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Development of Polyhydroxyalkanoate Nanoparticles for Cancer Therapy

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A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Abstract

Polyhydroxyalkanoates (PHAs), a class of biopolyesters produced and stored by bacteria, have garnered attention for a number of industrial and biomedical applications. The goal of the current study was to extend the use of PHAs to drug delivery for the treatment of cancer. As a first step, the cytotoxicity of poly(3-hydroxy-octanoate-co-10-undecanoate) (PHOU) towards the A549 lung carcinoma cell line was determined and nanoparticles were prepared via a single emulsion technique. Elution tests, whereby cells were grown in fluid extracts obtained from media incubated for 24 hours at 37°C with PHOU films, did not result in any significant changes in cellular appearance or proliferation, as determined by microscopy and a WST-8 cellular proliferation assay. In contrast, direct contact assays that required growth of the cells on the PHOU films, resulted in cell death after 24 hours, as indicated by Live-Dead cell staining. PHOU nanoparticles with a size of a ~202 nm and a zeta potential of ~2.7 mV, as established using a Zetasizer Nano, were successfully prepared by nanoprecipitation. Ultimately, consistent nanoparticle development through single emulsion using PHOU was unsuccessful so a miniemulsion technique was used for following nanoparticle development. After achieving consistent nanoparticle development through double emulsion, folate groups were to be attached to the nanoparticles to specifically target cancer cells, but due to polymer cross-linking occurring through that attachment process that prevented successful folate attachment, nanoparticle development with new PHA polymers was necessary. PHO-N3 was successfully used to develop nanoparticles through mini-emulsion, a shorter and more efficient process than previous development methods. Through the use of a Zetasizer Nano, the size and zeta potential for the PHO-N3 nanoparticles were established as ~108 nm and -80 mV; suitable values for drug delivery. Although further assessment is required to establish the cytoxicity of the nanoparticles, the results provide preliminary evidence that nanoparticles of an appropriate size for drug delivery can be prepared.

Executive Summary

A successful cell cycle requires a large number of signals to initiate cellular activities when necessary, end the cellular activities when appropriate, prevent unregulated cell division, and check that all cellular activities are running properly. Any cell that does not meet those constraints is considered diseased, such as cancer. Cancer is characterized by uncontrolled cell growth and the potential to metastasize or invade adjacent tissue. Because of these mutations, cancer is a challenging disease to treat, an issue illustrated by mortality rates.

According to the Center for Disease Control and Prevention, cancer is the second leading cause of death in United States. In 2013 alone, there were 580,350 reported deaths as a result of cancer as well as 1,660,290 new cases according to the statistics released by the American Cancer Society. These statistics reaffirm the need for continuous study of novel cancer treatment methods.

Current treatment strategies for cancer are focused on the removal or destruction of diseased tissue often through means of radiation and chemotherapy. These treatment options are accompanied by a significant level of toxicity which has great potential to further medical issues in the afflicted patient. Nanomedicine has become a frequent topic of discussion with regard to cancer therapeutics. Utilizing cancer drug delivery systems such as liposomes, micelles, or nanoparticles can increase an encapsulated drug's circulatory stability thereby increasing the probability of the drug arriving at the diseased tissue. Molecules that target specific receptors on the tumorous tissue can be attached to polymers called polyhydroxyalkanoates (PHAs) in order to enhance specific targeting of the

diseased tissue and increase efficacy of the drug. The combination of circulatory stability and targeting results in increased efficacy and reduced toxicity.

Although there has been success in the use of nanocarriers for treatments, application of this in a clinical setting is difficult due to the lack of biodegradability and biocompatability. In addition, preparation of current drug delivery systems is difficult as well as modifying the systems to express desired properties.

The long-term goal of this project is to develop a modifiable drug delivery system to treat cancer based on bacteria-produced, environmentally-friendly polymers [polyhydroxyalkanoates (PHAs)] with high biodegradability and inherent biocompatibility. The objective is to create a PHA-based carrier system that can be easily modified to incorporate a targeting molecule, in this case folate, that improves selectivity for cancer tissue and that is effective at encapsulating and improving the efficacy of an existing anti-cancer drug, paclitaxel (PTX). My hypothesis is that environmentally-friendly, cancer-targeted, PHA nanoparticles can be prepared with ease and that these particles can be used to enhance the therapeutic efficacy of encapsulated PTX. My hypothesis will be tested by pursuing the following specific aims: (1) synthesize and characterize PHA and PHA-folate (PHAF) nanoparticles and (2) establish the therapeutic efficacy of PTX-loaded PHA and PHAF nanoparticles.

The respective anticipated results are: (1) biocompatible, cancer-targeted nanoparticles can be prepared from PHA and PHAF; and (2) the therapeutic efficacy of PTX can be enhanced through incorporation into PHA and PHAF

nanoparticles. Also, PTX loaded into actively targeted, PHAF nanoparticles is anticipated to be more effective than if loaded into PHA nanoparticles.

The proposed research provides the foundation for the development of a new, effective cancer treatment strategy based on the use of environmentally-friendly, bacteria produced polymers than can be easily modified to incorporate a variety of therapeutics and target many cancer types.

The materials of interest is from a class of biopolymers called polyhydroxyalkanoates (PHAs). PHAs are synthesized and stored by an engineered form of *E. coli* and have received significant attention for industrial and biomedical applications. The preliminary steps, verifying that the PHA is a suitable polymer for cancer drug delivery and nanoparticle development, have been completed.

The goal of the project is to develop an effective PHA-based drug carrier system characterized by high biodegradability and biocompatibility that can encapsulate an existing anti-cancer drug called paclitaxel and be modified by adding a folate molecule in order to target cancer tissue. In order to do this, the current process for developing PHA nanoparticles has been optimized. After failing to achieve consistant nanoparticle development through nanoprecipitation and double emulsion techniques, a miniemulsion technique was found to produce consistent PHA nanoparticles and was optimized for nanoparticle development.

Previous research in the Nomura Laboratory at SUNY ESF using engineered E. Coli to produce PHA copolymers was extended beyond saturated

fatty acids to include 10-undecenoic, 10-undecynoic acid, and 10-azidodeanoic acid monomers.The method currently being used to develop nanoparticles is a miniemulsion technique dissolving these PHA copolymers in chloroform and adding the polymer-chloroform solution to a separate aqueous SDS solution. The mixture is stirred then ultrasonicated and then stirred again to evaporate off excess chloroform. By varying the amount of each component used, the nanoparticles can be optimized to the proper size and surface charge ideal for delivery and cellular uptake.

In order to assess cellular uptake of nanoparticles, a competitive binding assay was conducted by incubating lung carcinoma cells with fluorescently-tagged nanoparticles and then viewing them by microscopy using a Zeiss Axiovert 40 CFL microscope. Due to overlyconfluent cells and the presence of folates in the media used, uptake was difficult to correctly assess, although it was observed.

Nanoparticles prepared with PHA copolymers that included terminal alkynes were reacted with an azide containing fluorescent tag via click chemistry. By attaching azide functional groups to PHA nanoparticles, additional chemistry can be used to ultimately attach the folate groups needed for targeting cancer cells.

In addition, the efficacy of the drug delivery system is being evaluated *in vitro* via a WST-8 cell proliferation assay. The anti-cancer effect of PHA-N3 nanoparticles, PHA nanoparticles, PHA nanoparticles with a folate attached [PHAF], free PTX, PTX-loaded, PHA nanoparticles, PTX-loaded, PHAF

nanoparticles will be determined using this assay. Currently, anticipated toxicity has been experienced in PHA-N3 nanoparticles due to the interactions the azide functional groups have with the cells. Other test materials are currently undergoing experiment planning or testing for cytotoxicity,

Following the optimization of the nanoparticle development method, PTX will be encapsulated into the PHA nanoparticles and assessed to determine the drug encapsulation efficiency. The amount of drug left in the supernatant liquid remaining after the nanoparticles are developed will be determined using high performance liquid chromatography. Using this value, the loading capacity (mass of PTX/mass of nanoparticle) and loading efficiency (percentage of PTX loaded into nanoparticle/amount added to polymer solution) can be calculated.

Overall the purpose of this study is to synthesize and characterize PHA and PHAF nanoparticles as well as establish the therapeutic efficacy of drug-loaded PHA and PHAF nanoparticles. The knowledge gained from this study is essential for the development of targeted, environmentally friendly, biopolymer-based drug delivery systems as a method of cancer therapy characterized by increased efficacy and reduced toxicity. In addition to this study's significance with respect to cancer treatment, the overall reach extends to treatment methods for other diseases due to the drug carrier system's ease of manipulation.

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Acknowledgements

I would like to thank the Bader and Nomura Laboraties and in particular Professors Bader and Nomura, my capstone advisor and reader respectively. I would also like to thank Professor Martin Forstner from the Syracuse University Physics department and Patricia Wardwell for their contributions.

Chapter 1: Introduction

Current treatment strategies for cancer often employ a combination of established methods to achieve the greatest anti-cancer effect. While these methods are effective at destroying cancer cells, they are often accompanied by a significant amount of toxicity. Polyhydroxyalkanoates (PHAs) are a class of biodegradable biopolyesters produced and stored by bacteria that have received significant attention for industrial biomedical applications. The number of carbon atoms in the individual PHA monomers is the greatest determinant of a PHA polymer's physical properties. These PHA monomers are incorporated by bacterial enzymes into polymer chains (figure 1). PHA polymers can either be of short-chain-length (SCL) with 3 – 5 carbons or medium-chain-length (MCL) with 6 – 14 carbons[1,2]. PHA copolymers have proven to have material properties similar to polyethylene and polypropylene and are of special interest for their biocompatibility[1,2]. Currently, PHAs have been used in biomedical applications such as sutures, screws, surgical mesh, stents[3], and drug delivery[4-10] and this study aims to expand their role in drug delivery.

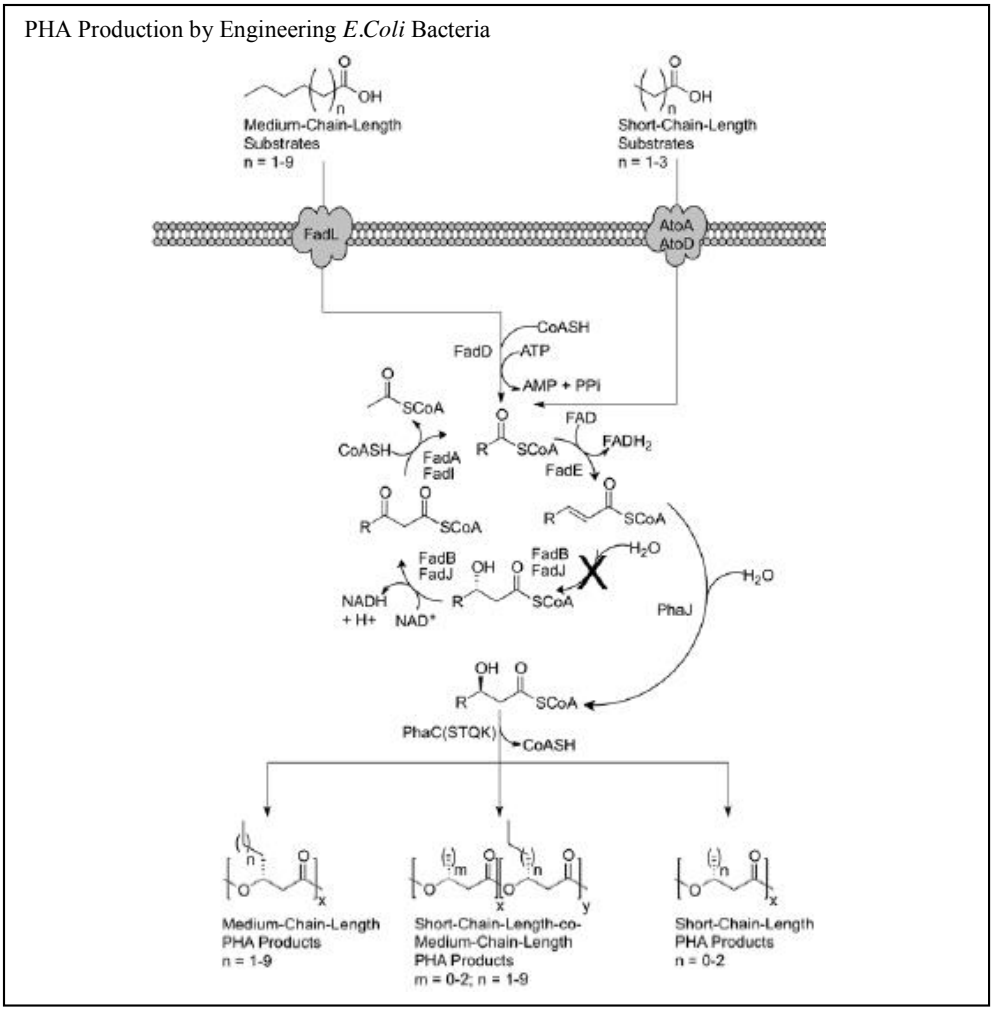


Figure 1. **Production of PHA Monomers by Engineering *E. coli*** Engineered *E. coli* can produce PHA copolymers from fatty acids with defined repeating unit compositions and material properties.[1]

PHAs were originally found present in bacteria in the 1920s[11] and since then, the process has been adapted for the laboratory environment. As shown in figure 2, bacteria are fed fatty acids of varying chain lengths to produce PHA monomers of different sizes. In fermentation chambers, PHAs are developed within the bacteria, which are then lysed for retrieval and purification of the PHA. The resulting product is a bioplastic with material properties largely determined by the monomer composition of the PHA. One such property, biodegradability, is

inherent of all PHA polymers and an important characteristic of environmentally-friendly plastics.

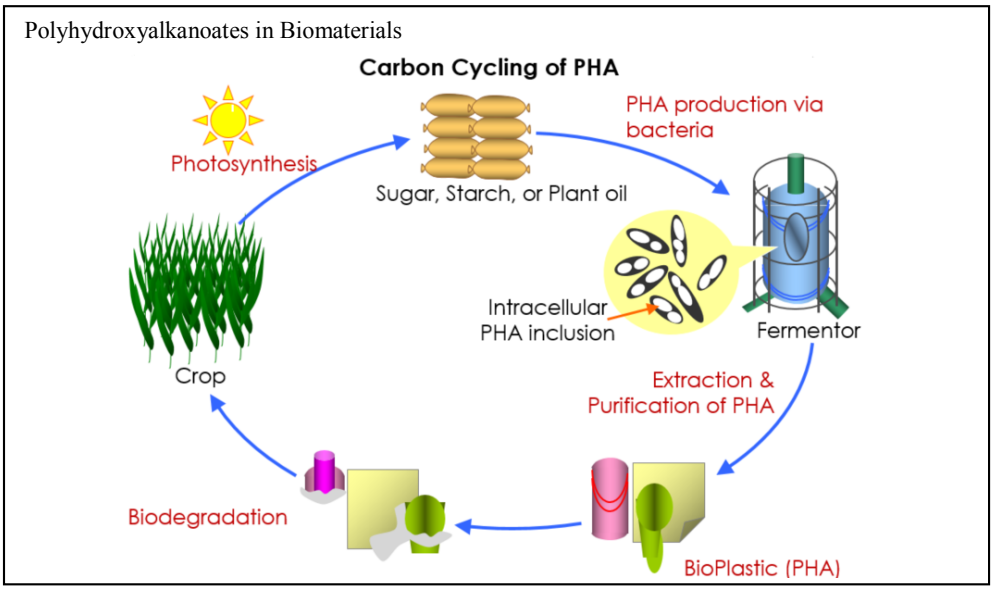


Figure 2. **Life Cycle of PHA Polymers in Biomaterials.** PHAs are produced within bacteria in both nature and the laboratory environment. The polymer is retrieved and purified and the resulting bioplastic is used for biomaterial applications. Ultimately, the polymer is biodegradable and breaks down back into the environment where it is nontoxic.

The Nomura Research Group at SUNY ESF engineered a strain of *Escherichia coli* to produce PHA polymers with specifically defined repeating chains from fatty acids[12,13]. Having the ability to produce consistent repeating polymer composition allows for the development of PHA polymers with desired material properties. Additionally, functionalized PHAs can be modified through thiol-ene and copper-catalyzed click chemistry to attach fluorescent tags or folate groups to visualize uptake by the cells or specifically target cancer[12]. Nanoparticles designated for drug delivery applications, like the ones used in this study, require polymers with biodegradable or biocompatible properties, such as PHA.

Polymer nanoparticles can be developed through a variety of methods[15], but the two main methods used in this study were nanoprecipitation and miniemulsification. Nanoprecipitation, a solvent displacement method, involves dissolving the polymer from which you wish to make nanoparticles in an organic solvent, before slowly adding the organic solution to an aqueous solution of non-solvent with surfactant. The nanoparticles are produced when the polymer solvent quickly diffuses in the non-solvent phase as drops of the organic solution are added. The tension between the solvent and non-solvent phases decreases when the two phases are mixed so the nanoparticle surface area increases and nanoparticles precipitate out. Miniemulsification involves dissolving the polymer in an organic solvent and mixing it with a solution of water and a surfactant. The mixture is then subjected to high shear through ultrasonication to produce the emulsified nanoparticles. The organic solvent is removed by heating the solution to evaporate the solvent out. Anti-cancer drugs such as paclitaxel[21-24] can be incorporated into the nanoparticles simply by dissolving the drug along with the polymer in the first step of the nanoparticle development process.

One of the greatest challenges of cancer treatment is tempering the anti-cancer effect of the treatments used such that healthy cells aren't damaged. One solution to this is to develop nontoxic treatment methods and ensure that only the diseased site is effected. Using a family of chemical reactions referred to as click chemistry, targeting groups and other functional moieties can be directly attached to PHA polymers that contain functional groups[18,19], before or after nanparticle development. Breast cancer, for example, overexpresses folate receptor α so by

attaching folate groups to PHA nanoparticles, breast cancer tissue can be specifically targeted[16,17]. Since PHA nanoparticles are biocompatible and biodegradable, toxicity is limited as paclitaxel is housed within the PHA nanoparticle, protecting healthy cells as the drugs move through the body. Furthermore, the addition of folate groups increases the anti-cancer drug concentration at the diseased tissue rather than healthy tissue.

Brain cancer, on the other hand, is significantly more difficult to treat due to the presence of the blood-brain barrier preventing the transportation of necessary anti-cancer drugs to the diseased site. Penetration and transportation across the blood-brain barrier using drug-loaded carrier systems can be facilitated and enhanced by using multi-function PHA nanoparticles[25] coated with lactoferrin and transferrin, receptors overexpressed by brain endothelial cells [26]. Additionally, by using certain surfactants in the nanoparticles development process, the absorption of apolipoprotein A-1 in the blood by the nanoparticles can be encouraged, further enhancing blood-brain barrier transport[27]. Finally, anti-epidermal growth factor antibodies and angiopep-2 ligands can be attached to PHA nanoparticles for additional blood-brain barrier transport enhancement and the specific targeting of brain cancer due to the overexpression of angiopep-2 ligand receptors in glioblastoma cells[28-33]. Ultimately, the result is a nontoxic, environmentally-friendly, targeted drug delivery system.

Chapter 2: Materials and Methods

The first step in this study was to establish the cytotoxicity of PHA polymers themselves before nanoparticles development. Indirect contact assays were used to determine whether elutions of the polymer in cell culture media are toxic to the cells. For this assay, PHOU was the PHA polymer used along with lung cancer cells of A549 cell line which uses F-12K media with 10% fetal bovine serum (FBS). Cells were cultured in four wells of a 24-well culture plate and incubated for 24 hours. The polymer was sterilized by submerging it a 70% ethanol solution for 30 minutes and then dried on a hot plate so that the ethanol was no longer present. After drying, the polymer was submerged in 15 mL of the culture medium and incubated for 24 hours at 37^{o}C. After 24 hours, the culture media was removed from the wells of the well plate and replaced with elutions of the polymer-contaminated media for two of the wells, and non-contaminated media for the other two wells. The cells in the well-plate were incubated again for 24 hours.

The indirect contact assay (figure 3) was performed using green (for live cells) and red (for dead cells) fluorescent dyes from the Life Technologies Live/Dead Assay which included calcein AM and ethidium homodimer-1. A Live/Dead stain solution was made from the two dyes along with Dulbecco's phosphate-buffered saline (D-PBS). The media was removed and replaced with Live/Dead stain for each well. After incubating the cells again for 45 minutes, the Live/Dead stain was removed and cells were rinsed with D-PBS. Using

fluorescence microscopy, images of live and dead cells can be obtained with green cells representing healthy cells and red cells representing dead cells.

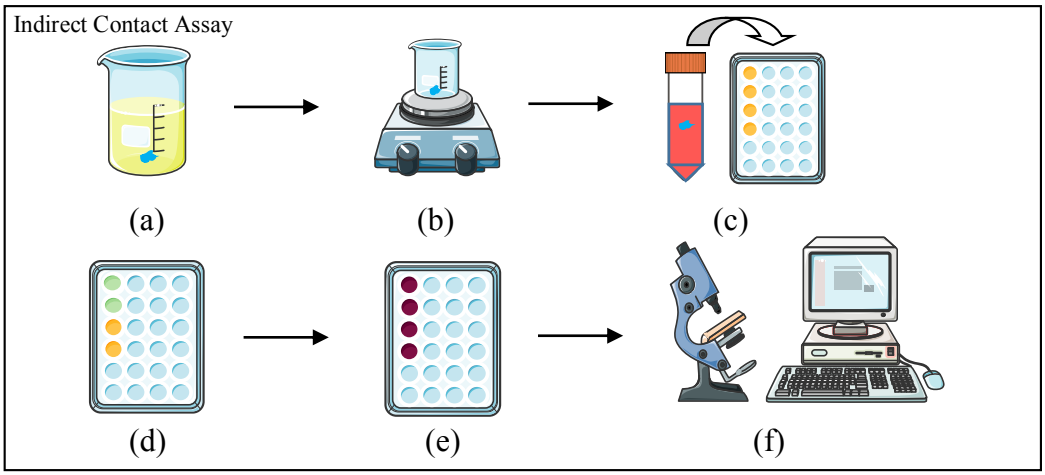


Figure 3. **Indirect Contact Assay for PHA and A549 Lung Carcinoma Cells** (a) Sterilization of polymer in 70% EtOH for 24 hours (b) Drying PHA to remove EtOH (c) Addition of PHA-contaminated media elutions to two wells of A549 lung carcinoma cells. Incubate 24 hours at 37°C. (d) Two wells of cells containing PHA-contaminated media and two wells containing uncontaminated media. Incubate 24 hours at 37°C (e) Media from all wells replaced with Live/Dead Stain. Incubate 45 minutes at 37°C. (f) Observe live and dead cells with fluorescence microscopy

The next step was to conduct a direct contact assay (figure 4) to determine whether cytotoxicity was experienced for cells cultured directly on PHA. The polymer was initially sterilized in the same manner as in the indirect contact assay and then adhered to the bottom of a 6 well plate. Cells of A549 lung carcinoma cell line were cultured on top of the polymer and incubated for 24 hours at 37°C. After 24 hours, the media was removed and the same Live/Dead stain from the indirect contact assay was added. The cells were again incubated at 37°C for 45 minutes and then rinsed with D-PBS. The polymer was carefully removed from the well plate with sterilized tweezers and placed on a glass slide for observation using fluorescence microscopy.

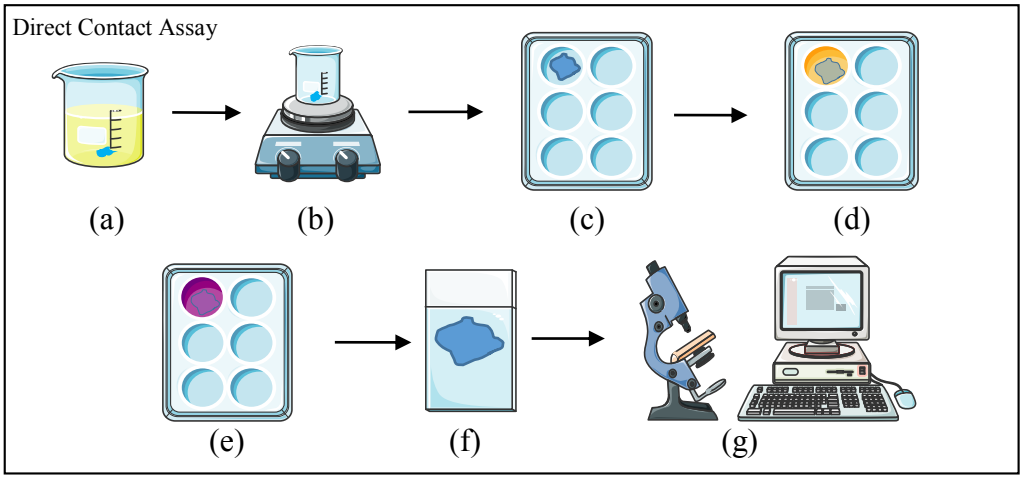


***Figure 4*. Direct Contact Assay for PHA and A549 Lung Carcinoma Cells** (a) Sterilization of polymer in 70% EtOH for 24 hours (b) Drying PHA to remove EtOH (c) Adherence of PHA to well of 6-well plate (d) Cells cultured on PHA in well. Incubate for 24 hours at 37ºC (e) Media replaced with Live/Dead Stain. Incubate 45 minutes at 37ºC. (f) PHA placed on glass slide for microscopy (g) Observe live and dead cells with fluorescence microscopy

After establishing PHA cytotoxity, successful nanoparticle development was the next step of the study. Intially, PHA nanoparticles were developed using a solvent displacement method known as nanoprecipitation (figure 5). PHOU was dissolved in acetone and then added dropwise to aqueous solution (PBS/Pluronic F-68 or Water/Pluronic F-68 was used). The mixture was stirred for 24 hours at room temperature at 1200 rpm using a stir bar and magnetic stir plate to allow the acetone to evaporate out. After 24 hours, the mixture was filtered using a 0.45 micron nylon filter and then ultracentrifuged for 1 hour at 51354 x G and 4ºC. The supernatant was discarded and the nanoparticles were resuspended in 9 mL deionized water for additional rinsing by centrifuging again under the same conditions. Following the second centrifugation, the supernatant was discarded and the nanoparticles were resuspended in 3 mL of deionized water.

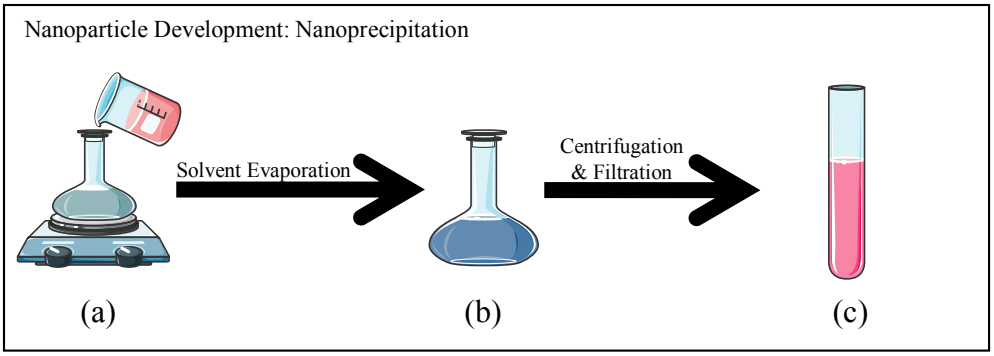


***Figure 5*. Nanoparticle Development through Nanoprecipitation Method.** (a) Organic Phase added drop-wise to aqueous phase (b) Polymeric Nanoparticles suspended in Aqueous Phase (c) Polymeric Nanoparticles suspended in deionized water

Ultimately, due to inconsistencies with the nanoparticles developed through the nanoprecipitation method, other methods of nanoparticle development had to be explored. Using the miniemulsion method shown in figure 6, 60 mg of PHA polymer was dissolved in 1.0 mL of chloroform which was then added to 2.73 mL aqueous sodium dodecyl sulfate (SDS) (4.44 mg/mL). The mixture was then stirred at room temperature for an hour on a magnetic stir plate resulting in monodisperse chloroform droplets containing the dissolved polymer, separated from water by the SDS. Ultrasonication was then used (40% amplitude, 4 second pulse, 1 minute) to emulsify the droplets and form the PHA nanoparticles. Chloroform is then removed by heating the emulsion at 70^{o}C for 2 hours, leaving the PHA nanoparticles suspended in water.

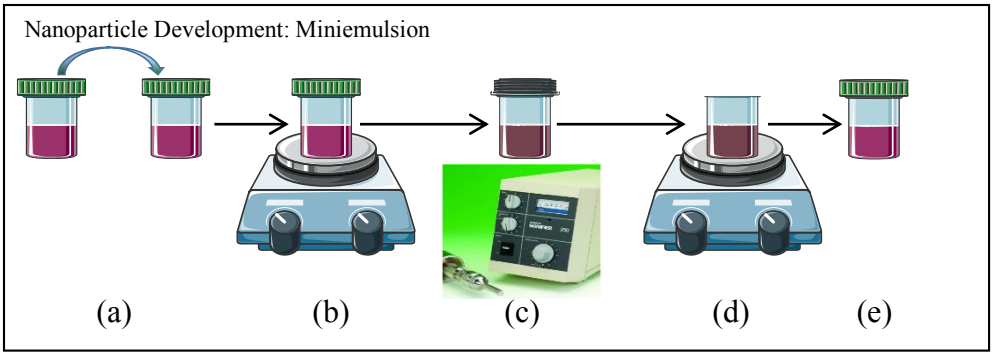


***Figure 6*. Nanoparticle Development through Miniemulsions.** (a) PHA polymer dissolved in Chloroform added to aqueous SDS (b) Pre-emulsification: Mixture stirred at RT for 1 hour (c) Ultrasonication: Mixture emsulsified forming PHA nanoparticles (d) Emulsion heated for 2 hours at 70°C to remove chloroform (e) PHA nanoparticles dissolved in water

Following nanoparticle development, it was necessary to characterize the nanoparticles. This was done by quantitatively measuring size and zeta potential (the surface electric charge of the nanoparticle) through dynamic light scattering using a Zetasizer Nano device. Atomic force microscopy and scanning electron microscopy was used to observe the shape of the nanoparticle and confirm the size.

Once the nanoparticles were found to be of appropriate size and zeta potential for drug delivery applications, functional groups were attached using click chemistry. Fluorescent tags were attached to PHA nanoparticles and then added to the media used to culture breast cancer cells of the MT3 cell line (figure 7). After incubating the cells with the PHA nanoparticle-contaminated media for 24 hours at 37°C, fluorescent microscopy was used to observe the PHA nanoparticles with the cells.

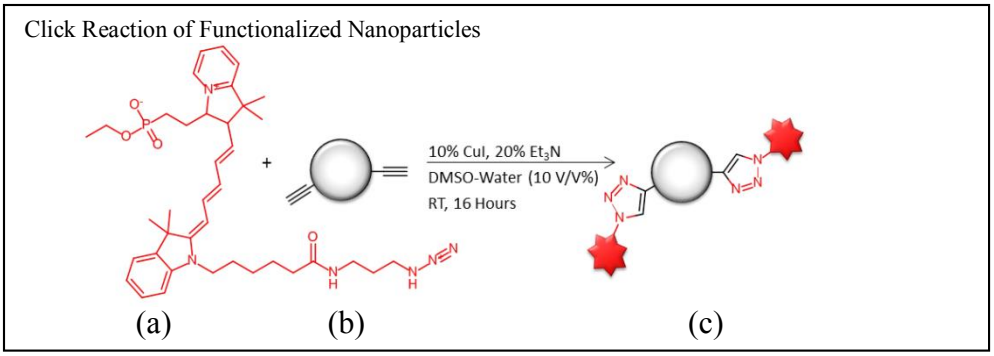


***Figure 7*. Copper Catalized Click Reaction to produce Fluorescently-Tagged Nanoparticles.** (a) Red fluorescent tag (b) Alkyne-modified PHA nanoparticles (c) Fluorescently-tagged PHA nanoparticles

Another step after nanoparticle development is to establish the cytotoxicity of the nanoparticles themselves. Using a WST-8 assay depicted in figure 9, nanoparticle cytotoxicity can be determined based on the metabolic activity of the cells. Lung or breast cancer cells were cultured in two rows of 7 wells in a 96-well plate and incubated for 24 hours at 37ºC. A dilution series (figure 8) was made by adding 200 µL of PHA nanoparticle solution to 800 µL of cell culture media in a 1.5 mL microcentrifuge tube. 500 µL of cell culture media was added to 5 additional 1.5 mL microcentrifuge tubes as well. After mixing the PHA nanoparticle solution in media well, 500 µL was removed and mixed with the edia of the next tube in succession. This process was repeated until the final tube contained 1000 µL total volume.

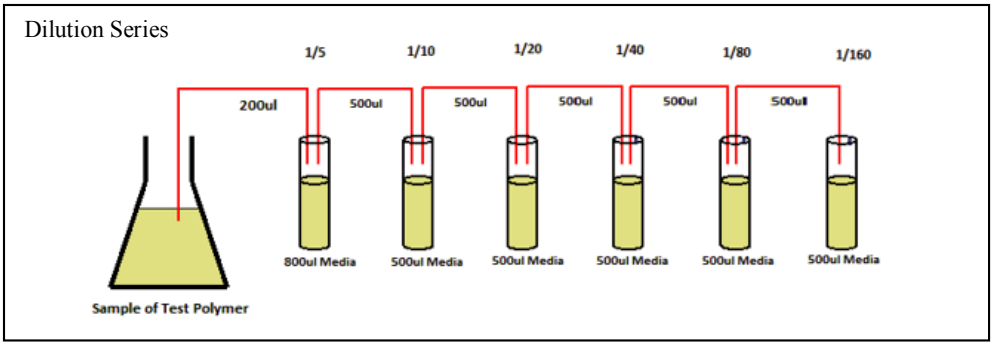


***Figure 8*. Dilution Series of PHA Nanoparticles in Cell Culture Media.** The volume labels beneath the graphics depict initial microcentrifuge tube contents and volume labels within the red lines show the movement of the PHA nanoparticles and nanoparticle/media mixture through the dilution. The result is 6 tubes containing an exact fraction of the initial nanoparticle. For each tube, the concentration compared to the original nanoparticle solution respectively is **(1)** 1/5 **(2)** 1/10 **(3)** 1/20 **(4)** 1/40 **(5)** 1/80 **(6)** 1/160

The dilution series was added in 200 µL aliquots to each respectively numbered experimental well of the 96-well plate. The 7th well of each row is the control well and media was replaced with 200 µL of normal culture media. The 8th well was designated as a blank well and remained on touched as this point. The cells were then incubated again for 24 hours at 37^{o}C. After 24 hours, the media and nanoparticles within each well were removed and replaced with 100 µL of media and 10 µL of CCK-8 reagent from Dojindo's Cell Counting Kit-8. After incubating once more for 1 hour, absorbance values were obtained using a plate reader and a wavelength of 450 nm.

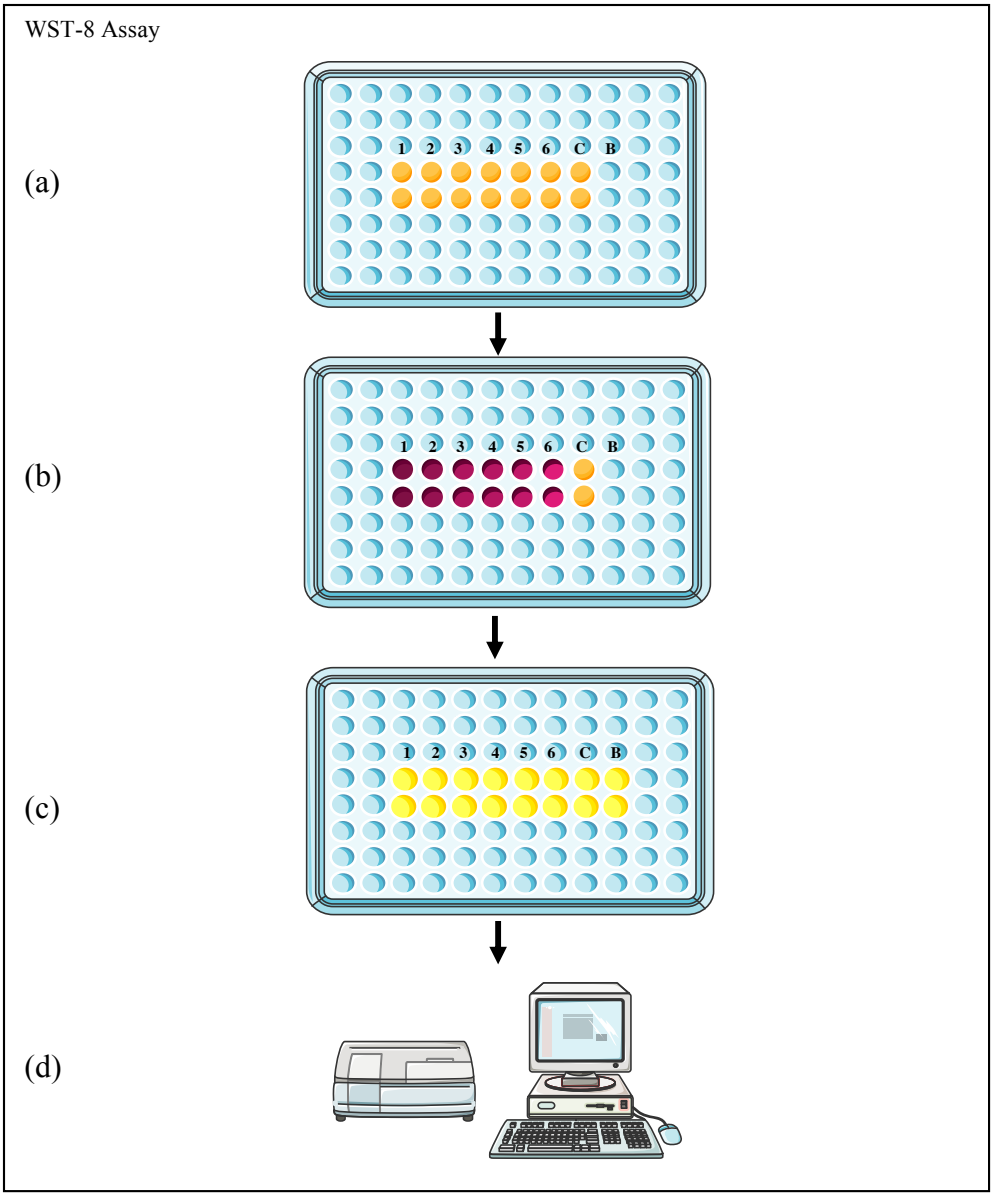


***Figure 9*. WST-8 Assay for PHA Nanoparticles and Cancer Cells.** (a) Cells cultured in two rows of 7 wells each and incubated for 24 hours at 37°C (b) Dilution series added to experimental wells and media replaced in control well (c) 10 µL of CCK-8 reagent and 100 µL culture media added to each well including blank well. Incubate 1 hour at 37°C (d) Obtain absorbance values using plate reader at 450 nm wavelength

Chapter 3: Results

The polymer, poly(3-hydroxy-octanoate-co-10-undecanoate) (PHOU), was shown to be nontoxic to the A549 cell line through the indirect contact assay. Live and dead cells are indicated by green and red fluorescence, respectively. This is as a result of the components used to develop the Live/Dead stain. Calcein AM, for example, is well retained in live cells, and produces green fluorescence in those cells. Ethidium homodimer-1, however, associates with damaged cell membranes that are present in dead cells and fluoresces with the nucleic acids that are no longer protected by the damaged cell membranes. As a result of these dyes and their associated reactions, only live cells can be depicted as green and only dead cells can show red. As seen in figure 10, lung cancer cells of the A549 cell line fluoresce green and no red fluorescence is observed, signifying a lack of cytotoxicity through indirect contact.

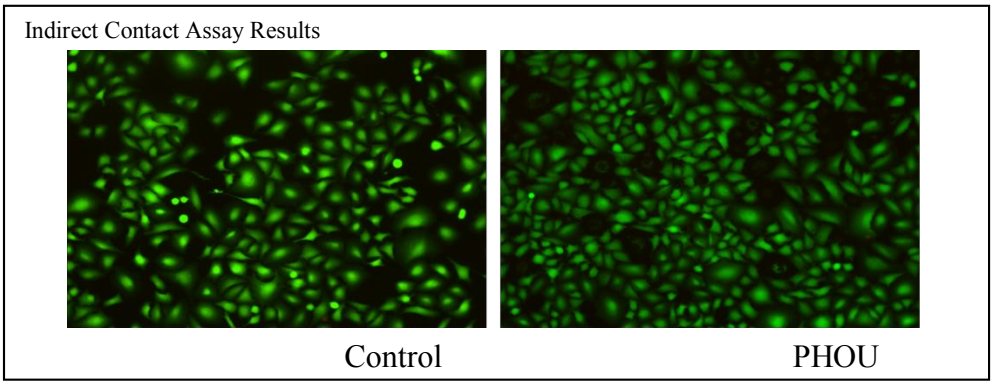


***Figure 10*. Live/Dead Assay for PHOU and A549 lung carcinoma cells.** Indirect contact assays were conducted with live/dead staining and fluorescence microscopy was used to show that elution tests did not result in any changes in cellular appearance or proliferation after incubation for 24 hours at 37°C.

PHOU was shown to result in cell death for the A549 cell line through the direct contact assay. Since the stain used for this assay is the same as the indirect contact assay, resulting pictures from fluorescence microscopy can be analyzed in

the same manner. As seen in figure 11, lung cancer cells of the A549 cell line fluoresce red with very little green fluorescence present, signifying severe cell death.

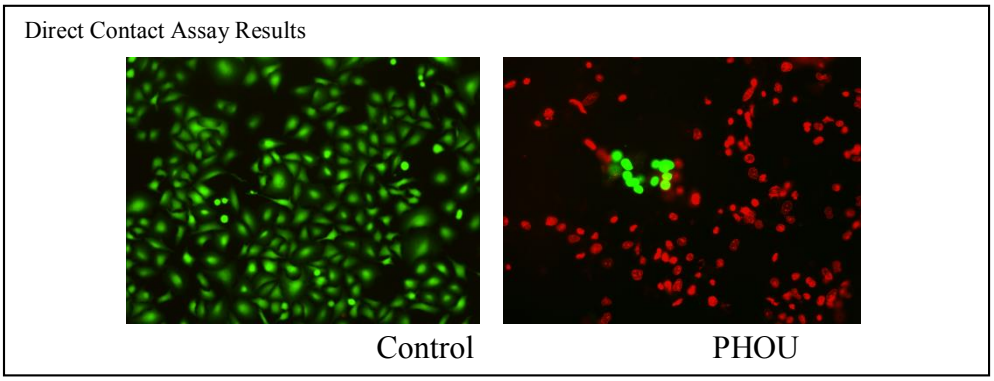


Figure 11. Direct Contact Assay for PHOU and A549 Lung Carcinoma Cells. Cells were cultured directly on PHOU. Direct contact assays were conducted with Live-Dead staining. Fluorescent microscopy was used to show severe death after incubation at 37°C for 24 hours.

Nanoparticles were characterized by their size and zeta potential using a Zetasizer Nano. The Zetasizer Nano uses dynamic light scattering to determine particle size. By shining a monochromatic light source through a clear solution of dissolved particles, the resulting light intensity patterns are captured by a photoreceptor and can be correlated to particle size which is calculated and displayed along with the size profile of the particle by a computer and accompanying software. The Zetasizer Nano measures the Brownian motion of the particles through dynamic light scattering and uses the Stokes-Einstein equation to relate the motion of the particles to particle size. For nanoparticles to be suitable for drug delivery applications, they must ideally must be 100 nm in diameter (size) and have an absolute zeta potential value greater than 30 mV (real zeta potential value >30mV or <-30 mV). Nanoparticles were successfully

developed using the nanoprecipitation method, but consistent sizes and ideal zeta potential values were unable to be achieved as shown in table 1.

PHOU Nanoparticle Characterization: Size and Zeta Potential

PHOU (mg)	Acetone (mL)	Pluronic F-68 (wt%)	PBS (mL)	Water (mL)	Average Size (nm)	Zeta Potential (mV)	PDI
25.0	5.0	4.0	15.0	0.0	248.70	-15.30	0.2523
9.0	3.0	4.0	10.0	0.0	186.75	-1.83	0.2495
9.0	3.0	5.0	10.0	0.0	175.40	-19.00	0.2510
9.0	3.0	10.0	10.0	0.0	256.65	-25.50	0.0870
15.0	3.0	10.0	10.0	0.0	306.40	-4.560	0.1050
9.0	3.0	1.0	0.0	10.0	143.39	-19.23	0.0287
9.0	3.0	10.0	0.0	10.0	194.27	-12.82	0.0295
9.0	9.0	0.1	0.0	10.0	90.51	-10.17	0.1022
9.0	9.0	0.5	0.0	10.0	46.43	-7.53	0.4353
9.0	9.0	1.0	0.0	10.0	142.02	-19.77	0.0317
9.0	9.0	10.0	0.0	10.0	109.30	-11.90	0.0560

***Table 1*. Size and Zeta Potential Measurements for PHOU Nanoparticles Developed Through Nanoprecipitation.** After a variety of attempts at nanoparticle development through nanoprecipitation with size and zeta potential ideal for drug delivery (~100 nm; >30 mV or < -30mV), consistent and appropriately sized and charged nanoparticles were not achieved.

Atomic Force Microscopy (AFM) was used to confirm the presence of PHOU nanoparticles and verify the relative size (Figure 12). A small glass slide the size of a penny was prepared for AFM by placing a 10 μL drop of PHOU nanoparticle solution on the slide and allowing the water to evaporate, leaving PHOU nanoparticles dried on the slide. The nanoparticles were observed using an atomic force microscope which develops a picture of the material on the AFM slide through the force response of a needle interacting with the test material. A scale can be used to associate the diameter of the particles in the pictures to the real particle size.

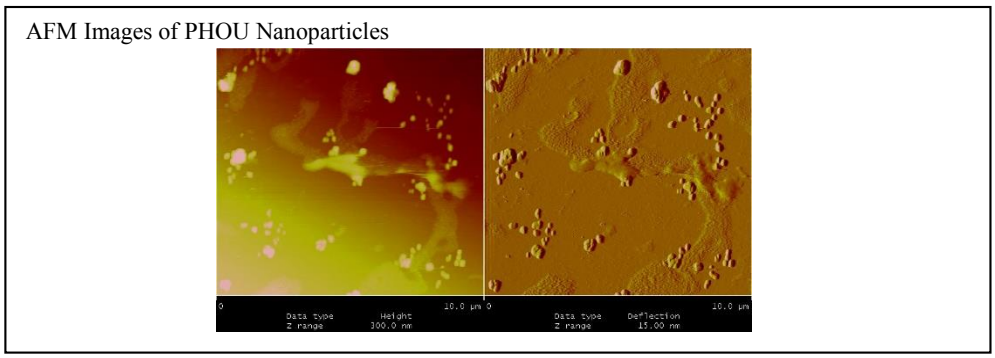


***Figure 12*. Atomic Force Microscopy Images of PHOU Nanoparticle Developed Through Nanoprecipitation.** The presence of PHOU nanoparticles was confirmed and relative size verified by AFM.

Scanning Electron Microscopy was also used to observe the shape and confirm the presence of PHOU nanoparticles. PHOU nanoparticles were freeze-dried and prepared on SEM sample holders and then observed using a scanning electron microscope. As shown in figure 13, SEM images of PHOU nanoparticles confirm inconsistent nanoparticle development.

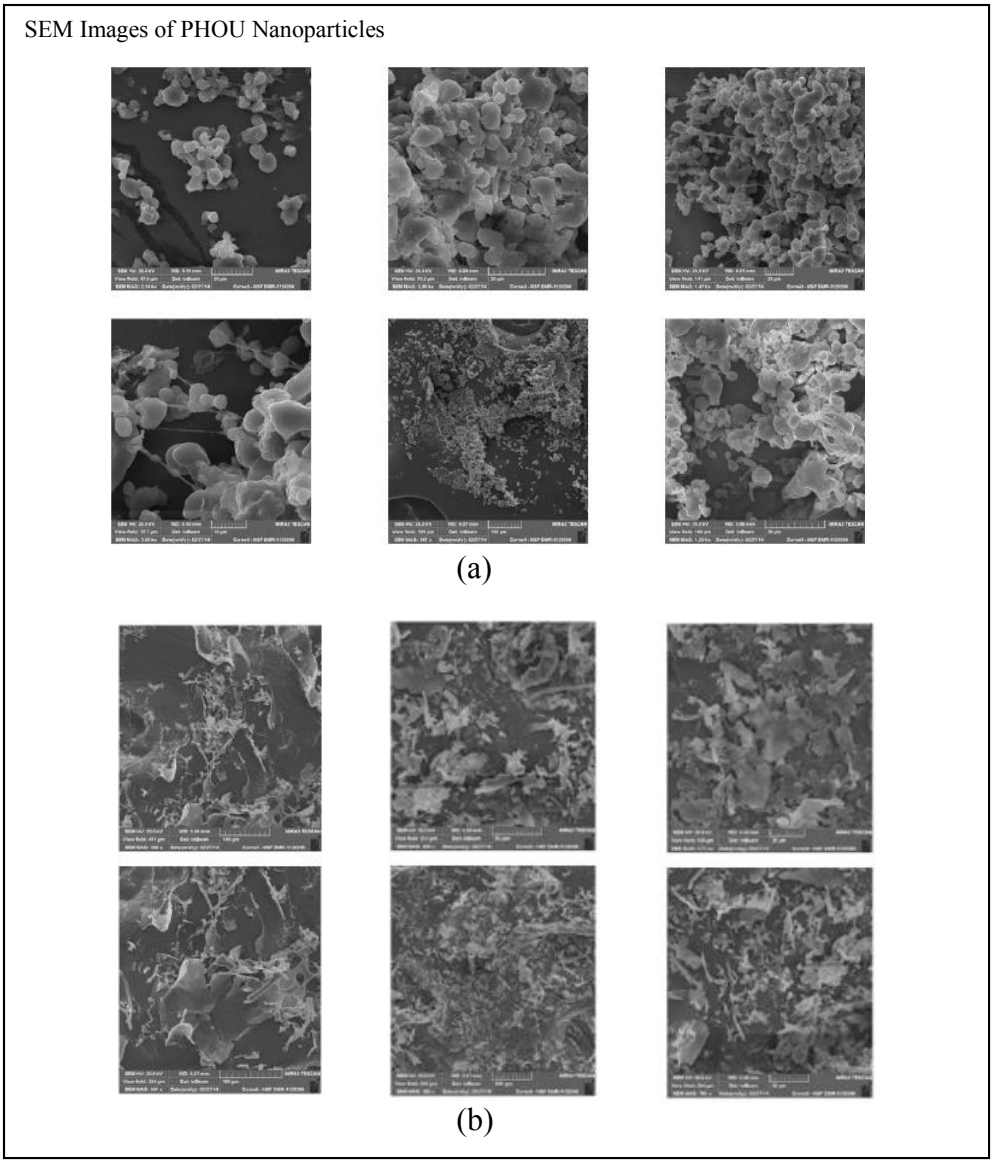


***Figure 13*. Scanning Electron Microscope Images of PHOU Nanoparticles Developed Through Nanoprecipitation.** SEM confirms inconsistent nanoparticle development with PHOU nanoparticles developed through nanoprecipitation. (a) The spherical shape of the nanoparticles shows nanoparticle development however agglomeration suggests the freeze-drying procedure could be improved. (b) The unrecognizable shape suggests that the formation cannot sustain its shape and therefore is not ideal for drug delivery.

After PHOU nanoparticles developed through nanoprecipitation proved to be too inconsistent for drug delivery applications, a new method was used called miniemulsion that successfully achieved consistent nanoparticle development with nanoparticles of appropriate size and zeta potential for drug delivery. PHA nanoparticles developed using miniemulsions used the PHA polymer, PHO-N3,

an azide-terminated PHA. These nanoparticles were measured using a Zetasizer Nano in the same manner as the previous nanoparticles. As shown in Table 2, PHO-N3 nanoparticles developed through miniemulsions achieved sizes and zeta potentials ideal for drug delivery.

PHO-N3 Nanoparticle Characterization: Size and Zeta Potential

PHO-N3 (mg)	Chloroform (mL)	Aqueous SDS (mL) [4.44 mg/mL]	Average Size (nm)	Average Zeta Potential (mV)	PDI
60.0	1.0	2.73	103.4	-79.53	0.0477

***Table 2*. Size and Zeta Potential Measurements for PHO-N3 Nanoparticles Developed Through Miniemulsions.** Consistent PHO-N3 nanoparticles of appropriate size and zeta potential for drug delivery were achieved and measured using a Zetasizer Nano.

Functional group-modified PHA nanoparticles were developed using a several different functional groups through miniemulsion with low polydispersity and appropriate size for drug delivery applications (table 3). Although it's not shown, zeta potential for each functionalized nanoparticle was found to be around -70 mV which is consistent with the charge of the SDS incorporated in the nanoparticle solution.

Functionalized PHA Nanoparticle Characteristics

Polymer	Functional Group	Size (nm)	PDI
PHO	Alkene	88.4	0.09
	Alkyne	136.8	0.09
	Azide:Alkyne (1:1)	133.5	0.016

***Table 3*. Size and Zeta Potential of Functionalized PHA Nanoparticles.** Successful development of functionalized PHA nanoparticles was achieved with low polydispersity and suitable sizes for drug delivery applications.

As a demonstration of the ability to perform click reactions on functionalized nanoparticles, fluorescent tags were attached (figure 5) and cellular uptake was observed. Figure 14 depicts red, fluorescently tagged, alkyne modified PHA nanoparticles within breast cancer cells of the MT3 cell line.

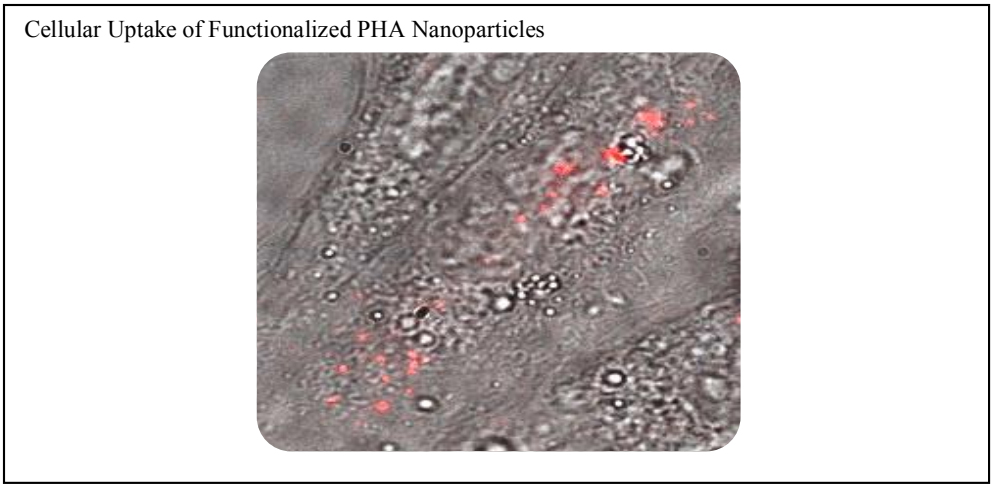
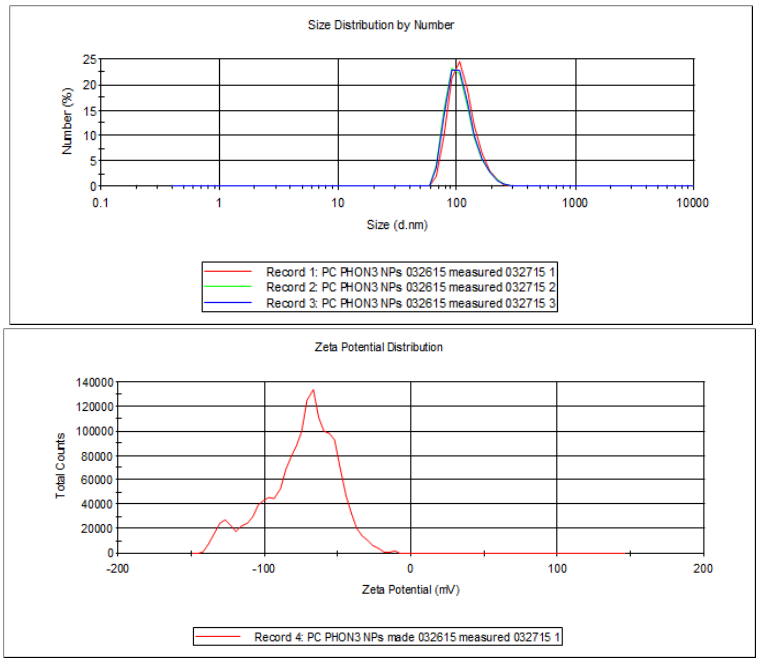


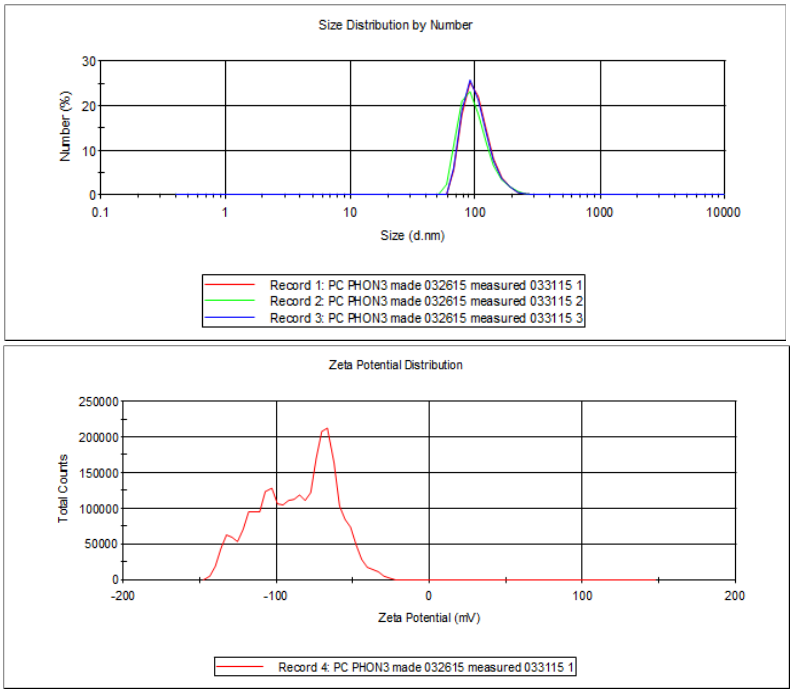
***Figure 14*. Cellular Uptake of Fluorescently Tagged PHA Nanoparticles by Breast Cancer cells.** Alkyne-modified PHA nanoparticles, fluorescently-tagged through click chemistry were observed within breast cancer cells of the MT3 cell line using fluorescence microscopy

Nanoparticle measurements were taken again 3 days after initial nanoparticle development to assess stability. Zetasizer Nano results show comparable results for size and zeta potential for both periods (figure 15).

Size and Zeta Potential: 24 and 72 Hours



(a)



(b)

***Figure 15*. Size and Zeta Potential of PHO-N3 Nanoparticles at 24 and 72 hours.** Size and zeta potential remain consistent (a) Size and zeta potential at 24 hours (b) size and zeta potential at 72 hours

After successful nanoparticle development through miniemulsions, the cytotoxicity of the functionalized nanoparticles needed to be established. Initial WST-8 assays resulted in an IC50 curve (figure 16) that allowed us to determine the concentration at which 50% of cell death is observed, or the IC50 concentration. For PHO-N3 nanoparticles, the IC50 concentration value was found to be 2.6815 mg/mL. From additional WST-8 assay results, no cytotoxicity of was shown for PHO-N3 nanoparticles toward the MDA-MB-231 breast cancer cell line despite increasing concentration (figure 17).

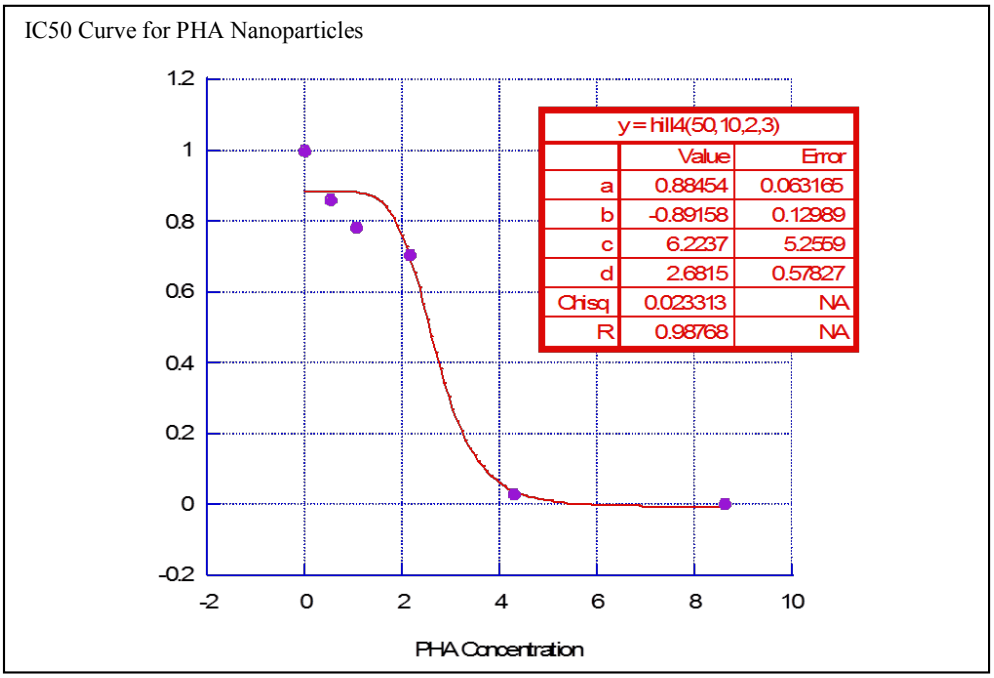


***Figure 16*. IC50 Curve for PHO-N3 Nanoparticles and MDA-MB-231 Breast Cancer Cells.** Based on the absorbance values from a WST-8 Assay assessing the cytotoxicity of PHO-N3 nanoparticles and MDA-MB-231 cells, an IC50 curve was established and an IC50 value of 2.6815 mg/mL was obtained. IC50 represents the concentration at which 50% of cell death occurs. On y axis, 1 represents no cytotoxicity and 0 represents total cell death. Values above 0.8 are considered nontoxic.

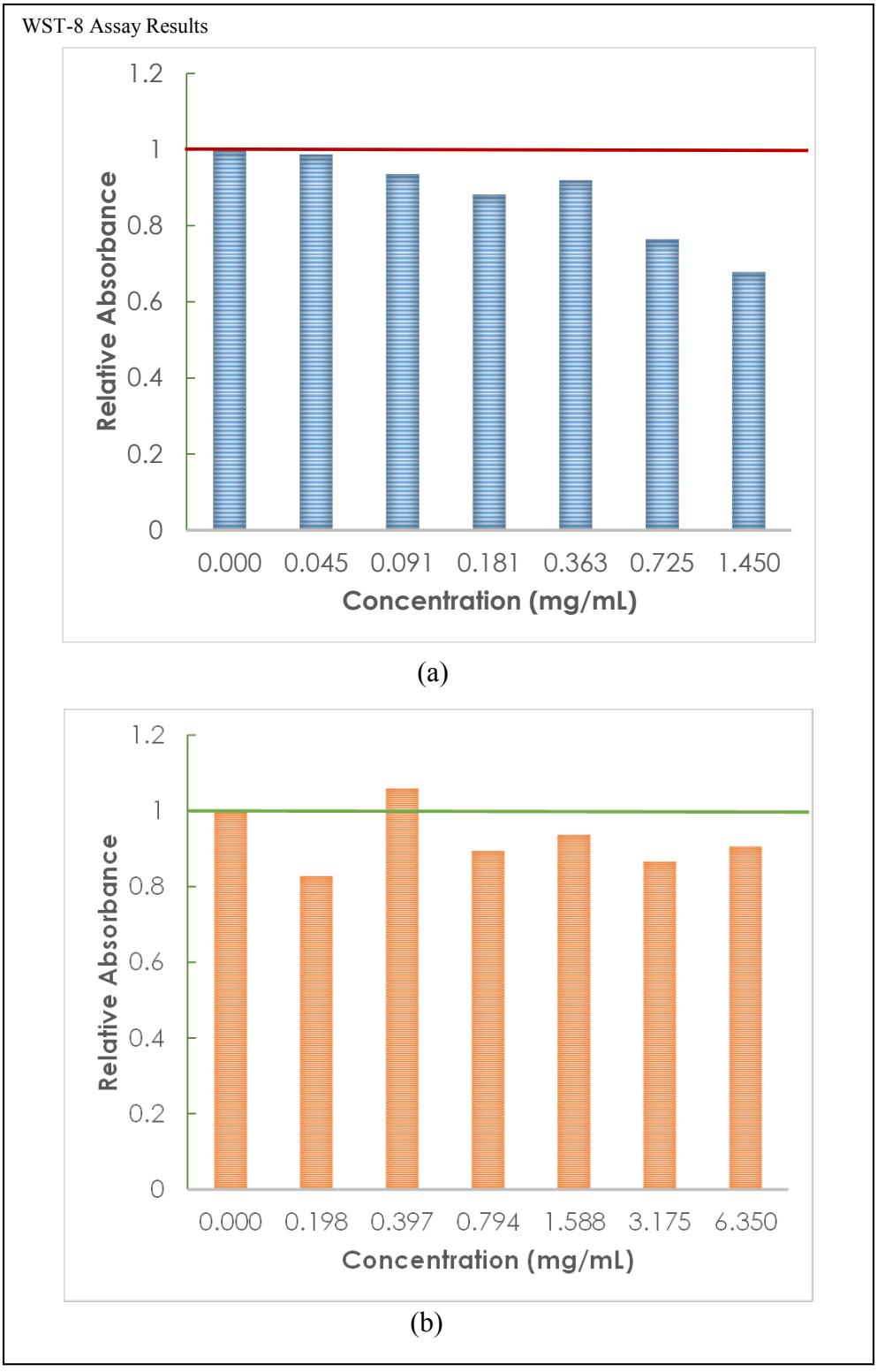


***Figure 17*. WST-8 Assay Results for PHO-N3 Nanoparticles and MDA-MB-231 Cell Line.** PHO-N3 nanoparticles have shown no cytotoxicity (absorbance < 0.8) with increasing concentration compared to widely-accepted PLGA nanoparticles.

Chapter 4: Discussion

While cells of the A549 cell line experienced no cytotoxicity through the indirect contact assay, cell death was observed when the cells were cultured directly on top of the PHOU polymer. This is believed to be as a result of the presence of bacterial microbes present within the folds of the polymer which protected the microbes from removal when the polymer was purified before the assay. The presence of toxic components within the polymer as well as cross-linking within the polymer structure and in general a lack of consistency among results for PHOU nanoparticles prompted nanoparticle development using another PHA, polyhydroxyoctanoate (PHO).

Although the material itself is partially responsible for the inconsistent nanoparticle development, the process of nanoprecipitation itself was found to be an inconsistent method altogether. Through nanoprecipitation, a wide range of sizes were produced even among nanoparticles developed identically. Zeta potential values were inconsistent and did not fall within the suitable range for drug delivery applications. The miniemulsion process, however, was not only an effective method for consistent nanoparticle development that produced appropriately sized and charged particles for drug delivery, but also a more efficient method.

The miniemulsion process requires a max development time of 4 hours from preparation through chloroform removal compared to the 28 plus hours required for nanoprecipitation. Steps for nanoprecipitation, such as centrifugation was the primary inconsistent element of the nanoparticle development process

since high speeds were required to separate out the nanoparticles. Often, the resulting nanoparticle pellet would quickly begin dissolving back into the supernatant after centrifugation, resulting in loss and ocacasionally negating the centrifugation step altogether. Monodisperse chloroform droplets containing dissolved PHA, separated from water by SDS are readily made in the miniemulsion process due to the kinetic behavior of the reagents involved and then a high shear energy source (in this case ultrasonication) is used to complete the emulsion and form the nanoparticles. At this point the nanoparticle solution can be purified simply by boiling out the chloroform. The result was a more consistent, ideally characterized nanoparticle carrier system than that developed by nanoprecipitation.

With regards to the cellular uptake of the fluorescently-tagged PHA nanoparticles, initial challenges were presented as a result of the presence of folates in the culture media used for the cells. Since breast cancer cells overexpress folate receptors, folate groups are an ideal targeting group for nanoparticle carrying drugs intended to treat breast cancer. However due to the presence of folates in the fetal bovine serum component of the culture medium, the presence of folate-functionalized nanoparticles within the breast cancer cells was not as pronounced.

The WST-8 assays provided some challenges as well. Initially, the assay was conducted successfully resulting in an IC50 curve and the establishment of an IC50 value (figure 16). Following attemps, however, resulted in unusable results in which absorbance values for experimental wells were found to be greater than

that of the control well. One potential cause of the unusual absorbance values found for experimental wells was the presence of nanoparticles within the wells during analysis with a plate reader. The media containing the test material for WST-8 assays is supposed to be removed before the addition of the CCK-8 reagent, however wells were inadequently rinsed, resulting in the presence of nanoparticles within the wells still during plate reading. The presence of nanoparticles skewed the absorbance values determined by the plate reader implying that cell proliferation was greater for experimental wells than in the control wells. Additional assays were performed with adequate removal of PHA nanoparticles and usuable result were obtained again that displayed no cytotoxicity (figure 17). Normally, azide would be toxic to cells but by conjugating the azide to form PHO-N3, cytotoxicity is eliminated.

Chapter 5: Conclusion and Future Work

Consistent development of PHA nanoparticles with size and zeta potential suitable for drug delivery was achieved. Despite initial concerns regarding PHA cytotoxicity, functionalized PHA nanoparticles were found to display no cytotoxicity to MDA-MB-231 breast cancer cells. Highly efficient click chemistry reactions were used to successfully attach functional groups to PHA nanoparticles. Through click chemistry, fluorescent tags were attached to alkyne-modified PHA nanoparticles and fluorescence microscopy was used to observe cellular uptake of these functionalized nanoparticles by the MT3 breast cancer cell line. While further work is needed to assess the drug-loading efficiency, stability, perform differential scanning calorimetry, and further establish PHA nanoparticle cytotoxicity, this study has shown that functionalized PHA nanoparticles with applications in targeted drug delivery can be developed.

Further work must be done to assess the stability of the PHA nanoparticles. By taking size and zeta potential measurements of PHA nanoparticles daily for a week and then once a week for a 3 months, size and zeta potential stability can be assessed. Thermal stability and material characteristics of the PHA nanoparticles can be established through differential scanning calorimetry. A thermal analyzer called a differential scanning calorimeter is used to determine material properties such as glass transition temperature and melting point. By knowing the thermal properties of the PHA nanoparticles, determinations can be made regarding the stability of the nanoparticles under a

variety of conditions. The most of relevant of which being the biological conditions found present in the human body.

Anti-cancer drugs such as PTX can be loaded within the PHA nanoparticles by dissolving the drug simulataneously with the polymer in chloroform when developing the nanoparticles through miniemulsions. The amount of drug left in the supernatant liquid remaining after the nanoparticles are developed will be determined using high performance liquid chromatography. Using this value, the loading capacity (mass of PTX/mass of nanoparticle) and loading efficiency (percentage of PTX loaded into nanoparticle/amount added to polymer solution) can be calculated.

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