Thermoresponsive iron oxide-polymer composite nanoparticles for multimodal cancer therapy

Sreekanth Purushotham

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THERMORESPONSIVE IRON OXIDE – POLYMER COMPOSITE NANOPARTICLES FOR MULTIMODAL CANCER THERAPY

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School of Materials Science and Engineering

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ABSTRACT

Magnetic nanoparticles possess unique properties that make them attractive materials for biomedical applications, such as cancer therapy, current modalities of cancer therapy suffer from several drawbacks. Multimodal cancer therapy by simultaneous hyperthermia and chemotherapy can synergistically enhance the therapeutic efficacy compared to individual/sequential application of these techniques.

In the current work, drug loaded thermoresponsive composite nanoparticles (CNP) were synthesized, characterized by transmission electron microscopy (TEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), UV-visible spectroscopy (UV-Vis), vibrating sample magnetometry (VSM), modulated differential scanning calorimetry (MDSC) and dynamic light scattering (DLS) techniques. They were evaluated by *in vitro* and *in vivo* tests for multimodal cancer therapy applications. γ -Fe₂O₃ as well as Fe₃O₄ MNP were synthesized by wet chemical methods. CNP with a Fe₃O₄ or γ -Fe₂O₃ MNP core and thermoresponsive crosslinked poly-n-isopropylacrylamide (PNIPAM) shell were synthesized by polymerization of monomers in a ferrofluid. CNP were loaded with the anti-cancer drug doxorubicin (dox).

The MNP exhibited good magnetic properties which were preserved after coating with PNIPAM and loading of dox. Swollen dox loaded Fe₃O₄ CNP exhibited temperature dependent drug release, with an initial burst phase followed by slow release. The lower critical solution temperature (LCST) of the shell could be used

as an on/off switch to decrease drug release below the transition temperature, enabling controlled drug release. The dox loaded CNP exhibited excellent *in vitro* heating in an alternating magnetic field (AMF) as well as simultaneous drug release. *In vitro* cytotoxicity tests on human bronchial endothelial cells with Fe_3O_4 CNP suggested low cytotoxicity of these particles. *In vivo* magnetic drug targeting, magnetic resonance imaging (MRI) and histology studies on a buffalo rat model indicated successful targeting of dox loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP to the liver tumor with negligible systemic distribution.

Modeling of dox release from CNP of different sizes was performed to study the effects of particle size on release kinetics, the optimum particle size for AMF induced heating of iron oxide MNP for *in vivo* hyperthermia applications was studied. Modeling of tumor heating by such particles was also performed to estimate MNP concentration and AMF parameters required for achieving hyperthermia. The quantities of dox loaded CNP for chemotherapy and hyperthermia are similar, supporting the feasibility of these particles for multimodal applications. This work shows that dox loaded thermoresponsive polymer coated iron oxide magnetic nanoparticles have excellent potential for combined drug targeting, hyperthermia and controlled drug release treatment of cancer.

TABLE OF CONTENTS

Acknowledgements

ABSTRACT

TABLE OF CONTENTS..... i

LIST OF ABBREVIATIONS..... xiv

CHAPTER 1: INTRODUCTION 1

1.1 Introduction..... 1

1.2 Cancer: the motivation..... 5

1.3 Inadequacy of existing treatments: the problem. 8

1.4 Targeted multimodal therapies: the solution..... 11

1.5 Objective 14

1.6 Scope of the project 14

1.7 Methodology 20

1.9 Organization of the thesis. 21

CHAPTER 2: BACKGROUND AND MOTIVATION..... 23

2.1 Introduction..... 23

2.2 Magnetic nanoparticles 23

2.2.1 Magnetic behavior of fine particles 23

2.2.2 Single domain particles..... 24

2.2.3 Coercivity of single domain particles 25

2.2.4 Coherent rotation of the magnetization..... 26

2.2.5 Superparamagnetism 28

2.3 Iron oxides for biomedical applications..... 30

2.3.1 Magnetite (Fe₃O₄) 30

2.3.2 Maghemite (γ-Fe₂O₃) 30

i

2.4 Factors affecting the <i>in vivo</i> applications of magnetic nanoparticles.	31
2.4.1 Particle properties	32
2.5 Surface coatings	34
2.6 Magnetic drug targeting and drug delivery	36
2.6.1 <i>In vivo</i> magnetic drug targeting	37
2.6.2 <i>In vitro</i> studies of magnetic nanoparticles for MDT	40
2.7 Hyperthermia	43
2.8 Magnetically mediated hyperthermia	44
2.8.1 Heat generation from magnetic nanoparticles by an alternating magnetic field	45
2.8.2 Types of magnetically mediated hyperthermia.....	50
2.8.3 Intracellular hyperthermia.....	52
2.8.5 Operational parameters for clinical hyperthermia in humans	55
2.8.6 First human clinical trials of magnetically mediated hyperthermia.....	56
2.9 Stimuli-responsive release systems.....	58
2.10 Thermoresponsive magnetic nanoparticles for biomedical applications	60
2.11 Simultaneous hyperthermia and chemotherapy from magnetic nanoparticles	62
2.12 Nanoparticle Synthesis.....	65
2.12.1 Precipitation from solution	65
2.12.2 High temperature synthesis from colloidal solutions.....	66
2.12.3 Co-precipitation in aqueous solutions.....	67
2.13 Coating of magnetic nanoparticles with thermoresponsive polymers and drug loading.....	68
2.14 Innovative aspects of the current work	69
CHAPTER 3: EXPERIMENTAL PROCEDURES.....	71

3.1 Synthesis of iron oxide and PNIPAM – iron oxide composite nanoparticles...	71
3.1.1 Synthesis of γ -Fe ₂ O ₃ magnetic nanoparticles by the high temperature decomposition method	71
3.1.2 Synthesis of PNIPAM – γ -Fe ₂ O ₃ composite nanoparticles by dispersion free-radical polymerization	73
3.1.3 Synthesis and processing of Fe ₃ O ₄ magnetic nanoparticles	74
3.1.3.1 Synthesis of 6 nm Fe ₃ O ₄ nanoparticles by the co-precipitation method	74
3.1.3.2 Synthesis of 13 nm Fe ₃ O ₄ nanoparticles by the co-precipitation method	75
3.1.4 Synthesis of PNIPAM – Fe ₃ O ₄ composite nanoparticles by dispersion free-radical polymerization	76
3.2 Drug loading of PNIPAM – iron oxide composite nanoparticles with doxorubicin	77
3.3 Characterization and <i>in vitro</i> tests.....	78
3.3.1 Transmission electron microscopy	78
3.3.2 Dynamic light scattering	79
3.3.3 Atomic Force Microscopy	79
3.3.4 X-ray diffraction	79
3.3.5 Thermal gravimetric analysis.....	79
3.3.6 Modulated differential scanning calorimetry.....	80
3.3.7 Magnetic measurements.....	81
3.3.8 <i>In vitro</i> hyperthermia	81
3.3.9 <i>In vitro</i> simultaneous hyperthermia and drug release	83
3.3.10 <i>In vitro</i> drug release without an alternating magnetic field	84

3.3.11 Controlled drug release	84
3.3.12 <i>In vitro</i> cell cytotoxicity	85
CHAPTER 4: RESULTS AND DISCUSSION	88
4.1 Physical properties and magnetic properties	88
4.1.1 Physical properties of γ -Fe ₂ O ₃ nanoparticles	88
4.1.2 Polymer content and drug loading of γ -Fe ₂ O ₃ nanoparticles	90
4.1.4 Physical properties of Fe ₃ O ₄ nanoparticles	91
4.1.5 Polymer content and drug loading of Fe ₃ O ₄ composite nanoparticles	95
4.1.6 Lower critical solution temperature of Fe ₃ O ₄ composite nanoparticles	97
4.2 Magnetic properties	110
4.2.1 Magnetic properties of γ -Fe ₂ O ₃ nanoparticles	110
4.2.2 Magnetic properties of Fe ₃ O ₄ nanoparticles	112
4.3 <i>In vitro</i> hyperthermia	119
4.3.1 <i>In vitro</i> hyperthermia of γ -Fe ₂ O ₃ nanoparticles	119
4.3.2 <i>In vitro</i> hyperthermia of Fe ₃ O ₄ nanoparticles	120
4.4 <i>In vitro</i> simultaneous hyperthermia and drug release	123
4.4.1 <i>In vitro</i> simultaneous hyperthermia and drug release from dox loaded γ -Fe ₂ O ₃ composite nanoparticles	123
4.4.2 <i>In vitro</i> simultaneous hyperthermia and drug release from dox loaded Fe ₃ O ₄ composite nanoparticles	124
4.5 <i>In vitro</i> drug release without magnetic field	128
4.5.1 <i>In vitro</i> dox release from γ -Fe ₂ O ₃ composite nanoparticles without magnetic field	128
4.5.2 <i>In vitro</i> dox release from Fe ₃ O ₄ composite nanoparticles without magnetic field	129

4.5.3 Controlled drug release from swollen dox loaded Fe₃O₄ composite nanoparticles 131

4.6 *In vitro* cytotoxicity..... 140

4.7 *In vivo* magnetic drug targeting and magnetic resonance imaging..... 146

4.8 Modeling 149

4.8.1 Modeling of power output from iron oxide magnetic nanoparticles 149

4.8.2 Modeling of tumor heating by optimized magnetic nanoparticles 156

4.8.3 Modeling of drug release from doxorubicin loaded PNIPAM – Fe₃O₄ composite nanoparticles..... 164

CHAPTER 5 168

CONCLUSIONS..... 168

5.1 Conclusions..... 168

REFERENCES 173

APPENDIX – A..... 204

In vivo magnetic drug targeting and magnetic resonance imaging..... 204

PUBLICATIONS AND CONFERENCES..... 206

LIST OF FIGURES

Figure 1.1 Examples of nanocarriers under development for clinical applications..	2
Figure 1.2 Global incidence and mortality rates for cancer in 2002.....	5
Figure 1.3 Overview of thermal therapy treatment options for cancer.....	8
Figure 1.4 Schematic representation of magnetic drug targeting to a tumor.....	12
Figure 1.5 Schematic diagram of drug release from poly-n-(isopropylacrylamide) encapsulated iron oxide nanoparticles below and above the lower critical solution temperature of the polymer.....	13
Figure 1.6 Overview of the thesis.....	19
Figure 2.1: The variation of coercivity with particle size for fine magnetic particles	26
Figure 2.2 (a-c): Hysteresis loop for (a) applied fields and H to the easy axis for a single domain particle with uniaxial anisotropy (b) bulk ferromagnetic material (c) an ensemble of uniaxial, single-domain particles with random easy axes	27
Figure 2.3 The potential clearance sites for nanoparticles in the body after intravenous injection.....	32
Figure 2.4 Schematic illustration of (a) active targeting to the vasculature around the tumor (b) active targeting to the tumor itself (c) passive targeting to the tumor using nanoparticles loaded with therapeutic agents	37
Figure 2.5 An example of Néel and Brownian relaxation time constants v/s particle size for Fe_3O_4 MNP. The smaller of the two relaxation times determines the effective relaxation time	48

Figure 2.6 The MFH 300F Magnetic field applicator suitable specifically designed for magnetic hyperthermia and used in the first human clinical trials recently conducted in Germany	57
Figure 2.7 (a) Chemical formula of PNIPAM (b) schematic illustration of the phase transition of PNIPAM by expulsion of hydrophobically bound water above the lower critical solution temperature	59
Figure 2.8 Mechanism of formation of uniform particles in solutions	66
Figure 2.9 The overall scheme for the high temperature synthesis of iron oxide from iron oleate	67
Figure 3.1 (a) Synthesis and processing of dox loaded PNIPAM – iron oxide composite nanoparticles; chemical formula of (b) BAAM crosslinked PNIPAM (c) doxorubicin.....	72
Figure 3.2 <i>In-vitro</i> hyperthermia test setup.....	82
Figure 4.1 Bright field transmission electron micrographs of uncoated γ -Fe ₂ O ₃ MNP of average size (a) 12 nm (b) 14 nm (c) 19 nm (d) 43 nm. Aggregation of the particles is due to inter-particle magnetic forces and the absence of surfactant.....	89
Figure 4.2 X-ray diffraction patterns of uncoated γ -Fe ₂ O ₃ MNP. The particles are highly crystalline and primarily γ -Fe ₂ O ₃	90
Figure 4.3 TGA weight loss plot for γ -Fe ₂ O ₃ -CNP. The PNIPAM content of the CNP is 6 wt%.	91
Figure 4.4 Bright field transmission electron micrographs of uncoated Fe ₃ O ₄ MNP of average size (a) 6 nm (b) 13 nm. Aggregation of the particles is due to inter-particle magnetic forces and the absence of surfactant	92

Figure 4.5 X-ray diffraction patterns of bare Fe_3O_4 MNP of average size 6 nm and 13 nm.	92
Figure 4.6 Dynamic light scattering intensity plots for dilute colloidal solutions of (a) Fe_3O_4 MNP (b) dehydrated Fe_3O_4 CNP (c) swollen Fe_3O_4 CNP (d) dehydrated dox loaded Fe_3O_4 CNP and (e) swollen dox loaded Fe_3O_4 CNP in water.....	93
Figure 4.7 Atomic force microscopy surface scan image of dehydrated Fe_3O_4 CNP clusters.	94
Figure 4.8 TGA weight loss plots for (a) bare MNP and Fe_3O_4 -CNP, the PNIPAM content of the CNP is 1.3 wt % (b) doxorubicin hydrochloride powder (c) dox loaded CNP of ~ 3 wt% PNIPAM and 6 wt % dox in the swollen and dehydrated conditions; plots have been normalized for water content, difference in dox content between dehydrated and swollen particles is ~ 0.9 wt%.	96
Figure 4.9 Swollen Fe_3O_4 CNP (a) dispersed in water (b) attracted from the suspension with a magnet.	97
Figure 4.10 MDSC thermograms of (a) swollen Fe_3O_4 -CNP (b) swollen dox loaded Fe_3O_4 -CNP (c) dehydrated dox loaded Fe_3O_4 -CNP (d) dox and MNP in PBS	98
Figure 4.11 Room temperature magnetization curves for 12 nm, 14 nm, 19 nm and 43 nm $\gamma\text{-Fe}_2\text{O}_3$ MNP and PNIPAM coated 43 nm CNP	111
Figure 4.12 Room temperature magnetization curves for 6 nm Fe_3O_4 MNP, 13 nm Fe_3O_4 MNP and dox loaded 13 nm core Fe_3O_4 -CNP	113

Figure 4.13 Heating characteristics of γ -Fe ₂ O ₃ ferrofluids in a 375 KHz, 1.7 kA.m ⁻¹ AMF. Ambient $T = 30\text{ }^{\circ}\text{C}$. Particle conc. = 10 mg.ml ⁻¹ . Dashed horizontal lines indicate the hyperthermia range of 41 – 48 $^{\circ}\text{C}$	119
Figure 4.14 Heating characteristics of Fe ₃ O ₄ ferrofluids in a 375 KHz, 4 kA.m ⁻¹ AMF. Ambient $T = 30\text{ }^{\circ}\text{C}$. Particle conc. (except 6 nm) = 2 mg.ml ⁻¹ . Dashed horizontal lines indicate the hyperthermia range of 41 – 48 $^{\circ}\text{C}$	121
Figure 4.15 Heating curve from <i>in-vitro</i> simultaneous drug release and hyperthermia test of dox loaded γ -Fe ₂ O ₃ -CNP in a 375 kHz, 0.1 – 4 kA.m ⁻¹ AMF with (a) heating (b) hyperthermia and (c) cooling segments. Particle conc. = 2.5 mg.ml ⁻¹	124
Figure 4.16 Heating curve from <i>in-vitro</i> simultaneous drug release and hyperthermia test of dox loaded Fe ₃ O ₄ -CNP dispersed in PBS and exposed to a 375 kHz, 0.1 – 5 kA.m ⁻¹ AMF for 35 min. Particle conc.= 1 mg.ml ⁻¹	125
Figure 4.17 <i>In-vitro</i> drug release from dox loaded PNIPAM – γ -Fe ₂ O ₃ CNP in PBS (pH 7.4) at 24 $^{\circ}\text{C}$, 37 $^{\circ}\text{C}$ and 42 $^{\circ}\text{C}$. Inset is an expanded view of release for the first 12 h	129
Figure 4.18 <i>In-vitro</i> drug release from dox loaded Fe ₃ O ₄ -CNP in PBS (pH 7.4) and tested at 24 $^{\circ}\text{C}$, 37 $^{\circ}\text{C}$ and 43 $^{\circ}\text{C}$, (a) dehydrated particles (b) swollen particles	130
Figure 4.19 Controlled drug release from swollen, dox loaded Fe ₃ O ₄ -CNP in PBS (pH 7.4) as a function of temperature. (a) cumulative dox release (b) dox release rates	132
Figure 4.20 Schemes for drug release from PNIPAM gels adapted from Ref. [84]. (a) relies on pressure generated during gel collapse to push out drug molecules from the matrix, (b) and (c) are Fickian processes, process (d) has not been	

extensively studied but is believed to be governed by the molecular properties of the drug and interaction between the drug and polymer network.	134
Figure 4.21 (a) LCST of swollen dox loaded Fe_3O_4 -CNP in cell culture medium (b) Zeta potential of bare Fe_3O_4 MNP, Fe_3O_4 -CNP and dox loaded Fe_3O_4 -CNP in PBS (pH 7.4) at 37 °C	142
Figure 4.22 Cell viability of BEAS-2B lung endothelial cells incubated with different concentrations of nanoparticles for (a) 24 h (b) 48 h at 37 °C	143
Figure 4.23 MRI scan of the Buffalo Rat implanted with HCC (a) baseline scan before injection of dox loaded $\gamma\text{-Fe}_2\text{O}_3$ -CNP (b) 30 min after injection. Particles are seen as new, dark regions in the HCC. (c) Histology slide of the HCC showing particles as dark deposits.....	147
Figure 4.24 Relaxation times for (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles as a function of magnetic particle size D_M and polymer shell thickness	151
Figure 4.25 The influence of magnetic particle size D_M and AMF frequency f on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ MNP for field strength $H_0 = 4 \text{ kA.m}^{-1}$	153
Figure 4.26 The influence of magnetic particle size D_M and field strength H_0 on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ MNP for frequency $f = 375 \text{ kHz}$	153
Figure 4.27 The influence of magnetic particle size D_M and anisotropy constant K on the SAR of Fe_3O_4 MNP with respect to (a) frequency f for field strength $H_0 = 16 \text{ kA.m}^{-1}$ and (b) field strength H_0 for frequency $f = 375 \text{ kHz}$	153
Figure 4.28 The influence of particle hydrodynamic diameter D_H on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles for a frequency $f = 375 \text{ kHz}$ and field strength $H_0 = 4 \text{ kA.m}^{-1}$	155

Figure 4.29 (a) Schematic of the model used for calculation of temperature rise in the tumor from AMF induced MNP heating. Temperature profile in tumor tissue (b) heated by a 12.5 mW point heat source for 30 min (c) as a function of time and distance from point source..... 158

Figure 4.30 Field strength and MNP concentration requirements for complete 42 °C thermal coverage of tumors with 375 kHz AMF. Guide lines are power-law fits to data points. 160

Figure 4.31 MNP mass and field strength requirements for complete hyperthermia coverage in tumors of various sizes with an AMF of $f = 375$ kHz and $H_0 \leq 20$ kA.m⁻¹ for (a) Fe₃O₄ and (b) γ -Fe₂O₃. Guide lines are power-law fits to data 161

Figure 4.32 Comparison of 42 °C and 40 °C thermal coverage of tumors with 1 g of Fe₃O₄ MNP and 375 kHz AMF..... 161

Figure 4.33 Predicted *in-vitro* dox release from dox loaded PNIPAM – iron oxide composite nanoparticle in PBS at (a) 24 °C (b) 37 °C and (c) 43 °C. Filled circles indicate experimental values. 165

Figure A.1 *In-vivo* magnetic drug targeting experiments on a Buffalo rat: (a) injection of dox loaded γ -Fe₂O₃-CNP (b) Buffalo rat in the custom built MRI coil..... 205

LIST OF TABLES

Table 2.1 Estimated single-domain size for spherical particles.....	25
Table 2.2 Examples of synthetic and natural polymers used for coating the surface of magnetic nanoparticles	34
Table 3.1 Dox loading parameters for PNIPAM – iron oxide CNP	78
Table 3.2 <i>In-vitro</i> hyperthermia test parameters.....	82
Table 3.3 <i>In-vitro</i> simultaneous hyperthermia and drug release test parameters ...	83
Table 3.4 Test parameters for <i>in-vitro</i> drug release in the absence of a magnetic field	85
Table 4.1 Hydrodynamic diameter of Fe ₃ O ₄ MNP and PNIPAM – Fe ₃ O ₄ CNP in water.....	94
Table 4.2 Physical and magnetic properties of γ -Fe ₂ O ₃ nanoparticles.	112
Table 4.3 Properties of Fe ₃ O ₄ MNP and PNIPAM – Fe ₃ O ₄ CNP.	113
Table 4.4 Comparison of magnetic properties of particles from the current work with selected literature for <i>thermoreponsive</i> magnetic particles.....	116
Table 4.5 Comparison of magnetic properties of particles from the current work with selected literature for <i>non-thermoreponsive</i> magnetic particles.	118
Table 4.6 Comparison of results of <i>in-vitro</i> drug release during AMF induced hyperthermia from current work with selected literature.	127
Table 4.7 Comparison of <i>in-vitro</i> drug release (without AMF) results of the current work with selected literature for <i>thermoreponsive</i> particles.	137
Table 4.8 Comparison of <i>in-vitro</i> drug release (without AMF) results of the current work with selected literature for <i>non-thermoreponsive</i> particles.....	138
Table 4.9 Comparison of <i>in-vitro</i> cytotoxicity results of current work with selected literature.	144

Table 4.10 Parameters used for modeling of power output from iron oxide MNP in
a ferrofluid exposed to an AMF..... 150

Table 4.11 Parameters used for modeling the temperature of AMF induced heating
from MNP distributed in the tumor. 159

Table 4.12 Comparison of the diffusion coefficient of doxorubicin D_{dox} of the
current work with calculated values from literature for various temperatures.
..... 166

LIST OF ABBREVIATIONS

AA	Acrylic acid
AAEM	Acetoacetoxylethyl methacrylate
AMF	Alternating magnetic field
APS	Ammonium persulphate
APTMS	3-aminopropyltrimethoxysilane
BAAm	N,N-methylene(bis)acrylamide
BSA	Bovine serum albumin
CIPAAm	2-carboxyisopropylacrylamide
CNP	Composite nanoparticle
DLS	Dynamic light scattering.
DMAAm	n,n-dimethlyacrylamide
DMEM	Dulbecco's Modified Eagle's Medium
dox	Doxorubicin
DPCC	Dipalmitoylphosphatidylcholine
EPR	Enhanced permeability and retention
FBS	Fetal bovine serum
HCC	Hepatocellular carcinoma
LCST	Lower critical solution temperature
MCL	Magnetic cationic liposomes
MD	Multi-domain
MDSC	Modulated differential scanning calorimeter/calorimetry
MDT	Magnetic drug targeting
MMH	Magnetically mediated hyperthermia

MNP	Magnetic nanoparticle(s)
MPS	Mononuclear phagocyte system
MRI	Magnetic resonance imaging
MTX	Methotrexate
NIPAM	n-isopropylacrylamide
NP	Nanoparticle
PAA	Polyacrylic acid
PBS	Phosphate buffer solution
PDADMAC	Poly(diallyldimethylammonium chloride)
PDI	Polydispersity index
PEG	Polyethylene glycol
PLGA	Poly(lactic-co-glycolic acid)
PNIPAM	Poly-n-(isopropylacrylamide)
PSS	poly(sodium 4-styrenesulfonate)
PTX	Paclitaxel
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
RES	Reticuloendothelial system
SAR	Specific absorption ratio
SD	Single domain
SPIO	Superparamagnetic iron oxide
TEGDMA	Tetraethyleneglycol dimethacrylate
TEM	Transmission electron microscope
TEMED	N,N,N',N''-tetramethylenediamine

TGA	Thermal gravimetric analysis
UV-Vis	Ultraviolet-visible
VCL	n-vinylcaprolactum
VSM	Vibrating sample magnetometer/magnetometry
XRD	X-ray diffraction

Chapter 1 – Introduction

CHAPTER 1

INTRODUCTION

1.1 Introduction

Nanotechnology is the science and engineering of materials with functional dimensions typically ranging from a few nanometers up to several hundred nanometers. Engineering nanomaterials are on the threshold of molecular and atomic regimes, which provides the ability to design and synthesize nanomaterials with novel and unique chemical and physical properties not found in bulk materials. Nanotechnology is multi-disciplinary, integrating traditional disciplines such as physics, materials science, biology and chemistry for the development of novel materials and technologies.

Nanotechnology promises novel and improved solutions for therapeutics and diagnostics in medicine and is a growing area of research [1-4]. The early focus of nanomedical research was the improvement of existing diagnostic and therapeutic modalities, however recent efforts have concentrated on the development of original and innovative platforms for medical applications. A major thrust of research in nanomedicine has been the development of nanocarriers for passively or actively targeted drug delivery, e.g., for cancer therapy such carriers can minimize systemic toxicity due to conventional chemotherapy or radiotherapy [3, 5-10]. Passive targeting utilizes the physiological conditions in the tumor environment. The leaky tumor vasculature enhances extravasation of the nanocarriers into tumor interstices, increasing drug concentration in the tumor, the undeveloped lymphatic drainage system favors increased retention of nanocarriers in the tumor. This phenomenon is known as the enhanced permeability and

Chapter 1 – Introduction

retention (EPR) effect [11, 12]. In active targeting, the nanocarrier surface is functionalized with biological agents such as antibodies, peptides, nucleic acid aptamers, carbohydrates and small molecules, alternatively, an external guidance system, e.g., a magnetic field, is used to direct the nanocarriers to the tumor site. A few examples of nanocarriers for targeted delivery are shown in Fig.1.1.

Figure 1.1 Examples of nanocarriers under development for clinical applications [7] .

Nanoparticles (NP), in the size range of 1 – 100 nm and with appropriate surface functionalization, have several distinguishing features that can lead to better cancer therapeutics compared to existing modalities [3]:

Chapter 1 – Introduction

- NPs can be loaded with a relatively large quantity of drugs without degradation of the drug or changes in their pharmacological properties.
- Multiple targeting ligands or multiple types of drug molecules can be attached to the NP due to the large surface area to volume ratio of the NP.
- The release kinetics of drugs from NP can be tuned to respond to external stimuli or environmental conditions in the tumor.
- NPs enter the tumor cell through endocytosis. Endocytosis involves the formation of a ‘pocket’ by the cell plasma membrane (with or without receptor mediation), enclosure of extra-cellular material such as a NP by the membrane pocket and its transport into the cell in an intracellular vesicle. This potentially allows NPs to avoid mechanisms of multidrug resistance involving cell-surface protein pumps.

Magnetic nanoparticles (MNPs) can exhibit ferrimagnetism, ferromagnetism or superparamagnetism. Superparamagnetic particles are magnetized in the presence of an applied magnetic field and behave like ferromagnets under the influence of the field. In the absence of a field their magnetic moment can be randomized by ambient thermal energy resulting in a ‘paramagnetic’ state with zero remanence and coercivity [13, 14]. An ensemble of superparamagnetic particles typically exhibits an anhysterical sigmoidal magnetization curve. This phenomenon is not observed in bulk magnetic materials, and is useful in reducing aggregation of MNP.

Chapter 1 – Introduction

MNPs have additional useful properties that increase their potential for applications in nanomedicine [15-19].

- The size of MNPs is tunable from a few nm to tens of nm, their surfaces can be functionalized by either polymers or inorganic materials to which drugs or biological agents can be attached. The design of particles can therefore be optimized to match specific biological features and environments.
- The magnetic nature of the particles and the ability of magnetic fields to penetrate human tissue enable active *in vivo* manipulation of the MNP by external magnetic field gradients to specific sites of interest such as a tumor.
- MNP have the unique ability to generate heat when an alternating magnetic field (AMF) is applied [20-22] ; this heat can be transferred to surrounding tumor tissue for thermal therapy. This enables the use of functionalized MNP as multimodal therapeutic agents, and is a major advantage over non-magnetic particles.

The intelligent exploitation of these properties enables the development of MNP based systems for improved drug targeting and drug delivery [16, 17, 19, 23-28], hyperthermia [16, 17, 19, 21, 22, 29-34], magnetic resonance imaging (MRI) [16, 17, 19, 35-39], biosensing [40, 41] and magnetic separation [16, 17, 42]. The synthesis, characterization, property evaluation and modeling of multi-functional core-shell magnetic nanoparticles for combined drug targeting, drug delivery and hyperthermia applications is described in this thesis.

Chapter 1 – Introduction

1.2 Cancer: the motivation

Cancer is a leading cause of death, accounting for nearly 7.6 million (13%) of all global deaths in 2005 [43-45]. Deaths from cancer are projected to rise steadily to an estimated 9 million in 2015 and 11.4 million in 2030. The statistics for the global incidence and mortality rates for major types of cancer in the year 2002 are shown in Fig.1.2 [45].

Figure 1.2 Global incidence and mortality rates for cancer in 2002 [45].

Cancer is characterized by the uncontrolled division and spread of abnormal cells [46] that have suffered DNA damage, resulting in mutation of the genes controlling

Chapter 1 – Introduction

cell division. The spread of abnormal cells occurs through invasion (direct growth into neighboring tissue) or metastasis (transport to distant sites via the bloodstream or lymphatic system). The exact mechanisms of formation and spreading of cancer cells are not fully understood, but six acquired capabilities of cancer cells – self sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, tissue invasion and metastasis are believed to be controlling factors [46]. External factors such as physical carcinogens (ultraviolet and ionizing radiation), chemical carcinogens (asbestos, tobacco) and biological carcinogens (virii, bacteria, fungi) can act as triggers for cancer. There are many different types of cancers classified according to localization, cell type, malignancy etc, but all share a common trait – if undetected and untreated at an early stage, the risk of death is high [45].

Cancer treatment depends on the type, level of advancement and location of the tumor. Common therapies include [47-49]:

- **Surgical resection** – excision of the tumor by surgical methods.
- **Chemotherapy** – destruction of tumor cells with powerful cytotoxic drugs that alter intra-cellular processes of actively reproducing cells.
- **Radiotherapy** – destruction of tumor cells with ionizing radiation which alters intra-cellular processes of actively reproducing cells.
- **Thermal therapy** – heating up the tumor to temperatures to selectively deactivate tumor cells, while sparing healthy tissue. When temperatures used are in the range of $\sim 41 - 47$ °C, the condition is known as hyperthermia and cells are destroyed by apoptosis; above this temperature

Chapter 1 – Introduction

both healthy as well as cancer cells are destroyed by necrosis from thermal ablation [31, 48-52]. Fig. 1.3 gives an overview of the thermal therapy options for cancer treatment [49].

Use of multimodal therapies, i.e., simultaneous application of two or more of the above methods, can be used to increase efficacy of treatment [48, 50, 51, 53-57]. Often chemotherapy, radiation therapy or another secondary therapy is given after primary treatment to control recurrence of cancer, this is called adjuvant therapy.

Chapter 1 – Introduction

Figure 1.3 Overview of thermal therapy treatment options for cancer [49].

1.3 Inadequacy of existing treatments: the problem.

Chapter 1 – Introduction

Conventional cancer therapies suffer from numerous and significant drawbacks and their effectiveness is strongly dependent on early detection of the disease before it has metastasized. The treatment may be curative or palliative, the former is applicable to early stage cancers that may be successfully cured, while the latter is used for advanced stage cancers with the aim of improving the patient's quality of life.

Surgical resection, the oldest form of cancer treatment, is usually an invasive procedure and generally limited to early stage solid tumors. It cannot be used when resection of the tumor leads to degradation in organ function. Apart from the risk of complications during surgery, there is also the possibility of post-operative problems such as infection, bleeding, blood clots, slow recovery of organ functions, etc [58].

In chemotherapy, highly cytotoxic drugs are administered parenterally, usually via intravenous injection and transported to the tumor site through the bloodstream. These powerful drugs have a narrow therapeutic window and the dosage is normally based on the patient's body weight and/or body surface area. Treatment involves a schedule of regular administrations distributed over several days, the specific schedule depends on the drug and type of cancer. Non-specific administration of the drug leads to systemic distribution which can result in insufficient drug concentration at the tumor, decreasing the effectiveness of treatment. More importantly, these drugs are non-specific in nature, i.e., they can kill both healthy as well as cancerous cells, resulting in serious side effects such as low blood cell count due to bone marrow damage, anorexia, diarrhea, fatigue,

Chapter 1 – Introduction

nausea and vomiting, heart damage, nerve damage, hair loss, mucosal ulcerations, liver damage, lung damage, etc [58].

Radiation therapy can be applied via external beam radiation, internal radiation using radioactive container implants (brachytherapy) or by radiopharmaceuticals (drugs with radioactive components). As in conventional chemotherapy, radiation therapy is non-specific i.e., it destroys healthy reproducing cells as well as cancerous cells. Side effects from radiation therapy can be severe and long-term; these include fatigue, damage to the skin, lungs, digestive tract, mouth and throat, changes in brain function, decrease in fertility, and in some cases triggering of secondary cancers [58].

Hyperthermia can be broadly classified into (a) regional or whole body hyperthermia when a part of the body or the whole body is heated to the range of 39 – 41 °C, (b) local hyperthermia when only the tumor is heated to 41 – 47 °C and (c) thermal ablation when the tumor is heated to 47 – 50 °C. Common hyperthermia techniques include radiofrequency capacitance hyperthermia, phased arrays of microwave antennae, whole body hyperthermia and isolated hepatic perfusion hyperthermia [31, 48, 59]. Due to the non-specificity of these methods, attainable tumor temperatures are limited to minimize the risk of collateral organ damage. There is high risk of death from organ failure due to the uniformly elevated body and hepatic temperatures particularly in the case of whole body hyperthermia and isolated hepatic perfusion hyperthermia [31]. Other techniques such as radiofrequency probes, microwave probes, interstitial laser photocoagulation and direct tumor injection of hot water suffer from drawbacks

Chapter 1 – Introduction

such as very specific deposition of heat, the need for accurate tumor localization and physical access to the tumor [31]. With these localized techniques, there is limited diffusion of heat through the tissue, necessitating probe repositioning or electromagnetic field pattern modification for tumors larger than 5 cm [31].

Thus the dosage and duration of conventional chemotherapy, radiation therapy and hyperthermia are restricted, resulting in inadequate treatment of the cancer.

1.4 Targeted multimodal therapies: the solution

Targeted therapies have the potential to greatly improve cancer treatment by accurately delivering drugs to the tumor site and minimizing systemic toxicity [9, 60]. As a result higher drug concentration in the tumor as well as minimal damage to healthy cells from these highly cytotoxic agents is obtained. Subsequent application of heat to raise the tumor temperature above 41 °C during drug release can further improve overall therapeutic efficiency of treatment compared to individual or tandem administration of hyperthermia and chemotherapy [48, 50, 51, 53-57].

Drug loaded polymer coated magnetic nanoparticles offer a solution for magnetic drug targeting (MDT) and synergistic simultaneous hyperthermia and drug release therapy. Iron oxide nanoparticles encapsulated in a suitable biocompatible polymer can be loaded with chemotherapeutic drugs, suspended in a carrier fluid and then injected into the bloodstream. By application of an external static magnetic field in the tumor region, the nanoparticles are trapped by the magnetic field gradient at the tumor [16, 17, 23-27], thus minimizing the distribution of drugs in healthy tissue

Chapter 1 – Introduction

(Fig. 1.4). Drug release occurs following localization of the nanoparticles in the tumor; subsequent application of an external AMF induces heat generation in the particles [17, 20, 21, 29-33] resulting in *simultaneous magnetically mediated hyperthermia (MMH) as well as chemotherapy*, thus utilizing the synergistic effects of thermal therapy and chemotherapy. If the polymer shell is responsive to environmental stimuli (such as temperature or pH), drug release can be triggered and controlled by manipulation of stimuli [61-64], enhancing drug release during hyperthermia and reducing it when the tumor cools following the hyperthermia session (Fig. 1.5). Iron oxide MNP deposited in the tumor can be retained for weeks [29, 65], enabling repeated application of hyperthermia at pre-determined intervals after the initial injection.

Figure 1.4 Schematic representation of magnetic drug targeting to a tumor [66].

Magnetic drug targeting is suitable for most solid tumors and especially for hepatocellular carcinoma (HCC); targeting to a liver tumor is easier since these

Chapter 1 – Introduction

tumors receive their blood supply from the hepatic arterial system unlike normal liver tissues which receive ~70% of their blood supply from the portal venous system [67]. Although systemic chemotherapy offers no survival benefit for cases of advanced HCC, targeted chemotherapy is the best chance for improved survival and quality of life for these patients [68-70], therefore improvements in this area are highly desirable. In addition to targeted therapies, thermochemotherapy may also lead to improved palliative treatment [71].

Figure 1.5 Schematic diagram of drug release from poly-n-(isopropylacrylamide) encapsulated iron oxide nanoparticles below and above the lower critical solution temperature of the polymer.

Chapter 1 – Introduction

1.5 Objective

The objective of this work is to study drug loaded thermoresponsive polymer encapsulated magnetic nanoparticles particles relevant to multimodal cancer treatment. Previous work, including human clinical trials [27, 29, 65, 72], has mostly studied magnetic drug targeting and hyperthermia separately, little work has been performed on MNP for combined, simultaneous application of these two modalities [73-78].

Various targeted drug delivery systems such as liposomes, pegylated liposomes, polymer-drug conjugates, antibody-drug conjugates, protein-drug conjugates, polymer-protein conjugates, polymeric micelles etc are being studied or are in clinical use [9, 60, 79]. However, these systems address only the targeting issue; hyperthermia still has to be performed in the conventional manner with all its drawbacks. In comparison, drug loaded magnetic composite nanoparticles (CNP) are a multifunctional and efficient single materials system, drug dosages can be drastically reduced by up to 80% of systemic dosage to achieve equivalent tumor [24].

1.6 Scope of the project

Iron oxides γ -Fe₂O₃ (maghemite) and Fe₃O₄ (magnetite) are biocompatible in the doses required for therapeutic applications and are currently in routine clinical use as magnetic resonance imaging (MRI) contrast enhancement agents [16, 17, 35-38, 80], hence these nanoparticles were chosen as the magnetic component of the CNP. Depending upon the particle size, environmental factors and AMF parameters, iron oxide MNP generate heat primarily by hysteresis, Néel or Brownian relaxation

Chapter 1 – Introduction

mechanisms [20, 21, 81]. Non-superparamagnetic Single domain MNP with finite coercivity and remanence exhibit a hysteresis loop; the heat generated by such particles is proportional to the area enclosed by this loop which in turn is indicative of the energy absorbed by the material during an AMF cycle. In contrast, superparamagnetic particles generate heat by rotation of the magnetic moment internally in the particle (Néel relaxation) or physical rotation of the particle in the carrier fluid to align the moment with the external field (Brownian relaxation) during an AMF cycle. Hence a study of the effects of particle size and shell thickness is necessary to optimize the particles for *in vivo* hyperthermia applications. Particle size and polymer shell properties also play an important role in the fate of the CNP when injected into the bloodstream, larger particles with hydrophobic coatings are likely to be labeled as foreign objects, opsonized and cleared by the reticulo endothelial system (RES), the body's immune system, more rapidly than smaller particles with hydrophilic coatings [23, 82].

Poly-n-(isopropylacrylamide) (PNIPAM), a well known thermoresponsive polymer was chosen as the polymer component [61, 62, 64, 83, 84]. PNIPAM is biocompatible [85-87] and has a lower critical solution temperature (LCST) of 30 – 35 °C in water [84, 88-91], this is close to the human body temperature of 37 °C, and is tunable by polymerization with an appropriate copolymer. Below the LCST, PNIPAM absorbs water and swells, this property can be used to load the composite nanoparticles with water soluble drugs such as doxorubicin (dox). During hyperthermia, the temperature of the polymer shell is raised above the LCST by the temperature rise of the magnetic core. Above the LCST, the hydrophobically bound water is expelled [88], which results in shrinkage of the polymer coating and

Chapter 1 – Introduction

rapid release of the loaded drug [61, 62, 83, 84]. The shrinkage is reversible, the polymer returns to its swollen state in the aqueous environment when cooled below the LCST and restricts drug release. The LCST is sensitive to the presence of solutes in PNIPAM as well as the solvent media [84, 90, 92, 93], if the LCST of the drug loaded PNIPAM – iron oxide particles is suppressed or higher than the maximum hyperthermia temperature (47 °C) temperature, the thermoresponsive property cannot be utilized.

Hence it is important to investigate the effect of drug loading on the LCST of the CNP.

In vivo testing of magnetic drug targeting in a buffalo rat model was performed using an external permanent magnet. MRI imaging using a custom built MRI coil was performed along with histology, the cell line used for inducing HCC in the rat was Morris Hepatoma 7777.

Since the effectiveness of these particles is sensitive to particle size, modeling *in vivo* heating behavior and drug release of these particles is useful for comparison with our *in vitro* drug release and hyperthermia experimental studies. Rosensweig's model for heat generation in ferrofluids during hyperthermia was used to study the effect of particle size and shell thickness [20]. The relevant heat transfer relationships were used to calculate the temperature rise in the tumor from the nanoparticles, modeling of the diffusion of the drug was used to estimate drug release from drug loaded CNP.

Chapter 1 – Introduction

Characterization techniques used for the current work included transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction (XRD), dynamic light scattering (DLS), thermal gravimetric analysis (TGA), UV-visible spectrophotometry (UV-Vis) and vibrating sample magnetometry (VSM).

Fig 1.6 outlines the logical flow of the thesis. The problem of conventional cancer therapy is identified and the objective of developing a magnetic nanoparticle system for combined drug targeting, hyperthermia and controlled drug release is defined. A materials system that meets safety as well as performance requirements is selected after extensive survey of the state of the art: iron oxide is the magnetic component for drug targeting and hyperthermia, poly-n-(isopropylacrylamide) is the thermoresponsive polymer component for control of drug release and doxorubicin is the anti-cancer drug. Suitable synthesis routes for iron oxide magnetic nanoparticles are explored; γ -Fe₂O₃ as well as Fe₃O₄ magnetic nanoparticles are synthesized and characterized. Particle sizes producing the best magnetic properties and *in vitro* hyperthermia performance are identified. Selected batches of high performance particles are encapsulated with PNIPAM and loaded with doxorubicin. The properties of these drug loaded particles are extensively characterized using a diverse array of techniques ranging from spectroscopy to microcalorimetry. Several aspects of their *in vitro* hyperthermia and drug release performance are evaluated. Processing conditions that promote or hinder thermoresponsive behavior are investigated. *In vitro* cytotoxicity and thermoresponsive properties in biological media are also studied to help in predicting *in vivo* performance. Concurrently, modeling of heat generation from γ -Fe₂O₃ and Fe₃O₄ particles in an alternating magnetic field is performed to identify

Chapter 1 – Introduction

the optimum particle size ranges for *in vivo* applications. Fe_3O_4 particles within this size range are synthesized and studied as detailed above. Tumor heating by optimized high performance $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 particles is modeled for several tumor sizes. For selected temperatures and particle sizes, drug release is also modeled. *In vivo* magnetic drug targeting to liver tumors is examined using a buffalo rat model. The results from experimental as well as theoretical work are compiled, presented and discussed in the thesis.

Chapter 1 – Introduction

Figure 1.6 Overview of the thesis.

Chapter 1 – Introduction

1.7 Methodology

A literature survey was carried out to examine the choices for MNP synthesis and subsequent polymer coating. The following synthesis routes were used to produce particles of different average sizes:

- high temperature decomposition of organometallic complexes in organic solvents – average size of 12 nm, 14 nm, 19 nm and 43 nm γ -Fe₂O₃ MNP.
- co-precipitation – average size 6 nm and 13 nm Fe₃O₄ MNP.

The bare MNP were characterized by X-ray diffraction (XRD), vibrating sample magnetometry (VSM), and transmission electron microscopy (TEM) techniques, ferrofluids were tested *in vitro* in an AMF to determine hyperthermia behavior.

Depending on their magnetic properties and *in vitro* hyperthermia performance, selected batches of particles were coated with PNIPAM and loaded with dox. The PNIPAM content of the CNP was determined by thermo-gravimetric analysis (TGA), LCST before and after drug loading was determined by modulated differential scanning calorimetry (MDSC). UV-visible spectrophotometry (UV-Vis) was used to calculate dox loading and drug release. *In vitro* drug release from the particles was measured under hyperthermia conditions in an AMF. Drug release at temperatures above and below the LCST of PNIPAM in the absence of a magnetic field was also determined. The ability of the PNIPAM coating to control drug release above and below the LCST was studied by cycling the temperature between 24 °C, 37 °C and 43 °C.

Chapter 1 – Introduction

In vitro cytotoxicity tests were carried out on BEAS-2B cells with bare MNP, CNP and dox loaded CNP. *In vivo* magnetic targeting experiments on buffalo rats implanted with HCC were carried out with selected batches of drug loaded particles, MRI was used to visualize the particle distribution after magnetic targeting.

Modeling of heat output from nanoparticles, the temperature rise in tumors of various sizes, and drug release from composite nanoparticles was carried out to compare experimental results with theoretical predictions and to provide a framework for future analysis.

In summary, the synthesis, characterization, performance evaluation and modeling of drug loaded magnetic core – thermoresponsive polymer shell nanoparticles for cancer treatment was performed. The potential for combined magnetic drug targeting, drug release and hyperthermia treatment of cancer using such core-shell composite nanoparticles was examined.

1.9 Organization of the thesis.

The thesis consists of 6 chapters.

- **Chapter 1** is the *Introduction*. Limitations of conventional cancer treatment are highlighted, the motivation for improved methodologies of cancer treatment is outlined and possible solutions suggested. The objective and scope of the thesis are presented along with a brief description of methodology and significant results.

Chapter 1 – Introduction

- **Chapter 2, *Background and Motivation*** contains an overview of selected topics in fine particle magnetism and its relevance to medical applications. The state of the art for magnetic drug targeting, drug release and magnetically mediated hyperthermia is described to provide the motivation for improving cancer treatment modalities.
- **Chapter 3** describes the *Experimental Procedures* for synthesis, characterization and *in vitro* as well as *in vivo* testing of iron oxide MNP, CNP and drug loaded CNP.
- **Chapter 4** contains the *Results and Discussion* of the modeling, characterization and performance evaluation of the iron oxide MNP, CNP and drug loaded CNP synthesized in this work.
- **Chapter 5** presents the *Conclusions*.

In the next chapter, selected topics in magnetism, magnetic drug targeting and magnetic hyperthermia are discussed. An overview of fine particle magnetism and mechanisms of magnetically mediated hyperthermia is provided. The requirements of magnetic nanoparticles for *in vivo* applications are listed, the properties of iron oxides and thermoresponsive systems as well as synthesis methods are discussed. Prior work and the state of the art for magnetic drug targeting, thermoresponsive magnetic carriers and magnetic hyperthermia is presented.

Chapter 2 – Background and Motivation

CHAPTER 2

BACKGROUND AND MOTIVATION

2.1 Introduction

In this chapter, the magnetic behavior of fine particles and its relevance to magnetic drug targeting and magnetically mediated hyperthermia is briefly discussed. Past developments and the state of the art for MDT and MMH are summarized with specific examples. The magnetic and structural property requirements for MDT and MMH are listed since these properties are critical in determining the performance of MNP. Particle properties are dependent on the synthesis and coating methods, therefore selected methods for the production of iron oxide MNP and iron oxide – polymer CNP are also described.

2.2 Magnetic nanoparticles

In this section, concepts relating to magnetism of ferromagnetic and fine particle systems will be discussed. Particle size greatly influences magnetic properties, which in turn decides the suitability for specific biomedical applications, hence it is important to understand magnetic behavior of particles in the nano size range. A more comprehensive treatment of these topics and fundamental concepts of magnetism is available in Refs [13, 14, 94, 95].

2.2.1 Magnetic behavior of fine particles

The magnetic behavior of fine ferromagnetic particles, typically below 1 μm , is dominated by two important features:

Chapter 2 – Background and Motivation

- Below a critical size limit, the particle prefers to be in a single domain state since multi-domain formation is energetically unfavorable.
- If the particle is small enough, thermal energy can decouple the magnetization moment from the particle, resulting in superparamagnetism.

2.2.2 Single domain particles

The magnetostatic energy of a ferromagnetic particle can be decreased by formation of multiple domains, but there is a limit to this process since domain wall formation costs energy [13, 14]. Below a certain size, the homogenous single domain (SD) particle magnetization state, which has a relatively large demagnetizing energy but very low exchange energy, is energetically more favorable than a closed multi-domain (MD) magnetization configuration with low demagnetizing energy but high exchange energy. The critical size for a particle to become single domain can be estimated as follows [13]:

- The magnetostatic energy E_{dem} in a particle of diameter D is proportional to $M_S^2 D^3$. M_S is the saturation magnetization.

$$E_{dem} \sim M_S^2 D^3 \quad (\text{Eq. 2.1a})$$

- Domain walls are required for domain formation in a MD particle. Since the domain wall is an area, the total domain wall energy E_{wall} is proportional to γD^2 where γ is the domain wall energy per unit area.

$$E_{wall} \sim \gamma D^2 \quad (\text{Eq. 2.1b})$$

- At smaller sizes the D^2 term dominates over the D^3 term, hence the increase in domain wall energy is greater than the decrease in magnetostatic energy

Chapter 2 – Background and Motivation

from domain wall formation. Below the critical single domain size D_{crit} , domain walls are not formed.

- Ignoring the proportionality constant and equating E_{dem} and E_{wall} allows the estimation of D_{crit} .

$$M_S^2 D^3 \sim \gamma D^2 \tag{Eq. 2.2}$$

$$D_{crit} \sim \gamma / M_S^2 \tag{Eq. 2.3}$$

The estimated single domain sizes for spherical particles of selected magnetic materials are listed in Table 2.1 [13].

Table 2.1 Estimated single-domain size for spherical particles [13].

Material	Single domain size (nm)
Fe	14
Co	70
Ni	55
Fe ₃ O ₄	128
γ-Fe ₂ O ₃	166

2.2.3 Coercivity of single domain particles

Since there are no domain walls in a SD particle, magnetization reversal occurs by spin rotation. This results in higher coercivity in SD particles compared to MD particles, because magnetization rotation is more difficult than domain wall movement. The magnetization vector can simply rotate by coherent motion of the atomic spins, but in chains of SD particles, other motions, e.g., fanning and curling can also occur [13, 95]. Coherent rotation is discussed below. Fig. 2.1 shows the

Chapter 2 – Background and Motivation

relationship between coercivity and particle size around the single domain limit [13].

Figure 2.1: The variation of coercivity with particle size for fine magnetic particles [13].

2.2.4 Coherent rotation of the magnetization

The magnetic response of a material to an external magnetic field is affected by various anisotropies. If the applied field, H , is perpendicular to the easy axis of a single domain particle with uniaxial anisotropy K , an anhysteretic magnetization curve is obtained (Fig 2.2a) [13]. When the applied field is parallel to the easy axis, saturation magnetization M_S is reached at a field strength value of $2K/M_S$. As the direction of the field is reversed i.e., antiparallel to M_S , initially there is no rotation of the magnetic moment since the field applies no torque. However, with increasing H , the magnetic energy for reversal eventually becomes so large that the minimum in energy shifts from the original M_S antiparallel to H , to M_S parallel to H , at $2K/M_S$. The resultant hysteresis loop is square (Fig 2.2a) in contrast to the

Chapter 2 – Background and Motivation

hysteresis loop of a multi-domain bulk ferromagnetic material (Fig. 2.2b). It may be noted that $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 do not exhibit uniaxial anisotropy.

The two cases above, i.e., the initial perpendicular and parallel orientations represent extremes of the possible hysteresis curves – anhysteretic and square respectively. Most often, when dealing with an ensemble of particles, the easy axes are randomly oriented with respect to the applied field, and a hysteresis curve which is an average over all orientations results (Fig. 2.2c).

Figure 2.2 (a-c): Hysteresis loop for (a) applied fields $\perp$ and $\parallel$ to the easy axis for a single domain particle with uniaxial anisotropy (b) bulk ferromagnetic material (c) an ensemble of uniaxial, single-domain particles with random easy axes [13].

Chapter 2 – Background and Motivation

2.2.5 Superparamagnetism

Below the Curie temperature, the moment of a ferromagnet or ferrimagnet is rigidly bound (with energy KV_M where K is the anisotropy constant and V_M is the volume of the particle) to the particle by various anisotropies. With decreasing particle size, the energy barrier to decoupling of total moment from the particle decreases to a point where the thermal energy $k_B T$ can overcome it. When this occurs, above the temperature known as the blocking temperature T_b , the moment is free to respond to an applied field and can be large (thousands of Bohr magnetons). The applied field tries to align this giant moment, but thermal energy tries to oppose it, just as in a paramagnet. This phenomenon is called superparamagnetism [13, 14]. If the anisotropy is zero or very weak, the total moment may point in any direction, and if K is significantly greater than zero, it tends to align in one of the two directions along the easy axes.

Above T_b , the total moment of the system in the absence of an external field is zero because each particle moment can be oriented with equal probability in two opposite directions (along the easy axes). In fine particle systems, the transition to a superparamagnetic state with increasing temperature occurs gradually due to variations in particle size and shape. These variations lead to a spread of energy barrier heights, resulting in different transition temperatures for different particles. Two characteristics of a superparamagnetic system are (i) lack of hysteresis and (ii) when magnetization values for different temperatures are plotted against H/T , they superimpose to form a universal curve.

Chapter 2 – Background and Motivation

The Néel relaxation time τ of the net magnetization of a superparamagnetic particle is given by

$$\tau = \tau_0 \exp\left(\frac{KV_M}{k_B T}\right) \quad (\text{Eq. 2.4})$$

where T the temperature, k_B the Boltzmann constant, and τ_0 the attempt time.

For non-interacting particles, the attempt time is of the order of 10^{-9} to 10^{-12} s. Observations of superparamagnetism are dependent not only on temperature, but also on measurement time, τ_m of the experimental technique used. If $\tau \ll \tau_m$ the moment rotation is fast relative to the experimental time window, and the particles appear to be paramagnetic. On the other hand, if $\tau_m \ll \tau$, rotation is slow and quasi-static properties are observed. The blocking temperature T_b is the temperature at which $\tau = \tau_m$. For a typical attempt time scale of $\tau_0 = 10^{-9}$ s and $\tau \cong 100$ s, the critical volume is

$$V_{SP} = \frac{25k_B T}{K} \quad (\text{Eq. 2.5})$$

If the particle has a volume lower than V_{SP} , it will behave superparamagnetically on a 100 s experimental time scale. Using the K values from Ref [20] and $T = 300$ K, the superparamagnetic size limit for spherical $\gamma\text{-Fe}_2\text{O}_3$ MNP is ~ 28 nm and for Fe_3O_4 MNP it ranges from ~ 14 nm to 17 nm, particles smaller than these sizes will exhibit superparamagnetic behavior.

Chapter 2 – Background and Motivation

2.3 Iron oxides for biomedical applications

The most commonly used magnetic nanoparticles in clinical applications are iron oxides – magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), hence it is useful to understand their properties.

2.3.1 Magnetite (Fe_3O_4)

Magnetite is one of the earliest known magnetic materials, occurring naturally in the earth's crust as iron ore. It is a black, ferrimagnetic material with a density of 5180 kg.m^{-3} and a bulk saturation magnetization value of $90 - 92 \text{ emu.g}^{-1}$ [96, 97]. Fe_3O_4 contains both Fe^{2+} and Fe^{3+} ions in an inverse spinel structure [97], with a face-centered cubic unit cell based on 32 O^{2-} ions that are regularly cubic close packed along the [111] direction. There are 8 formula units per unit cell and the unit cell edge length is 0.839 nm. Magnetite differs from most other iron oxides because it contains both divalent and trivalent Fe ions, with the formula written as $\text{Y}[\text{XY}]\text{O}_4$ where $\text{X} = \text{Fe}^{2+}$ and $\text{Y} = \text{Fe}^{3+}$; the brackets represent octahedral sites.

2.3.2 Maghemite ($\gamma\text{-Fe}_2\text{O}_3$)

Maghemite is a red-brown oxide, commonly produced from the oxidation of other Fe oxides, especially magnetite, and has a structure similar to magnetite. $\gamma\text{-Fe}_2\text{O}_3$ is also ferrimagnetic, with a density of 4600 kg.m^{-3} and bulk saturation magnetization value of $\sim 76 - 80 \text{ emu.g}^{-1}$ [96, 97]. Fine Fe_3O_4 particles, especially in the nanometer size range can easily get oxidized to $\gamma\text{-Fe}_2\text{O}_3$ even at room temperature [97, 98]. The major difference from Fe_3O_4 is that all or most of the Fe is present as Fe^{3+} , with cation vacancies compensating for the oxidation of Fe^{2+} [97].

Chapter 2 – Background and Motivation

The next subsection discusses requirements of nanoparticles for *in vivo* biomedical applications, and compares these requirements to the properties of iron oxide based composite nanoparticles.

2.4 Factors affecting the *in vivo* applications of magnetic nanoparticles.

Nanoparticles intended for *in vivo* applications must be able to circumvent filtration and clearance at the kidneys, and evade detection, opsonization and clearance by the reticulo endothelial system (RES, also known as the MPS-mononuclear phagocyte system) until they are localized at the tumor site [16, 23, 35, 82, 99, 100]. The RES is part of the immune system and is a diffuse system of phagocytic cells associated with the macrophage ('scavenger') Kupffer cells of the liver and the reticular connective tissue framework of the lymph nodes and spleen. Most intravenously injected particles are rapidly detected, identified as 'foreign' and coated with plasma proteins by the circulatory components in the bloodstream [23, 82, 99]. This process is called opsonization, and it renders these plasma protein-coated particles vulnerable to endocytosis/phagocytosis by the circulating monocytes or organ based macrophages. Endocytosis is a process by which the cell membrane undergoes invagination, engulfs the particulate matter and transports the engulfed particle to the cell interior in an intracellular vesicle. Phagocytosis is a special case of endocytosis where macrophage cells remove foreign particles, cell debris and dead cells from circulation. The endocytic process is followed by removal from circulation and deposition in organs with high phagocytic activity such as the liver and spleen. The opsonization and elimination process can be rapid, in the range of minutes after injection, with particles having a typical final *in*

Chapter 2 – Background and Motivation

in vivo distribution of 80 – 90 % in the liver, 5 – 8 % in the spleen and 1 – 2 % in the bone marrow [35]. The particle size, toxicity and surface characteristics such as hydrophilicity and surface charge strongly influence opsonization and hence the clearance and tissue distribution of intravenously injected nanoparticles. Fig. 2.3 depicts the fate of nanoparticles injected into the body [82].

Figure 2.3 The potential clearance sites for nanoparticles in the body after intravenous injection [82].

2.4.1 Particle properties

Magnetic particles used for biomedical applications are broadly classified as:

- Magnetic nanospheres: encapsulated (i.e., coated) magnetic particles ranging from 10 to 500 nm.
- Magnetic microspheres: magnetic particles from just below 1 μm to 500 μm .

Chapter 2 – Background and Motivation

- Ferrofluids: stable colloidal suspensions of single domain iron oxide particles.

Toxicity, particle size, surface charge and hydrophilicity are important properties for *in vivo* use of magnetic particles. When ferrofluids are used their pH should be close to 7.4 and the nanoparticle surfaces should be hydrophilic; particles with a hydrophobic surface are opsonized sooner and cleared by the RES [16, 23, 82]. Toxicity can be evaluated by *in vitro* studies of cell cultures incubated with the particles. *In vitro* cytotoxicity is usually higher than *in vivo* since degradation products responsible for toxicity are continuously eliminated from the application site *in vivo*. *In vivo* tests on animals [24, 25, 67, 101, 102] as well as clinical human trials [27, 29, 33, 65, 72] have shown excellent biocompatibility for iron oxide magnetic particles. Superparamagnetic iron oxide nanoparticles are already in widespread clinical use as contrast enhancement agents in MRI applications [37, 38].

Other properties remaining the same, larger particles are more susceptible to opsonization and clearance by the RES compared to smaller particles [16, 19, 23, 82, 99]. For long circulation times, a neutral surface is also desirable, as highly charged particles are quickly removed from circulation [23, 100]. Particles with negative charge are cleared by the phagocytes of the RES from the bloodstream at a faster rate than neutral particles, leading to increased uptake in the liver. Positively charged particles may form large aggregates in the presence of negatively charged blood serum proteins, leading to transient embolisms in the liver with subsequent dissociation, clearance by the RES and uptake in the liver.

Chapter 2 – Background and Motivation

Despite much work in the area, complete evasion of the RES by injected MNP has not yet been achieved [99].

The next subsection discusses the need for surface functionalization and coating of iron oxide MNP with polymers for MDT and controlled drug release.

2.5 Surface coatings

Therapeutic agents such as drug molecules, DNA, anti-bodies etc, cannot be attached directly to the MNP surfaces, hence the surface has to be functionalized with organic or inorganic coatings that can reversibly bind the agents to be transported [16, 19, 82]. Polymeric coatings of synthetic or natural polymers are most commonly used. Table 2.2 lists some examples of polymers commonly used for coating magnetic nanoparticles [16].

Table 2.2 Examples of synthetic and natural polymers used for coating the surface of magnetic nanoparticles [16].

Polymer	Properties/advantages
Gelatin	Gelling agent, emulsifier, hydrophilic, biocompatible.
Dextran	Improves colloidal stability, circulation times
Chitosan	Cationic linear polymer, biocompatible, hydrophilic.
Polyethylene glycol (PEG)	Non covalent immobilization of PEG on the surface improves the biocompatibility, blood circulation time and internalization efficiency of the NP.
Polyvinylpyrrolidone	Improves colloidal stability, blood circulation time.

Chapter 2 – Background and Motivation

(PVP)	
Polyvinyl alcohol (PVA)	Prevents coagulation of particles, gives rise to monodisperse particles
Polyacrylic acid (PAA)	Improves stability and biocompatibility, helps in bioadhesion
Poly(N-isopropylacrylamide) (PNIPAM)	Thermoresponsive behavior enables thermally controlled drug release, cell separation.

In addition to their role as binding or adsorption sites for therapeutic agents, polymeric coatings can also provide hydrophilic surfaces which reduce agglomeration of the otherwise hydrophobic iron oxide nanoparticles, thus improving colloidal stability in aqueous environments. A hydrophilic coating also aids in RES evasion, especially if the coating consists of PEG which is known to increase circulation times [99, 100].

Among inorganic coatings, gold is widely studied due to its biocompatibility, and its ability to bind biological agents. Synthesis and characterization of hybrid nanoparticles consisting of magnetic core and Au shell have been previously reported [103-106].

In the next subsection, magnetic drug targeting using magnetic nanoparticles is discussed, including selected examples of *in vivo* and *in vitro* work from literature.

Chapter 2 – Background and Motivation

2.6 Magnetic drug targeting and drug delivery

The science of drug delivery may be described as the application of chemical and biological principles to control the *in vivo* temporal and spatial location of drug molecules for clinical benefit. In conventional drug delivery, there are two modes of targeting – active and passive. The physical properties of the particles determine the final biodistribution in passive targeting, while in active targeting, specific cells or organs are recognized via receptors and markers on the particles. Fig. 2.4 illustrates the active and passive modes of targeting to a tumor via the vasculature [10].

MDT offers a third mode – it is a physical mode of drug delivery that utilizes external influences to facilitate distribution of drugs and carrier systems in the body.

In MDT, nano or micron size coated magnetic particles with drugs attached to them are injected into the bloodstream and guided to the target site by an external magnetic field gradient and immobilized there for a controlled treatment period [16, 17, 24-27, 107]. The treatment can then be passive or active. In the passive mode, the drug is slowly released over a period of time through mechanisms such as diffusion. In the active mode, drug release is triggered by manipulation of environmental variables like temperature, pH, osmolarity etc.

Chapter 2 – Background and Motivation

Figure 2.4 Schematic illustration of (a) active targeting to the vasculature around the tumor (b) active targeting to the tumor itself (c) passive targeting to the tumor using nanoparticles loaded with therapeutic agents [10].

The advantage of MDT is that, compared to existing therapies, drug concentration is significantly enhanced in the target region while overall drug dosage required to achieve therapeutic local concentrations is reduced. More importantly, drug concentration in surrounding healthy tissue is drastically lowered, thus reducing the risk of systemic toxicity due to indiscriminate dispersion of powerful cytotoxic drugs in the body.

2.6.1 *In vivo* magnetic drug targeting

Widder and Senyei [108] were the first to successfully demonstrate the concept of *in vivo* MDT. They used 1- 2 μm magnetite particles encapsulated with albumin and loaded with doxorubicin (an anti-cancer drug) to target a distinct region of a

Chapter 2 – Background and Motivation

rat's tail. The dosage achieved was more than 200 times that which could be achieved by intravenous injection of the free drug.

One of the earliest clinical trials of MDT in humans was conducted in the 1990s in the former Soviet Union by Kuznetsov *et al.* [109, 110]. 0.01 – 0.1 μm ferro-carbon particles called 'cefesorb' loaded with carminomycine or rubomycine were used to treat more than 150 patients with advanced stages of cancer. It was claimed that some of the patients fully recovered, but detailed information of the outcome of the trial has not been made available. Similar ferro-carbon particles were used by Goodwin *et al* in a swine model [111]. The magnetic particles were retained in the areas of interest to which they were magnetically guided. Even after removal of the external field, there was no significant redistribution of the particles, with retention up to at least 6 days in the targeted liver. Histopathology indicated high particle densities in the areas that were targeted, but some particle presence was also detected in the interstitium and intra-arterially.

In 2000, Alexiou *et al.* [24] targeted VX-2 squamous cell carcinomas in the hind legs of rabbits with a ferrofluid injection of 100 nm multi-domain starch coated magnetite particles bound with mitoxrantrone and guided with a 1.7 T external magnetic field. Complete tumor remission was observed after 12 -57 days for rabbits treated with MDT, while the control groups (non-MDT) experienced tumor growth.

Significant results were obtained by Lübke *et al.* [27, 107] using 100 nm MNP coated with polymeric glucose partially functionalized with epirubicin. Ferrofluid

Chapter 2 – Background and Motivation

(conc. 15 mg.ml^{-1}) corresponding to 5 % of the overall blood volume was injected into the femoral vein of rats under the influence of an external magnetic field (0.2 T for 5, 10 and 30 min). An irreversible thrombus (i.e., blood clot in an intact blood vessel) formed within the capillary bed of the muscle after 10 min of field application, suggesting the feasibility of this method for inducing microembolization of tumors. In related work, Lübke *et al.* reported complete tumor regression of malignant, aggressively metastasizing adenocarcinomas and hypernephromas that were implanted into ears or abdomen of mice [112]. Iron and iron-oxide MNP coated with starch and partially functionalized with epirubicin were injected into the tail vein while an external magnetic field was applied in the tumor region. Tumor embolization could be achieved only with physiologically unfeasible ferrofluid doses of 10% of total blood volume, but tumor regression was achieved with ferrofluid of only 0.5 % of total blood volume. Side effects were observed to be minimal unless the amount of ferrofluid injected exceeded 10 – 20 % of total blood volume.

Based on the results of *in vivo* animal trials, the same group conducted clinical trials in humans [27, 107] using 100 nm core, anhydroglucose polymer coated particles functionalized with epirubicin to test for MDT related toxicity. Ferrofluid corresponding to 0.5 % of blood volume was injected into a vein located contralaterally to the tumor while an external magnetic field (0.5 – 0.8 T) was applied over the tumor for 60 – 160 min. In 6 out of 14 patients, the MNP were successfully concentrated in the tumor. In others, the MNP were detected in the liver, and in some cases the concentration of epirubicin in the tumor was less than what could be achieved by systemic administration. Hafeli [18] attributes this to

Chapter 2 – Background and Motivation

the possibly inadequate magnetic properties of the particles and the mode of injection used to administer the ferrofluid. However, the study showed iron oxide MNP related toxicity is not a serious problem for future application of MDT in humans.

2.6.2 *In vitro* studies of magnetic nanoparticles for MDT

The synthesis, characterization and *in vitro* studies on cell cultures of iron oxide MNP with various surface modifications have been previously reported [113-132]. In an *in vitro* study of human fibroblasts, Berry *et al.* demonstrated that iron oxide nanoparticle surface derivatisation plays an important role in cell – nanoparticle interactions: compared to bare MNP which were rapidly endocytosed and eventually caused cell death, albumin derivatised MNP underwent partial endocytosis and induced cell proliferation [113].

Zhang *et al.* studied PEG, folic acid and PEG – folic acid coated iron oxide MNP [124, 125] and MNP conjugated with the anti-folate anti-cancer drug methotrexate (MTX) [122, 123]. *In vitro* testing on BT20 human breast cancer cells (which overexpress folate receptors on the cell surface) revealed that the uptake of PEG – folic acid coated MNP was five times higher than that of MNP coated with PEG alone [125], the uptake of folic acid coated MNP was lower than that of PEG coated MNP [124]. *In vitro* tests of RAW 264.7 mouse macrophage cells showed a higher uptake of bare MNP compared to PEG coated and folic acid coated MNP. The uptake of coated particles in the macrophage cells was much lower than that in the BT20 cells, suggesting that PEG modification of the surface can help avoid protein adsorption and detection by the RES and facilitate uptake by specific

Chapter 2 – Background and Motivation

cancer cells [124]. Studies for MDT and MRI applications on 9L glioma cells with MTX loaded PEG coated MNP [122] showed that particles were internalized by the cells and the crystal structure retained for up to 144 h. The cytotoxicity of these drug loaded particles *in vitro* was higher than free MTX indicating the potential of these particles for dual MRI and MDT applications.

PEGylated iron oxide MNPs of average hydrodynamic diameter 100 nm and average magnetic core diameter of 8 nm, PEG content of 90 wt% were prepared by inverse emulsion polymerization [115]. Ferrofluids of similar particles with high colloidal stability over a pH range of 4 to 10 have also been synthesized by Hervé *et al.* [132]. Neither of these studies has reported drug loading and release.

The interaction of poly vinyl alcohol (PVA) coated MNPs with cell culture media as well as with different cell types has been studied [119, 126-131]. Superparamagnetic iron oxide MNP of average diameter 9 nm and coated with PVA, or PVA functionalized with thiol, carboxylate or amino groups were tested *in vitro* on human melanoma cells (Me 237 and Me275) [129]. Only PVA coatings functionalized with the amino group were taken up by the cells, the particle – cell interactions depended on the polymer to iron oxide ratio, and maximum uptake was of coated particles in the 24 to 32 nm average hydrodynamic diameter range. The cell-specific interaction of these particles as well as dextran coated MNP with brain derived cells was also studied [127]. Neither dextran nor the various PVA coated particles were cytotoxic to rat brain derived endothelial EC219 cells and murine microglial N9 and N11 cells. However, as before, the uptake of amino functionalized PVA coated MNP was much higher than other types of particles.

Chapter 2 – Background and Motivation

Munnier *et al.* have reported a novel method of loading doxorubicin onto superparamagnetic iron oxide nanoparticles by preparing a dox – Fe^{2+} complex and incubating it with a citrate treated ferrofluid containing MNP of average diameter 8 nm [118]. Drug loading was 14 wt% of the particles and binding was reversible under physiological pH conditions. *In vitro* 85 % of the loaded dox was released in 1 h at pH 7.4 while at pH 4, almost all the dox was released immediately. The released dox was free from iron, and was nearly as cytotoxic to HeLa cells *in vitro* as free dox solution.

Jain *et al.* have developed iron oxide nanoparticles of average diameter ~ 9 nm coated with oleic acid (OA) and Pluronic F-127 for combined MRI and MDT applications [116, 117, 120, 121]. *In vivo* biodistribution and clearance tests on Sprague-Dawley rats indicated no adverse effects due to long term changes in liver enzyme levels or oxidative stress from the injected Fe_3O_4 dose of 10 mg.kg^{-1} of body mass [117]. *In vitro* cytotoxicity studies on PC3 prostate cancer and MCF-7 breast cancer cells with dox loaded particles revealed that the cytotoxic effect was dose dependent and slightly lower than that of free dox solution of equivalent concentration [116]. *In vitro* studies were also carried out with particles loaded with hydrophobically modified dox and paclitaxel (PTX) individually as well as together [121]. Interestingly, when comparing the *in vitro* cytotoxic effect of free drug solutions on MCF-7 cells, the IC_{50} of PTX-dox was lower than that of PTX alone or dox alone. However, when the effect of drug release from the MNP was considered, the IC_{50} of PTX alone was the lowest, followed by PTX-dox and dox alone.

Chapter 2 – Background and Motivation

Misra *et al.* have reported magnetite nanoparticles of average size ~7 nm with folic acid functionalized PEG coating and loaded with doxorubicin [133]. *In vitro* drug release was initially rapid, (~65 % of loaded drug after ~12 h) followed by steady state release.

2.7 Hyperthermia

Hyperthermia [48-52] is the process of deactivating cancer cells by heating to temperatures in the range of 41 °C to 47 °C. Heating to the target temperatures causes moderate cellular inactivation in a dose dependent manner, however the critical target of thermal inactivation in the cell is not yet established [48, 51, 52]. Probably there is no individual cellular target of hyperthermia unlike well known DNA damage after radiotherapy. The molecular-biological mechanisms of these effects are still under investigation, various targets such as membranes, cytoskeleton, macromolecular synthesis, gene expression and DNA repair are affected by the temperature rise, thereby inducing cell apoptosis [51, 52]. In the case of combined hyperthermia and radiation, the combined effect occurs at the cellular level and can be attributed to the heat induced malfunction of repair processes after DNA irradiation damage. This synergistic effect is weaker if heat is induced either after or before irradiation. In addition, human tumors have increased blood flow under hyperthermia and this effect persists for a few hours after application of heat. Hence there is increased perfusion in the tumor region leading to higher oxygen concentration improving drug delivery and the efficacy of radiotherapy by increased reoxygenation [51, 52].

Chapter 2 – Background and Motivation

Early simplified clinical trials, and uncritically transferred data from preclinical trials suggested a minimum temperature of 43 °C for effective hyperthermia [48]. This was subsequently abandoned after many clinical studies showed greater effectiveness with hyperthermia at lower temperatures and current clinical trials focus mainly on the optimization of temperature homogeneity at moderate temperatures (42 – 43 °C) in the target volume [48, 49]. Attaining temperatures above the systemic temperature in the target tumor volume is technically challenging due to difficulties with tumor targeting, heat loss and improper heat distribution in surrounding tissues, perfusion induced cooling in the tumor etc. Temperature rise in the tissues is induced by applying a power-density specific absorption rate (SAR), measured in W.kg^{-1} . The human basal metabolic rate is above 1 W.kg^{-1} , and the rise in temperature is counteracted by perfusion. In order to heat up at least parts of such tumors to a temperature of 42 °C, with common perfusion rates in tumors in the range of 5 - 15 ml per 100 g.min⁻¹, a SAR of 20 - 40 W.kg^{-1} is required in the tumor [48]. The problem is compounded by technological limitations leading to lack of precise control and monitoring of temperatures *in vivo*, especially for regional hyperthermia. Common conventional hyperthermia techniques such as radiofrequency capacitance hyperthermia and whole body hyperthermia suffer from numerous drawbacks (section 1.2); magnetically mediated hyperthermia overcomes many of these shortcomings.

2.8 Magnetically mediated hyperthermia

Magnetically mediated hyperthermia [21, 22, 29-31, 34] is a mainly experimental modality that has the potential to address the targeting, attainable temperature and heat distribution drawbacks of other hyperthermia approaches. MMH consists of

Chapter 2 – Background and Motivation

localization of magnetic particles or seeds within tumor tissue followed by application of an external AMF to induce heating by hysteresis losses, Néel relaxation, Brownian rotation or eddy currents.

2.8.1 Heat generation from magnetic nanoparticles by an alternating magnetic field

The fundamental feature of MMH is that energy is absorbed from an alternating magnetic field and transformed into heat by means of a special target material, most commonly magnetic particles. There are three different mechanisms [20, 21, 81]:

1. Generation of eddy currents in low resistivity materials.
2. Magnetization rotation losses inside magnetic materials, i.e., hysteresis and Néel relaxation.
3. Rotation of the magnetic material (usually particle) relative to its surroundings, i.e., Brownian rotation.

The simultaneous action of two of these major mechanisms is also possible, particularly in the case of single domain magnetic particles. In hyperthermia, heat generation occurs within the volume of the material.

Eddy currents are generated as a consequence of the law of electromagnetic induction, and not restricted to magnetic materials, therefore eddy currents can be generated in tissue as well at high field frequencies although the specific conductivity of tissue (0.6 ohm-m^{-1}) is much lower than that of a conductor such as copper ($6 \times 10^7 \text{ ohm-m}^{-1}$). However, significant heating effects are found only in metallic materials of macroscopic size [81] since high electrical conductivity of

Chapter 2 – Background and Motivation

metals produces considerable current in all closed circuits in an alternating magnetic flux. Eddy currents are a major contributing mechanism only in interstitial implant hyperthermia which involves the use of surgically implanted macroscopic ferromagnetic or metallic seeds. For other types of hyperthermia, the particles used are very fine and the AMF frequencies are too low for substantial eddy current generation.

Ferromagnetic materials exhibit hysteresis loss when exposed to AMF, the amount of heat generated per unit volume, P_{FM} is given by the product of the frequency and the area of the AMF hysteresis curve [81]

$$P_{FM} = \mu_0 f \int H dM \quad (\text{Eq. 2.6})$$

where f is the frequency and μ_0 the permeability of free space.

In principle, substantial hysteresis heating of ferromagnetic particles may be obtained using magnetic particles with high anisotropy, but constraints on the usable magnitudes of H are such that fully saturated loops cannot be obtained. Only minor, i.e., unsaturated loops can be used, and these would yield much poorer heating effects. Ideally, the hysteresis loop should be square to achieve maximum energy loss and heating, but this would require an ensemble of uniaxial single domain particles perfectly aligned along the field direction. Such a configuration would be nearly impossible to achieve *in vivo*, and a more realistic system of randomly aligned ferromagnetic particles would produce only about 25% of the theoretical maximum performance.

Chapter 2 – Background and Motivation

Excellent heating performance can be obtained by using a colloidal suspension of superparamagnetic particles [20]. When the external field applied to such a ferrofluid is reduced to zero, its magnetization decreases to zero due to ambient thermal energy, this relaxation can be Brownian or Néel. These relaxation processes occur many times when the AMF varies between positive and negative values of its maximum amplitude. The relaxation processes are characterized by relaxation times. For Brownian relaxation, the relaxation time τ_B is given by

$$\tau_B = \frac{3\eta V_H}{k_B T} \quad (\text{Eq. 2.7})$$

where η is the viscosity of the fluid, T the temperature, and V_H the hydrodynamic volume of the particle. The Néel relaxation time τ_N is given by

$$\tau_N = \frac{\sqrt{\pi}}{2} \tau_0 \frac{\exp\left(\frac{KV_M}{k_B T}\right)}{\sqrt{\frac{KV_M}{k_B T}}} \quad (\text{Eq. 2.8})$$

where K is the anisotropy constant, V_M is the magnetic volume and $\tau_0 = 10^{-9}$ s.

Since Brownian and Néel relaxation processes occur in parallel, the effective relaxation time is given by

$$\frac{1}{\tau} = \frac{1}{\tau_B} + \frac{1}{\tau_N} \quad (\text{Eq. 2.9})$$

The shorter relaxation time term dominates the effective relaxation time for a given particle size. τ_B is determined in part by the hydrodynamic properties of the fluid, while τ_N is governed by the magnetic anisotropy energy relative to thermal energy.

Both Brownian and Néel processes may be present in a ferrofluid, but only Néel

Chapter 2 – Background and Motivation

relaxation occurs in constrained (i.e., immobilized) superparamagnetic particles. Relaxation times depend on particle size, and for a given size, losses due to Brownian rotation are usually maximized at a lower frequency than Néel losses. A graphical relationship between the relaxation times and particle size is shown in Fig. 2.5.

Figure 2.5 An example of Néel and Brownian relaxation time constants v/s particle size for Fe₃O₄ MNP. The smaller of the two relaxation times determines the effective relaxation time.

In superparamagnetic particles, the finite change of M in a ferrofluid means that it will lag behind H , so there is a conversion of internal energy to heat during relaxation. The volumetric power dissipation can be expressed as [20]

Chapter 2 – Background and Motivation

$$P = \pi\mu_0\chi_0 H_0^2 f \frac{2\pi f\tau}{1 + (2\pi f\tau)^2} \quad (\text{Eq. 2.10})$$

where μ_0 is the permeability of free space, χ_0 is the equilibrium susceptibility, H_0 is the AMF field strength, f is the AMF frequency and τ is the effective relaxation time.

χ_0 can be calculated from the following expressions:

$$\chi_0 = \chi_i \frac{3}{\xi} \left(\coth \xi - \frac{1}{\xi} \right) \quad (\text{Eq.2.11})$$

$$\chi_i = \frac{\mu_0 \phi M_d^2 V_M}{3k_B T} \quad (\text{Eq 2.12})$$

$$\xi = \frac{\mu_0 M_d H V_M}{k_B T} \quad (\text{Eq 2.13})$$

where ϕ is the volume fraction of nanoparticles in the ferrofluid, M_d is the domain magnetization of the nanoparticle.

Experimentally, quantification of heat generation from magnetic particles is given by the specific absorption rate (SAR) which can be calculated as

$$SAR = C \left(\frac{\Delta T}{\Delta t} \right) \frac{\text{mass}_{\text{ferrofluid}}}{\text{mass}_{\text{nanoparticles}}} \quad (\text{Eq. 2.14})$$

where C is the mass weighted heat capacity of the ferrofluid and $(\Delta T/\Delta t)$ is the slope of the initial section of the temperature v/s time curve.

Most ferromagnetic materials require applied field strengths of about 100 kA.m^{-1} or more before they can yield fully saturated loops, only minor loops can be achieved with operationally feasible fields of about 15 kA.m^{-1} , with consequently lower

Chapter 2 – Background and Motivation

SAR values. In contrast, superparamagnetic particles are capable of generating high SAR values [20, 21, 134] at lower fields. It has been recently suggested that single domain particles in the transitional size range between single-domain and superparamagnetic, e.g. 30-40 nm bacterial magnetosomes [21], may produce higher power output than purely superparamagnetic particles, but this is also dependent upon specific field parameters and environmental conditions.

2.8.2 Types of magnetically mediated hyperthermia

Gilchrist *et al.* were the first to describe and test the concept of MMH [135]. Their objective was the treatment of lymphatic metastases of large bowel cancer by heat using ferromagnetic particles to embolize lymph nodes draining the primary cancer site. This was followed by heating of the particles with an external AMF. A concentration of 5 mg of magnetite per g of lymph node tissue was found to produce a temperature rise of 14 °C in 3 min.

MMH has developed along four general paths, all sharing the common principle of deposition of magnetic material within or adjacent to tumor tissue, followed by exposure to an external AMF to induce heating.

- a) Interstitial implant hyperthermia [31] consists of implantation of ferromagnetic seeds a few mm in size, or rods directly into the tumor tissue followed by exposure to an AMF to cause heating by induced eddy currents.
- b) Arterial embolization hyperthermia [31, 67, 101] utilizes the arterial supply of a tumor to selectively embolize a tumor with magnetic particles, followed by AMF application to cause heating. Although this technique has

Chapter 2 – Background and Motivation

- not been clinically tested in humans to date, it promises to be well-suited to treatment of hepatic malignancies since macroscopic liver tumors derive virtually all their blood supply from the hepatic arterial system, making it easier to preferentially target liver tumors by arterial injection of MNP [67].
- c) Direct injection hyperthermia [29, 31] consists of directly injecting a ferrofluid into the tumor tissue with the subsequent application of an alternating magnetic field to cause heating. Unlike in case of the arterial embolization technique where heat originates from particles deposited within blood vessels, in direct injection hyperthermia the heat originates from extra-cellular deposits of particles.
- d) Intracellular hyperthermia is the most relevant to this project since it offers the possibility of concurrent hyperthermia and chemotherapy using the same particles. It involves delivery of surface modified magnetic particles to the tumor cells by arterial embolization or direct injection [31]. The particles used are in the nanometer range, have a surface coating, and may optionally have attached tumor specific antibodies. Upon injection, the particles are ingested by tumor cells and uptake by healthy tissue is minimal especially when magnetic targeting of the particles to the tumor is used. When exposed to an AMF, heat is produced from within tumor cells due to the ingested particles. Healthy cells, which have a much smaller quantity of particles within them, produce less heat and are reasonably protected from thermal damage.

Chapter 2 – Background and Motivation

2.8.3 Intracellular hyperthermia

The earliest studies on intracellular hyperthermia were by Gordon *et al.* [136] using 2.6 – 6 nm magnetite particles suspended in a sucrose solution. The study was conducted on Sprague-Dawley rats with implanted mammary carcinomas in the tail. 100 mg of the particles were injected into the tail vein of the rats, and exposed to 450 kHz AMF for 12 min, an increase in tumor temperature of ~ 8 °C was recorded. Particle related toxicity and side effects were reported to be absent, and histological evidence of MNP uptake by the tumor cells was presented. However, substantial deposits of MNP were also found outside the tumor cells – in the liver, spleen and kidney, suggesting that heat generation may not have been solely due to intracellularly deposited particles but also from particles outside the tumor.

In 1999, Shinkai *et al.* [137] conducted an *in vivo* study on F344 rats with implanted femoral subcutaneous gliomas. Magnetite cationic liposomes (MCL) with 10 nm magnetite cores were subcutaneously injected into the tumors and the rats were exposed to a 30.6 kA.m^{-1} , 118 kHz AMF for 30 min. Tumor temperatures over 43 °C were recorded and histology showed that 90 % of the tumors had completely regressed after three rounds of hyperthermia application.

A study on the treatment of large tumors (~ 15 mm) using MCLs was performed by Ito *et al.* [138]. Large MM46 mammary tumors implanted in C3H/HeN mice were targeted with 10 nm core MCLs, and tumor temperatures of 45 – 46 °C were observed with a 118 kHz AMF (field strength not mentioned) after 5 min of application. Complete tumor regression was achieved in all the mice treated with MMH.

Chapter 2 – Background and Motivation

Jordan *et al.* have studied the interactions between tumor cells and magnetic nanoparticles [22, 30, 34, 139-141]. They showed that human colonic adenocarcinoma cells accumulated up to 1.1 pg of iron oxide per cell when the cells were cultured in a medium containing the ferrite particles [141]. However, after exposure to an AMF and generation of temperatures over 43 °C, no improvement in cytotoxicity was found compared to a water bath exposure at 43 – 45 °C. It was suggested that this problem was due to intracellular digestion of the particle coating.

Jordan *et al.* investigated the cellular uptake and biological effect of both dextran and aminosilane coated magnetite particles [140]. The dextran-magnetite particles had a 3.3 nm core with a negative surface charge, the aminosilane-magnetite particles had a 13.1 nm core and highly positive surface charge. Normal human cell types (fibroblasts and neurons) and carcinomas (colonic adenocarcinoma and glioma) were cultured in separate mediums, each containing differently coated magnetite particles. The cells were subjected to MMH with a 520 kHz, 13.2 kAm^{-1} AMF. For comparison purposes, both types of cells were heated with a water bath in ferrofluid-free mediums. Increased uptake of iron - up to 400 pg per cell for gliomas with aminosilan-magnetite, and 500 pg per cell for fibrobaslts with dextran-magnetite, and greater cell cytotoxicity with hyperthermia as compared to a water bath were reported.

Intracellular hyperthermia has all the potential advantages associated with arterial embolization hyperthermia and direct injection hyperthermia [31], depending on

Chapter 2 – Background and Motivation

the route of nanoparticle delivery to the tumor. Intracellular hyperthermia can localize the heat generated to the tumor region, improving heating efficiency. An additional advantage is that when tumor cells which have taken up MNP undergo division, there is a high probability that the daughter cells will contain some of the particles that were present in the parent cell [140]. This could make newly formed cells susceptible to inactivation by hyperthermia without the need for additional nanoparticle delivery. Also, since particles used in intracellular hyperthermia are usually single domain (and often superparamagnetic), magnetic field strengths required are moderate compared to multi-domain ferromagnetic particles that require high field strengths to produce fully saturated hysteresis loops. Finally, cytotoxic drugs can be attached to the MNP coating, enabling simultaneous chemotherapy and hyperthermia.

The disadvantages of intracellular hyperthermia are similar to those of arterial embolization and direct injection hyperthermia [59]. The issue of the relative heat contributions from intracellularly and extracellularly deposited heat models has not been fully resolved in a live tumor model. For fully effective treatment, a very high fraction of the tumor cells would have to ingest the MNP to ensure adequate intracellular heating. Those tumor cells which have not ingested the particles also need to be heated by extracellular particle deposits to cause necrosis. For very large or poorly vascularized tumors, this may not be feasible if arterial embolization delivery is used. In tumors with poor vasculature and semi-necrotic cores that harbor viable tumor cells, delivery of particles might not be feasible. If a direct injection approach is taken, particle distribution may be sub-optimal.

Chapter 2 – Background and Motivation

2.8.4 *In vitro* magnetically mediated hyperthermia

Significant amount of *in vitro* work has been performed on the synthesis, characterization and property evaluation of magnetic particles for MMH [15, 17, 19, 21, 33, 76, 77, 134, 142-158]. However, due to the wide range of materials, particle sizes, ferrofluid concentrations, carrier fluids, test temperatures, AMF parameters etc., used in the reports, it is difficult to directly compare the results.

2.8.5 Operational parameters for clinical hyperthermia in humans

Apart from the magnetic particles to be used, MMH also requires an AMF applicator system. For biomedical purposes, the frequency of the AMF used in MMH has to be higher than 50 kHz to avoid neuromuscular electrostimulation in humans and lower than 10 MHz for appropriate penetration of the radiofrequency field and to avoid undesirable eddy current heating of tissue [159]. In most of the laboratory *in vitro* and *in vivo* experiments, generators with a frequency between 50 kHz and 1 MHz, field strengths of a few tens of kA.m^{-1} and induction coils or air gap applicators have been used [35]. These parameters were selected more on the availability of generators and ancillary equipment rather than on theoretical considerations or modeling.

For clinical applications, a large air volume, at least $5000 - 10,000 \text{ cm}^3$ has to be enveloped by an AMF with very high field homogeneity because field gradients can produce thermal gradients even if particle distribution *in vivo* is uniform. Since it is easier to achieve field homogeneity in air than homogenous particle distributions in tissue, the field should be as homogenous as possible. Heating of a small quantity of MNP ($\sim 10 \text{ mg per g of tumor}$) requires field amplitude of the

Chapter 2 – Background and Motivation

order of 10 kA.m^{-1} , combined with the requirements of volume and field homogeneity, this makes the AMF applicator system power intensive [30]. Considering these requirements and limitations, inductive coils are not appropriate for clinical MMH treatment, only a ferrite-core based applicator system is suitable. A field applicator system called the MFH[®] 300F has been developed by MFH Hyperthermiesysteme GmbH, Germany and installed at the Clinic for Radiation Medicine, Charité Campus Virchow-Klinikum, Berlin, for use in human clinical trials. A description of the equipment has been provided by Gneveckow *et al.* (Fig.2.6) [33, 160].

2.8.6 First human clinical trials of magnetically mediated hyperthermia

Jordan *et al.* conducted a clinical phase II trial combining magnetic hyperthermia/thermoablation and radiation therapy with promising results [29, 33, 65, 72, 161]. For example, for treatment of prostate tumors [29], a ferrofluid of aminosilane coated iron oxide particles with a 15 nm core (Fe concentration of 120 mg.ml^{-1}) was injected as 24 depots (subcutaneous or intramuscular deposit of pharmacological agents for long term action) covering the prostate; nanoparticles were retained in the prostate for the entire treatment period of 6 weeks. Treatment was carried out in the MFH[®]300F magnetic field applicator system with a field strength of $0 - 18 \text{ kA.m}^{-1}$ and frequency of 100 kHz. Intra-prostatic temperatures in the range of 39.4°C to 48.5°C were attained at field strengths of $4 - 5 \text{ kA.m}^{-1}$.

Patients were also treated for cervical, rectal and prostate carcinomas, as well as for chondrosarcoma, rhabdomyosarcoma, glioblastoma and liver metastasis [33, 65, 72, 161]. Sessions were for 1 h, and after local injection of the magnetic particles,

Chapter 2 – Background and Motivation

the tumor temperature increased to 43 – 45 °C for AMF parameters of 100 kHz, 3 – 9.5 kA.m⁻¹. The hyperthermia treatment was repeated from 2 to 11 times without any additional injections, and the MMH treatment was well tolerated. Two patients showed complete remission 9 and 14 months after treatment, while the other six patients showed local control with no recurrent growth.

Figure 2.6 The MFH 300F Magnetic field applicator suitable specifically designed for magnetic hyperthermia and used in the first human clinical trials recently conducted in Germany [29, 65, 160].

The next subsection discusses the utilization of stimuli responsive polymeric systems for biomedical applications.

Chapter 2 – Background and Motivation

2.9 Stimuli-responsive release systems

Stimuli-responsive release systems offer a means of triggering release of therapeutic agents after their localization in the tumor by utilizing specific environmental conditions in the tumor such as pH and temperature [61, 62, 64, 162]. The advantage of using stimuli-responsive systems is that therapeutic payload of the delivery system is maintained relatively intact during transport to the tumor site and released only when the carrier is localized in the tumor, thus (a) minimizing wastage of the payload from systemic distribution, (b) minimizing systemic toxicity and (c) maximizing dosage in the tumor. Thermoresponsive systems are of particular interest due to the additional advantage of being able to modulate drug release by external means.

Poly n-(isopropylacrylamide) (Fig. 2.7a) is a thermoresponsive polymer [61-64, 84, 162] with a lower critical solution temperature of between 30 – 35 °C in water; at this transition point, the polymer undergoes a rapid and reversible volume change. Below the LCST, the polymer chains are stabilized by a hydrophobically hydrated shell arising from extensive hydrogen bonding interactions of water molecules with amide groups, this is the swollen state. As the temperature is raised above the LCST, the hydrogen bonding network is broken and replaced with intra- and intermolecular amide – amide hydrophobic bonds, leading to expulsion of the hydrophobically bound water and collapse of the polymer [84]. Fig. 2.7b is a schematic representation of this process [62].

Chapter 2 – Background and Motivation

Figure 2.7 (a) Chemical formula of PNIPAM (b) schematic illustration of the phase transition of PNIPAM by expulsion of hydrophobically bound water above the lower critical solution temperature [62].

The dehydration process has two stages [90], the driving force is the increase in entropy of the phase separated polymer – water system compared to the polymer in solution above the LCST. Hydrophobic bond formation occurs in stage I, followed by water – polymer hydrogen bond dissociation in stage II, leading to disruption of the hydration shell surrounding the hydrophobic portion and rapid dehydration and aggregation of the polymer chains. Enthalpy changes for both stages are endothermic. The change in entropy is relatively low for stage I, but for stage II release of bound water molecules and polymer collapse results in significantly increased configurational entropy. During cooling of the sample below the LCST, rehydration does not begin until rehydration shells are reestablished following breaking of the hydrophobic bonds.

Chapter 2 – Background and Motivation

The reversible phase transition for PNIPAM is relatively insensitive to the molecular weight and concentration, but strongly influenced by the type of solvent and additives such as salts, surfactants, co-solvents etc [84, 92, 93]. The additives can alter the energy barrier for the formation of hydrophobic bonds by changing the structure of the hydration shell and consequently the hydration behavior of the hydrophobic segments. This can lead to shifting or disappearance of the transition temperature. NIPAM can also be copolymerized with the appropriate hydrophobic or hydrophilic monomers to yield a thermoresponsive polymer with the desired LCST [61, 62, 64, 84, 162].

2.10 Thermoresponsive magnetic nanoparticles for biomedical applications

Due to the biocompatibility of PNIPAM [85-87, 163] and the proximity of its LCST to the human body temperature, it has been studied extensively for biomedical applications such as drug delivery and cell separation applications [61, 62, 64, 83, 84, 87, 162, 163]. Thermoresponsive magnetic carriers consisting of iron oxide and PNIPAM homopolymer or copolymer have been previously reported at micron [77, 164-169] and nano [74, 78, 170-184] size scales.

Ramanujan *et al.* have studied the thermoresponsive behavior and PNIPAM – magnet composite systems containing micron sized Fe_3O_4 and Fe magnetic particles [77, 167]. Upon heating the PNIPAM gels loaded with water soluble dye a distinct volume transition occurred at the LCST of PNIPAM (34 °C), leading to collapse of the gel and dye release [77].

Chapter 2 – Background and Motivation

Aoyagi *et al.* have reported the synthesis and characterization of thermoresponsive composite nanoparticles with a 130 nm average size magnetite core coated with n-isopropylacrylamide-co-2-carboxyisopropylacrylamide (NIPAM-co-CIPAM) functional copolymer [74]. At temperatures higher the LCST of 34 °C, the particle hydrodynamic diameter was found to increase with time as a consequence of aggregation induced by the hydrophobic surface of the polymer above the LCST. When exposed to an alternating magnetic field, the particles heated up and exhibited reversible hydrophobic and hydrophilic behavior. Similar particles were also synthesized using a layer-by-layer method [172].

Heparinized CNP prepared by coating 6-8 nm average diameter magnetite particles with PNIPAM via atom-transfer radical polymerization followed by immobilization of heparin on the polymer coating have been reported [179]. After PNIPAM coating and heparinization, the M_s values dropped to 33 emu.g⁻¹ from the initial 51 emu.g⁻¹ of the bare MNP. Higher biocompatibility was achieved with heparin functionalization; uptake by RAW 264.7 mouse macrophage cells after 8 h was only 30% of the uptake of bare MNP and PNIPAM coated CNP, suggesting better *in vivo* RES evasion capability for the heparinized CNP.

Misra *et al.* have developed stimuli responsive CNPs with magnetite core of ~ 5 nm average diameter conjugated with doxorubicin by acid labile hydrazone bonds and encapsulated by a stimuli responsive polymer consisting of either chitosan-g-poly(n-isopropylacrylamide-co-n,n-dimethylacrylamide) [176] or dextran-g-poly(n-isopropylacrylamide-co-n,n-dimethylacrylamide) [173]. For both systems, the LCST of the polymer alone was ~ 38 °C in PBS, and drug release was

Chapter 2 – Background and Motivation

significantly higher above the LCST, especially in mildly acidic conditions (pH 5.3) that lead to breaking of the hydrazone bond.

MNPs with PNIPAM based coating have also been studied for *in vitro* magnetic separation of biological agents, such as adsorption/desorption of bovine serum albumin [175] and capture/release of biological agents in microfluidic devices [171, 180, 181].

The next subsection deals with the benefits of combined hyperthermia and chemotherapy in cancer therapy, and the utilization of polymer – magnetic composite nanoparticles for such applications.

2.11 Simultaneous hyperthermia and chemotherapy from magnetic nanoparticles

Multimodal therapy, i.e., hyperthermia during chemotherapy can improve the efficacy of the treatment [48, 50-57, 185, 186] and this approach is often used for multimodal and adjuvant therapy. The synergistic effect of simultaneous application of heat and drug is greater than that obtained from sequential application.

Magnetic carriers with thermoresponsive coatings can be used for achieving simultaneous hyperthermia and drug release, additionally, the LCST of the polymer can act as an on/off switch to trigger drug release during hyperthermia and “switch off” release when the tumor cools below the hyperthermia temperature range. So

Chapter 2 – Background and Motivation

far, only a few studies have examined such magnetic carriers for multimodal therapy [73, 75-78, 142, 187, 188].

MNP of average magnetic core size 10 – 20 nm coated with PNIPAM via a water-in-oil suspension polymerization method and loaded with methylene blue or rhodamine B as mock drugs have been previously reported [78]. Drug release from the ferrofluid upon heating up with application of an external AMF (20 kA.m⁻¹, 360 kHz) was 5 to 8 times higher than that without the AMF.

In vitro release of vitamin B₁₂ and methylene blue from hydrogel discs of PNIPAM crosslinked with tetra (ethylene glycol) dimethacrylate and loaded with 5 wt% of 25 nm Fe₃O₄ MNP has been reported [182]. The release could be modulated by cycling of the temperature above and below the LCST using a 297 kHz, 5.3 kA.m⁻¹ AMF to induce heating of the MNP and collapse of the hydrogel.

Babinec *et al.* have reported large unilamellar magnetoliposomes containing 8 nm average size maghemite MNP and loaded with dox [76]. When exposed to an AMF of frequency 3.5 MHz and field strength 1.5 mT, the embedded MNP generated heat, raising the temperature of the phospholipid layer above its transition temperature of 42 °C, resulting in rapid drug release. Dox loaded magnetoliposomes with a Fe concentration of 1.2 mg.ml⁻¹ released ~70 % of the loaded dox in just 6 min; however it must be noted that heat generated was confined to the phospholipid layer, macroscopic heating of the magnetoliposome suspension was insignificant. The same group reported *in vitro* tests on BP6 rat sarcoma cell cultures with cisplatin loaded starch polymer coated iron oxide

Chapter 2 – Background and Motivation

nanoparticles and showed that a combination of cisplatin and hyperthermia was much more effective in killing the cells compared to individual modalities [142].

Other studies on dextran-magnetite based thermosensitive magnetoliposomes (TM) for combined hyperthermia and drug release applications have also been reported [73, 75, 188, 189]. When 5-fluororacil loaded TMs were exposed to an external AMF [73], the temperature of the lipid layer was raised to 42 °C resulting in a four fold increase in drug release compared to release at 37 °C without an AMF. Importantly, drug release could be repeated multiple times by switching the AMF on or off as desired, to heat the TM to 42 °C or cool them to body temperature. The same group also reported complete tumor remission in AH60C tumor bearing Donryu rats treated with magnetically mediated hyperthermia using these TMs (without drug loading) [75].

Brusentsov *et al.* have also reported tumor control of non-metastatic P388 tumors in BDF₁ mice treated with Melphalan loaded dextran-ferrite based ferrofluids with hyperthermia as part of a larger treatment procedure involving tumor slime aspiration [190].

Nanoparticle properties depend on the synthesis and processing methods. For materials for *in vivo* biomedical applications, selection of the optimum methods for iron oxide nanoparticle synthesis and coating with polymer are critical.

Chapter 2 – Background and Motivation

2.12 Nanoparticle Synthesis

Nanoparticle synthesis methods can be generally classified into those that produce magnetic nanoparticles from solutions or aerosol/vapor phases and those that yield composites consisting of MNP dispersed in submicron-sized organic or inorganic matrices [16, 94, 191-193]. The first category includes particle synthesis via co-precipitation, reverse-micelle, polyol, spray pyrolysis, laser pyrolysis and high temperature decomposition of organometallic precursors. The co-precipitation and high temperature decomposition methods are of relevance to this thesis, and will be discussed.

2.12.1 Precipitation from solution

These are relatively simple processes that yield particles with good size and shape distribution. Uniform particles are usually prepared via a homogeneous precipitation reaction, a process which involves the separation of nucleation and growth stages. Briefly, in homogeneous precipitation, a short single burst of nucleation occurs when the concentration of constituent species reaches critical supersaturation. These nuclei are allowed to grow uniformly by diffusion of solutes from the solution to their surface until the final size is attained. To obtain monodisperse particles, the nucleation and growth stages must be separated and nucleation should be prevented during the growth period (curve I, Fig.2.8) [192]. Aggregation of much smaller subunits leading to reasonably uniform nanoparticles can also be achieved. (curve II, Fig.2.8). Uniform particles may also be obtained by multiple nucleation events, with the uniformity of the final product determined by Ostwald ripening (curve III, Fig.2.8).

Chapter 2 – Background and Motivation

Figure 2.8 Mechanism of formation of uniform particles in solutions [192].

2.12.2 High temperature synthesis from colloidal solutions

In this method, MNP are produced by thermal decomposition at elevated temperatures (240 – 320 °C) of an organometallic precursor in a high boiling organic solvent in the presence of one or more surfactants. The final MNP are highly crystalline and monodisperse, this process offers control of size by controlling the temperature of reaction, boiling point of the organic solvent, aging time and quantity of surfactants. In the case of iron oxide MNP, the precursor is an iron-oleate complex, the surfactants are usually oleic acid or oleylamine and the organic solvents are 1-hexadecane (b.p. 274 °C), octyl ether (b.p. 287 °C), 1-octadecene (b.p. 317 °C) etc.

Nanocrystal formation is by the following mechanism (Fig.2.9) [194]. Nucleation occurs at 200 – 240 °C triggered by the dissociation of an oleate ligand from the Fe(oleate)_3 precursor by CO_2 elimination. Although slow growth can occur below 250 °C, significant growth occurs around 300 °C and is initiated by the dissociation

Chapter 2 – Background and Motivation

of the remaining two oleate ligands from the iron-oleate complex. Since growth and nucleation stages are separated, the size distribution of the particles is narrow. Size tuning can be achieved in several ways: particle size can be increased with increased boiling points of the organic solvent (and consequently the aging temperature) due to the increased reactivity of the iron oleate complex with a higher boiling solvent. Longer aging times also result in larger particles. Another option for increasing size is by increasing the surfactant concentration. This synthesis process offers advantages apart from monodispersity, crystallinity and size control. The reactants used in the process are non-toxic and environmentally friendly compared to other methods such as pyrolysis. Large quantities (few tens of grams) can be produced in one batch.

Figure 2.9 The overall scheme for the high temperature synthesis of iron oxide from iron oleate [194].

2.12.3 Co-precipitation in aqueous solutions

Co-precipitation is one of the easiest methods to produce iron oxides, and offers reasonable control of size and shape [16, 191, 192]. Stoichiometric mixtures of

Chapter 2 – Background and Motivation

ferrous and ferric hydroxides are aged in aqueous media, yielding Fe_3O_4 particles which are homogeneous in size. Spherical, crystalline Fe_3O_4 particles are formed by aggregation of primary particles formed within a $\text{Fe}(\text{OH})_2$ gel. Final particle size can be tuned by varying the pH, ionic strength of the medium and synthesis temperature [195-198].

2.13 Coating of magnetic nanoparticles with thermoresponsive polymers and drug loading

Almost all MNP used for biomedical applications are coated with natural or synthetic polymers for the reasons mentioned in Section 2.5 and several coating methods have been reported [16, 193, 199]. However, for the preparation of iron oxide – PNIPAM composite nanoparticles [74, 165, 171-175, 177], surfactants may undesirably alter the LCST of PNIPAM [84, 92, 93] and leave residual chemicals that may not be biocompatible. For this work, the free radical dispersion polymerization method [77, 166, 200] was chosen to coat iron oxide MNP with PNIPAM due to its simplicity, absence of surfactants, and the ability to coat relatively large quantities of particles in a single step. In this method, *n*-isopropylacrylamide (NIPAM) monomer is polymerized in the presence of an aqueous suspension of iron oxide MNP using a cross-linker, an initiator and an accelerator. The mixture is ultrasonicated or stirred at high speed to ensure dispersion of the nanoparticles in the polymerizing solution and subsequently, uniform coating of the particles.

Drug loading may be carried out either during the polymerization process by (a) incorporating the drug in the monomer solution prior to polymerization, or (b)

Chapter 2 – Background and Motivation

incubating the coated nanoparticles in the drug solution after polymerization [201]. The latter approach is used in the current work since polymerization may alter the properties of the drug. Additionally, drug loading during polymerization restricts further processing (such as washing/purification) of the composite particles.

2.14 Innovative aspects of the current work

From the literature survey, it is evident that the potential for applications of MNP in cancer therapy is vast. Despite the significant progress made in the fields of MDT and MMH individually, there is insufficient work, both *in vitro* and *in vivo*, on the development of CNP for combined MMH and MDT. Hence, the current work was undertaken.

1. Synthesis and processing of thermoresponsive composite nanoparticles with an iron oxide core and PNIPAM shell. Most of the prior work on thermoresponsive CNP has involved complex PNIPAM based copolymers without studying the basic MNP – PNIPAM system, the influence of MNP on the properties of PNIPAM is also not well understood. Prior work has not examined in detail the influence of MNP and drugs on the LCST of PNIPAM – iron oxide nanoparticles.
2. Simultaneous drug release and MMH from PNIPAM – iron oxide CNP loaded with a clinically relevant anti-cancer drug has not yet been demonstrated. Only dyes have been used in prior work on PNIPAM – iron oxide nanoparticles [78], hence the drug release behavior of this system under AMF induced heating is unknown. The current work will clearly demonstrate the loading of drug onto CNP and *in vitro* simultaneous drug release and MMH from these particles.

Chapter 2 – Background and Motivation

3. The drug release behavior of drug loaded PNIPAM – iron oxide CNP has not been studied so far. The current work will investigate the release of doxorubicin from these particles at different temperatures and time scales to establish a better understanding of the release properties of this system.
4. Thermoresponsive controlled release of drugs is a primary attraction of the PNIPAM – iron oxide CNP, however, prior work has not clearly demonstrated this behavior. The ability of these nanoparticles to regulate drug release by utilizing the LCST as an on/off switch will be established in the current work.
5. So far, there have been few reports on the modeling of the heating behavior [202-207] and drug release from CNP. The present work will add to existing knowledge by modeling the effect of magnetic core size and polymer shell thickness on the power output of CNP, the heating of the tumor by these particles, and drug release from these particles.
6. The cytotoxicity of PNIPAM – iron oxide CNP has not yet been reported. *In vitro* cytotoxicity of MNP and CNP will be studied with human epithelial cells.
7. *In vivo* magnetic drug targeting has not been studied so far with the PNIPAM – iron oxide CNP. In the current work, the effectiveness of targeting drug loaded CNP to a tumor in a Buffalo rat model will be demonstrated with MRI and histology.

The next chapter describes the experimental procedure used for synthesis, processing, characterization and property evaluation.

Chapter 3 – Experimental Procedures

CHAPTER 3

EXPERIMENTAL PROCEDURES

This chapter describes the experimental procedures for the synthesis, characterization, property evaluation of bare as well as drug loaded PNIPAM – iron oxide composite nanoparticles used in the current work. *In vitro* cytotoxicity tests and *in vivo* magnetic drug targeting, MRI and histology investigation protocols are also described.

3.1 Synthesis of iron oxide and PNIPAM – iron oxide composite nanoparticles

Two types of iron oxides – γ -Fe₂O₃ (average size 12 nm, 14 nm, 19 nm and 43 nm) and Fe₃O₄ (average size 6 nm and 13 nm) were studied as the magnetic core of the composite nanoparticles. The particles with the best *in vitro* hyperthermia performance were coated with N,N-methylene(bis)acrylamide (BAAm) – crosslinked PNIPAM for further investigation. The overview of the processing for the particles is shown in Fig 3.1a, the chemical formulae of PNIPAM crosslinked with BAAm (Fig. 3.1b) and dox (Fig. 3.1c) are also shown.

3.1.1 Synthesis of γ -Fe₂O₃ magnetic nanoparticles by the high temperature decomposition method

γ -Fe₂O₃ MNP of average size 12 nm, 14 nm, 19 nm and 43 nm were synthesized by a modified high temperature thermal decomposition method described in detail elsewhere [194]. In a typical experiment to synthesize 14 nm MNP, 10.8 g of iron chloride (FeCl₃·6H₂O, 120 mmol, Aldrich, 98%) and 36.5 g of sodium oleate (120 mmol, TCI, 95%) were dissolved in a solvent mixture of 80 ml ethanol, 60 ml distilled water and 140 ml hexane. The solution was heated in a round bottom flask

Chapter 3 – Experimental Procedures

to 70 °C under reflux conditions and held for 4 h. At the end of the reaction, the dark red upper organic layer containing the iron oleate complex was washed several times with 30 ml of distilled water in a separatory funnel and dried under vacuum for 72 hours to yield a dark red, waxy solid.

Figure 3.1 (a) Synthesis and processing of dox loaded PNIPAM – iron oxide composite nanoparticles; chemical formula of (b) BAAM crosslinked PNIPAM (c) doxorubicin.

Chapter 3 – Experimental Procedures

In a round bottom flask with a reflux condensation set-up, 36 g (40 mmol) of the iron oleate complex from the above procedure and 5.7 g of oleic acid (20 mmol, Aldrich, 90%) were dissolved in 200 g of the organic solvent 1-octadecene (Aldrich, 90%). The mixture was heated in argon to 320 °C at a heating rate of approximately 3.5 °C.min⁻¹. Upon reaching the reaction temperature slightly above the boiling point of 1-octadecene (b.p. 317 °C), a vigorous reaction occurred and the initial transparent red solution began to turn brownish-black. The mixture containing the MNP was aged at 320 °C for 2 h and cooled to room temperature, the nanoparticles were precipitated by adding 500 ml of ethanol and extracted from the liquid with the help of a permanent magnet. The synthesized particles were coated with oleic acid and residual organic solvent both of which are not biocompatible. To evaporate these organic residues from the surfaces of the particles, in a departure from the procedure in [194], the particles were heated to 400 °C and the temperature maintained for 1 h. The MNP were washed several times with ethanol, acetone and distilled water, dried and stored under vacuum.

3.1.2 Synthesis of PNIPAM – γ -Fe₂O₃ composite nanoparticles by dispersion free-radical polymerization

The 43 nm size MNP were found to possess the best magnetic properties and heating behaviour (Chapter 4) and were selected for coating with PNIPAM using the dispersion free-radical polymerization method [77, 166]. Polymerization was performed in nitrogen within a glove-box and chemicals were used as received. In a typical synthesis 410 mg of the 43 nm MNP, 600 mg NIPA (Aldrich, 97%), 5.1 mg BAAM (Fluka 98%) and 7.2 μ l N,N,N',N''-tetramethylethylenediamine (TEMED) (Lancaster, 99%) were mixed in 40 ml of ultrapure MilliQ water and ultrasonicated

Chapter 3 – Experimental Procedures

for 15 min. After a uniform suspension formed, 100 μl of ammonium persulfate (APS) (J.T. Baker, 98.5%) was added drop wise. Ultrasonication continued for a further 2 h after which the composite magnetic nanoparticles were separated by centrifugation. The composite magnetic particles were re-dispersed in milliQ water, ultrasonicated for 15 min and collected with a permanent magnet. The supernatant containing non-magnetic components such as free PNIPAM or unreacted polymerization reagents was discarded. This washing/magnetic separation procedure was performed several times to ensure that only magnetically responsive composite nanoparticles were used for further processing.

3.1.3 Synthesis and processing of Fe_3O_4 magnetic nanoparticles

The co-precipitation method was used to synthesize 6 nm and 13 nm Fe_3O_4 magnetic nanoparticles.

3.1.3.1 Synthesis of 6 nm Fe_3O_4 nanoparticles by the co-precipitation method

The co-precipitation method was adapted from the work by Kim *et al.* [196]. Ferric chloride hexahydrate – $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (>99%, Aldrich), ferrous chloride tetrahydrate - $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (>99%, Aldrich), sodium hydroxide – NaOH (>99%, Aldrich) and hydrochloric acid – HCl (>37%, Aldrich) were used as received. Ultra-pure water was de-oxygenated by bubbling nitrogen gas through it for 1 h prior to use. Stock solutions of 1.28 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.64 M $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.4 M HCl were prepared as a source of iron by dissolving the relevant quantities of the chemicals in deoxygenated water under vigorous stirring. Concurrently, stock solutions of 0.9 – 1.5 M NaOH were prepared as alkali sources. An aqueous dispersion of MNP was made by mixing an aqueous mixture of ferric and ferrous

Chapter 3 – Experimental Procedures

salts with NaOH at room temperature, while N₂ gas was continuously bubbled through the mixture. 25 ml of iron source was added dropwise into 250 ml of alkali source under mechanical stirring (2000 rpm) for 30 min at room temperature. A pH of 11 was maintained. At the end of the reaction, the particles were separated by centrifugation at 8000 rpm for 10 min, washed with water, acetone and ethanol numerous times to remove any undesirable chemicals from their surface. After washing several times with MilliQ water and ethanol to remove residual reactants, the MNP were ultrasonically redispersed in 500 ml of ethanol until a dark suspension was obtained. A 0.8 T block permanent magnet was placed on the outer wall of the beaker to attract the most magnetically responsive particles in the suspension. The supernatant was extracted with a peristaltic pump (Simon, Manostat). The responsive particles were redispersed in ethanol and the process was repeated thrice. The magnetically separated particles were dried in vacuum and stored in a vacuum oven at room temperature.

3.1.3.2 Synthesis of 13 nm Fe₃O₄ nanoparticles by the co-precipitation method

The co-precipitation method adapted from Aono *et al.* [195] was used to obtain particles with an average size between 10 to 14 nm. In a typical experiment, FeCl₃·6H₂O (0.1 M, 27 g) (Merck) and FeCl₂·4H₂O (0.05M, 10 g) (Merck) were dissolved in 200 ml of ultrapure MilliQ water and heated to 80 °C. The synthesis was performed inside a glove-box in an inert atmosphere, nitrogen was bubbled through all solutions for 30 min before use. Under vigorous stirring, 140 ml of NaOH solution (0.14 M, 5.6 g in 140 ml) (Merck) was dripped into this solution at a rate of 6 ml.min⁻¹ upon which the solution turned black, indicating the precipitation of Fe₃O₄ (magnetite). The mixture was maintained at 80 °C for 30

Chapter 3 – Experimental Procedures

min, cooled to room temperature and centrifuged at 8000 rpm for 15 min to extract the particles. The particles were washed, magnetically separated and stored as described in section 3.1.3.1.

3.1.4 Synthesis of PNIPAM – Fe_3O_4 composite nanoparticles by dispersion free-radical polymerization

Composite nanoparticles were prepared, in the presence of MNP, by dispersion free radical dispersion polymerization of NIPAM monomer in water with BAAM as the cross-linker, TEMED as the accelerator and APS as the initiator [77, 166]. This procedure does not involve surfactants which can alter the LCST [84, 92, 93]. Sources of chemicals are listed in Sec. 3.1.2. Nitrogen was bubbled through MilliQ water for 15 min prior to use. NIPAM was purified by recrystallization from hexane and dried in vacuum for 24 h before use. In a typical experiment 35 ml of ferrofluid containing 850 mg of MNP was added to a solution of 960 mg of NIPAM in 30 ml water and ultrasonicated for 10 min. The mixture was transferred to a beaker and stirred at 24000 rpm by an IKA-Werke T25 Ultra Turrax high speed dispersion unit while 2.5 ml of BAAM solution (8 mg in 5 ml) and 0.5 ml of APS solution (180 mg in 5 ml) were added to the mixture. Finally 1 ml of TEMED was added drop-wise while stirring was continued. After 20 min of stirring, the mixture was left undisturbed for 2 h after which the CNP were magnetically separated and washed several times with MilliQ water. Some of the CNP was dried in vacuum for 24 h resulting in dehydration of the particles (Fig 3.1a), the remainder was used as-synthesized in the swollen state for dynamic light scattering and microcalorimetry experiments.

Chapter 3 – Experimental Procedures

3.2 Drug loading of PNIPAM – iron oxide composite nanoparticles with doxorubicin

The water-soluble anti-cancer drug doxorubicin (dox) was chosen as a model drug. Typically, a loading solution of doxorubicin hydrochloride (loading target ~10 wt% of CNP) in MilliQ water was prepared by ultrasonication for 10 min. Dehydrated CNP was dispersed in this solution by ultrasonication for an additional 10 min. The bottle was sealed and gently shaken in a rotary shaker in darkness overnight to enable reswelling of the PNIPAM shell in the aqueous dox solution as well as to facilitate doxorubicin uptake. The loading parameters are listed in Table 3.1. After loading, the dox-loaded CNP were extracted by centrifugation and magnetic separation. To determine residual dox content, the supernatant was analyzed in a UV-visible spectrophotometer (Shimadzu UV1700) and compared with reference plots of doxorubicin in MilliQ water at a wavelength of 482 nm. Drug loading of dox loaded CNP was determined from the difference between initial loading and dox content of the supernatant using Eq. 3.1.

$$Wt \% \text{ Dox content of CNP} = \frac{W_{LS} - W_{SN}}{W_{CNP}} \times 100 \quad (\text{Eq. 3.1})$$

where W_{LS} is the mass of dox in loading solution (concentration measured by UV-visible spectroscopy), W_{SN} is the mass of dox in post-loading supernatant (concentration measured by UV-visible spectroscopy) and W_{CNP} = mass of CNP used for dox loading.

For Fe_3O_4 CNP, part of the batch of prepared particles was dried in vacuum for 48 h to investigate the effect of dehydration on particle performance; the remainder was used in the swollen condition for experiments.

Chapter 3 – Experimental Procedures

Table 3.1 Dox loading parameters for PNIPAM – iron oxide CNP.

CNP type	PNIPAM – γ -Fe ₂ O ₃ CNP	PNIPAM – Fe ₃ O ₄ CNP
CNP in loading solution (mg)	315	245
Dox in loading solution (mg)	36	26.5
Volume of loading solution (ml)	200	20
pH of loading solution	5.5	5
Loading temperature (°C)	22	24
Loading time (h)	16	24

3.3 Characterization and *in vitro* tests

Iron oxide and PNIPAM – iron oxide nanoparticles were characterized by transmission electron microscopy, x-ray diffraction, vibrating sample magnetometry and thermal gravimetric analysis. Selected batches of Fe₃O₄ particles were studied with modulated differential scanning calorimetry and dynamic light scattering. *In vitro* hypothermia and drug release tests were carried out; for selected batches of Fe₃O₄ particles *in vitro* cytotoxicity was also studied.

3.3.1 Transmission electron microscopy

Drops of dilute suspensions of the uncoated MNP were placed on carbon coated copper grids and dried under vacuum for transmission electron microscopy. Imaging was conducted using a JEOL 2010F TEM operating at 200 kV. ImageJ image processing software was used to measure particle size from the micrographs. The size of the CNP particles could not be reliably examined by the TEM since the electron beam caused ablation of the polymer shell.

Chapter 3 – Experimental Procedures

3.3.2 Dynamic light scattering

DLS was used to measure the hydrodynamic diameter of aggregates of Fe_3O_4 MNP, Fe_3O_4 CNP and dox loaded Fe_3O_4 CNP in MilliQ water. Dilute suspensions ($< 0.2 \text{ mg.ml}^{-1}$) were transferred to low volume quartz cuvettes and investigated using a Malvern Instruments Zetasizer Nano DLS unit. The samples were equilibrated at 25°C for 5 min before each measurement.

3.3.3 Atomic Force Microscopy

An Asylum Research MFP-3D atomic force microscope (AFM) was used to study the size of PNIPAM – Fe_3O_4 CNP. Drops of dilute suspension of CNP in water were placed on a silicon dioxide substrate and evaporated in vacuum at room temperature. The particles were scanned using tapping mode.

3.3.4 X-ray diffraction

XRD scans were performed on dried powders of bare MNP using a Shimadzu 6000 X-ray diffractometer with $\text{Cu K}\alpha$ radiation of wavelength $1.54056 \text{ \AA}$, in the 2θ range of 10° to 90° at a scan rate of 1° min^{-1} . Phase identification was performed by matching peak positions and relative intensities to reference JCPDS files. The crystallite size was calculated using the Scherrer formula.

3.3.5 Thermal gravimetric analysis

Thermal gravimetric analysis was used to measure polymer content of the composite nanoparticles. 20 mg of dehydrated CNP powder was placed in a ceramic pan, heated to 600°C at a rate of $20^\circ\text{C.min}^{-1}$ under nitrogen flow in a TA Instruments TGA Q500 thermo gravimetric analyzer, the weight loss was measured

Chapter 3 – Experimental Procedures

as a function of temperature. Weight loss of bare MNP was measured under identical conditions for reference. The decrease in dox content of drug loaded CNP after vacuum dehydration was estimated by analyzing the weight loss of dehydrated as well as swollen dox loaded particles. CNP with higher PNIPAM content (~ 3 wt %) and dox content (~ 6 wt %) were also prepared and weight loss up to 900 °C was measured, weight loss of doxorubicin hydrochloride powder was also analyzed.

3.3.6 Modulated differential scanning calorimetry

Determination of LCST for PNIPAM aqueous solutions is often performed by optical light transmission methods (e.g. UV-visible spectrophotometry), or microcalorimetry [89, 92, 93]. However, for composite magnetic nanoparticles optical methods do not yield reliable results at concentrations of interest ($> 1 \text{ mg.ml}^{-1}$), hence LCST measurements for Fe_3O_4 CNP were performed with a TA Instruments DSC 2920 modulated differential scanning calorimeter (MDSC). Swollen Fe_3O_4 CNP as well as dox loaded Fe_3O_4 CNP, and dehydrated dox loaded Fe_3O_4 CNP dispersed in PBS were studied. Dehydrated particles were dispersed in PBS and refrigerated at 8 °C for 4 h before testing to enable reswelling of the polymer shell. Reference pans contained Fe_3O_4 MNP in PBS without any polymer. A few microliters of the sample suspensions were placed in hermetically sealed aluminum pans and heated to 50 °C at a scanning rate of $0.5 \text{ }^\circ\text{C.min}^{-1}$ with modulation amplitude of $0.2 \text{ }^\circ\text{C min}^{-1}$. Pans were equilibrated in the DSC cell at 20 °C before a heating run. The MDSC thermograms were analyzed by TA Universal Analysis software, scans of dox and MNP were also conducted.

Chapter 3 – Experimental Procedures

3.3.7 Magnetic measurements

A LakeShore 7404 vibrating sample magnetometer was used to measure magnetic properties at room temperature of MNP and CNP in an applied magnetic field range of 0 to 10 kG.

3.3.8 *In vitro* hyperthermia

Ferrofluids for testing *in vitro* hyperthermia performance were prepared by ultrasonically dispersing MNP and CNP in MilliQ water or PBS. Glass bottles containing the ferrofluids and thermally insulated with ceramic wool were placed within a 6 loop water-cooled copper coil driven by an Inductelec A.C. generator (Sheffield, UK) (Fig. 3.2). The field strengths produced by the generator were previously calibrated using a custom built AC gaussmeter. Table 3.2 lists the test parameters for the γ -Fe₂O₃ and Fe₃O₄ ferrofluids. The ambient temperature was 30 °C and a Luxtron M600 fiber optic thermometry unit connected to a computer was used to measure ferrofluid temperature. The specific absorption rate, SAR, of the

particles was calculated using Eqn. 2.14 i.e.. $SAR = C \left(\frac{\Delta T}{\Delta t} \right) \frac{mass_{ferrofluid}}{mass_{nanoparticles}}$

For the current work, the SAR has been expressed in terms of W.kg⁻¹ of iron oxide MNP or PNIPAM – iron oxide CNP according to the tested material. The slope of the time – temperature curve i.e., $\Delta T/\Delta t$, was calculated by a linear fit to the initial section of the curve up to a time period of 120 s. The mass of particles and the mass of the carrier fluid was weighed in a precision balance (Mettler Toledo) prior to ultrasonic dispersal; these values were used in the calculation of the mass weighted specific heat capacity *C*. The heat capacity values used were as follows: 0.620 J.g⁻¹K⁻¹ for Fe₃O₄, 0.650 J.g⁻¹K⁻¹ for γ -Fe₂O₃ and 4.184 J.g⁻¹K⁻¹ for water.

Chapter 3 – Experimental Procedures

Figure 3.2 *In vitro* hyperthermia test setup.

Table 3.2 *In vitro* hyperthermia test parameters.

Particle type	$\gamma\text{-Fe}_2\text{O}_3$	Fe_3O_4
MNP average size (nm)	12, 14, 19, 43	6, 13
AMF frequency, f (kHz)	375	375
AMF strength, H_0 (kA.m ⁻¹)	1.7	4
Ferrofluid volume (ml)	5	5 (6 nm), 3 (13 nm)
Particle concentration in ferrofluid (mg.ml ⁻¹)	10	10 (6 nm), 5 (6 nm), 2 (13 nm)

Chapter 3 – Experimental Procedures

3.3.9 *In vitro* simultaneous hyperthermia and drug release

For studying drug release during hyperthermia, additional experiments were carried out using dehydrated dox loaded CNP dispersed in PBS. The AMF strength was manually varied to raise the temperature rapidly and maintain the temperature in the range in the hyperthermia range between 41 and 47 °C for various time intervals up to 60 min. The parameters for the tests are provided in Table 3.3. At the end of each test, particles were immediately separated from the ferrofluid by centrifugation and magnetic separation, drug release was quantified by measuring dox content in the supernatant via UV-visible spectrophotometry. The loss of dox during vacuum dehydration was factored in to calculate the effective drug loading of dehydrated particles (section 3.3.5).

Table 3.3 *In vitro* simultaneous hyperthermia and drug release test parameters.

CNP type	Dox loaded γ -Fe ₂ O ₃ – CNP	Dox loaded Fe ₃ O ₄ - CNP
Magnetic core average size (nm)	43	13
AMF frequency, f (kHz)	375	375
AMF strength, H_0 (kA.m ⁻¹)	Variable, 0.1 – 4	Variable, 0.1 – 5
Ferrofluid volume (ml)	5	5
Particle concentration in ferrofluid (mg.ml ⁻¹)	2.5	1
Hyperthermia exposure time (min)	40	10, 30, 60

Chapter 3 – Experimental Procedures

3.3.10 *In vitro* drug release without an alternating magnetic field

Drug release for extended time periods without a magnetic field was studied at temperatures of 24 °C (below the LCST), 37 °C (body temperature, near the LCST) and 42 °C (above the LCST). Dox loaded CNP were weighed on a microbalance, ferrofluids were prepared by ultrasonically dispersing the particles in PBS (pH 7.4) in glass test tubes. A minimum of three samples were prepared for testing at each temperature. Sealed test tubes were placed in preheated water baths (Fisher Isotemp 202) and the temperature stabilized (± 1 °C) at 24 °C, 37 °C and 42 °C for the test duration. Periodically, 1 ml of the release medium, free of nanoparticles, was extracted and replaced with fresh PBS. Dox content of the extracted solution was measured by UV-visible spectrophotometry and cumulative dox release calculated. The loss of dox during vacuum dehydration was factored in to calculate the effective drug loading of dehydrated particles (section 3.3.5). Table 3.4 summarizes the details of the test.

3.3.11 Controlled drug release

The ability of swollen dox loaded Fe_3O_4 CNP to modulate drug release by varying the temperature was studied. The experimental details are described in section 3.3.10; the temperature was cycled between 24 °C, 37 °C and 43 °C for various time periods and dox release measured via UV-visible spectrophotometry.

Chapter 3 – Experimental Procedures

Table 3.4 Test parameters for *in vitro* drug release in the absence of a magnetic field

CNP type	Dox loaded γ -Fe ₂ O ₃ – CNP	Dox loaded Fe ₃ O ₄ – CNP
MNP average size (nm)	43	13
Release medium	PBS (pH 7.4)	PBS (pH 7.4)
Ferrofluid volume (ml)	5	5
Particle concentration in ferrofluid (mg.ml ⁻¹)	2	1
Test temperature (°C)	24, 37, 42	24, 37, 43
Test duration (h)	101	29

3.3.12 *In vitro* cell cytotoxicity

Zeta potential of the Fe₃O₄ MNP, Fe₃O₄ CNP and swollen dox loaded Fe₃O₄ CNP in PBS (pH 7.4) at 37 °C was measured using the Malvern Instruments Zetasizer Nano DLS unit in a procedure similar to that described in section 3.3.2 for size measurement. The ferrofluids were contained in zeta potential measurement cells (with embedded electrodes) instead of quartz cuvettes and equilibrated at 37 °C for 5 min before measurement.

The LCST of the dox loaded PNIPAM – Fe₃O₄ CNP in the cell culture medium Dulbecco's Modified Eagle's Medium (DMEM) was tested by MDSC using the procedure described in Sec. 3.3.5. Reference pans contained bare Fe₃O₄ MNP in DMEM.

Chapter 3 – Experimental Procedures

Cytotoxicity of Fe_3O_4 MNP, Fe_3O_4 CNP and swollen dox loaded Fe_3O_4 CNP was assessed for the human bronchial epithelial cell line BEAS-2B for particle concentrations of 0.05 mg.ml^{-1} and 0.1 mg.ml^{-1} and exposure times of 24 h and 48 h. Cells were cultured in T-75 flasks (Corning) with DMEM (PAA, Austria) containing 10% fetal bovine serum (FBS) (PAA), 1 % antibiotics (PAA) and 1% L-Glutamine at 37°C in a humidified 5 % CO_2 atmosphere incubator (Sanyo). Subculturing was performed when the cells attained 70% confluency. Cells were seeded into 24-well plates (Corning) at a density of 5×10^4 cells per well and incubated at 37°C for 24 h prior to testing. A $50 \mu\text{l}$ suspension of the nanoparticles of relevant concentration dispersed in PBS (pH 7.4) and $450 \mu\text{l}$ of growth medium were added to each well. Wells containing cells without nanoparticles, and wells containing only $500 \mu\text{l}$ of medium (blanks) were used as controls. At the end of the incubation period, the growth medium was removed from the wells and cells were washed with PBS thrice.

For testing cell viability, $25 \mu\text{l}$ of Cell Counting Kit-8 (Dojindo), which is a tetrazolium salt based colorimetric cell viability assay, and $225 \mu\text{l}$ of growth medium was added to each well. The plates were incubated for 2 h at 37°C ; the colorimetric assay from the 24 well plate was transferred into a 96 well plate and the absorbance at 450 nm for each well was read with a microplate reader (Tecan Infinite 200). Absorbance of the blanks was subtracted, and the cell viability was calculated as the ratio of the absorbance of test wells to the absorbance of the cell control wells.

Chapter 3 – Experimental Procedures

The results of the experiments described in this chapter as well as the modeling performed in the current work are presented and discussed in the next chapter.

Chapter 4 – Results and Discussion

CHAPTER 4

RESULTS AND DISCUSSION

The results of the synthesis, characterization, *in vitro* and *in vivo* tests, and modeling of drug loaded polymer coated iron oxide composite nanoparticles are described in this chapter. γ -Fe₂O₃ and Fe₃O₄ MNP were synthesized, encapsulated with the thermoresponsive polymer PNIPAM and loaded with the anti-cancer drug doxorubicin. These particles were characterized and *in vitro* as well as *in vivo* performance was evaluated. Modeling of heat generation from the MNP and heating of tumor with the MNP in an AMF was also performed.

4.1 Physical properties and magnetic properties

This subsection discusses the physical properties, PNIPAM coating and drug loading of γ -Fe₂O₃ and Fe₃O₄ particles.

4.1.1 Physical properties of γ -Fe₂O₃ nanoparticles

γ -Fe₂O₃ MNP of average size 12 nm (SD 3), 14 nm (SD 3.5), 19 nm (SD 4.9) and 43 nm (SD 5) as measured from transmission electron micrographs (Fig. 4.1) were synthesized by the modified high temperature decomposition method. The particles are observed to be irregular in shape.

The powders were determined to be γ -Fe₂O₃ (maghemite) from X-ray diffraction patterns (Fig. 4.2). Peak breadth decreases with increasing particle size, sharp and clearly defined peaks indicate high degree of crystallinity. The nanoparticles were brown, consistent with interpretation of the X-ray data that these nanoparticles are

Chapter 4 – Results and Discussion

primarily $\gamma\text{-Fe}_2\text{O}_3$ and not Fe_3O_4 which is black. The nanoparticles also contained minor quantities of the $\alpha\text{-Fe}_2\text{O}_3$ phase. The average crystallite size as calculated by the Scherrer formula was found to be 9.3 nm, 13.6 nm, 19.5 nm and 22 nm for the MNP with average size (TEM) 12 nm, 14 nm, 19 nm and 43 nm respectively.

Figure 4.1 Bright field transmission electron micrographs of uncoated $\gamma\text{-Fe}_2\text{O}_3$ MNP of average size (a) 12 nm (b) 14 nm (c) 19 nm (d) 43 nm. Aggregation of the particles is due to inter-particle magnetic forces and the absence of surfactant.

Chapter 4 – Results and Discussion

Figure 4.2 X-ray diffraction patterns of uncoated γ - Fe_2O_3 MNP. The particles are highly crystalline and primarily γ - Fe_2O_3 .

4.1.2 Polymer content and drug loading of γ - Fe_2O_3 nanoparticles

Analysis of the TGA data (Fig. 4.3) showed that weight loss between 200 °C and 440 °C corresponding to the PNIPAM content [208] of the γ - Fe_2O_3 CNP is ~ 6 wt %. From the UV-visible spectrophotometry data at 482 nm (not shown) of the post-loading dox solution the dox content of the dox loaded γ - Fe_2O_3 CNP was calculated as 2.5 wt %. After vacuum dehydration, the effective drug loading was 2.1 wt%.

Chapter 4 – Results and Discussion

Figure 4.3 TGA weight loss plot for $\gamma\text{-Fe}_2\text{O}_3$ CNP. The PNIPAM content of the CNP is 6 wt%.

4.1.4 Physical properties of Fe_3O_4 nanoparticles

Fig. 4.4 shows a typical transmission electron micrograph of bare Fe_3O_4 MNP. The average size of bare nanoparticles synthesized by co-precipitation was measured from transmission electron micrographs to be 6 nm (standard deviation = 1) and 12.9 nm (~13 nm) (standard deviation = 3) respectively. The XRD pattern (Fig. 4.5) indicates that particles are crystalline, peak matching with reference JCPDS files shows that the particles are primarily Fe_3O_4 (magnetite).

Chapter 4 – Results and Discussion

Figure 4.4 Bright field transmission electron micrographs of uncoated Fe_3O_4 MNP of average size (a) 6 nm (b) 13 nm. Aggregation of the particles is due to inter-particle magnetic forces and the absence of surfactant.

Figure 4.5 X-ray diffraction patterns of bare Fe_3O_4 MNP of average size 6 nm and 13 nm.

The average hydrodynamic diameter (D_H) and polydispersity index (PDI) of the particles in water at 25 °C was calculated from an exponential fit to the intensity

Chapter 4 – Results and Discussion

correlation function of the light scattering data obtained from DLS experiments; the data is presented in Fig. 4.6 and Table 4.1. Bare Fe_3O_4 MNP had an average D_H of 203 nm (PDI of 0.301), swollen Fe_3O_4 CNP showed D_H of 158 nm (PDI 0.168) which increased to 180 nm (PDI 0.229) for dehydrated CNP. The D_H for dox loaded Fe_3O_4 CNP in the swollen and dehydrated states was 252 (PDI 313) and 466 nm (PDI 0.343) respectively.

Figure 4.6 Dynamic light scattering intensity plots for dilute colloidal solutions of (a) Fe_3O_4 MNP (b) dehydrated Fe_3O_4 CNP (c) swollen Fe_3O_4 CNP (d) dehydrated dox loaded Fe_3O_4 CNP and (e) swollen dox loaded Fe_3O_4 CNP in water.

Chapter 4 – Results and Discussion

Table 4.1 Hydrodynamic diameter of Fe₃O₄ MNP and PNIPAM – Fe₃O₄ CNP in water.

Sample	Hydrodynamic Diameter D_H (nm)	Polydispersity Index
Fe ₃ O ₄ MNP	203	0.301
PNIPAM – Fe ₃ O ₄ CNP (dehydrated)	180	0.229
PNIPAM – Fe ₃ O ₄ CNP (swollen)	158	0.168
Dox loaded PNIPAM – Fe ₃ O ₄ CNP (dehydrated)	466	0.343
Dox loaded PNIPAM – Fe ₃ O ₄ CNP (swollen)	252	0.313

A typical AFM surface scan of dehydrated CNP clusters is shown in Fig. 4.11. The size of dehydrated PNIPAM – Fe₃O₄ CNP was measured to be in the range of 35 – 60 nm.

Figure 4.7 Atomic force microscopy surface scan image of dehydrated Fe₃O₄ CNP clusters.

Chapter 4 – Results and Discussion

4.1.5 Polymer content and drug loading of Fe_3O_4 composite nanoparticles

The TGA weight loss vs. temperature plot for both Fe_3O_4 MNP and Fe_3O_4 CNP is shown in Fig 4.8a. Heating in the range of room temperature to 125 °C results in expulsion of water from the polymer, PNIPAM decomposition occurs between 200 °C and 440 °C [208]. From the TGA data, PNIPAM content of CNP is estimated to be 1.3 wt %. The MNP sample shows less than 0.5 % weight loss confirming that weight change for the CNP sample beyond 200 °C is due to PNIPAM degradation. The TGA plot of pure doxorubicin hydrochloride indicates that decomposition starts at 185 °C and weight loss continues up to 750 °C (Fig. 4.8b). For CNP with 6 wt % dox loading, vacuum dehydration results in a decrease in dox content of ~ 0.9 wt %, compared to the swollen condition (Fig. 4.8c).

UV-visible spectrophotometry results (not shown) revealed that drug loading of dox loaded Fe_3O_4 CNP used in the current work was 4.16 wt. %. The effective drug loading for dehydrated Fe_3O_4 CNP was 3.54 wt %.

Chapter 4 – Results and Discussion

Figure 4.8 TGA weight loss plots for (a) bare MNP and Fe₃O₄ CNP, the PNIPAM content of the CNP is 1.3 wt % (b) doxorubicin hydrochloride powder (c) dox loaded CNP of ~ 3 wt% PNIPAM and 6 wt % dox in the swollen and dehydrated conditions; plots have been normalized for water content, difference in dox content between dehydrated and swollen particles is ~ 0.9 wt%.

Chapter 4 – Results and Discussion

Fig. 4.9 shows an example of a dispersion of Fe_3O_4 CNP in water and the particles in suspension attracted by a magnet.

Figure 4.9 Swollen Fe_3O_4 CNP (a) dispersed in water (b) attracted from the suspension with a magnet.

4.1.6 Lower critical solution temperature of Fe_3O_4 composite nanoparticles

Modulated DSC thermograms for the swollen Fe_3O_4 CNP, swollen dox loaded Fe_3O_4 CNP and dehydrated dox loaded Fe_3O_4 CNP are shown in Fig. 4.10. The LCST was measured as the onset temperature, since unlike the peak maximum temperature, onset temperature is relatively insensitive to sample weight related artifacts [89]. The swollen particles exhibit two distinct endothermic peaks (Figs. 4.10a-b) at the LCST. For the swollen CNP (Fig. 4.10a) the onset of the first endothermic event is at 35.3 °C, and the endothermic peak is at 35.8 °C. The second transition has an onset temperature of 39.4 °C and endothermic peak at 39.9 °C. For the swollen dox loaded CNP the onset temperatures of the events are 35 °C and 42 °C, with the peaks at 35.5 °C and 43.5 °C respectively. The thermogram of

Chapter 4 – Results and Discussion

the dehydrated particles (Fig. 4.10c) lacks the distinct peaks exhibited by those of swollen particles, indicating the absence of a well-defined transition temperature, thermograms of dox, MNP and a mixture of both also do not show distinct peaks (Fig. 4.10d).

Figure 4.10 MDSC thermograms of (a) swollen Fe₃O₄ CNP (b) swollen dox loaded Fe₃O₄ CNP (c) dehydrated dox loaded Fe₃O₄ CNP (d) dox and MNP in PBS.

Chapter 4 – Results and Discussion

Iron oxide MNP of average size 12 nm, 14 nm, 19 nm and 43 nm (Fig 4.1) synthesized by the high temperature decomposition were highly crystalline, composed of primarily $\gamma\text{-Fe}_2\text{O}_3$ with minor quantities of $\alpha\text{-Fe}_2\text{O}_3$ (Fig. 4.2). The MNP synthesized by this method are expected to be Fe_3O_4 for particle size larger than ~ 5 nm and $\gamma\text{-Fe}_2\text{O}_3$ for smaller particles [194]. After synthesis, as per the published protocol, excess of a polar solvent (ethanol) was added to the colloidal solution to precipitate and separate the particles from the octadecene synthesis medium, these precipitated particles were coated with oleic acid as well as octadecene, both of which are unsuitable for biomedical applications. Attempts to remove the organic residues by repeated washing/ultrasonication with several polar and non-polar solvents (deionized water, ethanol, hexane, acetone, etc) as well as vacuum drying at room temperature were unsuccessful. Hence the particles were exposed to $400\text{ }^\circ\text{C}$ for 1 h in air, above the boiling point of the organic residues, to remove them by evaporation. This heat treatment resulted in the transformation of Fe_3O_4 to $\gamma\text{-Fe}_2\text{O}_3$ with trace amounts of $\alpha\text{-Fe}_2\text{O}_3$ (hematite). The $\gamma\text{-Fe}_2\text{O}_3$ phase is more stable than Fe_3O_4 , due to the high surface area to volume ratio for nanoparticles there is a strong tendency for oxidation of the Fe_3O_4 MNP surface layer to $\gamma\text{-Fe}_2\text{O}_3$ even at room temperature [97, 98]. Heat treatment in air accelerates this process, in the temperature range of $150 - 500\text{ }^\circ\text{C}$ Fe_3O_4 is converted to $\gamma\text{-Fe}_2\text{O}_3$ [98, 209, 210]. At temperatures above $500\text{ }^\circ\text{C}$ the $\gamma\text{-Fe}_2\text{O}_3$ phase can be completely transformed to $\alpha\text{-Fe}_2\text{O}_3$ with an attendant change of the crystal structure, this conversion can begin at temperatures as low as $270\text{ }^\circ\text{C}$ [209]. The transformation behavior described above explains the phase transformation

Chapter 4 – Results and Discussion

from Fe_3O_4 for as-synthesized particles to $\gamma\text{-Fe}_2\text{O}_3$ with minor amounts of $\alpha\text{-Fe}_2\text{O}_3$ after heat treatment at 400 °C in air. The size distribution also arises from the heat treatment stage during which non-uniform particle growth may occur as the organic residues evaporate at high temperature, leading to a dynamic variation in the ratio of residual surfactant and solvent with respect to the nanoparticles.

Irregular-shaped (Fig. 4.4), crystalline (Fig. 4.5) Fe_3O_4 MNP of average size 6 nm and 13 nm were produced by the coprecipitation method, this synthesis technique was simpler than the high temperature decomposition method. Large quantities of nanoparticles (~few grams) were produced in a single step and minimal additional processing was required post-synthesis; heat treatment was not needed since there were no residual reagents after several water/ethanol washes. A wide range of particle sizes ranging from ~ 2 nm to 30 nm can be synthesized by the coprecipitation method [195, 196, 198]. In theory, the high temperature method has the advantage of relatively narrower size distribution for as-synthesized particles compared to coprecipitation. In practice, magnetic separation of the Fe_3O_4 MNP enabled the extraction of the most magnetically responsive particles as well as narrowing of the size distribution (Std. Dev. 1 - 3), this processing step was not used for the $\gamma\text{-Fe}_2\text{O}_3$ MNP due to the relatively lower yields. The additional heat treatment stage for the $\gamma\text{-Fe}_2\text{O}_3$ MNP led to uncontrolled particle growth and variation in particle size (Std. Dev. 3 – 5) and negated the advantage of initial monodispersity of the as-synthesized MNP. Hence, for this work, the coprecipitation method was found to be better for producing the MNP intended for biomedical applications.

Chapter 4 – Results and Discussion

The particle sizes used in this work were selected on the basis of *in vivo* requirements as well as materials properties necessary for heat generation in magnetic hyperthermia. The smallest capillaries in the body have a diameter of about 4 μm , larger particles or particle aggregates will be captured and withheld in the lungs. Particles that are smaller than 10 nm are well suited for tumor penetration [19], but are rapidly cleared by renal filtration and extravasation. Particles in the size range of 50 – 150 nm are typically cleared by the Kupffer cells of the liver and those that are larger than 200 nm are likely to be filtered by the venous sinuses of the spleen [16, 19, 23]. For *in vivo* applications particles in the size range of 10 – 100 nm may be suitable for relatively long circulation times and retention in the liver. However, the requirements for MDT and MMH are not identical, this leads to constraints in specifying an optimal size for multi-functional particles. The saturation magnetization of the MNP increases with size [96], larger particles (approx. > 100 nm) have higher magnetization and are therefore easier to guide to the tumor by an external magnetic field. However, this larger size decreases circulation time since larger particles are likely to be opsonized sooner [23]. For MMH, the ideal magnetic particle core size for heat generation is in the 10 – 25 nm range [16, 19, 20], which is lower than the ideal size for MDT, but larger than the ideal size for tumor penetration (approx. < 10 nm). Iron oxide MNP smaller than 25 nm can be superparamagnetic and if coated with a suitable hydrophilic coating, the tendency to increase effective size by aggregation is reduced [16], this also improves circulation times by delaying opsonization. *Hence, particles in the 10 – 45 nm size range, with hydrophilic coating provide a good balance of circulation times, ease of manipulation by an external magnetic field and heat generation for hyperthermia [19].*

Chapter 4 – Results and Discussion

CNP comprising of a core of MNP and shell of PNIPAM were successfully synthesized using 43 nm γ -Fe₂O₃ and 13 nm Fe₃O₄ MNP by dispersion free-radical polymerization. Macková *et al.* have systematically studied the effect of reaction parameters on the synthesis of PNIPAM – iron oxide microspheres by dispersion polymerization [166]. The synthesis route for the CNP used in the present work was adapted from their work. The iron oxide to PNIPAM ratios, stirring speed, etc were modified according to the requirements for the current work to yield nano sized CNP instead of micron sized particles. Due to differences in MNP size, dispersion technique (ultrasonication for γ -Fe₂O₃ v/s high speed stirring for Fe₃O₄), and concentrations of reactants, the PNIPAM content was different (6 wt% and 1.3 wt %) for γ -Fe₂O₃ and Fe₃O₄ CNP. The underlying principle remains the same [77, 166, 200, 211]: the reactants are dissolved in water, and APS dissociates to produce S₂O₈²⁻ ions which initiate polymerization of the NIPAM monomer around iron oxide ‘seed’ nanoparticles. Growing oligomeric chains adsorbed on the iron oxide surface undergo gelation by crosslinking due to the presence of BAAM, the degree of crosslinking depends on the ratio of BAAM to the monomer. Since polymerization occurs at a temperature below the LCST and the non-crosslinked polymeric chains are water soluble, they do not form part of the PNIPAM shell around the iron oxide MNP core. TEMED accelerates the process and gelation occurs in a few minutes at room temperature [211]. Uniform dispersion of the iron oxide MNP in the reaction mixture, especially with a ultra-high speed dispersion tool as in the case of the Fe₃O₄ CNP, yields CNP with a narrow size distribution (PDI 0.168 by DLS). The size of the PNIPAM shell can be tuned by changing the

Chapter 4 – Results and Discussion

cross-linking density, the reaction temperature, and the concentration of monomer and initiator [200].

The PNIPAM shell is beneficial in several ways. In addition to providing drug loading ability as well as a hydrophilic (below the LCST) surface for the MNP to postpone RES uptake, the shell stabilizes the particles in aqueous media thereby decreasing the tendency for aggregation of the particles [16]. This is reflected in the smaller hydrodynamic diameters and lower polydispersity index (Fig. 4.6 and Table. 4.1) of swollen Fe_3O_4 CNP ($D_H = 158$ nm, PDI = 0.168) compared to bare Fe_3O_4 MNP ($D_H = 203$ nm, PDI = 0.301). Upon loading with dox, the D_H increases to 252 nm (PDI = 0.313) due to the presence of dox molecules in the PNIPAM shell and surface that alters the interaction with water. Dehydration of the shell increases D_H as well as PDI of Fe_3O_4 CNP ($D_H = 180$ nm, PDI = 0.229) and dox loaded Fe_3O_4 CNP ($D_H = 466$ nm, PDI = 0.343). This can be attributed to the reduced shielding from inter-particle magnetic attraction and increased clustering as a result of the thinner dehydrated PNIPAM shell. Generally, the measured hydrodynamic diameter in a fluid is larger than the actual particle size as seen by the ~35 – 60 nm size dehydrated Fe_3O_4 CNP (measured by AFM) exhibiting a D_H of 180 nm in water.

Despite the advantages of the polymeric shell, magnetization of composite particles can be inferior to those of bare nanoparticles due to the non-magnetic organic component; this can degrade the responsiveness of particles to a magnetic field gradient, increasing the challenges for *in vivo* targeting. Hence the polymer content must be kept to the minimum required for drug loading. *In the current work, dox*

Chapter 4 – Results and Discussion

loading of 2.5 wt % and 4.1 wt % of the CNP weight was achieved for $\gamma\text{-Fe}_2\text{O}_3$ CNP with 6 wt % PNIPAM content and Fe_3O_4 CNP with 1.3 wt % PNIPAM content respectively. These are therapeutically relevant quantities of drugs since in MDT 20 % of a conventional intravenously injected dosage is sufficient for the equivalent therapeutic effect [24]. When drug loading is expressed in terms of only the PNIPAM weight, the dox content is equivalent to 320 wt % of PNIPAM for the Fe_3O_4 CNP and 41.6 wt % of PNIPAM for the $\gamma\text{-Fe}_2\text{O}_3$ CNP. These high values are due to the large surface area to volume ratio of the polymer shell in the nano size range, which enhances adsorption of dox. Drug loading for $\gamma\text{-Fe}_2\text{O}_3$ CNP was lower than Fe_3O_4 CNP due to lower concentration of dox in the drug loading solution (0.18 mg.ml^{-1} v/s 1.3 mg.ml^{-1}) as well as shorter loading time (16 h v/s 24 h). Dox solubility in water is dependent on pH, and decreases with increasing pH, therefore changing pH of the loading solution to higher values ($\sim \text{pH } 9$) can also increase dox loading of CNP by promoting diffusion of dox into PNIPAM [164].

As described previously (sec. 2.9), the LCST of PNIPAM is sensitive to a number of factors such as solvent type, salt concentration, incorporation of hydrophobic or hydrophilic agents, cross-linking density etc [84, 92, 93, 200] all of which can affect interactions between water molecules and the polymer network. It has been reported that the LCST increases with an increase in degree of cross-linking [200]. The ‘dual’ LCST of the swollen Fe_3O_4 CNP (Fig. 4.10a) may be attributed to local variation in cross-linking density in the PNIPAM shell during the polymerization process, leading to transitions at temperatures of 35.3°C and 39.4°C . Dox is a hydrophilic drug, loading of dox in PNIPAM can occur by hydrogen bonding [164] to the amide group of the PNIPAM chain when the pH of the dox loading

Chapter 4 – Results and Discussion

solution is low. This can decrease the driving force for hydrophobic interactions between the amide groups, thereby increasing the transition temperature. It is likely that a greater fraction of dox molecules are bound to polymer chains in areas of higher cross-link density, leading to a shift in the second endothermic peak from 39.4 °C to 42 °C after dox loading, while areas with lower cross-link density (35 °C endothermic peak) have a lower dox concentration (Fig. 4.10b).

Dehydrating the dox loaded CNP in vacuum at room temperature (below the LCST) leads to suppression of thermoresponsive behavior (Fig. 4.10c). Removal of water molecules that were bound to PNIPAM chains leads to changes in interaction between hydrophobic groups and bound dox molecules. When dehydrated particles are dispersed in aqueous solvent for re-swelling, dox molecules may interfere with the establishment of the hydration shell around the hydrophobic groups, thus affecting the degree of re-swelling as well as thermoresponsive behavior. In addition, dehydrating dox loaded CNP results in a decrease in dox content (Fig. 4.8c) and may cause convection driven partitioning of the drug inside the PNIPAM shell leading to highly inhomogenous drug distribution [212], further affecting rehydration of PNIPAM chains.

Thermoresponsive polymer coated magnetic carriers for biomedical applications have been previously reported [74, 78, 165, 173, 175, 176, 179, 183, 184, 213]. Aoyagi *et al.* used 3-aminopropyltrimethoxysilane (APTMS) coated 130 nm Fe₃O₄ MNP as the core for the CNP [74]. A co-polymer of n-isopropylacrylamide and 2-carboxyisopropylacrylamide was prepared separately and bound to the MNP surface by ultrasonication with 1-ethy-3-(3-dimethylaminopropyl)carbodiimide

Chapter 4 – Results and Discussion

hydrochloride as the conjugation agent. The LCST of the co-polymer (without MNP) was measured to be 34 °C in water and 28 °C in 100 mM NaCl solution, the hydrodynamic diameter in water at 20 °C was ~220 nm. The polymer content, LCST, magnetic properties, cytotoxicity and drug loading/release studies of the composite particles were not reported.

Uddin *et al.* have reported the synthesis and characterization of PNIPAM coated 9.3 nm Fe₃O₄ particles [175]. The Fe₃O₄ MNP surface was functionalized with thiodiglycolic acid and 4-vinylaniline; these double surfactant coated MNP were coated with cross-linked PNIPAM via precipitation polymerization. The size of the composite particles were measured by a conventional TEM to be ~12 nm in diameter, however this measurement is unlikely to be accurate since the high energy electron beam causes ablation of the polymer shell. The particles were loaded with bovine serum albumin, adsorption efficiency was found to depend on temperature and pH, and a maximum of 28 wt % loading was achieved at 40 °C, pH 4.66. The polymer content, LCST and cytotoxicity of the composite particles were not reported.

Wuang *et al.* have reported heparinized PNIPAM coated composite magnetic nanoparticles for applications requiring prevention of thrombosis [179]. Fe₃O₄ MNP of average size 6 – 8 nm were surface functionalized with the initiator 2-(4-chlorosulfonylphenyl) ethyl trichlorosilane, PNIPAM was coated onto the particles via atom transfer radical polymerization. The hydrodynamic diameter in water for the PNIPAM coated MNP was >300 nm and this decreased to ~23 nm after

Chapter 4 – Results and Discussion

immobilization of heparin on the PNIPAM surface due to the higher hydrophilicity of the heparin. The polymer content and LCST of the particles were not reported.

Müller-Schulte *et al.* have reported the synthesis and characterization of thermoresponsive polymer coated magnetic particles for hyperthermia triggered drug release [78]. Iron oxide MNP of average size 10 - 20 nm were coated with PNIPAM via a water-in-oil suspension polymerization method involving the use of surfactants. A high speed dispersion/stirring tool (Ika-Werke T25) identical to the one used in this work was used to disperse water droplets in the oil phase. Stirring speeds of 800 – 2000 rpm resulted in composite beads in the size range of 10 – 200 μm , while speeds greater than 5000 rpm were necessary to reduce the size to below 10 μm . Rhodamine B and methylene blue were incorporated into the beads during the polymerization stage, and drug loading was 2 wt % of the composite beads. The magnetic properties, LCST and actual PNIPAM content of the composite beads were not reported.

Dual stimuli responsive magnetic carriers developed by Misra *et al.* had a Fe_3O_4 core of ~ 5 nm average diameter conjugated with doxorubicin by acid labile hydrazone bonds and encapsulated by a stimuli responsive polymer consisting of either chitosan-g-poly(n-isopropylacrylamide-co-n,n-dimethylacrylamide) [176] or dextran-g-poly(n-isopropylacrylamide-co-n,n-dimethylacrylamide) [173]. For both systems, the LCST of the polymer alone was ~ 38 °C in PBS, LCST and hydrodynamic diameter of the composite particles was not reported. TGA indicated that the chitosan based composite particles and dextran based particles

Chapter 4 – Results and Discussion

contained 30 wt % and 40 wt % of “organic substance” (i.e., dox and polymer) respectively. The weight fraction of the dox in the composite particles was 9 wt% for the dextran based particles and 10 wt% for the chitosan based particles. Magnetic properties and cytotoxicity tests were not reported.

From the above examples, it is seen that several important properties of thermoresponsive CNP such as the LCST for the actual CNP, the polymer content, hydrodynamic diameter, magnetic properties, drug loading and drug release, cytotoxicity etc are often not studied or reported. In the current work, these properties have been systematically investigated and reported for PNIPAM – iron oxide CNP. In addition, synthesis and processing methods reported earlier are often complex, involving several steps (For e.g., [165, 173, 176, 213]), this increases the difficulty for production of the necessary quantities of composite particles needed for extensive testing and characterization. Additionally, for drug loading, some of the procedures [78, 173, 176] involve incorporation of drugs during the synthesis or processing stages, this may adversely affect the chemical and pharmacological properties of the drug and limit the ability to process/purify the composite particles [201]. Since *in vitro* cytotoxicity of these complex particles has not been reported, their suitability for biomedical applications is unknown. In contrast, in the current work, relatively simple synthesis and processing methods have been used to enable production of sufficient quantity of CNP with a single in-situ polymerization step of NIPAM monomers in a ferrofluid. Drug loading of therapeutically relevant quantities of dox is performed after washing of the CNP, and the chemical/pharmacological properties of the dox are preserved due to the facile physical loading process at room temperature instead of a chemical loading

Chapter 4 – Results and Discussion

process. This permits the characterization, testing and analysis of the behavior of an already complex dox – PNIPAM – iron oxide system without introducing additional complexity.

Rubio-Retama *et al.* have reported particles with a different morphology: acrylic acid (AA) functionalized surfaces of PNIPAM spheres decorated with MNP [165]. The AA – PNIPAM spheres were separately synthesized by surfactant free radical polymerization, and hybrid particles were prepared via co-precipitation synthesis of MNP (~6.4 nm diameter) in an aqueous suspension of the polymer spheres. By modifying the feed ratios, hybrid particles containing 82 wt% polymer and 62 wt% polymer were obtained, these had a hydrodynamic diameter of ~550 nm and ~440 nm respectively. LCST was determined by DLS measurements of the hydrodynamic diameter; it was observed that addition of acrylic acid to the PNIPAM resulted in a shift of the LCST from 33 °C to 36 °C. Incorporation of 18 wt % MNP resulted in a further increase in the LCST to 40 °C, however no LCST was observed for the particles with 38 wt% MNP. Drug loading and release were not reported.

Similar work by Wong *et al.* reported layer by layer assembly of a shell of poly(sodium 4-styrenesulfonate) (PSS) coated γ -Fe₂O₃ MNP on a core of PNIPAM microgel which itself was surface functionalized with a positively charged poly electrolyte poly(diallyldimethylammonium chloride) (PDADMAC) [213]. The PSS content of the PSS-MNP was estimated to be 7-8 wt% but the weight fraction for the actual final hybrid particle was not reported. The LCST and hydrodynamic diameter of the hybrid particles with the PNIPAM-PDADMAC core and PSS-MNP

Chapter 4 – Results and Discussion

shell as measured by DLS were reported to be 32 °C and ~ 400 nm respectively. It was stated that the volume transition at the LCST was reversible and did not result in the detachment of the MNP from the microgel core. Drug loading and release were not reported.

The morphology reported in the above two examples is said to facilitate the tuning of the properties of individual layers and thus tune the properties of the final particles [213]. However, there are also disadvantages compared to the ‘conventional’ magnetic core – polymer shell morphology for drug delivery and hyperthermia applications for the following reasons: (i) the presence of MNP on the surface of the PNIPAM microgel core is likely to interfere with drug release when the drug loaded microgel collapses above the LCST (ii) hybrid particles will have a greater tendency to aggregate due to inter-particle magnetic attraction arising from the MNP on the shells (unless the MNP are superparamagnetic) (iii) the MNP may detach from the microgel core during magnetic hyperthermia as the MNP attempt to physically align with the field, and (iv) the MNP may detach from the microgel core during thermally induced shrinkage above the LCST.

4.2 Magnetic properties

This subsection describes the magnetic properties of γ -Fe₂O₃ and Fe₃O₄ particles.

4.2.1 Magnetic properties of γ -Fe₂O₃ nanoparticles

Room temperature magnetization curves of γ -Fe₂O₃ particles are shown in Fig. 4.11. The saturation magnetization (M_S) and coercivity (H_C) increases with particle size. The M_S values were 10 emu.g⁻¹, 25 emu.g⁻¹, 45 emu.g⁻¹ and 58 emu.g⁻¹ for the

Chapter 4 – Results and Discussion

12 nm, 14 nm, 19 nm and 43 nm MNP respectively, the corresponding H_C values were 29 G, 11 G, 41 G and 70 G, respectively. Coating with PNIPAM did not significantly reduce the magnetic properties of the 43 nm CNP, the M_S of the CNP decreased by 7 emu.g^{-1} to 51 emu.g^{-1} , the H_C increased from 70 G to 108 G. The physical and magnetic properties of $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles used in this work are summarized in Table 4.2.

Figure 4.11 Room temperature magnetization curves for 12 nm, 14 nm, 19 nm and 43 nm $\gamma\text{-Fe}_2\text{O}_3$ MNP and PNIPAM coated 43 nm CNP.

Chapter 4 – Results and Discussion

Table 4.2 Physical and magnetic properties of γ -Fe₂O₃ nanoparticles.

Composition	γ -Fe ₂ O ₃ (trace: α - Fe ₂ O ₃)	γ -Fe ₂ O ₃ (trace: α - Fe ₂ O ₃)	γ -Fe ₂ O ₃ (trace: α - Fe ₂ O ₃)	γ -Fe ₂ O ₃ (trace: α - Fe ₂ O ₃)	γ -Fe ₂ O ₃ (trace: α - Fe ₂ O ₃) + 6 wt% PNIPAM
Average size (nm) (TEM)	12 (Std. Dev.=3)	14 (Std. Dev.=3.5)	19 (Std. Dev.=4.9)	43 (Std. Dev.=5)	43 nm MNP + PNIPAM
Average size (XRD)	9.3	13.6	19.5	22	-
M_s (emu.g ⁻¹)	10	25	45	58	53
H_c (G)	29	11	41	70	108

4.2.2 Magnetic properties of Fe₃O₄ nanoparticles

Magnetization curves of Fe₃O₄ MNP and dox loaded Fe₃O₄ CNP at room temperature are shown in Fig 4.12. The bare 13 nm Fe₃O₄ MNP exhibits a saturation magnetization (M_s) of 61 emu.g⁻¹, this decreases to 56.8 emu.g⁻¹ for the dox loaded CNP. Coercivity H_c of the dox loaded CNP is 8.5 G, lower than H_c of 43 G exhibited by bare MNP. In comparison, 6 nm MNP has a lower M_s of 43.4 emu.g⁻¹ as well as a lower H_c of 3.4 G. The physical and magnetic properties of Fe₃O₄ MNP and Fe₃O₄ CNP used in this work are summarized in Table 4.3.

Chapter 4 – Results and Discussion

Figure 4.12 Room temperature magnetization curves for 6 nm Fe_3O_4 MNP, 13 nm Fe_3O_4 MNP and dox loaded 13 nm core Fe_3O_4 CNP.

Table 4.3 Properties of Fe_3O_4 MNP and PNIPAM – Fe_3O_4 CNP.

Composition	Fe_3O_4	Fe_3O_4	$\text{Fe}_3\text{O}_4 + 1.3 \text{ wt\% PNIPAM}$
Average size (nm) (TEM)	6 (Std. Dev. = 1)	12.9 (Std. Dev. = 3)	12.9 + PNIPAM
Average size (nm) (XRD)	5.5	11.9	-
M_s (emu.g^{-1})	43.4	61	56.8
H_C (G)	3.4	43 (± 8)	8.5 (± 1.5)

Chapter 4 – Results and Discussion

The magnetic properties were found to be particle size dependent, the M_S and H_C were well within the range required for in vivo applications. For $\gamma\text{-Fe}_2\text{O}_3$ MNP (Fig. 4.11), the highest M_S of 58 emu.g^{-1} was exhibited by the 43 nm MNP followed by 45 emu.g^{-1} for the 19 nm MNP. In the case of the Fe_3O_4 MNP (Fig. 4.12) too the M_S increased with particle size, for 6 nm particles the M_S was 43.4 emu.g^{-1} while for the 13 nm MNP it was 61 emu.g^{-1} . Decrease in saturation magnetization with decrease in particle size [96, 214] can be due to ferromagnetic interactions between sublattices inducing random canting of spins, these spins cannot be oriented even with high field strengths [215]. The origin of these canted spins has been attributed to surface effects [216] or finite size effects [217], and is still a subject of investigation.

There are two reasons for the lower M_S of the $\gamma\text{-Fe}_2\text{O}_3$ MNP compared to the Fe_3O_4 MNP despite the smaller sizes of the latter particles: (i) the bulk M_S of $\gamma\text{-Fe}_2\text{O}_3$ is $\sim 76 - 80 \text{ emu.g}^{-1}$ compared to the bulk M_S of Fe_3O_4 which is $90 - 92 \text{ emu.g}^{-1}$ [96, 97] (ii) the $\gamma\text{-Fe}_2\text{O}_3$ MNP synthesized in this work contain minor amounts of $\alpha\text{-Fe}_2\text{O}_3$ which is antiferromagnetic with a very low bulk M_S of less than 1 emu.g^{-1} [97].

The critical size for superparamagnetic behavior of $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 spherical particles at room temperature is $\sim 28 \text{ nm}$ and $\sim 14 - 17 \text{ nm}$ respectively (sec. 2.2.5). All the MNP studied in this work are below the single domain size limit, and all except the 43 nm $\gamma\text{-Fe}_2\text{O}_3$ MNP are superparamagnetic. The coercivity observed in the magnetization curves is due to the particle size distribution, some of the particles are larger than the superparamagnetic size.

Chapter 4 – Results and Discussion

For effective *in vivo* magnetic guidance of the particles to the tumor through the bloodstream and magnetic hyperthermia from particles localized in the tumor, the M_S must be high since it strongly influences the magnetic force experienced by a particle [218, 219] as well as the power output in an AMF [20, 21]. *After encapsulation with PNIPAM, the decrease in M_S was minor and magnetic properties were preserved with the $\gamma\text{-Fe}_2\text{O}_3$ CNP showing 51 emu.g^{-1} and the Fe_3O_4 - CNP showing 56.8 emu.g^{-1} , confirming the magnetic suitability of these particles for MDT.*

Unfortunately, many of the existing studies on thermoresponsive magnetic nanoparticles do not report magnetic properties [78, 166, 173, 174, 176, 183, 184]. Tables 4.4 and 4.5 are a comparison of the magnetic properties of the particles synthesized in the current work with selected references from literature for thermoresponsive and non-thermoresponsive particles respectively. The M_S values for the 43 nm $\gamma\text{-Fe}_2\text{O}_3$ and 13 nm Fe_3O_4 MNP as well as CNP used in the current work are similar to reported values.

The surfactant coated 9.3 nm MNP reported by Uddin *et al.* [175] exhibited a high M_S of 76 emu.g^{-1} dropping to 52 emu.g^{-1} after PNIPAM encapsulation. Similarly, in the work of Wuang *et al.*, bare MNP exhibited 51 emu.g^{-1} , decreasing to 44 emu.g^{-1} after PNIPAM coating, and finally to 33 emu.g^{-1} after heparinization of the particles [179]. The magnetic properties of the PNIPAM-PDADMAC microgel core – PSS-MNP shell particles of Wong *et al.* [213] were not reported, the M_S for only the PSS-MNP was stated to be 42 emu.g^{-1} , slightly lower than the 46 emu.g^{-1}

Chapter 4 – Results and Discussion

of the bare MNP. Similar particles reported by Rubio-Retama *et al.* [165] exhibited relatively low saturation magnetization even with high applied field strengths of 20 kOe: 10 emu.g^{-1} for composite particles with 82 wt% polymer and 20 emu.g^{-1} for those with 62 wt% polymer. *This underlines the requirement of minimum polymer content in the CNP to retain sufficient saturation magnetization in the CNP.*

Table 4.4 Comparison of magnetic properties of particles from the current work with selected literature for *thermoreponsive* magnetic particles.

Materials system	Avg. Size of MNP (nm)	Polymer content of CNP (wt%)	MNP M_s (emu.g^{-1})	CNP M_s (emu.g^{-1})	Comments	Ref.
Fe_3O_4 – PNIPAM	13	1.3	61	56.8	For combined hyperthermia and drug release	Current work [220]
$\gamma\text{-Fe}_2\text{O}_3$ – PNIPAM	43	6	58	53	For combined hyperthermia and drug release	Current work [221]
Fe_3O_4 – PNIPAM	10 – 20	NR	NR	NR	For combined hyperthermia and drug release	Müller-Schulte <i>et al.</i> [78]
Fe_3O_4 – PNIPAM – Heparin	6 – 8	NR	51	44 33	33 emu.g^{-1} is after heparinization	Wuang <i>et al.</i> [179]
$\gamma\text{-Fe}_2\text{O}_3$ – PSS on PDADMAC-PNIPAM core	5 – 7	7 – 8	46 ¹	42 (for PSS-MNP only)	Magnetic data for hybrid particles NR	Wong <i>et al.</i> [213]
$\gamma\text{-Fe}_2\text{O}_3$ – AA on PNIPAM core	6.4	82 62	NR	10 20	Micron sized hybrid particles	Rubio-Retama <i>et al.</i> [165]
Fe_3O_4 – dox – dextran-g-poly(NIPAM-co-DMAAm)	5	40 (dox + polymer)	NR	NR		Misra <i>et al.</i> [173]

Chapter 4 – Results and Discussion

Fe ₃ O ₄ – dox – chitosan-g-poly(NIPAM-co-DMAAm)	5	30 (dox + polymer)	NR	NR		Misra <i>et al.</i> [176]
Fe ₃ O ₄ – PNIPAM	9.3	NR	76	52		Uddin <i>et al.</i> [175]
Fe ₃ O ₄ – APTMS – poly(NIPAM –co- CIPAAm)	130	NR	NR	NR		Aoyagi <i>et al.</i> [74]
Fe ₃ O ₄ – poly(NIPAM-co-CIPAAm)	130	NR	NR	NR		Aoyagi <i>et al.</i> [172]
γ-Fe ₂ O ₃ – PNIPAM	4.9	NR	NR	8	For microfluidic separation applications	Stayton <i>et al.</i> [171]
Fe ₃ O ₄ – PNIPAM	33	NR	~0.05 emu	NR	Mass specific M_S was not reported	Krishnan <i>et al.</i> [178]
Fe ₃ O ₄ – biotinylated PNIPAM	10	NR	NR	NR		Krishnan <i>et al.</i> [181]
Fe ₃ O ₄ – PNIPAM	NR	14 – 70	55	45 – 18	Hollow ‘microcontainers’ of PNIPAM with MNP + drug in cavity	Wang <i>et al.</i> [164]
Fe ₃ O ₄ – AAEM-co-VCL	7 – 20	94.8 to 88.4	75	1.25 to 1.9	Thermoresponsive + electrically conducting	Pich <i>et al.</i> [222]
Fe ₃ O ₄ – PAA – PNIPAM	5	> 45	NR	NR		Hatton <i>et al.</i> [170]
NR = Not reported						

Chapter 4 – Results and Discussion

Table 4.5 Comparison of magnetic properties of particles from the current work with selected literature for *non-thermoreponsive* magnetic particles.

Materials system	Avg. Size of MNP (nm)	Polymer content of CNP (wt%)	MNP M_s (emu.g ⁻¹)	CNP M_s (emu.g ⁻¹)	Comments	Reference
Fe ₃ O ₄ – PNIPAM	13	1.3	61	56.8	For combined hyperthermia and drug release	Current work [220]
γ-Fe ₂ O ₃ – PNIPAM	43	6	58	53	For combined hyperthermia and drug release	Current work [221]
Fe ₃ O ₄ – OA – Pluronic F-127	9.3	15.4 (OA) 14.5 (Pluronic)	45.1	59.2	CNP M_s is per g of Fe ₃ O ₄	Labhasetwar <i>et al.</i> [116]
Fe ₃ O ₄	7.5 to 416	None	7.5 to 25	None		Gu <i>et al.</i> [158]
Fe ₃ O ₄ – starch	250 (D_H)	25	NR	32	For combined hyperthermia and drug release	Babinec <i>et al.</i> [142]
Fe ₃ O ₄	100	None	45.75	None		Lee <i>et al.</i> [155]
γ-Fe ₂ O ₃ – PEG – MA	8	~89	NR	NR		Dumas <i>et al.</i> [115]
Fe ₃ O ₄ – PEG – MA	13	NR	50	40		Gupta <i>et al.</i> [223]
γ-Fe ₂ O ₃ – silica	13	NR	67	30		Gaberšček <i>et al.</i> [224]
Fe ₃ O ₄ – PEG	8	> 90	57.4	4.5		Hervé <i>et al.</i> [132]
Fe ₃ O ₄ – PVA	9	NR	63.2	NR		Petri-Fink <i>et al.</i> [119]
NR = Not reported						

Chapter 4 – Results and Discussion

4.3 *In vitro* hyperthermia

This subsection discusses the physical properties, PNIPAM coating and drug loading of $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 particles

4.3.1 *In vitro* hyperthermia of $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles

The *in vitro* heating behaviour of the bare $\gamma\text{-Fe}_2\text{O}_3$ ferrofluids of concentration 10 mg.ml^{-1} subjected to a 375 kHz , 1.7 kA.m^{-1} AMF is shown in Fig. 4.13. The 43 nm MNP exhibited the fastest heating, reaching 41°C in $\sim 120 \text{ s}$, this temperature was reached by the 19 nm , 14 nm and 12 nm particles in 180 s , 210 s and 465 s respectively. The specific absorption ratio was calculated to be 42.8 W.g^{-1} for the 43 nm , 30.1 W.g^{-1} for the 19 nm , 22.4 W.g^{-1} for the 14 nm and 11.6 W.g^{-1} for the 12 nm MNP.

Figure 4.13 Heating characteristics of $\gamma\text{-Fe}_2\text{O}_3$ ferrofluids in a 375 KHz , 1.7 kA.m^{-1} AMF. Ambient $T = 30^\circ\text{C}$. Particle conc. = 10 mg.ml^{-1} . Dashed horizontal lines indicate the hyperthermia range of $41 - 48^\circ\text{C}$.

Chapter 4 – Results and Discussion

4.3.2 *In vitro* hyperthermia of Fe₃O₄ nanoparticles

The temperature rise from ambient temperature plotted against time for Fe₃O₄ ferrofluids exposed to a 375 kHz, 4 kA.m⁻¹ AMF is shown in Fig. 4.14. 13 nm bare Fe₃O₄ MNP reaches hyperthermia temperature (41 °C) in 335 s while Fe₃O₄ CNP and dox loaded Fe₃O₄ CNP attain this temperature more rapidly, in 295 s and 255 s respectively.

From the ferrofluid concentration and slope of the initial segment of the heating curve, SAR was calculated to be 41.2 W.g⁻¹ for 13 nm Fe₃O₄ MNP, 72 W.g⁻¹ for Fe₃O₄ CNP and 74.1 W.g⁻¹ for dox loaded Fe₃O₄ CNP. In contrast, 6 nm Fe₃O₄ MNP exhibit relatively poorer heating performance even at higher concentrations (5 and 10 mg.ml⁻¹) with 10 mg.ml⁻¹ ferrofluid reaching 41 °C in 660 s with a SAR of 8.2 W.g⁻¹, the 5 mg.ml⁻¹ ferrofluid was unable to attain 41 °C even after 1200 s.

The 19 nm and 43 nm γ-Fe₂O₃ MNP and the 13 nm Fe₃O₄ MNP showed excellent heating behavior in an AMF even at conservative concentrations and field strengths (Figs. 4.13 and 4.14). For the γ-Fe₂O₃ particles, the SAR increased with particle size from 11.6 W.g⁻¹ for the 12 nm MNP to 42.8 W.g⁻¹ for the 43 nm MNP. Since the 43 nm MNP are single domain but not superparamagnetic, hysteresis loss is the dominant loss mechanism [21, 81]. Only minor saturation loops are produced with a 1.7 kA.m⁻¹ AMF, greater heating can be achieved at higher AMF strengths by fully saturated hysteresis loops.

Chapter 4 – Results and Discussion

Figure 4.14 Heating characteristics of Fe_3O_4 ferrofluids in a 375 KHz, 4 kA.m^{-1} AMF. Ambient $T = 30^\circ\text{C}$. Particle conc. (except 6 nm) = 2 mg.ml^{-1} . Dashed horizontal lines indicate the hyperthermia range of 41 – 48 $^\circ\text{C}$.

For 13 nm Fe_3O_4 particles, SAR was measured to be 41.2 W.g^{-1} for the MNP, 72 W.g^{-1} for CNP and 74.1 W.g^{-1} for the Dox CNP samples. *This confirms that with appropriate choice of magnetic core size, there is no decrease in power as D_H of the CNP increases (Sec. 4.8.1.2).* Since heating performance of the 6 nm Fe_3O_4 MNP is relatively poor these particles are not the best choice for *in vivo* hyperthermia applications.

For superparamagnetic $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 MNP, the experimentally measured SAR was found to follow the trends predicted by the modeling of power output

Chapter 4 – Results and Discussion

from superparamagnetic MNP (Sec. 4.8.1); the quantitative differences in SAR when compared to predicted values arise from finite particle size distribution especially for the γ -Fe₂O₃ MNP and viscous losses not accounted for in the model (viscous losses will not contribute significantly to the SAR of MNP that are internalized and immobilized in the tumor cells *in vivo*). Viscous losses can occur [225] in *in vitro* tests of magnetic particles dispersed in a fluid, and for all particle sizes regardless of superparamagnetic behavior [21, 81]. However modeling viscous losses requires precise measurements of the particle hydrodynamic diameter, the magnetic volume contributing to the hydrodynamic diameter, fluid viscosity, remanent magnetization and field strength experienced by particles as input data, otherwise errors in calculation are significant. Empirical estimation of viscous losses can be performed by measuring the difference between SAR of MNP immobilized in a matrix and the SAR of the same MNP dispersed in a fluid.

Although direct comparisons cannot be made due to the difference in the materials properties and AMF parameters, after accounting for the AMF frequency and field strength, *the SAR values for the particles used in this work exceed or compare favorably to reported values [19, 35, 74, 147, 158]*. The SAR can be readily increased by increasing the concentration, field strength or frequency, for e.g., under the same field conditions, the predicted *in vitro* SAR value for 13 nm Fe₃O₄ in a 20 mg.ml⁻¹ ferrofluid is 284 W.g⁻¹.

In vitro hyperthermia experiments are a very useful guide to the performance of the particles, *in vivo* heating will differ from *in vitro* values due to tissue thermal conductivity, immobilization of the particles in the cells (which prevents Brownian

Chapter 4 – Results and Discussion

relaxation and viscous losses) cooling of the tumor by perfusion and heat loss from surrounding tissues. It is important to maximize the power output of the particles to achieve therapeutic temperatures in the tumor with minimum quantity of particles. *In vivo* conditions favor hyperthermia for larger liver tumors; although particle deposition in large tumors was lower than in small tumors, heating rates in larger tumors were higher due to poorer cooling from low blood perfusion and better heat conduction from tumor vasculature structure [67, 226].

4.4 *In vitro* simultaneous hyperthermia and drug release

This section describes the *in vitro* simultaneous hyperthermia and drug release performance of dox loaded γ -Fe₂O₃ and Fe₃O₄ composite nanoparticles.

4.4.1 *In vitro* simultaneous hyperthermia and drug release from dox loaded γ -Fe₂O₃ composite nanoparticles

The temperature vs. time plot for a magnetic hyperthermia cycle of (a) heating, (b) temperature stabilization and (c) cooling for dox loaded γ -Fe₂O₃ CNP is shown in Fig. 4.15. With field strength in the range of 0.1 to 4 kA.m⁻¹ and a starting temperature of 37 °C, the ferrofluid heated up rapidly, reaching 41 °C in just 50 s. Due to the higher field strengths, heating rate was rapid even with 2.5 mg.ml⁻¹ of the ferrofluid, lower than the 10 mg.ml⁻¹ used for hyperthermia performed at a field strength of 1.7 kA.m⁻¹ (Fig. 4.13). Temperature stabilization within the hyperthermia range was successfully attained by variation of the field strength; switching off the AMF resulted in the ferrofluid cooling down to 37 °C in 16 min. 17.3 % of the loaded dox was released after a total of 47 min, i.e., 30 min of

Chapter 4 – Results and Discussion

exposure to hyperthermia temperatures (41 °C – 48 °C) and 17 min above body temperature (37 °C) during the heating and cooling stages.

Figure 4.15 Heating curve from *in vitro* simultaneous drug release and hyperthermia test of dox loaded γ -Fe₂O₃ CNP in a 375 kHz, 0.1 – 4 kA.m⁻¹ AMF with (a) heating (b) hyperthermia and (c) cooling segments. Particle conc. = 2.5 mg.ml⁻¹.

4.4.2 *In vitro* simultaneous hyperthermia and drug release from dox loaded Fe₃O₄ composite nanoparticles

A typical *in vitro* simultaneous hyperthermia and drug release curve, in this case for 30 min of hyperthermia exposure for dox loaded Fe₃O₄ CNP, is shown in Fig. 4.16. Compared to the heating behavior with 2 mg.ml⁻¹ ferrofluids, at lower (1 mg.ml⁻¹) concentration the ferrofluid of dox loaded Fe₃O₄ CNP reaches 41 °C in 240 s due to the higher applied field strength and ambient temperature. After

Chapter 4 – Results and Discussion

exposure to hyperthermia temperatures (41 °C – 47 °C) for 10 min, 30.7 % of bound dox was released from the particles, increasing to 38.6 % for 30 min and 42.4 % for 60 min of exposure.

Figure 4.16 Heating curve from *in vitro* simultaneous drug release and hyperthermia test of dox loaded Fe_3O_4 CNP dispersed in PBS and exposed to a 375 kHz, $0.1 - 5 \text{ kA.m}^{-1}$ AMF for 35 min. Particle conc.= 1 mg.ml^{-1} .

The dox loaded 43 nm $\gamma\text{-Fe}_2\text{O}_3$ CNP as well as the Fe_3O_4 CNP successfully achieved simultaneous hyperthermia and *in vitro* drug release (Figs. 4.15 and 4.16). The heating rate was higher than that for the *in vitro* hyperthermia tests despite the lower ferrofluid concentration due to the higher field strengths used. Hyperthermia temperatures were rapidly reached and temperature could be stabilized in the 41 °C – 48 °C range for clinically relevant time periods by adjusting the field strength, as is currently done in clinical human trials [29]. For dox loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP the drug release during 47 min of magnetic hyperthermia

Chapter 4 – Results and Discussion

closely matches the drug release in 45 min from the 42 °C release tests for dehydrated particles (Fig. 4.17), with a cumulative release of 17.3 % and 17.6 % of loaded dox respectively. For dox loaded Fe₃O₄ CNP, cumulative release after 10 min (30.7 %), 30 min (38.6 %) and 60 min (42.4 %) agrees closely with the release from dehydrated particles at 43 °C (Fig. 4.18a) after 10 min (31.1 %), 30 min (37 %) and 60 min (39.8 %). *Even with the low concentrations of dox loaded CNP used for the in vitro simultaneous hyperthermia and drug release tests, the mass of dox release was therapeutically relevant for chemotherapy.* The results of the current work are compared with references from literature in Table 4.6. However, most of the literature does not report drug loading and drug release values, hence direct comparison with the current work is difficult.

Chapter 4 – Results and Discussion

Table 4.6 Comparison of results of *in vitro* drug release during AMF induced hyperthermia from current work with selected literature.

Materials system	Drug	Drug in CNP (wt%)	AMF f (KHz) H_0 (kA.m ⁻¹)	Drug Release		Comments	Ref.
				Release Time (min)	Cumul. release (%)		
Fe ₃ O ₄ – PNIPAM	Doxorubicin	4.1	375 5	10 30 60	30.7 38.6 42.4	PBS pH 7.4	Current work [220]
γ-Fe ₂ O ₃ – PNIPAM	Doxorubicin	2.5	375 4	40	17.3	PBS pH 7.4	Current work [221]
γ-Fe ₂ O ₃ – phospholipid	Doxorubicin	20	3500 1.2	6	70	Unilamellar magnetoliposomes	Babinec <i>et al.</i> [76]
Fe ₃ O ₄ – starch	Cisplatin	“32 μM”	3 1.2	20	98		Babinec <i>et al.</i> [142]
Fe ₃ O ₄ – DPCC	5- Fluoracil	19.3	500 NR	120	2.54	Sörensen buffer + 50 % calf serum pH 7.4	Horikoshi <i>et al.</i> [73]
Fe ₃ O ₄ – PNIPAM	Rhodamine B	NR	360 20	14	1.6 % of CNP wt	Reported % release was not with respect to drug loading	Müller-Schulte <i>et al.</i> [78]
	Methylene blue				1 % of CNP wt		
Fe ₃ O ₄ – PNIPAM – TEGDMA	Vitamin B ₁₂	NR	297 5.3	2880	“ $M_t/M_\infty = 0.6$ ”	PBS. 15 mm x 1.5 mm nanocomposite discs. M_∞ not actual drug loading	Hilt <i>et al.</i> [182]

NR = Not reported.
Release medium and pH are listed in the **Comments** column.

Chapter 4 – Results and Discussion

4.5 *In vitro* drug release without magnetic field

This subsection describes drug release from dox loaded γ -Fe₂O₃ CNP and Fe₃O₄ CNP at different temperatures in the absence of a magnetic field.

4.5.1 *In vitro* dox release from γ -Fe₂O₃ composite nanoparticles without magnetic field

The dox release profiles at 24 °C, 37 °C and 42 °C for dehydrated dox loaded γ -Fe₂O₃ CNP in PBS (pH 7.4) are shown in Fig. 4.17. The inset is an expanded view of the release for the first 12 h. Cumulative dox release for 101 h at 24 °C was 34 % of loaded dox, increasing to 42.7 % at 37 °C and 48.2 % at 42 °C. Drug release occurs relatively rapidly until 10.75 h, with 29.1 % release at 24 °C, 31.8 % at 37 °C and 33.1 % at 42 °C, after which release slows down, this effect is most prominent at 24 °C.

Chapter 4 – Results and Discussion

Figure 4.17 *In vitro* drug release from dox loaded PNIPAM – γ -Fe₂O₃ CNP in PBS (pH 7.4) at 24 °C, 37 °C and 42 °C. Inset is an expanded view of release for the first 12 h

4.5.2 *In vitro* dox release from Fe₃O₄ composite nanoparticles without magnetic field

Plots for cumulative dox release in PBS without an applied magnetic field for dehydrated as well as swollen dox loaded Fe₃O₄ CNP at 24 °C, 37 °C and 43 °C are shown in Fig. 4.18a and Fig. 4.18b respectively. At the end of 29 h, maximum bound dox released by dehydrated particles was 56.4 % at 43 °C followed by 46.5 % at 37 °C and 36.8 % at 24 °C. The corresponding values for the swollen particles are 78.1 % at 43 °C, 63.7 % at 37 °C and 42.6 % at 24 °C.

Chapter 4 – Results and Discussion

Figure 4.18 *In vitro* drug release from dox loaded Fe_3O_4 CNP in PBS (pH 7.4) and tested at 24 °C, 37 °C and 43 °C, (a) dehydrated particles (b) swollen particles.

Chapter 4 – Results and Discussion

4.5.3 Controlled drug release from swollen dox loaded Fe_3O_4 composite nanoparticles

Dox release from swollen Dox CNP in PBS under temperature switching is shown in Fig. 4.19a, corresponding drug release rates with respect to initial dox loading are shown in Fig. 4.19b. After a total exposure of 20 h at 24 °C, and 4 h each at 37 °C and 43 °C, 65.4 % of loaded dox was released. The maximum release rate was 35.5 %. h^{-1} for the first 1 h of exposure at 37 °C, this dropped drastically for 24 °C exposures during the 2 h to 4 h and 6 h to 24 h time periods.

Chapter 4 – Results and Discussion

Figure 4.19 Controlled drug release from swollen, dox loaded Fe₃O₄ CNP in PBS (pH 7.4) as a function of temperature. (a) cumulative dox release (b) dox release rates.

Chapter 4 – Results and Discussion

For drug release from dehydrated dox-loaded γ -Fe₂O₃ CNP in PBS in the absence of an external magnetic field, there is an initial relatively rapid release phase for times of up to ~ 10.75 h by which time the particles release 29.1 %, 31.8 % and 33.1 % of the loaded dox at 24 °C, 37 °C and 42 °C respectively (Fig. 4.17). This is followed by slow release behavior up to 101 h, with cumulative dox release of 34 % (24 °C), 42.7 % (37 °C) and 48.2 % (42 °C). A similar result is obtained for dehydrated dox loaded Fe₃O₄ CNP (Fig. 4.18a) which also shows limited influence of temperature on release behavior. There is an initial rapid release phase up to 2 h during which 25.7 %, 37.4 % and 45 % of loaded dox is released at 24 °C, 37 °C and 43 °C respectively followed by steady release.

Fig. 4.20 is a representation of schemes of drug release from dehydrated PNIPAM gels [84]. Since the gel is not hydrated, there is no burst release by squeezing action from dehydrated particles above the LCST; the collapsed hydrophobic (above the LCST) surface of the PNIPAM shell will obstruct rehydration of the shell and transport of dox molecules through the polymer network. Convection driven migration of the drug inside the PNIPAM matrix during the water evaporation stage of the vacuum dehydration process leads to higher drug concentration near the surface [212], release of this drug will be Fickian. After the drug concentration near the surface is reduced, the release is expected to slow down significantly. This decrease in release rate is clearly observed at 37 °C and higher temperatures for both dehydrated γ -Fe₂O₃ CNP and dehydrated Fe₃O₄ CNP. If temperature is subsequently lowered below the LCST to enable shell rehydration and reswelling and is then raised above the LCST, a burst phase is expected due to shell collapse.

Chapter 4 – Results and Discussion

Below the LCST, since surface hydrophobicity is absent, the dehydrated shell will be rehydrated relatively easily and dox release occurs by transport of the hydrophilic drug molecules into the release medium (osmotic pressure driven) during hydration [227]. The shell dimensions are in the nano size range, hence this is expected to occur relatively quickly and result in distinctly slower release once the swelling has reached equilibrium and the driving force for drug transport is reduced [78, 182].

Figure 4.20 Schemes for drug release from PNIPAM gels adapted from Ref. [84].

(a) relies on pressure generated during gel collapse to push out drug molecules from the matrix, (b) and (c) are Fickian processes, process (d) has not been extensively studied but is believed to be governed by the molecular properties of the drug and interaction between the drug and polymer network.

Chapter 4 – Results and Discussion

However, there is no significant difference observed between the release profiles at tested temperatures for a period of up to 10 h for the dehydrated dox loaded γ - Fe_2O_3 CNP and 2 h for the dehydrated dox loaded Fe_3O_4 CNP. This suggests that shell rehydration below the LCST is not occurring as expected, particularly in the case of the γ - Fe_2O_3 MNP. *Therefore, drug release utilizing thermoresponsive behavior is not feasible from particles in the dehydrated state; vacuum dehydration appears to result in structural changes to the PNIPAM, altering its re-swelling behavior.* However, dehydration is a simple method to achieve sustained long term slow release from drug loaded PNIPAM – iron oxide CNP, indicating their potential for applications that require this type of release behavior [87, 163].

In contrast, swollen dox loaded Fe_3O_4 CNP shows pronounced thermoresponsive behavior for drug release in PBS without an AMF (Fig. 4.18b). The rapid release phase extends up to 4 h resulting in release of 33.7 %, 50.1 % and 65.7 % of the loaded dox at 24 °C, 37 °C and 43 °C respectively; this is followed by steady state release. There is a greater difference in drug release at various temperatures as well as higher cumulative release above the LCST (63.7 % at 37 °C and 78.1 % at 43 °C for 29 h) compared to the dehydrated dox loaded Fe_3O_4 CNP, e.g., dox release from the swollen particles at 43 °C in just 1 h (51.2 %) is equal to that from dehydrated particles after 8 h (51.2 %). Higher release from swollen particles is due to the collapse of the PNIPAM shell above the LCST and squeezing of the dox molecules into the release medium along with expulsion of hydrophobically bound water. After the initial burst phase, drug release slows since drug molecules entrapped in the interior of the shell cannot easily travel through the collapsed polymer structure, this effect is seen after 4 h for the swollen Fe_3O_4 CNP.

Chapter 4 – Results and Discussion

Rehydration will occur if the particles are cooled below the LCST, priming the particles for another phase of burst release. Below the LCST (24 °C), drug release slows down due to the lower driving force for hydration and consequently, osmotic pressure, since the particles are already swollen with water. Hence drug release occurs only by slow diffusion and primarily of dox near the surface. It has been reported that PNIPAM microgels can possess a core-shell structure [228], this shell may affect the release kinetics of drug molecules from the CNP by introducing an interface barrier, but the exact mechanism is not clear. Another point of interest is that tumor tissues can be slightly more acidic (~ pH 5.3 – 7.0) than blood (pH 7.4) [229] and dox release in acidic environments will be faster than in a neutral environment [118, 164, 173, 176].

The results obtained in the current work are compared with *in vitro* drug release without a magnetic field for thermoresponsive particles and non-thermoresponsive particles in Tables 4.7 and 4.8 respectively. The dox release results of the current work for swollen CNP are consistent with those reported by Misra *et al.* [173, 176] with dox release above the LCST significantly higher than that below the LCST. The dox release reported by Wang *et al.* [164] for 24 h at 20 °C in PBS at pH 7.4 is similar to the 24 °C release results of the current work; it was also reported that cumulative release at 40 °C (~ 41 %) was unchanged from release at 20 °C. They attributed this to the solubility limit of dox in PBS at pH 7.4 which resulted in almost identical cumulative release at the tested temperatures after 24 h; however the initial release rate was much higher at 40 °C. When pH was decreased, release was rapid at 20 °C. In the current work, the volume of PBS buffer solution used as

Chapter 4 – Results and Discussion

a sink was relatively large and the concentration of dox in the release medium was not high enough for the solubility limit to become a factor.

Table 4.7 Comparison of *in vitro* drug release (without AMF) results of the current work with selected literature for *thermoreponsive* particles.

Materials system	Drug / agent	Drug in CNP (wt%)	Release Temp. (°C)	Cumul. release (%)	Time (h)	Comments	Ref.
Fe ₃ O ₄ – PNIPAM	Doxorubicin	4.1	24 37 43	36.8 46.5 56.4	29	Dehydrated CNP PBS pH 7.4	Current work [220]
			24 37 43	42.6 63.7 78.1		Swollen CNP PBS pH 7.4	
γ-Fe ₂ O ₃ – PNIPAM	Doxorubicin	2.5	24 37 42	34 42.7 48.2	101	Dehydrated CNP PBS pH 7.4	Current work [221]
Fe ₃ O ₄ – PNIPAM	Bovine serum albumin	28	30 40	80 60	2	NaCl soln. pH 9.35	Uddin <i>et al.</i> [175]
Fe ₃ O ₄ – dox – dextran-g-poly(NIPAM-co-DMAAm)	Doxorubicin	9	20 37 40	28 65 85	48	Na ₂ HPO ₄ – KH ₂ PO ₄ buffer pH 7.4	Misra <i>et al.</i> [173]
Fe ₃ O ₄ – dox – chitosan-g-poly(NIPAM-co-DMAAm)	Doxorubicin	10	20 37 40	22 44 77	48	Na ₂ HPO ₄ – KH ₂ PO ₄ buffer pH 7.4	Misra <i>et al.</i> [176]
Fe ₃ O ₄ – PNIPAM microcontainer	Doxorubicin	50	20 40 20	[#] 40 [#] 41 *80	24	PBS [#] pH 7.4 *pH 4.6	Wang <i>et al.</i> [164]
γ-Fe ₂ O ₃ – SBA-15 – PNIPAM	Gentamicin	28	20 30 40 50	17 30 65 74	6	PBS pH 7.4	Zhu <i>et al.</i> [230]
Fe ₃ O ₄ – dextran – DPPC	Calcein	40	30 35 42	5 9 45	0.08	Thermosensitive magnetoliposomes TES buffer	Horikoshi <i>et al.</i> [231]
NR = Not reported. Release medium and pH are listed in the Comments column.							

Chapter 4 – Results and Discussion

Table 4.8 Comparison of *in vitro* drug release (without AMF) results of the current work with selected literature for *non-thermoreponsive* particles.

Materials system	Drug	Drug in CNP (wt%)	Release Temp. (°C)	Cumul. release (%)	Time (h)	Comments	Ref.
Fe ₃ O ₄ – PNIPAM	Doxorubicin	4.1	24 37 43	36.8 46.5 56.4	29	Dehydrated CNP PBS pH 7.4	Current work [220]
			24 37 43	42.6 63.7 78.1		Swollen CNP PBS pH 7.4	
γ-Fe ₂ O ₃ – PNIPAM	Doxorubicin	2.5	24 37 42	34 42.7 48.2	101	Dehydrated CNP PBS pH 7.4	Current work [221]
Fe ₃ O ₄ – OA – Pluronic F-127	Doxorubicin	8.2	37	28 62	48 168	PBS pH 7.4	Labhasetwar <i>et al.</i> [116]
Fe ₃ O ₄ – OA – Pluronic F-127	Paclitaxel	9.5	37	25 60	48 168	PBS pH 7.4	Labhasetwar <i>et al.</i> [121]
γ-Fe ₂ O ₃ – silica	Naproxen	30	37	73	2	Buffer solution	Gaberšček <i>et al.</i> [224]
γ-Fe ₂ O ₃ – Fe ₃ O ₄ – Fe ²⁺ – citrate	Doxorubicin	14	37	85 #100	1 #Inst ant	Tris Buffer pH 7.4 #Acetate buffer pH 4.0	Chourpa <i>et al.</i> [118]
Fe ₃ O ₄ – PEG	Doxorubicin	50	37	68	24	Sterilized water pH 7.0	Misra <i>et al.</i> [133]
Fe ₃ O ₄ – starch	Mitoxantrone	1.3	37	98	1	Also used <i>in vivo</i> for MDT	Alexiou <i>et al.</i> [24]
Fe ₃ O ₄ – PLGA	Dexamethone	2.5	37	82 100	72 192	PBS pH 7.4	Butoescu <i>et al.</i> [126]
NR = Not reported. Release medium and pH are listed in the Comments column.							

Chapter 4 – Results and Discussion

This thermoresponsive ability of the swollen dox loaded CNP is demonstrated by the controlled release tests (Figs.4.19 a-b). Initial thermal exposure at 37 °C for 1 h results in release of 35.5 % of the loaded dox, followed by a 43°C, 1 h exposure yielding 48.5 % release. When the temperature is reduced below the LCST to 24 °C for 2 h, dox release is drastically reduced. When the temperature is again increased to 37 °C or 43 °C drug release resumes, a drop to 24 °C (6 h to 24 h) causes drug release to reduce once again, suggesting that reswelling of PNIPAM below the LCST is relatively rapid. Thus, temperature acts like an on/off switch to regulate drug release above and below the LCST.

Comparison of the drug release curve of the swollen dox loaded Fe₃O₄ CNP at 24 °C (Fig. 4.18b) and the controlled release curve segments at 24 °C (Fig. 4.19a) shows that release exhibited by the former is much higher (>20 % initial release) than that of the latter. Dox on the PNIPAM shell surface rapidly diffuses into the release media (PBS) when the particles are dispersed at 24 °C, independent of the thermoresponsive behavior. For the controlled release test, the swollen Dox CNP is initially held at temperatures of 37 °C and 43 °C for 1 h each, surface release occurs rapidly during this period. Additionally, due to PNIPAM shrinkage above the LCST, dox from the polymer matrix interior is also released, resulting in a lower dox concentration in PNIPAM at the end of the first 2 h. This decreases the driving force for dox release when the temperature is reduced to 24 °C. Release rate is negligible at this temperature as the shell swells quickly, preventing further transport of dox through the matrix into the release medium. Only an increase in temperature above the LCST, inducing matrix shrinkage, triggers further release. With each subsequent temperature switch above the LCST, the concentration of

Chapter 4 – Results and Discussion

drug in the polymer shell is reduced, thereby decreasing the quantity of drug released in the ‘pulse’.

In previous reports, AMF controlled pulsatile release of Vitamin B₁₂ and methylene blue from 15 x 1.5 mm nanocomposite discs of PNIPAM embedded with Fe₃O₄ MNP was demonstrated by Hilt *et al.* [182]. When AMF was applied, there was a sharp pulse of drug release resulting in distinctly higher release rates, as the AMF was shut off the release slowed down significantly. Temperature controlled pulsatile release of model molecules from macroscopic PNIPAM hydrogels has also been reported previously [227].

The drug release results confirm the potential of thermoresponsive dox loaded PNIPAM – iron oxide CNP for pulsatile drug release applications such as in combined hyperthermia and chemotherapy treatment.

4.6 *In vitro* cytotoxicity

This subsection describes the *in vitro* cytotoxicity tests of Fe₃O₄ MNP, Fe₃O₄ CNP and dox loaded Fe₃O₄ CNP.

The LCST of swollen dox loaded Fe₃O₄ CNP in cell culture medium is shown in Fig. 4.21a. The onset temperature for the first transition was 38.2 °C with the peak temperature of 38.7 °C. For the second endothermic event, the corresponding temperatures were 42.8 °C and 43.7 °C. The LCST of swollen dox loaded Fe₃O₄ CNP in the cell culture medium is higher than the 35 °C in PBS (Fig. 4.10b) due to the difference in the solvent. This implies that at the test temperature of 37 °C, the

Chapter 4 – Results and Discussion

particles are likely to be below the LCST and present a hydrophilic surface to the cells.

The zeta potential of the nanoparticles in PBS (pH 7.4) at 37 °C is shown in Fig. 4.21b. The bare Fe₃O₄ MNP display an average zeta potential value of -32.4 mV, after PNIPAM coating the zeta potential changes to -29.9 mV. Dox loading of the CNP results in a positive zeta potential value of +2.2 mV. This suggests that uptake of the positively charged dox loaded particles by cells will be faster than uptake of the MNP and CNP since the cell membrane is negatively charged [23].

In vitro cell viability of BEAS-2B lung endothelial cell cultures exposed to Fe₃O₄ MNP, Fe₃O₄ CNP, dox loaded Fe₃O₄ CNP and free dox solution at 37 °C for 24 h and 48 h is shown in Figs. 4.22a and 4.22b respectively. After 24 h exposure, cell viability for bare MNP (conc. 0.1 mg.ml⁻¹ and 0.5 mg.ml⁻¹) is in the range of ~ 85 % to 87 %, this remains largely unchanged for the 48 h exposure. Cells incubated with Fe₃O₄ CNP show higher cell viability for the same concentrations and exposure times, in the range of ~ 88 % to 98 %. There is a reduction in cell viability with all tested concentrations of dox-loaded Fe₃O₄ CNP, after 24 h only 18.1 % to 25.13 % of the cells survive. As the exposure time is increased to 48 h, the proportion of live cells decreases to ~ 2 % – 3.7 % of the control cells.

The cytotoxicity of the Fe₃O₄ CNP on human cells showed interesting results. Table 4.9 compares the *in vitro* cytotoxicity results of the current work with selected references from literature for non-cancerous cells. The *in vitro* cytotoxicity

Chapter 4 – Results and Discussion

results of the current work are comparable with those reported by others in similar *in vitro* studies [87, 118, 121, 123, 223, 232, 233].

Figure 4.21 (a) LCST of swollen dox loaded Fe₃O₄ CNP in cell culture medium
(b) Zeta potential of bare Fe₃O₄ MNP, Fe₃O₄ CNP and dox loaded Fe₃O₄ CNP in PBS (pH 7.4) at 37 °C.

Chapter 4 – Results and Discussion

Figure 4.22 Cell viability of BEAS-2B lung endothelial cells incubated with different concentrations of nanoparticles for (a) 24 h (b) 48 h at 37 °C.

Chapter 4 – Results and Discussion

Table 4.9 Comparison of *in vitro* cytotoxicity results of current work with selected literature.

MNP type	Coating	Cell type and cells per well	Particle conc. (mg.ml ⁻¹)	Exposure time (h)	Avg. cell viability (%)	Reference
13 nm Fe ₃ O ₄	None	BEAS-2B 5 x 10 ⁴	0.1 0.05	24	85.2 87	Current work
			0.1 0.05	48	85.3 86.3	
	PNIPAM		0.1 0.05	24	93.1 97.7	
			0.1 0.05	48	88.1 97.9	
Ferumoxtran -10 SPIO	Dextran	HMM 1 – 2 x 10 ⁶	0.1 – 1	24 -72	> 90 for all	Müller <i>et al.</i> [234]
30 nm Fe ₃ O ₄	None	BRL-3A Confluent in 6, 24 well plates	0.01 0.05 0.1 0.25	24	> 100 95 90 85	Hussain <i>et al.</i> [235]
47 nm Fe ₃ O ₄			0.01 0.05 0.1 0.25		>100 97 87 68	
207, 289 and 1430 nm SPIO	Dextran	Jurkat clone E6-1 2 x 10 ⁶	0.0138 0.069 0.138	4	Negligible decrease in cell viability	Tsourkas <i>et al.</i> [232]
13 nm Fe ₃ O ₄	None	hTERT-BJ1 1 x 10 ⁴	0.1 0.25 0.5 1	24	85 72 68 50	Gupta <i>et al.</i> [223]
	MA-PEG		0.1 0.05 0.5 1		No decrease in cell viability	
13.6 nm Fe ₃ O ₄	None	hTERT-BJ1 1 x 10 ⁴	0.05 0.1 0.25 0.5 1 2	24	80 72 61 49 45 39	Gupta <i>et al.</i> [80]
	Pullulan		0.05 0.1 0.25 0.5 1 2		> 100 100 97 95 90 90	

Chapter 4 – Results and Discussion

In vitro cell viability tests on human lung endothelial cells reveal that bare Fe_3O_4 MNP as well as Fe_3O_4 CNP show good cell viability at the tested concentrations of 0.1 mg.ml^{-1} and 0.05 mg.ml^{-1} . Cytotoxicity of the bare MNP remains essentially unchanged for 24 h and 48 h exposures as well as for both the tested concentrations, with cell viability remaining at $\sim 86 \%$. The cytotoxicity of Fe_3O_4 CNP is lower than that of bare Fe_3O_4 MNP for the concentration of 0.05 mg.ml^{-1} ; for 24 h and 48 h exposures the cell viability is $\sim 97\%$. There is a slight drop in cell viability for the 0.1 mg.ml^{-1} CNP concentration at 48 h (88.1%) compared to 24 h (93.1%), but it remains higher than that for bare MNP. These results indicate that cell viability will not be affected by the particles but instead depend on the cytotoxic drug loaded onto the particles. This is confirmed by the tests on dox loaded Fe_3O_4 CNP; at all tested concentrations and exposure times, there is a drastic reduction in cell viability due to cytotoxic dox killing endothelial cells. After 24 h, maximum cell viability is only 25.1% , as exposure time is increased to 48 h, the cytotoxic effect is increased and maximum cell viability is only 3.7% .

In vitro studies by Vihola *et al.* [233] on the response of Caco-2 and Calu-3 cell lines to PNIPAM have shown that the polymer is well tolerated up to relatively high concentrations (10 mg.ml^{-1}) in its hydrophilic form, cell viability decreases as the hydrophobicity increases. Hsiue *et al.* have also reported high tolerance of PNIPAM by corneal epithelial cells of New Zealand white rabbits [87]. Gupta *et al.* observed that cell viability of human fibroblasts was consistently higher by $40\% - 100 \%$ for PEG coated MNP compared to bare MNP [223]; a similar result was obtained in the current work for BEAS-2B cells, but the difference in cytotoxicity

Chapter 4 – Results and Discussion

between the bare MNP and CNP was not as drastic. Chourpa *et al.* reported that dosing MCF-7 cancer cells with citrate functionalized MNP loaded with 0.3 μM of dox for 24 h resulted in a cell viability of 72 %, increasing the dox concentration to 8.6 μM decreased cell viability to 20 % [118].

4.7 *In vivo* magnetic drug targeting and magnetic resonance imaging

This subsection describes the *in vivo* magnetic drug targeting and magnetic resonance imaging using dox-loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP.

Dox-loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP were successfully targeted to the HCC in a Buffalo rat model. Fig. 4.23a is the pre-injection MRI baseline scan of the rat, the HCC is uniformly grey. After ferrofluid injection and guidance by an external magnet, the dox-loaded MNP were immobilized in the HCC as seen by the dark regions (Fig. 4.23b) and confirmed by histology (Fig. 4.23c), staining of HCC tissue clearly revealed iron oxide deposits. Histology of tissues from surrounding organs such as the kidney and spleen did not show iron oxide deposits, suggesting that the dox-loaded particles were accurately targeted to the HCC and not systemically distributed.

Chapter 4 – Results and Discussion

Figure 4.23 MRI scan of the Buffalo Rat implanted with HCC (a) baseline scan before injection of dox loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP (b) 30 min after injection. Particles are seen as new, dark regions in the HCC. (c) Histology slide of the HCC showing particles as dark deposits.

In vivo magnetic drug targeting experiments showed deposition of dox loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP in the HCC, this was confirmed by MRI as well as by histology of the HCC sections. The ferrofluid volume (0.5 ml) and concentration (0.36 mg.ml^{-1}) used were optimized for MRI visualization in order to track the particles *in vivo*. For clinical drug delivery [27, 107] and hyperthermia applications [29, 65] ferrofluid volume and particle concentration will be two orders of magnitude higher, however, these high particle concentrations are not suitable for MRI visualization [37, 38]. In previous work, Lübke *et al.* used anhydroglucose coated 50 -150 nm MNP loaded with epirubicin to target tumors in rats; at a ferrofluid concentration of 15 mg.ml^{-1} a dosage of 0.5 % of blood volume was sufficient for tumor regression [112]. Importantly, desorption of the bound drug occurred in 30

Chapter 4 – Results and Discussion

min after targeting, indicating that once particles are localized in the tumor, cytotoxic action of the drug can commence.

Healthy parenchyma of the liver contain Kupffer cells of the reticulo endothelial system, while tumors lack these macrophage cells [38]. Intravenous administration of iron oxide MNP for MRI of the liver results in rapid clearance of particles from the bloodstream, these particles are then deposited in the Kupffer cells. The presence of MNP decreases the T2 weighted signal intensity, resulting in a contrast between healthy tissue which contains Kupffer cells (and the MNP) and the tumor (which lacks the MNP) [37, 38]. In the current work, the dox loaded γ -Fe₂O₃ CNP were injected via the hepatic artery and magnetic guidance was used to deposit them in the tumor. Hence, the T2 weighted signal intensity from the tumor decreased (Fig. 4.23b) without changing signal intensity from healthy tissue. Histology of spleen, kidneys, and stomach confirmed that particles were absent in these organs, *highlighting the effectiveness of magnetic field targeting to the tumor*. While normal liver tissues obtain about 70% of their blood supply from the portal venous system, the hepatic arterial system is the main source of blood supply for liver tumors, which makes preferential targeting of liver tumors via the hepatic artery much easier [67]. This is also consistent with the results of Alexiou *et al.* in which starch coated mitoxantrone loaded MNP of average size 100 nm (D_H) were targeted to VX-2 tumors in the limbs of New Zealand white rabbits [24]. A high magnetic field strength of 1.7 T was used for guidance, intra-arterial and intravenous targeting were compared; it was established via MRI that intra-arterially targeted tumors retained a very high concentration of MNP and the particles were uniformly distributed in the tumor. Complete tumor remission was

Chapter 4 – Results and Discussion

achieved with MDT using the equivalent of only 20 % and 50 % of the systemic dosage of mitoxantrone.

4.8 Modeling

The details of the modeling performed to study the heat output from MNP in an AMF, the heating of the tumor by MNP and the drug release from dox loaded PNIPAM – iron oxide CNP are presented in this section.

4.8.1 Modeling of power output from iron oxide magnetic nanoparticles

The Rosensweig model [20] (section 2.8.1) was used to examine the effect of particle size on the heating power (SAR) of γ -Fe₂O₃ and Fe₃O₄ nanoparticles in a ferrofluid exposed to an AMF. The nanoparticles were assumed to be monodisperse and spherical. For bare MNP, the magnetic particle diameter D_M was taken to be the hydrodynamic diameter D_H ; for CNP, the particle diameter including the polymeric shell was considered as D_H except where stated. The analysis does not incorporate polydispersity, viscous and hysteresis losses, inter-particle interactions and changes in viscosity with increasing temperature. The parameters used for modeling were adapted from Ref.[20] and are listed in Table 4.10.

Particle properties such as D_M , D_H and K strongly influence the power output, as do AMF field strength and frequency. From the plots of τ_N , τ_B and τ for Fe₃O₄ MNP (Fig. 4.24a) and γ -Fe₂O₃ MNP (Fig. 4.24b) the influence of D_M and polymer shell thickness (which contributes to D_H) is clearly observed. As D_M increases, τ_N

Chapter 4 – Results and Discussion

increases very rapidly, while the change in τ_B due to an increase in D_H is less rapid; this is due to the exponential dependence of τ_N on the magnetic volume and the linear dependence of τ_B on the hydrodynamic volume (Eqs. 2.7 and 2.8). The effect of K is also prominent; the lower K of $\gamma\text{-Fe}_2\text{O}_3$ (4.6 kJ.m^{-3} v/s 32 kJ.m^{-3} for Fe_3O_4) results in lower τ_N and τ for the same D_M . This is because thermal energy $k_B T$ can ‘unlock’ the magnetic moment more easily by overcoming the lower anisotropy energy KV_M (Eq. 2.8).

Table 4.10 Parameters used for modeling of power output from iron oxide MNP in a ferrofluid exposed to an AMF.

Nanoparticle type	$\gamma\text{-Fe}_2\text{O}_3$	Fe_3O_4
MNP concentration in the ferrofluid (mg.ml^{-1})	10	10
Mass fraction of MNP in ferrofluid	1.93×10^{-4}	3.86×10^{-4}
Domain magnetization, M_d (kA.m^{-1})	414 [20]	446 [20]
Anisotropy constant, K (kJ.m^{-3})	4.6 [20]	23, 32, 41 [20]
Temperature, T (K)	310	310
Viscosity of carrier fluid, η (N.S.m^{-2})	6.92×10^{-4}	6.92×10^{-4}
AMF frequency f , (kHz)	50 – 800	50 – 800
AMF field strength, H_0 (kA.m^{-1})	4 – 20	4 – 20

Chapter 4 – Results and Discussion

Using the materials properties listed in Table 4.10, typical attempt time scale τ_0 of 10^{-9} s and observational time scale of 100 s, the superparamagnetic size limit can be calculated [13] as 14 – 17 nm for Fe_3O_4 MNP and 28 nm for $\gamma\text{-Fe}_2\text{O}_3$ MNP. In the superparamagnetic regime, at low AMF frequencies ($f\tau \ll 1$), power output increases as the square of the frequency. When $f\tau \gg 1$, the power loss is insensitive to frequency, the maximum power output occurs when $f\tau = 1$ [21], for e.g., τ of a 13 nm Fe_3O_4 MNP is 1.65×10^{-6} s, increasing f beyond 606 kHz will not significantly increase SAR.

Figure 4.24 Relaxation times for (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles as a function of magnetic particle size D_M and polymer shell thickness

Figs. 4.25a and 4.25b are plots of power output as a function of D_M and f for Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ MNP, respectively. The highest SAR is obtained for particle sizes in a narrow range of D_M . Within this range, the optimum D_M is inversely proportional to frequency. This inverse dependence is due to relaxation time (τ); $\tau_B < \tau_N$ at larger D_M and there is a lag between f and τ . As f increases, so does the lag, and τ_B

Chapter 4 – Results and Discussion

(and consequently τ) becomes independent of frequency. Therefore, as f increases, high power output is obtained from particles with smaller D_M and the dominant heat generation mechanism is Néel relaxation. For 375 kHz, the highest SAR is for bare $\gamma\text{-Fe}_2\text{O}_3$ MNP in the 23 – 24 nm D_M size range (Fig. 4.26a), and for bare Fe_3O_4 MNP D_M is in the 16 – 18 nm range (Fig. 4.26b). The SAR of Fe_3O_4 MNP is observed to be lower than that of $\gamma\text{-Fe}_2\text{O}_3$. The large difference between the K values (and therefore, τ) of the two types of iron oxides results in a difference in optimum size and power output. Examining the influence of K on the SAR of Fe_3O_4 MNP (Figs. 4.27a and 4.27b), an increase in K results in a drop in power output and a shift towards smaller D_M for maximum power output (in the regime where Néel relaxation dominates). Higher field strengths are required to ‘unlock’ the magnetic moment from the anisotropy and smaller values of D_M results in lower values of τ_N .

Figure 4.25 The influence of magnetic particle size D_M and AMF frequency f on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ MNP for field strength $H_0 = 4 \text{ kA.m}^{-1}$.

Chapter 4 – Results and Discussion

Figure 4.26 The influence of magnetic particle size D_M and field strength H_0 on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ MNP for frequency $f = 375 \text{ kHz}$.

Figure 4.27 The influence of magnetic particle size D_M and anisotropy constant K on the SAR of Fe_3O_4 MNP with respect to (a) frequency f for field strength $H_0 = 16 \text{ kA.m}^{-1}$ and (b) field strength H_0 for frequency $f = 375 \text{ kHz}$.

Chapter 4 – Results and Discussion

For simultaneous hyperthermia and drug release applications, a polymeric surface coating around the MNP is essential; however, this coating will increase D_H compared to a single bare MNP. After synthesis of MNP and coating with polymer, it is likely that the MNP will be present as clusters encapsulated in the polymer, or bare MNP clustered together due to inter-particle magnetic or other interactions; this will also increase D_H . In addition, changes in solvent, viscosity etc will change D_H and SAR. *When the particles are immobilized in tumor cells, heating mechanisms will be restricted to Néel relaxation losses and hysteresis heating [153, 205], heat generation from viscous losses and Brownian relaxation will be negligible. Therefore the D_M that maximizes Néel losses should be selected.* Figs. 4.28a and 4.28b show the effect of D_H on the SAR for fixed D_M of Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ CNP respectively at 375 kHz. MNP with 22 - 24 nm D_M for $\gamma\text{-Fe}_2\text{O}_3$ and 12 - 13 nm D_M for Fe_3O_4 are optimum for *in vivo* applications, they rely on Néel relaxation for power output and there is limited drop in SAR as D_H increases for such particles. The effect of polydispersity on the reduction of SAR has been previously modeled [20, 236] and experimentally studied [144, 236].

The importance of heating by Néel relaxation has been highlighted by other researchers [144, 153, 205, 236], their conclusions support the results of the current work. Gazeau *et al.* studied the effects of field parameters and materials properties on the SAR of $\gamma\text{-Fe}_2\text{O}_3$ and CoFe_2O_4 MNP [236], optimum particle size for maximum SAR shifted to smaller sizes for MNP with higher K . Brownian heating was available from a broader range of particle sizes, however Néel heating within the narrow optimum size range produced substantial heating. Néel heating from larger MNP produced higher SAR compared to smaller particles; this is also clearly

Chapter 4 – Results and Discussion

predicted in the current work where Néel heating of larger $\gamma\text{-Fe}_2\text{O}_3$ MNP (23 nm) yielded higher SAR than smaller Fe_3O_4 MNP (12 nm) (Fig. 4.28). Importantly, the same group also demonstrated that once the MNP are internalized by cells, heating occurs almost exclusively by Néel relaxation [153].

Figure 4.28 The influence of particle hydrodynamic diameter D_H on the SAR of (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles for a frequency $f = 375$ kHz and field strength $H_0 = 4$ kA.m^{-1}

Interestingly, Majetich *et al.* have reported that colloidal suspensions consisting of superparamagnetic iron oxide MNP clusters produce higher heating than dispersions of discrete MNP, a ‘blocking frequency’ above which the MNP exhibit superparamagnetic hysteresis behavior was also observed [152].

Chapter 4 – Results and Discussion

4.8.2 Modeling of tumor heating by optimized magnetic nanoparticles

The temperature rise and thermal coverage of tumors from AMF induced heating of MNP was modeled to determine the quantity of MNP required to raise tumor temperatures to hyperthermia levels. The following methodology was used:

- Tumors were assumed to be spherical with uniform distribution of CNP clusters in the tumor volume (Fig. 4.29a)
- Upon AMF application, a MNP cluster acts as a point heat source and heats up the surrounding tissue. The equation for heat conduction from a point source in a spherical volume (Eq. 4.1) [237] was used to calculate the temperature rise in the tumor after heating for 30 min for a range of power values of the point source. The maximum temperature in the tissue at a microscopic distance of 80 μm from the point heat source was fixed at thermoablative levels of 60 $^{\circ}\text{C}$. The point heat source model deviates from real-world behavior as the distance from the point source decreases to small values, hence a distance of 80 μm from the point source was chosen for fixing maximum tumor tissue temperature. Assuming a typical cell size of $\sim 10 - 20 \mu\text{m}$ [226], this corresponds to a distance of $\sim 4 - 8$ cells from the point source. The following equation was used to model the temperature distribution in the region surrounding the point heat source:

$$T_r = T_{\infty} + \frac{Q}{4\pi k_t r} \left[1 - \text{erf} \left(\frac{r}{2\sqrt{\alpha t}} \right) \right] \quad (\text{Eq. 4.1})$$

where T_r is the temperature at distance r from the point source, T_{∞} is the steady state temperature at the boundary of the localized spherical volume, Q is the power output from the MNP cluster point source, k_t is the thermal conductivity of the

Chapter 4 – Results and Discussion

tumor tissue, the thermal diffusivity $\alpha = \frac{k_t}{\rho_t C_t}$, ρ_t is the density of tumor tissue, C_t

is the specific heat capacity of the tumor tissue, and t is the time. The values used are listed in Table 4.11.

- Using Eq. 4.1, the power output from a single point source required to heat tumor tissue in the temperature range of 42 °C – 60 °C in 30 min was determined to be 12.5 mW by an iterative method. The temperature profile is shown in Fig. 4.29b.
- Using Eq. 4.1, the radius corresponding to ≥ 42 °C temperature coverage in the tumor from this 12.5 mW source was calculated to be 350 μm (Fig. 4.29b). The change in temperature with time at various distances from the point source was also calculated using Eq. 4.1 (Fig. 4.29c).
- The mass of MNP clusters required at each point source to achieve the required power output of 12.5 mW was calculated on the basis of the SAR of the MNP (Sec. 4.8.1). Particle sizes yielding maximum SAR were used for this calculation, i.e., 23 nm for $\gamma\text{-Fe}_2\text{O}_3$ and 12 nm for Fe_3O_4 (Sec. 4.8.1).
- For clinically relevant [29, 65] quantities (0.5 – 4 g) of MNP distributed uniformly in the tumor, the microscopic volume heated to a minimum temperature of 42 °C by each point source was summed to obtain the total tumor volume at hyperthermia temperatures.
- The volume corresponding to the total mass of MNP was subtracted from the overall tumor volume to yield the effective tumor volume heated by the MNP. The tumor volume at hyperthermia temperatures was divided by the

Chapter 4 – Results and Discussion

effective tumor volume to obtain the fraction of tumor volume experiencing hyperthermia.

Figure 4.29 (a) Schematic of the model used for calculation of temperature rise in the tumor from AMF induced MNP heating. Temperature profile in tumor tissue (b) heated by a 12.5 mW point heat source for 30 min (c) as a function of time and distance from point source.

Chapter 4 – Results and Discussion

Table 4.11 Parameters used for modeling the temperature of AMF induced heating from MNP distributed in the tumor.

AMF frequency (kHz)	375
AMF field strength (kA.m ⁻¹)	1 – 20
Exposure time, <i>t</i> (s)	1800
Tumor diameter (cm)	1 – 4
Normal tissue temperature, <i>T</i> _∞ (°C)	37 (310 K)
Hyperthermia tissue temperature, <i>T</i> _r (°C)	≥ 42 (≥ 315 K)
Thermal conductivity of tumor tissue, <i>k</i> _t (W.m ⁻¹ .K ⁻¹)	0.57 [238]
Density of tumor tissue, <i>ρ</i> _t (kg.m ⁻³)	1.04 x 10 ³ [238]
Specific heat capacity of tumor tissue, <i>C</i> _t (J.kg ⁻¹ .K ⁻¹)	3500 [204]

Modeling of tumor heating by MNP dispersed uniformly in the tumor reveals that hyperthermia coverage is dependent on the MNP concentration in the tumor and the applied field strength (Fig. 4.30). An increase in MNP concentration implies decreased field strength requirements. For Fe₃O₄ a field strength of 19.5 kA.m⁻¹ is required for hyperthermia in the tumor when MNP concentration in the tumor of ~0.12 g.ml⁻¹; this drops to 4 kA.m⁻¹ as the concentration increases to 1.13 g.ml⁻¹. A similar trend is seen for γ-Fe₂O₃ MNP but with lower *H*₀ requirements: 18 kA.m⁻¹ for 0.06 g.ml⁻¹ dropping to 4 kA.m⁻¹ for 0.3 g.ml⁻¹.

Chapter 4 – Results and Discussion

Figure 4.30 Field strength and MNP concentration requirements for complete 42 °C thermal coverage of tumors with 375 kHz AMF. Guide lines are power-law fits to data points.

As tumor size increases, increasing either the MNP mass or the applied AMF field strength can maintain hyperthermia temperatures (Fig 4.31). For instance, 0.5 g of Fe₃O₄ MNP fully heats up a 1.5 cm tumor when exposed to 11 kA.m⁻¹, increasing MNP mass to 1 g the same heating can be achieved with ~ 7 kA.m⁻¹ (Fig. 4.31a). Since their SAR is higher, tumors can be heated with lower concentrations of γ -Fe₂O₃ MNP (Fig. 4.31b) than that of Fe₃O₄ MNP.

Additionally, field strength and MNP mass requirements decrease significantly as target temperature is reduced. For low temperature hyperthermia in the temperature range of 39 °C – 41 °C (Fig. 1.3), a wide range of tumor sizes can be heated to 40 °C with MNP mass and AMF strengths much lower than that for target temperatures of 42 °C. An example is illustrated in Fig. 4.32; with 1 g of Fe₃O₄

Chapter 4 – Results and Discussion

MNP, a 2 cm tumor heats up completely to 42 °C with ~ 12.5 kA.m⁻¹, while full 40 °C coverage requires only 6 kA.m⁻¹.

Figure 4.31 MNP mass and field strength requirements for complete hyperthermia coverage in tumors of various sizes with an AMF of $f = 375$ kHz and $H_0 \leq 20$ kA.m⁻¹ for (a) Fe_3O_4 and (b) $\gamma\text{-Fe}_2\text{O}_3$. Guide lines are power-law fits to data

Figure 4.32 Comparison of 42 °C and 40 °C thermal coverage of tumors with 1 g of Fe_3O_4 MNP and 375 kHz AMF.

Chapter 4 – Results and Discussion

The temperature at the tumor boundary should not exceed 42 °C to prevent damage to healthy tissue surrounding the tumor. The safety requirement of a maximum temperature of 42 °C at the tumor boundary is included in the present work by explicitly limiting the temperatures of the localized spherical volume heated by the point source in the range of 42 °C – 60 °C. At the tumor boundary, outside this ‘localized hyperthermia volume’ ($r > 350 \mu\text{m}$), the temperature is below 42 °C (Fig. 4.29b) and decreases to 37 °C, hence blood perfusion effects in this region are implicitly accounted for by the 37 °C boundary temperature.

Modeling of hyperthermia in a tumor from AMF induced heating of MNP has been performed previously [202-204, 206, 207, 239-241]. In previous work, Rabin showed that intracellular hyperthermia was not feasible when MNP clusters are embedded in a single cell (size 10 – 20 μm) and surrounded by a large cellular network of MNP-free cells [226]. In the current model, the uniform distribution of MNP clusters and localized volume of tumor heated by each MNP cluster is used to determine thermal coverage of the tumor (Fig. 4.29a).

Andra *et al.* modeled the spatial distribution of temperature in a tumor from hysteresis heating of micron sized magnetic particles [202]. Using a finite concentric sphere model, Johnson *et al.* [203] calculated analytical and numerical solutions to the transient bioheat equation to optimize heating from MNP. For tumor heating with 13.8 mg of Fe_3O_4 per g of tumor tissue (vol fraction = 0.003) and 500 kHz, 6.5 kA.m^{-1} AMF, the steady state maximum temperature at the center was 45 °C, dropping to 40 °C at a distance of 1 cm. Dughiero *et al.* have used a finite element model to calculate temperature rise in tumors containing different

Chapter 4 – Results and Discussion

concentrations of MNP with optimized particle size [204] and 300 kHz, 3.3 kA.m^{-1} AMF. With 15 nm MNP and 3 wt % concentration, the temperature in a 3 cm tumor after 420 s of heating was 51.4°C , at the boundary of the tumor the temperature rapidly dropped to 42°C .

McHenry *et al.* have modeled the volumetric power loss from Fe_3O_4 , $\gamma\text{-Fe}_2\text{O}_3$, Fe, FePt, Co and FeCo in a 300 kHz, 39.7 kA.m^{-1} AMF using the Rosensweig model [242]. FeCo was found to yield the highest power output. It was also suggested that the presence of cobalt ferrite shell on the FeCo particles would provide a stable, biocompatible surface. Self regulated heating of ferrofluids with temperature dependent magnetization and anisotropy has also been modeled [243]. McHenry *et al.* have also modeled heating of a 10 ml ($r = 2.15 \text{ cm}$) tumor with FeCo MNP ferrofluid in a 267 kHz, 29.8 kA.m^{-1} AMF using analytical and finite element methods[241]; it was predicted that temperatures inside the tumor would reach thermoablation levels while the edges would undergo hyperthermia.

In human clinical trials [29, 65] a ferrofluid containing aminosilane coated 15 nm core MNP was used; the frequency was 100 kHz, and the highest tolerated field strength was greater than 10 kA.m^{-1} (in the head region). For tumor volumes ranging from 1.8 ml to 49.8 ml, the ferrofluid used was in the range of 3 ml to 12.5 ml. For an average MNP concentration of $\sim 64 \text{ mg.ml}^{-1}$ in tumor tissue, full hyperthermia coverage of greater than 42°C was achieved only in $\sim 30\%$ of the tumors, while 40°C coverage was achieved in more than 90 % of the tumors [65]. The authors suggested that increasing the field strengths would improve heating. This is consistent with the observations from the current model that for a given

Chapter 4 – Results and Discussion

mass of MNP, hyperthermia coverage can be increased in a tumor by increasing the tumor concentration or field strength.

4.8.3 Modeling of drug release from doxorubicin loaded PNIPAM – Fe₃O₄ composite nanoparticles

The drug release from dox loaded composite nanoparticles was modeled for different particle sizes at temperatures of 24 °C, 37 °C and using experimental data from drug release tests (section 4.5.2). The following methodology was used:

- The drug loaded composite nanoparticles were assumed to be spherical and monodisperse. The hydrodynamic diameter measured by DLS (Sec. 4.2.1) was taken as the particle diameter.
- The drug release was governed by diffusion, the diffusion coefficient D_{dox} was determined by fitting the experimental data to the equation for drug release (Eq. 4.2) from a monolithic spherical particle [244, 245].

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(-\frac{D_{dox} n^2 \pi^2 t}{R^2}\right) \quad (\text{Eq. 4.2})$$

where M_t is the cumulative mass of drug released in time t , M_∞ is the cumulative mass of drug released at infinite time, i.e., taken as the total drug loading of the particles, and R is the radius of the composite nanoparticle. The D_{dox} values calculated for each cumulative release period t and the related M_t/M_∞ values were used in Eq. 4.2 to calculate the corresponding release times for particles of various sizes.

Chapter 4 – Results and Discussion

The predicted dox release profiles from iron – oxide PNIPAM particles at 24 °C, 37 °C and 43 °C in PBS for various particle sizes are shown in Fig. 4.33. An initial burst release phase is seen, followed by slower, steady state release. The burst release phase becomes less prominent as particle size increases, and overall drug release also becomes slower. The experimental values are also shown for comparison.

Figure 4.33 Predicted *in vitro* dox release from dox loaded PNIPAM – iron oxide composite nanoparticle in PBS at (a) 24 °C (b) 37 °C and (c) 43 °C. Filled circles indicate experimental values.

Chapter 4 – Results and Discussion

Modeling of dox release from swollen dox loaded PNIPAM was performed to understand the effect of CNP size on the release kinetics. The model is based on diffusion controlled release from a spherical monolithic particle. It suggests that as CNP diameter increases, for a given release time, the cumulative mass fraction of released drug is lower at all three temperatures (Fig. 4.33). Hence, for slower release of the drug over an extended time period after the initial burst phase (Sec. 4.5), increasing the CNP diameter will be useful. This can be done by increasing the PNIPAM shell thickness, although it will result in decreased saturation magnetization of the CNP. However, this may be more attractive than dehydrating the dox loaded CNP, since thermoresponsive behavior can be retained.

The diffusion coefficient (D_{dox}) values calculated from experimental data of the current work (Fig. 4.18b) were comparable to values calculated from experimental data of previous reports (Table 4.12) using the above model.

Table 4.12 Comparison of the diffusion coefficient of doxorubicin D_{dox} of the current work with calculated values from literature for various temperatures.

D_{Dox} at 20 °C ($\text{cm}^2.\text{s}^{-1}$)	D_{Dox} at 37 °C ($\text{cm}^2.\text{s}^{-1}$)	D_{Dox} at 40 °C ($\text{cm}^2.\text{s}^{-1}$)	Reference
1.01×10^{-17}	4.13×10^{-17}	5.63×10^{-17}	Current work
1.7×10^{-16}	-	1.86×10^{-16}	Wang <i>et al</i> [164]*
1.92×10^{-18}	9.19×10^{-18}	1.42×10^{-17}	Misra <i>et al</i> [176]**
2.24×10^{-18}	1.75×10^{-17}	1.76×10^{-17}	Misra <i>et al</i> [173]**
*Hydrodynamic diameter = 589 nm ** Hydrodynamic diameter assumed to be 158 nm			

Chapter 4 – Results and Discussion

For clinical chemotherapy, the dox dosage administered intravenously is based on body surface area of the patient and is usually in the range of 60 – 75 mg.m⁻² every three weeks [246]. However, with MDT, equivalent chemotherapeutic effect can be achieved with doses as low as 20 – 50 % of the conventional dose [24]. For a patient of height 160 cm and weight 55 kg, body surface area can be calculated as 1.56 m² using the Mosteller formula [247]. Assuming a 70 mg.m⁻² dox dosage, the quantity of dox required for intravenous administration is ~ 110 mg. Conservatively assuming 50 % of this dosage (55 mg) for equivalent therapeutic efficiency in MDT, and 4 wt % dox loading of the PNIPAM – iron oxide CNP, the quantity of CNP required is 1375 g. Making another conservative assumption that only 75 % of the CNP reach the targeted tumor, the total quantity of CNP required for MDT is ~1835 mg. These are conservative assumptions and the actual quantity of drug loaded CNP required is likely to be lower, especially for smaller tumors, and also depend on the actual cytotoxic drug loaded on to the particles. The mass of MNP required for hyperthermia of tumors smaller than ~2.5 cm (Fig. 4.31) with clinically relevant AMF parameters is in the same range (~ 2 g) as that required for MDT. *These calculations suggest that the quantity of dox loaded CNP required for hyperthermia and chemotherapy treatments match closely, increasing the feasibility of these particles for combined hyperthermia and drug release applications.* For large tumors that require higher a higher mass of MNP for hyperthermia, the drug loading on the particles can be easily decreased as per the dosage requirements for chemotherapy.

The major conclusions of the present work are summarized in the next chapter.

Chapter 5 – Conclusions

CHAPTER 5

CONCLUSIONS

This chapter lists the significant findings of the present work.

5.1 Conclusions

Cancer therapy utilizing the synergistic effect of simultaneous hyperthermia and drug delivery in the tumor has the twin advantages of minimizing systemic toxicity as well as improving therapeutic efficacy. In the present work, drug loaded thermoresponsive magnetic nanoparticles comprising of an iron oxide core and PNIPAM shell loaded with the anti-cancer drug doxorubicin were studied for combined magnetic drug targeting, hyperthermia and controlled drug release applications. These particles were synthesized by chemical methods and extensively characterized using TEM, VSM, XRD, TGA MDSC, AFM and DLS. Their *in vitro* hyperthermia and controlled drug release as well as simultaneous hyperthermia and drug release performance was investigated. *In vitro* cytotoxicity of the particles was studied using BEAS-2B human epithelial cells. *In vivo* magnetic drug targeting to a liver tumor was performed on a Buffalo rat model. The heat generation of these particles in an alternating magnetic field, the heating of tumors to hyperthermia temperatures and drug release from these particles was modeled. The conclusions of the study are summarized:

- γ -Fe₂O₃ nanoparticles of average size 12 nm, 14 nm, 19 nm and 43 nm were synthesized by a high temperature decomposition process; Fe₃O₄ nanoparticles of average size 6 nm and 13 nm were prepared by co-

Chapter 5 – Conclusions

precipitation. The co-precipitation method was better suited for producing nanoparticles necessary for biomedical applications.

- The 43 nm γ -Fe₂O₃ and 13 nm Fe₃O₄ particles were coated with the thermoresponsive polymer poly-n-(isopropylacrylamide) via a simple, facile and easily scalable coating process. Encapsulation of the particles by PNIPAM was confirmed by TGA, VSM and DLS. The PNIPAM - γ -Fe₂O₃ composite particles contained 6 wt% of PNIPAM and the PNIPAM – Fe₃O₄ particles contained 1.3 wt % of PNIPAM. These particles were loaded with therapeutically relevant quantities of the cytotoxic drug doxorubicin in the range of 2.5 – 4.1 % of particle weight using a simple physical process of incubating the particles in dox solution. Swollen PNIPAM – Fe₃O₄ particles clearly exhibited thermoresponsive behavior; this was preserved with drug loading. The PNIPAM shell of the particles underwent a reversible volume shrinkage at a lower critical solution temperature of ~ 35 °C. Drug loading did not alter this thermoresponsive behavior. In contrast, vacuum dehydration of drug loaded particles was found to be detrimental to thermoresponsive behavior.
- The synthesized particles exhibited excellent magnetic properties with a maximum saturation magnetization of 61 emu.g⁻¹ and low coercivity. The magnetic properties were not significantly affected by the PNIPAM shell or by drug loading, saturation magnetization values exceeded, or compared favorably to values reported in literature. These magnetic properties suggest their suitability for magnetic drug targeting applications.

Chapter 5 – Conclusions

- The particles exhibited excellent *in vitro* hyperthermia performance in a 375 kHz alternating magnetic field even with low particle concentrations and applied field strength. This was partly due to careful selection of particle sizes for experimental studies, based on the results of modeling of heat generation from iron oxide nanoparticles in an alternating magnetic field. Iron oxide particles with a PNIPAM shell showed better heating performance than bare iron oxide particles.
- When an alternating magnetic field was applied, simultaneous *in vitro* hyperthermia and therapeutically relevant drug release from dox loaded PNIPAM coated iron oxide nanoparticles was clearly demonstrated. Up to 42.4 % of the loaded dox was released in 1 h of hyperthermia, strongly suggesting the suitability of these particles for multimodal cancer therapy.
- In the absence of a magnetic field, swollen dox loaded PNIPAM – iron oxide nanoparticles demonstrated temperature dependent drug release in phosphate buffer saline (pH 7.4) at 24 °C, 37 °C and 43 °C. Drug release was significantly higher at temperatures above the LCST of the PNIPAM shell, demonstrating the thermoresponsive nature of these particles, with 63.7 % of the loaded dox released in 29 h at 37 °C. In contrast, drug release from vacuum dehydrated dox loaded particles was less dependent on temperature due to the absence of a distinct LCST. In all cases the release mechanism was found to be not governed solely by diffusion.
- Controlled drug release tests involving cycling of temperature above and below the LCST demonstrated that the LCST could be used as an ‘on/off’

Chapter 5 – Conclusions

switch to regulate drug release from swollen dox loaded PNIPAM – iron oxide particles. Below the LCST, drug release was nearly zero, release could be triggered by raising the temperature above the LCST, again demonstrating the thermoresponsive nature of these particles.

- Low cytotoxicity of the PNIPAM coated Fe_3O_4 nanoparticles were suggested by *in vitro* cytotoxicity tests on BEAS-2B human lung endothelial cells. At concentrations of 0.1 mg.ml^{-1} and 0.05 mg.ml^{-1} , 48 h cell viability was in the range of $\sim 88\% - 97\%$. In contrast, dox loaded particles killed the majority of the cells.
- *In vivo* magnetic drug targeting of dox loaded 43 nm core PNIPAM – $\gamma\text{-Fe}_2\text{O}_3$ particles to a liver tumor in a Buffalo rat was successfully demonstrated. The localization of the particles in the tumor without systemic distribution in other organs was confirmed via MRI and histology. In conjunction with *in vitro* cytotoxicity tests, this result suggests considerable promise for magnetic drug targeting applications.
- Modeling of heat generation from iron oxide nanoparticles in an alternating magnetic field showed that for a 375 kHz field frequency, the optimum particle size for Fe_3O_4 particles is in the 12 – 13 nm range and for $\gamma\text{-Fe}_2\text{O}_3$ particles in the 23 – 24 nm range. For these particle sizes heating power will not decrease in the absence of Brownian relaxation when the particles are immobilized in tumor cells. These particles generate heat primarily by Néel relaxation, therefore an increase in shell thickness due to polymer coating will not adversely affect heat generation. $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles

Chapter 5 – Conclusions

generate more heat (but at different optimum sizes) than Fe_3O_4 nanoparticles.

- Modeling of alternating magnetic field induced heating of tumors by iron oxide nanoparticles uniformly distributed in the tumor reveals that the nanoparticle concentration as well as field strength plays an important role in determining thermal coverage. Tumors can be heated up to hyperthermia temperatures by increasing either the nanoparticle concentration in the tumor tissue or by increasing the field strength.
- Modeling of dox release from dox loaded PNIPAM – iron oxide particles shows that dox release occurs faster as polymer shell thickness decreases. Calculations of the nanoparticle quantities required to deliver chemotherapeutic doses of dox via magnetic drug targeting in a clinical situation suggest that the overall particle quantities required for hyperthermia and chemotherapy are similar, favoring the use of these particles for combined hyperthermia and chemotherapy applications.

In summary, this work has demonstrated that drug loaded thermoresponsive poly-n-(isopropylacrylamide) coated iron oxide nanoparticles hold great promise for combined drug targeting, hyperthermia and controlled chemotherapy treatment of cancer.

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Appendix – A

APPENDIX – A

***In vivo* magnetic drug targeting and magnetic resonance imaging.**

In vivo MDT and MRI studies with dox loaded γ -Fe₂O₃ CNP were carried out at the Dept. of Experimental Surgery and the Radiology Dept. of Singapore General Hospital [221, 248] by Ms. Kee I.H.C and Dr. H. Rumpel. To visualize the particles *in vivo*, preliminary *in vitro* MRI scans were performed on γ -Fe₂O₃ MNP immobilized in a polyvinyl alcohol gel to determine the concentration of the ferrofluid required for optimum MRI signal, this concentration was found to be 0.36 mg.ml⁻¹. Male buffalo rats (Charles River, USA) were laparotomized and Morris Hepatoma 7777 cells (ATCC, USA) were implanted into the liver to induce development of the hepatocellular carcinoma. Two weeks after implantation, MRI of the rat was performed using a custom-built MRI coil in a Siemens MRI scanner (Magnetom Allegra) to confirm that the HCC had developed in the liver, this scan also served as the baseline pre-treatment scan. A few days after the baseline scan, 0.5 ml of the ferrofluid containing dox loaded γ -Fe₂O₃ CNP (conc. 0.36 mg.ml⁻¹) was injected via the main hepatic artery of the rat (Fig A.1a). An external magnetic field was simultaneously applied by a permanent magnet (field strength 0.8T) placed externally over the liver. 30 min post injection MRI was performed to check for particle distribution (Fig A.1b). The rat was euthanized after the MRI scan and the intra-abdominal viscera were harvested to check for the presence of iron oxide particles.

Appendix – A

Figure A.1 *In vivo* magnetic drug targeting experiments on a Buffalo rat: (a) injection of dox loaded $\gamma\text{-Fe}_2\text{O}_3$ CNP (b) Buffalo rat in the custom built MRI coil.

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