MEDUSA PARTICLES: A MAGNETICALLY TRIGGERED DRUG DELIVERY SYSTEM CONSISTING OF PEG-PCL DIBLOCK POLYMER BRUSHES BOUND TO THE SURFACE OF SINGLE CRYSTAL MAGNETITE NANOPARTICLES

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ABSTRACT

Current cancer chemotherapy treatment involves intravenous administration of highly toxic drugs relying on the strategy that some of the chemotherapy agent will reach the site of cancer and effectively kill those cells. This method leads to mutation and damage of healthy cells, which can manifest into side effects including fatigue, alopecia, and death.

To combat these side effects and to target cancer cells preferentially, we aimed to develop a drug delivery system in which chemotherapy agents, such as doxorubicin, are entrapped within a semi-crystalline polymer shell covalently attached to the surface of magnetic nanoparticles. Not only could these “medusa particles” be directed to specific sites of cancerous tissue using targeting ligands attached to an external polymer corona, but their release of drug could also be temporally controlled by a magnetically triggered thermal induction mechanism. Once the particles reach the sites of cancer, an external radio frequency alternating current magnetic field would be applied to heat the nanoparticles causing the polymer shell to melt and allowing the drug to diffuse out of the core. This mechanism could have extensive biomedical applications due to the wide variety of targeting ligands and drugs one could utilize in the drug delivery system.

In this thesis, we have demonstrated synthesis of magnetite nanoparticles verified by the x-ray photoelectron spectroscopy in the Fe 2p binding energy region, and were determined to be 11 nm in diameter as seen by transmission electron microscopy. Doxorubicin was successfully loaded into medusa particles at about 2-3% by total weight on average. Magnetically triggered
release of doxorubicin from medusa particles was demonstrated and monitored using UV-Vis spectroscopy, electrochemical methods including linear sweep and differential pulse voltammetry, and cell studies involving CHO-K1 cells.
DEDICATION

For James D. McCormick.
LIST OF ABBREVIATIONS AND SYMBOLS

°C degrees Celsius
CL caprolactone
cRGDfk cyclic (arginine-glycine-aspartic acid-D-phenylalanine-lysine)
δ chemical shift
DLS dynamic light scattering
DMF dimethylformamide
DOX doxorubicin
DPV differential pulse voltammetry
EE encapsulation efficiency
EG ethylene glycol
EPR Enhanced Permeation and Retention Effect
G Gauss
GC glassy carbon
kHz kilohertz
LBW percent drug loading by weight
LSV linear sweep voltammetry
MEK methyl ethyl ketone
MFH magnetic field heating
MeO  methoxy
μM  micromolar
mL  milliliter
mM  millimolar
$M_n$  number average molecular weight
MΩ  meghoms
MW  molecular weight
MWCO molecular weight cutoff
nm  nanometer
NMR  nuclear magnetic resonance
OH  hydroxyl
PEG  poly(ethylene glycol)
PCL  poly(caprolactone)
PGP  permeability glycoprotein
RGD  arginylglycylaspartic acid
THF  tetrahydrofuran
UP  ultrapure
V  volts
λ  wavelength
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CHAPTER 1
INTRODUCTION

1.1 Statement of Problem

Cancer is one of the most prevalent causes of death in the world today. Malignant neoplasms caused 23.3% (over half a million) of the deaths in the United States in 2010.\textsuperscript{1} Chemotherapy drugs are used to combat cancer; however, many of these treatments cause significant side effects because the drug is spread systemically when administered intravenously, and these drugs often require high doses over long periods of time to be effective. Thus, we need systems to deliver more specific toxicity to cancer cells at a lower dosage without producing the nocuous side effects. These problems have led to the research and use of drug delivery systems.

1.2 Drug Delivery Systems

Many types of drug delivery systems have been employed as an alternative to direct administration of drugs. These systems often use metals or polymers to hold a drug until release is pushed by an external source, such as magnetic or laser heating, light, or pH, or some endogenous mechanism.\textsuperscript{2,3} Often, these systems use amphiphilic molecules that self-assemble into nanostructures, such as micelles or liposomes.\textsuperscript{4} In certain solvents, such as ultrapure water, the hydrophobic and hydrophilic portions of the amphiphilic molecule will aggregate together in a predictable manner to create the nanostructures listed. Drugs can be trapped in one of these areas based on the drug’s intermolecular interactions. For example, if a drug is hydrophobic, it
can be trapped in the hydrophobic portion of a self-assembling nanostructure during formation. Using these concepts, we wish to synthesize a new type of polymer brush nanostructure.

1.2.1 Loaded Polymer Micelles

Our previous experiments have involved the use of polymer micelles made from poly(ethylene glycol-b-caprolactone) diblock copolymers. The hydrophobic polycaprolactone entraps magnetic nanoparticles and chemotherapy agents on the interior of the micelle, while the poly(ethylene glycol) block creates a hydrophilic corona on the exterior of the micelle. Ideally, the drug would remain inside the core of the micelle until heated above the melting point of the semi-crystalline polycaprolactone core. However, this micelle system has shown to leak the chemotherapy drug prematurely.

1.2.2 Doxorubicin

Doxorubicin (DOX) is a commonly used chemotherapy agent used against several cancers including large B cell lymphomas and early stage breast cancer, and is the main drug used in our drug delivery system experiments. DOX is in a cell cycle non-specific class of drugs called anthracyclines, which are derived from a species of *Streptomyces* bacteria. The specific mechanism by which it kills cancer cells is highly disputed but the most common explanations include intercalation of DNA, free oxygen radical damage of DNA, and inhibition of topoisomerase II enzymes; however, it is notorious for its serious side effects, such as cardiotoxicity, which is likely a result of free radical formation. Despite these side effects, doxorubicin is extremely effective is was listed in the World Health Organization’s list of essential medicines in 2015. In addition to its importance internationally for cancer treatment, we have chosen doxorubicin as the primary drug of study because it has a visible chromophore
peak near 485 nm and exhibits fluorescence, and these characteristics can be used to determine DOX concentration.

1.3 Medusa Particles as a Drug Delivery System

We seek to build a targeted, magnetically triggered drug delivery system for cancer therapy. We aim to synthesize “medusa particles” in which poly(ethylene glycol-\textit{b}-caprolactone) diblock copolymers would be covalently bound to the surface of magnetite nanoparticles creating a polymer brush system (Figure 1.3). PEG and PCL are compatible for use in humans and are known to be biodegradable.\textsuperscript{2} When dispersed in aqueous media, we expect the polycaprolactone block to form a semi-crystalline shell around the magnetite core. We predict that this crystallinity will trap the drug within the polymer shell. The poly(ethylene glycol) block would form a hydrophilic corona, allowing the medusa particles to disperse collectively in water. A targeting ligand, such as a cyclic RGD peptide, would be bound to the termini of several poly(ethylene glycol) blocks in order for the medusa particles to travel preferentially to cancer cells.\textsuperscript{10} Doxorubicin would be trapped in the semi-crystalline hydrophobic core of the medusa particles. Drug loaded medusa particles would be injected intravenously, where they could travel to cancer locations and bind via the targeting ligands. Application of an external radio frequency ac magnetic field would then heat the medusa particles via magnetic induction, whereupon the entrapped chemotherapy agent would be released at the site of cancer.\textsuperscript{11} In previous studies, semi-crystalline PCL has been found to have a melting point of about 43°C, which is at the lower end of hyperthermia.\textsuperscript{12}
An extensive amount of literature exists regarding the biomedically purposed surface modification of magnetic iron oxide nanoparticles, such as magnetite (\(\text{Fe}_3\text{O}_4\)) and maghemite (\(\gamma\text{-Fe}_3\text{O}_4\)), including cancer treatment\(^{13-15}\). Nanoparticle surfaces are often PEGylated to enhance biocompatibility, particularly due to their decreased opsonization by various cells of the immune system when PEG is on the surface\(^6\). Nanoparticles also typically go undetected by the immune system due to their small size, as renal and hepatic clearance mechanisms do not typically filter out nanoscale molecules and nanostructures typically evade Pgp efflux\(^7\). This allows nanoparticle drug delivery systems to reach tissues as they move from blood vessels to tissues, including cancerous tissues, without detection and remain for extended periods, which is known as the Enhanced Permeation and Retention Effect (EPR)\(^8\). The blood vessels providing the

Figure 1.3 Medusa particle model representation showing the diblock copolymer, polycaprolactone (red) and poly(ethylene glycol) (blue), covalently attached to an iron oxide nanoparticle (black) with a targeting ligand (green) attached to the PEG corona. Doxorubicin would be contained within the PCL shell.
vascular supply for cancer often have wider gaps than the size of typical blood vessel gaps, which allows for the increased extravasation leading to EPR. It has been shown that ideal size for drug delivery systems utilizing EPR is an average diameter of 200 nm or less.\textsuperscript{19}

Modification of nanoparticles surfaces with polymers can be achieved either by a “grafting to” or “grafting from” approach. The “grafting to” approach reacts a previously synthesized polymer chain with determined structure with a functional group on the surface of the particle. The “grafting from” approach involves growing a polymer chain from the surface of the particle after the particle has been functionalized with an initiator.\textsuperscript{20-24} In the synthesis of medusa particles, we have used the “grafting to” approach by binding the PEG-PCL diblock copolymer to the surface of magnetite using a silane coupling agent, which will allow us to bind previously characterized polymers to the particle surface.

Our desire to use an external trigger for drug release is based on several factors. First, it allows for temporal control of drug release. If the drug is targeted and released specifically at the tissues sites of cancer, then the undesirable side effects associated with chemotherapy may be avoided, as the DNA intercalation caused by doxorubicin would be preferentially targeted at the cancerous cell sites and surrounding tissue, which could also disrupt the vascular supply for the growing neoplasm. This would also lead to greater efficiency in treatment, as a greater portion of the drug would be present at the site of cancer at one time, instead of circulating systemically throughout the body. Finally, there are positive effects in using magnetic induction to heat the iron oxide-based drug carriers. Heating these particles can cause hyperthermia in cells, which would be desirable if the cells are attached to cancer cells. Hyperthermia in cells is usually induced between the temperatures of 41 and 46°C.\textsuperscript{25} The survival of Chinese hamster ovary cells,
in particular, decreases exponentially as they are exposed to hyperthermic temperatures from 43 to 46°C.\textsuperscript{26}

1.4 Targeting of Medusa Particles

In previous studies, cRGDfk (Arginine-Glycine-Aspartate-D-Phenylalanine-Lysine) peptides have been attached to the surface of poly(ethylene glycol) molecules for the purpose of drug delivery (Figure 1.4). RGD peptides bind specifically to $\alpha V\beta_3$ and $\alpha V\beta_5$ integrins, which are heavily expressed on the endothelial cells of cancers. Furthermore, using RGD targeting in conjunction with a drug delivery system has shown to increase the facilitation of intracellular uptake of drug by the tumor cells via integrin-mediated endocytosis.\textsuperscript{10}

![Figure 1.4. Structure of cRGDfk peptide used as targeting ligand to bind overexpressed $\alpha V\beta_3$ and $\alpha V\beta_5$ integrins on various cancer cell lines](image)

Our group has shown compatibility of RGD peptides with polymer micelles, and those same polymers are used in medusa particles. The previously synthesized PEG-PCL can be terminated with a maleimide. Then, a cyclic RGD peptide can be attached to the maleimide
group via a Michael Addition. We expect the synthesis to be equally successful with medusa particles, although the route of functionalization would be slightly different.

First, the maleimide would be attached to the end of PEG in the PEG-PCL diblock. Then, the silane coupling agent would be attached to the alcohol terminus of PCL, which would be reacted with iron oxide nanoparticles to produce medusa particles. A cyclic RGD peptide would then be attached to the maleimide via a Michael Addition. Finally, the normal drug loading procedure would create DOX-loaded medusa particles functionalized with a surface cRGDFk peptide expressed on the terminal PEG corona.

Accordingly, based on earlier experiences in our group, we are confident that we can bind targeting groups to the periphery of medusa particles. However, targeting was not conducted as a part of this thesis, as the primary focus was to demonstrate magnetically triggered release.
2.1 Source of Chemicals and Materials

All chemicals were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, Missouri, United States), at least reagent grade, and used as received unless otherwise noted. Polyethylene glycol monomethyl ether (MeO-PEG, MW 2,000) was dried by heating to 60 °C under vacuum overnight stored in a desiccator containing Drierite®. The ε-caprolactone was distilled from calcium hydride and stored under nitrogen over molecular sieves (4 Å). Dialysis tubing, Spectra/Por® 7 MWCO 50 kDa standard pre-treated regenerated cellulose, was purchased from VWR International (Radnor, Pennsylvania, United States). 18.2 MΩ ultrapure water was obtained from a Thermo Fisher Scientific EasyPure II reservoir feed water purification system (Waltham, Massachusetts, United States).

10x PBS buffer was created by dissolving sodium chloride (80 g), potassium chloride (2 g), disodium phosphate (14.4 g), and monopotassium phosphate (2.4 g) in 800 mL ultrapure water, adjusting the pH to 7.4 with HCl, and then bringing the volume to one liter. The 10x PBS buffer was then diluted to 1x with ultrapure water for experimental use.

Cell lines based on CHO-K1 cells were purchase from ATCC (Manassas, Virginia, United States). Chemically defined EXCELL CD-CHO media from Sigma-Aldrich Chemical Co. (Saint Louis, Missouri, United States), supplemented with Penicillin-Streptomycin, 2 mM L-Glutamine and 1% anti-clumping agent from Life Technologies (Carlsbad, California, United
States), were used for the suspended CHO-K1 cells. Trypan blue stain, 0.4%, was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, United States).

2.2 Synthesis of Medusa Particles

2.2.1 Synthesis of Magnetite (Fe$_3$O$_4$) Nanoparticles

Magnetic nanoparticles were prepared by thermal decomposition of iron (III) oleate in refluxing 1-octadecene with modification using the synthesis developed by Hyeon and coworkers.\textsuperscript{28} X-ray diffraction curves of the nanoparticles were measured on a Bruker Advance D8 Powder Diffractometer with GAADS (Billerica, Massachusetts, United States) using a Co K$_\alpha$ source.

2.2.2 Synthesis of Diblock Copolymer

The diblock copolymer, MeO-EG$_{43}$-CL$_{20}$-OH, was synthesized by the tin(II)-catalyzed ring opening polymerization of $\varepsilon$-caprolactone from the alcohol terminus of poly(ethylene glycol) monomethylether, $M_a \sim 2,000$. The polymer was purified via recrystallization using a mixture of acetone and hexane.\textsuperscript{12}

2.2.3 $^1$H NMR Results for Diblock Copolymer

MeO-(EG)$_{43}$-(CL)$_{20}$-OH $^1$H NMR (CDCl$_3$) $\delta$ (ppm) 1.36 (m, 42 H), 1.61 (m, 80 H), 2.28 (t, 40 H), 3.36 (s, 3 H), 3.62 (s, 172 H), 4.04 (t, 37 H), 4.20 (t, 3 H).
2.2.4 Addition of Silane Coupling Agent

The silane coupling agent, 3-isocyanatopropyltriethoxysilane, was bound to the alcohol terminus of the diblock copolymer using a modification of a well-established procedure, as shown in Figure 2.2.4 below.²⁹,³⁰ To a 100 mL round-bottom flask was added 5.0 g of MeO-EG₄₃-CL₂₀-OH, 750 microliters of 3-isocyanatopropyltriethoxysilane, 10 mL of dry dimethylformamide, and 80 microliters of dibutyltin dilaurate catalyst. The reaction flask was heated in a silicon oil bath at 78°C and allowed to stir under nitrogen gas for 19 hours. The reaction was allowed to cool to room temperature before adding 40 mL of diethyl ether. The mixture was placed in a -30°C freezer overnight whereupon the polymer crystallized. The polymer was isolated via vacuum filtration using a Büchner funnel. The collected precipitate was repeatedly washed with diethyl ether to remove residual DMF and allowed to dry overnight under vacuum at room temperature. The addition of silane coupling agent to the alcohol terminus of MeO-EG₄₃-CL₂₀-OH can also be performed without DMF solvent. Using the same molar equivalencies, the diblock copolymer was melted and the silane linker and tin catalyst were added, resulting in the same product as verified by ¹H NMR. This synthesis route eliminates the need for removing residual solvent, although purification is still necessary to remove unreacted reagents.
2.2.5 \( ^1\)H NMR Results for Silane-Terminated Diblock

\[
\text{MeO-(EG)}_{43-\text{(CL)}}_{20-\text{OH}} \quad ^1\text{H NMR (CDCl}_3) \delta \text{ (ppm)} \quad 0.85 \text{ (t, 2 H), 1.23 (m, 29 H), 1.35 (m, 13 H), 1.61 (m, 20 H), 2.29 (t, 9 H), 3.36 (s, 1 H), 3.62 (s, 43 H), 3.70 (s, 2 H), 4.04 (t, 9 H)}.
\]

2.2.6 Addition of Silane-Terminated Diblock to Magnetite Nanoparticle Surface

The silane-terminated diblock copolymer (4.00 g) was then added to the surface of 11 nm nanoparticles (0.1993 g) in 35 mL of dry toluene (distilled from calcium hydride) in a 125 mL round-bottom flask while stirring under nitrogen for 42 hours at room temperature. After this period of time, the particles had dispersed in the toluene, creating a dark brown solution and no solid precipitant. Three volumes of hexane were added per volume of particle solution to precipitate the medusa particles, which were then filtered via vacuum filtration. The particles were dried at room temperature under vacuum overnight. Further purification of the medusa particles was required. The particles were dissolved in 15 mL THF in an Erlenmeyer flask. The
flask was placed on a high field neodymium iron boron permanent magnet. After sitting for 30 minutes, the particles were driven by the magnet out of the THF solution. This process was performed five times before the final precipitation, yielding approximately 60% from the crude product. The magnetic precipitation significantly expedites the purification process.

Transmission electron micrographs were acquired on an FEI Technai F20 transmission electron microscope (Hillsboro, Oregon, United States). Samples were deposited onto holey carbon coated copper TEM grids after dissolution in THF. Infrared spectra were obtained on a KBr pellet, containing about 5 mg of the medusa particles and 100 mg of dry KBr, on a JASCO FT/IR-4100 infrared spectrometer (Easton, Maryland, United States).

Dynamic light scattering was performed to determine the number average hydrodynamic diameter using a Malvern ZEN3600 obtained from Malvern Instruments (Malvern, Worcestershire, England), which employs a 632.8 nm helium neon laser.

2.3 Drug Loading of Medusa Particles

Doxorubicin was then loaded into the medusa particles. Doxorubicin HCl (3.9 mg) was added to 2 mL of dimethyl sulfoxide and 25 microliters of triethylamine. The TEA base was used for removal of the hydrochloride salt, encouraging the DOX to be trapped in the hydrophobic PCL core. This solution was combined with a solution of medusa particles (21.4 mg) in 5 mL methyl ethyl ketone. The resulting solution was added dropwise with probe sonication to 10 mL of 18.2 MΩ ultrapure water. The MEK was allowed to evaporate for 48 hours. The solution was then placed in dialysis tubing The unbound in 1 L of ultrapure water for two 3-hour intervals in a cold room in which the ultrapure water dialysate was replaced after the first interval in order to remove the unbound doxorubicin and DMSO. The loaded medusa
particles were then filtered through 0.45-micron syringe filters obtained from VWR International (Radnor Pennsylvania, United States). Ultrapure water was added to reach a constant solution volume of 25 mL. The concentration of doxorubicin was determined by measuring the absorbance of the solution using UV-Vis spectroscopy ($\lambda = 485$ nm), using a UV-2401PC UV-Vis Recording Spectrometer from Shimadzu Scientific Instruments (Columbia, Maryland, United States). The extinction coefficient of doxorubicin was 9380 M$^{-1}$cm$^{-1}$. Due to the amphiphilic nature of the medusa particles, they have low solubility in most solvents; thus, different solvents, including acetone, THF, and methyl ethyl ketone, were tried separately for drug loading in order to determine if the solvent used affected the drug loading efficiency.

2.4 Drug Release Studies

Drug release was determined using multiple methods. First, the particles were heated by magnetic induction to melt the semi-crystalline polycaprolactone shell in order to evaluate the magnetically triggered release of entrapped doxorubicin, which was monitored by both electrochemistry and UV-Vis spectrometry. This simulates the external ac magnetic field one could utilize for in vivo studies. Furthermore, an isothermal release profile was determined for medusa particles by placing samples in water baths set at different temperatures, and the release of drug was measured by UV-Vis spectrometry to determine the nature of release at various temperatures.

2.4.1 Electrochemical Monitoring of Drug Release

An electrochemical cell was setup to monitor release of doxorubicin on a nanomolar scale. 3 mL of sample was placed in a glass cylinder. A 2 mm diameter glassy carbon working
electrode, platinum wire counter electrode, and Ag/AgCl reference electrode were placed in the solution. The GC working electrode required polishing after each reading because the doxorubicin forms a monolayer on the electrode surface preventing sequential detection of oxidation peaks. Polishing was done manually on an alumina pad soaked in UP water. Linear sweep voltammetry and differential pulse voltammetry, using a CH Instruments Model 1000C Series Multi-Potentiostat (Bee Cave, Texas, United States), were employed to detect DOX release. The oxidation peaks were monitored using the following parameters for linear sweep voltammetry: \([0 \text{ Initial } E (V), 0.8 \text{ High } E (V), 0 \text{ Low } E (V), \text{ Positive Initial Scan Polarity}, 0.01 \text{ Scan Rate } (V/s), 4 \text{ Sweep Segments}, 0.001 \text{ Sample Interval } (V), 2 \text{ Quite Time } (s), 1e-5 \text{ Sensitivity } (A/V)]\). For differential pulse voltammetry, the following parameters were used: \([0.2 \text{ Initial } E (V), 0.6 \text{ High } E (V), 0.004 \text{ Increase Potential } (V), 0.05 \text{ Amplitude } (V), 0.05 \text{ Pulse Width } (s), 0.0167 \text{ Sample Width } (s), 0.5 \text{ Pulse Period } (s), 2 \text{ Quite Time } (s), 1x10^{-5} \text{ Sensitivity } (A/V)]\).

2.4.2 Drug Loading of Medusa Particles for Electrochemical Determination of Release

Using a similar method as mentioned above (2.3), doxorubicin was loaded into medusa particles for electrochemical monitoring of release. The electrochemical cell requires an ionic electrolyte and PBS buffer was adequate. Doxorubicin HCl (4.0 mg) was added to 2.0 mL of DMSO and 25 microliters of triethylamine. This solution was combined with a solution of medusa particles (20.5 mg) in methyl ethyl ketone. The DOX/medusa particle solution was then added dropwise with ultraprobe sonication to 10 mL of 1x PBS buffer. The MEK was allowed to evaporate for 48 hours. The unbound doxorubicin was removed by dialyzing against 1 L of 1x PBS buffer for six hours in a cold room, replacing the 1x PBS dialysate after the first three hours. The loaded medusa particles were then filtered through 0.45-micron syringe filters. 1x PBS
buffer was then added to reach a constant solution volume of 25 mL. The concentration of doxorubicin was then determined using UV-Vis spectroscopy ($\lambda = 485$ nm).

2.4.3 Setup of Hyperthermia Coils for Magnetic Release

Magnetic heating was conducted using custom hyperthermia coils from Induction Atmosphere (Rochester, New York, United States). A Novastar 5-kW power supply from Ameritherm Inc. (Scottsville, New York, United States) was connected to the coils (Figure 2.4.1). 18°C water was circulated through the coils using a chiller bath (Model JT1000, Koolant Koolers, Kalamazoo, Michigan, United States). The coils had 4 total turns with pairs of two coil turns separated by enough space to place a petri dish. Each turn is 0.6 cm in height, ranging 4.4 cm from top to bottom, and the coils had a 4 cm inner diameter and each set of two turns was 1.2 cm in height separated from the other two turns by 2 cm. Depending on the size of the experimental samples, the samples were placed in 10 mL centrifuge tubes (Figure 2.4.2) or a 3D-printed custom plastic vessel that can hold three 1 mL Eppendorf tubes (Figure 2.4.3). The samples were placed in the center of a section of turns to maximize interaction with the magnetic field. Although the coils were chilled, they did heat slightly during the course of an experiment. This required insulation of the sample from the coils. The sample container was insulated with foam to prevent dissipation of heat from the coil surface to the sample solution; thus, all heat produced in solution should ideally be a result of magnetic heating via induction.

The magnetic coils were set to the following standard parameters: 750 V, 430 kHz, 480 G. These were the base internal parameters used for all magnetic heating experiments with the hyperthermia coils. If an isothermal or maximum temperature was desired in an experiment, the
voltage applied to the copper coils could be manually lowered to a fraction of the 750 V maximum preset.

An FLIR E40 Infrared Thermal Imaging Camera (Wilsonville, Oregon, United States) was positioned directly above the magnetic coils to monitor the temperature. The camera should not be too close to the experimental setup or the magnetic field generated by the coils will damage the camera. Multiple focal points could be used simultaneously to monitor the temperature of the coils and multiple regions within the sample itself. The infrared camera was attached via USB to a monitor for real time observation of temperature.

![Figure 2.4.1 Novastar 5 kW power supply used for hyperthermia coils. The display selector allows manual manipulation of settings. To maintain an isothermal bulk solution temperature, we could adjust the voltage to a fraction of the 750 V preset, such as 85% shown here.](image)
2.4.4 Isothermal Release Studies

DOX loaded medusa particle samples in 1x PBS were also analyzed for temperature dependent release of drug. In triplicate, 3.5 mL samples were placed in dialysis tubing in jars containing 350 mL of pure 1x PBS buffer, which were placed in a Thermo Fisher Scientific Precision Shaking Water Bath (Waltham, Massachusetts, United States), as shown in Figure 2.4.4. The 350 mL PBS solutions were allowed to sit in the water baths for 24 hours prior to administering the drug loaded samples. This allowed the water bath solutions to come to the desired release temperatures. Isothermal release studies were conducted for five temperatures: 6°C, 27°C, 32°C, 37°C, 47°C, and 58°C. 1.5 mL aliquots were taken from each dialysis bag at
various time points and the UV-Vis absorption ($\lambda = 485$ nm) was recorded to calculate the concentration over a period of time. The aliquots were placed back in the dialysis tubing after readings were done. Absorption readings were conducted at 0 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, and 24 hours in triplicate, i.e. measurement on three replicate samples. Temperature was maintained using the programmed settings of the water bath and verified using both a mercury thermometer and a Sper Scientific 800005 Advanced Thermocouple Thermometer (Scottsdale, Arizona, United States).

Figure 2.4.4 Experimental setup of water bath with jars containing 350 mL of ultrapure water and one dialysis bag containing 3.5 mL of DOX loaded medusa particles. The water bath maintained the isothermal temperature digitally and was further verified via mercury and electronic thermometers.
2.4.5 Magnetically Triggered Release of Doxorubicin

DOX loaded medusa particle samples in 1x PBS buffer (10 mL) were placed in the magnetic coils for 30-120 minutes depending on the experiment. To quantify the amount of doxorubicin released, two primary methods were used: electrochemical monitoring and UV-Vis absorbance. For experiments monitored electrochemically, 1.5 mL aliquots were removed from the solution after each 30-minute interval and electrochemically analyzed via linear sweep or differential pulse voltammetry to determine release of doxorubicin, as the height of the oxidation peak correlates to the concentration of DOX detected. A control reading was taken prior to magnetic heating to determine the concentration of free doxorubicin. The aliquot’s oxidation peaks were compared to a calibration curve of pure doxorubicin in 1x PBS buffer at different concentrations ranging from about 3 to 225 μM doxorubicin for linear sweep voltammetry and about 2 to 121 μM doxorubicin for differential pulse voltammetry.

To create these calibration curves, DOX was dissolved in 1x PBS and then the initial concentration was determined using UV-Vis (λ = 485 nm) to determine the concentration, which was concluded to be 225 and 121 μM for the respective experiments. The remaining samples were then created by simply diluting half of the preceding sample with pure 1x PBS buffer to reach the desired concentrations. The concentrations used for the linear sweep voltammetry calibration curve were 3.5, 7.0, 14.1, 28.2, 56.3, 112.7, and 225.3 μM DOX-PBS, the concentrations for the differential pulse voltammetry calibration curve were 1.9, 3.8, 7.6, 15.1, 30.2, 60.4, and 121 μM DOX-PBS. Each of these samples were then monitored by the respective electrochemical method in triplicate to establish a calibration curve.

For the UV-Vis absorbance monitoring experiments, 1 mL aliquots were taken after each 30-minute interval of magnetic heating for 90 minutes, in addition to a control aliquot, and both
experimental and control samples were placed in 1 mL, 50 kD MWCO Spectra/Por® G2 Float-A-Lyzers obtained from VWR International (Radnor, Pennsylvania, United States). On the day of experimentation, the Float-A-Lyzers were prepared as instructed in the packing, including a 20-minute wash with a 10% ethanol solution followed by a 15-minute wash in ultrapure water. The Float-A-Lyzers were placed in a 1x PBS sink (1 L) for six hours allowing the free, released DOX to be removed. After this time, the retentate concentrations were determined using UV-Vis to monitor the absorbance at 485 nm.

2.5 Cell Study

Cell studies were conducted on CHO-K1 cells as a method to analyze the non-specific targeting of DOX loaded medusa particles. The cell studies were also useful as a further method of tracking the release of DOX from medusa particles, supplementing the electrochemical and UV-Vis absorption monitoring methods.

2.5.1 Preparation of Cells

CHO cells in the exponential growth phase were used at about $10^6$ cells per mL cell density. Suspended CHO cells were maintained in 125 mL shaker flasks placed on a 15 mm diameter mini shaker set at 120 rpm in a Symphony™ Water Jacketed CO$_2$ Incubator maintained at 37°C and obtained from VWR International (Radnor, Pennsylvania, United States). Cells were passaged every three days and the viability of the cells in the suspension culture was maintained at greater than 90 percent. For each experiment, 200 microliters of suspended cells were placed into a 96-well plate and then the experimental and control groups were made.
2.5.2 Live/Dead Assay

In order to determine the viability of CHO cells, trypan blue stain was employed. 50 microliters of suspended cells were mixed with 10 microliters of trypan blue stain. Live and dead cell counts were performed using a Brightline Hemacytometer obtained from Hausser Scientific (Horsham, Pennsylvania, United States), ensuring that at least 100 cells were counted per reading if present. The cell depth was 0.100 mm deep, and the glass slide was 1.5 mm thick with a 0.1 mm slide cover.

2.5.3 Experimental Design of Cell Study

To determine the effect of magnetically heated DOX loaded medusa particles on CHO-K1 cells, we exposed the cells to a variety of experimental conditions and analyzed the cell viability and density percentages. For all cells experiments, the control and experimental samples were mixed with CHO cells at a known concentration. Autoclaved DI water was used as a negative control. As a positive control, a dose-response curve was created for CHO cells in pure DOX-PBS. In addition to these positive and negative control groups, a control for exposure to the magnetic field was employed to ensure that the magnetic field itself was not inducing toxicity. This magnetic field control consisted of CHO cells in autoclaved DI water exposed to the magnetic field for 60 minutes. Furthermore, we included further control groups for the 1x PBS buffer used for all of the experimental groups and the DOX dose-response curve. These two control groups were exposed to 1x PBS buffer (4%) with magnetic heating for 60 minutes and without magnetic heating, respectively. The experimental groups consisted of DOX loaded medusa particles with 60 minutes of magnetic heating and without magnetic heating, respectively.
All experimental and control groups consisted of 4% sample (8 microliters) and 96% suspended CHO cells (192 microliters). After exposing the magnetic heating groups to the field for 60 minutes, all experimental and control groups were placed on the shaker in the incubator for 32 hours. After the 32 hours, live/dead assays were performed on the cells, and the percent cell viability and cell density values were determined.

2.5.4 Dose-Response Curve

A dose-response curve was constructed for doxorubicin against CHO-K1 cells. For each sample, 96% (192 microliters) CHO-K1 cells in suspension were exposed to 4% (8 microliters) doxorubicin-PBS samples at known concentrations. The concentrations used were 6.47, 12.93, 25.86, 51.73, 103.45, and 206.90 μM. The first sample was made and back calculated using UV-Vis spectroscopy (λ = 485 nm) to determine its concentration, which was concluded to be 206.90 μM. The remaining samples were then made by simply diluting half of the previous sample with pure 1x PBS buffer to reach the desired concentrations.
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Characterization of Iron Oxide Nanoparticles

Thermal decomposition of iron (III) oleate in 1-octadecene gave spherical particles with an average diameter of 11 +/- 2 nm, as determined by TEM. The size distribution plot is shown in Figure 3.1.1. Magnetite and maghemite have similar unit cell sizes, which can make it difficult to differentiate between the two phases. Gorski and Sherer demonstrated that the phase composition could be determined by either Mossbauer spectroscopy or X-ray diffraction. Figure 3.1.2 displays the data from Table 3.1 of the x-ray diffraction results for the iron oxide nanoparticles, which compares peak position resulting unit cell size for our particles versus literature results. The peak positions for the particles reported here were very close to the literature values for magnetite, and a value of 836.89 nm was calculated for the unit cell size; however, this was not sufficient evidence to differentiate between magnetite and maghemite.
Figure 3.1.1 TEM image (left) and histogram (right) of particles sizes for the particles. A total of 150 particles were counted and the average particle diameter was 11 +/- 2 nm.

Figure 3.1.2 X-ray diffraction curve for iron oxide nanoparticles
Table 3.1. Comparison of the X-ray diffraction peaks and the unit cell size from this work with those observed in the literature for magnetite.

<table>
<thead>
<tr>
<th>Diffraction Peak</th>
<th>Our work</th>
<th>Gorski and Scherer$^{32}$</th>
<th>Nakagiri, et al.$^{33}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>35.14°</td>
<td>35.12°</td>
<td>35.11°</td>
</tr>
<tr>
<td>311</td>
<td>41.60°</td>
<td>41.24°</td>
<td>41.43°</td>
</tr>
<tr>
<td>400</td>
<td>50.59°</td>
<td>50.33°</td>
<td>50.50°</td>
</tr>
<tr>
<td>511</td>
<td>67.35°</td>
<td>67.12°</td>
<td>67.30°</td>
</tr>
<tr>
<td>440</td>
<td>74.25°</td>
<td>74.02°</td>
<td>74.21°</td>
</tr>
<tr>
<td>Unit cell size (pm)</td>
<td>836.89</td>
<td>839.4</td>
<td>839.6</td>
</tr>
</tbody>
</table>

The nanoparticles were further characterized using x-ray photoelectron spectroscopy (XPS). An XPS spectrum for the Fe 2p binding energy region will show satellite peaks in the maghemite phase not found in the magnetite phase. In Figure 3.1.3, an XPS spectrum for the 11 nm iron oxide particles shows small satellite peaks near 718 and 730 eV, indicative of a mixed phase between magnetite and maghemite. Particles on the smaller size of the distribution (around 5 nm) tend to prefer the maghemite phase, whereas larger particles (around 25 nm) tend to prefer the magnetite phase.
3.2 Characterization of Polymers

The MeO-EG-CL-OH diblock was previously characterized by our group using $^1$H NMR and the degree of polymerization was determined to be 43 for the EG block and 20 for the CL block (Figure 3.2.1).\textsuperscript{5}
The silane-terminated diblock was also characterized using $^1$H NMR (Figure 3.2.2). Integration and identification of several peaks was difficult due to overlap as well as the small concentration of silane coupling agent compared to polymer. The methylene closet to the Si group can be seen at 0.85 ppm. There are also traces of DMF, acetone and water in the sample. Other silane coupling agent hydrogens are likely buried under the PCL and PEG peaks.
Characterization of Medusa Particles

3-isocyanatetriethoxysilane was bound to the alcohol terminus of MeO-(PEG)$_{43}$-(PCL)$_{20}$-OH resulting in MeO-(PEG)$_{43}$-(PCL)$_{20}$-O(CH$_2$)$_3$SiOEt$_3$, enabling covalent attachment to iron oxide nanoparticles. The silane-terminated diblock polymer was attached to the surface of magnetite nanoparticles.$^{29,30}$ In Figure 3.3.1, transmission electron microscopy images demonstrate the spherical structure of the medusa particles. However, the TEM images only display the particle, as the polymer shell is transparent to the electron beam. A high resolution TEM was conducted to attempt visualization of the Si-O shell around the surface of the particle, but a sharp image could not be obtained due to interference by the polymer. Future work should involve obtaining a sharper high resolution TEM image using plasma etching on a silicon
monoxide grid to etch away the polymer, presumably leaving the iron oxide and silicate shell intact.

Figure 3.3.1 TEM image of medusa particles with 20 nm bar (right) and high resolution TEM with 5 nm bar (left) show the size of the spherical particles without the polymer shell. It was not possible to obtain a sharp high resolution image due to polymer coating interference.

Figure 3.3.2 Triangular model representation of three oxygen atoms used to determine the geometrical surface area of a silane coupling agent

Figure 3.3.3 Tripod of oxygen atoms on silane coupling agent covalently binding to the magnetite surface.
Plane geometry was used to estimate the surface area of one silane coupling agent. A self-assembly model was assumed with the silane bound to the particle surface through three oxygen atoms, as modeled in Figures 3.3.2 and 3.3.3. The oxygen atoms form a triangle. Assuming the O-Si-O bond angle is a 109.5° tetrahedron and the Si-O bond length is 161 pm, the distance between the oxygen atoms can be determined. The three oxygen atoms form a triangle (blue triangle) with an inter-nuclear distance of 262 pm. The area occupied by the black triangle is assumed to be the area occupied by a silane coupling agent. To calculate the value of this area, the length of the base of the black triangle and the height of the black triangle must be determined. Both the blue triangle and the black triangle share the same central point. The distance from this point to one of the corners of the black triangle (the red line) is the sum of the distance from the center to the corner of the blue triangle plus the radius of the oxygen atom. The distance from the center point to the corner of the blue triangle can be calculated. Galeoti and coworkers functionalized the surface of 20 nm (average diameter) magnetite particles with silane coupling agents and used TGA to determine the surface coverage. Assuming the ideal conditions in which the silane coupling agents form a monolayer on magnetite nanoparticles with three oxygen atoms on the surface, we estimate there to be 15 silane molecules per nanometer.

To obtain an experimental estimate of the number of polymer chains bound to the surface of each iron oxide nanoparticle, a carbon hydrogen combustion analysis was performed on the medusa particles in duplicate by Atlantic Microlab, Inc. (Norcross, Georgia, United States). This gave values of the percent carbon (50.95, 50.85) and percent hydrogen (7.51, 7.44). Using the average diameter of the iron oxide nanoparticles as determined by TEM imaging, we can estimate the volume and surface area of the nanoparticles. By systematically varying the number of polymer molecules in our calculations, we can estimate the percent composition of various
elements within the medusa particle structure based on the PEG\textsubscript{43}-PCL\textsubscript{20} diblock and the iron oxide nanoparticle core. Based on these calculations and the carbon hydrogen combustion analysis indicating an average of 55.90% carbon and 7.47% hydrogen for the medusa particles, we estimate there to be about 2000-2500 polymer molecules bound to the surface of each particle. The full list of calculations can be found in Appendix B.

Infrared spectroscopy was performed on two different medusa particle batches based on the same synthetic route, resulting in the same peak expression. Magnetite nanoparticles generally show a peak near 565 cm\textsuperscript{-1}.\textsuperscript{34-36} The peaks in Figure 3.3.4 were dominated by the presence of MeO-PEG\textsubscript{43}-PCL\textsubscript{20}-OH diblock, which may have obscured peaks due to the iron oxide particles. The methylene group symmetric and antisymmetric stretching modes were seen in the range of 2800-3000 cm\textsuperscript{-1}. A strong peak at 1112 cm\textsuperscript{-1} was due to the aliphatic ether stretching mode (C-O-C). A moderately strong peak was due to the ester stretching mode (C-O-C). Two small, sharp peaks at 1042 cm\textsuperscript{-1} and 1061 cm\textsuperscript{-1} likely indicate the expected Si-O-Si stretching, although they are obscured by the PEG and PCL C-O-C stretching. The intense, sharp peak at 1724 cm\textsuperscript{-1} indicates the carbonyl (C=O) stretching. This peak was also shown in the Raman spectra of polycaprolactone and was assigned to the crystalline phase. A peak at 1733 cm\textsuperscript{-1} was assigned to the carbonyl (C=O) stretching of the amorphous phase. Furthermore, the sharpness of the crystalline peak and the positioning at 1724 cm\textsuperscript{-1} suggests a semi-crystalline polycaprolactone block with a high level of crystallinity. It was concluded that in the solid state the polycaprolactone block was highly crystalline, presumably forming a semi-crystalline layer around the magnetite nanoparticle.
Dynamic light scattering performed on the medusa particles indicated a number average hydrodynamic diameter of 18.17 nm (Figure 3.3.5). This diameter was larger than the 11 nm diameter as determined by TEM imaging. This value increases due to the covalently attached polymer shell that was transparent to the electrons of the TEM beam. The particles tended to assemble into clusters. Using an equation from Devanand and Selser to determine the radius of gyration for PEG based on its molecular weight, we determined the radius of gyration for our PEG-2000 to be about 1.8 nm.\(^{37}\) Using this radius of gyration, the hydrodynamic average of the medusa particles and the average diameter of the iron oxide nanoparticles, we estimate that the PCL core in which we trap the drug is approximately 1-2 nm in diameter.
Drug Loading Results for Medusa Particles

Using doxorubicin, drug loading experiments were performed in tandem with drug release experiments. The release experiments being performed on the sample determined which experimental method to use. For the electrochemical monitored release experiments, it was necessary to use 1x PBS buffer, whereas UV-Vis absorption monitoring did not require this ionic presence. However, there were several experiments in which 1x PBS buffer was used for both electrochemical and UV-Vis monitored release of drug. Doxorubicin HCl was added to DMSO and TEA, and then combined with medusa particles dissolved in various organic solvents. Medusa particles showed the best dispersion in methyl ethyl ketone (about 2.5 mg/mL), although they were successfully dispersed in acetone (1.7 mg/mL) and tetrahydrofuran (1.5 mg/mL) as well. The combined DOX/medusa particle solution was then added dropwise with probe sonication to ultrapure water, which demonstrated to be significantly more effective for dispersion in water than typical stirring techniques. Using the Beer’s Law equation \(A = \varepsilon bc\), we determined the concentration of doxorubicin based on the absorbance \((\lambda = 485 \text{ nm})\) reading determined by UV-Vis spectroscopy and the extinction coefficient for doxorubicin \((\varepsilon = 9380 \text{ M}^{-1}\text{cm}^{-1})\) with a 1 cm path length. A linear slope following the initial nanoparticle peak was used to
reach a true baseline for absorbance, as the concentration of DOX would appear much higher than actual without subtracting the baseline.

Loading of doxorubicin into medusa particles was successful. The process of dialyzing followed by syringe filtering the samples ensures that all DOX not loaded into the particles is removed; therefore, peaks in UV-Vis at 485 nm must be a result of DOX trapped within the medusa particles.

Loading calculations were completed by either determining the percentage of the total weight of the medusa particles or by the percentage of the weight of the polymer core. These loading determinants are calculated using the following equations (Eq. 1-5):

\[
\frac{\text{Absorbance}}{\text{Extinction coefficient} \times \text{Path length}} = \text{Concentration} \quad (\text{Eq. 1})
\]

\[
\text{Concentration} \times \text{Volume of sample} = \text{mol of DOX} \quad (\text{Eq. 2})
\]

\[
\text{mol DOX} \times \text{MW DOX} = \text{grams of DOX} \quad (\text{Eq. 3})
\]

\[
\frac{\text{grams DOX}}{\text{grams medusa particles used}} \times 100 = \% \text{ loading by total weight} \quad (\text{Eq. 4})
\]

\[
\% \text{ loading by total weight} \times \frac{\text{Percent PCL of medusa particles}}{100} = \% \text{ loading by weight of core} \quad (\text{Eq. 5})
\]

The encapsulation efficiency was also calculated by comparing the amount of DOX actually loaded into the medusa particles versus the amount of DOX used in the loading procedure. Table 3.4.1 shows the average drug loading results in the three organic solvents used, and Table 3.4.2 shows the average drug loading results in ultrapure water versus 1x PBS buffer. No significant difference was seen between samples that used water versus 1x PBS buffer. Ultrapure water showed an average encapsulation efficiency of 14.3 +/- 0.2 and an average loading by percent total weight of 2.5 +/- 0.1. 1x PBS buffer showed an average encapsulation efficiency of 16 +/- 5 and an average loading by percent total weight of 3 +/- 1. In consideration
of organic solvents used to dissolve the medusa particles, MEK showed the highest
capsulation efficiency and loading by percent core weight. This may be a result of MEK’s
ability to disperse the medusa particles better than the other solvents. Although the standard
deviation appears high for the MEK values, this comes as a result of two loading experiments
that resulted in significantly higher values for all three loading determinants. There was also a
qualitative color difference when using different solvents. THF and MEK both produced bright
orange-red solutions, whereas acetone produced a deep purple solution. All of the experimental
data, including the amount of particles and what solvents were used in each loading experiment,
can be found in the Appendix.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Encapsulation Efficiency (%)</th>
<th>Loading (% by total weight)</th>
<th>Loading (% by core weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>13 +/- 2</td>
<td>2 +/- 1</td>
<td>4 +/- 1</td>
</tr>
<tr>
<td>Methyl Ethyl Ketone</td>
<td>17 +/- 5</td>
<td>3 +/- 1</td>
<td>6 +/- 2</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>14</td>
<td>2.5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.4.2. Average drug loading results in UP water versus 1x PBS buffer

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Encapsulation Efficiency (%)</th>
<th>Loading (% by total weight)</th>
<th>Loading (% by core weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrapure Water</td>
<td>14.3 +/- 0.2</td>
<td>2.5 +/- 0.1</td>
<td>4.9 +/- 0.3</td>
</tr>
<tr>
<td>1x PBS Buffer</td>
<td>16 +/- 5</td>
<td>3 +/- 1</td>
<td>6 +/- 3</td>
</tr>
</tbody>
</table>
3.5 Isothermal Release from Medusa Particles in Triplicate

Isothermal release was induced by placing 3.5 mL DOX loaded medusa particle samples into dialysis bags inside jars within 350 mL of pure ultrapure water or 1x PBS buffer depending on the experiment. The 10x volume (350 mL) dialysate samples were used to ensure a sufficient sink for release of drug. These jars were contained within an isothermal shaker bath set to various temperatures. 27°C was used to simulate release at ambient temperatures. 37°C was used to simulate body temperature. 32°C was used merely as a data point between ambient and body temperatures. 47°C and 58°C were used to simulate temperatures above the melting point of the semi-crystalline polycaprolactone polymer. 6°C was used as a negative control to show the release profile when drug loaded medusa particles were kept in a refrigerator. At fixed time intervals, UV-Vis absorption readings (λ = 485 nm) were taken on 1.5 mL aliquots to monitor the release of drug over time at the various temperatures, and then the aliquots were replaced in the dialysis bags. Ideally, no drug would be released from the medusa particles below the melting point of the polymer (about 41°C). The isothermal release profiles of 37°C and 58°C demonstrate significant temperature dependence of release (Figure 3.5.1). However, in Figure 3.5.2, the isothermal release profiles normalized to the 6°C release are shown, showing significant release of drug below the ideal 37°C temperature.

Crank’s model of late time release from a polymer sphere was applied to the isothermal release values for each temperature. This model is effective for predicting the diffusion coefficient from spherical molecules at times after 40% of the maximum release has been reached based on the radius and infinite release of the sphere, and the equation is shown below (Eq. 6). The release data fit to this model is shown in Table 3.5.
\[ M_t = M_\infty \left( 1 - \frac{6}{\pi^2} x e^{-\frac{\pi^2 \Delta t}{r^2}} \right) \]  
(Eq. 6)

Table 3.5. Release data fit to Crank’s model for late time release from a polymer sphere

<table>
<thead>
<tr>
<th>Temperature</th>
<th>( M_\infty )</th>
<th>( D/r^2 ) (hr(^{-1}))</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6°C</td>
<td>63 +/- 3</td>
<td>0.0228 +/- 0.005</td>
<td>0.8574</td>
</tr>
<tr>
<td>27°C</td>
<td>70 +/- 11</td>
<td>0.0133 +/- 0.085</td>
<td>0.7295</td>
</tr>
<tr>
<td>32°C</td>
<td>96 +/- 2</td>
<td>0.0307 +/- 0.003</td>
<td>0.9579</td>
</tr>
<tr>
<td>37°C</td>
<td>97 +/- 3</td>
<td>0.0395 +/- 0.007</td>
<td>0.9445</td>
</tr>
<tr>
<td>47°C</td>
<td>101 +/- 3</td>
<td>0.0526 +/- 0.009</td>
<td>0.9428</td>
</tr>
<tr>
<td>58°C</td>
<td>100 +/- 3</td>
<td>0.0590 +/- 0.009</td>
<td>0.9476</td>
</tr>
</tbody>
</table>
Figure 3.5.1 Isothermal release profiles of DOX from medusa particles at 37°C and 58°C throughout first six hours shows difference between body temperature and severely hyperthermic temperatures with error bars cut off below 0 and above 100% release.

Figure 3.5.2 Isothermal release profiles of DOX from medusa particles highlighting the dependence of release on increasing temperature with error bars cut off below 0 and above 100% release.
3.6 Magnetic Heating of Medusa Particles

DOX loaded medusa particles were magnetically heated via induction by the custom hyperthermia coils as described. The bulk solution temperature was measured using an infrared camera, allowing simultaneous monitoring of multiple regions in the solution as well as the coil temperature (Figure 3.6). The maximum bulk solution temperature resulting from magnetic induction was concluded to be about 75°C after two hours of heating at the maximum voltage. After the two hours, the temperature remained roughly constant. The initial temperature of the bulk solution in various experiments ranged from 18-22°C. This demonstrates a significant bulk solution temperature increase, although a large increase in bulk solution temperature was not seen in every experiment, especially those with small volumes in Eppendorf tubes. It is important to note that the coil temperature remained at about 24-28°C throughout all experiments, which shows that the bulk solution temperature increase was not merely a result of dissolution of heat from the coils, but rather from magnetic induction of the iron oxide nanoparticles.

![Figure 3.6 Thermal image of real time magnetic heating using IR camera attached to monitor in which the temperature of multiple regions in the sample (1/2) and the coils (3) are simultaneously monitored](image)

Figure 3.6 Thermal image of real time magnetic heating using IR camera attached to monitor in which the temperature of multiple regions in the sample (1/2) and the coils (3) are simultaneously monitored.
3.7 Electrochemical Monitoring Results for Magnetically Triggered Release

Doxorubicin concentration can be monitored electrochemically, as its concentration is proportional to oxidation/reduction currents produced. The hydroquinone in DOX can undergo oxidation and the quinone can undergo reduction (Figure 3.7.1). Throughout our experiments, we only measured the oxidation currents because this method does not require purging the sample with nitrogen to remove oxygen from the air; however, measuring reduction current is also an effective method for predicting DOX concentration and could be utilized in further experiments. The setup of our electrochemical cell is shown in Figure 3.7.2.

Figure 3.7.1 Redox reaction of doxorubicin measured by LSV or DPV and the structure of doxorubicin-HCl showing hydroquinone and quinone groups
Using linear sweep and differential pulse voltammetry, magnetically triggered release of doxorubicin from medusa particles was monitored. To quantify the concentration of released drug, a calibration curve was created for each method using pure DOX in 1x PBS buffer (Figure 3.7.3). The plot of peak current as a function of concentration was linear in the concentration range of 1.9 to 60.4 μM. The data point at a higher concentration fell below this line, which may be indicative of adsorption of DOX, or its oxidative products, onto the glassy carbon electrode, which would block the electrode surface, thereby decreasing the peak current.

![Electrochemical cell setup](image)

**Figure 3.7.2** Electrochemical cell setup used for both linear sweep voltammetry and differential pulse voltammetry showing the glassy carbon working electrode (green), Ag/AgCl reference electrode (white) and platinum counter electrode (red)

Electrochemical monitoring of DOX release using linear sweep voltammetry was difficult to interpret. The non-faradaic current created a significant sloping baseline that made the oxidation peak hard to resolve, and thus, irreproducible and unreliable. Only at concentrations above 30 μM could the peak be distinguished from the charging current. The current produced
by magnetically triggered DOX release from medusa particles as measured by linear sweep voltammetry fell below this concentration. As a result, we used differential pulse voltammetry instead as a more effective way of monitoring DOX release electrochemically. DPV superimposes a square wave onto the sweeping potential, which helps to remove the non-faradaic current. This gave more consistent results at lower concentrations, and the oxidation peak could be clearly resolved from the baseline. Figure 3.7.4 shows the difference in LSV and DPV curves.

![Graph showing DOX calibration curve determined using DPV when compared to Ag/AgCl reference electrode. The parameters of DPV can be found in Chapter 2.4.1. The error bars were cut off below zero microamps.](image)

Figure 3.7.3 Plot of DOX calibration curve determined using DPV when compared to Ag/AgCl reference electrode. The parameters of DPV can be found in Chapter 2.4.1. The error bars were cut off below zero microamps.
Magnetically triggered release of doxorubicin from medusa particles was demonstrated and monitored using DPV (Figure 3.7.5). The current shown for all of these groups has been baselined to a control group whose current was determined before experimentation to factor out
any free DOX that had been released prior to heating. The Room 30 and Room 60 secondary control groups were left covered on the benchtop at room temperature for 30 or 60 minutes, respectively, while the Water Bath 30 and Water Bath 60 experimental groups were placed in a water bath at 43°C for the respective amounts of time and the MFH 30 and MFH 60 experimental groups were placed in the magnetic field setup at maximum voltage for the respective amounts of time. The bulk solution temperature of the magnetically heated samples never reached 43°C in this experiment, but instead remained in the range of 30-34°C. This shows that the amount of drug released from medusa particles is not merely a result of the bulk solution temperature, as the MFH groups released more DOX than the water baths groups at both 30 and 60 minutes. However, as can be seen in the room temperature secondary control currents, there is release of DOX below the desired febrile temperature indicating premature leakage of drug.

Figure 3.7.5 Plots of oxidation currents produced by DOX when compared to Ag/AgCl reference electrode as measured by DPV demonstrating magnetic release of DOX from medusa particles after 30 or 60 minutes in a magnetic field as compared to control samples at room temperature outside of field.
3.8 UV-Vis Absorbance Monitoring Results from Magnetically Triggered Release

DOX-loaded medusa particles were heated for 30 minutes either in a centrifuge tube in a water bath set to 43°C or by magnetic induction using the hyperthermia coils maintaining a bulk solution temperature of 43°C. After 30 minutes, the samples were immediately dialyzed for 3 hours against pure 1x PBS buffer in Float-A-Lyzers placed in a jar in a cold room to remove released DOX. The absorbance of each sample was then measured using UV-Vis spectroscopy to determine the concentration remaining, and thus, the amount of released DOX in each experimental group (Figure 3.8).

![Figure 3.8 Plot of UV-Vis monitored magnetic release of DOX from medusa particles](image-url)
3.9 Comparison of Electrochemically Monitored and UV-Vis Monitored Release of DOX

Using both UV-Vis and differential pulse voltammetry, magnetically triggered release of doxorubicin from medusa particles was demonstrated. In addition to wanting to measure release using multiple methods for the purpose of verification of results, we also used both methods because they are quite different in how they determine DOX concentration. UV-Vis measures the absorbance at 485 nm, the approximate chromophore wavelength of doxorubicin, which can be used to extrapolate an estimate of the concentration of DOX in a sample. However, UV-Vis cannot distinguish between DOX within the core of medusa particles and DOX free in solution that has been released by medusa particles.

DPV measures the oxidative current produced by doxorubicin, and this current is proportional to the concentration of doxorubicin free in solution. Using this method, we can distinguish between DOX within the core of medusa particles and DOX free in solution because the electrode monitoring current must come within about 10 angstroms of the doxorubicin to detect its current. As shown in Chapter 3.3, the medusa particles used have an average hydrodynamic diameter of about 18 nm; therefore, the electrode should not be able to detect the current of DOX bound to the core of the particles. This leads us to conclude that the DPV results may be more accurate in monitoring drug release than UV-Vis, although both methods have their limitations.

When using UV-Vis to monitor release of DOX from medusa particles, we dialyze samples after inducing release to remove unbound DOX. As shown earlier in this chapter, the concentration of DOX appears to go down even in samples kept in a refrigerator when dialyzed, which may indicate that DOX loaded medusa particles are adhering to the dialysis membrane.
Furthermore, there is a potential for dilution of samples as a fraction of the dialysate may leak into the sample as during removal simply as a result of human error.

On the other hand, while DPV can monitor the concentration of DOX directly from an experimental sample, the glassy carbon working electrode must be polished between each reading as the doxorubicin forms a monolayer on the electrode during oxidation. The degree to which the electrode is polished between each reading as well as variations in concentrations of DOX leading to different kinetics in monolayer formation lead to potential error in DPV. However, by performing five replicates for each group in DPV this potential error was reduced.

There was a significant amount of release even for the control group (purple) after 30 minutes. This may be a result of the medusa particles prematurely leaking drug below the desired febrile temperatures, as the control sample was allowed to sit at room temperature for the time interval. There was a greater amount of release in both the water bath and magnetic heating groups than the control group after 30 minutes as predicted because the temperature of these samples were held at 43°C. Furthermore, the magnetic release sample had the greatest amount of drug release. This result indicates that the local temperature of the magnetically heated samples is higher than the water bath, despite having the same bulk solution temperature. An increased local temperature at the surface of the nanoparticles could cause greater or faster melting of the polymer, which would lead to a greater amount of drug release. This finding is significant as it shows that drug release from medusa particles can be triggered by an external ac radio frequency magnetic field, although the release of drug seen in the Control 30 sample is not desired.
3.10 Cell Study Results

3.10.1 Cell Viability and Statistical Data

Cell viability was monitored using a hemocytometer with trypan blue stained suspended cells. The percent viability of cells was calculated using the following equation:

\[
\% \text{ Cell Viability} = \frac{\text{Live Cells}}{\text{Total Number of Cells}} \times 100
\]

(Eq. 7)

\[
\text{Cell Density} = \frac{\text{Cell Count per Square} \times \text{Dilution Factor}}{\text{Volume of Square}}
\]

(Eq. 8)

All cell experiments were conducted in triplicate measurements of control and experimental groups. The percent viability and density were normalized to the control. For our cell studies, the dilution factor was 1.2 and the volume was \(10^{-4}\) m\(^3\). A standard deviation calculation was performed to assess the distribution of cell viability data, and the normalized sample standard deviation was determined using the following equation:

\[
\frac{dN}{N} = \sqrt{\left(\frac{dC}{C}\right)^2 + \left(\frac{dE}{E}\right)^2}
\]

(Eq. 9)

For Equation 9, N represents the normalized cell viability, C represents the control sample cell viability, E represents the experimental sample cell viability, and dN, dC, and dN represent the standard deviations for N, C, and E, respectively.

3.10.2 Dose-Response Curve for CHO-K1 Cells

A dose-response curve was created for CHO-K1 cells treated with pure doxorubicin in 1x PBS buffer (Figure 3.10). The values were normalized to a control group without exposure to DOX. The EC\(_{50}\) was determined to be approximately 75 +/- 5 \(\mu\)M.
Table 3.10 Normalized percent cell viability and percent cell density with normalized standard deviations in CHO-K1 cell study

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<th>Normalized Percent Viability</th>
<th>Normalized Percent Density</th>
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<td>DOX-loaded medusa particles with heating</td>
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Figure 3.10 Dose-response curve for CHO-K1 cells exposed to pure DOX-PBS for 32 hours
As shown in Table 3.10, the viability of CHO cells was not affected by 1x PBS buffer, as the two experimental groups with PBS had almost identical viability results as the control group that used autoclaved DI water. For reference, the control maintained about 79.2 percent viability during the 32-hour period, which was normalized to 100 percent. The two DOX-loaded medusa particle experimental groups showed significant death. Over 96 percent of the cells undergoing magnetic heating with DOX-loaded medusa particles were killed, whereas about 82 percent of the cells exposed to the sample medusa particle sample without magnetic heating were killed. This shows that the medusa particles are prematurely leaking doxorubicin at 37°C, which would not be desirable in a drug delivery system. We do see, however, that the heated 1x PBS buffer did not kill the cells, which means the magnetic coils aren’t inducing bulk solution temperature increase, but rather the coils are inducing magnetic induction of the magnetite nanoparticles in the core of the medusa particles.

Furthermore, the percent cell density of the experimental groups was significantly decreased, as DOX inhibits cell division. There is also a decrease in the 1x PBS buffer groups percent cell density; however, we predict this is a result of the CHO-K1 cells remaining in the lag phase as they adjust to the new environmental conditions, as opposed to a foreshadowing of diminished viability. As shown in Table 3.10, the viabilities of the 1x PBS buffer secondary control groups were not significantly different than the pure media control group. Conversely, the DOX-loaded medusa particles experimental groups had significant decreases in both viability and density, which is indicative of release of DOX from the medusa particles.

Unloaded medusa particles with and without heating were also used in experimentation, but the samples were compromised by contamination and residual solvent. Further cell studies should incorporate these experimental groups.
CHAPTER 4
CONCLUSIONS AND FUTURE WORK

Medusa particles were successful synthesized in multiple experiments and characterized using various techniques. $^1$H NMR and IR were used to validate the synthesis. TEM was used to visualize the nanostructure of the magnetic core of particles and determined the diameter to be 11 +/- 2 nm. DLS established the number average hydrodynamic diameter of about 18 nm, giving a holistic idea of the size of the medusa particles. XPS showed that the particles were a mixed phase of $(\text{Fe}_3\text{O}_4)_x - (\gamma\text{-Fe}_2\text{O}_3)_{1-x}$.

In order to evaluate the applicability of the medusa particles to a drug delivery system, drug loading and release experiments were performed. We chose doxorubicin as the primary drug for investigation of loading and release because the general properties of the drug are well-documented in literature and it is a commonly used chemotherapy agent. Release was demonstrated by both UV-Vis spectroscopy and linear sweep and differential pulse voltammetry.

Finally, we conducted a cell study evaluating the effects of medusa particles and release of doxorubicin from medusa particles on CHO cells in order to establish non-specific targeting of medusa particles against these cells and to further confirm the magnetothermal control of drug release from medusa particles.

Magnetic heating of DOX loaded medusa particles was demonstrated to be effective; however, the medusa particles were leaking drug below desirable hyperthermic conditions. Leaking DOX at or below body temperature would lead to similar systemic side effects as
current chemotherapy treatments. As can be seen in the cell study, the DOX loaded medusa particles kill the majority of the cells without any magnetic heating, although the magnetic heating experimental group killed significantly more cells. This premature leaking has been found in previous experiments with micelles. In order to be used as a drug delivery system, medusa particles need to be optimized to hold the drug until magnetically heated to 43-46°C.

Medusa particles could be optimized in several ways. Different polymer combinations or ratios could be used. We predict that a higher PCL to PEG ratio could increase the drug loading capacity of medusa particles.

Furthermore, experiments involving specific targeting of cancerous cells using various ligand attachments need to be performed in order to optimize the specific toxicity of the particles towards cancer cells. As described in Chapter 1, we have previously attached cRGDfk peptides to polymeric micelles via a PEG-maleimide polymeric reagent. The synthetic route for adding these targeting peptides to medusa particle would be quite similar. Future work on medusa particles should involve adding the targeting peptides to medusa particles and creating a comparative evaluation of the cell viability as a result of specific targeting to the non-specific targeting studies shown in Chapter 3.

It would also be useful to monitor the local temperature of nanoparticles instead of the bulk solution temperature to get a better understanding of how the PCL core may be affected locally by magnetically heated nanoparticles, even when the bulk solution temperature is below the melting point of the polymer as was shown in the magnetically triggered release study.

Future work could also involve pH-induced release of drugs from medusa particles to predict the behavior of release in various cellular compartments, or the incorporation of other drugs into medusa particles. The premature leaking of drug may be the result of doxorubicin’s
interaction with the polymer phases, and not merely a result of a failed system. We predict a more hydrophobic drug, like paclitaxel, may be held within the polymer core more effectively than doxorubicin. Our system could be applied to many drugs and therapeutic applications, but modification of the medusa particles may be needed to maximize drug loading efficiency and to create more desirable temporal release.
REFERENCES


(23) Galeotti, F.; Bertini, F.; Scavia, G.; Bolognesi, A. A controlled approach to iron oxide nanoparticle functionalization for magnetic polymer brushes. *Colloids and Surfaces B: Biointerfaces* 2011, 360, 540-547.


## APPENDIX A

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## APPENDIX B

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