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Magnetically induced release of fluorescein isothiocyanate (FITC) from polymer nanoparticle composites (PNCs)

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MAGNETICALLY INDUCED RELEASE OF FLUORESCEIN ISOTHIOCYANATE (FITC) FROM POLYMER NANOPARTICLE COMPOSITES (PNCS)

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To my family, friends, and mentors.
Thank you for your support and belief in me.

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Abstract

Magnetically induced drug release can be used as a site-specific, minimally invasive pharmaceutical treatment. Its purpose is to increase the efficacy of drug therapies to diseased or damaged tissue and to decrease the amount of unnecessary damage to healthy, surrounding tissue. To prove the concept of drug release by a magnetic field, this study focused on the release of a fluorescent molecule from magnetic polymeric nanoparticle composites (PNCs) via induction of an alternating current (AC) magnetic field. Fluorescent magnetic PNCs used were 250 μm or less in size, and were made of poly(methyl methacrylate) (PMMA) containing either the magnetic material magnetite nanoparticles or cobalt nanoparticles, and the fluorescent dye fluorescein isothiocyanate (FITC). Characterization of the composites included transmission electron microscopy (TEM) and scanning electron microscopy (SEM) for size and morphology, fluorescent microscopy for fluorescent images, elemental analysis for iron and cobalt content, and superconducting quantum interference device (SQUID) magnetometer readings for saturation magnetization measurements and field profiles of each particle type. Magnetic release of FITC from the composites was induced by applying an AC magnetic field to the PNCs in phosphate buffered saline (PBS) at various frequencies in the range of 44-430 Hz at the corresponding voltage of 15-123 V, magnetic field strength of approximately 465 G and current of 11 A. The PNCs were exposed to the magnetic field for various amounts of time ranging from 5 minutes to 3 hours and at temperatures of 4°C, 22°C, and 43°C. For each experiment, a control sample that was not exposed to the magnetic field was also tested for release. Fluorescence released was measured using a fluorospectrometer following filtration and sample dilution. The investigations demonstrated that the release of FITC was not significantly dependent on the frequency of the magnetic field, the experimental duration, nor the presence of

the AC magnetic field. The study demonstrated, however, that greater release of FITC was dependent on higher temperatures and that magnetite-PNCs released more FITC than cobalt-PNCs. This research potentially leads the way to the biological applications of *in-vitro* and *in-vivo* studies of magnetically induced, controlled drug release from magnetic polymeric structures.

Chapter 1 – Background

Magnetic polymeric composites can be used in a variety of applications, including magnetically induced drug release and the heat therapies of thermal ablation and hyperthermia. The term magnetic polymer nanoparticle composites, or magnetic PNCs, describe structures that contain magnetic material inside a polymer matrix. The materials used and their characteristics will differ according to the application of interest. Polymers used for biomedical applications should be biocompatible; however, they may or may not be biodegradable. The magnetic material incorporated into the polymer is dependant upon the magnetic characteristics necessary for the particular application, and the active component encapsulated by the polymer is specific to the treatment needed. The following chapter will discuss the importance of magnetically induced drug release via magnetic field.

1.1 Magnetically Induced Drug Release

Magnetic particles have been shown to aid in the release of encapsulated molecules from inside of a polymer matrix when magnetically induced by an oscillating or alternating current (AC) magnetic field (De Paoli et al. 2006; Edelman et al. 1985; Ikehara et al. 2006; Lu et al. 2005; Rana et al. 2007) (Figure 1.1). Magnetic release can control the activity of a drug delivery system in several ways, including decreasing the amount of unnecessary damage to healthy tissue, increasing the efficacy of the drug, and treating ailments in a minimally invasive way. Unnecessary damage to healthy tissue can be decreased by using an external magnet to guide polymeric particles containing magnetic material and drugs to cells that need to be treated; (Kumar et al. 2004)an external magnetic field can then be used to release the drugs at the target site (Arias et al. 2001; Asmatulu et al. 2005; Kim and Park 2005; Tan et al. 2005). The efficacy of the drugs can be increased by using a magnetic field to induce drug release in conjunction

with other treatments, such as hyperthermia, which creates a dual attack on tumor cells by combining the effect of the drug treatment with the heat-treatment of cells (Gu et al. 2005; Guedes et al. 2005; Ikehara et al. 2006; Ito et al. 2003; Tanaka et al. 2005). Certain ailments can be treated in a minimally invasive way by using magnetic release of biomolecules from implantable drug-encapsulated polymer structures, such as orthopedic implants that contain anti-inflammatory agents and antibiotics (Faber et al. 2003). These examples exhibit the positive effect from the use of a magnetic field and polymeric system for delivery and release of drugs.

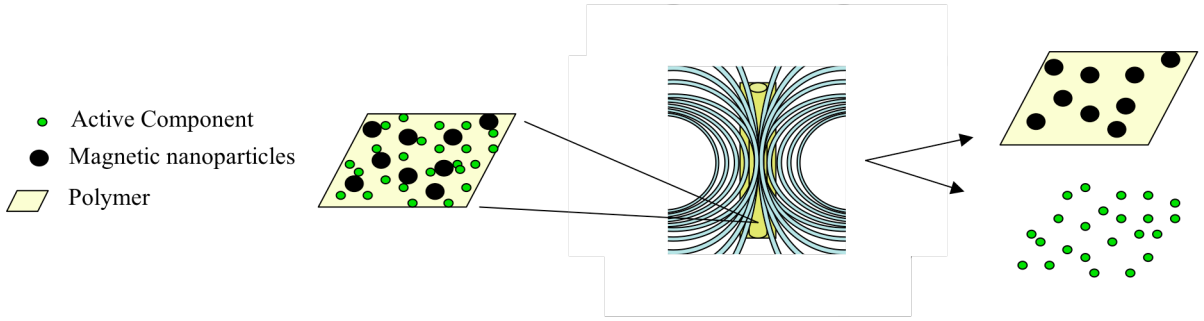

Figure 1.1 Diagram of theoretical magnetically induced drug release

Constituent release from polymers via oscillating magnetic fields has been shown to occur at different ranges of magnetic field frequencies and strengths, duration in the magnetic field, release medium, composition and size of particles, which include the polymer, active component, and magnetic material. The following are studies that show magnetically induced release.

Collagen gels (1 cm thick) were prepared with magnetite (10 nm) for the release of rhodamine-labeled dextran (Dex-R) using an oscillating magnetic field. Magnetic field release studies were performed in phosphate buffer solution for up to four days with a magnetic field strength of 14,000 G and frequency of 0.3 Hz. Higher release of Dex-R was found in the presence of the magnetic field compared to in its absence. Higher release was also found with the

use of higher magnetic particle content over 12 hours and with a lower molecular weight of Dex-R over 24 hours of magnetic field application (De Paoli et al. 2006).

Ethylene-vinyl acetate copolymer (EVAc) bars (1 cm x 2 cm) containing 1.4 mm diam by 1.4 mm long cylinders of samarium cobalt ($SmCo_5$) permanent magnets were created to release bovine serum albumin (BSA). Particles were put into phosphate buffer as release medium and subjected to a magnetic field for 2 hours at 5 Hz at magnetic field strength of 870, 1300, or 1800 G and 0.87-11 Hz at 1800 G. Results showed that higher magnetic field and frequency yielded an increase in release of BSA (Edelman et al. 1985).

Polyelectrolyte microcapsules (5 μm) made of poly(sodium styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) bilayers containing ferromagnetic gold-coated cobalt (3 nm) were synthesized to uptake FITC-dextran in order to determine permeability, a transport phenomenon similar to release. Tris buffer was used as the release medium, the duration in the magnetic field was 30-60 minutes at frequencies of 100-1000 Hz, and the magnetic field induction remained constant at 1200 Oe for all experiments. Results showed that permeability of FITC-dextran into the polymer complex was greatest between 100-300 Hz (Lu et al. 2005).

Oligomannose-coated liposomes (OMLs) of less than 1 μm in size, made from dipalmitoylphosphatidylcholine (DPPC) and containing magnetite (10 nm), were produced to release the anti-tumor agent, 5-fluorouracil (5-FU). The particles were injected in the peritoneal cavity of mice and were subjected to a magnetic field for 30 minutes at a frequency of 118 kHz and magnetic field induction of 125 Oe. This study resulted in the reduction of tumor growth due to the combination of the presence of 5-FU and magnetite compared to the presence 5-FU or magnetite alone (Ikehara et al. 2006).

Magnetoliposomes (~ 1 μm) made from DPPC containing dextran-magnetite (DM) (8 nm) at various concentrations were made to release 5-FU. Particles in 50% calf serum in Sorensen buffer were planed in a magnetic field of 15,000 G and 500 KHz for 120 minutes for release studies. Release of 5-FU was found to be higher with a magnetic field than without it due to an increase in sample temperature that was produced (Viroonchatapan et al. 1997).

Nickel ferrite particles (5-8 nm) coated with poly(methacrylic acid) (PMAA) were created to release the anti-cancer drug doxorubicin (Dox). A magnetic field of 1000 Oe was applied for release studies for up to 10 days. Higher release of Dox was found in the presence of the magnetic field than in its absence (Rana et al. 2007).

Based on these studies, release via magnetic field occurs at both low and high frequencies and magnetic field strengths, from different polymeric structure and sizes, containing different magnetic material and sizes, and using different release media.

1.2 Magnetic Fields

Magnetic material can be controlled with either a direct current (DC) magnetic field or an alternating current (AC) magnetic field. A DC magnetic field will cause the net magnetic moments of a magnetic material to align parallel to the magnetic field and to move in the same direction as the magnetic field (Ferreira et al. 2005; Liu et al. 2006; Pankhurst et al. 2003; Viroonchatapan et al. 1997). For instance, a permanent magnet can be used to separate magnetic material. An oscillating or AC magnetic field produces a different effect. The current of this type of field rotates or switches directions at a specified frequency. This rotation inhibits the particles from moving in one particular direction. Instead, they oscillate, generating mechanical friction and heat, which is why magnetically induced magnetic material is used in hyperthermia (Rida and Gijs 2004). It is suggested that under the influence of an alternating magnetic field,

electromagnetic energy is supplied to the magnetic material; heat is generated due the relaxation of the magnetic moment, or loss of hysteresis, when the magnetic field is removed (Ito et al. 2005; Moroz et al. 2002). The hypothesis of the current study of magnetically induced drug release from polymeric, magnetic particles is that heat produced locally by magnetic particles in AC magnetic fields loosens the polymer strands surrounding the particles at temperatures above the glass transition temperature (Tg) of that particular polymer, allowing the encapsulated constituent to be released. There are several types of magnetic material that can be controlled by magnetic fields for drug release applications, along with others biomedical applications.

1.3 Magnetic Material

Magnetic materials are used for a variety of biomedical applications (Ito et al. 2005; Molday and MacKenzie 1982; Safarik and Safarikova 2002; Tartaj et al. 2003). These applications include myocardial tissue engineering (Shimizu et al. 2007); cell labeling and magnetic separation (Freeman 2005; Lea et al. 1988; Seesod et al. 1997; Ugelstad et al. 1993); MRI contrast agents (Kohler et al. 2005; Suwa et al. 1998); hyperthermia and thermal ablation (Gu et al. 2005); gene therapy (Freeman 2005; Gu et al. 2005; Xu et al. 2005); and site-specific drug targeting, delivery, and controlled release (De Paoli et al. 2006; Kohler et al. 2005; Rana et al. 2007; Yang et al. 2006b; Zhang et al. 2002).

Commonly used magnetic substances include stainless steel magnetic alloy (Edelman et al. 1985); various cobalt particles including cobalt ferrite ($CoFe_2O_4$) (Wilhelm et al. 2003), gold-coated cobalt (Co@Au) (Lu et al. 2005), and samarium cobalt (Edelman et al. 1985); and the family of iron oxides, including nickel ferrites (Rana et al. 2007), hematite (Fe_2O_3), maghemite (γ-Fe_2O_3) (Horak et al. 2004; Safarik and Safarikova 2002; Yang et al. 2006b), and magnetite (Fe_3O_4).

1.3.1 Magnetite

Magnetite is a natural mineral made of iron oxide (Fe_3O_4) with a molecular weight of 231.54 Daltons, found inside igneous rock and in brain tissue of some forms of bacteria, bees, homing pigeons, and salmon (Barnes 1992; Safarik and Safarikova 2002; Wilhelm et al. 2003). Magnetite has also been known to have low toxicity (Arias et al. 2001; Gu et al. 2005; Iannone et al. 1991; Yang et al. 2006b). A simple lab set-up can also be used to chemically synthesize it to produce nano-sized particles of 6 to 50 nm for use in biomedical systems (Gu et al. 2005; Kumar et al. 2004). The small size of magnetite coincides with a large surface area to volume ratio (Zhang et al. 2002). It also allows magnetite to be superparamagnetic. Superparamagnetism is an important magnetic property because it allows the nanoparticles to be magnetized under the influence of a magnetic field, but not to retain residual magnetism in its absence (De Paoli et al. 2006; Kim and Park 2005; Pankhurst et al. 2003; Safarik and Safarikova 2002; Tartaj et al. 2003). Superparamagnetism is denoted by the lack of hysteresis at room temperature (300 K), but the presence of a hysteresis at low temperatures, such as at 10 K (Liu et al. 2003).

1.3.2 Cobalt

Cobalt (Co) is a metal with a molecular weight of 58.93 Daltons. It is found naturally in the earth, underground water, in some plants and animals in trace amounts, and is present in vitamin B12 at 4%. Cobalt has a fairly low toxicity level; however it can be carcinogenic at relatively high concentrations (Valko et al. 2005). Cobalt is also superparamagnetic at the nano-level. It can be chemically synthesized and is frequently made in conjunction with other molecules for biomedical and chemical purposes to produce particles such as the following: Co nanowires of 10-20 nm in diameter (Gu et al. 2005), gold-coated cobalt (Co@Au) of 3 nm in

diameter (Lu et al. 2005), and cobalt ferrite ($CoFe_2O_4$) of 4.6-165 nm in diameter (Baldi et al. 2007; Lee et al. 2007; Wilhelm et al. 2003).

1.4 Polymers

There are many ways in which polymers can be classified. The classification of biodegradable or non-biodegradable is important in biomedical applications. Biodegradable polymers break down inside the body over time, allowing the body to absorb them. The rate of degradation depends on the chemical make-up and the structure of the polymer. Biodegradable polymers can be used as temporary implants that are needed to hold their structure for a limited time, such as with absorbable sutures and staples (Shikanov et al. 2005), cell scaffolding for bone regeneration (Thomson et al. 1995), gene delivery (Khan et al. 2004; Li and Huang 2004), and drug delivery systems (Ahlin et al. 2002; Arias et al. 2001; Liu et al. 2002; Rana et al. 2007; Sparnacci et al. 2005; Yang et al. 2006b).

Common biodegradable polymers used in many of these applications include poly(lactic-co-glycolic acid) (PLGA) (Ahlin et al. 2002; Khan et al. 2004; Liu et al. 2002; Sparnacci et al. 2005), poly(ethyl-2-cyanoacrylate) (PECA) (Arias et al. 2001; Yang et al. 2006a), poly(ε-caprolactone) (PCL) (Elvira et al. 2004; Savic et al. 2003; Yang et al. 2006b), poly(ethylene oxide) (PEO) (Savic et al. 2003), poly(ethylene glycol) (PEG) (Liu et al. 2005; Owais and Gupta 2005; Rana et al. 2007; Zhang et al. 2002), collagen (De Paoli et al. 2006), and alginate (Chan et al. 2005).

Non-biodegradable polymers do not get absorbed into the body as time progresses. These materials are normally implanted and allowed to remain as stable structures, holding their form for a certain application and replaced when needed, or they are surgically removed after the application has been completed. Poly(hydroxyethyl methacrylate) (PHEMA), an insoluble

hydrogel used for short-term implants, was studied for use as drug delivery and release carriers (Gursel et al. 1998; Horak et al. 2004; Tan et al. 2005). Polystyrene (PS) has been used in conjunction with fluorescent dyes to study the uptake of particles into the human intestine to investigate the bioavailability of certain orally administered drugs (Pietzonka et al. 2002). One of the most widely used non-biodegradable polymer in biomedical applications is poly(methyl methacrylate) (PMMA) .

PMMA is a hydrophobic polymer that has a molecular weight of 950, 000 Daltons, and it is biocompatible, but non-biodegradable. Some of its biomedical applications include bone cement (Sivakuma and Rao 2002) and cell labeling (Nagao et al. 2006). Other, non-biomedical applications of PMMA include paints, adhesives, textiles, applications in food processing, colloid science, and physics and rheology studies (Bosma et al. 2002; Dullens et al. 2004).

PMMA is widely studied for its use in drug delivery systems, in which the encapsulation of the drugs provides protection from biomolecules inside the body prior to reaching the target site (Owais and Gupta 2005). The following drug delivery systems utilize PMMA as a drug carrier or host, and they incorporate a variety of drugs and therapeutics for many different applications: verapamil for stomach ailments (Streubel et al. 2003); trypsin as a model protein for the HIV-1 Tat protein vaccine (Sparnacci et al. 2005); cisplatin as a chemotherapeutic agent for testicular cancer, ovarian cancer, lymphoma, and glioma (Yan and Gemeinhart 2005); cholesterol as a model for low-solubility steroids (Elvira et al. 2004), ibuprofen (Feng and Li 2007), antisense oligonucleotides (Tondelli et al. 2001), enalaprilat as a model for low bioavailability of orally administered drugs (Ahlin et al. 2002); and gentamicin, daptomycin, and Dhvar-5, as antibiotics and anti-microbial agents incorporated into bone cement to prevent and treat orthopedic infections (osteomyelitis) during orthopedic surgeries, such as implants,

prosthetics, and bone replacements (Faber et al. 2003; Sivakuma and Rao 2002). A few studies are reviewed in depth to show the release of drugs from PMMA.

The release studies of ibuprofen and gentamicin from PMMA were conducted in PBS at 37°C. In one set of studies, PMMA was modified by adding carboxyl groups. Release was highest from the modified PMMA than regular PMMA due to an increase in porosity and ability to encapsulate more drug. Release of gentamicin (72%) was higher than release of ibuprofen (54%), from modified PMMA. However, the release rate of ibuprofen (≤ 15% in 2 hours) was greater than that for gentamicin (≤ 5% in 2 hours). Ibuprofen was thought to become physically entrapped in the pores of PMMA, which allowed it to release relatively quicker than gentamicin. However, the amino groups of gentamicin allowed it to couple to the added carboxylic groups of the modified PMMA, increasing the amount of drug that could be entrapped, thereby increasing the amount of drug that was available to be released (Sivakuma and Rao 2002).

The release studies of verapamil HCl from PMMA were conducted in N HCl at 37°C with shaking at 75 rpm. The effect of drug loading and release from two different polymers were studied. PMMA that contained 9.6% and 19.2% verapamil, released ≤ 3% and ≤ 60%, respectively, after 2 hours. Eudragit RS that contained 9.6% and 18.6% loading of verapamil, released ≤ 90% for both drug loadings after 2 hours. This study showed that the type of polymer used can dramatically change the release of an encapsulated substance. It can be assumed that Eudragit RS was more porous and allowed a more complete drug release to occur in a shorter amount of time than PMMA (Streubel et al. 2003).

Release studies of tebucozanole from PMMA were conducted in water at room temperature with magnetic stirring. PMMA was modified with phthalic anhydride (PA), which resulted in an increase in release of tebucozanole. PMMA containing 0%, 10%, 30%, and 50%

PA were shown to release about 12%, 17%, 40%, and 68% drug, respectively, in 500 hours.. The glass transition temperature (Tg) of PMMA was also studied. PMMA containing 30% PA had a lower Tg (58.4°C) than PMMA (95.5°C). When 40% tebuconazole was added to PMMA-PA (30%), Tg decreased to 26.3°C. This study showed that a decrease in Tg occurred with the addition of PA and drug, which allowed an increased release rate even at room temperature due to an increase in polymer porosity. The effect of molecular weight of PMMA on release was also tested. PMMA with molecular weight of 350,000 Da released 10% tebuconazole, while PMMA of 120,000 MW released 20 %. Clearly the lower molecular weight released more drug (Asrar et al. 2004).

1.5 Active Component

The entrapment of one or more active components inside of a polymer allows the polymeric structure to become a vehicle for release. The current study tests the release of a fluorescent dye, fluorescein isothiocyanate (FITC). FITC is a hydrophilic bio-fluorescent molecule that has a molecular weight of 389.38 Daltons. It is frequently used for staining antibodies (Rekhi et al. 2005), distinguishing between apoptotic and necrotic cells using FITC-annexin V label and propidium iodide stain (Hsieh et al. 2001), and has other uses as an immunochemical stain (Kim and Shukla 2005). FITC has also been used to label magnetic nanoparticles for visualization and tracking in cells (Won et al. 2005), and it has been used to label magnetic silica microspheres for magnetic targeting (Deng et al. 2005). FITC will be used as the active component for release in the current study in order to clearly quantify its release from polymeric microparticles via oscillating magnetic fields.

1.6 Objectives

The objectives for this study were to create micro-sized, fluorescent, magnetic, polymer nanoparticle composites (PNCs) using FITC, magnetite or cobalt nanoparticles, and PMMA; to successfully release FITC from these composites by using an AC magnetic field; and to compare the effects of magnetic field frequency, duration, temperature, and magnetic material on the release. The significance of this study was to test the concept of magnetically induced release of pharmaceuticals from a magnetic polymer controlled by using a magnetic field. The theory behind the use of an alternating current magnetic field for release is that heat locally produced by the magnetic particles due to oscillations caused by the magnetic field may reach temperatures above polymer's glass transition temperature (Tg), which may loosen the polymer strands surrounding the magnetic particles and allow the encapsulated component to be released. This study leads the way to potentially control the release of drugs from polymeric structures *in-vitro* and *in-vivo*.

1.7 Design Considerations

Several considerations were taken into account when designing the magnetic polymeric nanoparticle composites and the controlled release parameters to be used for the present study of magnetically induced release. The polymer for the composites was chosen to be PMMA (Table 3.2) because it is non-biodegradable. Particles for the long-term release of certain drugs are more appropriately used if they do not break apart easily *in vivo*. For example, in the prevention and treatment of osteomyelitis, best results are achieved by loading the drugs into bone cement implemented as an implant that does not biodegrade. PMMA is widely used in bone cement for this property. The addition of magnetic material in the PMMA and the concept of magnetically induced release of the drugs is practical for this long-term application.

The magnetic materials used in the present study were magnetite and cobalt (Table 3.2) They were chosen particularly because they are superparamagnetic. Superparamagnetic particles are necessary because they do not retain magnetism after the removal of a magnetic field. This concept is important for *in vivo* drug delivery systems to allow control of the release by controlling the magnetic field. For practical applications, it is important for the drug delivery system to be as small as possible, starting with the entrapped magnetic material, to inhibit their detection from macrophages *in vivo*. Both magnetic materials chosen, magnetite and cobalt, are relatively simple to synthesize at the nanoscale. A comparison of the two different particles was studied to determine if the thermal behavior produced by the magnetic field would be different for the two of them, and thereby affect release.

The active component chosen was a fluorescent dye in order to clearly quantify its release using a spectrofluorimeter. FITC (Table 3.2) is a hydrophilic fluorescent dye that was specifically chosen because it fluoresces at a range of wavelengths that could be detected by the spectrofluorimeter and fluorescent microscope available for the use of this study. It was also chosen because it was able to dissolve in a common solvent with the chosen polymer, (PMMA).

The solvent, methanol, was selected after a series of tests were deigned to determine which, out of several different solvents, would best dissolve PMMA, magnetite, and FITC. PBS was chosen as the release media for the controlled released studies because it mimics *in vivo* conditions. It is also commonly used in release studies, allowing a basis for comparison to other works.

Following the design of the PNCs, the parameters studied for the controlled release experiments were carefully selected as follows. An AC magnetic field was chosen instead of a DC magnetic field because the oscillating of the AC field is what causes the magnetic moments

of the superparamagnetic nanoparticles to switch directions at a set frequency. This switching is what causes mechanical vibration and, in turn, heat. The loosening or swelling of the surrounding polymer strands due to the increase in temperature is what allows release media to enter the polymer matrix and dissolve the entrapped active component. The driving force behind the release of FITC toward the medium is the large concentration gradient (high concentration of FITC/PBS inside polymer to low concentration of FITC/PBS outside of polymer).

The magnetic field strength of 465 G was used because it is the largest field strength that could be created with an affordable power supply. The current and voltage of 11A and 123 V, respectively, were the maximum that could induce the strongest field. A lower field strength would weaken the magnetic moment. Higher field strength would require a more expensive power supply.

The frequencies of 44, 230, 430 Hz were selected for the frequency dependent studies because they are the minimum, a median, and the maximum frequencies that could be attained at the highest field strength and current (465 G, 11 A). This range of frequencies was also similar to the studies from Lu et al. (2006), which found that frequencies of 100-300 Hz were better than 300-1000 Hz for controlling the porosity of a polymer matrix.

The frequency of 430 Hz was chosen for the majority of the release experiments because application of magnetic field at 430 Hz released the highest amount of FITC in preliminary tests. The durations of 30, 60, 120, 180 minutes were chosen for the release experiments because they are relatively short time periods that seemed appropriate for a treatment of drug release, but are long enough to allow sufficient time for sample processing.

Table 1.1. Main components of magnetically induced release studies

Polymer	**Active Component**	**Magnetic Material**	**Magnetic Material**
Poly(methyl methacrylate) (PMMA)	Fluorescein 5-isothiocyante (FITC)	Magnetite	Cobalt
$C_5H_8O_2$ (monomer)	$C_{21}H_{11}NO_5S$	Fe_3O_4	Co
MW 950,000 Daltons	MW 389.38 Daltons	MW 231.54 Daltons	MW 58.93 Daltons
Drug delivery[1], bone cement[2], cell labeling[3]	Antibody stain[4], apoptotic cell labeling[5]	Mag release[6], hyperthermia[7], MRI contrast agent[8]	Mag release[9], mag permeability[10], bio-templated nanowires[11]

1. Feng and Li, 2007
2. Faber et al., 2003
3. Nagao et al., 2006
4. Rekhi et al., 2005
5. Hsieh et al., 2001
6. Ikehara et al., 2006
7. Gu et al., 2005
8. Suwa et al., 1998
9. Edelman et al., 1985
10. Lu et al., 2005
11. Gu et al., 2005

Chapter 2. Materials and Methods

2.1 Materials

Iron II chloride ($FeCl_2$•$4H_2O$) 98%, iron III chloride ($FeCl_3$) 97%, ammonium hydroxide (NH_4OH) 29.05%, cobalt II acetate tetrahydrate 98%, N-dodecyl-N,N-dmethyl-3-ammonia-1-propanesulfonate (SB12), sodium borohydride ($NaBH_4$) 98%, fluorescein 5-isothiocyanate (FITC) isomer I 90%, Dubecco's phosphate buffered saline (HyClone HyQ DPBS), and ethanol were purchased from Sigma Aldrich. Methanol was purchased from EMD Chemicals. 950 polymethyl methacrylate A10 (10% in Anisole) was graciously donated by Dr. Yoonyoung Jin. Air-free nanopure water was be made under nitrogen by distilling nanopure water made with a Barnstead NanoPure Water System.

2.2 Experimental Procedures

2.2.1 Magnetic Polymer Nanoparticle Composites

Two processes to create fluorescent magnetite-polymer nanoparticle composites were utilized: the LIGA process (X-ray lithography, electroplating, and molding) and the batch process. LIGA was used to control the size and shape of the composites by using masks with particular dimensions. The batch process, a simpler, yet less exact process, did not allow for a strict control in shape and size. Both processes utilized the same PNC mixture containing PMMA, magnetite, and FITC in methanol.

- **Synthesis of Magnetite Nanoparticles**

Magnetite nanoparticles were made using procedures from Kumar at el. (2004) (Figure 2.1). 1.622 g $FeCl_3$ and 0.994 g $FeCl_2$•$4H_2O$ were weighed using a AG204 Delta Range Mettler Toledo scale and combined in a three-necked 100-ml RB flask, which was previously purged and

filled with nitrogen gas three times to remove oxygen. The iron salts were dissolved in 25 ml of air-free nanopure water and stirred magnetically on a magnetic stirrer (Corning Stirrer/Hot Plate) with a 2 cm magnetic stirring bar (VWR Spinbar Magnetic Stirring Bars). To this solution, 29.05% NH_4OH was added until the solution was completely black. A black precipitate was obtained by decanting the supernatant after settling on a permanent magnet (Eriez Series S). The precipitate was then heated at 80˚C for 30 minutes using a hot plate (Corning Stirrer/Hot Plate), washed three times with 25 ml water and twice with 20 ml ethanol, and dried under nitrogen flow (Kumar et al. 2004).

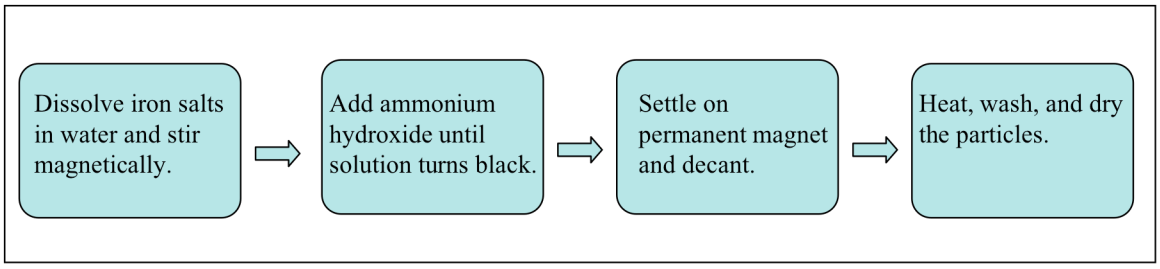

Figure 2.1. Synthesis of magnetite nanoparticles

- **Synthesis of Cobalt Nanoparticles**

Cobalt nanoparticle were made using procedures from Son et al. (2002) by combining 5.25 g cobalt II acetate and 2.184 g N-dodecyl-N,N-dmethyl-3-ammonia-1-propanesulfonate (SB12), a surfactant, in 700 ml air-free nanopure water in a three-necked 1000-ml flask by sonicating under nitrogen until dissolved (Figure 2.2). A reducing agent was made separately by combining 1.05 g sodium borohydride ($NaBH_4$) with 210 ml air-free nanopure water under nitrogen. The reducing agent was added drop-wise to the cobalt and surfactant solution. A black precipitate was obtained by decanting the supernatant after settling on a permanent magnet for several hours until completely settled. The particles were washed three times with 100 ml ethanol, dried under nitrogen, and kept in a glove box due to air sensitivity (Son et al. 2002).

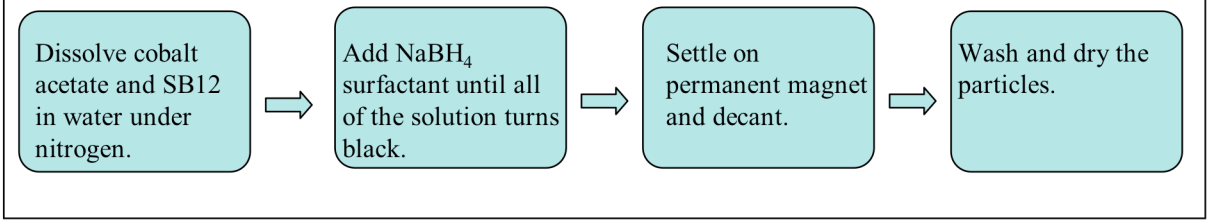

Figure 2.2. Synthesis of cobalt nanoparticles

- **Production of Fluorescent Magnetite-PNCs Using LIGA**

The process of making fluorescent magnetite-polymer nanocomposites was initiated by combining 800 mg crushed magnetite (10% w/w of PMMA) with 8 ml methanol and sonicating (VWR 750D Sonicator) for 1.5 hours (Figure 2.3). Prior to creating the polymeric microparticles, magnetite nanoparticles were crushed to a fine powder using a mortar and pestle. Next, 8 g of PMMA was weighed in a 50 ml beaker. An overhead stirrer (IKA-Werke RW16 basic) was set up using a 5 mm ACE Precision ground shaft and corresponding ACE mini-blade stirrer blade. While stirring, 400 μl of FITC solution (0.05 mg/ml; 0.0025% w/w of PMMA) was added at a time, until a total of 4 ml FITC solution was added. The time interval between each addition of 400 μl of FITC was 15 seconds, to allow enough time for the polymer and methanol to mix completely before adding more methanol. Next, the magnetite and methanol suspension was also added 400 μl at a time allowing 15 seconds to stir. Once all of the magnetic material had been added, the polymer mixture was transferred into a 250 μl round bottom, three-necked flask. To increase the thickness of the mixture, some of the methanol was allowed to evaporate by stirring on a lower setting with a nitrogen flow for 30 minutes. A 3 μm-layer of the mixture was spin coated with a spinner onto a silicon wafer that had been electroplated with a 10 nm-layer of chromium and a 50-nm layer of gold using an electron beam evaporator. It was allowed to cure briefly at 180°F on a high-temperature burner for 1.5 minutes. A small portion of the substrate

was then covered with three masks made of a graphite membrane of 100 μm thick and a gold absorber of 15 μm thick, which were used to make two sizes of square composites of 100 μm x 100 μm and 400 μm x 400 μm, as well as one rectangular composite of 100 μm x 400 μm. All of the composites were made to be 3 μm thick due to the thickness of the mixture after spin coating. Another mask made of silicon nitrate membrane of 1 μm thick and gold absorber of 1.5 um thick was used to make rods of 3 μm long and 700 nm in diameter. The substrate and mask was then exposed to an X-ray beam, followed by a developing process to remove the composites from the wafer substrate.

- **Production of Fluorescent Magnetite-PNCs Using a Batch Process**

The batch process of making the PNCs followed the same general procedure as that of the LIGA process of dissolving FITC and magnetite into methanol (separately), slowly adding the FITC/methanol to PMMA while stirring with an overhead mechanical stirrer, adding the magnetite/methanol mixture to the PMMA/FITC, and allowing the PNC mixture to thicken by evaporation of methanol with nitrogen (Figure 2.3). The thickened polymer composite mixture was spread as thinly as possible by turning the glass flask in all directions until the inside of the flask was completely coated. The composites were dried under a flow of nitrogen for several hours and removed by scraping the sides of the flask. Finally, the composites were crushed and sieved to under 250 μm.

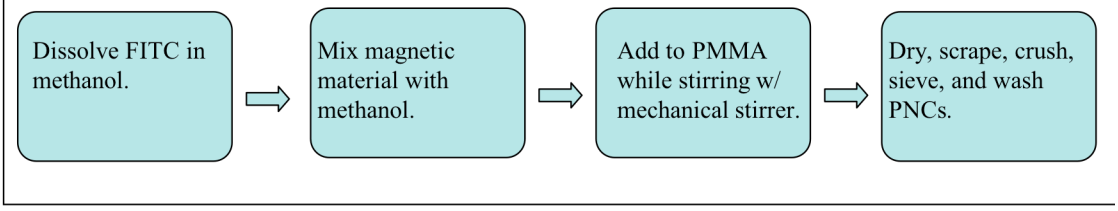

Figure 2.3. Production of polymer nanoparticle composites

- **Production of Fluorescent Cobalt-PNCs Using a Batch Process**

The process of making fluorescent cobalt-polymer nanocomposites was initiated by combining 680 mg crushed cobalt nanoparticles (10% w/w of PMMA) with 6.8 ml methanol by swirling in a three-necked flask kept under nitrogen. Next, 6.8 g of PMMA was weighed in a 50 ml beaker and transferred to another three-necked flask, to which the overhead stirrer, ground shaft, and stirrer blade was then attached. While stirring, 400 μl of FITC solution (0.05 mg/ml; 0.0025% w/w of PMMA) was added at a time, until a total of 3.4 ml FITC solution was added. The time waited in between each addition of 400 μl of FITC was 15 seconds to allow enough time for the polymer and methanol to mix completely before adding more methanol. Next, the cobalt and methanol suspension was also added 400 μl at a time allowing 15 seconds to stir. The polymer composite mixture was spread as thinly as possible in the flask, dried under nitrogen for several hours, and removed by scraping the sides of the flask. The composites were then crushed and sieved to under 250 μm using a mortar and pestle.

- **Removal of Surface Fluorescence**

Both the magnetite-PNCs and the cobalt-PNCs were washed to remove FITC from the surface of the particles. The washes were made to mimic the same concentration gradient of the release studies. 300 mg of the dry composites were placed in a 50 ml centrifuge tube with 50 ml of PBS, vortexed, and settled on a magnet. The washings were removed from the particles and 50 ml of clean PBS was added for the next wash. The particles were washed in series twelve times by vortexing for 1 minute and three more times by vortexing for 5 minutes. After each step, the washings were filtered, diluted, and measured for fluorescence to determine the total amount of FITC that was removed.

2.2.2 Characterization

- **Size and Morphology**

A transmission electron microscope (TEM) (JOEL100X TEM) at 80 kV and 100 kV, and scanning electron microscope (SEM) (Cambridge S-260 SEM) were used to characterize the size and morphology of the magnetite, cobalt, and polymeric composites made by the lab process.

For the preparation of TEM samples, nanoparticles were dispersed in water through sonication, and 1 µl was pipetted onto a copper grid (Electron Microscopy Science 400 mesh copper grid). The particles were allowed to air-dry and settle for 5 minutes prior to removing the excess liquid by wicking with filter paper. The loaded grid was placed into the sample holder of the TEM after filling with liquid nitrogen. SEM was used to show detailed surface structure of the PNCs and to confirm their size.

For the preparation of the SEM sample, dry composites were mounted on conductive adhesive tabs on aluminum specimen mounds and sputter coated with 60% gold and 40% palladium in an Edwards S-10 Sputter Coater. Both TEM and SEM images were taken with the instruction of Cindy Henk, at the Socolofsky Microscopy Center in the Department of Biological Science, LSU, Baton Rouge, Louisiana.

- **Fluorescence Imaging**

The fluorescent images of the FITC-entrapped PNCs were taken using a fluorescent microscope (TS100 Nikon Eclipse) and camera (Photometrics CoolSnap Roper Scientific). Dry composites were imaged on a glass slide and again after 1 um PBS was added. Fluorescence was imaged using a FITC filter and were taken with a bin number of 2 and exposure of 10 seconds. Brightfield images were also taken for comparison. All images were taken at a magnification of 10x.

- **Magnetization**

Magnetic data was generated by a superconducting quantum interference device (SQUID magnetometer) (Quantum Design MPMS-5S). For sample preparation, 3 mg magnetite, 3 mg of magnetite-PNCs, 4.5 mg cobalt, and 5 mg cobalt-PNCs were weighed then put into plastic capsules. The capsules were stuffed with cotton to keep the loose sample from dispersing, and then each was pushed into the middle of a clear plastic straw. The straws containing the sample were separately put into the magnetometer. Magnetic measurement were taken at 10 K and 300 K. SQUID magnetometer measurements were taken by Dr. Laurence Henry in the Department of Physics at Southern University A & M College (SU), Baton Rouge, Louisiana and by Dr. John DiTusa in the Department of Physics at LSU.

- **Magnetic Material Purity and Content**

The purity of the synthesized magnetite and cobalt and the amount of magnetite and cobalt present inside the composites was determined by using elemental analysis. A sample of each particle type, magnetite, magnetite-PNCs, cobalt, and cobalt-PNCs, weighing 5 mg each, were sent to Galbraith Laboratories, Inc., Knoxville, Tennessee, for analysis. The purity of magnetite was calculated by dividing the measured amount of iron in the magnetite sample by the theoretical amount of iron in magnetite (Equation 1). The theoretical amount of iron in magnetite was calculated by dividing the molecular weight of iron that is in magnetite (Fe_3O_4) by the molecular weight of magnetite (Equation 2). The amount of magnetite in the magnetite-PNCs was calculated by dividing the measured amount of magnetite that was in magnetite-PNCs by the theoretical amount of iron in magnetite (Equation 3). The following equations were used in the calculations for magnetic material purity and content:

purity of Fe_3O_4 = (A/B) x 100, where [1]

A = measured amount of Fe in Fe_3O_4, and

B = theoretical amount of Fe in Fe_3O_4

B = (MW Fe in Fe_3O_4/MW Fe_3O_4) x 100 [2]

amount of Fe_3O_4 in magnetite-PNCs = (C/B) x 100, where [3]

C = measured amount of Fe in magnetite-PNCs

The purity of cobalt and the amount of cobalt in cobalt-PNCs was measured and no calculations were needed.

2.2.3 Release Experiments

The objectives of the release experiments were to determine what effects different parameters had on the release of FITC from the prepared PMMA-based PNCs. The effects of frequency, duration, temperature, magnetic material, and presence of magnetic field were studied. The samples were methodically processed after each experiment to make sure that every sample was treated in the same manner for quality of comparison. The magnetic field generator used to test the magnetic field experiments is described below.

- **Magnetic Field Generator**

The magnetic field was created by generating power using a power supply (P1351 Behlman Electronics Power Passport AC Power Supply) to control the magnetic field inside of a copper coil (Alpha-Core Inc.), based on the specifications made by Toby Miller at CAMD, LSU. The coil had an outer diameter of 6.75 inches and an inner diameter of 0.6875 inch. It was 0.5 inch thick and contained 258 turns. Each experimental sample was placed inside the coil.

The average magnetic field applied to the particles was 465 G, which was measured using a Gaussmeter. The maximum parameters that can generate this field were a frequency of 430 Hz and voltage of 123 V at a current of 11 A. The lowest parameters that could generate the

same field were 44 Hz, 15 V at 11 A. A median frequency of 230 Hz and 67 V at 11 A also created the same field. These three frequencies, 44, 230, and 430 Hz, were used to study the effects of frequency on the release of FITC from the composites for the durations of 5, 30, and 60 minutes.

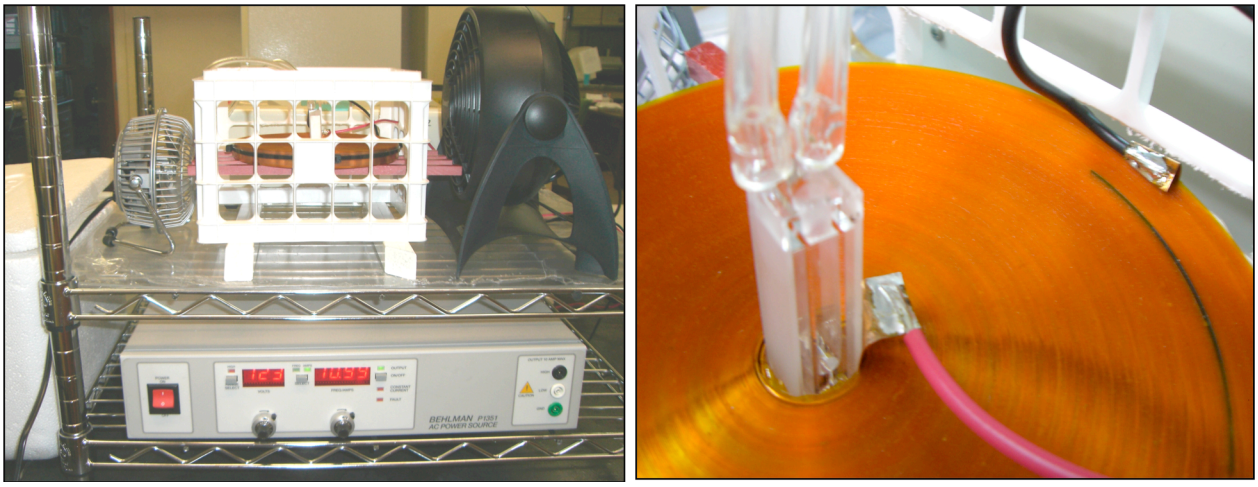

Figure 2.4 Magnetic field generator and coil

- **Fluorescence Detection**

The sample processing for the magnetic field release studies is illustrated in Figure 2.2 Each sample consisted of 3 mg of PNCs and 500 μl of PBS. Samples that were to be exposed to the magnetic field were put into a water-jacketed cuvette (Starna Cells, Constant Temperature Quartz Cell); samples that were not to be exposed to the magnetic field were put into a 1.5 ml Eppendorf tube. To maintain the temperature of all of the samples, two fans blew air over the coil and water was pumped through the water-jacketed cuvette. Ice water was used to maintain the temperature of the sample at 4°C. Room temperature water was used for the 22°C samples. Samples released at 43°C were maintained without using water.

The magnetic field-induced samples were exposed to the appropriate frequency in the magnetic field by placing each sample in the center of the copper coil, where the field was the

strongest. After the appropriate duration, the samples were removed from the field and settled on a Neodymium magnet. A gel pipettor was used to remove 450 µl of the media. This media was filtered using a 1ml syringe (BD 309 syringe), needle (BD 5 26 G 5/8" syringe needle), and syringe filter (National Scientific PTFE syringe filter; 4mm diameter, 0.2 µm pore size). The filtered medium was diluted by a 1:25 ratio in triplicate.

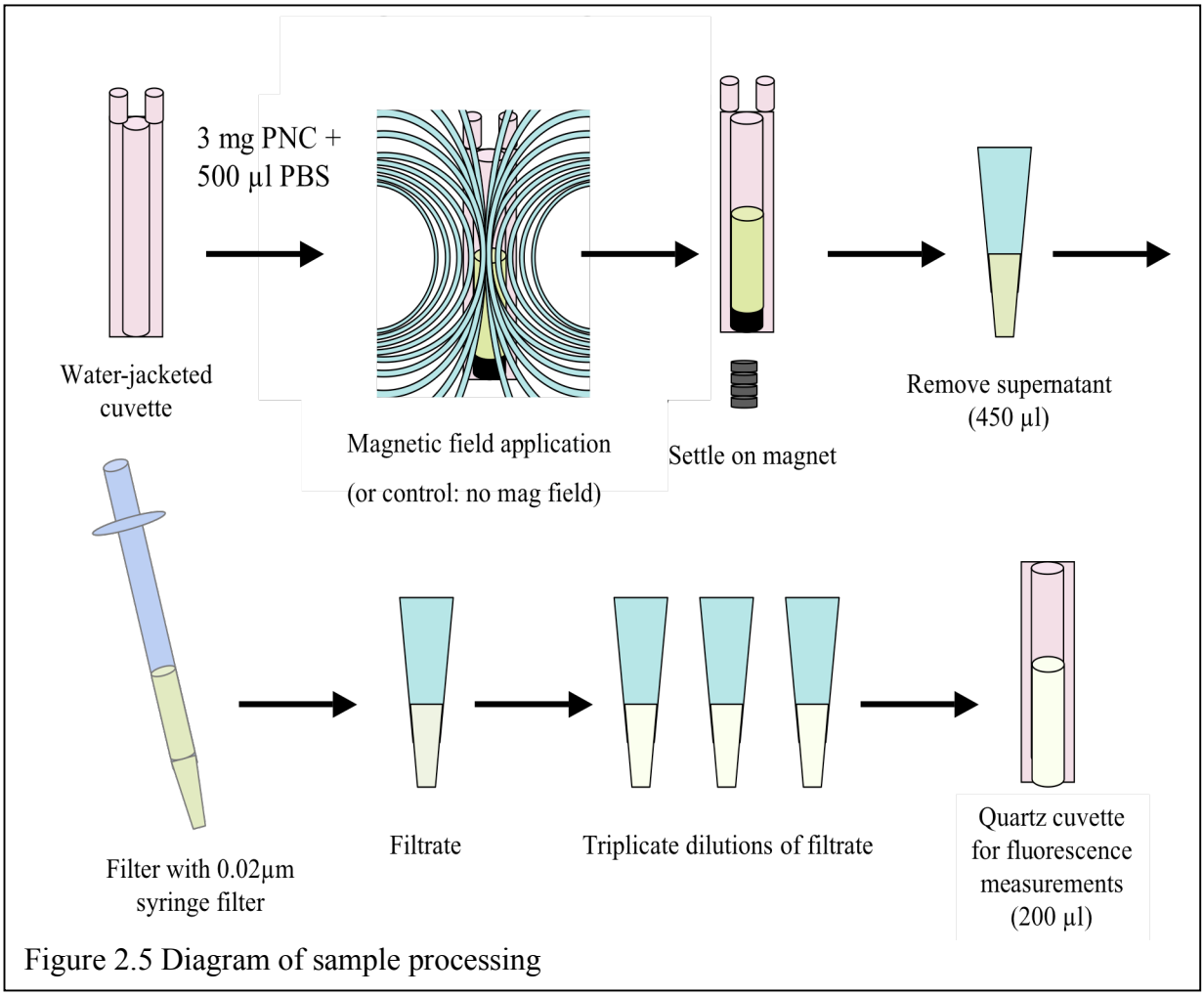

Figure 2.5 Diagram of sample processing

The fluorescent content of each dilution was measured using a spectrofluorimeter (LS55 Luminescence Spectrometer, Perkin Elmer Instruments). A quartz cuvette (Starna Cells, Quartz Fluorimeter Cell) was filled with 200 µl of the filtered medium. Excitation and emission wavelengths were set to 492 nm and 518 nm respectively, the excitation and emission to detect

FITC. Slit widths were set to excitation/emission wavelengths of 10 nm /15 nm to increase the sensitivity of detection. All settings were controlled using FL Winlab Software.

- **Effect of Frequency on Release**

This first set of magnetic field release study tests was designed to compare the effect of three different frequencies on the release of FITC from the PNCs. The effect of frequency on release was studied at 44, 230, and 430 Hz for experimental durations of 5, 30, and 60 minutes using five replicate samples. The samples were processed and measured for fluorescence as stated above.

- **Effects of Duration, Temperature, and Presence of Magnetic Field on Release**

This set of magnetic field studies tested the release of FITC for four separate durations, at three separate temperatures, and with and without the presence of the magnetic field. The effect of the duration that the samples were allowed to release was studied by testing release for 30, 60, 120, and 180 minutes. The effect of temperature was studied at the sample environmental temperatures of 4°C, 22°C, and 43°C. These temperatures were measured over a period of 3 hours at 430 Hz using a thermocouple and a multimeter. The samples were tested for the percentage of FITC released under each of these parameters in the magnetic field at 430 Hz and outside of the magnetic field (0 Hz) as a control. Each experiment was completed in triplicate with the exception of the 43°C samples, which were tested in duplicate. After each experiment, the composites were settled on a magnet and 450 μl of the medium was removed. The medium was filtered, diluted, and fluorescent measurements were completed.

- **Effect of Magnetic Material on Release**

The effect of magnetic material on release was studied by comparing release from magnetite-PNCs and cobalt-PNCs. Samples were run for 60 minutes at 4°C, 22°C, and 43°C. Samples magnetically induced at 430 Hz and controls not subjected to the magnetic field were completed in triplicate. The composites were then settled on a magnet, the medium was removed, filtered, diluted, and measured for fluorescence.

- **Natural Release**

Two samples were used to test natural release at room temperature. One washed sample of magnetite-PNCs was tested for natural release at room temperature to determine an approximate amount of FITC that could be released in one week. The other sample was a sample of un-washed magnetite-PNCs that was tested for release for 2.5 hours. The samples were processed and measured for fluorescence.

- **Standard Concentration Curve**

A standard concentration curve was made by dissolving 3 mg FITC in 60 ml PBS (0.05 mg/ml). Fluorescence was measured in triplicate at various concentrations ranging from 0.0001 μg/ml to 0.01 μg/ml using the spectrofluorimeter at excitation and emission wavelengths of 492 nm and 518 nm, and excitation and emission slit widths of 10 nm and 15 nm. The concentration curve was analyzed for precision and accuracy using Equations 4 and 5 (Thejavathi et al. 1995).

Precision = relative standard deviation = (standard deviation/mean) x 100 [4]

Accuracy = measure concentration x (100/actual concentration) [5]

The measurements for the above calculations were made in triplicate using a concentration of 0.0009 ug/ml of FITC in PBS. The graph for the standard concentration curve of FITC in PBS is shown in Appendix A.

2.3 Statistical Analysis

Values for percent FITC released are indicated as mean ± standard deviation. Statistical analysis was completed by using SAS software. The effect of frequency on release was analyzed using a two-way ANOVA. The statistical analysis for the remaining studies was analyzed using two three-way ANOVAs. Significance was determined by the Tukey adjusted p-value of $p \leq 0.05$.

Chapter 3 – Results

3.1 Characterization

3.1.1 Size and Morphology

TEM micrographs of magnetite and cobalt showed that both types of nanoparticles are roughly spherical in shape and that cobalt was more monodispersed than magnetite, which appeared agglomerated (Figure 3.1). The monodispersity of cobalt was expected due to the addition of a surfactant during synthesis, specifically, SB12 (Wu et al. 2004). The average diameters of magnetite and cobalt were 11.21 nm and 4.65 nm, respectively. They were estimated with the aid of MetaVue software using 300 nanoparticles each. The size distribution of magnetite was wider than the size distribution of cobalt (Figure 3.2). This uniformity in size was also attributed to incorporation of SB12. SEM images of magnetite-PNCs and cobalt-PNCs showed composites of irregular shapes and sizes; however, they generally appeared flat and less than 250 μm as expected since they were crushed and sieved to 250 μm or less (Figure 3.3).

3.1.2 Fluorescence Imaging

Fluorescent microscope images of dry magnetite-PNCs did not appear fluorescent; however, fluorescence was detected with an added drop of PBS, indicating that FITC was contained within the PNCs (Figure 3.4a). The fluorescence of the dry particles could not be seen using a fluorescent microscope due to the lack of FITC on the particle surface, which was removed during washing. The dry cobalt-PNCs, however, appeared fluorescent with and without the addition of PBS, indicating surface fluorescence that was not fully removed during washing (Figure 3.4b). This suggestion is reinforced by the amount of FITC removed from the particle surface of magnetite-PNCs compared to cobalt-PNCs by the washing process (Section 3.2.6,

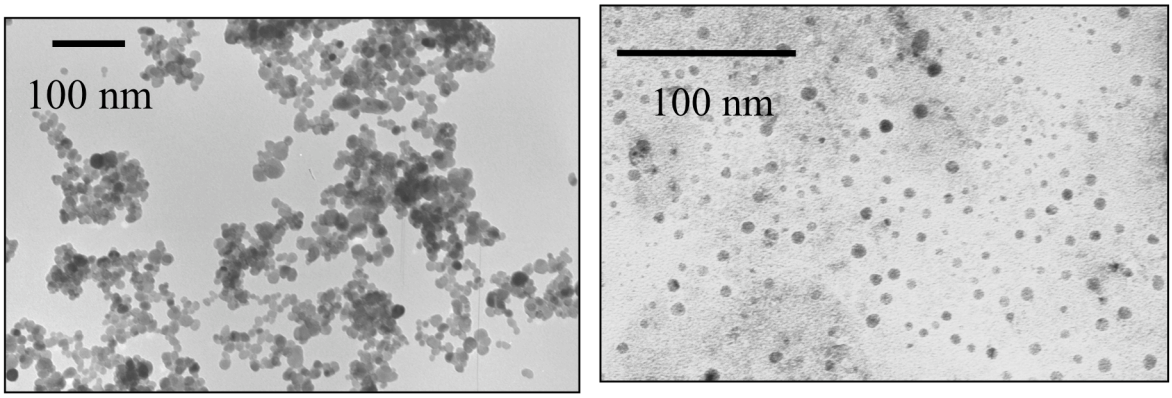

Figure 3.1. TEM images of magnetite (left) and cobalt (right).

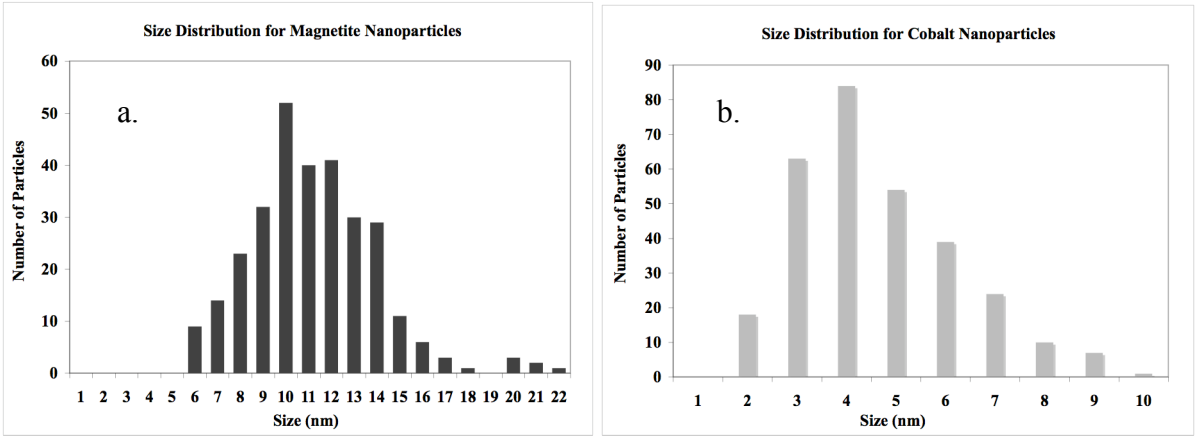

Figure 3.2. Size distributions of magnetite (a) and cobalt (b).

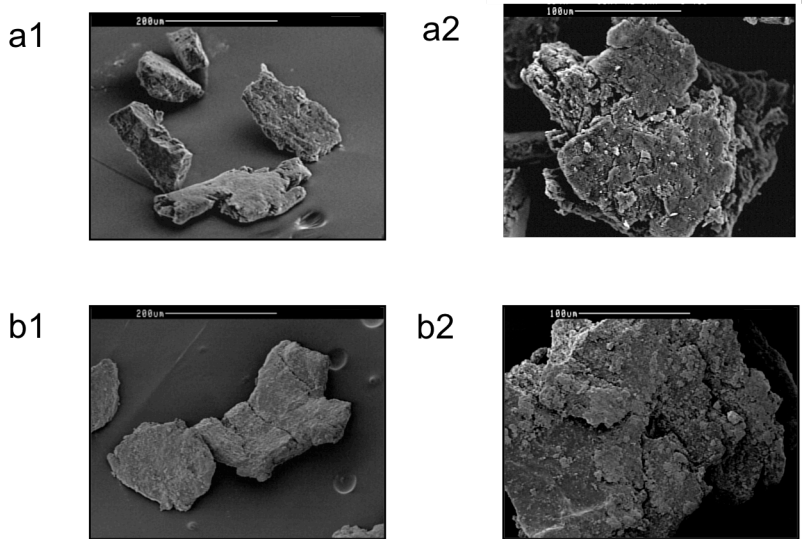

Figure 3.3. SEM images of magnetite-PNCs at 200X (a1), 400X (a2), and cobalt-PNCs at 200X (b1), 400X (b2).

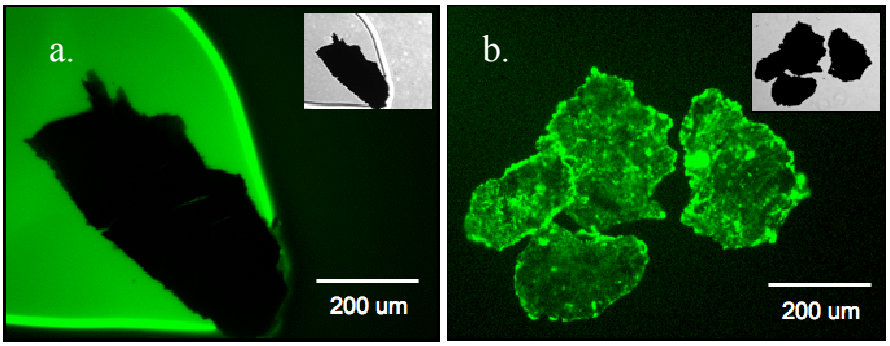

Figure 3.4. Fluorescent microscopy images of magnetite-PNC (a) and cobalt-PNCs (b). Inset: Brightfield image of magnetite-PNC (a) and cobalt-PNCs (b).

Figure 3.12). The remaining surface fluorescence of cobalt-PNCs was attributed to possible interactions of FITC with the surfactant SB12 used in the synthesis of cobalt nanoparticles.

3.1.3 Magnetization

SQUID magnetometer data for magnetite and cobalt showed hysteresis at 10 K and a lack of hysteresis at 300K, signifying that they were superparamagnetic (Liu et al. 2003). Similarly, the magnetic data for both magnetite-PNCs and cobalt-PNCs showed hysteresis at 10 K, but not at 300 K. Despite the increase in overall particle size, it was expected that the polymer-coated magnetic particles would remain superparamagnetic (Chatterjee et al. 2004; Zheng et al. 2005). Superparamagnetism is an important feature of particles used for magnetic release because it allows the particles to be magnetized under a magnetic field, but to return to their non-magnetic, relaxed state after the removal of the magnetic field (Kim and Park 2005).

3.1.4 Magnetic Material Content

The amount of magnetic material inside the polymeric microparticles was determined by elemental analysis. Based on the theoretical and measured amount of iron in magnetite (72.36% and 62.4%, respectively), the calculated magnetite content in the synthesized magnetite was 86.34%. The measured amount of iron in magnetite-PNCs was 23.3%, and the calculated magnetite content of the magnetite-PNCs was 32.2%. The measured cobalt content in the synthesized cobalt was 88.3% and the amount in cobalt-PNCs was 28.4 %.

3.2 Release Analysis

3.2.1 Effect of Frequency on Release

The percentage of FITC (mean ± standard deviation) released from five samples of magnetite polymer nanoparticle composites in the presence of the magnetic field for 5 minutes at

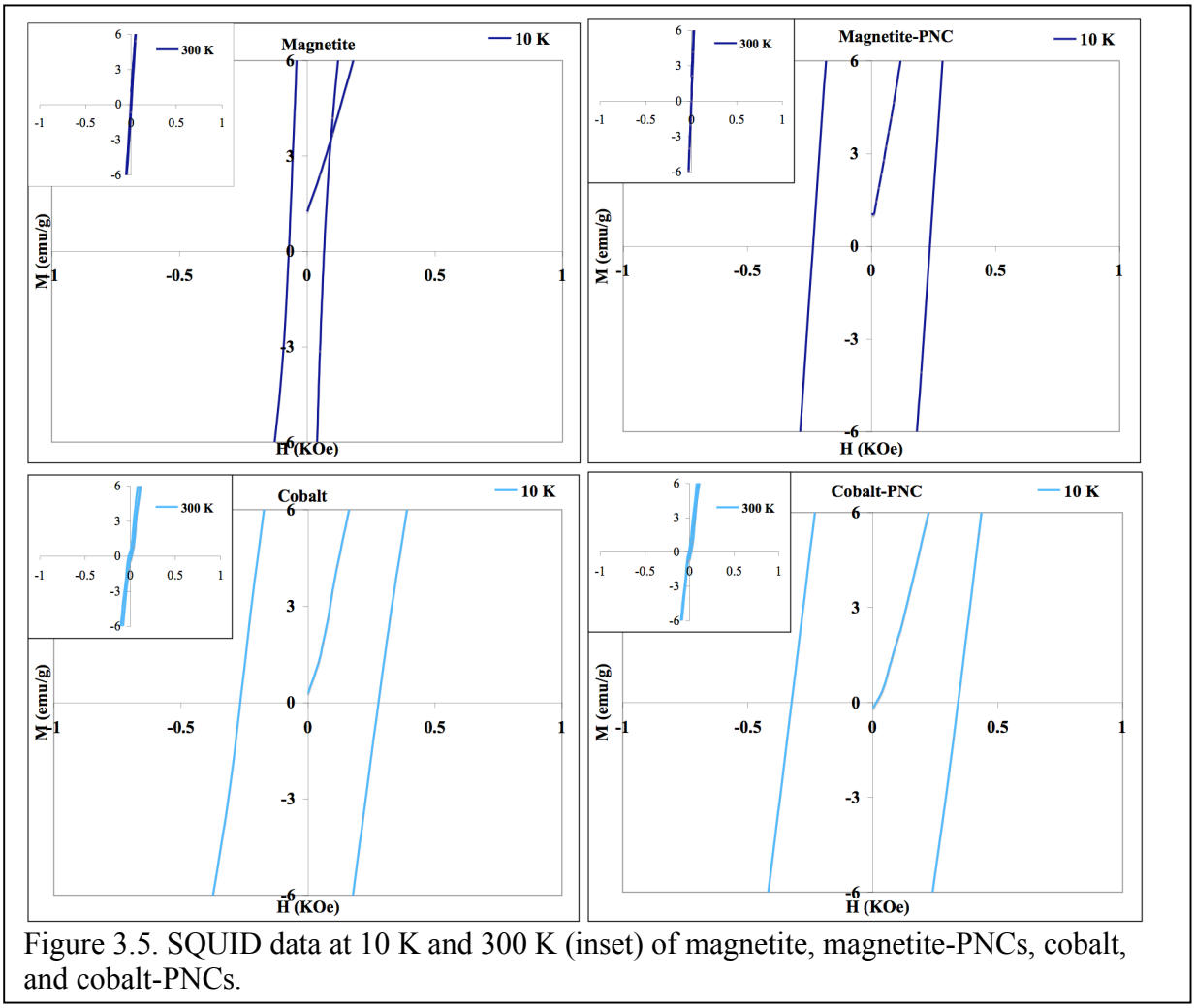

Figure 3.5. SQUID data at 10 K and 300 K (inset) of magnetite, magnetite-PNCs, cobalt, and cobalt-PNCs.

frequencies of 44, 230, and 430 Hz was 1.44±0.39%, 1.47±0.28%, and 1.52±0.23%, respectively. The mean percentage of FITC released from five samples after they were exposed to the magnetic field for 30 minutes at frequencies of 44, 230, and 430 Hz was 2.20±0.53%, 2.70±0.19%, 2.87±0.38%, respectively. The percentage of FITC (mean ± standard deviation) released from five samples after exposure to the magnetic field for 60 minutes at frequencies of 44, 230, and 430 Hz was 3.54±0.42%, 2.81±0.45%, and 3.84±0.54%, respectively (Figure 3.6).

The release was insignificantly different as a function of frequency with the exception of the release tested at 230 and 430 Hz for 60 minutes (adj p=0.0056). The frequency of 430 Hz was chosen for the remaining magnetic field tests because the release at 44, 230, and 430 Hz was generally similar to each other, and the largest amount of FITC was released at 430 Hz.

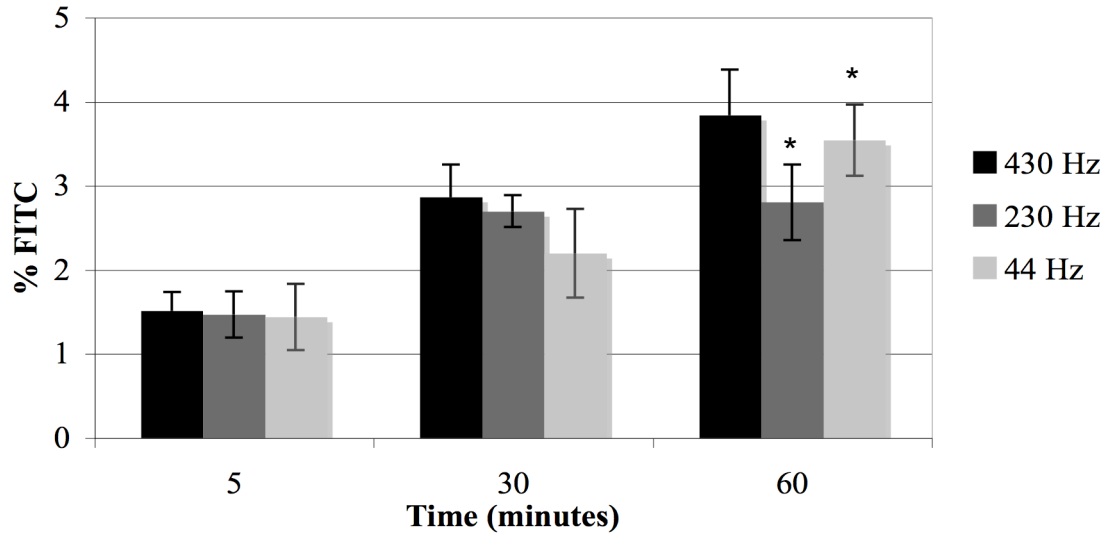

Figure 3.6. Percent of FITC released over time at 44, 230, and 430 Hz. Statistically different results are indicated by asterisks (*).

The difference in release of FITC at frequencies of 44 Hz and 430 Hz is similar to what has been done in other works. Lu et al. (2005) showed that frequencies above 300 Hz was found

to have a weaker ability to allow dextran-FITC to permeate into the polymeric particles, unlike the current study, which showed very little difference between release at 230 Hz and 430 Hz. Several other studies have shown a release at frequencies lower than 44 Hz. De Paoli et al. (2006) released Dex-R from collagen gels containing magnetite at 0.3 Hz, while Edelman et al. (1985) released BSA from ethylene-vinyl acetate copolymer containing samarium cobalt at a range of 0.87 – 11 Hz. These studies are an indication that release of FITC from PMMA may also occur below 44 Hz. Viroonchatapan et al. (1997) showed release at a high frequency of 500 kHz. Increasing the frequency to the kilohertz range may also be a possibility to increase the amount of FITC released.

3.2.2 Effect of Duration on Release

The percentage of FITC (mean ± standard deviation) released from three samples of magnetite-PNCs that were exposed to the magnetic field at 4°C were 2.39±0.17% at 30 minutes; 2.48±0.41% at 60 minutes; 3.17±0.48% at 120 minutes; and 2.79±0.47% at 180 minutes. The corresponding controls not subjected to the magnetic field, but kept at 4°C, resulted in the release of 2.97±0.42% at 30 minutes; 2.43±0.25% at 60 minutes; 2.29±0.76% at 120 minutes; and 2.62±0.17% at 180 minutes (Figure 3.7, Table 3.1).

There was no statistical difference between the amount of FITC released from magnetite-PNCs that were exposed to the magnetic field at any combination of the durations of 30, 60, 120, or 180 minutes for samples run at 4°C. Similarly, there was no statistical differences of the release from the control samples that were not exposed to the magnetic field for any combination of the experimental durations at 4°C.

The percentage of FITC (mean ± standard deviation) released from three samples of magnetite-PNCs that were exposed to the magnetic field at 22°C were 3.04±0.26% at 30

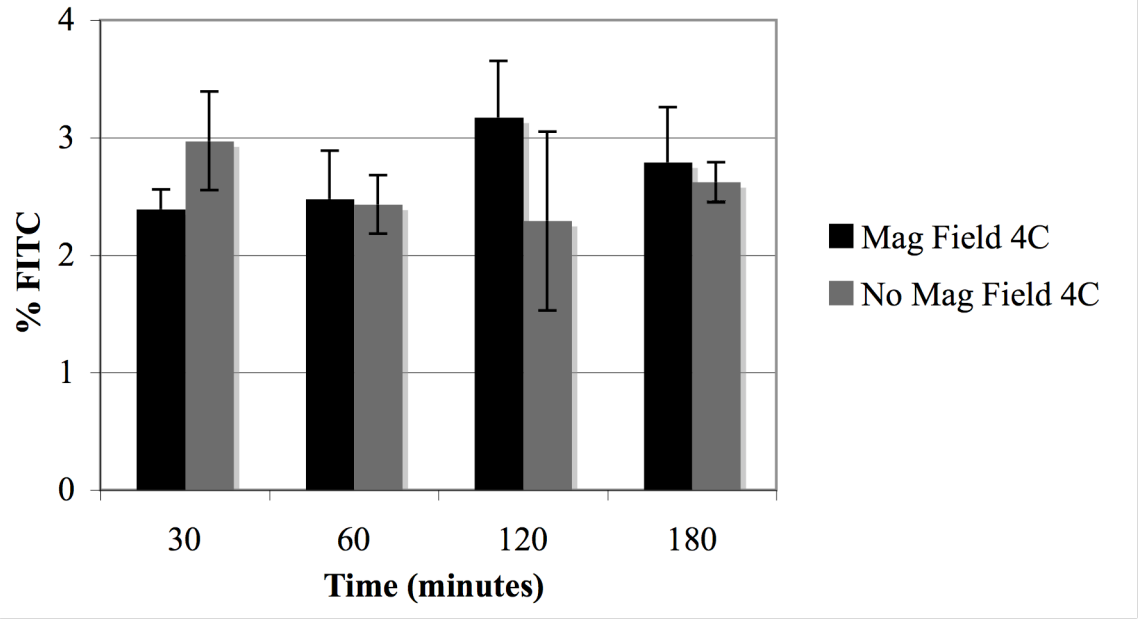

Figure 3.7. Percent of FITC released for 30, 60, 120, and 180 minutes with (430 Hz) and without (0 Hz) magnetic field at 4°C.

minutes; 3.80±0.45% at 60 minutes; 3.66±0.64% at 120 minutes; and 4.04±0.39% at 180 minutes. The corresponding controls not subjected to the magnetic field, but kept at 22°C, resulted in the release of 2.59±0.35% at 30 minutes; 2.83±0.62% at 60 minutes; 3.19±0.12% at 120 minutes; and 3.55±0.12% at 180 minutes (Figure 3.8, Table 3.1). Table 3.1 also shows the standard deviation of percent FITC released.

There was no statistical difference between the amount of FITC released from magnetite-PNCs as a function of time when then samples were exposed to the magnetic field for any combination of 30, 60, 120, or 180 minutes at 22°C. Similarly, there was no statistical difference between the release from the control samples for any combination of the experimental durations that were also run at 22°C.

The percentage of FITC (mean ± standard deviation) released from two samples of magnetite-PNCs that were exposed to the magnetic field at 43°C were 4.52±0.18% at 30

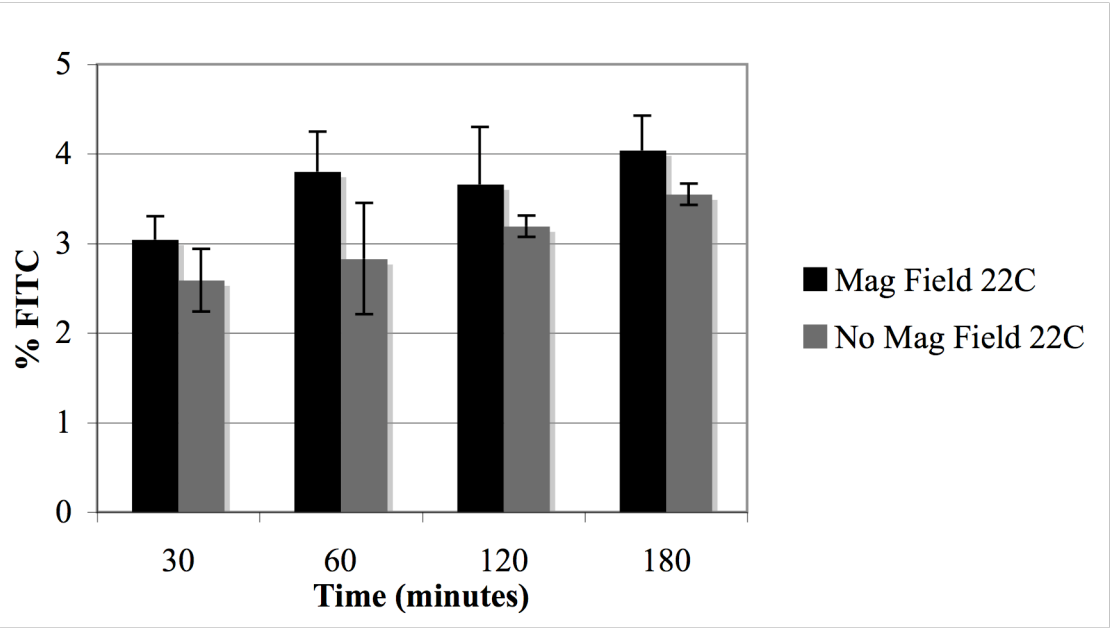

Figure 3.8. Percent of FITC released for 30, 60, 120, and 180 minutes with (430 Hz) and without (0 Hz) magnetic field at 22°C.

minutes; 5.28±0.01% at 60 minutes; 5.52±0.23% at 120 minutes; and 6.29±0.08% at 180 minutes. The corresponding controls not exposed to the magnetic field, but kept at 43°C, resulted in the release of 4.77±0.38% at 30 minutes; 5.33±0.14% at 60 minutes; 5.75±0.16% at 120 minutes; and 6.88±1.23% at 180 minutes (Figure 3.9, Table 3.1).

There was no statistical difference of FITC released from magnetite-PNCs between samples that were exposed to the magnetic field for 60 and 120 minutes, and any other duration at 43°C. Similarly, there was no statistical difference between the release of the control samples at 60 and 120 minutes, and any other duration at 43°C. There was, however, statistical difference between the release of FITC from magnetite-PNCs with magnetic field at 43°C for 30 minutes and those without it for 180 minutes (adj p=0.0034). Similarly, there was statistical difference for the control samples at 43°C between the durations of 30 and 180 minutes (adj p=0.0310).

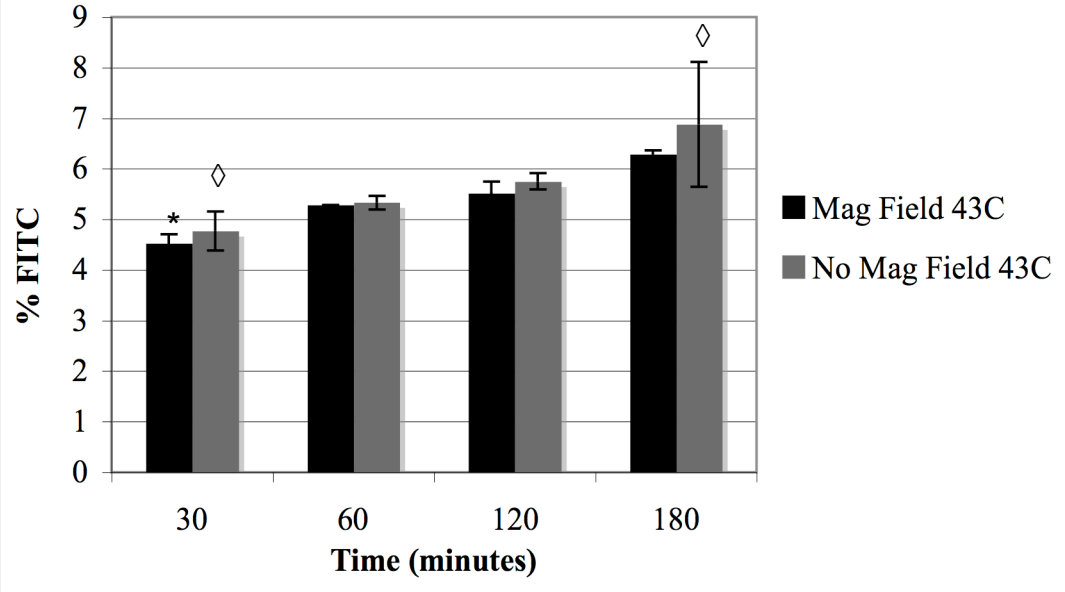

Figure 3.9. Percent of FITC released for 30, 60, 120, and 180 minutes with (430 Hz) and without (0 Hz) magnetic field at 43°C. Statistically different results are indicated by asterisks (*) and diamonds (◊).

Release of FITC from magnetite-PNCs was generally independent of experimental duration, with the exception of a slight difference in release at 30 minutes compared to release at 180 minutes for the 43°C samples. These results are similar to other studies of magnetically induced release. Viroonchatapan et al. (1997) exhibited a slightly higher release of 5-FU from magnetoliposomes at 42°C for 120 minutes (2.54%) than for 60 minutes (~2.3%) and 30 minutes (~2.2%).

Other studies, however, allowed release to occur for longer periods of time than 180 minutes. De Paoli et al. (2006) showed about 25% and 28% release of Dex-FITC from a collagen gel at 5 and 10 hours, respectively, but about 45% at 24 hours, 65% at 48 hrs, and about 80% at 72 hours. This data was normalized to 100% release at 96 hours. Based on the findings of this study, a higher release of FITC from PMMA may have occurred at longer time periods than the maximum that was test of 180 minutes.

Another factor of release that De Paoli et al. (2006) studied was the effect of the molecular weight of the entrapped molecule. At 180 minutes in the magnetic field, about 30% release of Dex-FITC with 3,000 MW compared to 10% with 10,000 MW, and 3% with 70,000 MW. These results may be help to determine why the release of FITC from PMMA was so low. FITC has a MW of less than 240 Daltons, which is quite small compared to the size of the active component in the studies from De Paoli et al. (2006). The low release of FITC is most likely not due to its small size. It is possible, however, that the high molecular weight of the polymer in a drug delivery system plays a significant role in the ability of the entrapped active component to be released. In the current study, the molecular weight of PMMA is 950,000 Da, which is quite high and may be resisting the release of FITC. Perhaps PMMA that has a lower molecular weight would increase the release of FITC.

3.2.3 Effect of Temperature on Release

For magnetite-PNCs samples that were exposed to the magnetic field for 30 minutes, (Figure 3.10), there was no statistical difference between the release of FITC at 4°C and 22°C, nor between 22°C and 43°C; however, there was statistical difference between release at 4°C and 43°C (adj $p=0.0007$). The 60-minute samples also showed no statistical difference between the release of FITC at 4°C and 22°C, nor between 22°C and 43°C; however, there was statistical difference between release at 4°C and 43°C (adj $p<0.0001$). The 120-minutes samples had no statistical difference between release at 4°C and 22°C; however, there was significance difference between release at 22°C and 43°C (adj $p=0.0055$), and between release at 4°C and 43°C (adj $p=0.0001$). Similarly, the 180-minutes samples had no statistical difference between release at 4°C and 22°C; however, there was also statistical difference between release at 22°C and 43°C (adj $p=0.0003$); and between release at 4°C and 43°C (adj $p<0.0001$).

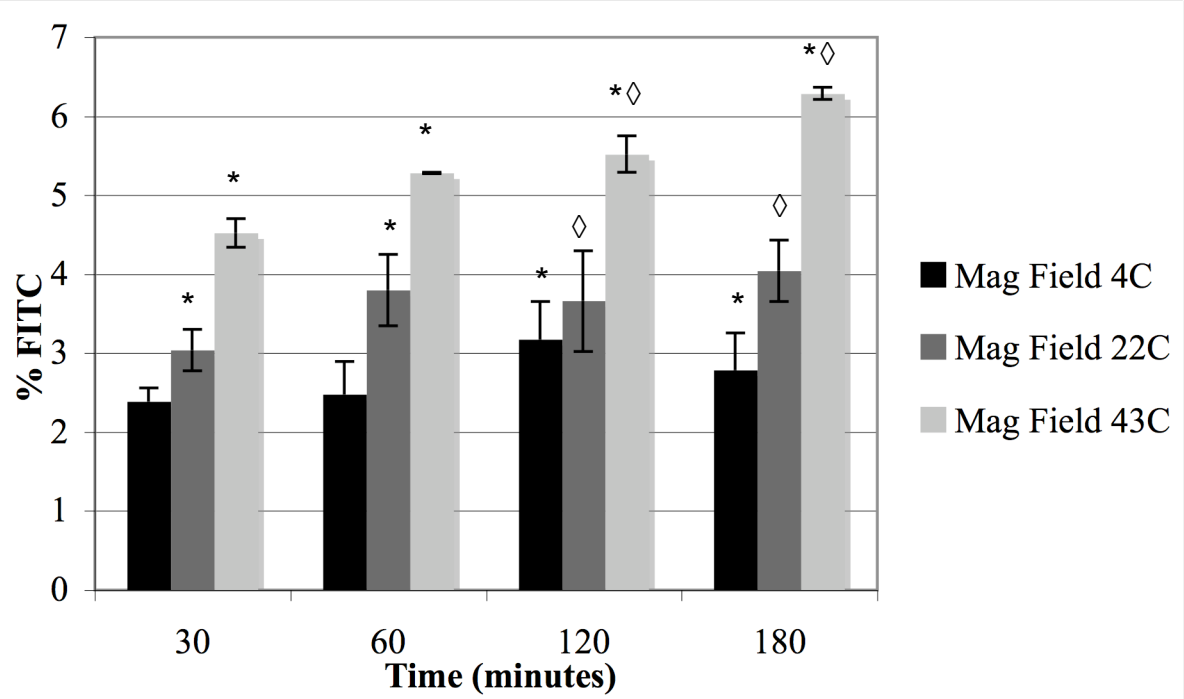

Figure 3.10. Percent of FITC released for 30, 60, 120, and 180 minutes with (430 Hz) magnetic field at 4°C, 22°C, 43°C. Statistically different results are indicated by asterisks (*) and diamonds (◊).

For the control magnetite-PNCs samples with experimental duration of 30 minutes (Figure 3.11), there was no statistical difference between the release of FITC at 4°C and 22°C; however, there was statistical difference between release at 22°C and 43°C (adj p=0.0005), and between release at 4°C and 43°C (adj p=0.0084). The 60-minute control samples had no statistical difference between the release of FITC at 4°C and 22°C; however, there was statistical difference between release at 22°C and 43°C (adj p<0.0001), and between release at 4°C and 43°C (adj p<0.0001). The 120-minute control samples had no statistical difference between the release of FITC at 4°C and 22°C; however, there was statistical difference between release at 22°C and 43°C (adj p=0.0001), and between release at 4°C and 43°C (adj p<0.0001). Finally, the 180-minute control samples had no statistical difference between the release of FITC at 4°C and

22°C; however, there was statistical difference between release at 22°C and 43°C (adj $p<0.0001$), and between release at 4°C and 43°C (adj $p<0.0001$).

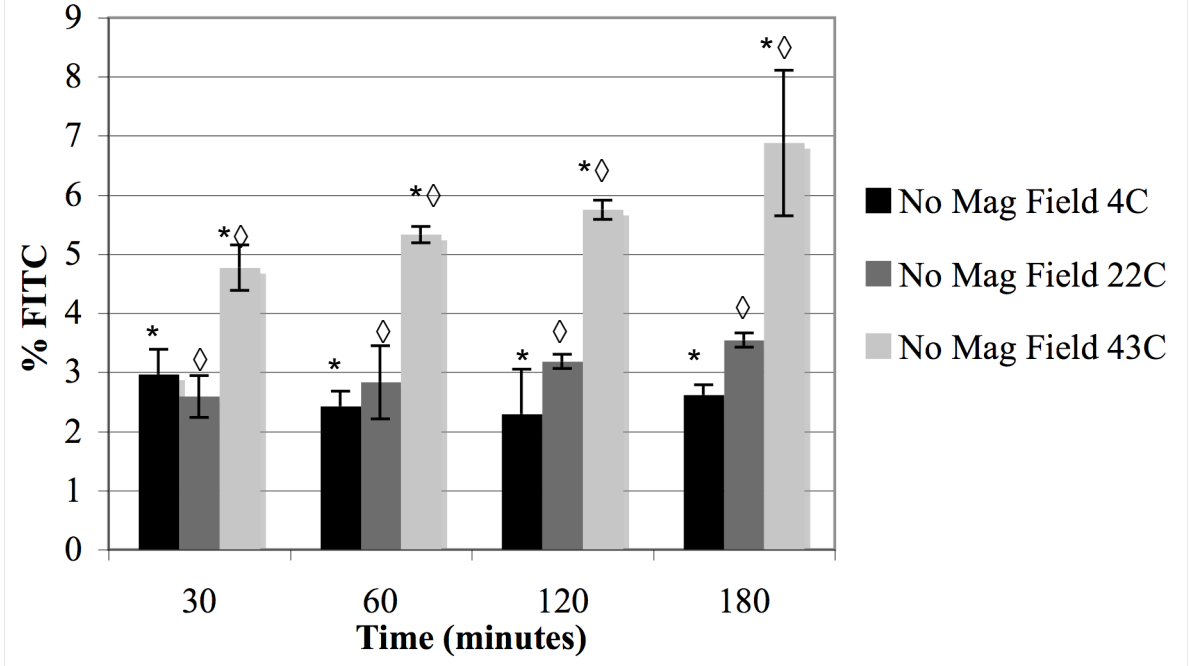

Figure 3.11. Percent of FITC released for 30, 60, 120, and 180 minutes without (0 Hz) magnetic field at 4°C, 22°C, 43°C. Statistically different results are indicated by asterisks (*) and diamonds (◊).

The most prominent factor for release from magnetite-PNCs proved to be the sample environmental temperature. These PNCs may have a thermoresponsive quality that can be seen by a generally higher release of FITC at 43°C than at 22°C. There was no difference in release between any samples at 4°C and 22°C. These results are in agreement with natural release studies form De Paoli et al. (2006), which showed 20% release of Dex-R at 37°C, compared to 10% release at 24°C. Both of these studies were done in the absence of a magnetic field. Similarly, thermoresponsive studies from Viroonchatapan et al. (1997) showed 2.25% release of 5-FU at 42°C attained using a magnetic field, compared to 1% release at 37°C without the use of

a magnetic field. These results of increase in release from 24°C to 37°C to 42°C are similar to the finding of the current study of an increase in release of FITC from 22°C to 43°C, and confirms the thermoresponsive quality of the release of FITC from PMMA. It also shows a possibility of a physiological thermal effect of the magnetic PNCs.

Table 3.1. Percent of FITC (mean ± SD) released from magnetite-PNCs for 30, 60, 120, and 180 minutes at 4°C, 22°C, and 43°C, with (430 Hz) and without (0 Hz) magnetic field

	30 min	60 min	120 min	180 min
No Mag Field 4°C	2.97±0.42	2.43±0.25	2.29±0.76	2.62±0.17
Mag Field 4°C	2.39±0.17	2.48±0.41	3.17±0.48	2.79±0.47
No Mag Field 22°C	2.59±0.35	2.83±0.62	3.19±0.12	3.55±0.12
Mag Field 22°C	3.04±0.26	3.80±0.45	3.66±0.64	4.04±0.39
No Mag Field 43°C	4.77±0.38	5.33±0.14	5.75±0.16	6.88±1.23
Mag Field 43°C	4.52±0.18	5.28±0.01	5.52±0.23	6.29±0.08

3.2.4 Effect of Magnetic Material on Release

The mean percentage of FITC released from three samples of cobalt-PNCs that were exposed to the magnetic field for 60 minutes were 0.69%±0.12 at 4°C; 0.83%±0.04 at 22°C; and 1.18%±0.04 at 43°C. The corresponding controls not subjected to the magnetic field, but kept at 4°C, 22°C, and 43°C for 60 minutes resulted in the release of 0.58%±0.01 at 4°C; 0.72%±0.22 at22°C; and 1.03%±0.1 at 43°C (Figure 3.12). A significant difference was found between the release of FITC from cobalt-PNCs and that from magnetite-PNC at all three temperatures for both the magnetic field samples and the control samples (adj $p < 0.0001$). The release from magnetite-PNCs was greater than that from cobalt-PNCs; the difference between the release from magnetite-PNCs and cobalt-PNCs was the most dramatic result of the studies. Release of

FITC from cobalt-PNCs, however, was independent of temperature and the presence of the magnetic field.

The low amount of release from cobalt is attributed to possible interactions between SB12 and FITC, which may be inhibiting the release of FITC. Other factors, such as the molecular weight of the polymer, the molecular weight of the active component, the hydrophobicity of the polymer and the active component with respect to the release medium, other interactions between the active component and the polymer or the magnetic material, the Tg of the polymer, the magnetic material content, sample temperature, sample duration, and discrepancies in the production of different batches of PNCs, are all possible contributors to the lack of release from the cobalt-PNCs compared to that of magnetite-PNCs.

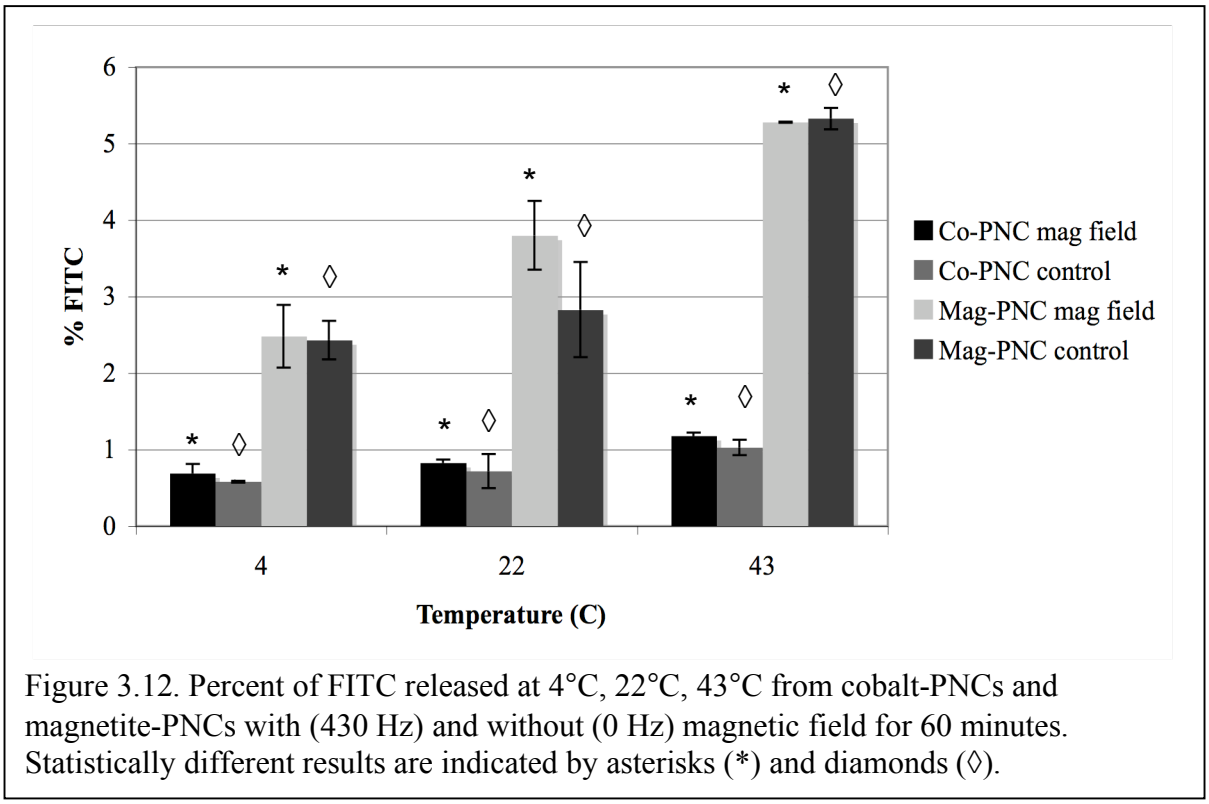

Figure 3.12. Percent of FITC released at 4°C, 22°C, 43°C from cobalt-PNCs and magnetite-PNCs with (430 Hz) and without (0 Hz) magnetic field for 60 minutes. Statistically different results are indicated by asterisks (*) and diamonds (◊).

3.2.5 Effect of Presence of Magnetic Field on Release

All samples that tested the effect of the presence of magnetic field were insignificantly different when compared with the controls. There was no statistical difference between the release of FITC from magnetite-PNCs nor from cobalt-PNCs in the presence and in the absence of the magnetic field at any of the environmental temperatures (4°C, 22°C and, 43°C) and for any of the four durations (30, 60, 120, or 180 minutes).

Since there were no differences between the release from particles that were magnetically induced and those that were not. It could be stated that the magnetic field at 465 G and 430 Hz did not sufficiently oscillate the magnetic material inside the PMMA-based particles to reach a local temperature of 96°C. If it had, the PMMA molecular bonds should have weakened, loosening the polymeric structure and allowing more FITC to be released.

Some factors that could be changed to possibly enhance release via magnetic field could be to increase the magnetic material content or the size of the magnetic nanoparticles, to increase the magnetic field strength while testing at low frequencies, to test a large range of frequencies over a range of field strengths, to use a polymer with a lower Tg than 96°C, and to increase sample duration.

The following studies show positive results of magnetically induced release: Viroonchatapan et al. (1997) showed approximately 56% increase in the release of 5-FU from magnetoliposomes using a magnetic field (2.25% release) for 2 hours than without using the magnetic field (1% release). The release was at a similar, low level to the release from PMMA in the present study. The particles were made with dextran-magnetite (~ 8 nm) of similar size to magnetite used in the present study (~ 11 nm), but were exposed to a much higher magnetic field strength of 15,000 G and 500 kHz.

Rana et al. (2007) showed a 60% increase in release of Dox from PMAA-coated nickel ferrite particles over 2 hour using a magnetic field (15% release) than without it (6% release). This release is at the higher end of release that was achieved in the present study at 3 hours. The magnetic field strength used to release Dox was 1000 Oe. The frequency used was not mentioned.

3.2.6 Analysis of Washings

The fluorescence measurements of the washings showed that 8.32% FITC was removed from cobalt-PNC compared to 30.6% that was removed from magnetite-PNCs (Figure 3.13). These results confirm the findings from the fluorescent images, which showed that there was more surface fluorescence left on the cobalt-PNCs than on the magnetite-PNCs after washing. The reason why this occurred is thought to be interactions between FITC and the surfactant used to synthesize cobalt, which hindered FITC release during washing and controlled release studies The low amount of release of FITC from cobalt-PNCs compared to the release from magnetite-

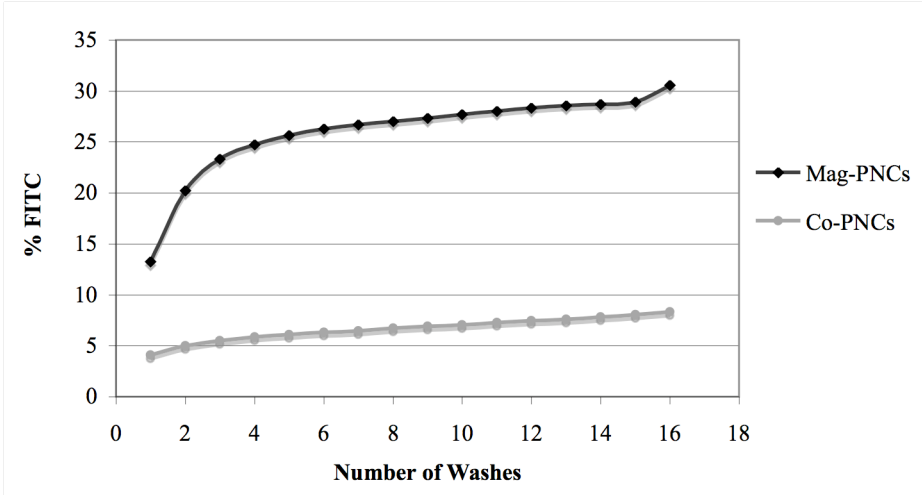

Figure 3.13. Percentage of FITC removed from washes of magnetite-PNCs and cobalt-PNCs.

PNCs in the release studies, also confirms this idea, especially considering that there may have been more FITC available to be released from the cobalt-PNCs than from the magnetite-PNCs after they were washed.

3.2.7 Natural Release Study

The natural release of FITC for one week was 6.5 ± 0.3% (n=3). This release is 45% higher than the comparable release of FITC at room temperature for 180 minutes (3.55 ± 0.12%). Un-washed magnetite-PNCs released 11.23% (n=1) FITC after 2.5 hours at room temperature. This release is about 72% and 68% higher than release for 2 and 3 hours, respectively, at room temperature from washed magnetite-PNCs. These results show that it is necessary to wash the particles and that the current washing system is sufficient to remove surface fluorescence from magnetite-PNCs.

Chapter 4 – Conclusions and Future Considerations

4.1 Conclusions

While the LIGA process created uniform composites of a controlled size, very small amounts could be made at one time. The batch process, therefore, was used to make the PNCs that were used for the experiments.

Release of FITC from magnetite-PNCs was generally dependent upon temperature and independent of both experimental duration and presence of magnetic field. Release of FITC from cobalt-PNCs was independent of temperature and the presence of the magnetic field. The release from magnetite-PNCs was greater than that from cobalt-PNCs.

Magnetic polymeric nanoparticle composites displayed a thermoresponsive behavior that was seen by the difference in release of FITC at 22°C and 43°C. A physiological thermal response may also exist for these particles.

Cobalt-PNCs did not release more FITC than magnetite-PNCs as would be expected since less FITC was removed during washing from cobalt-PNCs. The presence of a surfactant on cobalt nanoparticles, which was not present on magnetite, explains why less FITC was released from cobalt-PNCs than from magnetite-PNCs due to a possible interaction between the surfactant and FITC in cobalt-PNCs.

Magnetically induced release of FITC from magnetic PNCs was not attained during the course of this study. The reason that the release of FITC was the same with and without the magnetic field may be the use of PMMA as the polymer. The use of a different polymer with a lower Tg may help increase release by allowing the magnetic particles to sufficiently heat and loosen the polymer matrix.

Release of FITC with and without the magnetic field was fairly low. A range of release from 2.29% - 6.88% was achieved from magnetite-PNCs during the controlled release studies. A possible reason for this low release is the use of PMMA with a molecular weight of 950,000 Da. Natural release from PMMA 350,000 Da MW achieved 10% drug release, while PMMA 120,000 Da MW released 20% drug (Asrar et al. 2004). PMMA with a lower molecular weight may help achieve higher release either with or without the magnetic field.

4.2 Future Considerations

Future considerations include further investigation of a more efficient process to make uniform composites of controlled sizes in large quantities based on the LIGA technology.

Another important step for the future of this study is the achievement of greater release with the magnetic field than without the magnetic field, possibly by testing the release from PMMA with a lower molecular weight than 950 kDa. The use of a polymer with a lower Tg than that of PMMA may increase the magnetic field-induced release at the current magnetic field strength and frequency. Another idea is to use a stronger magnetic field and a higher frequency to locally attain the Tg of PMMA.

A study on the thermoresponsive quality of the magnetite-PNCs for a possible physiological response can be carried out by determining the difference in release at 37°C as compared 22°C and 43°C.

Eventually, *in-vitro* and *in-vivo* testing of the release of an active biomolecule to determine the efficacy of magnetically induced release in cells would complete the ultimate vision for a controlled drug delivery system using magnetic nanoparticles and a polymer matrix.

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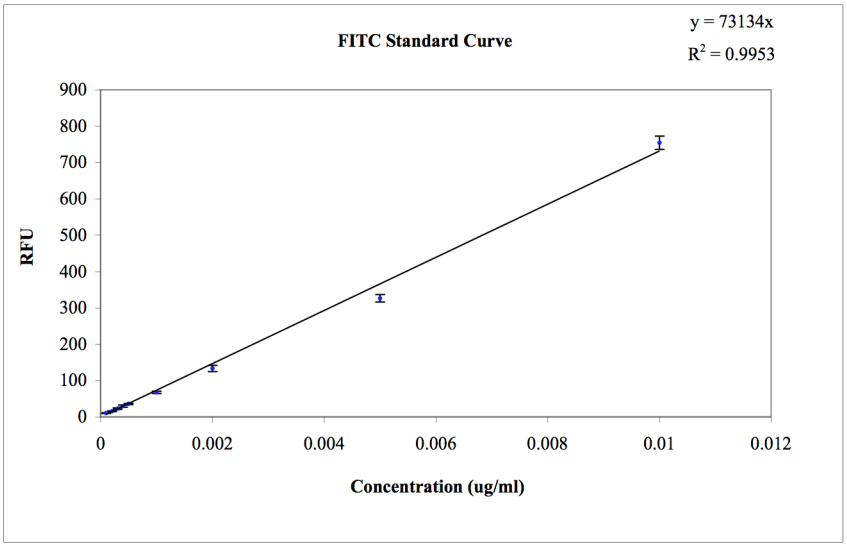
Appendix A: Standard Concentration Curve of FITC in PBS

The standard curve was used to determine the concentration of the FITC released from the PNCs. Its precision was ±2.84% and it was 96.84% accurate.



Standard concentration curve of FITC in PBS.

Appendix B: Calculations for Percentage of FITC Released

Concentration 1 is the concentration of fluorescence in a filtered and diluted sample from the release experiments:

C1 = RFU/73134, where

73134 is the slope from the standard curve of FITC in PBS

Concentration 2 is the concentration of the dilution of the release sample:

C2 = C1 x (v1/v2), where

v1 = v2 + 480 µl PBS = 500 µl = 0.5 ml

v2 = volume of released sample (filtered) = 20 µl = 0.02 ml

Amount FITC Released = C2 x v3 x 1000, where

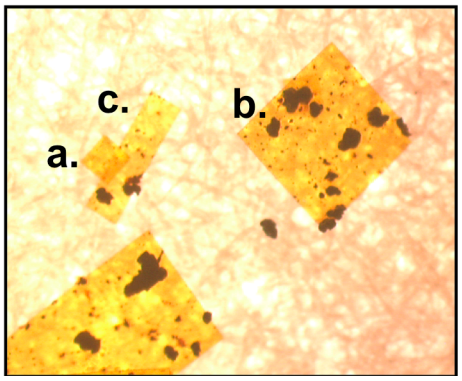
v3 = volume of sample measured for fluorescence

% FITC Released = (Amount FITC released / n) x 100, where

n = amount of FITC available for release in 3 mg of PNCs after washing

Appendix C: Light Microscope Images of PNCs Made with LIGA

Optical microscopy images of LIGA-processed PNCs showed composites of precise shape and size.



Optical microscopy images of magnetite-PNCs. Magnetite-PNCs made by the LIGA process of sizes of (a.) 100 x 100 x 3 μm, (b.) 400 x 400 x 3 μm, (c.) 100 x 40 x 3 μm, and (d.) 3 x 0.7 μm.

Vita

Michelle Urbina was born in New Orleans, Louisiana, in 1982, to John Cort and Yolanda Hurtado O'Brien. She grew up in Metairie and graduated from St. Mary's Dominican High School, in New Orleans, in May 2000. She moved to Baton Rouge, Louisiana, in August 2000 to start her college adventure at Louisiana State University in the Honors College, where she resided as a Residential Assistant for two years. She joined the Biological and Agricultural Engineering Department in August 2001, married Geber Enrique Urbina in January 2004, and received her Bachelor of Science in Biological and Agricultural Engineering in August 2005. She is currently a candidate for the degree of Master of Science in Biological and Agricultural Engineering, which she started in August 2005 and which will be awarded to her in August 2007.