Senior Honors Thesis:
Nanoparticle Drug Delivery of Antitumor Agents

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Abstract

My research in the lab of Dr. Raoul Kopelman has focused on the synthesis, preparation, characterization and *in vitro* testing of polyacrylamide (PAA) nanoparticles. The Kopelman lab has made both positively charged and negatively charged drug-loaded nanoparticles for chemotherapy.

Chemotherapy drug loaded nanoparticles have several intrinsic properties that aid in their effectiveness. First is a targeting ability known as enhanced permeability and retention (EPR), which enables the nanoparticles to be delivered and remain at a cancer tumor site with selectively, compared to other areas of the body. Second, the chemotherapy drug release rate for polyacrylamide nanoparticles is well suited for a biological system – not too fast as to cause release while particles are still in the circulatory system, but fast enough for cytotoxicity after contact and entry into cancer cells.

There are several distinct advantages in using an acrylamide based matrix for the synthesis of drug delivery nanoparticles. First is the biocompatibility and lack of toxicity of the nanoparticles, allowing for a reduction of chemotherapy drug side effects and an increase in the maximum tolerated dose of the drug. Second is the great versatility in engineering these nanoparticles. Nanoparticles are highly tunable, allowing for customizability in factors such as size, charge, surface characteristics, loaded drug concentrations, and drug release rates.

While the size of nanoparticles used in our studies are typically on the scale of 30 to 100 nm, the possible size range for these particles is far broader. We have found that post-

synthesis loading of chemotherapy drugs results in much higher loaded drug concentrations within the nanoparticles than when drugs are incorporated during synthesis. The optimal concentration for post-loading of cisplatin was found to be 2 mg/mL, incubated for 4 hours at 90^{o}C in an aqueous solution. Binding studies have confirmed that hyaluronic acid (HA) conjugated nanoparticles results in greater binding specificity between particles and CD44 expressing tumor cell lines. The cytotoxicity of nanoparticles was determined via an MTT assay, showing a low degree of toxicity in the case of blank particles and higher toxicity in the case of cisplatin loaded particles. The drug release rates of our nanoparticles were measured using release studies and MTT assays, confirming that a polyacrylamide based particle matrix has a loaded drug release period of over 1 week. This drug release profile demonstrates the advantage of a polyacrylamide matrix for drug delivery, showing a longer and more stable release of loaded drugs than nanoparticle drug formulations currently on the market.

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Introduction

At well over 500,000 lives lost per year, cancer is the second most common cause of death in the United States[1]. Most patients diagnosed with cancer undergo chemotherapy treatment; however, some cancer cells are known for their high resistance against chemotherapy drugs, so called multi-drug resistance (MDR)[2,3]. MDR often leads to relapse in cancer patients that have undergone chemotherapy treatment[4]. In addition to the complications associated with MDR, chemotherapy treatments lead to mild to severe side-effects in over 80% of patients, including nausea, hair loss, fatigue, increased risk of infection and anemia[5,6]. These side effects limit the dosages at which chemotherapy drugs may be used – a higher dose would more effectively kill chemotherapy drug-resistant cancer cells, but at the cost of greater unintended damages to the body. A strategy to overcome this dose limiting issue is localized high-concentration delivery of chemotherapy drugs to sites of cancerous growth. The proposed method of localized delivery that originated and is being studied in the Kopelman lab involves the use of drug loaded polyacrylamide based nanoparticles as a targeting and delivery system.

Current methods of cancer treatment involve an intrusive combination of chemotherapy and surgery. The degree of effectiveness of chemotherapy is directly related to how efficiently and effectively the treatment is able to destroy tumor cells while leaving healthy cells unharmed. Current methods of chemotherapy often fall short in terms of 1) being able to selectively target tumor cells without harming healthy cells, and 2) being able to effectively kill tumor cells without relapse. While newer, more effective drugs are beginning to overcome

these shortcomings, especially the second, and are thus able to combat tumor cells with greater efficiency and efficacy, the field of nanoparticle drug delivery provides further opportunities for the improved oncology, through delivery of both old and new chemotherapy agents[7].

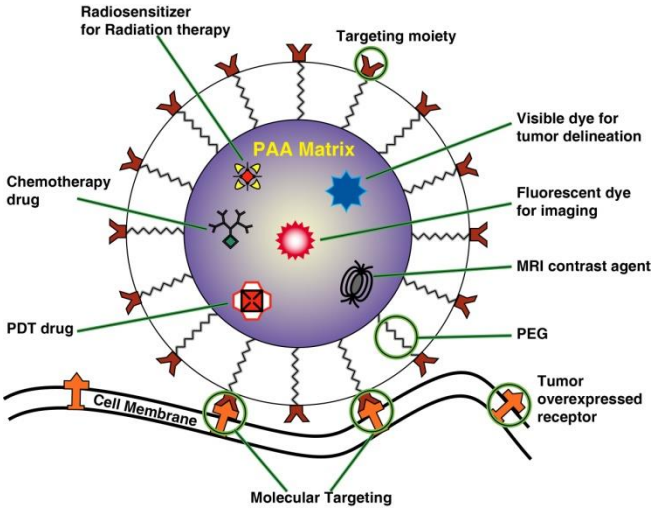

Figure 1: Schematic diagram of a molecularly targeted, multifunctional PAA NP with the many options applied so far for cancer detection and therapy[8].

In the 1950's and 1960's, the field of pharmaceuticals grew tremendously. As a result, drug delivery and controlled release quickly became a topic of interest. In 1969, Dr. Peter Paul Speiser, a Swiss researcher working at the Swiss Federal Institute of Technology, worked to develop drug delivery systems, including polyacrylic acid beads, microcapsules, and eventually the first nanoparticle for drug delivery[9,10]. Speiser first tested the retarded release properties of nanoparticles through delivery of vaccines that generally required multiple injections in order to build up a high enough level of antibodies. He hoped to show that the slow release rate properties of nanoparticles would require only a single injection in place of many injections

of the pure compound. In his lab was developed the technique of micelle polymerization, where surfactant is added in high concentrations to an aqueous phase, allowing a micelle environment in which monomers can polymerize. This same method is still used to this day for nanoparticle production[9,11].

In the late 1970's and 1980's, nanoparticle research began its focus on cancer therapy, with studies showing higher chemotherapy drug delivery efficiency with lower toxicities. In 1983, Grislain *et al.* demonstrated rapid clearing of 200 nm diameter nanoparticles from the blood stream following intravenous injection, with progressive accumulation in the primary tumor location of a Lewis Lung Carcinoma and in lung metastases[12]. This same effect was later observed and named in 1989 by Maeda and Matsumura as the enhanced permeability and retention (EPR) effect[2,13].

Due to the rapidly dividing nature of tumor cells, the nutrient diffusion-limiting size of a tumor is ~2 mm^3. Past this size, the tumor must recruit the formation of new vasculature in order to deliver adequate levels of nutrients for further growth. This often results in the formation of defective, or "leaky," vasculature surrounding the tumor due to the rapid vascularization needed to support tumor growth. These vasculature abnormalities result in extensive leakage of blood plasma components, including nanoparticles, into the tumor tissue. In addition, tumor vascularization results in poor lymphatic drainage. These two factors result in what is known as the EPR effect[7,14].

Through the EPR effect, it is possible to achieve concentrations of polymeric drugs 10-50 folds higher than those found in normal tissue within several days of delivery. While low

molecular weight drugs are not affected by the EPR effect due to their high rate of diffusion, the large molecular weight of drug loaded nanoparticles can be effectively used to exploit the EPR effect[14].

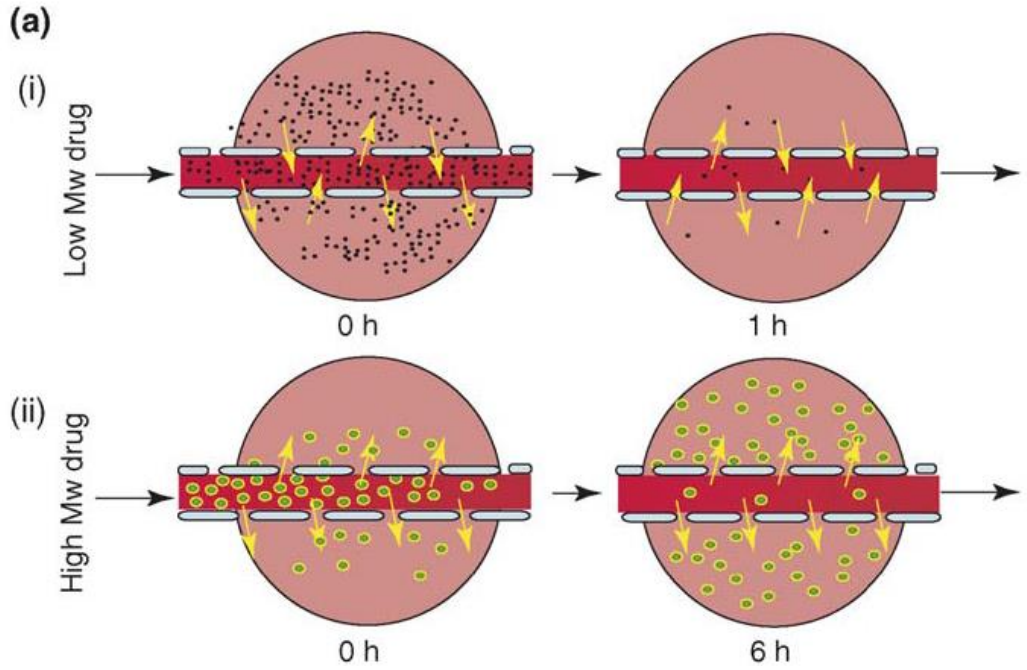

Figure 2: (a) Shows the diffusion of a low molecular weight (Mw) drug (black spots) in (i) and a high Mw drug (green spots with yellow circles) in (ii), from the blood vessels into the interstitium of tumor tissue (large pink circles). Note that low Mw drugs can diffuse freely in and out of the tumor blood vessels because of their small size and, hence, the effective concentration of the drug in the tumor diminishes after 1 h when the drug concentration in plasma becomes low (a; i), whereas the high Mw drug cannot easily diffuse back into the blood stream because of its large size. Thus, there is progressive accumulation of macromolecular drug in the tumor tissues with time by the enhanced permeability and retention (EPR) effect (a; ii)[14].

In 2005, Abraxane became the first Food and Drug Administration (FDA) approved nanoparticle to enter the commercial market. Abraxane is a human serum albumin based nanoparticle used for the delivery of paclitaxel for the treatment of various forms of cancer.

Abraxane has shown great promise for the clinical application of nanoparticle drug delivery, performing very well in comparison to its free drug counterpart. In comparison to patients that underwent chemotherapy, a recent Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT) study showed a 59% increase in the 1 year survival and doubling of the 2 year survival in pancreatic cancer patients who were treated with a combination of Abraxane and traditional chemotherapy[15].

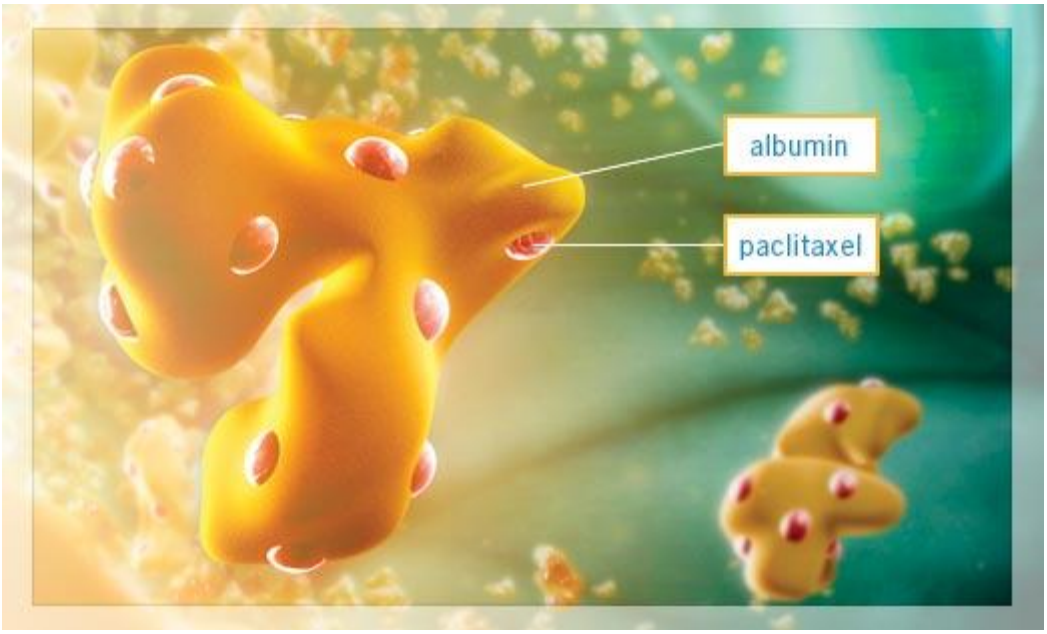

Figure 3: Albumin-bound paclitaxel. (Abraxane.pcdem)

With the current rise in clinical usage of nanomedicines, we can expect a continued growth and emphasis of nanoparticle drug delivery. The research goal of the Kopelman lab is to approach the field of nanoparticle drug delivery of chemotherapy agents from the perspective of acrylamide based particles. The matrix of the nanoparticles in the Kopelman group consists

of an acrylamide primary monomer. There are a several key advantages in using an acrylamide based matrix that makes it promising for chemotherapy drug delivery.

A primary advantage for the use of a polyacrylamide matrix is the low toxicity of acrylamide polymers, allowing for a high degree of biocompatibility. Although acrylamide in its monomeric form is a neurotoxin and carcinogen, polyacrylamide is not toxic. In addition, polyacrylamide in the presence of a cross-linker is very stable and can easily withstand physiologically relevant conditions without risk of degradation into monomers[4,16,17].

A second key advantage of an acrylamide matrix is its high engineerability. Due to the synthetic nature of these particles, their size can be tuned within a very broad range, from below the tens of nanometers in diameter upward. In addition to size, the charge of the nanoparticle can also be controlled by the composition of the matrix. In the Kopelman lab, two main types of particles are most commonly synthesized: positively charged nanoparticles containing N-(3-Aminopropyl)methacrylamide (APMA) as the secondary monomer and negatively charged nanoparticles containing acrylic acid (AA) as the secondary monomer.

In addition to size and charge, the surface characteristics of these particles can be adjusted as well, conjugating various ligands to affect the physical and chemical characteristics of the nanoparticle. Nanoparticle surface conjugation is performed for various reasons, depending on the ligand being used. Hyaluronic acid (HA) is a ligand for the CD44 cell surface antigen, and is conjugated onto the surface of nanoparticles for increasing the selectivity of cell uptake via a mechanism known as receptor-mediated endocytosis. While targeting ligands

such as HA do not increase the localization of nanoparticles to the tumor site due to the EPR effect, it does increase the internalization of the nanoparticle[18].

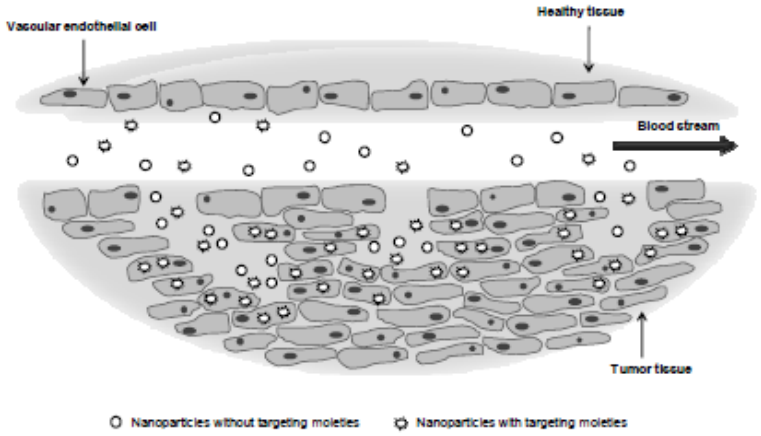

Figure 4: Targeting strategies for cancer therapy. Passive targeting can be achieved by enhanced permeation and retention, an effect involving leaky vascular structures. Active targeting mediated by targeting ligands specifically localizes drug carriers at desired cells or tissues. The decoration of the nanoparticles with ligands improves its internalization by endocytosis[18].

HA is used to target the CD44 cell-surface glycoprotein, which is overly expressed in many cancer cell lines, including the Hep3B and SKOV3 ovarian tumor cell lines[19,20,21]. HA conjugation results in an overall increase in negative charge on the nanoparticle surface, causing electrostatic repulsion of the particle from the cell surface and a slower rate of internalization in comparison to positively charged particles. However, due to the CD44 antigen's strong affinity for binding HA, surface conjugation of HA onto nanoparticle results in an increase in the specificity of binding to those cells that overly express CD44. This is an

example of specific binding interactions between the nanoparticle and the targeted cell, which also overcomes the global charge repulsion.

In contrast to the negatively charged HA ligand, another commonly used ligand for assisting nanoparticle conjugation is the neutrally-charged polyethylene glycol (PEG). The development of covalently attached PEG chains onto the surface of nanoparticles began in the 1980's and 1990's. Because nanoparticles are rapidly removed by macrophages in the reticuloendothelial system, their circulation time in the body is relatively short. Attachment of PEG onto the surface of nanoparticles, however, greatly reduces the rate by which they are eliminated in the body by neutralization of nanoparticle surface charge, creating a hydrophilic outer shell and thus reducing the particle surface charge interactions with the cellular membrane. This charge neutralization, along with steric repulsions from the large chain lengths, make PEG an effective "stealth agent" when conjugated onto the surface of nanoparticles[9,22]. This is useful for applications where particle-cell interactions are unwanted, such as when particle clearance via the reticular-endothelial system needs to be minimized[23]. For example, PEG coated Fe_3O_4 nanoparticles has been shown to reduce nonspecific uptake of the particle by macrophage cells and was shown to produce negligible aggregation under cell-culturing conditions (figure 5)[24].

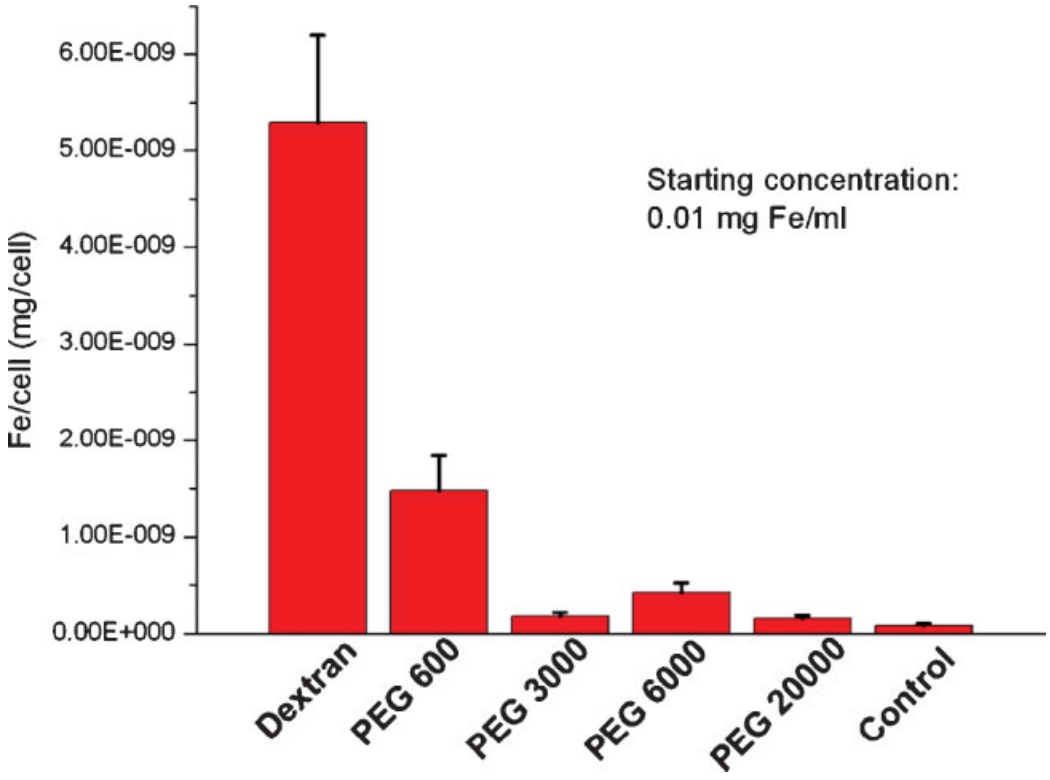

Figure 5: ICP-AES analysis of the result of the uptake of dopamine–PEG coated Fe_3O_4 nanoparticles by macrophage cells[24].

In addition to surface conjugation, acrylamide nanoparticles can be loaded with a broad range of molecules. Commonly loaded chemicals used in the Kopelman group are chemotherapy agents such as cisplatin and docetaxel. In addition, imaging agents may also be loaded or directly incorporated into the matrix of nanoparticles. For example, rhodamine containing nanoparticles are commonly used as a method of fluorescently tagging nanoparticles to study cell binding.

While a variety of small molecule drugs can be loaded into nanoparticles for delivery, cisplatin has been selected as a drug of choice in our studies for several reasons. First is its common usage in the treatment of various forms of cancer, including ovarian cancer, the key

focus of our cell studies. Second is that it is a small molecule which exhibits fast delivery characteristics. The dosage of the drug is limited by its toxicity to the kidneys and nervous system, as well as a variety of side effects, including nausea, hearing loss, electrolyte disturbance, and anemia[25]. The dose limiting aspect of cisplatin makes it a perfect candidate for nanoparticle drug delivery, which would not only allow for localized delivery of the drug to the tumor site but also a reduction of side effects as a result of the targeted delivery. Lastly, the platinum present in cisplatin allows for easy and accurate characterization of drug loaded nanoparticles via inductively coupled plasma – optical emission spectroscopy (ICP-OES).

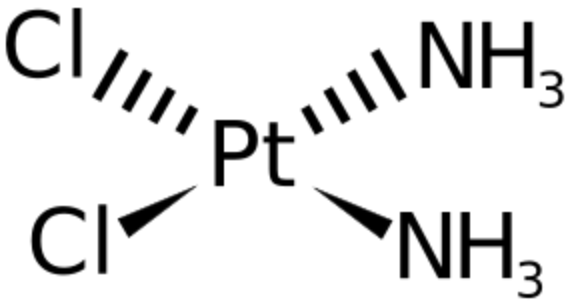

Figure 6: Chemical structure of cisplatin.

In addition to cisplatin, docetaxel is another common drug candidate for nanoparticle delivery. Mainly used for the treatment of ovarian, breast, prostate, and lung cancers, docetaxel interferes with cell mitosis[26,27,28]. Due to its mechanism of action, docetaxel affects cells in the body that have a high turnover rate, resulting in slow division of hair follicles, bone marrow, and other rapidly dividing cells in addition to tumor cells. For these reasons, localized nanoparticle delivery of docetaxel could avoid these germ cells, directing the drug to the tumor site.

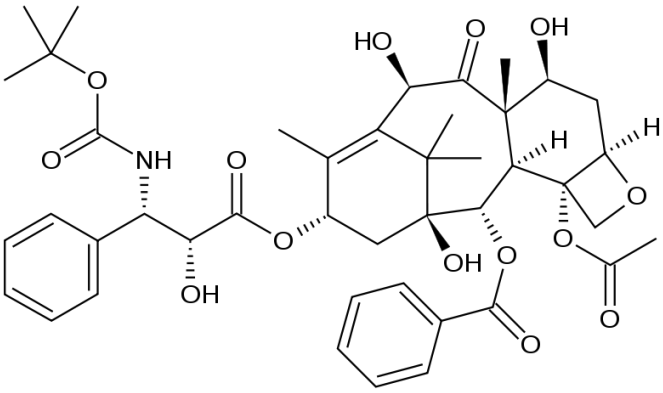

Figure 7: Chemical structure of docetaxel.

The rate at which these loaded chemicals are released is also highly tunable. The degree to which the nanoparticle is held together is largely determined by the ratio of cross-linkers to monomers. Fewer cross-linkers in the nanoparticle matrix result in a faster rate of release[4,16,17]. Nanoparticles contain cross-linkers that cleave under specific conditions, causing drug release. In the case of 3-acryloyloxy-2-hydroxy-propyl methacrylate (AHM) cross-linked nanoparticles, the cross-linker is cleaved by esterase. N,N'-Bis(acryloyl)cystamine (CBA) cross-linked nanoparticles are redox sensitive and cleave in the presence of a reducing agent such as glutathione (GSH) or dithiothreitol (DTT). Each molecule of AHM contains two ester bonds that can be readily cleaved by esterase while CBA contains a disulfide bond that is readily reduced to two thiol moieties (figure 8)[29,30].

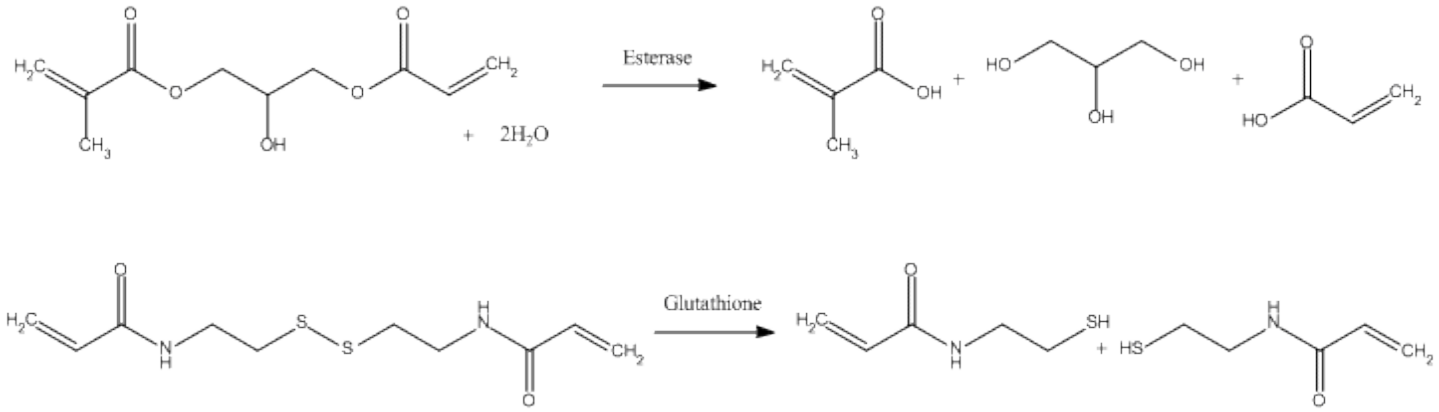

Figure 8: AHM hydrolysis via esterase (top) and CBA reduction via glutathione (bottom).

Following synthesis, several key analytical instruments are used for the characterization of our nanoparticles. Characterization of nanoparticles includes determination of size, surface charge, and total loaded drug content. These are determined via dynamic light scattering (DLS), electrophoretic light scattering (ELS), and ICP-OES, respectively.

The Beckman Coulter DelsaNano C is the primary instrument used for both particle sizing and surface charge determination. Particle sizing is accomplished using dynamic light scattering, measuring the rate of fluctuations in a laser that is passed through the sample. As light is passed through the sample, the photons are scattered in all directions via Rayleigh scattering. As the particles diffuse in the solution, the scattered photons constructively and destructively interfere with those from surrounding particles. The intensity of this scattering undergoes a time-dependent fluctuation due to the motion of the particles in the sample. Fluctuations at a higher frequency are due to smaller particles while lower frequency fluctuations are due to larger particles[31,32].

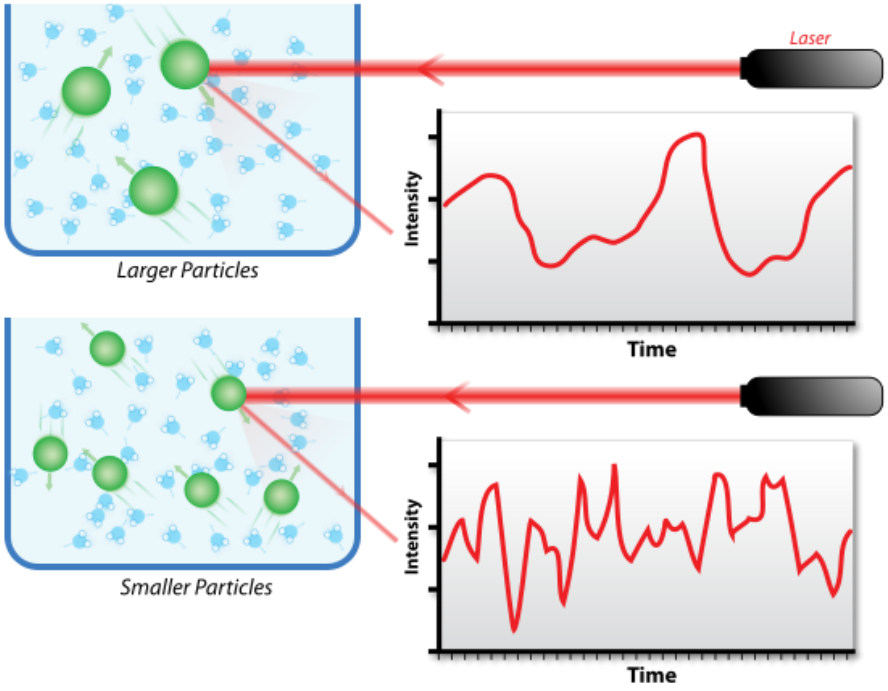

Figure 9: Hypothetical dynamic light scattering of two samples: Larger particles on the top and smaller particles on the bottom[31].

Surface charge is accomplished using electrophoretic light scattering (ELS) to calculate zeta potential. In ELS, the velocity of particles is determined in a similar fashion as in DLS, but under an applied electric field. As charged particles move through the sample solvent, a laser is passed through the sample. The frequency of the scattered light is detected, and the Doppler shift between the laser light and the scattered light is measured to determine the velocity of the particle. Since the velocity of the particle in an electric field is proportional to its charge, this can be used to calculate the zeta potential of the particle[32].

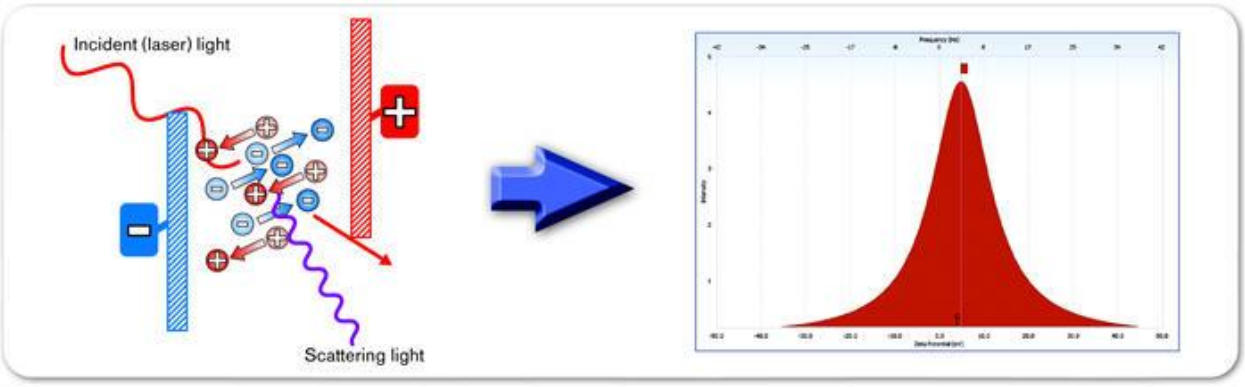

Figure 10: Schematic of Electrophoretic Light Scattering[32].

Total encapsulated cisplatin content is determined via ICP-OES, using an yttrium internal standard. Platinum concentrations are determined, corresponding to total cisplatin content of a nanoparticle. ICP-OES is an excellent method of detecting trace metals, such as platinum, owing to its low limit of detection and high degree of reproducibility. In ICP-OES, argon plasma is used to produce excited atoms and ions. These atoms and ions then emit incidental photons of a wavelength specific to each element, and the wavelength and intensity of the light is used to determine the type of element and its concentration within the sample[33].

Following the physical characterization of the nanoparticle, several *in vitro* tests are performed to determine the binding efficiency and cytotoxicity of the particles. In surface binding studies, cells are incubated in a solution of fluorescently tagged nanoparticles for several hours. After incubation, excess nanoparticles are rinsed away and the cell wells are measured for fluorescence output. Thus, groups of cells with a higher fluorescence correspond to a higher degree of surface binding of the nanoparticle to the cellular membrane. Binding of

nanoparticles to the cellular membrane eventually leads to endocytosis and internalization of the particle.

In addition to confirming nanoparticle surface binding, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays are used to determine the efficacy of nanoparticle drug delivery and to measure the overall cytotoxicity of particles. MTT assays measure the enzymatic activity of cells which reduces a soluble tetrazolium containing dye into a purple colored and insoluble formazan containing dye (figure 11)[34]. Rapidly dividing cells, such as cancer tumor cells, show a high degree of metabolic activity and thus show a high degree of MTT reduction. MTT is metabolized to its formazan product by accepting electrons from a cellular reducing equivalent such as NADH, NADPH, or succinate. This redox reaction is dependent upon the redox activity and mitochondrial function of the cell, indicating the cellular oxidative metabolic activity[35].

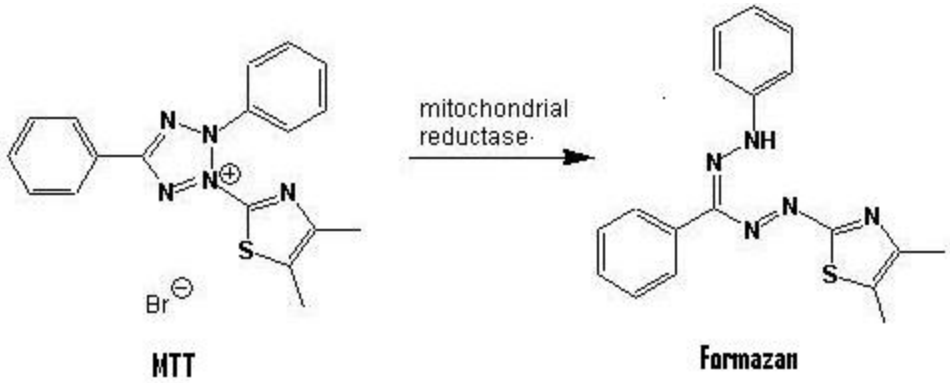

Figure 11: Reduction of a tetrazolium dye, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), into a formazan dye via cellular mitochondrial enzymatic activity[34].

Upon addition of MTT, a greater concentration of viable cells within a well result in greater conversion of MTT into the colored formazan dye. The high absorptivity of the formazan dye at 570 nm is then measured using a 96 well microplate reader to determine the concentration of cells alive at the time of the assay.

MTT assays are carried out following the incubation of cells with drug containing nanoparticles to test for drug delivery efficacy. Drug loaded nanoparticles are typically compared against non-loaded nanoparticles to ensure low particle cytotoxicity. In addition, a solution of the free drug is also used to compare the cytotoxicity of the drug loaded nanoparticles versus that of the unloaded drug.

In summary, we aim to create a robust and biocompatible nanoparticle platform that can selectively deliver a chemotherapy agent to the tumor site. Nanoparticle drug delivery is advantageous when compared to direct delivery of the drug in that it can prolong the timeframe of the effectiveness of the drug and can be directed to the tumor site via the EPR effect and selective targeting. Polyacrylamide nanoparticles are advantageous in that they are biocompatible and nontoxic. In addition, polyacrylamide nanoparticles are highly engineerable, allowing for variation in size, drug release rate, and surface conjugation.

Materials and Methods

Synthesis of Polyacrylamide Based Nanoparticles

Add 0.269 g AHM cross-linker and 0.539 g acrylamide to 2 mL of cisplatin solution. Additionally, add a desired amount of APMA (positively charged) or AA (negatively charged) secondary monomer, based on the properties you wish for your particles to have. Sonicate the solution for 5 minutes, and heat if necessary, until all cisplatin has dissolved.

In a 100 mL round bottomed flask, add 1.6 g of dioctyl sodium sulfosuccinate (AOT), 3.3 mL of Brij 30, and 45 mL of argon purged hexanes. Introduced a stir bar and rubber septum to the flask, and place it under an argon atmosphere, with argon bubbling out of solution. Stir for 20 minutes.

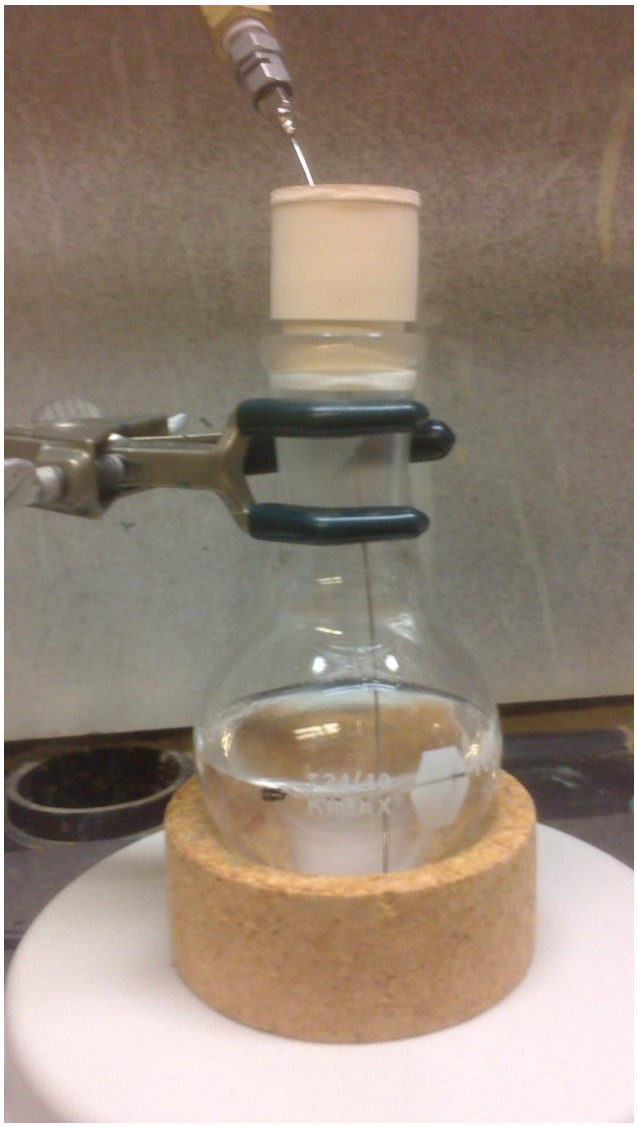

Figure 12: Reverse micelle polymerization reaction setup containing hexane and aqueous phase stirring under an argon atmosphere.

Sonicate the monomer solution for an additional 5 minutes, and transfer its contents to the round bottomed flask with a syringe. As cisplatin is sensitive to light, cover the reaction flask with foil, and continue stirring for 20 minutes.

Add 0.1 mL of 10% aqueous ammonium persulfate solution (APS) (w/v) and 0.1 mL of tetramethylethylenediamine (TEMED) to the round bottomed flask. Pull the argon purge needle from out of the solution, but still on the flask. Let the solution stir for 2 hours. After 2 hours, the solution should appear slightly milky white. Remove hexanes with rotary evaporation. The solution should now be viscous and milky white.

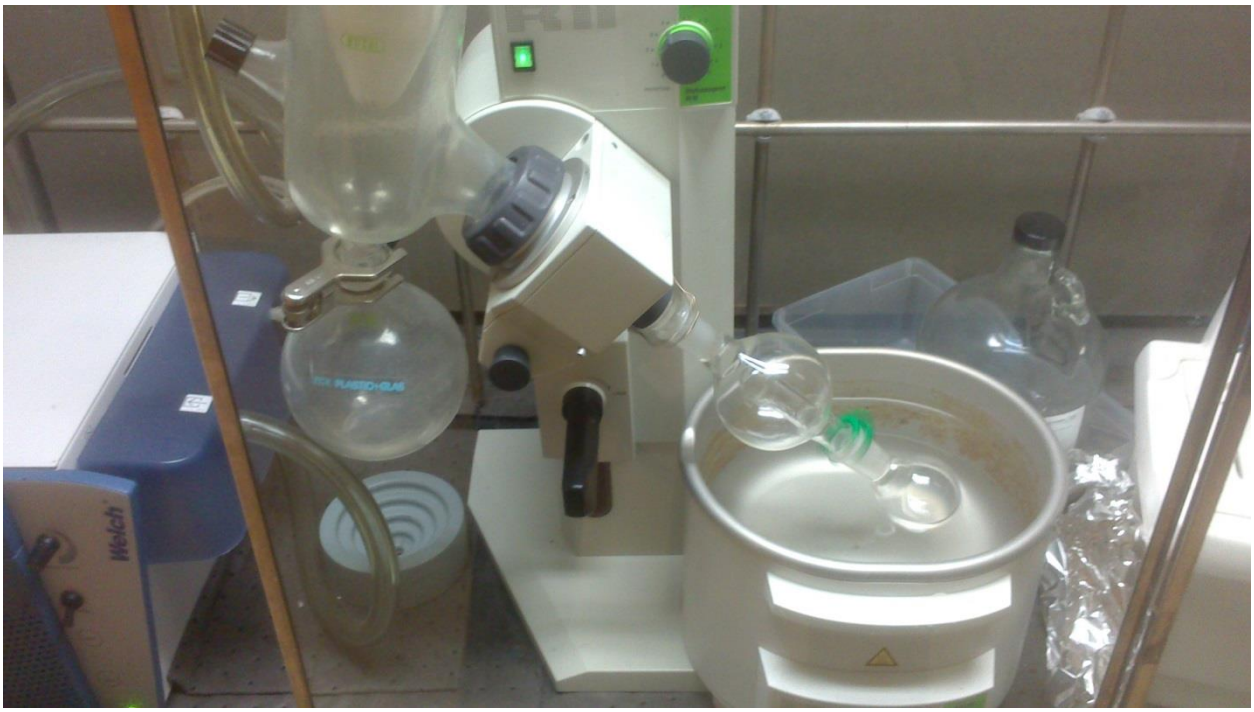

Figure 13: Rotary evaporation of polymerized nanoparticle reaction flask. The solution becomes milky white and viscous as hexane is pulled out of the mixture.

Wash nanoparticles via 300 kDaMWCO Amicon ultrafiltration cell, five times with ethanol and five additional times with water. This is to ensure removal of all surfactant and unreacted monomers. Freeze-dry washed nanoparticle solution via liquid nitrogen and freeze-dryer to collect solid nanoparticles.

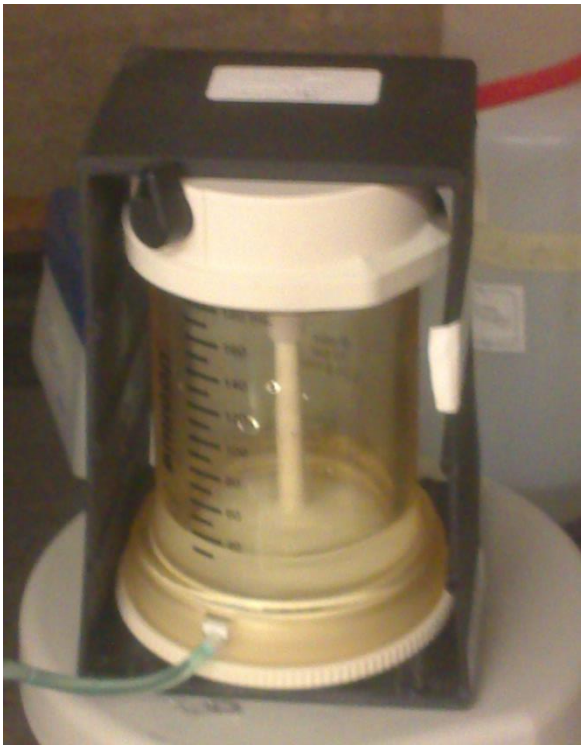

Figure 14: Amicon ultrafiltration cell unit set up for nanoparticle washing.

Post-Loading of Cisplatin

To 1 mL of H_2O is added 2 mg of cisplatin. The solution is sonicated until all cisplatin has been dissolved. 10 mg of nanoparticle is added to the solution, allowed to dissolve, and then stirred at 90^{o}C for four hours. After incubation, wash nanoparticles via 100 kDaMWCO Amicon ultrafiltration cell seven times with water to ensure all free cisplatin has been washed away.

Conjugation of Nanoparticles with Hyaluronic Acid

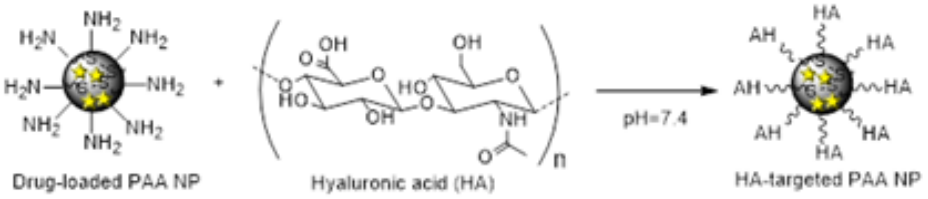

Figure 15: Reaction scheme for HA conjugation of cisplatin loaded nanoparticles.

Dissolve 8.5 mg of HA in 1 mL of pH 7.4 PBS. After all HA has been dissolved, add 10 mg of drug-loaded nanoparticles and allow the solution to stir for 2 hours at room temperature. After reaction for 2 hours, wash nanoparticles via either a 300 kDaMWCO Amicon ultrafiltration cell (when reacted with 90 kDa HA) or a 100 kDaMWCO Amicon Centrifugal filter unit (when reacted with 17 kDa HA). Wash seven times with PBS. Nanoparticles may then be freeze-dried to collect solid particles or maintained in a solution of PBS.

Methods of Analysis

Particle Sizing via dynamic light scattering (DLS): The size of nanoparticles is determined using a 2 mg/mL aqueous solution and a Beckman Coulter Delsa Nano C set to DLS.

Surface Charge Measurements via electrophoretic light scattering (ELS): The surface charge of nanoparticles is determined using a 2 mg/mL aqueous solution and a Beckman Coulter Delsa Nano C set to measure zeta potential.

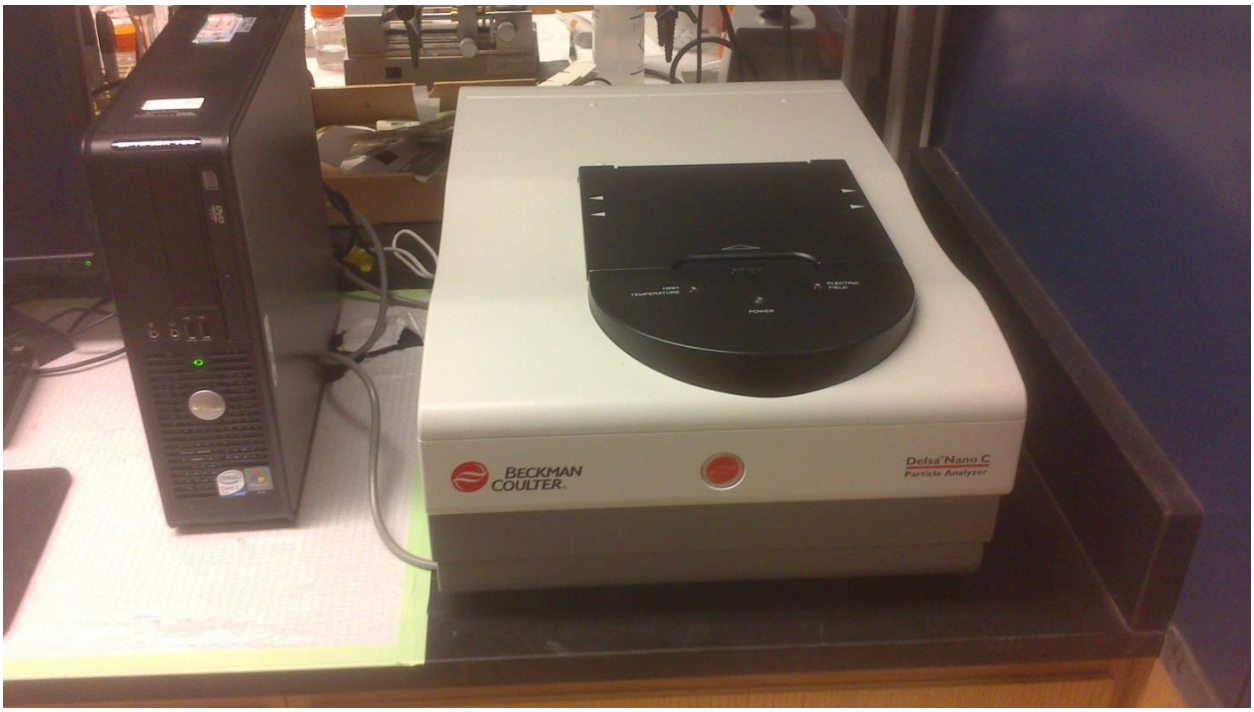

Figure 16: Beckman Coulter Delsa Nano C used for DLS and ELS measurements.

Encapsulated Drug Concentrations via inductively coupled plasma optical emission spectroscopy (ICP-OES): Nanoparticles solutions are prepared using PBS, with a particle concentration of 1 mg/mL. To 1 mL of nanoparticle solution is added 0.25 mL of 20 ppm yttrium stock solution in 5% HNO_3, and 3.75 mL of 5% HNO_3. A blank is made as control, containing PBS in place of nanoparticle solution. The ICP is set to monitor two emission wavelengths, 371.029 nm for the yttrium standard, and 265.945 nm for determination of platinum concentrations. Samples are fed through the ICP, and cisplatin concentrations are calculated based upon a platinum calibration curve.

Figure 17: ICP-OES system used for detection of cisplatin concentrations.

Drug Release Studies

Dissolve 1 mg of cisplatin loaded nanoparticles into 1 mL of PBS with a cleaving agent specific to the cross-linker. Incubate and stir the samples at 37°C, taking multiple nanoparticle incubation time points. Remove each sample after allotted incubation time and filter out nanoparticles using a 100 kDaMWCO Amicon ultrafiltration cell, keeping the filtrate. The filtrate is prepared for ICP-OES using the method outlined above. Measure cisplatin concentration of filtrate using ICP-OES platinum detection.

Binding Studies

Synthesize rhodamine containing nanoparticles using the normal particle preparation procedures (see above), but adding rhodamine 6G tetrafluoroborate into the initial monomer solution and replacing acrylic acid with *N*-Hydroxysuccinimide (NHS) conjugated acrylic acid. After synthesis with rhodamine, conjugate nanoparticles with hyaluronic acid (HA), using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a cross-linker.

Next, transfer cells into a 96 well plate and allow settling overnight. After incubation overnight, add 50 µL of 2 mg/mL nanoparticle solution into each well and allow incubation for 4 hours. After 4 hours, remove the nanoparticle solution from each well, and measure the fluorescence of cells, using a microplate reader.

Cell Studies

Using a hemocytomer, determine the concentration of tumor cells to be tested. Dead cells are excluded from counting using trypan blue staining. Introduce tumor cell line to 96 well cell culture plate at approximately 3000 cells per well, and allow for them to grow overnight with incubation at 37 °C.

Prepare cell media containing either drug loaded nanoparticles or free cisplatin. Replace cell media in the 96 well cell cultures with cell media containing each experimental

condition. Typically, 12 wells are tested for each condition, allowing for 8 conditions to be tested. Allow cells to incubate at 37°C for 2 days.

After incubation, remove the nanoparticle/cisplatin containing solution from the wells and add fresh fetal bovine serum (FBS) free cell media with 0.83 mg/mL thiazolyl blue tetrazolium bromide. Incubate cells at 37°C for 4 hours.

After 4 hours, remove the solution from the wells and add 100 µL dimethyl sulfoxide into each well. Absorbance is measured at 550 nm with a background of 610 nm, using a UV/Vis absorbance microplate reader after 1 hour of shaking.

Results and Discussion

General Characteristics of Polyacrylamide Nanoparticles

Synthesis of nanoparticles is accomplished via water in hexane polymerization, allowing for size control and uniformity of particles. Monomers and cross-linkers are soluble in the water phase, and react in nano-sized aqueous micelles formed as the solution is stirred in the oil phase.

Synthesis of nanoparticles can be accomplished with or without initial drug incorporation in the water phase. If cisplatin is not included in the synthesis of the nanoparticles, it is loaded afterward. This typically results in a higher loading efficiency as well as a greater total weight percent of the drug. Post loading of cisplatin is often performed at high temperatures (90^{o}C) in order to increase the loading speed and decrease incubation time. Cisplatin has been shown to maintain its cytotoxicity after incubation at these temperatures.

The ratio of water to hexane will determine the nanoparticle size by alteration of the aqueous micelles. Both the ratio of monomers to cross-linker as well as particle size affects the release rate of loaded drugs. Smaller particles, which have a higher surface area to volume ratio, are able to release drugs at a much faster rate than larger particles. In addition, particles with a higher polymeric molecular weight results in slower release of drugs[18].

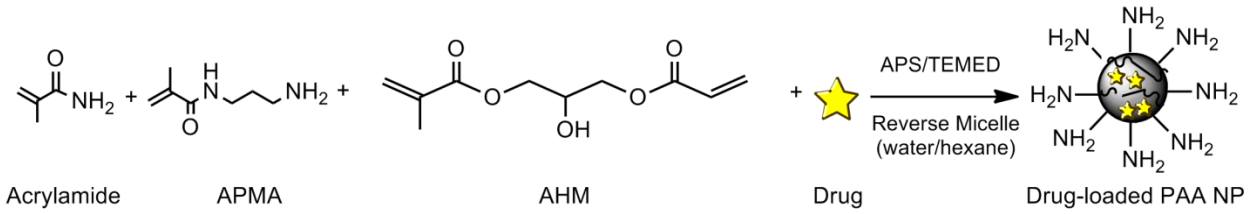

Figure 18: Representative synthetic scheme for APMA, AHM cross-linked polyacrylamide based nanoparticles with initial drug incorporation. APS and TEMED act as catalysts for polymerization, and water/hexane reverse micelles are used in the size control of nanoparticles.

Nanoparticles are characterized via determination of particle size, surface charge, total encapsulated cisplatin content, and cisplatin release profile. Particle sizing is determined by measurement of a 2 mg/mL aqueous nanoparticle solution in a DLS spectrometer. While typical particle sizes are on the order of tens to hundreds of nanometers, monodisperse particles with a Gaussian distribution in the size range of 30 to 100 nm are used for our *in vitro* cell studies. Nanoparticles are typically sized following surface conjugation, as well, which results in an increase in mean particle diameter. Below are typical results for particle sizing via DLS (figure 19).

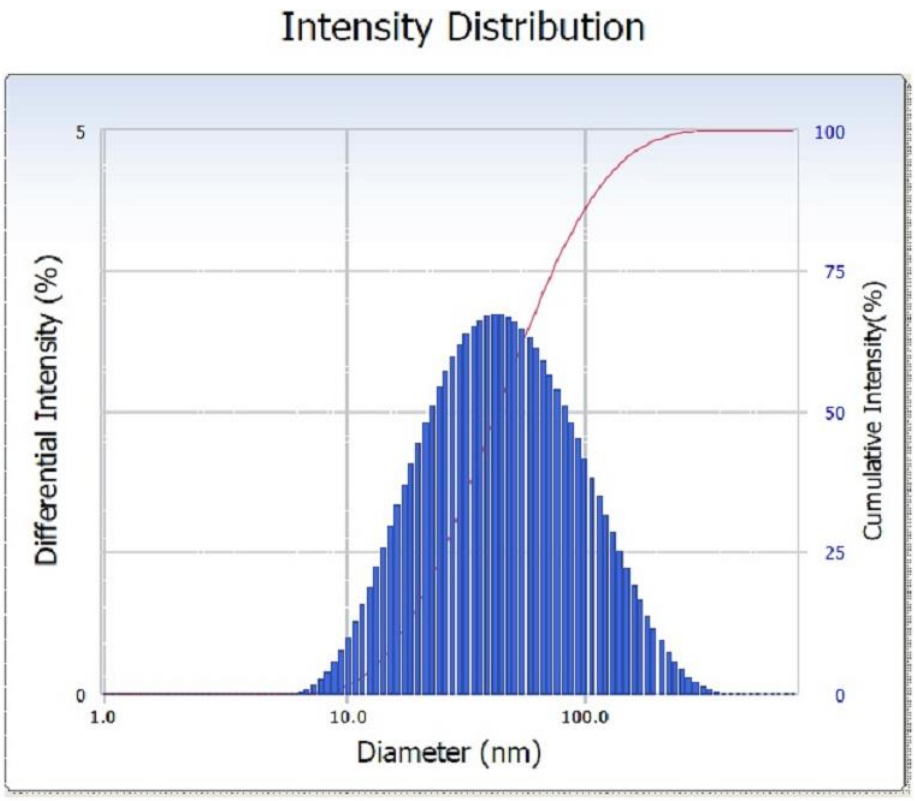

Figure 19: Representative particle sizing data for AHM cross-linked acrylamide-APMA nanoparticles, measured via Beckman Coulter Delsa Nano C particle analyzer.

Surface charge is determined by ELS. The surface charge of the particle is dependent upon what specific secondary monomer is used in its preparation. In the case of an acrylamide-APMA matrix, the positive charge is due to the amine moiety present on APMA. The acrylamide-acrylic acid (AA) matrix is negatively charged due to the carboxylic acid moiety present in acrylic acid. Following surface conjugation with HA, APMA particles become more negative in charge and AA particles remain negative. Conjugation with PEG results in charge neutralization for both APMA and AA nanoparticles. Below shows typical results for surface charge density via ELS (figure 20).

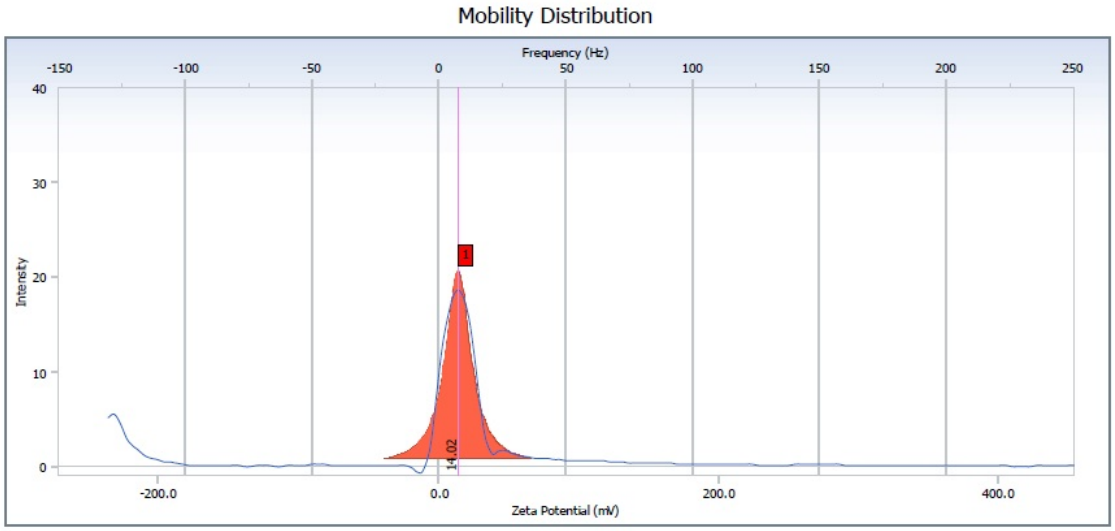

Figure 20: Representative particle ELS data for AHM cross-linked acrylamide-APMA nanoparticles, measured via Beckman Coulter Delsa Nano C particle analyzer.

Surface charge is an important factor in determining the stability of nanoparticles as well as determining how the particle interacts with the cell membrane. Charged surfaces increases the repulsive interactions between nanoparticles, leading to less aggregation of the particles when dissolved in solution. Suspensions of nanoparticles have been shown to be stabilized when the minimum absolute zeta potential of the particles is ± 30 mV. However, while PEG conjugation decreases the overall charge of the particles, the steric effects and hydration forces of adding a bulky polymer to the particle surface tends to increase overall stability of particles, as well[18].

Prior to HA conjugation, cisplatin is loaded into blank nanoparticles using the post-loading method described above. In order to quantify cisplatin concentration of nanoparticles, ICP-OES is used to detect platinum levels, using yttrium as an internal standard. Figure 21

shows typical platinum concentrations present in post-loaded nanoparticles, using various post-loading conditions.

	Cisplatin solution concentration	stir time	wt% loading	loading efficiency, %	Solution condition
1	0.5mg/mL	overnight	1.7	33	Clear
2	0.5 mg/mL	4 hrs	1.9	37	Clear
3	1 mg/mL	4 hrs	3.8	38	Clear
4	2 mg/mL	4hrs	11.5	58	clear
5	2.91 mg/mL	4 hrs	6.1	21	not suspendable

Figure 21: Platinum concentration and loading efficiency in cisplatin loaded AHM nanoparticles. All concentrations were determined via ICP-OES.

Typical loading efficiencies for nanoparticle are ~30-60%, with a maximum loading efficiency at 2 mg/mL cisplatin during the post loading. Since post loading is carried out in aqueous conditions, cisplatin's limited solubility in water restricts the amount which may be dissolved in the reaction solution. As a result, cisplatin concentrations higher than 2 mg/mL will become turbid unless the reaction is heated. Loading efficiency reaches a maximum around 4 hours of incubation, after which the loading efficiency no longer increases and in some cases is even lowered.

Drug Release Studies

Following the synthesis, drug loading, surface conjugation, and characterization of nanoparticles, drug release studies are performed to study the rate at which the loaded drug is released into the environment. Drug release studies are performed at biologically relevant pH and temperatures, and typically five data points are taken over a week-long time frame. Drug loaded nanoparticles are first dissolved in each of the PBS solutions containing cross-linker activators (DTT, GSH, or esterase) at intercellular concentrations. Additional samples that do not contain the activator serve as controls. These samples are then stored at $37^{o}C$ until their respective time points. The samples are then filtered through an Amicon centrifugal unit to remove nanoparticles, and the drug concentration in the filtrate is determined. Figure 22 shows typical results for the release profile of cisplatin loaded, AHM cross-linked nanoparticles over a period of one week. The importance of nanoparticle cross-linkers is twofold in nature: To stabilize the acrylamide matrix while the nanoparticles are circulating in the bloodstream, and to cleave and cause drug release after nanoparticles have been introduced to an intracellular condition.

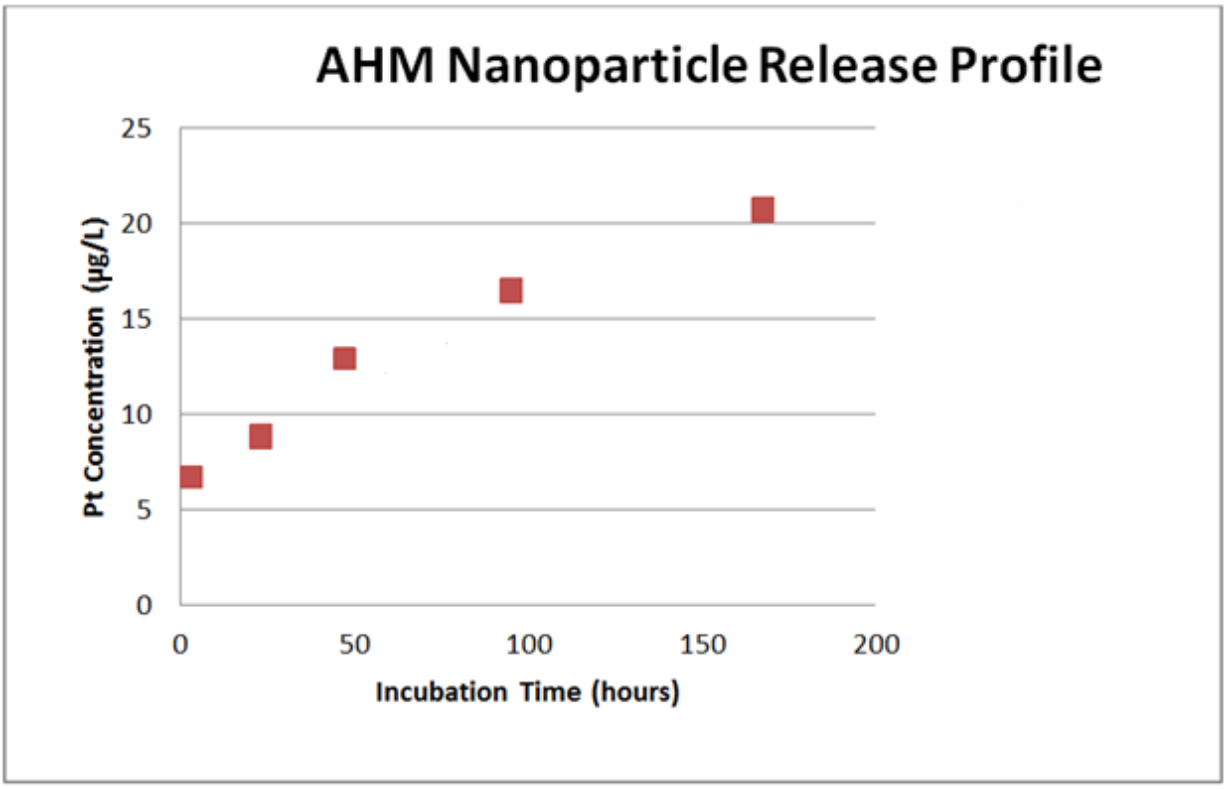

Figure 22: Release profile for cisplatin loaded AHM cross-linked nanoparticles.

One of the key advantages of using an acrylamide matrix for nanoparticle drug delivery is the stability of cross-linked polyacrylamide. This high stability results in a much slower drug release rate (order of 1 week) than the rate of vascular circulation and cell binding (order of 1 hour), allowing for the targeted delivery of the nanoparticles and steady release of the loaded drug at the tumor site.

Cell Binding Studies

Binding studies are used to determine the degree of specificity to which a nanoparticle will bind to a specific cell surface protein. In the case of HA coated nanoparticles, the HA ligands are used to target cells that have a high expression of the CD44 cell-surface glycoprotein. Figure 23 shows the results of a binding study in which two cell lines, one with high CD44 expression (Hep3B) and one with low CD44 expression (HepG2), were incubated with HA conjugated and non-conjugated nanoparticles which contain rhodamine dye, a strong fluorophore.

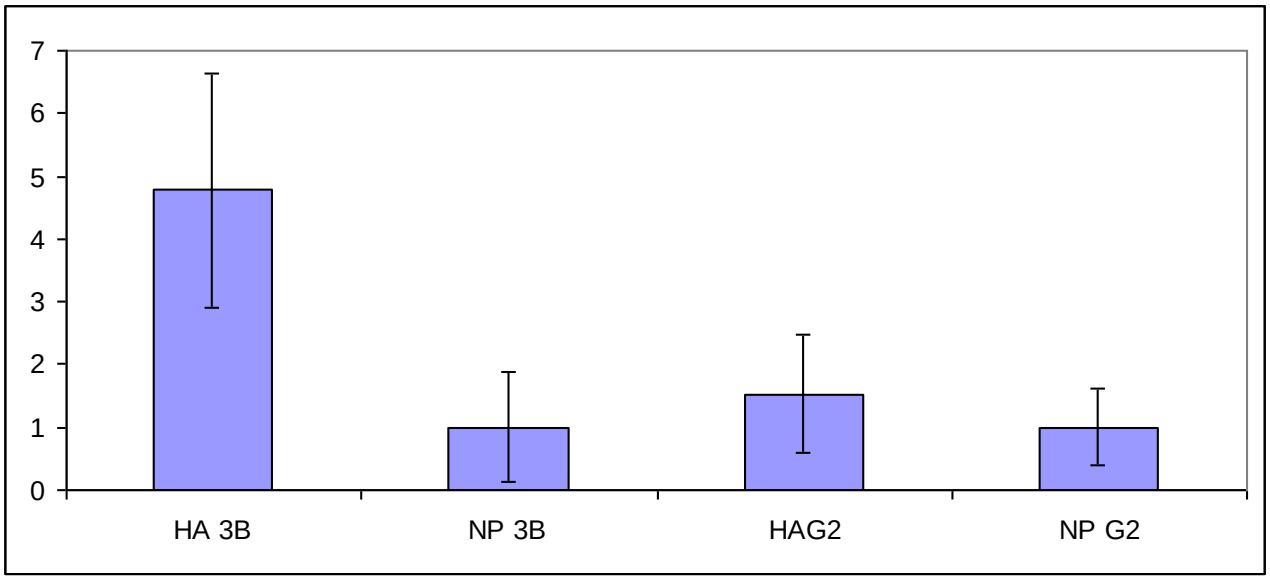

Figure 23: Binding study results using negatively charged nanoparticles. HA = Hyaluronic acid coated nanoparticles. NP = Non-coated nanoparticles. 3B = Hep3B cells (High CD44 expression). G2 = HepG2 cells (Low CD44 expression). Y-axis shows the relative number of bound nanoparticles per cell. 3B and G2 data sets were normalized independently.

After incubation, the cells are washed with Dulbecco's Phosphate-Buffered Saline (DPBS) to remove any excess nanoparticles, and levels of fluorescence are measured from each

cell well to determine the relative amount of cell bound nanoparticles. Because nanoparticles contain the rhodamine fluorophore, more surface-bound nanoparticles results in a greater degree of total fluorescence. In the provided study, HA conjugated nanoparticles preferentially bind to Hep3B while binding HepG2 to a far lesser degree. Non-conjugated nanoparticles did not show strong binding to either cell line due to the repulsive forces between the negatively charged nanoparticle and the cell membrane.

Cell binding studies are carried out using fluorescence spectroscopy and microscopy. Depending upon the surface charge and functional groups of a nanoparticle, its binding affinity for various cell lines will vary. In terms of nonspecific binding, positively charged nanoparticles will have attractive coulomb interactions with the negatively charged cellular membrane. This attraction allows stronger intrinsic binding between positive nanoparticle and the cell membrane, while negatively charged particles are more repelled. In addition to coulomb interactions based upon surface charge, there are also specific interactions between functional groups on the surface of nanoparticles and various characteristics of the cellular membrane.

Cell Toxicity Studies

After characterization of nanoparticles, we employ an MTT assay in order to determine cytotoxicity. A typical assay consists of cell groups introduced to several different growth media conditions: cell media containing cisplatin (free molecule), non-loaded (blank) nanoparticles, nanoparticles loaded with various concentrations of cisplatin, and media with no treatment to serve as a control. Figure 24 shows typical results for an MTT assay, performed on the HEY1 cell line incubated for four hours.

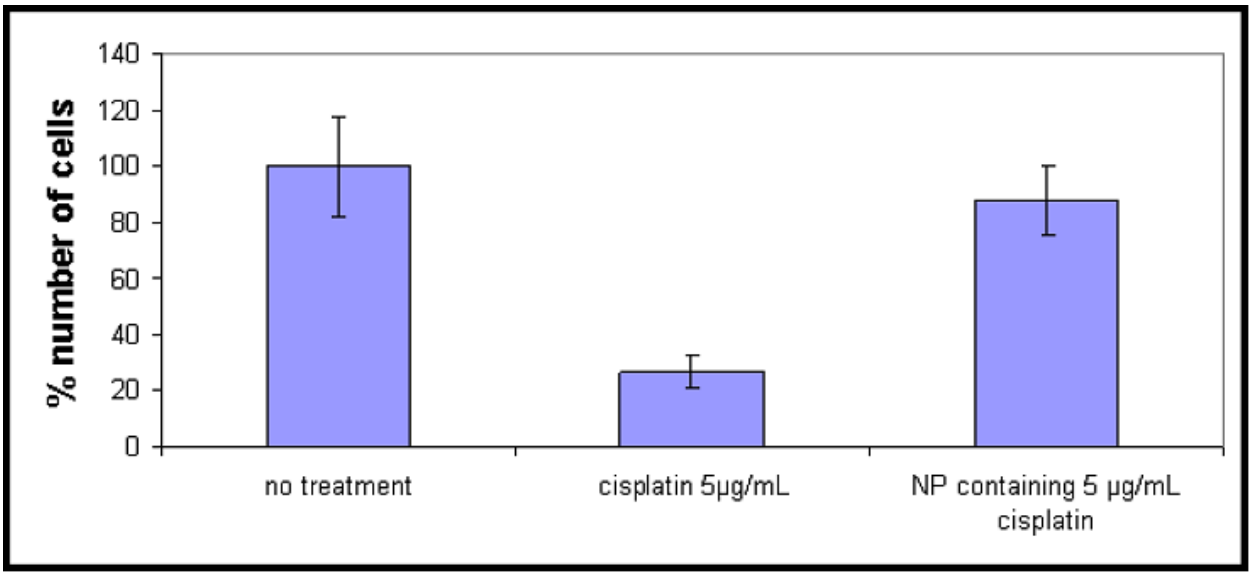

Figure 24: Representative results for an MTT assay using the HEY1 cell line, comparing the cytotoxicity of cisplatin loaded nanoparticles against that of free cisplatin.

In this example assay, the pure cisplatin incubated cells showed a high degree of cytotoxicity while the no treatment acted as the control for cytotoxicity. Cells that were

incubated for four hours with an equivalent concentration of cisplatin loaded nanoparticles showed a low degree of cytotoxicity after the incubation period, due to the timed drug release rate of polyacrylamide nanoparticles. Cytotoxicity between *in vitro* and *in vivo* studies will also differ due to physiological factors not present in the MTT assay. An example of this is both passive and active targeting, which would not be present for free cisplatin but would increase the cytotoxicity of cisplatin loaded functionalized nanoparticles. In addition, nanoparticles used for this study did not contain a targeting moiety on the particle surface, which would also enhance the selectivity of the cisplatin loaded nanoparticles.

Conclusion and Future Directions

The field of nanotechnology has grown rapidly in the past decades and has now matured to the point of making an impact within the medical field. FDA approved nanoparticle delivered chemotherapy agents are now available and increasingly used on the medical market, with more and more on the way. The most common types of nanoparticle delivery systems are composed of liposomal and protein based matrices, such as Doxil, a lysosome packaged form of doxorubicin surrounded by a layer of methoxypolyethylene glycol (MPEG), and Abraxane, an albumin packaged form of paclitaxel. The half-life of Doxil is approximately 55 hours, while that of Abraxane is 27 hours, greatly increasing the terminal half-lives of their free drug counterparts, 17.9 hours and 69 minutes, respectively[36,37].

While these nanoparticle drug formulations dramatically increase the half-life of the chemotherapy agent, the timescale of drug release is still not optimal. For example, paclitaxel is a cytoskeletal drug which targets tubulin, blocking the progression of mitosis, thereby preventing cell division and triggering apoptosis or reversion of cells to the G-phase of the cell cycle. As such, paclitaxel is a cell cycle dependent drug. In the case of carcinoma of the breast, it must be administered for several hours every two or three weeks, for four cycles, in order to have maximum effect. Thus, Abraxane's 27 hour half-life is still very short in comparison to the amount of time required to treat breast cancer[38,39].

The research and development of a polyacrylamide based nanoparticles for the purpose of chemotherapy drug delivery aims to overcome this issue of a short half-life. Polyacrylamide

matrices are durable and stable, allowing for longer circulation time and lower frequency of intravenous infusions required throughout the chemotherapy process. Polyacrylamide is also nontoxic and biocompatible, and it can readily withstand physiological conditions without degradation.

In addition to stability, polyacrylamide matrices also offer a higher degree of versatility and engineerability, allowing for incorporation of a wide variety of chemical groups upon the nanoparticle surface or within the matrix. For example, fluorescent dyes, such as rhodamine, can be readily incorporated into the acrylamide matrix, creating a fluorescent tag for tracking and quantification of nanoparticle concentrations. Surface chemistry is very accessible through reaction with the carbonyl site of the secondary monomer (AA or APMA), allowing for covalent linkage of ligands that serve a variety of purposes. Current chemotherapy nanoparticle formulations rely solely upon the EPR effect to localize drug delivery to the tumor site. While the EPR effect can greatly increase drug localization, there is a limit to the degree of specificity this can offer toward selective targeting of tumor cells. While the EPR effect draws nanoparticles into the tumor site, it does not select for the types of cells that the nanoparticles are being taken up by. In order to make this distinction, targeting moieties that interact with specific cell surface proteins are added to the surface of the nanoparticle, leading to greater uptake of the nanoparticle when it comes in contact with the specific cell of interest. This high degree of versatility has led to the use of polyacrylamide nanoparticles as both a targeted therapeutic and targeted diagnostic tool, often concurrently.

The stability, biocompatibility, and versatility of the polyacrylamide nanoparticle make it a promising candidate for chemotherapy drug delivery. Recognizing this, the Kopelman lab is pursing research of a polyacrylamide nanoparticle drug delivery system, with research spanning from synthesis of the particles to *in vivo* studies on tumor cell lines. Using reverse micelle polymerization, a primary acrylamide monomer and other secondary monomers (AA, APMA) are linked together with a cross-linking agent (CBA, AHM). In a hexane solution, nano-sized micelles of water and surfactant are formed, and the soluble monomers and cross-linkers react to form spherical nanoparticles ranging from 30-100 nm in diameter. Nanoparticles are then loaded with a chemotherapy drug (cisplatin, docetaxel, etc.) and conjugated with a surface ligand (PEG, HA, 5-FTSC, etc.), washed to remove any impurities and unreacted molecules, and freeze dried to solidity the nanoparticles. Before release and cell studies, nanoparticles are characterized for size via DLS, surface charge via ELS, and internal drug concentration via ICP. Drug release studies are carried out to study the release profile of the nanoparticle, testing the triggering mechanism of the nanoparticle when its cross-linker in in the presence of its respective cleaving agent (AHM via esterase, CBA via glutathione). Cell binding studies are carried out using fluorescently tagged nanoparticles. Tumor cells are incubated in the presence of these nanoparticles, and fluorescence spectroscopy and microscopy is then used to quantify the binding efficiency of the particular nanoparticle. Lastly, cytotoxicity tests are carried out using MTT assays.

As we move forward with our research on nanoparticle drug delivery of anti-cancer agents, there are several projects planned for future studies. These include testing of nanoparticles on tumor containing rats and mice, testing the effectiveness of new

chemotherapy drugs that might otherwise not be viable without a nanoparticle formulation, focusing efforts to target cancer stem cells, as well as overcoming their multi-drug resistance (MDR), and testing the use of photodynamic therapy in conjunction with drug delivery.

Animal studies

Following *in vitro* cell studies, the next logical step for testing nanoparticle efficacy is the use of an animal model. Mouse and rat models provide an effective method of mimicking human physiology and allow for pre-clinical testing of our nanoparticles. Currently, the Kopelman lab collaborates with the lab of Dr. Ronald Buckanovich (University of Michigan Ann Arbor, Medical School) on animal studies, where intravenous injections of chemotherapy drug loaded nanoparticles are tested on mice with ovarian tumors, as well as with the labs of Dr. Pedro Lowensein and Dr. Maria Castro on brain tumors. As the project advances, we plan to use animal studies to a greater extent, testing the efficacy of nanoparticles that target different types of tumor cells and deliver various types of chemotherapy drugs.

Other drugs

In addition to the use of cisplatin and docetaxel drug delivery, another future direction for nanoparticle research in the Kopelman lab is the delivery of other chemotherapy agents with high cytotoxicity that would otherwise not be a viable option for use in cancer treatments. Many experimental chemotherapy agents are very effective at destroying cancer cells but cannot be used for direct injection due to a high degree of toxicity. Nanoparticle drug delivery can potentially overcome this toxicity through localized delivery to the tumor site, greatly lessening the interaction between the drug and normal cells.

Cancer Stem Cells

Cancer stem cells (CSC) are cancer cells that possess the characteristics of normal stem cells. They are tumor forming and are the source of all cell types found within a tumor site. Similar to normal stem cells, CSCs are self-renewing and differentiate into tumor cells in order to initiate and sustain tumor growth. CSCs have been suggested to be the cause of many negative aspects associated with cancer such as MDR and relapse of the disease, as well as metastasis. Many cancer therapies ultimately do not completely eliminate the disease because CSCs remain within the body even after tumors have been destroyed, allowing for the eventual reemergence of new tumors. CSCs are more difficult to target than tumor cells for several reasons. First is their ability to remain dormant within the body at a slow metabolic rate, thus making them difficult to target using drugs that combat the quick proliferation rates of tumor cells. Second are their high expression levels of drug transporters, DNA mismatch repair enzymes, and detoxification enzymes, making them much more resistant to chemotherapy and radiation treatments, i.e. multi-drug and multi-treatment resistant[40,41].

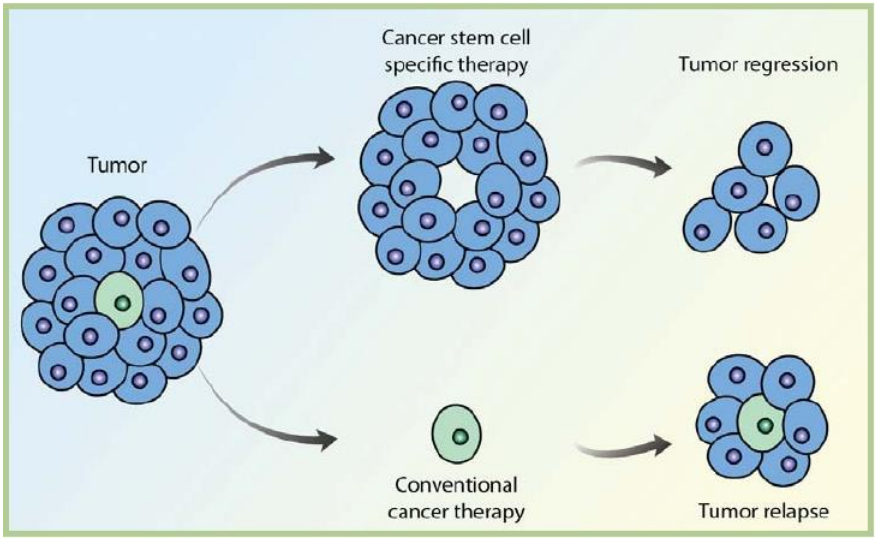

Figure 25: A new treatment strategy that specifically targets cancer stem cells, when combined with current treatments, may lead to a more complete and durable regression of malignant cancers[41].

If CSCs can be directly targeted for destruction, cancer can be much more efficiently and effectively eliminated from the body. As such, targeting CSCs is a highly advantageous method toward developing next generation cancer therapies. The strategy of nanoparticle drug delivery used in the Kopelman lab is a promising method for selected targeting of CSCs for several reasons. First is the ability of nanoparticle drug delivery to overcome MDR by bypassing or saturating drug elimination pathways of the CSC. Nanoparticles would be effective at overcoming this MDR barrier because nanoparticles deliver high concentrations of the chemotherapy drug after internalization into the cell, giving the potential to overpower drug elimination and DNA repair pathways of the cell. Due to the selective targeting ability of functionalized nanoparticles, chemotherapy agents can be delivered at concentrations much higher than allowed by direct injection of the drug, while eliminating many of the side effects associated with the drug[40,41].

Photodynamic Therapy

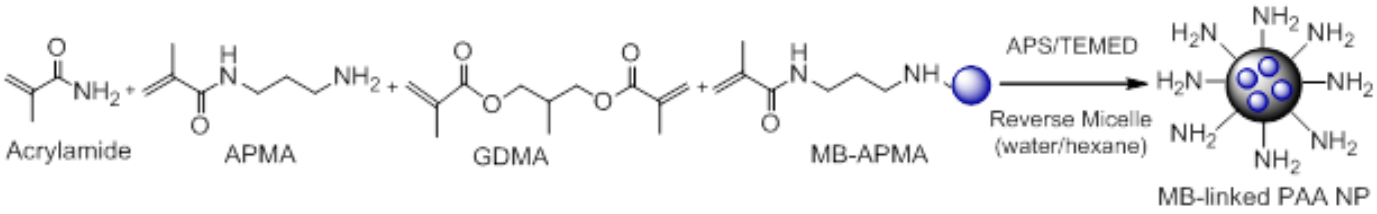

Figure 26: Reaction schematics for methylene blue linked nanoparticles.

In addition to drug delivery, nanoparticles can also be designed for photodynamic therapy. An example of this is nanoparticles that contain covalently linked methylene blue. These methylene blue moieties are bound to the nanoparticle matrix and are not released.

When exposed to oxygen and light, methylene blue can create singlet oxygen species. These singlet oxygen species, and the other reactive oxygen species (ROS) they create, are highly reactive and are able to interfere with and destroy the cell once the nanoparticle has been internalized and activated by light. The combination of targeted chemotherapy drug delivery and photodynamic therapy via nanoparticle would be an effective strategy for increasing cancer killing efficacy.

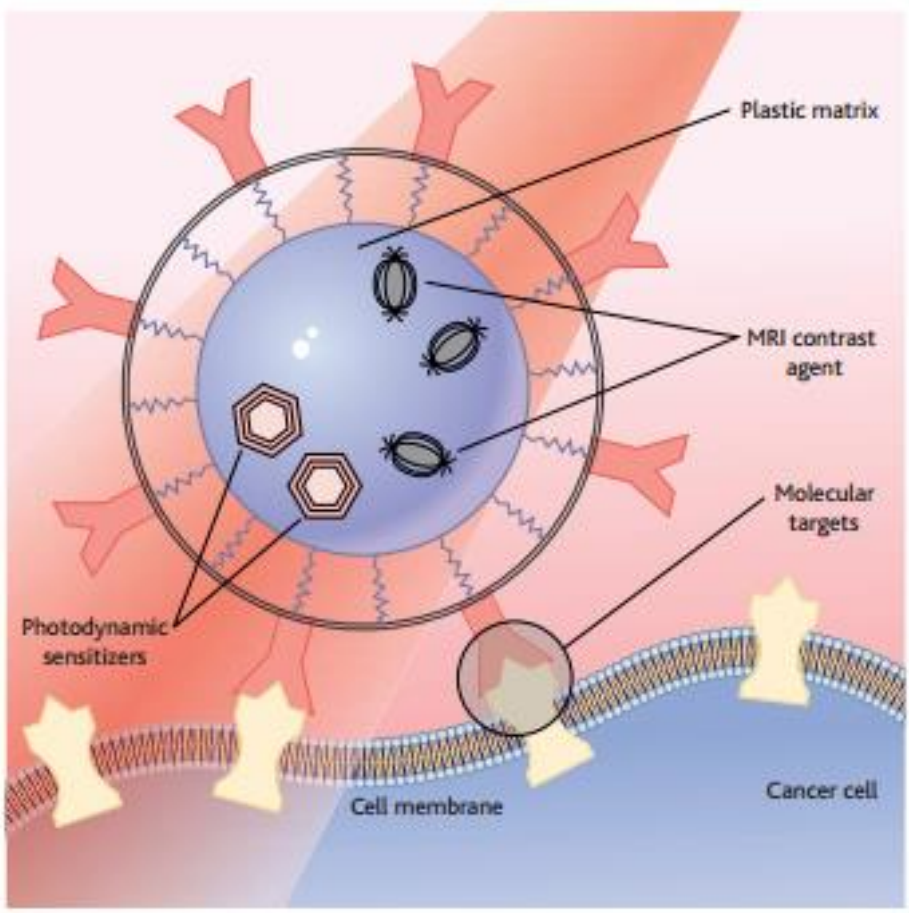

Figure 27: Nanoparticles are a flexible platform for the detection and treatment of cancer. In this example, photodynamic drugs and MRI contrast agents are part of the targeted multifunctional NP[42,43].

As is the case with the majority of medical research, the ultimate goal of this project is to be granted drug approval by the FDA so that these polyacrylamide nanoparticles may be used on human cancer patients. After synthesis, *in vivo*, and animal testing of the nanoparticles, the FDA's initial approval must be granted so as to begin human testing. The drug then enters three phases of clinical trials that are approximately 1, 2, and 3 years long, respectively, and increase in size of experimental patient population[44].

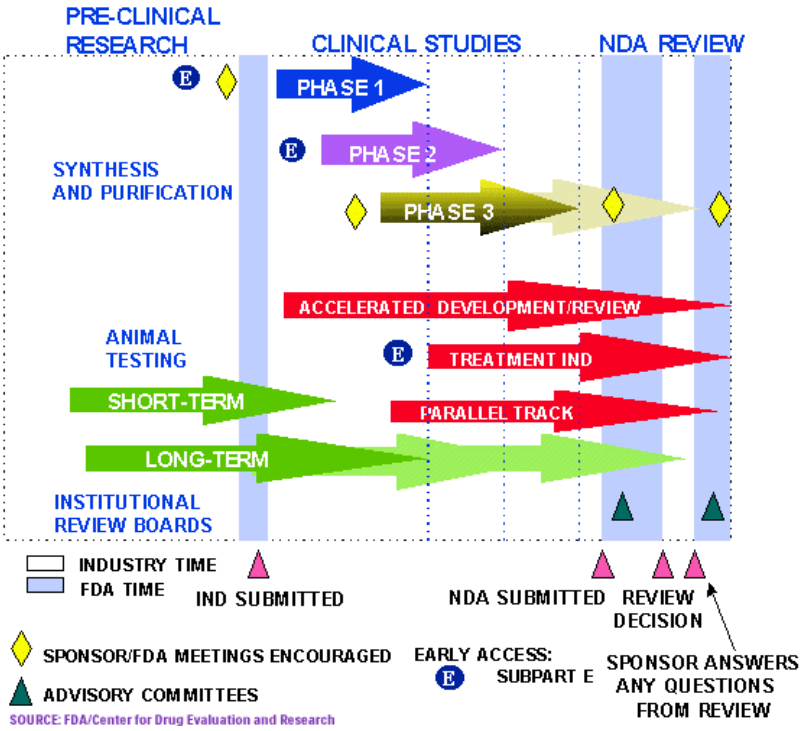

Figure 28: Steps and time frame for a research drug to pass FDA approval[44].

As cancer research continues to advance, more and more viable options for curing the disease begins to appear. Nanoparticle drug delivery has shown a great degree of promise in the past several years, and we believe that polyacrylamide nanoparticles have the potential to continue this promising trend, offering new technological advancements to the field of drug delivery and new hope toward the elimination of cancer.

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