MIP successfully rebounded only 20% ± 3% of paclitaxel and the control polymers rebounded 27% ± 6%. Unlike in dichloromethane, increasing the volume of toluene did not result in much of an increase in rebinding capacity, as only 23% ± 2% of paclitaxel rebounded in the MIPs and 25% ± 2% of paclitaxel rebounded in the control polymers in row 15.

6. **Failure to properly add initiator resulted in polymers failing to form.**

For rows 2, 3, 23, 26, and 27, the MIPs and control polymers failed to form. The polymers from rows 2 and 3 were early trials where we were learning to pipette small volumes, but rows 23, 26 and 27 were precipitation trials where the amount of initiator was reduced. Most bulk trials used 2 µL of AIBN for initiator, but the precipitation trials based off Ishkuh et al. (2014) were scaled down to 0.02 weight percent of the functional monomer. At the small scale level, scaling the amount of initiator to this level reduces the amount of initiator to less than 0.2 µL, which is less than 10 percent of the amount for the bulk trials. This small amount is difficult to pipette properly, leading to only some polymerizations occurring. Figure 16 illustrates failed formations of polymers.

Figure 16: MIP (two left vials) and control polymer (two right vials) contents, with only one control polymer forming

7. **The precipitation procedure was ineffective at producing functioning MIPs.**

No successful MIPs were formed from the precipitation reaction procedure based on Ishkuh et al. (2014). Some failure for the precipitation procedure can be blamed on the low amount of initiator, but another reason for the lack of success could be that the initial procedure by Ishkuh et al. (2014) utilizes miniemulsion polymerization, not precipitation polymerization. This procedure required the use of a surfactant and a hydrophobic agent, while our procedure was simply modified to not require either surfactant or hydrophobic agent. This approach has turned out to be ineffectual as it is and will need to be modified for it to work.

4.2 Analysis of Variance

To determine which varied components had the biggest impact on rebinding results for our MIPs and control polymers, our group performed an Analysis of Variance, or ANOVA. An ANOVA is a set of statistical methods that allows us to estimate the change caused by each varied component of our MIPs (Analysis of Variance, n.d.).

For our ANOVA, we tested the changes in rebinding capacity for the MIPs and control polymers caused by the following components: the type of solvent, the amount of solvent in relation to the template, the type of cross-linker, and the molar ratio between the template, functional monomer, and cross-linker. The results of the ANOVA for the MIPs and control polymers are shown by Table 2 and Table 3. According to the ANOVA, F values that are greater than the F-crit values indicate that the component had a significant impact on the rebinding capacity of the MIPs or control polymers, while F values that are lesser than the F-crit values indicate that the component did not have a significant impact on the rebinding capacity of the MIPs or control polymers. P-values with a value less than 0.05 indicate that there is a strong confidence in the results, while P-values with a value greater than 0.05 indicate that there is not a strong confidence in the results.

Table 2: MIP Rebinding ANOVA Results

MIP Formation Condition	F	P-Value	F-crit
Solvent	10.12	0.00498	4.26
Solvent Volume/mg Template	8.37	0.00883	4.26
Cross-Linker	0.07	0.797	4.96
Template:Functional Monomer:Cross-Linker Molar Ratio	8.32	0.00899	4.26

Table 3: Control Polymer Rebinding ANOVA Results

CTL Formation Condition	F	P-Value	F crit
Solvent	5.99	0.0257	4.46
Solvent Volume/mg Template	7.31	0.0156	4.46
Cross-Linker	1.50	0.251	5.12
Template:Functional Monomer:Cross-Linker Molar Ratio	6.73	0.0193	4.46

According to the ANOVA in Table 2, varying solvent had the greatest impact on the rebinding capacity of the MIPs, while varying cross-linker had the least impact on the rebinding capacity of the MIPs. For the solvents, an F value of 10.12 was calculated in comparison to an F-crit value of 4.26. In addition, the P-value is only 0.00498, which is less than 0.05, which indicates a high confidence in these results. For the cross-linkers, an F value of 0.07 was calculated in comparison to an F-crit value of 4.96, indicating a negligible impact on the rebinding capacity of the MIPs. However, the P-value of 0.797 is greater than 0.05, which indicates a low confidence in the accuracy of these results.

For the control polymers, similar trends were observed in the ANOVA in Table 3. The cross-linker was shown to be the only varied component to have a lower F value than F-crit value, but with a P-value greater than 0.05. Unlike the MIPs however, the ratio between solvent volume and mass of template had the greatest impact on the effectiveness of the control polymers, with the highest F value calculated to be 7.31.

Chapter 5: Conclusions and Recommendations

Over the course of the project, several conditions for the construction of a paclitaxel-specific MIP were investigated. In this section, the shortcomings for our experiments, a summary of what was studied, and ideas for future study will be discussed.

5.1 Experimental Shortcomings

While a few functioning MIPs were created, there are two main shortcomings that can be observed from our experiments that are stated as follows:

1. **The sample size for the working MIPs is small with non-definitive results.**

There were 27 MIP groups created for this project. Out of them, only six groups showed any rebinding capacity for the MIPs and control polymers. The working polymers are listed in Table 4.

Table 4: List of effective MIPs

	Date of Procedure	Temp late	Functional Monomer	Cross-Linker	Solvent	Molar Ratios	Method of Polymerization	Percent Rebounded in MIP	Percent Rebounded in CTL
11	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EGDMA	25 µL/mg DCM	1:4:20	Bulk	27% ± 3%	28% ± 2%
12	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EGDMA	50 µL/mg DCM	1:4:20	Bulk	37% ± 20%	41% ± 6%
13	12/5/2016	5 mg Taxol	5.96 µL MAA	59.8 µL TRIM	25 µL/mg DCM	0.25:3:8	Bulk	31% ± 9%	23% ± 2%
14	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EGDMA	25 µL/mg Toluene	1:4:20	Bulk	20% ± 13%	27% ± 6%
15	12/5/2016	5 mg Taxol	1.99 µL MAA	22.1 µL EGDMA	50 µL/mg Toluene	1:4:20	Bulk	23% ± 2%	25% ± 2%
17	1/23/2017	10 mg Taxol	1.99 µL MAA	22.1 µL EGDMA	40 µL/mg Chloroform	2:4:20	Bulk	70% ± 1%	57% ± 3%

Every polymer listed for each row was made in duplicate, so there are a total of only 12 working MIPs with 12 control polymers out of a total of 54 MIPS with 54 control polymers. With such a small sample size of working polymers, it is difficult to draw definitive conclusions about the best conditions for paclitaxel-specific MIP formation. This is especially true because the MIPs in rows 12, 13, and 14, the percent error for rebinding is high (between 9% and 20%). In addition, the most effective MIPs generated, the ones with the conditions in row 17, rebounded only 13% ± 4% more paclitaxel than its control polymer. Because the functional

monomers, cross-linkers, type and amount of solvent, molar ratios, and type of polymerization can all be varied in a myriad of ways, there are likely untested combinations that would result in more effective MIPs.

2. **High paclitaxel-selectivity was not achieved, but there is potential for selectivity to improve.**

The biggest drawback for our polymers, outside of the lack of a large sample size, was the lack of selectivity demonstrated by the MIPs and control polymers. As can be seen in Table 4, most MIPs that rebounded to paclitaxel at all rebounded less than 40% of 10 mg/L of paclitaxel in solution, and for rows 11, 12, 14, and 15 the control polymers actually rebounded a higher percentage of paclitaxel than the MIPs. Only the MIP conditions in row 17 resulted in a high rebinding capacity of 70% ± 1%, but with a high rebinding capacity in the control polymer of 57% ± 3% , the difference between the MIP and control polymer is only 13% ± 4%. As discussed previously, the paclitaxel imprinted polymers from Ishkuh et al. resulted in lower rebinding capacities but a higher rebinding difference between the MIPs and control polymers. Table 5 highlights these differences.

Table 5: Percentage of paclitaxel (PTX) rebinding by polymers and difference binding between MIPs & control polymers (NIP) with chloroform as solvent (Ishkuh et al., 2014)

Polymer	PTX (mmol)	MAA (mmol)	MMA (mmol)	EGDMA (mmol)	TRIM (mmol)	PTX rebinding MIP (%)	PTX rebinding NIP (%)	Difference binding between MIP & NIP
MIP1	1	4	–	16	–	18.5	2.2	16.3
MIP2	0.25	3	–	–	8	48.4	10	38.4
MIP3	0.25	3	–	–	4	35.2	11.8	23.4
MIP4	0.25	3	–	–	1	18.4	2.7	15.7
MIP5	0.25	3	6	–	1	23.6	5.6	18
MIP6	0.25	3	6	–	2	25.9	5	20.9

The lowest rebinding difference between the MIP and control polymer in the experiments by Ishkuh et al. (2014) is 15.7% for MIP 4, which is greater than the 13% ± 4% observed for our most effective polymer in row 17. Since these experiments show that differences of up to 38.4% for a paclitaxel-specific MIP are possible, this shows there is still plenty of room for improvement for the selectivity of our MIPs.

5.2 Recommendations for Future Work

Because of the limited timespan for our project, we were limited to how we could vary the conditions for forming the MIPs and control polymers. The variables that we tested, with the reason that we tested them, are listed as follows:

1. Molar ratios
    a. 1:4:20, tested 16 times
        i. Common "Golden Ratio" for the template:functional monomer:cross-linker (McClusky et al., 2007)
        ii. Example: Lanza and Sellergren (1999) paper for testing small scale MIP synthesis with terbuthylazine as template
    b. 2:4:20, tested 5 times
        i. Produced most effective MIP in our experiments
    c. 0.25:3:8, tested 4 times
        i. Most effective ratio in Ishkuh et al. (2014) using paclitaxel, MAA, and TRIM
    d. 1:4:16, tested 2 times
        i. Ratio used in Ishkuh et al. (2014) using paclitaxel, MAA, and EGDMA
2. Effects of functional monomer
    a. Only MAA used
        i. One of the most commonly used functional monomers
        ii. Effective in paper by Ishkuh et al. (2014) with paclitaxel as template
3. Effects of cross-linker
    a. EGDMA, tested 21 times
        i. One of the most commonly used cross-linkers (Vasapollo et al., 2011)
    b. TRIM, tested 6 times
        i. One of the most commonly used cross-linkers (Vasapollo et al., 2011)
        ii. Effective in paper by Ishkuh et al. (2014) with paclitaxel as template
4. Porogenic solvent
    a. Dichloromethane, tested 9 times
        i. One of the most commonly used cross-linkers (Vasapollo et al., 2011)
    b. Toluene, tested 6 times
        i. One of the most commonly used cross-linkers (Vasapollo et al., 2011)
    c. Chloroform, tested 12 times
        a. One of the most commonly used cross-linkers (Vasapollo et al., 2011)
        b. Effective in paper by Ishkuh et al. (2014) with paclitaxel as template

Because only one reasonably effective MIP was produced under these conditions, more MIP formation conditions should be tested to address the lack of sample size and effectiveness for our MIPs. Recommendations for different conditions are given in the following sections.

5.2.1 Molar Ratio Recommendations

The ratio between template:functional monomer:cross-linker has been proven to have a significant impact on MIP effectiveness. A molar ratio of 1:4:20 is a generally accepted starting point to begin experimentation, but this ratio does not always produce the most effective MIPs. For example, the experiments by Ishkuh et al. (2014) showed the most effective MIP for miniemulsion with paclitaxel as template had a molar ratio of 0.25:3:8, not 1:4:20.

The Ishkuh et al. (2014) experiments also demonstrated that decreasing the amount of cross-linker in relation to the template gradually resulted in less effective MIPs (see Table 5 in Chapter 5.1, MIPs 2, 3, and 4). In general, this trend follows the literature, which suggests an excess of cross-linking monomers in relation to the template and functional monomer is more favorable to a lack of cross-linking monomers. Papers by Liu, Dai, Li, Tang, and Jin (2014) and Song, Wang, and Shu (2009) support this claim. The experiments in Liu et al. (2014) used a template:cross-linker ratio of 1:35 that produced effective MIPs for the antibiotic drug enrofloxacin (ENRO), while the experiments used in Song et al. (2009) used a template:cross-linker ratio of 1:46.25 that produced effective MIPs for the flavonol quercetin. This may not always be the case, as a paper by Tom, Schneck, and Walter (2012) found that increasing the template:cross-linker ratio from 1:20 to 1:40 actually decreased the effectiveness of MIPs made for the antimicrobial medication sulfadimethoxine (SDM). However, this was acknowledged in the paper as unusual and not typical for MIPs.

The relation between the ratio of template:functional monomer is much less defined than the relation between the relation between template:cross-linker. A ratio between template:functional monomer of 1:4 is a typical starting point, but the most effective ratios usually depend on the nature of the template and functional monomer. For example, the experiments by Liu et al. (2014) found that a template:functional monomer ratio of 1:7 would be the most effective with ENRO as template and MAA as the functional monomer. Liu et al. (2014) theoretically predicted this ratio using geometry optimization, the bonding situation, and the binding energies involved between ENRO and MAA and then experimentally demonstrated that the 1:7 ratio was the most effective option. Figures 17 and 18 demonstrate the interaction between MAA and ENRO at ratios of 1:1 and 1:7, respectively, and Table 6 highlights the experimental results for Liu et al. (2014).

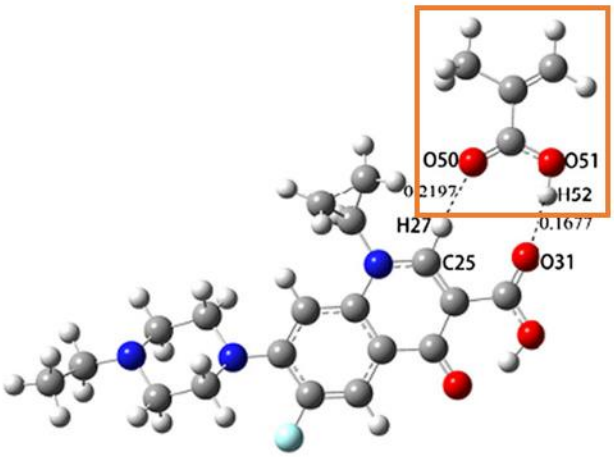

Figure 17: Template:Functional Monomer Ratio of 1:1 with ENRO and MAA (highlighted in orange) (Liu et al. 2014)

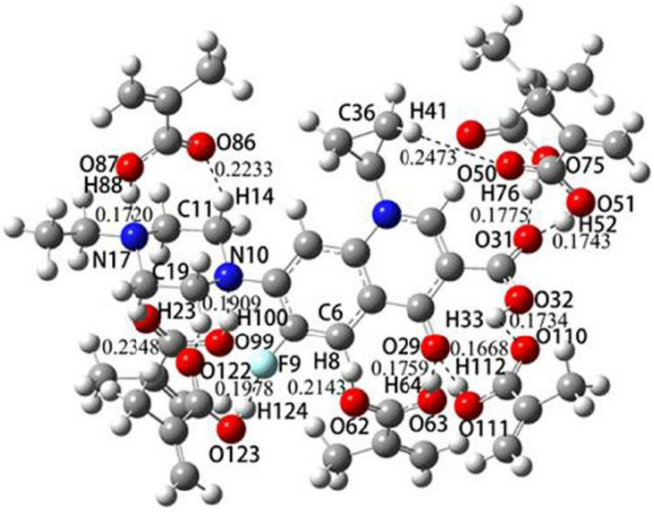

Figure 18: Template:Functional Monomer Ratio of 1:7 with ENRO and MAA (Liu et al. 2014)

Table 6: Rebinding capacities of ENRO specific MIPs with different template:functional monomer ratios (Liu et al. 2014)

Imprinting ratio	1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8
Q (mg/g)	1.45	2.15	3.06	4.16	4.79	5.42	6.37	5.77

Our experiments tested molar ratios of 1:4:20. 2:4:20 (equivalent to 1:2:10), 0.25:3:8 (equivalent to 1:12:32), and 1:4:16, with the most effective results occurring with a molar ratio of 2:4:20. Since the general trends suggest increasing cross-linker concentration increases MIP effectiveness, we suggest that future attempts to form paclitaxel-specific MIPs use an increased concentration of cross-linker. The most effective MIPs were formed with a cross-linker

concentration ten times greater than the template concentration, while MIPs formed in papers by Liu et al. (2014), Song et al. (2009), and Tom et al. (2012) made effective MIPs using cross-linker concentrations 35, 46.25, and 40 times the concentrations of the templates.

The ratio between template:functional monomer does not follow the same trends as the template:cross-linker ratio, making it more difficult to predict a ratio that would increase MIP effectiveness. Increasing functional monomer concentration to excess does not necessarily increase MIP effectiveness, as evidenced by the results reported by Liu et al. (2014) in Table 6, so optimizing a ratio is difficult. Since a template:functional monomer ratio of 1:2 produced an effective MIP with the other tested ratios relatively inconclusive, we recommend testing template:functional monomer ratios of 1:4 again, 1:6, and 1:15. These were the ratios tested by Tom et al. (2012), and depending on the results more specific ratios could be tested to optimize the template:functional monomer ratio provided the MIPs work effectively.

5.2.2 Functional Monomer Recommendations

The interaction between the template and functional monomer is important to consider when forming an MIP. It is generally assumed that the stronger or more stable the complex is between the template and functional monomer before polymerization occurs the more selective the MIP will be (Karim et al. 2005). This is why MAA is commonly used, and why we used it for our experiments, as the functional monomer for MIP formation; its ability to act as a hydrogen bond proton donor and hydrogen bond proton acceptor allow it strongly interact with compounds such as paclitaxel. Figure 19 demonstrates how MAA can interact with paclitaxel to form a stable complex.

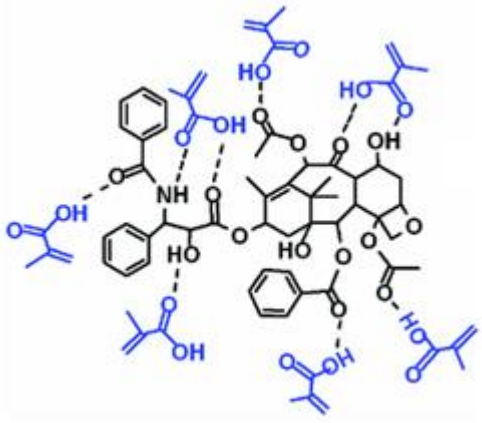

Figure 19: MAA hydrogen bonding with paclitaxel (Ishkuh et al., 2014)

However, Fu, Yang, Zhou, Lin, and Yang (2015) investigated the interaction strength between the flavone luteolin and the functional monomers 1-allyl-piperazine (1-ALPP), 4-vinylpyridine (4-VP), and acrylic acid (AA), and found that interaction strength does not necessarily correlate to more selective MIPs. While 1-ALPP has the strongest interaction with luteolin and AA has the weakest interaction, Fu et al. (2015) found that with luteolin as the

template, EGDMA as the cross-linker, and tetrahydrofuran (THF) as the porogenic solvent, MIPs with 4-VP as the functional monomer were the most selective, with 1-ALPP MIPs being the next most selective and AA MIPs as the least selective. These results suggest that the functional monomer interaction with the cross-linker and solvent is important to consider as well as the functional monomer interaction with the template.

Since the choice of functional monomer for paclitaxel-specific MIPs can depend on how it interacts with cross-linker and solvent as well as the template, we recommend continuing to utilize MAA as the monomer while using a cross-linker and solvent that has weak interactions with MAA. If adjustments to the functional monomer must be made, additional functional monomers are listed in Table 7.

Table 7: List of Functional Monomers

Acrylic Acid (AA)
P-vinylbenzoic acid
Itaconic acid
Acrylamide (AM)
2-(Trifluoromethyl)Acrylic Acid (TFMAA)
2-Hydroxyethyl Methacrylate (HEMA)
4-Vinylpyridine (4-VP)
1-Allyl-piperazine (1-ALPP)

5.2.3 Cross-Linker Recommendations

The interaction of the cross-linker with the other components of MIPs is far less studied than the roles of the template, functional monomer, and porogenic solvent, but nevertheless is plays an important role in the effectiveness and selectivity of MIPs. The group from Ishkuh et al. (2014) was able to produce working MIPs using TRIM as cross-linker, while our experiments produced successful MIPs using EGDMA as cross-linker.

However, research by Muhammad, Nur, Piletska, Yimit, and Piletsky (2012) suggests that the cross-linker should be chosen based on the strength of its interaction with the template. This research demonstrated a correlation between the binding energy of the cross-linkers EGDMA, TRIM, and divinylbenzene (DVB) with zidovudine (AZT), a drug used to treat HIV/AIDS, and the effectiveness of the MIPs produced using those cross-linkers. The DVB, which had the lowest binding energy with AZT, was found to be the most effective cross-linker for AZT-specific MIPs, while EGDMA, which had the highest binding energy with AZT, was found to be the least effective for AZT-specific MIPs. Figure 20 demonstrates the difference in

binding energy between the cross-linkers and AZT and Table 8 shows the imprinting factor Muhammad et al. (2012) calculated for the MIPs made with each cross-linker, with higher imprinting factors signifying more effective MIPs. These imprinting factors were calculated by dividing the binding capacity of the MIPs by the binding capacity of the control polymers.

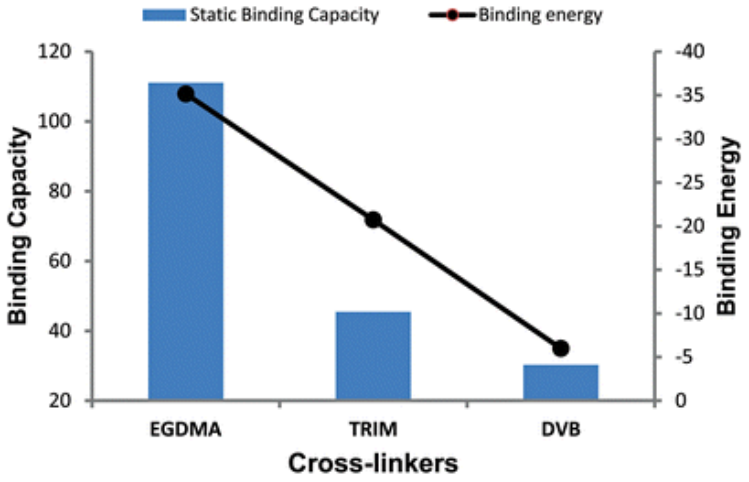

Figure 20: Binding energy of cross-linkers with AZT (Muhammad et al., 2012)

Table 8: Cross-linkers vs. Imprinting Factor for AZT specific MIPs

Cross-linker	Imprinting Factor
EGDMA	1.33
TRIM	1.45
DVB	1.85

The results from the research of Muhammad et al. (2012) suggest that cross-linkers that do not interact with the template, or have weak interactions with the template, are more favorable than cross-linkers that are capable of stronger interactions with the template. Since the carbonyl groups on EGDMA and TRIM have a partial negative charge around the oxygen atoms, they have the potential to interact with the hydrogen bonds on paclitaxel. Cross-linkers such as DVB do not have the potential to interact with the hydrogen bonds of paclitaxel in this way. Figures 21 and 22 illustrate the hydrogen bonding sites of paclitaxel and the carbonyl groups of EGDMA and TRIM, respectively.

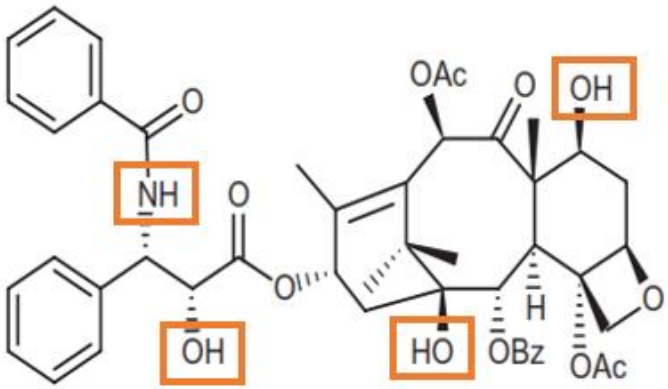

Figure 21: Hydrogen bonding sites on paclitaxel (highlighted in orange)

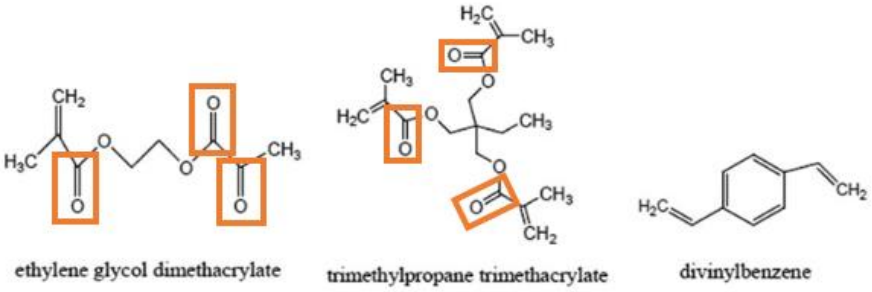

Figure 22: EGDMA (left) and TRIM (center) compared to DVB (right), with carbonyl groups in EGDMA and TRIM highlighted in orange (Cheong et al., 2013)

Because of these observations, we recommend future attempts to make paclitaxel-specific MIPs utilize cross-linkers such as DVB that would not interact with the hydrogen bonds of paclitaxel. 1,3-Diisopropenylbenzene (DIP) would also fit this description, since it does not contain any nitrogen or oxygen groups that would interact with hydrogen bonds. Other potential cross-linkers are listed in Table 9.

Table 9: List of Cross-linkers

Divinylbenzene (DVB)
1,3-Diisopropenylbenzene (DIP)
Tetramethylene Dimethacrylate (TDMA)
Pentaerythritol Triacrylate (PETRA)
Pentaerythritol Tetraacrylate (PETEA)
N,N’-Methylenediacrylamide
N,N’-1,4-Phenylenediacrylamide

5.2.4 Porogenic Solvent Recommendations

Selecting the proper porogenic solvent is an important factor for making effective MIPs because different solvents can interact differently with the template, functional monomer, and cross-linker. These interactions can impact the formation of the MIPs and their ability to function properly (Song et al., 2009).

Our experiments, and the experiments by Ishkuh et al. (2014), produced working MIPs using chloroform as the porogenic solvent, while our experiments using DCM and toluene produced much less effective MIPs. Experiments by Song et al. (2009), however, suggest that important aspects to consider for the solvent are its polarity and the amount of solvent used in relation to the template, functional monomer, and cross-linker. Song et al. (2009) researched the effects of using 1,4-dioxane, tetrahydrofuran (THF), acetone, and acetonitrile as solvent to produce MIPs specific to the flavonol quercetin, a polar molecule. The research found that THF, which has a medium polarity, was the best solvent for producing quercetin-specific MIPs. Table 10 shows the dielectric constant for each of these solvents, with higher constants representing higher polarity, in comparison to the imprinting factor for the MIPs produced by each solvent.

Table 10: Polarity of each solvent vs. Imprinting Factor

Porogenic Solvents from Song et al. 2009	Dielectric Constant (ε)	Imprinting Factor
1,4-Dioxane	2.25	1.05
THF	7.58	1.20
Acetone	20.7	1.07
Acetonitrile	37.5	1.03

Song et al. (2009) hypothesized that this trend occurred because the relatively high polarity for acetone and acetonitrile resulted in the template and the functional monomer interacting with the solvent more than with each other. This would lead to fewer quercetin-specific imprinting sites for the MIPs, which would decrease the specificity of the MIPs. Meanwhile, Song et al. (2009) hypothesized that the relatively low polarity for 1,4-dioxane lead to the MIPs falling precipitating out of solution quickly because polar molecules are less soluble in non-polar solvents. This was demonstrated in our experiments by the lack of solubility of paclitaxel in toluene and the poor performance of the MIPs formed using toluene. Song et al. (2009) hypothesized that because THF has a medium polarity in comparison to the other solvents mentioned, the template and functional monomers were able to act more properly for MIP formation.

The other trend that was realized by Song et al. (2009) was that when several volumes of solvent with the same amount of template, functional monomer, and cross-linker, an optimum

volume of solvent can be determined for the most effective MIPs. Figure 23 shows the effect that the volume of THF has on the MIPs' ability to adsorb quercetin according to Song et al. (2009). The results show an optimum volume of 5 mL of THF when using 0.13 g of quercetin as template, 0.2 g of acrylamide as functional monomer, and 3.5 mL of EGDMA as cross-linker. Song et al. hypothesized that solvent volumes below 5 mL resulted in the MIPs precipitating too early for proper imprinted sites to form. It was also hypothesized that volumes above 5 mL diluted the solution too much and caused imprinted sites to have more defects.

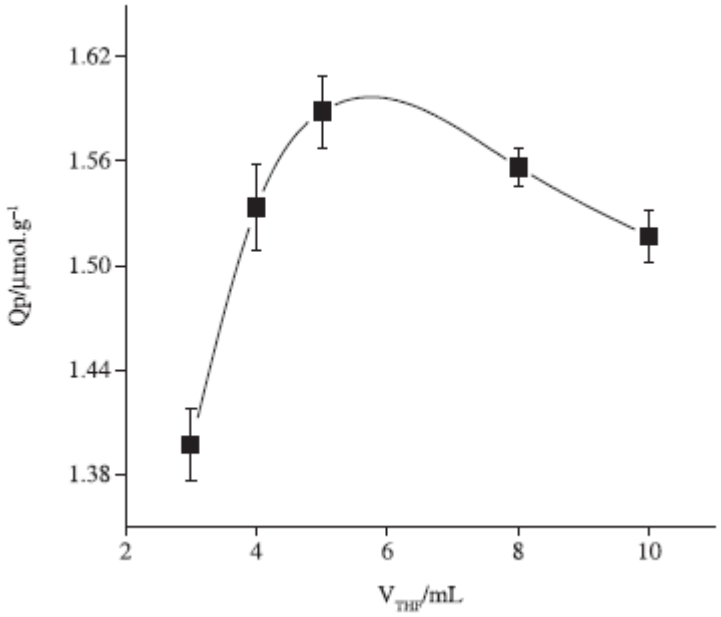

Figure 23: Effect of THF volume on adsorption of quercetin (Song et al., 2009)

The main trends from Song et al. (2009) suggest that solvents have an optimum polarity for forming MIPs, depending on the template, and that there is an optimum solvent volume that can be experimentally determined. Table 11 shows the dielectric constants of the solvents used in our experiments.

Table 11: Dielectric constants of tested solvents (Dielectric Constant, n.d.)

Porogenic Solvents from our trials	Dielectric Constant (ε)
Toluene	2.38
Chloroform	4.81
DCM	8.93

Chloroform, a solvent with a polarity that falls in between those of toluene and DCM, was the solvent that produced our most effective paclitaxel-specific MIPs. The working MIPs

formed by both toluene and DCM were not nearly as effective as the working ones formed in chloroform. This suggests that solvents with dielectric constants that lie around or between those of toluene, at 2.38, and DCM, at 8.93, could potentially be the most effective at forming paclitaxel-specific MIPs. Table 12 shows a few commonly used potential solvents that fall close to this range that we recommend testing.

Table 12: Dielectric constants of potential solvents for paclitaxel-specific MIPs (Dielectric Constant, n.d.)

Tetrahydrofuran (THF)	7.58
1,4-Dioxane	2.25
1,2-Dichloroethane	10.36

Song et al. 2009 found 5 mL of THF as solvent was the optimum volume for 130 mg of the template quercetin in solution. Based on the molecular weights of quercetin and paclitaxel, 130 mg of quercetin is the molar equivalent to 370 mg of paclitaxel. Since our experiments used only between 5 mg and 20 mg of paclitaxel as template due to working on the small scale, the optimum amount of solvent should be much lower than 5 mL. Proportionally, 5 mL of solvent per 370 mg of paclitaxel would be equivalent to 0.068 mL of solvent per 5 mg of paclitaxel and 0.27 mL of solvent per 20 mg of paclitaxel. Therefore, we recommend forming MIPs using a range between 0.068 mL and 0.27 mL (68 to 270 μL) of solvent to see if an optimum amount can be determined to form the best paclitaxel-specific MIPs.

5.2.5 Polymerization Method Recommendations

For our experiments, we tested two different types of polymerization: bulk polymerization and precipitation polymerization. We focused these polymerization methods due to their relative simplicity, and received some positive results in the MIPs formed from bulk polymerization. Unfortunately, all of the MIPs formed from precipitation polymerization did not produce any results. However, precipitation polymerization has produced favorable results in other experiments, as evidenced by Javanbakht, Mohammadi, Esfandyari-Manesh, and Abdouss (2010). In the experiments by Javanbakht et al. (2010), precipitation polymerization resulted in successful MIPs for the drug dipyridamole (DIP). Up to 79% ± 3.1% of DIP in solution rebound to the MIPs, compared to only 34% ± 2.5% for the control polymers, demonstrating both high rebinding capacity and selectivity for the template molecule. The full results of the experiments by Javanbakht et al. (2010) can be seen in Table 13.

Table 13: Recovery percentage of DIP from MIPs formed by precipitation polymerization (Javanbakht et al., 2010)

Polymer	Template : Monomer	Dipyridamole (mmol)	MAA (mmol)	EGDMA (mmol)	Recovery (%)
MIP-1	1 : 2	0.65	1.3	13.8	43 (± 1.4) [a]
MIP-2	1 : 4	0.32	1.3	13.8	56 (± 2.3)
MIP-3	1 : 6	0.22	1.3	13.8	79 (± 3.1)
MIP-4	1 : 8	0.16	1.3	13.8	58 (± 2.8)
MIP-5	1 : 10	0.13	1.3	13.8	72(± 4.2)
NIP	-	-	1.3	13.8	34 (± 2.5)

a Average of four determinations.

Since all of our favorable results came from bulk polymerization, we suggest that our method for bulk polymerization continue to be tested as it currently is. Since no results came from our precipitation polymerization method, it will need to be adjusted to produce working MIPs.

Our method for precipitation polymerization was based on the miniemulsion method used by Ishkuh et al. (2014) that used paclitaxel as template. Their miniemulsion polymerization was similar to precipitation polymerization except that it required the use of sodium dodecyl sulfate (SDS) as a surfactant and hexadecane (HD) as a hydrophobic agent. Our precipitation procedure was simply adjusted to not include SDS or HD as a surfactant or hydrophobic agent. Since this proved unsuccessful, we recommend basing the precipitation procedure on the one used by Javanbakht et al. (2010), which successfully produced effective and selective MIPs. This procedure used many of the same materials used in our experiments, including MAA as the functional monomer, EGDMA as the cross-linker, and chloroform as the porogenic solvent. The main difference is the use of dipyridamole as the template rather than paclitaxel. While DIP is not completely analogous with paclitaxel, they do share a few similar hydrogen bonding sites. These sites are highlighted by Figure 24.

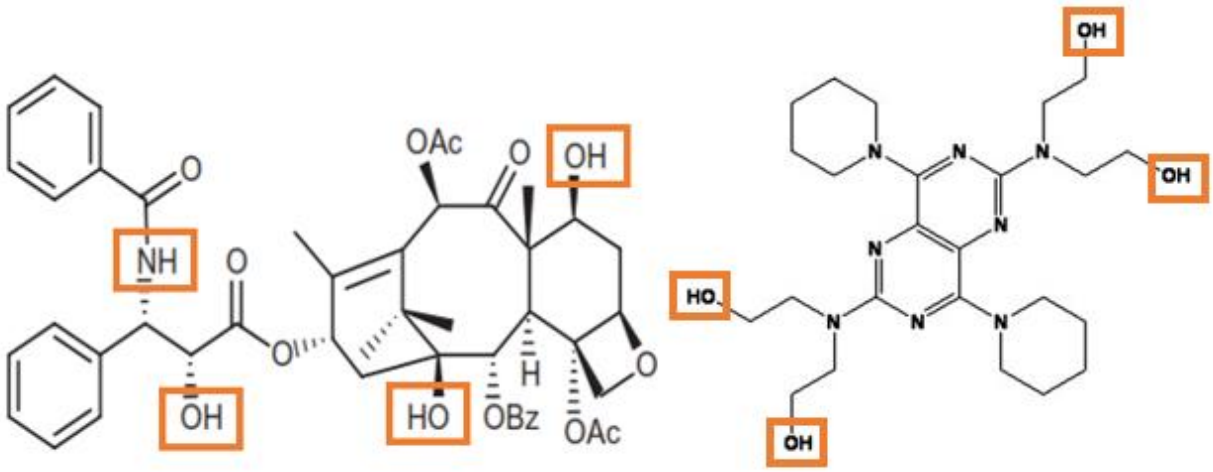

Figure 24: Hydrogen bonding sites of paclitaxel (left) and DIP (right), highlighted in orange

5.3 Conclusion

There were two initial goals for this project. The first goal was to find the conditions that would produce the most effective paclitaxel-specific MIPs. The second goal was to construct the most effective MIPs on a larger scale and test the binding capacities of the MIPs in a mixture of taxanes. While the second goal was not achieved, we were able to produce paclitaxel-specific MIPs, with the most effective MIP rebinding 70% ± 1% of a 10 mg/L solution of paclitaxel. Since the best difference of paclitaxel adsorbed between the MIP and control polymer is only 13% ± 4%, the effectiveness of the MIPs has room to improve based on the experimental results of Ishkuh et al. (2014). With thorough research we proposed guidelines for using different molar ratios, functional monomers, cross-linkers, solvents, and polymerization methods that we believe could improve the effectiveness of paclitaxel-specific MIPs. Therefore, we recommend testing the proposed guidelines to determine if they improve the effectiveness of the MIPs. Provided improvement occurs, we then suggest attempting to accomplish the second goal of our project: testing the effectiveness of the MIPs in a mixture of taxanes.

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Appendix A: General Procedure Example

Appendix A.1: Bulk MIP Synthesis, from Tsermentseli et al. (2013)

	1	2	3	4
1	1-1 Chloroform: 400 µL with Taxol 1.99 µL MAA 22.09 µL EDMA 2 µL AIBN	1-2 Chloroform: 400 µL with Taxol 1.99 µL MAA 22.09 µL EDMA 2 µL AIBN	1-3 Chloroform: 400 µL 1.99 µL MAA 22.09 µL EDMA 2 µL AIBN	1-4 Chloroform: 400 µL 1.99 µL MAA 22.09 µL EDMA 2 µL AIBN
2	2-1 Chloroform: 400 µL with Taxol 1.99 µL MAA 37.71 µL TRIM 2 µL AIBN	2-2 Chloroform: 400 µL with Taxol 1.99 µL MAA 37.71 µL TRIM 2 µL AIBN	2-3 Chloroform: 400 µL 1.99 µL MAA 37.71 µL TRIM 2 µL AIBN	2-4 Chloroform: 400 µL 1.99 µL MAA 37.71 µL TRIM 2 µL AIBN

1. Measure 40 mg Taxol in an Eppendorf tube (20 mg in two tubes if tubes aren't big enough)
2. Label UPLC vials like in chart
3. Pipette 1600 µL of chloroform to Eppendorf tube (800 µL if using two tubes)
4. Vortex Taxol/chloroform mixture in Eppendorf tube
5. Add Taxol/chloroform mixture to MIP vials: 1-1, 1-2, 2-1, 2-2
    a. 400 µL in each vial
6. Add pure chloroform to Control Vials: 1-3, 1-4, 2-3, 2-4
    a. 400 µL in each vial
7. Pipette monomer to MIP and Control vials
    a. Row 1, 1.99 µL of MAA
    b. Row 2, 1.99 µL of MAA
8. Pipette cross-linker to MIP and Control vials
    a. Row 1, 22.09 µL of EDMA
    b. Row 2, 37.71 µL of TRIM
9. Make initiator
    a. 10 mg AIBN/100 µL chloroform
10. Add initiator to each
    a. 2 µL AIBN/chloroform initiator in every vial
11. Crimp vials
12. Make nitrogen balloons
13. Add balloons and needles to vials for 10 minutes
14. Leave vials in heating block at 70 C for 24 hours

Appendix A.2: Precipitation MIP Synthesis, based on miniemulsion from Ishkuh et al. (2014)

	1	2	3	4
4	4-1 Chloroform: 400 µL with Taxol 1.99 µL MAA 22.09 µL EDMA 0.5 µL AIBN	4-2 Chloroform: 400 µL with Taxol 1.99 µL MAA 22.09 µL EDMA 0.5 µL AIBN	4-3 Chloroform: 400 µL 1.99 µL MAA 22.09 µL EDMA 0.5 µL AIBN	4-4 Chloroform: 400 µL 1.99 µL MAA 22.09 µL EDMA 0.5 µL AIBN
5	5-1 Chloroform: 400 µL with Taxol 1.99 µL MAA 37.71 µL TRIM 0.5 µL AIBN	5-2 Chloroform: 400 µL with Taxol 1.99 µL MAA 37.71 µL TRIM 0.5 µL AIBN	5-3 Chloroform: 400 µL 1.99 µL MAA 37.71 µL TRIM 0.5 µL AIBN	5-4 Chloroform: 400 µL 1.99 µL MAA 37.71 µL TRIM 0.5 µL AIBN

1. Label 8 Eppendorf tubes
2. Set Eppendorf tubes into 2 rows of 4
3. Add cross-linker to each Eppendorf tube
    a. 22.09 µL of EDMA to Row 4
    b. 37.71 µL of TRIM to Row 5
4. Add functional monomer to each Eppendorf tube
    a. 1.99 µL of MAA to Row 4
    b. 1.99 µL of MAA to Row 5
5. Measure ~10.0 mg of PTX on 4 plastic dishes and to the 4 MIP Eppendorf tubes, not in the 4 NIP Eppendorf tubes
6. Vortex Eppendorf tubes to ensure mixing
7. Sonicate Eppendorf tubes for 18 minutes
8. While sonication is occurring, make initiator
    a. 10 mg AIBN in 100 µL of chloroform
9. Add 400 µL of chloroform to each Eppendorf tube after sonication
10. Add AIBN/chloroform mixture to each Eppendorf tube
    a. 0.5µL to Row 4 (0.5 µL)
    b. 0.5 µL to Row 5 (0.5µL)
11. Vortex Eppendorf tubes to ensure mixing
12. Tape tubes to the shakers for 3 hours
13. Label UPLC vials
14. Carefully transfer Eppendorf tube contents to appropriately labeled UPLC vials by pipetting

15. Make nitrogen balloons
16. Purge UPLC vials with nitrogen for 5 minutes
17. Heat at 70 °C for 16 hours

Appendix A.3: Template Removal Procedure

1. Turn off heating block
2. Let block cool and then remove vials, keeping them in order
3. Photograph each row for future reference

Preparing Sonicator

4. Clean out inside of the sonicators if necessary
5. Collect ice from ice storage in green ice bucket
6. Fill sonicator approximately halfway with ice
7. Add water until ice begins to float

Preparing Polymers for Initial Solvent Wash

8. Remove MIP and Control polymer blocks from vials by breaking the vials with a hammer on paper towel (don't cut yourselves)
9. Add polymers and 1 mL of chloroform to Eppendorf tubes, marked as follows: (Identities marked in book)
    a. Date 1-1
    b. Date 1-2
    c. Date 1-3
    d. Date 1-4
    e. Date 2-1
    f. Date 2-2
    g. Date 2-3
    h. Date 2-4
10. Add marked Eppendorf tubes to sonicators for 20 minutes
11. Centrifuge when done

Chromatographic Evaluation for Initial Solvent Wash

12. Remove 10 μL of MIP/solvent and Control/solvent solutions with pipette and add to 990 μL 70/30 H2O/CAN in UPLC vials. Mark vials like the following
    a. Date Sol Wash, 1-1
    b. Date Sol Wash, 1-2
    c. Date Sol Wash, 1-3
    d. Date Sol Wash, 1-4
    e. Date Sol Wash, 2-1
    f. Date Sol Wash, 2-2
    g. Date Sol Wash, 2-3
    h. Date Sol Wash, 2-4

Preparing Polymers for First Methanol Wash

13. Remove solvents from Eppendorf tubes without removing polymers, by carefully pouring/pipetting into waste beaker in fume hood
14. Add 1 mL 90/10 MeOH/AcOH to the Eppendorf tubes with polymers
15. Add marked Eppendorf tubes to sonicators for 20 minutes
16. Centrifuge when done

Preparing Polymers for Second Methanol Wash

17. Remove MeOH/AcOH from Eppendorf tubes without removing polymers, by carefully pouring/pipetting into waste beaker in fume hood
18. Add 1 mL 90/10 MeOH/AcOH to the Eppendorf tubes with polymers
19. Add marked Eppendorf tubes to sonicators for 20 minutes
20. Repeat steps 17-19 as necessary

Chromatographic Evaluation for Final Methanol Wash

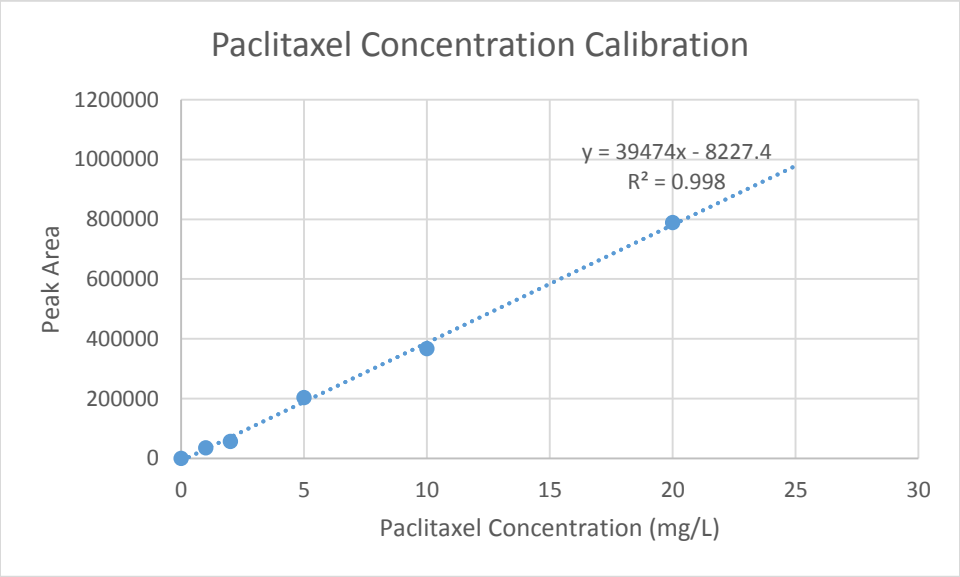
21. Remove 10 μL of MIP/MeOH/AcOH and Control/MeOH/AcOH solutions with pipette and add to 990 μL 70/30 H2O/CAN in UPLC vials. Mark vials like the following:
    a. Date MeOH Wash, 1-1
    b. Date MeOH Wash, 1-2
    c. Date MeOH Wash, 1-3
    d. Date MeOH Wash, 1-4
    e. Date MeOH Wash, 2-1
    f. Date MeOH Wash, 2-2
    g. Date MeOH Wash, 2-3
    h. Date MeOH Wash, 2-4

Appendix A.4: Template Rebinding Procedure

1. Add 1 mL of chloroform to the MIPs and controls in Eppendorf tubes
2. Create a 1 mg/mL sample of Taxol and MeOH
    a. Weigh out 1 mg of Taxol in an Eppendorf tube
    b. Add 1 mL of MeOH to the Eppendorf tube
3. Add 10 μL of Taxol/MeOH solution to each MIP and control in Eppendorf tubes
4. Sonicate mixtures for 20 minutes
5. Remove 500 μL of each MIP and control solution and add to fresh Eppendorf tubes
6. Place tubes in evaporative centrifuge for 30 minutes
7. Add 500 μL of 70/30 H2O/CAN to each Eppendorf tube and sonicate for 6 minutes
8. Transfer entirety of the contents of each Eppendorf tube to UPLC vials and run them on UPLC

Appendix B: Rebinding Data Example

Polymer Number	Concentration Remaining (mg/L)	Retention time	Area	Concentration Removed (mg/mL)	Average Concentration Removed (mg/mL)	Type of Polymer	Average Percentage Rebinded to Polymer (%)
1	7.63	3.892	292917	2.37	2.68	MIP Duplicates	26.8
2	7.02	3.488	268839	2.98			
3	6.98	3.499	267298	3.02	2.85	CTL Duplicates	28.5
4	7.32	3.482	280850	2.68			
5	4.33	3.479	162884	5.67	3.70	MIP Duplicates	37.0
6	8.27	3.49	318158	1.73			
7	6.53	3.487	249576	3.47	4.11	CTL Duplicates	41.1
8	5.24	3.482	198811	4.76			
9	5.96	3.481	227195	4.04	3.11	MIP Duplicates	31.1
10	7.82	3.487	300442	2.18			
11	7.93	3.487	304868	2.07	2.25	CTL Duplicates	22.5
12	7.56	3.49	290296	2.44			
13	6.66	3.478	254814	3.34	2.08	MIP Duplicates	20.8
14	9.18	3.482	354019	0.82			
15	6.79	3.475	259809	3.21	2.66	CTL Duplicates	26.6
16	7.88	3.479	303011	2.12			
17	7.86	3.473	301908	2.14	2.31	MIP Duplicates	23.1
18	7.53	3.47	288928	2.47			
19	7.35	3.505	282075	2.65	2.48	CTL Duplicates	24.8
20	7.68	3.48	294827	2.32			



Appendix C: ANOVA Results

Appendix C.1: Solvent Variations for MIPS

	Solvents		
	Chloroform	DCM	Toluene
Mean	69.7	31.6	21.9
Standard Deviation	0.4	13.4	9.0

ANOVA

F	*P-value*	*F crit*
10.12	4.98E-03	4.26

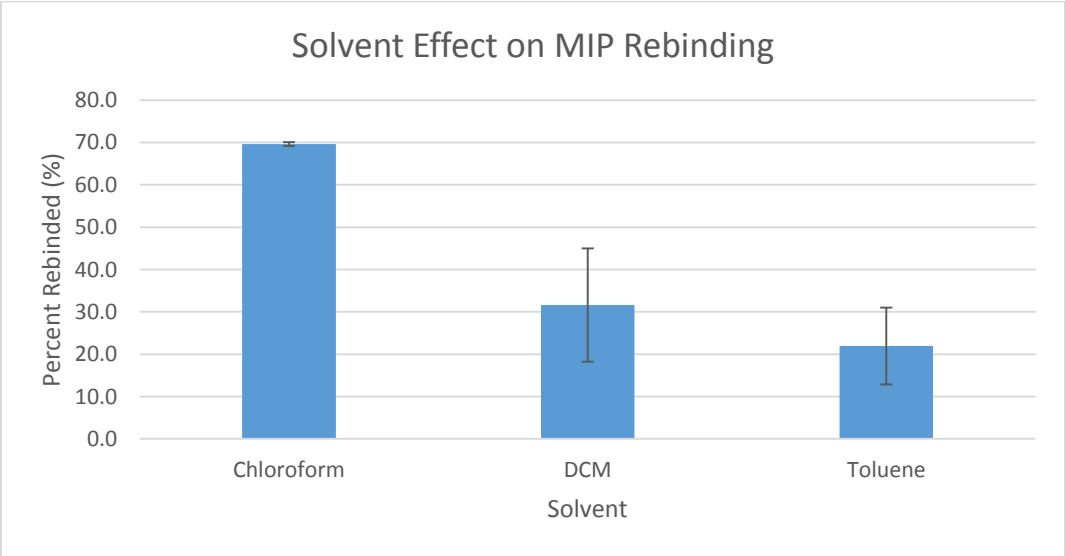
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Chloroform	2	139	69.65	0.405
DCM	6	190	31.6167	214.7257
Toluene	4	87.7	21.9325	109.0682

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3152.07382	2	1576.04	10.12271	0.004975	4.256495
Within Groups	1401.23801	9	155.693			
Total	4553.31183	11				



Appendix C.2: Solvent Variations for Control Polymers

	Solvents		
	Chloroform	DCM	Toluene
Mean	57.0	30.7	25.8
Standard Deviation	0.0	8.7	4.1

ANOVA

F	*P-value*	*F crit*
5.99	2.57E-02	4.46

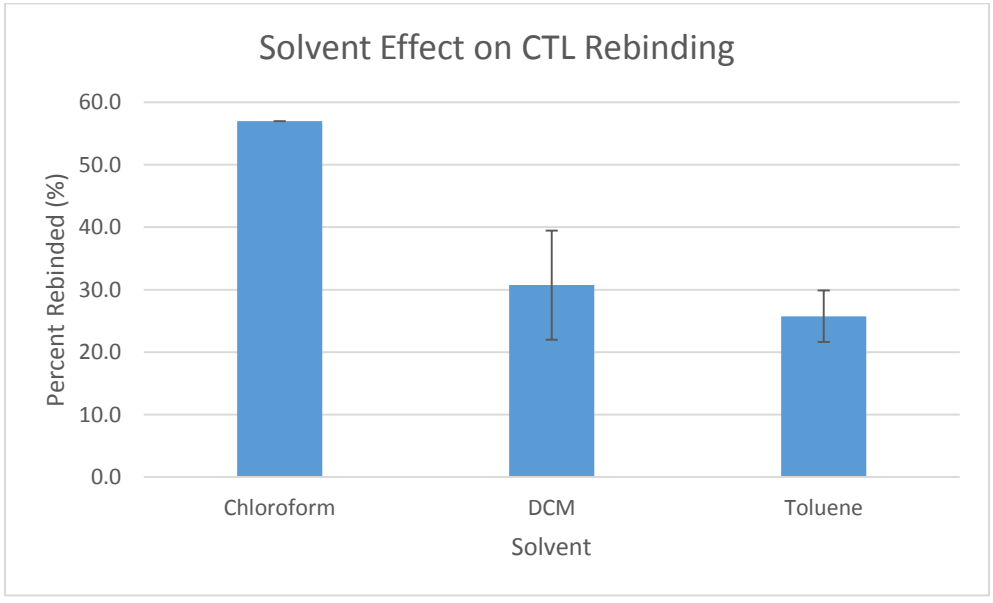
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Chloroform	1	57	57	#DIV/0!
DCM	6	184.4	30.73333333	91.35066667
Toluene	4	103	25.75	22.69666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	785.6257576	2	392.8128788	5.987506806	0.025728331	4.458970108
Within Groups	524.8433333	8	65.60541667			
Total	1310.469091	10				



Appendix C.3: Cross-linker Variations for MIPs

	Cross-linkers					
	EGDMA	TRIM				
Mean	35.5	31.1				
Standard Deviation	20.9	9.3				

ANOVA

F	*P-value*	*F crit*
0.069842758	0.796932602	4.964602744

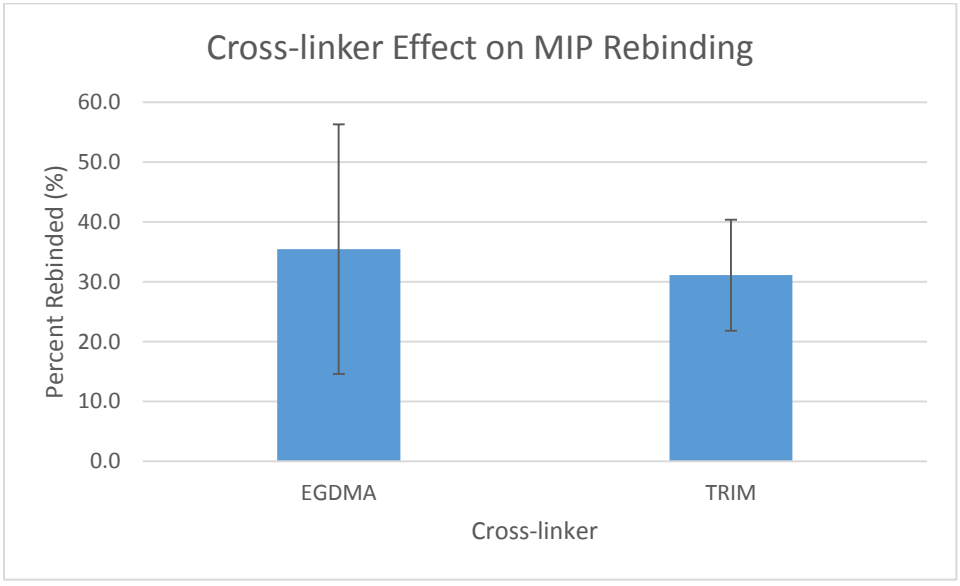
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
EGDMA	10	354.53	35.453	483.1945
TRIM	2	62.2	31.1	172.98

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31.581015	1	31.58102	0.069843	0.796933	4.964603
Within Groups	4521.73081	10	452.1731			
Total	4553.311825	11				



Appendix C.4: Cross-linker Variations for Control Polymers

	Cross-linker	
	EGDMA	TRIM
Mean	33.3	22.6
Standard Deviation	11.1	1.9

ANOVA

F	*P-value*	*F crit*
1.50	2.51E-01	5.12

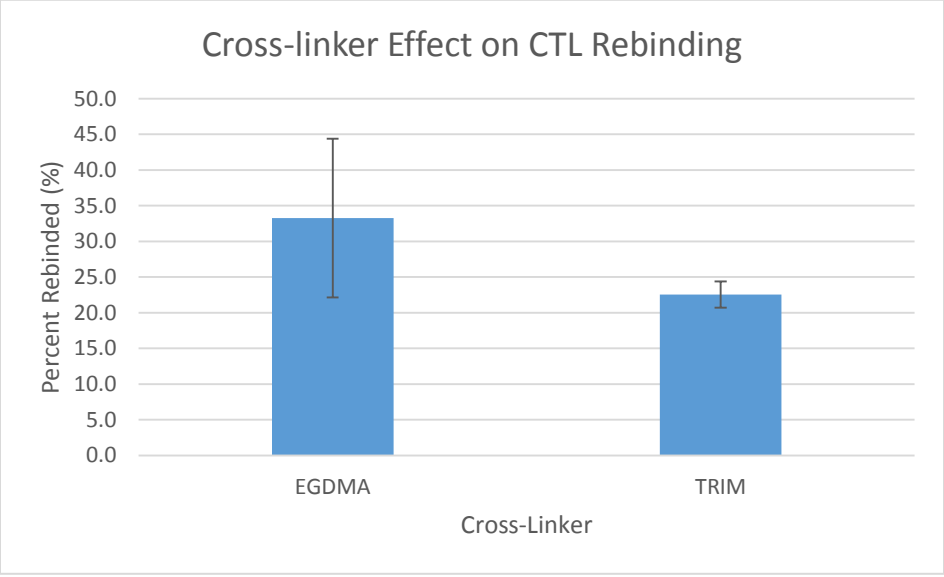
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
EGDMA	9	299.3	33.25556	139.5103
TRIM	2	45.1	22.55	6.845

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	187.5418687	1	187.5419	1.503104	0.251305	5.117355
Within Groups	1122.927222	9	124.7697			
Total	1310.469091	10				



Appendix C.5: Molar Ratio Variations for MIPs

	Molar Ratio		
	1;04;20	0.25;3;8	2;04;20
Mean	26.9	31.1	69.7
Standard Deviation	13.3	9.3	0.4

ANOVA

F	*P-value*	*F crit*
8.32	8.99E-03	4.26

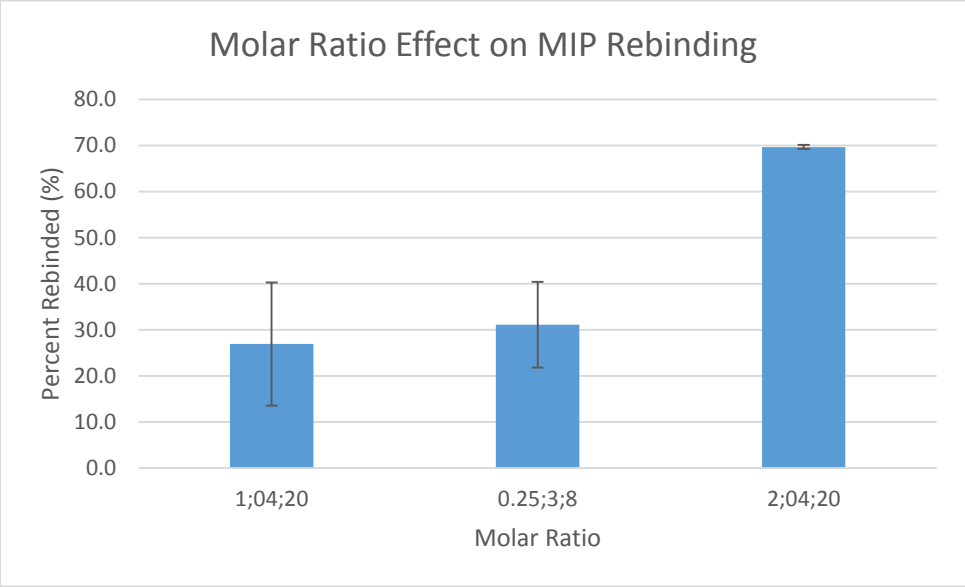
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1;04;20	8	215.23	26.904	203.537
0.25;3;8	2	62.2	31.1	172.98
2;04;20	2	139.3	69.65	0.405

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2955.168037	2	1477.6	8.321064	0.008991	4.256495
Within Groups	1598.143788	9	177.57			
Total	4553.311825	11				



Appendix C.6: Molar Ratio Variations for Control Polymers

	Molar Ratio		
	1;04;20	0.25;3;8	2;04;20
Mean	30.3	22.6	57.0
Standard Deviation	7.8	1.9	0.0

ANOVA

F	*P-value*	*F crit*
6.73	1.93E-02	4.46

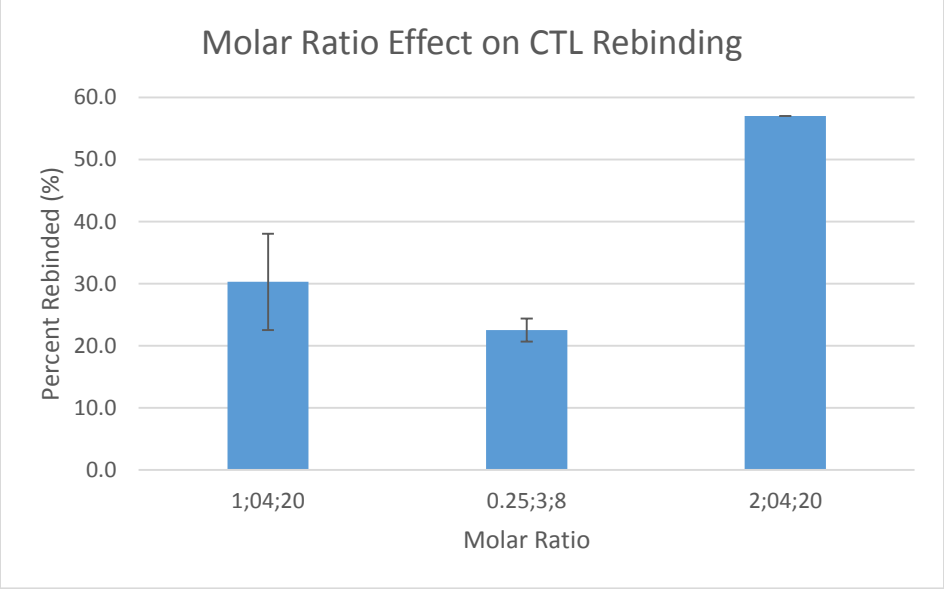
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1;04;20	8	242.3	30.288	68.82982
0.25;3;8	2	45.1	22.55	6.845
2;04;20	1	57	57	#DIV/0!

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	821.815	2	410.91	6.727179	0.019333	4.45897
Within Groups	488.654	8	61.082			
Total	1310.47	10				



Appendix C.7: Solvent Volume/Template Mass Variations for MIPs

	Solvent/Template Ratios		
	25 µL/mg	40 µL/mg	50 µL/mg
Mean	26.2	69.7	30.0
Standard Deviation	10.12397	0.45	15.62232

ANOVA

F	*P-value*	*F crit*
8.373772118	0.008826	4.256495

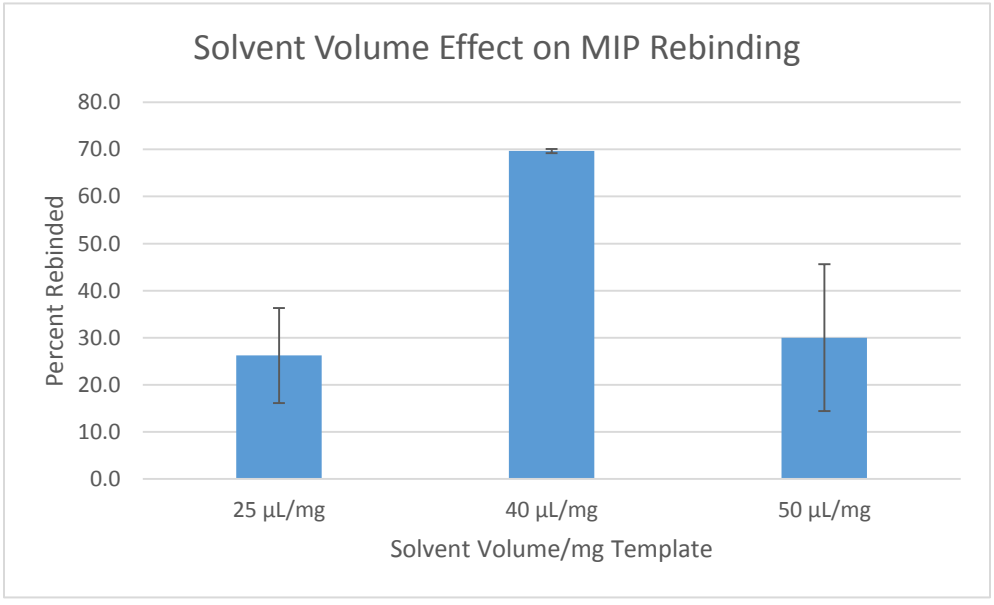
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	157.33	26.22167	122.9936
Column 2	2	139.3	69.65	0.405
Column 3	4	120.1	30.025	325.4092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2961.711	2	1480.856	8.373772	0.008826	4.256495
Within Groups	1591.601	9	176.8445			
Total	4553.312	11				



Appendix C.8: Solvent Volume/Template Mass Variations for Control Polymers

	Solvent/Template Ratios		
	25 µL/mg	40 µL/mg	50 µL/mg
Mean	25.9	57.0	33.0
Standard Deviation	4.264583	0	9.41196

ANOVA

F	*P-value*	*F crit*
7.310310196	0.015644	4.45897

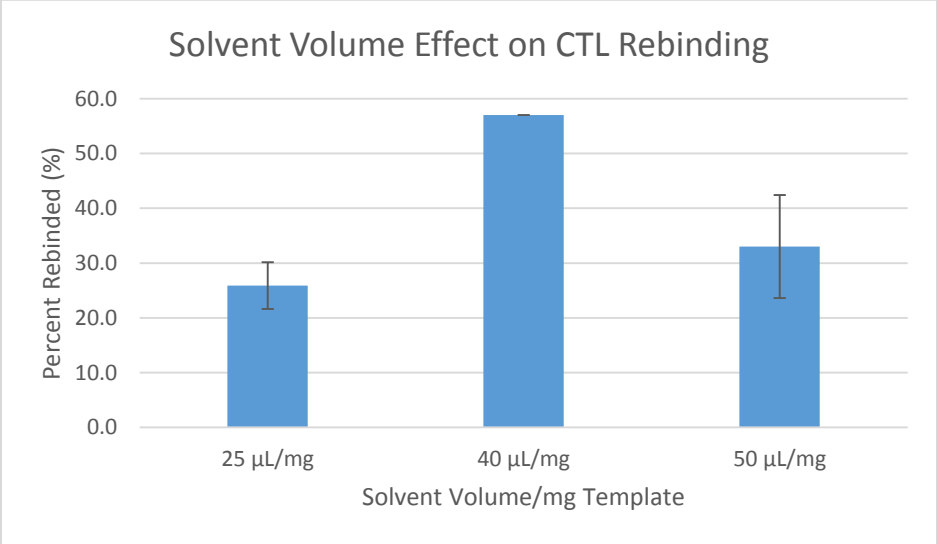
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	155.4	25.9	21.824
Column 2	1	57	57	#DIV/0!
Column 3	4	132	33	118.1133

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	847.0091	2	423.5045	7.31031	0.015644	4.45897
Within Groups	463.46	8	57.9325			
Total	1310.469	10				



Appendix C.9: ANOVA Results Summary

MIP ANOVA Results

MIP Formation Condition	*F*	*P-value*	*F crit*
Solvent	10.12	4.98E-03	4.26
Cross-linker	0.07	7.97E-01	4.96
Molar Ratio	8.32	8.99E-03	4.26
Solvent Volume/mg Template	8.37	8.83E-03	4.26

CTL ANOVA Results

CTL Formation Condition	*F*	*P-value*	*F crit*
Solvent	5.99	2.57E-02	4.46
Cross-linker	1.50	2.51E-01	5.12
Molar Ratio	6.73	1.93E-02	4.46
Solvent Volume/mg Template	7.31	1.56E-02	4.46

Appendix D: Experimentally Varied Components

Molar Ratios	Functional Monomers	Cross-Linkers	Porogenic Solvents
1:4:20 (16 Times)	MAA (27 times)	EGDMA (21 times)	DCM (9 times)
2:4:20 (5 times)		TRIM (6 times)	Toluene (6 times)
0.25:3:8 (4 times)			Chloroform (12 times)
1:4:16 (2 times)			

Appendix E: Potential MIP Testing Summary

Untested functional monomers	**Untested cross-linkers**	**Untested Porogenic Solvents (use polar solvents)**	**Untested molar ratios**
Acrylic Acid (AA)	Divinylbenzene (DVB)	Acetonitrile (ACN)	0.25:4:20
P-vinylbenzoic acid	1,3-Diisopropenylbenzene (DIP)	1-4 Dioxane	1:4:10
Itaconic acid	Tetramethylene Dimethacrylate (TDMA)	Tetrahydrofuran (THF)	1:4:8
2-(Trifluoromethyl)Acrylic Acid (TFMAA)	Pentaerythritol Triacrylate (PETRA)	Acetone	1:1:10
2-Hydroxyethyl Methacrylate (HEMA)	Pentaerythritol Tetraacrylate (PETEA)	Acetonitrile/Chloroform (1:1, v/v) (Exfandyari-Manesh et al.)	1:5:10
Methyl Methacrylate (MMA)	N,N'-methylenediacrylamide	1,2-dichloroethane	1:3:10
Acrylamide (AM)	N,N'-1,4-phenylenediacrylamide	Methanol	1:7:46.25

Appendix F: Ishkuh et al. (2014) Miniemulsion MIP Synthesis for Paclitaxel

1. Mix the functional monomer (MAA or MMA), cross-linker (EGDMA or TRIM), and hexadecane together (hexadecane removed for our precipitation procedure)
2. Add the template molecule (paclitaxel) to the mixture (do not add to control polymers)
3. Sonicate the mixture for 18 minutes
4. Add solvent (chloroform) to the mixture
5. Add AIBN (0.02 wt% of the total amount of functional monomer and cross-linker) as initiator
6. Stir the solution for 3 hours
7. Slowly pour the contents into water containing sodium dodecyl sulfate (1 wt%) using a high-speed homogenizer at 24000 rpm for 5 minutes (this step was skipped for our precipitation procedure)
8. Purge the contents with nitrogen gas for 5 minutes
9. Heat the contents at 70 ^{o}C for 16 hours
10. Separate the formed MIPs and control polymers from reaction medium by centrifugation for 30 minutes at 17000 rpm
11. Freeze-dry the MIPs and control polymers at -40 ^{o}C for 48 hours (replaced this step with air-drying for 2 hours for our precipitation procedure for time purposes)
12. Remove the template molecule via batch-mode solvent extraction with methanol containing 10 % acetic acid (v/v) five times, until no template can be detected

Appendix G: Javanbakht et al. (2010) Precipitation MIP Synthesis for Dipyridamole

1. Mix dipyridamole (template, could replace with paclitaxel) and MAA (functional monomer) in chloroform (solvent) (do not add template to the control polymers)
2. Place the solution in room temperature for 5 hours
3. Add EGDMA (cross-linker) and AIBN (initiator) to the mixture
4. Sonicate the mixture for 5 minutes
5. Purge the mixture with nitrogen gas for 3 minutes in sealed containers
6. Heat the mixture at 60 ^{o}C for 22 hours
7. Filter the polymers using a Whatman filter and wash with acetone and methanol
8. Remove the template by washing the polymers with methanol/acetic acid solution (9:1, v/v) five times, each for 1.5 hours, and then four times in pure water for 1.5 hours