Syntheses of Iron Oxide and Other Transition Metal Oxide Nanoparticles, and Their Modifications for Biomedical Applications

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Abstract

Superparamagnetic iron oxide (Fe₃O₄ and γ-Fe₂O₃) nanoparticles (IONPs) are of great interest as a diagnostic and/or therapeutic aid. Several IONPs with biocompatible polymer coatings have been approved for clinical use, as MRI contrast agents. IONPs conjugated to targeting ligands and therapeutic agents are being investigated for targeted drug delivery applications. The superparamagnetic properties of IONPs are also helpful for magnetic field assisted localization to specific target sites and for in situ MRI applications. This thesis primarily focuses on the synthesis and surface modifications (with biocompatible polymers including dextran, poly(ethylene glycol) (PEG), dextran, poly(ethyl methacrylate) (PEMA), poly(hydroxyethyl methacrylate) (PHEMA), etc.) of IONPs. The IONPs were prepared following the classical co-precipitation method and a novel reduction-hydrolysis method. Initial studies used bovine serum albumin (BSA) to examine the capabilities of polymer coated IONP to deliver a model protein therapeutic. Gel migration studies using BSA physisorbed onto polymer coated IONP under gradient magnetic field of an MRI showed that the IONPs had limited control in transporting the protein. Covalent linking of therapeutics to IONP core can improve the time window of formers controllability using magnetic field. To facilitate covalent conjugations, functional silane coated IONPs (with surface amino and carboxylic acid) were prepared as general precursors. The utility of silane coated IONPs for bioconjugations was demonstrated by cova-
lently linking PEG diacid through surface amino groups and by linking of BSA through surface carboxylic acid groups. The biocompatibility of the IONPs synthesized following the novel reduction-hydrolysis method were assessed in vitro on cell culture models using toxicity assays. The versatile reduction-hydrolysis method was further extended, as a general method to prepare several early transition metal oxide NPs (manganese oxide (Mn$_3$O$_4$), cobalt oxide (Co$_3$O$_4$), nickel/nickel oxide (Ni/NiO), copper/copper oxide (Cu/Cu$_2$O) and zinc oxide (ZnO) NPs), silica nanoparticles with surface IONPs, and iron/iron oxide nanosheets.
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Dedicated to my parents and my wife...
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# List of Abbreviations and Symbols

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<tbody>
<tr>
<td>$\Delta G^*$</td>
<td>Critical free energy</td>
</tr>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>AEM</td>
<td>Analytical EM</td>
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<tr>
<td>AmS</td>
<td>Aminosilane</td>
</tr>
<tr>
<td>APTES</td>
<td>3-Aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
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<tr>
<td>BrMPA</td>
<td>2-Bromo-2-methyl propionic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Caco2</td>
<td>Caucasian colon adenocarcinoma cell-line</td>
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<tr>
<td>CED</td>
<td>Convection-Enhanced Delivery</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>Co(acac)$_3$</td>
<td>Cobalt(III) acetylacetonate</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>$D_H$</td>
<td>Hydrodynamic diameter</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>Abbreviation</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>DP</td>
<td>Diffraction Pattern</td>
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<tr>
<td>DRIFT</td>
<td>Diffused Reflectance Infrared Fourier Transform</td>
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<tr>
<td>$D_{SD}$</td>
<td>Critical diameter (Single-domain)</td>
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<tr>
<td>$E^\circ$</td>
<td>Standard Electrode Potential</td>
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<tr>
<td>EDLC(s)</td>
<td>Electric Double-Layer Capacitor(s)</td>
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<td>EDS</td>
<td>Energy-Dispersive Spectrometry</td>
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<td>Electron-Energy Loss Spectrometry</td>
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<td>EM</td>
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<td>EMA</td>
<td>Ethyl methacrylate</td>
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<tr>
<td>ET</td>
<td>Elevated Temperature</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FDA</td>
<td>Food and Drug Administration (USA)</td>
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<td>Fe(acac)$_3$</td>
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<tr>
<td>FID</td>
<td>Free Induction Decay</td>
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<td>Flourescein isothiocyanate</td>
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<td>FT-IR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<td>GA</td>
<td>L-Glutamic acid</td>
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<td>$G_{FE}$</td>
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<td>$G_{SS}$</td>
<td>Slice-Selection Gradient</td>
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<tr>
<td>$H$</td>
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<tr>
<td>$H_c$</td>
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<td>HE</td>
<td>Hemotoxylin-Eosin</td>
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<td>HepG2</td>
<td>Liver Hepatocellular cell-line</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HR-TEM</td>
<td>High Resolution TEM</td>
</tr>
<tr>
<td>$I$</td>
<td>Nuclear spin quantum number</td>
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<tr>
<td>ICCD</td>
<td>International Centre for Diffraction Data</td>
</tr>
<tr>
<td>IES</td>
<td>Interendothelial cell slits</td>
</tr>
<tr>
<td>IO</td>
<td>Iron Oxide</td>
</tr>
<tr>
<td>IO cs-NPs</td>
<td>Iron Oxide core–shell Nanoparticles</td>
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<td>IONP(s)</td>
<td>Iron Oxide Nanoparticle(s)</td>
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<td>IR</td>
<td>Infrared</td>
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<td>IS</td>
<td>Isomer Shift</td>
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<td>L-Arginine</td>
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<td>LC</td>
<td>Liquid crystal</td>
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<td>$M$</td>
<td>Magnetization</td>
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<td>MCT</td>
<td>HgCdTe</td>
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<td>MDDC</td>
<td>Magnetically Directed Drug Convection</td>
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<td>Mn(acac)$_3$</td>
<td>Manganese(III) acetylacetonate</td>
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<tr>
<td>MONP(s)</td>
<td>Metal Oxide Nanoparticle(s)</td>
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<tr>
<td>$M_r$</td>
<td>Remnant magnetization</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>MRM</td>
<td>Magnetic Resonance Microscopy</td>
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<tr>
<td>MRN</td>
<td>Magnetic Resonance Navigation</td>
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<tr>
<td>$M_s$</td>
<td>Saturation Magnetization</td>
</tr>
<tr>
<td>MT</td>
<td>Magnetic field assisted Targeting</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
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<tr>
<td>Ni(acac)$_2$</td>
<td>Nickel(II) acetylacetonate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP(s)</td>
<td>Nanoparticle(s)</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>OAm</td>
<td>Oleylamine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Silane</td>
</tr>
<tr>
<td>PC</td>
<td>Precursor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
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<tr>
<td>PEMA</td>
<td>Poly(ethyl methacrylate)</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PGA</td>
<td>Poly(glycerol monoacrylate)</td>
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<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
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<tr>
<td>PMDETA</td>
<td>N,N,N',N',N''-Pentamethyl diethylenetriamine</td>
</tr>
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<td>POM</td>
<td>Polarized Optical Microscopy</td>
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<tr>
<td>PVDF</td>
<td>Poly(vinylidene fluoride)</td>
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<tr>
<td>QS</td>
<td>Quadrupole Shift</td>
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<tr>
<td>$r^*$</td>
<td>Critical radius</td>
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<tr>
<td>$R_1$</td>
<td>Longitudinal relaxation rate</td>
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<tr>
<td>$r_1$</td>
<td>Longitudinal Relaxivity</td>
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<tr>
<td>$R_2$</td>
<td>Transverse relaxation rate</td>
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<tr>
<td>$r_2$</td>
<td>Transverse Relaxivity</td>
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<tr>
<td>$R_2^*$</td>
<td>Net transverse relaxation rate</td>
</tr>
<tr>
<td>RARE</td>
<td>Rapid Acquisition with Relaxation Enhancement</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
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</table>
$R_H$  
Hydrodynamic radius

$R_N$  
Rate of homogeneous nucleation

RPM  
Rotations Per Minute

RT  
Room Temperature

$S$  
Supersaturation

$S^*$  
Critical Supersaturation

SAED  
Selected Area Electron Diffraction

SAXS  
Small-angle X-ray scattering

SD  
Single-Domain

SEM  
Scanning electron microscopy

siRNA  
Small interfering RNA

$T_1$  
Longitudinal relaxation time

$T_2$  
Transverse relaxation time

$T_2^*$  
Net transverse relaxation time

$T_B$  
Blocking Temperature

TE  
Echo Time

TEM  
Transmission electron microscopy

TEOS  
Tetraethyl orthosilicate

TGA  
Thermogravimetric Analysis

TGS  
Tri-glycine sulfate

THF  
Tetrahydrofuran

TJ  
Tight Junction

TMAOH  
Tetramethylammonium hydroxide

TN(s)  
Theranostic(s)

TR  
Slice-selection Pulse Repeat time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>Powder X-ray Diffraction</td>
</tr>
<tr>
<td>Zn(acac)_2</td>
<td>Zinc(II) acetylacetonate</td>
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Chapter 1:

General Introduction
1.1 Nanoscience: How it came to be?

Nanoscience refers to the science of materials or objects that has at least one dimension falling inside the nanoscale. The International Organization for Standardization (ISO) in its publication ‘ISO TS 8004–1 Nanotechnologies — Vocabulary — Part 1: Core Terms’ defines nanoscale as the size range of 1 to 100 nm. Health Canada in its working definition of nanomaterials considers “any manufactured substance or product and any component material, ingredient, device or structure to be nanomaterial if: (a) it is at or within the nanoscale (1-100nm) in at least one external dimension or has internal or surface structure at the nanoscale; or (b) it is smaller or larger than the nanoscale in all dimensions and exhibits one or more nanoscale properties/phenomena”.¹

1 nm is one billionth of a meter; to put nanoscale into perspective, the diameter of a human hair is ≈100,000 nm, a red blood cell is ≈8,000 nm, small blood capillaries are ≈5,000–10,000 nm, and the human immunodeficiency virus (HIV) is ≈130 nm (Figure 1.1). Many biological processes involve nanoscale materials, e.g., DNA (diameter ≈2 nm), Haemoglobin (5.5 nm), etc. Haemoglobin, made up of a protein with four highly folded polypeptide chains encompassing four iron-containing heme groups, is an example for an ideal nanocarrier; it reversibly binds and transports the otherwise plasma-insoluble oxygen through blood and also removes and transports carbon dioxide as by-product through blood. In terms of human endeavours, we have prepared nanomaterials since ancient times. Analysis of Bronze Age (1200 – 1000 BC) stained glass, excavated from Northern Italy reveal the use of copper based nanocrystals for producing color.² The di-
1.1 Nanoscience: How it came to be?

Chromatic Lycurgus cup from 400 AD Roman era had silver/gold nanoparticles (NPs) in the glass, and displayed interesting optical effects by appearing wine-red in transmitted light and pea-green in reflected light (Figure 1.2). In the modern age (post industrial revolution), Michael Faraday published one of the earliest studies on the optical properties of colloidal gold, in which he described the observed color as “ruby”. These optical effects observed in nanoscale Cu, Ag and Au particles are based on their now well understood surface plasmon resonance effect. It was only after the invention of transmission electron microscopy (TEM, Section 1.3.2) in the 1930s, that the literal visualization and studies on size-dependent properties of nanomaterials was made possible.
Figure 1.1. Comparison of nanoscale with sizes of some known objects: A) atomic force microscopy image of a pentacene molecule; atoms and small molecules measure less than 1 nm, B) high resolution TEM image of crystalline magnetite (Fe₃O₄) nanoparticle (∼5 nm) with visible layers of Fe atoms in 311 planes; nanomaterials have at least one dimension falling inside the nanoscale (1–100 nm), C) TEM image of a HIV (Dartmouth Electron Microscope facility); sizes of virus range from 10 to few 100 nm; D) SEM image of red blood cells (Dartmouth Electron Microscope facility), E) SEM image of a single strand of human hair (diameter ∼ 67000 nm or 0.07 mm) (University of Minnesota, Duluth, Research Instrumentation Laboratory) and F) photograph of a worker honeybee (∼10 mm) in Manitoba, Canada (Photograph by Vinith Yathindranath); Insects measure from around 1 mm to few 10 mm.
Richard Feynman, in his famous 1959 talk “There’s Plenty of Room at the Bottom” presented perhaps the earliest idea of atomic scale arrangement and manipulation while manufacturing devices. There was the awareness then that a small cluster of atoms may experience quantum confinement effects and exhibit unique physical and chemical properties that are non-existent in their bulk-size counterparts. The term nanotechnology was coined by Prof. Norio Taniguchi in 1974, and it was in the late 20th century that nanoscience and nanotechnology was truly launched into its present day trajectory. With an arsenal of new tools and instrumental techniques including TEM, scanning electron microscopy (SEM, Section 1.3.2), scanning tunnelling microscopy (STM), atomic force microscopy (AFM), etc., scientists have made tremendous strides in studying nanomaterials and their size dependent properties. The nanosize-dependent optical (surface Plasmon resonance of gold NPs), electrical (semiconductor properties of CdSe quantum dots), and magnetic (superparamagnetism in Fe$_3$O$_4$ NPs) properties of materials have been widely
investigated. Another key aspect of a nanomaterial is its high surface area compared to the equivalent quantity of bulk material; e.g. when a gold sphere of 1 cm in diameter (Surface area: 3.14 cm$^2$) is broken down into tiny nanospheres of 5 nm diameter, it will have a collective surface area of $\approx 595$ m$^2$ (6,400 square feet). The large surface area of nanomaterials enhances among others their surface reactivity and binding property.

At present, nanoscience has its presence in most branches of science and engineering. It is widely seen as a field that has the potential to improve the quality of life of people, by benefiting fields including biomedical, green energy, energy storage, sensors, catalysis, environmental remediation, etc. Since the year 2000, governments and businesses in more than sixty countries have established their own nanotechnology initiatives. United States, China, Germany and Japan are leading in terms of government spending on nanoscience research and volume of publication (2008–2009). United States through its National Nanotechnology Initiative (NNI) has invested a total of 18 billion US dollars (USD) since 2001, which includes around 1.8 billion USD allocated during 2013 budget period alone. Among developing economies, the Indian government in its eleventh five year plan (2007–2012) allocated 250 million USD for India’s “Nano Mission”. The sustained investment through global recession of 2008 shows the continuing commitment and high hopes that the scientific community, policy makers and the society at large have on nanoscience.

With the hype surrounding nanotechnology, sceptics have their reservations on its growth predictions, practicality and safety. Nanotechnology industries in some parts of the world have faced attacks from eco-anarchist groups. The concern with nanotech-
nano1ogy lies with the fact that novel physicochemical properties exhibited by nanomateri-als may cause novel human health or environmental hazard. In the past, Biotechnologies, especially genetically modified food and stem cell research faced greater public aversion mainly due to lack of awareness. Similarly, nanotechnologies may face public aversion if effective early risk assessments and policies are not put in place. A meta-analysis of data compiled from surveys published in 22 papers between 2004 and 2009 on nanotechnology risk perception across United States, Canada, Europe and Japan revealed that more than 51% knew ‘nothing at all’ about nanoscience. Understanding public perception on nanotechnologies for effective policy making is an ongoing process. The Government of Canada and USA have already created a Regulatory Cooperation Council in 2011 to create common policies to regulate and oversee actual and emerging applications of nanotechnology and nanomaterials. Such proactive Government policies and effective risk assessments across the globe is crucial to prevent any unanticipated catastrophe and boost public confidence.

Nanoscience has seen considerable growth in the 21st century; it is already a well-established area of research in academia and industries and is more likely to enjoy greater prominence in the years to come.

1.2 Superparamagnetic iron oxide nanoparticles

Superparamagnetic iron oxide (Fe\textsubscript{3}O\textsubscript{4}/\gamma-Fe\textsubscript{2}O\textsubscript{3}) nanoparticles (IONPs) are one of the widely studied nanomaterials. Magnetite (Fe\textsubscript{3}O\textsubscript{4}) and maghemite (\gamma-Fe\textsubscript{2}O\textsubscript{3}) phases are
biocompatible (FDA approved), chemically stable, and possess high magnetic moments after elemental iron, which makes them a highly desirable material for biomedical applications (Section 1.2.3). Iron under ambient conditions and at the nanoscale is unstable and readily oxidizes/rusts to haematite and goethite which are weakly magnetic. Magnetite at ambient conditions may oxidize to form stable maghemite phase. In this thesis, in the places where the presence of a single magnetite or maghemite phase is known with certainty based on characterization or from data published in the literature, the actual phase name will be used to introduce them; in places where there is uncertainty, the material will be introduced as iron oxide (IO). It is a common practice especially in the biomedical field to describe magnetite and maghemite phases as just IO though iron forms other oxide phases (FeO – wustite, $\alpha$–Fe$_2$O$_3$ – hematite, etc.). Some papers may use abbreviations like SPIONs (superparamagnetic iron oxide NPs), USPIONs (ultrasmall superparamagnetic iron oxide NPs), or MIONs (magnetic iron oxide NPs) to describe magnetite and maghemite NPs.

Researchers have been preparing IONPs well before modern applications were realized. Elmore in 1938 precipitated magnetite particles and peptized them by washing the particles with 0.01N HCl and adding the particles to aqueous soap (sodium oleate) solution. He observed that some of the particles showed substantial demagnetization depending on particle concentration.33 This is believed to be the earliest reference to superparamagnetic behaviour, but the work predated TEM usage to study the sizes of the particles. In 1959, Bean and Livingston first coined the term superparamagnetism to describe the
unique nanosize dependent magnetic behaviour of very small ferromagnetic particles;\(^9\) the term superparamagnetism and its characteristics will be discussed later in this section. Papell S. S., in 1965 prepared oleic acid stabilized magnetite (100–200 nm) colloidal suspension in rocket fuel, following a top-down ball milling method.\(^{34}\) Kaiser and Miscolcz adapted Papell’s top-down method to prepare magnetite NPs (diameter < 10 nm, from TEM) with sub-single domain sizes by ball-milling magnetite particles in carrier fluids (kerosene, fluorocarbon or water) for more than 41 days.\(^{35}\) It is among the earliest reports where TEM was used to study the size dependant superparamagnetic properties of magnetite NPs. In the early and mid twentieth century, unique properties/applications of magnetic fluids or ferrofluids (colloidal magnetic particle suspension stable even in the presence of strong magnetic field) including magnetocaloric power cycle,\(^{36}\) the magnetoviscous effect,\(^{37}\) the dynamic stability of ferrofluid interfaces, buoyancy in magnetic fluids,\(^{38}\) magnetic-fluid seals,\(^{39}\) etc. were widely investigated. Modern bottom-up synthetic approaches and recent advances in preparing superparamagnetic IONPs will be discussed later in section 1.2.1.

Magnetite in bulk scale is a ferrimagnetic mineral and an abundant natural ore (72.36% Fe) for iron. Tiny magnetite crystals are naturally formed in some life forms such as homing pigeons (beak),\(^{40}\) honey bee (stomach),\(^{41}\) sharks\(^{42,43}\) and magnetotactic bacteria.\(^{44}\) These creatures use the biogenic magnetite for sensing and mapping of the earth’s magnetic field for navigational purposes. Magnetite has an inverse-spinel structure in which oxygen atoms form a close-packed face-centred-cubic (ABCABC type) lat-
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tice. The interstitial tetrahedral sites are occupied by Fe$^{3+}$ and the octahedral sites are occupied by both Fe$^{3+}$ and Fe$^{2+}$ ions (Figure 1.3A and B). The tetrahedrally coordinated Fe$^{3+}$ and octahedrally coordinated Fe$^{2+}$ and Fe$^{3+}$ atoms are arranged in alternative coplanar layers perpendicular to the $hkl = 111$ (Miller index, Section 1.3.1) plane (Figure 1.3C) and for every tetrahedral Fe$^{3+}$, there are corresponding octahedral pair comprising Fe$^{3+}$ and Fe$^{2+}$. The atomic magnetic moments of iron cations within a layer are aligned whereas between the layers are anti-parallel. As a result, the magnetic moments of Fe$^{3+}$ [5 $\mu_B$ (Bohr magneton)] in tetrahedral and octahedral sites cancel each other and the net magnetization arises from octahedral Fe$^{2+}$ (4 $\mu_B$) (Figure 1.3D).
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**Figure 1.3.** Magnetite (Fe₃O₄) showing two types of Fe layers in 111 plane: A) Fe ions in bulk octahedral sites and B) Fe ions in bulk octahedral and tetrahedral sites. C) The arrangement of Fe layers and O in face-centred-cubic (ABCABC) planes. Reproduced with permission from reference 45, copyright © 1996, The American Physical Society. D) In the inverse-spinel structure, tetrahedral sublattice is occupied by Fe³⁺ and octahedral sublattice is occupied by equal number of Fe³⁺ and Fe²⁺ and has a net magnetic moment resulting from Fe²⁺ magnetic moments.

Bulk scale IO is comprised of several uniform magnetization domains separated by domain walls (Figure 1.4A and B). The formation of domain walls is governed by the magnetostatic and domain wall energies. Magnetostatic energy increases proportionally with the size of the material and domain wall energy increases proportionally with the interfacial area. When a particle size is reduced below a critical diameter ($D_{SD}$), it is energetically favourable to have a single-domain (SD) of magnetization than to have domain
walls (Figure 1.4D and E). The SD threshold for Fe$_3$O$_4$ is 76 nm.$^{46}$ The two orientations of magnetization (parallel and antiparallel, ↑ and ↓) of SD particles (with uniaxial anisotropy and diameter less than $D_{SD}$) along the easy axis ($hkl = 111$ for IONPs) are separated by an energy barrier $E = KV$, where $E$ is the magnetic anisotropy energy, $K$ is the anisotropic constant and $V$ is the NP volume (Figure 1.5A). The average time for flipping between two magnetization directions or Néel relaxation time ($\tau_N$) is given by Néel-Arrhenius law:

$$\tau_N = \tau_0 e^{KV/kT} \quad \text{Eq. 1.1}$$

where $\tau_0$ is the intrinsic magnetization reversal time ($\tau_0 \approx 10^{-9}$ s), $K$ is the magnetic anisotropy constant, $V$ is the volume of the NP, $k$ is the Boltzmann constant and $T$ is the temperature.$^{47}$ On bulk scales, $KV/kT \gg 1$ and relaxation time $\tau_N$ is extremely long. When the diameter of the SD IO is further reduced below their superparamagnetic threshold ($D_{SP}$, 50 nm for Fe$_3$O$_4$), $KV$ is comparable to $kT$ (thermal energy), above blocking temperature ($T_B$) and magnetization vector flips rapidly along easy axis.$^{47}$ The rapid flipping of superparamagnetic NPs cannot be resolved with magnetization instrument which usually has an acquisition time ($\tau_a = 1$ s) larger than $\tau_N$. In spite of having the atomic magnetic moment coupled together, superparamagnetic NPs do not show characteristic ferro/ferrimagnetic behaviour during magnetization studies. The illustrative magnetization ($M$) vs. magnetic field ($H$) curves for bulk ferro/ferrimagnetic and superparamagnetic materials are shown in Figure 1.4 C and F, respectively. Superparamagnetic NPs exhibit little remnant magnetization ($M_r$) and coercivity ($H_c$) post perturbation, as if
they are paramagnetic, but contrary to paramagnetic materials possess a high saturation magnetization ($M_s$). This unique nanoscale magnetic property of IONPs is the basis for its application as susceptibility contrast agents for MRI, heat mediators for tumour thermo-therapy and drug carriers for magnetically directed drug delivery applications.

**Figure 1.4.** A) Bulk scale ferrimagnetic IO with randomly oriented domain magnetization vectors. B) In the presence of an applied magnetic field ($H$), the magnetization vectors in every domain align parallel to the direction of $H$, at saturation magnetization ($M_s$). C) Illustration of the magnetization vs. magnetic field (M–H) curve of ferrimagnetic IO showing hysteresis. D) Single domain IONPs at zero field have no magnetic anisotropy. E) In the presence of an applied magnetic field ($H$), single-domain IONPs align parallel to $H$. F) Illustration of the M–H curve of superparamagnetic IONPs at room temperature.

When IONPs form a colloidal suspension in liquid media, both Néel (Figure 1.5B) and Brownian relaxation (Figure 1.5C) govern the return of magnetization to equilibrium.
Brownian relaxation occurs by the rotation of the entire NP against the viscous drag of solvent. The net relaxation rate for colloidal IONPs is the sum of Néel and Brownian relaxation rates given by

\[
\frac{1}{\tau} = \frac{1}{\tau_N} + \frac{1}{\tau_B}
\]  

Eq. 1.2

where \(\tau\) is the net magnetic relaxation time and \(\tau_B\) is the Brownian relaxation time, given by

\[
\tau_B = \frac{3V_H \eta}{kT}
\]  

Eq. 1.3

where \(\eta\) is the viscosity of the carrier medium and \(V_H\) is the hydrodynamic volume of IONP. For colloidal IONPs, \(\tau_B\) is shorter than \(\tau_N\) as Brownian relaxation is proportional to NP volume and Néel relaxation (Eq. 1.1) is an exponential function of NP volume. Hence the Brownian relaxation contributes more towards net magnetic relaxation of colloidal IONPs.
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Figure 1.5. A) Illustration of magnetic energy ($E$) change with respect to the direction of magnetization vector against easy axis. Two modes of magnetic relaxation: B) Néel relaxation in which magnetic moment flips between two easy directions and C) Brownian relaxation occur by the rotation of the IONPs in colloidal suspension.

1.2.1 Synthesis of superparamagnetic iron oxide nanoparticles

Before moving into examples on IONP synthesis from the literature, some prevalent theory behind the formation of NPs in liquid-phase will be helpful to better appreciate the topic. An understanding of NP formation pathways is crucial for designing reactions to manipulate NP properties including size, size-distribution, shape, functionality, etc.

Like any chemical reaction, bottom-up syntheses of NPs involve the reaction of precursors to form the building blocks (atoms or molecules) of a NP. As time proceeds, the
concentration of these building blocks increase, leading to the nucleation and precipitation of NPs. Nucleation is the process by which a new phase (sparingly soluble) is formed from the parent phase (precursor solution). When nucleation occurs from homogeneous parent phase, it is termed as homogeneous nucleation and heterogeneous nucleation defines the instance when nuclei forms on a substrate/impurity phase. According to classical thermodynamic nucleation theory, the nucleus is considered as the aggregate of several solute molecules in equilibrium with parent phase at the supersaturation condition. Based on its size, the nucleus will have certain degree of solubility and the system as a whole will be thermodynamically unstable due to the large interfacial area and the high interfacial energy. In order to reduce the interfacial area, the nuclei will undergo growth possibly by Ostwald ripening. Among nuclei there exists a critical radius \( r^* \) given by

\[
r^* = -\frac{2\sigma_{SL}v}{kT\ln S}
\]

where \( \sigma_{SL} \) is the solid-liquid interfacial energy, \( v \) is the volume of atom/molecule forming the cluster, \( k \) is the Boltzmann constant, \( T \) is the temperature, \( S \) is the supersaturation (\( S = \frac{C}{C_{eq}} \), where \( C \) and \( C_{eq} \) are the concentrations of solute at saturation and equilibrium, respectively). The critical free energy \( \Delta G^* \) corresponding to \( r^* \) is given by

\[
\Delta G^* = \frac{16\pi\sigma_{SL}^3 v^2}{3k^2T^2\ln^2 S}.
\]
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Nuclei at the verge of critical parameters \((r^*\text{ and } \Delta G^*)\) have the greatest probability to grow.\(^{50}\) Nuclei with \(r > r^*\) will grow and \(r < r^*\) will dissolve (Figure 1.7A). The rate of homogeneous nucleation \((R_N)\) is the rate of addition of atoms/molecules to a nucleus at the verge of critical parameters and is given by

\[
R_N = A e^{-\frac{(\Delta G^*)}{kT}} \quad \text{Eq. 1.6}
\]

where \(A\) is a composite term usually taken as \(10^{25} \text{ s}^{-1} \text{ m}^{-3}.\(^{50}\) The \(R_N\) is an exponential function of \(S\); hence is negligible until a certain critical supersaturation \(S^*\) is reached, beyond which it increases rapidly. The formation of monodisperse NPs can be effectively explained by the LaMer and Dinegar diagram.\(^{56}\) Initially, at the onset of the reaction, the concentration of the product/solute increases (Figure 1.6, stage I) to supersaturation \((S)\) and later to critical supersaturation \((S^*)\) conditions. Under supersaturation condition, \(R_N\) increases (Figure 1.6, stage II) and at \(S^*\), \(R_N\) increase rapidly forming the maximum number of nuclei. Nucleation at \(S^*\) can only occur when the reaction medium is completely free from impurity phases and usually results in maximum number of NPs and a sharp decrease in their sizes. Following burst nucleation, the solute concentration drops below \(S^*\) and \(R_N\) drops immediately to zero. Post nucleation, stable nuclei will grow into discrete NPs by diffusion of dissolved solute to nuclei surface (Figure 1.6, Stage III). During stage III, the \(S\) condition is still present but there is a balance between the rate of formation of solute from reaction and their consumption by nuclei in the growth stage. The final growth rate is hence governed by the rate of the reaction.
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Figure 1.6. Illustration of the different stages involved in the formation of monodisperse NPs by LaMer and Dinegar diagram, adapted with permission from reference 56, copyright © 1950, American Chemical Society. Stage I: the concentration of solute increases rapidly. Stage II: At critical supersaturation ($S^*$), burst nucleation followed by immediate loss of $S^*$ condition and fresh nucleation subsides ($R_N = 0$). Stage III: nuclei grow into monodisperse NPs by a diffusion-limited process.

When the primary particles grow into larger single crystalline NPs by solute-by-solute attachment and unit cell replication (Ostwald ripening), it is said to follow a classical mechanism of crystallization (Figure 1.7B and C). Spherical/quasi-spherical NPs are usually formed following a classical mechanism and require stabilization to prevent aggregation. Another growth mechanism termed as non-classical crystallization involves aggregation and coalescence of primary particles. NPs formed by a non-classical mechanism may be polycrystalline with grain boundaries or have iso-oriented grains showing long-range order. Such a growth by aggregation and coalescence is termed non-classical crystallization (Figure 1.7D and E). In a non-classical pathway, primary particles un-
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NPs with 1 dimensional (D), 2D and 3D structural features are usually formed following a non-classical pathway.\textsuperscript{57}

**Figure 1.7.** Schematic illustration showing the pathways involved in nanocrystal formation. A) At supersaturation conditions, NP phase (nuclei) is in equilibrium with the parent phase (reaction medium). B) and C) Nuclei with radius \((r)\) greater than critical radius \((r^*)\) will grow into monodisperse single-crystalline NPs following classical mechanism. D) and E) Primary particles may aggregate and coalesce following a non-classical mechanism to form iso-oriented crystalline NPs.

The NPs at growth stage or post growth have a strong tendency to agglomerate in order to reduce the surface to volume ratio and the interfacial energy. Agglomeration in NPs can be prevented by introducing steric or electrostatic repulsion between NPs (Figure 1.8). Steric repulsion can be introduced by bound polymers, surfactants or other bulky organic molecules to the NP surface, and electrostatic repulsion can result from the adsorbed charged species such as H\(^+\) or OH\(^-\) on the NP surface. Examples on NP stabiliza-
tion by charged surface layer and stabilizing molecules will be presented later in this section.

Figure 1.8. Stabilization of NPs by A) steric repulsion from bulky surface molecules and B) electrostatic repulsion from surface adhering charged species.

Coprecipitation method:
Coprecipitation is a mild aqueous-phase method for preparing biocompatible IONPs. IONPs are precipitated from the aqueous mixture of ferric and ferrous salts in 2:1 ratio, respectively, under basic conditions. Massart for the first time actually developed this classical synthesis to prepare 12 nm magnetite NPs (size from XRD). A stable ferrofluid was obtained by adding tetramethylammonium hydroxide or perchloric acid to as-synthesized IONPs. The general chemical reaction leading to the formation of magnetite NPs is given by the following equation:

\[
2\text{FeO(OH)} + \text{Fe(OH)}_2 \xrightarrow{\text{pH} > 10} \text{Fe}_3\text{O}_4 + 2\text{H}_2\text{O} \xrightarrow{\text{O}_2} \gamma\text{-Fe}_2\text{O}_3
\]

Under basic conditions, the iron salts undergoes a hydrolysis reaction forming Fe(II) hy-
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Hydroxide and Fe(III) oxyhydroxide. It is the condensation reaction between these two hydroxides that results in the formation of magnetite phase, its nucleation under supersaturation conditions and growth to yield magnetite NPs. Under ambient conditions, magnetite NPs stored in water are known to undergo a slow topotactic oxidation and form maghemite (magnetite and maghemite have very similar physical properties). Maghemite has Fe$^{3+}$ cations occupying both octahedral and tetrahedral sites of the inverse spinel oxygen lattice and unlike magnetite has several vacancies within the octahedral sites. Both magnetite and maghemite NPs exhibit superparamagnetic behaviour, but the saturation magnetization value of magnetite NPs decreases when maghemite is formed due to the latter’s smaller magnetic moment (4.1 µB for magnetite and 2.3 µB for maghemite). The oxidation mechanism is believed to occur by the diffusion of iron cation to the surface of NPs before oxidation. Heating magnetite NPs above 500 °C under ambient conditions on the other hand forms the weakly magnetic hematite ($\alpha$–Fe$_2$O$_3$) phase. The presence/formation of maghemite phase along with magnetite phase does not drastically affect the usefulness of IONPs, however it is advantageous to obtain and preserve the magnetite phase for its higher magnetic moment.

L. Babes et al. synthesized uncoated IONPs following aqueous coprecipitation method using tetramethylammonium hydroxide (TMAOH) as a base. They investigated the effect of various reaction parameters including temperature, iron concentration (FeCl$_2$ and FeCl$_3$), iron ratio (Fe$^{2+}$/Fe$^{3+}$), reagent injection fluxes and reaction atmosphere (ambient and inert) on the IONPs sizes and their relaxivities. The sizes of the obtained IONPs
were always below the 10 nm mark. It was observed that the Fe$^{2+}$/Fe$^{3+}$ ratio of 0.5 yielded best results in terms IONPs yield and relaxivities (crucial for MRI contrast). J. P. Jolivet et al. demonstrated both size and shape control of magnetite NPs synthesized by coprecipitation method by varying the pH and ionic strength in the reaction medium. Magnetite NPs were synthesized from Fe(NO$_3$)$_3$ and FeCl$_2$ in the presence of NaNO$_3$ salt. The sizes of the as-synthesized magnetite NPs decreased from 13 nm to 1.3 nm with increasing pH (9.5, 10, 10.5, 11, 11.5 and 12) of the reaction medium. Shape control of NPs was also achieved by controlling the pH and surface charge density.

When IONPs are coated with hydrophilic and biocompatible inorganic or organic shell, it may enhance the colloidal stability and biocompatibility of IONPs and in some cases may form a protective layer around the IO core. Surface coatings comprising of various inorganic or organic molecules have been accomplished in situ or ex situ. C. C. Berry et al. synthesized magnetite NPs surface modified with dextran following the coprecipitation method.$^{62}$ The as-synthesized magnetite NPs synthesized without dextran measured around 10 nm and with dextran (in situ) measured around 8 nm. The coprecipitation method has been used to prepare IONPs with several surface coating such as graft copolymers,$^{68-70}$ poly(vinyl alcohol),$^{71}$ silica,$^{72}$ and silanes,$^{73}$ among others.

The advantages of the coprecipitation method include: a) It is an aqueous phase synthesis and does not require organic solvents, b) the reaction can be carried out at room temperature or temperatures $<100 \, ^\circ$C, c) the reaction does not require additional stabilizer
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molecules as the particles are stabilized by electrostatic repulsion at high pH or ions from added salts, d) the use of simple iron precursors, e) it can be easily scaled up for bulk production, f) the as-synthesized IONPs are hydrophilic and biocompatible. Some of the drawbacks of the coprecipitation method includes: a) The as-synthesized IONPs are usually polydisperse (broad size distribution), which will affect their size dependent magnetic properties, and b) any variation in the ratio of ferric and ferrous salts may result in the formation undesirable oxide phases which will lower the magnetic moment of the IONPs.

High temperature methods:

IONPs with narrow size distribution have been prepared by thermal decomposition of iron precursors such as Fe(acac)$_3$, Fe(CO)$_5$, Fe(Cupferronate)$_3$, etc. in organic solvents and stabilizers. The Alivisatos group prepared monodisperse maghemite NPs from iron cupferon complex [Fe(Cupferronate)$_3$]. The iron precursor in octylamine was rapidly added to trioctylamine at 300 °C under vigorous stirring and inert atmosphere to yield maghemite NPs of around 7 nm diameter. S. Sun et al. reported an organic phase high temperature synthesis of magnetite NPs. In this method, Fe(acac)$_3$ in phenyl ether in the presence of oleic acid, oleylamine and 1,2-hexanediol was heated to reflux (265 °C) under nitrogen to yield 4 nm magnetite NPs. In high temperature syntheses, oleylamine and 1,2-hexanediol acts as reducing agents to form Fe$^{2+}$ from Fe$^{3+}$ and other reducing agents such as hydrazine and citrate ions have also been used. Larger magnetite NPs were synthesized from smaller magnetite NPs following a seed-mediated growth. In the seed-mediated synthesis, the thermal decomposition reaction was carried out with differ-
ent seed (smaller magnetite NPs) concentrations to obtain larger magnetite NPs of 8, 12 and 16 nm (Figure 1.9) diameter. Size control was also achieved by varying the reaction time; S. Sun et al. prepared 6 nm magnetite NPs by refluxing Fe(acac)_3, oleic acid, oleylamine and 1,2-hexanediol in benzyl ether at 298 °C for 2 hours against 30 minutes which yielded 4 nm magnetite NPs. The as-synthesized magnetite NPs were hydrophobic and were subsequently transformed into hydrophilic NPs by shaking the NP dispersion in hexane and a suspension of tetramethylammonium 11-aminoundecanoate in dichloromethane. Hyeon’s group reported a high temperature synthesis of magnetite NPs from Fe(oleate)_3. During synthesis, iron oleate and oleic acid in 1-octadecene were heated at 320 °C for 30 minutes to yield magnetite NPs of 12 nm diameter. In this method, size control was achieved by varying the reaction temperature with the aid of different high boiling point (b.p.) solvents. Magnetite NPs of around 5, 9, 12, 16 and 22 nm diameter were obtained using 1-hexadecene (b.p. 274 °C), octyl ether (b.p. 287 °C), 1-octadecene (b.p. 317 °C), 1-eicosene (b.p. 330 °C) and trioctylamine (b.p. 365 °C), respectively.
N. Pinna et al. proposed a high temperature synthesis of magnetite NPs by using benzyl alcohol as solvent and stabilizer.\textsuperscript{79} The iron precursor Fe(acac)\textsubscript{3} in benzyl alcohol were heated in an autoclave at 175 °C for 2 days to yield magnetite NPs of 12 nm diameter. The reaction mixture heated at 200 °C for 2 days yielded magnetite NPs measuring ≈25 nm. To transform the hydrophobic magnetite NPs into hydrophilic, a ligand exchange was carried out by sonicating the as-synthesized magnetite NPs in the presence of dopamine. Hydrophilic magnetite NPs were directly prepared following the high temperature decomposition of Fe(acac)\textsubscript{3} in 2-pyrrolidone.\textsuperscript{80} In this method a solution of Fe(acac)\textsubscript{3} in 2-pyrrolidone was refluxed (245 °C) to yield ≈5 nm magnetite NPs.

Though the high temperature decomposition methods yield highly monodisperse
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IONPs (important for homogeneous magnetic response), there are some drawbacks: a) as-synthesized IONPs usually carry organic stabilizers and/or residues on their surface making them hydrophobic and unsuitable for biomedical applications, b) further ligand exchange or addition steps are required to render the IONPs hydrophilic and biocompatible, c) The solvents and stabilizers are usually expensive and toxic, d) high temperature requirements make the synthesis less energy efficient, e) It is expensive to scale-up for bulk productions.

Other synthetic methods:
The aqueous coprecipitation method and non-aqueous high temperature decomposition methods are most widely followed to prepare IONPs. In addition, there are other methods that may share the principle of coprecipitation and high temperature methods, but with modifications in terms of special metal precursors, reagents and/or special equipment to generate the required reaction conditions including temperature.

Magnetite NPs measuring ≈40 nm were prepared by the hydrothermal treatment of magnetite NPs following a coprecipitation method.\(^{81}\) In this synthesis, magnetite NPs measuring ≈12 nm were prepared from FeCl\(_3\) and FeCl\(_2\) in the presence of N(CH\(_3\))\(_4\)OH. The reaction mixture after 20 minutes was placed in an autoclave and heated at 250 °C for 24 hours to yield larger 40 nm magnetite NPs. In another hydrothermal synthesis, W. Cheng et al. prepared magnetite hollow spheres (240 nm) formed by smaller 20 nm NPs.\(^{77}\) Here, a mixture of FeCl\(_3\), sodium citrate, urea and polyacrylamide were heated in
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a Teflon lined autoclave at 200 °C for 12 hours to yield magnetite hollow spheres. At high temperature, the sodium citrate acts as a reducing agent and reduces Fe\(^{3+}\) to Fe\(^{2+}\). At the same time NH\(_3\) generated by urea provides the basic condition for the formation of magnetite phase. H. Cai et al. prepared poly(ethyleneimine) (PEI) coated magnetite NPs from FeCl\(_2\) following the hydrothermal method.\(^{82}\) The mixture of FeCl\(_2\) and NH\(_4\)OH was stirred vigorously in the presence of air to oxidize Fe\(^{2+}\) to Fe\(^{3+}\). Later the mixture along with PEI in DI water was heated to 134 °C at 2 bar pressure in an autoclave. Size control of PEI magnetite NPs (11.5–18.9 nm) was achieved by varying the iron precursor concentration. T. Yonezawa et al. prepared gelatine stabilized IONPs (≈2.8 nm) from aqueous solution of FeCl\(_3\) and gelatine.\(^{83}\) Aqueous NaBH\(_4\) was used to reduce Fe\(^{3+}\) to Fe\(^{2+}\) for the formation of IONP phase.

IONPs have also been prepared following sol-gel methods. The sol-gel process can be defined as the conversion of a precursor solution into an inorganic solid following a chemical reaction. Metal precursors such as metal salts (halide, acetate, nitrate, etc.) or metal alkoxides are traditionally used as precursors. The steps involved include hydrolysis and condensation reactions. The final gel obtained is then dried at high temperatures. Y. K. Gun'ko et al. prepared IONPs from the iron precursor Fe(OBu')\(_2\) that was initially prepared from FeBr\(_2\).\(^{84}\) The precursor in a THF and water mixture was sonicated for one hour and the precipitate was collected by filtration. The collected material was dried at 300 °C for 30 minutes to yield magnetite NPs measuring around 15 nm.
IONPs have been synthesized in constrained environments formed by surfactants. Surfactant molecules form micelles or water in oil emulsions, and can act as a tiny nanoreactor suitable for forming IONP phase, for example by the coprecipitation method. S. Santra et al. prepared silica coated IONPs following the microemulsion method. A microemulsion composed of surfactant/oil: Brij–97/cyclohexane, Igepal CO–520/n-heptane or Triton X–100/cyclohexene and nitrogen purged water were prepared. Two portions of surfactants carrying the iron precursors (FeCl₃ and FeCl₂) and the base (NaOH and NH₄OH) were prepared. Later under sonication, microemulsion with base was added to the microemulsion with iron precursors and sonicated for 2 hours to yield IONPs that were less than 5 nm in diameter. Silica coated IONPs were prepared adding tetraethyl orthosilicate (TEOS) to both portions of the microemulsion.

Also microwaves were used as the energy source in IONP synthesis. All domestic microwave ovens and microwave reactors operate at the frequency of 2.45 GHz (wavelength 12.24 cm) and do not break chemical bonds. Microwave reactions are based on the efficient fast heating of the materials (solvent and/or reagents). The electric component of the electromagnetic radiation generates heat by dipolar polarization and ionic conduction mechanisms. Hu et al. prepared monodisperse magnetite NPs (6 nm) by microwave assisted heating (250 °C) of a mixture of Fe(acac)₃, oleic acid and 1,2-hexadecanediol in dibenzyl along with ionic liquid [bmim][BF₄]. The ionic liquid was used as stabilizer and to absorb the microwave radiation. E. A. Osborne et al. reported the synthesis of dextran coated IONPs from FeCl₃ using microwave heating at 100 °C. Sonochemical
methods have been used to synthesize several nanomaterials including IONPs.\textsuperscript{92,93} Ultrasound typically spans the frequency range of around 15 kHz to 10 MHz (acoustic wavelength $\lambda \approx 10$ to 0.01 cm) and hence does not directly couple with a material at the molecular level. Sonochemistry occurs from the physical effect of ultrasound on the liquid reaction medium (e.g., solvent, reagent). Ultrasound waves in liquid travel with a velocity of around 1500 m$\text{s}^{-1}$ and causes acoustic cavitation involving bubble nucleation, growth and implosion. Acoustic cavitation generate local hot spots with temperatures of around 5000 K (heating and cooling rate of $10^{9}$ Ks$^{-1}$) and pressures of around 500 atmosphere.\textsuperscript{94} A. Gedanken’s group prepared several metal oxide NPs (Co$_3$O$_4$, CuO and ZnO) including magnetite NPs by sonochemistry.\textsuperscript{95} In this synthesis, metal acetate [Fe(II)acetate] in water-DMF solvent system was exposed to ultrasound waves (20 kHz) using an ultrasonic horn (Ti-horn) under Ar atmosphere for 3 hours. The magnetite NPs obtained measured around 20 nm. Ultrasound irradiation of colloidal NP over a short span of time (few seconds) can break down agglomerates and is widely used during NP purifying or processing steps.\textsuperscript{96}

\subsection*{1.2.2 Surface modifications of iron oxide nanoparticles}

If IONPs are to be developed as a material for biomedical applications with long blood-circulation half-life, they have to be surface modified to mask any undesirable physical/chemical properties. Liquid-phase (aqueous and non-aqueous) syntheses of IONPs and other transition metal oxide NPs (MONPs) are usually carried out following co-precipitation and high temperature hydro/solvo-thermal, sol-gel and microemulsion
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methods. IONPs as such are biocompatible but its pharmacokinetics when administered intravenously depends on its size and hydrophobic-hydrophilic balance. Hydrophobic and charged NPs non-specifically bind to cells, and in circulation are prone to opsonisation and removal by the reticuloendothelial system (RES). Depending on the chosen synthesis, uncoated IONPs or IONPs with hydrophilic or hydrophobic stabilizers are obtained. Hence, the synthetic strategies have to be adapted to obtain predominantly hydrophilic IONPs with biocompatible polymer or other organic/inorganic shell, in situ. When the synthetic procedure does not favour in situ coating, post-synthetic surface modification steps are necessary to enhance hydrophilicity. Literature presents an overwhelming number of combinations of IONP synthesis, surface modifications and bioconjugations. Out of the apparently never-ending list of surface stabilizers/coatings reported, this section will focus on the few that are well established or have greater potential in biomedical applications. The choice of the coating material is crucial and depending on the intended application, it should prevent undesirable aggregation and if necessary provide anchoring points for the attachment of therapeutic and diagnostic (theranostic, TN) agents.

Surface iron atoms in IONPs are highly reactive and act as Lewis acids and coordinate with functional groups that can donate lone-pair electrons. In an aqueous colloidal dispersion, surface iron atoms in IONPs coordinate with water molecules and forms surface hydroxyl groups around them. Surface hydroxyl groups on IONPs are amphoteric and can react with acids and bases. Carboxylic acid groups when adsorbed on IONPs, readily coordinate by forming carboxylate species following the elimination of surface
hydroxyl groups. Coordination of amine groups to the IONP surface is complex and occurs through two pathways depending on the amount of surface hydroxyl group: a) In the case of high temperature synthesis when surface hydroxyl groups are low, amine coordinates by donating its lone-pair to an uncoordinated surface iron cation and b) when surface hydroxyl groups are abundant, a small fraction of adsorbed amine binds via a proton exchange with surface hydroxyl groups (forms positively charged amine and deprotonated oxide surface). Small molecules such as oleic acid, citric acid, 2-bromo-2-methylpropionic acid, etc. with carboxylic acid groups are known to attach to IONP surfaces. Hydroxyl groups have been shown to coordinate to surface iron cations of IONPs as demonstrated by the IONP coating by polyols such as dopamine, catechaldehyde, triethylene glycol, poly(vinyl alcohol) (PVA), etc.

IONPs may be coated with biocompatible polymers, in situ or ex situ depending on the reaction conditions and reagents. IONPs coated with PEI have been prepared in situ by simple adsorption in a fashion shown in Figure 1.10A, by carrying out a hydrothermal synthesis in the presence of PEI. N-Alkyl-PEI coated IONPs were prepared following a high temperature synthesis via hydrophobic interactions between surface oleic acid on IONPs and alkyl chain (dodecane) linked to PEI. PEI is a branched polymer with several amino groups and has been covalently linked to IONPs with amine reactive surface functional groups. PEI with its cationic charge, binds negatively charged species such as DNA and RNA, non-specifically and enters the cell via endocytosis.

Poly(ethylene glycol) (PEG) is a linear hydrophilic polymer that is widely used as ex-
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cipients in approved recombinant protein therapeutics to minimize proteolytic inactivation and enhance their blood circulation half-lives.\textsuperscript{109} PEG coated IONPs like PEGylated protein therapeutics have enhanced colloidal stability in blood and evade RES.\textsuperscript{110} J. Xie et al. transformed hydrophobic oleic acid and oleyl amine coated IONPs from high temperature synthesis into hydrophilic PEG coated IONPs following a ligand exchange procedure.\textsuperscript{111} For this, a carboxylic acid terminus of PEG diacid was coupled with dopamine via dicyclohexylcarbodiimide/N-hydroxysuccinimide (DCC/NHS) coupling and the mixture in CHCl\textsubscript{3} was stirred overnight with hydrophobic IONPs, to achieve ligand exchange. N. Kohler et al. carried out a ligand exchange on oleic acid coated IONPs (from high temperature synthesis) with PEG-trifluoroethylester silane by sonication for 4 hours. This method yielded IONP with a thin silane shell, and PEG brush (Figure 1.10C) with trifluoroethyl ester terminal which on further treatment with excess ethylene diamine yielded IONP with a silane shell and PEG brushes with surface amine groups for further conjugations.\textsuperscript{112} PEG has been widely used as a coating material or as a spacer around IONPs in combination with TNs or targeting ligands.\textsuperscript{113–119}
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**Figure 1.10.** Fabricating strategies of magnetic NP for biomedical applications: A) polymer adsorbed on IONP surface B) polymer grafted from monomeric functional group attached to IONP surface, C) polymer linked through thin functional shell (e.g., silica, aminosilane) around IONP, D) a single IONP core encapsulated in thick shell (e.g., silica), E) multiple IONPs encapsulated within a shell (e.g., silica, polymer) and F) silica NPs with surface IONPs.

Copolymer coated IONPs are prepared to take advantage of the distinct properties of different polymers. PEG-PEI-chitosan copolymer coated IONPs were prepared in situ following coprecipitation method from aqueous FeCl$_3$ and FeCl$_2$ solution in the presence of ammonium hydroxide. Here PEG enhances the biocompatibility of the copolymer, and PEI and chitosan are known to bind DNA.$^{120}$ Graft poly(glycerol monoacrylate)-g-poly(PEG methyl ether acrylate) (PGA-g-PEG) coated IONPs have been prepared in situ following the coprecipitation method and size control was achieved (4–18 nm) by varying the graft density.$^{68}$ Poly(lactic-co-glycolic acid) (PLGA) coating on IONPs with oleic acid coating from high temperature synthesis were accomplished by a ligand exchange procedure.$^{121}$
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Dextran, a branched polysaccharide made of α-D-glucopyranosyl units, has been widely used to coat IONPs for its biocompatibility and hydrophilicity. Many of the clinically approved IONPs based MRI contrast agents have dextran coating. Ferumoxide and ferumoxtran-10 have dextran coating,\textsuperscript{122} ferucarbotran has a carboxydextran coating\textsuperscript{123–124} and ferumoxytol have carboxymethyl dextran.\textsuperscript{125} In most cases, dextran coating is carried out in situ during IONP synthesis by the coprecipitation method.\textsuperscript{126–127}

Silica has been extensively used to encapsulate IONPs for it is hydrophilic, biocompatible and easy to functionalize. Encapsulation of IONPs is usually carried out by the Stöber method or the reverse microemulsion method. In the Stöber method, a silica shell is formed around the IONP-seed by the hydrolysis and condensation of a sol-gel precursor (e.g., TEOS) under basic conditions (NH\textsubscript{4}OH).\textsuperscript{128} In the reverse microemulsion method, the IONP-seed is entrapped within the micelle where the silica sphere is formed.\textsuperscript{129} IONPs with a silica shell (resembling the design shown in Figure 1.10D) of varying thickness can be prepared by varying the concentration of both sol-gel precursor and base. M. Zhang et al. prepared IONPs with a silica shell of 2 nm or 20 nm thickness by varying the concentration of NH\textsubscript{4}OH and TEOS in a reverse microemulsion synthesis;\textsuperscript{130} lower and higher concentrations of TEOS and NH\textsubscript{4}OH yielded a thin and thick silica shell, respectively. The number of IONPs encapsulated (single or cluster as in Figure 1.10E) can be controlled by controlling the size of the micelles, which in turn is dependent on the surfactant (Igepal CO–520) and NH\textsubscript{4}OH concentration. Silica NPs with IONPs immobilized on their surface similar to the design shown in Figure 1.10F have been pre-
pared.\textsuperscript{131} The silica NP was prepared following the Stöber method\textsuperscript{128} and its surface was modified with 3-aminopropyltrimethoxysilane to form surface amino groups. The IONPs (7 nm) was prepared separately following thermal decomposition method\textsuperscript{132} and the surface oleic acid groups were replaced with 2-bromo-2-methylpropionic acid following ligand-exchange\textsuperscript{133} to form surface bromide group. Later, a nucleophilic substitution reaction between amino terminal silica NPs and bromo terminal IONPs yielded silica NPs with immobilized IONPs. Selected examples of the synthesis of IONP with a variety of surface coatings and their core sizes and hydrodynamic diameter are presented in table 1.1.

**Table 1.1.** IONPs synthesis and surface modification: The methods followed for IONP phase synthesis and surface modification along with the particle sizes from TEM and hydrodynamic diameter from dynamic light scattering are given. In the size column, different numbers or numbers connected by the symbol “–” indicate demonstrated size control. Size distribution is described as numbers connected by the symbol followed by the term “range”.

<table>
<thead>
<tr>
<th>Core IONP synthesis</th>
<th>Core Size TEM (nm)</th>
<th>Surface coating</th>
<th>Coating method IS\textsuperscript{a}, PS\textsuperscript{b}, Other</th>
<th>D\textsubscript{H} (nm)</th>
<th>Purpose</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Fe\textsubscript{3}O\textsubscript{4}</td>
<td>Coprecipitation</td>
<td>4–8</td>
<td>Poly(TMSMA-co-PEGMA)\textsuperscript{d}</td>
<td>IS and PS</td>
<td>16, 12</td>
<td>MRI</td>
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<td>Fe\textsubscript{3}O\textsubscript{4}/\gamma-Fe\textsubscript{2}O\textsubscript{3}</td>
<td>Coprecipitation</td>
<td>4, 5</td>
<td>PEG\textsuperscript{e}</td>
<td>PS</td>
<td>5, 12, 73</td>
<td>Drug delivery</td>
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<td>10, 8</td>
<td>Albumin, Dextran</td>
<td>IS</td>
<td>–</td>
<td>NP-cell interaction</td>
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<tr>
<td>Fe\textsubscript{3}O\textsubscript{4}/\gamma-Fe\textsubscript{2}O\textsubscript{3}</td>
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<td>–</td>
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<td>Fe\textsubscript{3}O\textsubscript{4}</td>
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<td>4, 7, 9, 18</td>
<td>PGA-co-PEG\textsuperscript{f}</td>
<td>IS</td>
<td>–</td>
<td>Size control</td>
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<td>Poly(OEGMA-co-MAA)⁵</td>
<td>IS</td>
<td>10, 15, 18, 24</td>
<td>MRI</td>
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<td>PMPC-PGMA⁴</td>
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<td>PS</td>
<td>49, 53</td>
<td>Stabilization</td>
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<td>Stöber method</td>
<td>268</td>
<td>Fe₃O₄/γ-Fe₂O₃-TSA¹</td>
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<td>Fluorescent tagging</td>
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<td>γ-Fe₂O₃</td>
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<td>6–7</td>
<td>Residual Octylamine/trioctylamine</td>
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<tr>
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<td>IS</td>
<td>310</td>
<td>Conjugations, MRI</td>
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<td>SiO₂</td>
<td>IS</td>
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<td>–</td>
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<td>Microwave assisted</td>
<td>6, 17</td>
<td>Dextran</td>
<td>IS and PS</td>
<td>39</td>
<td>MRI</td>
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<tr>
<td>Fe₃O₄</td>
<td>Microwave assisted</td>
<td>6</td>
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<td>Fe₃O₄</td>
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<td>[EMIm][BF₄]²</td>
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<td>–</td>
<td>–</td>
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<td>IS</td>
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<td>BMPA</td>
<td>Ligand exchange</td>
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<td>Conjugations</td>
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<td>15</td>
<td>Dopamine-PEG</td>
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<td>67</td>
<td>Antibody conjugation</td>
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<td>7</td>
<td>Ethylene, dithylene, Triethylene and tetraethylene glycol</td>
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<tr>
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<td>PEG 600, 3000, 6000, 20000</td>
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<td>40, 50, 70, 90</td>
<td>Stabilization, conjugation</td>
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<td>Multimodal imaging, glioma targeting</td>
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<td>–</td>
<td>MRI, Drug delivery</td>
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<td>Silane-PEG</td>
<td>PS</td>
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<td>PEG-Chlorotoxin</td>
<td>Multistep</td>
<td>–</td>
<td>Glioma targeting</td>
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<td>Polyol (Reaction in PEG)</td>
<td>5–10 (range)</td>
<td>PEG-exendin4</td>
<td>Multistep</td>
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<td>102, 90</td>
<td>MRI, gene transfection</td>
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<tr>
<th>Fe₂O₃/γ-Fe₂O₃</th>
<th>Thermal decomposition</th>
<th>SiO₂</th>
<th>PS</th>
<th>Stem cell labeling</th>
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<tr>
<td>7</td>
<td>124</td>
<td>129</td>
<td></td>
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</table>

*In situ, †Post synthesis, ‡Hydrodynamic diameter, §Poly(trimethoxysilyl)propyl methacrylate-co-PEG methacrylate, †Poly(ethylene glycol)-co-fumarate, ‡Poly(glycerol monoacrylate)-g-poly(PEG methyl ether acrylate), §Poly[oligo(ethylene glycol)methacrylate-co-methacrylic acid], †Poly[2-(methacryloxy)ethyl phosphorylcholine]-block-(glycerol monomethacrylate), ‡N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride, †1-Ethyl-3-methylimidazolium tetrafluoroborate, †Atom transfer radical polymerization

IONPs with biocompatible coatings are further conjugated with custom TNs and targeting molecules for targeted drug delivery and diagnosis. The strategies followed for TN bioconjugations are presented in section 1.2.3.

1.2.3 Biomedical applications

Superparamagnetic properties and biocompatibility of IONPs are crucial for biomedical applications. Most of the biomedical applications of IONPs are concerned with its magnetization and relaxation behaviour in a magnetic field, but once the magnetic field is removed, the induced magnetization is lost, thereby removing agglomeration and preventing possible capillary embolization. IONPs are being used in clinical medicine as $T_1$ and $T_2/T_2^*$ enhancing MRI contrast agents (diagnosis) and are widely investigated for their potential therapeutic (drug delivery and in situ tracking using MRI) applications. IONPs induced hyperthermia for tumor thermotherapy is seen as a better alternative to other hyperthermia mediums due to their scope to target (passive, active and/or magnetic targeting) cancer cells.\(^{134}\) In the broader field termed as nanobiotechnology, IONPs containing beads (polymer, silica) are used for efficient magnetic bioseparation of cells, proteins, nucleic acids, etc. for analysis and diagnosis.\(^{135}\) One of the major interests in NPs for TN...
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applications is the opportunity to accomplish multiple tasks in a single administration. Multifunctional IONP-TN (Figure 1.11) with targeting ligands, therapeutic agents, imaging aid (fluorescent, IR dye, quantum dots, gold NPs, radiolabel) can be designed to target specific tissue types for therapy and multimodal imaging (MRI, fluorescent, Positron Emission Tomography – PET) for in situ tracking and diagnosis.

Figure 1.11. Schematic illustration of multifunctional magnetic NPs: A) The IONP core is grafted with biocompatible polymer and conjugated with photoluminescent material for multimodal imaging, targeting ligand and therapeutic agent forming a corona. B) The IONP core is encapsulated in a thick shell of biocompatible material (polymer or silica) with entrapped photoluminescent dye and therapeutic agent. The surface is grafted with biocompatible polymer and conjugated to targeting ligand. C) Biocompatible polymer or silica NP serving as support for IONPs and the surface grafted with biocompatible polymer with conjugated targeting ligand, therapeutic agent and photoluminescent dye forming the corona.

Many of the IONP-TNs are designed for non-invasive intravascular administration and hence the primary task is to have long blood-circulation half-lives. Long-circulating IONP-TNs have a better chance to accumulate at the intended site and provide adequate
time window for imaging. To have a long circulation half-life, IONP-TNs have to overcome the physiological barriers presented by the human RES including phagocytic monocyte and macrophages (Kupffer cells in Liver; red pulp, marginal zone and white pulp in spleen). Dextran coated IONPs when administered intravenously, showed prolonged blood circulation half-life of 3–4 hours, in a rat model; by evading detection by the Kupffer cells and splenic macrophages. The long circulation half-life can be attributed to their small size and hydrophilicity; eventually NPs are cleared following opsonisation. During circulation, the IONP-TNs will have to pass through the interendothelial cell slits (IES – 200–500 nm) in sinusoidal spleen. NPs with rigid diameter of less than 200 nm may pass freely whereas larger particles will be trapped by IES.\textsuperscript{136} Red blood cells pass through IES by its ability to deform and hence NP-TNs larger than 200 nm have to be deformable to escape splenic filtration. Rigid NPs larger than 200 nm may serve as splenotropic agents.\textsuperscript{136} On the other hand, very small hydrophilic NPs with hydrodynamic diameter less than 10 nm are rapidly removed from circulation via extravasation and renal clearance.\textsuperscript{137,138} Hence an ideal IONP-TN meant for intravenous administration should have the right hydrophilic-hydrophobic balance to avoid opsonization, and a hydrodynamic diameter of 10–200 nm (rigid), to have a long blood-circulation half life. There are few studies that have evaluated the effect of shape (spherical vs. anisotropic) on blood circulation half lives of NPs.\textsuperscript{139,140} In these studies, anisotropically shaped NPs were shown to have longer blood circulation half lives than their spherical counterparts.\textsuperscript{140,141} Though the results are promising, the volume of work on such anisotropically shaped NPs is very small and further extensive studies are needed.
The specificity of long-circulating IONP-TN towards a particular tissue type is crucial to reduce the drug dosage and avoid possible toxicity risk to healthy tissue. The non-specific cell binding can be minimized by designing the IONP-TN to target a disease site by passive, active and/or magnetic targeting. Figure 1.12A–D is the diagrammatic representation of different types of capillaries showing the extent and sizes of the fenestrations or pores in them. Compared to capillaries in other body parts, brain capillary endothelial cells forming the BBB are particularly restrictive to xenobiotics including NPs, due to the physical (tight junction, TJ) and metabolic (efflux pumps) barriers. Almost 98% of small-molecule drugs and 100% of large-molecule drugs under current pharmacological development have significant mass transport barriers at the BBB and restricted CNS entry. As a rule of thumb, only lipophilic molecules, not exceeding molecular weights of ≈500 Da, are capable of partitioning into the CNS and crossing the BBB.

Passive and active targeting:

Long-circulating NPs of suitable sizes can undergo rapid paracellular transport or extravasation in organs/tissues with fenestrated capillaries or in areas with capillary dysfunction. This type of accumulation of NPs is termed as passive targeting. Passive targeting strategies have been used in IONP enhanced MR imaging of liver, metastatic lymph nodes, and blood pool. The TJ in brain capillaries present a formidable barrier for paracellular diffusion, but pathological conditions such as stroke, multiple sclerosis (MS) and tumor compromise their integrity leading to cerebrovascular leakage and macrophage infiltration. IONPs enhanced MRI of monocytes/macrophages have been used to evaluate inflammatory and degenerative processes associated with stroke.
atherosclerosis,\textsuperscript{152} multiple sclerosis (MS),\textsuperscript{153} and metastatic lymph nodes\textsuperscript{154}. The integrity of endothelium is also compromised from inflammatory processes concerning rheumatoid arthritis, infarction and infections, paving ways for the paracellular diffusion of IONPs.\textsuperscript{155} Tumor tissues are known to have a leaky vasculature and can accumulate long circulating NPs by enhanced permeation and retention (EPR) effect.\textsuperscript{156} IONPs with surface targeting moieties including small molecules,\textsuperscript{112,119,157–159} peptides,\textsuperscript{160,161} proteins,\textsuperscript{162,163} polysaccharides, etc. can attach to specific cell types with complimentary antigens or receptors, by active targeting (Figure 1.13E).
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Figure 1.12. Diagrammatic illustration of the different types of blood capillaries (A–C). A) The capillary endothelial cells have no fenestrations and the basal lamina is continuous (e.g., brain capillary forming BBB have restrictive tight junctions between adjacent endothelial cells with openings <0.1 nm). B) The endothelial cells have fenestrations and the subendothelial basal lamina is continuous (e.g., capillaries in kidney, intestine, endocrine and exocrine organs are fenestrated (openings <50–60 nm)). C) The endothelial cells in sinusoid capillaries in liver, spleen and bone marrow have large pores (100–1000 nm) and a discontinuous basal lamina. The fenestrae in capillary endothelial cells facilitate paracellular exchanges between luminal (blood) and abluminal (tissue) sides as in the case of B and C. D) Cross-sectional view of the brain capillary (BBB); small lipophilic molecules crosses the BBB via passive transcellular diffusion and other vital molecules undergo active transport via carrier or receptor mediation. Shematic illustration of E) receptor and F) charge mediated endocytosis of NPs. In receptor mediated endocytosis,
NPs modified with targeting ligands, selectively bind to the cell membrane through the ligand-receptor complexing and internalized into endosomes via caveolae. In the charge mediated endocytosis, NPs with positive surface charge, non-selectively bind to the negatively charged cell membrane and are internalized through caveolae.

**Vectorization of IONPs:**

A variety of strategies including physical interactions (electrostatic, affinity) and covalent bonding have been used to attach targeting ligands and theranostics to IONP surfaces (Figure 1.13). The strategy is usually determined by the properties and the reactivity of the conjugant. Usually NPs can be designed to meet the specific conjugation needs (physical interaction or covalent linkage) through suitable surface modifications. IONPs modified to carry a positive surface charge have been used to attach negatively charge gene (DNA and siRNA for gene delivery applications)\(^{106,120}\) and TN agent (Doxorubicin)\(^{164}\) through electrostatic interactions (Figure 1.13A). Hydrophobic interactions were also used to attach moieties with hydrophobic block to IONPs with surface hydrophobic coating.\(^{165}\) Electrostatic and hydrophobic interactions are sensitive to pH and hydrophilicity of the carrier medium. A more robust physical interaction is the affinity interactions of the likes of streptavidin and biotin, which is specific and among the strongest non-covalent bindings. Streptavidin and biotin interactions have been used to immobilize Neisseria gonorrhoeae F62 enzyme to IONP surface.\(^{166}\)
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Figure 1.13. Modes of attachment of targeting ligands and TNs to IONP surface: (A) electrostatic attraction, (B) affinity, (C) Direct attachment to IONP surface iron cations, (D) covalent attachment through a linker.

Targeting ligands and TNs are more often linked covalently as they are strong and allow control over the linking site through special functional groups. Conjugations can be established through functional groups on IONPs or through functional groups on a linker (e.g. polymer chain) attached to IONPs. A variety of functional group combinations and linking chemistry have been used in the conjugation of targeting ligands and TNs to the IONP surface (Figure 1.14). TNs with primary amine or carboxylic acid group can be conjugated to IONPs with surface carboxylic acid or primary amine group, respectively, through $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide/$N$-hydroxysuccinimide (EDC/NHS) coupling (Figure 1.14B). EDC/NHS forms the labile NHS-ester with carboxylic acid groups, for easy substitution reactions with primary amine group carrying moieties (NPs, proteins, peptides, polymers, etc.). X. Li et al. linked octreotide peptide (targeting ligand for breast cancer cells) to PEG coated IONP with surface carboxylic acid groups, following EDC/sulfo-NHS coupling. $^{167}$ Sulfo-NHS (N-
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hydroxysulfosuccinimide sodium salt) has the same reactivity as NHS, but has SO$_3^-$Na$^+$ group that enhances its water solubility for aqueous coupling reactions. W. Chen et al. prepared magnetite NPs coated with meso-2,3-dimercaptosuccinic acid and later conjugated rotavirus capsid surface protein –VP4 (for enhanced cell uptake) through EDC/NHS coupling.\textsuperscript{168} Doxorubicin (Anticancer drug with –NH$_2$ group) was conjugated to the surface VP4 protein, again by EDC/NHS coupling. A. Tsourkas et al. prepared multifunctional IONPs with crosslinked dextran coating and surface Cy5.5 (far red and near infrared dye) and monoclonal antibody anti-VCAM-1 following EDC/NHS coupling.\textsuperscript{169} The IONP with surface cross-linked dextran had surface amine groups that attached to Cy5.5-NHS. The Anti-VCAM-1 antibody was conjugated through a linker octanedioic acid by EDC/NHS coupling. Similarly, small molecules including methotrexate,\textsuperscript{158,170} folic acid,\textsuperscript{112} etc. were also coupled to IONPs following carbodiimide/NHS chemistry. Epoxides are highly susceptible to nucleophilic attack and can react with amines, thiols and hydroxyls via a ring opening reaction (Figure 1.14C). IONPs modified to have surface epoxide groups have been used to conjugate trypsin,\textsuperscript{171} but was found to be less efficient than thiol-maleimide and carbodiimide/NHS coupling, due to unwanted ring opening reactions. One of the most widely used fluorescent labels, fluorescein isothiocyanate is readily conjugated to IONPs with surface amino groups through the formation of thiourea (Figure 1.14D).\textsuperscript{172–174}
Figure 1.14. Examples of functional group combinations for NPs and ligands and theranostics for conjugations are given. Conjugations may also be achieved via a linker.
like biocompatible PEG chain (between NPs and TNs) with smart selection of reactable functional groups.

Maleimide activated ligands react with thiol groups to form thioether linkages (Figure 1.14F). L. Maurizi et al. prepared IONPs modified with meso-2,3-dimercaptosuccinic acid (DMSA) having surface thiol groups and conjugated the fluorescent dye tetramethylrhodamine-5-maleimide. More recently, “click” chemistry involving copper catalyzed azide-alkyne cycloaddition reaction (Figure 1.14I) have been used in the bioconjugations of IONPs. Azide-alkyne chemistry is advantageous as reactions of azide and alkyne containing moieties are very specific towards one another and form a highly stable linkage. “Click” reactions are fast and can be carried out in aqueous medium at room temperature, making it well suited for bioconjugations. M. Das et al. conjugated propargyl folate (for targeting) to multifunctional IONPs containing surface azide groups. M. Mazur et al. demonstrated the conjugation of propargyl mannopyranoside to IONPs coated with azide terminated dopamine. Though there are many reports on “click” reaction based IONP bioconjugations, there are few limitations that has to be considered while using them: So far the products of the “click” chemistry have not been well investigated within biological systems. Effective catalyst removal post conjugation and the in vivo toxicity of the final products have to be established before this conjugation method can be widely accepted.

**Magnetic resonance navigation of IONPs:**

As a non-ligand based targeting strategy, magnetically directed drug convection (MDDC) has a great potential for site-specific drug delivery. One of the earliest reports on magnet-
ic field assisted targeting (MT) of magnetic materials in vivo was published three decades ago.\textsuperscript{179} In the study, external magnets were used to focus and enhance the delivery of doxorubicin using magnetite and observed better tumor reduction in rats. Liu et al. used focused ultrasound (locally disrupts blood-brain barrier) and MT using externally positioned magnets (tied to rat’s head) in synergy to enhance concentration of epirubicin linked magnetite NPs across the blood-brain barrier (BBB).\textsuperscript{180} Using external magnets for focusing might work in small animals but will be challenging in humans as the efficiency depends on the proximity between magnet and target tissue. Magnetic resonance navigation (MRN) is proposed as a viable option for deep tissue MT. MRN can be achieved using conventional MRI scanner with modified gradient coils and software to facilitate 3D magnetic focusing. A magnetic field of 1.5 T or higher in MRI scanner overcomes the challenge of deep tissue magnetic focusing and hence can be used for MT anywhere in the human body.\textsuperscript{181} Also, it is advantageous to develop a clinical MRI for MRN and MT, for it is widely available in hospitals across the world. Mathiue and Martel used propulsion gradient coils in an MRI scanner with 1.5 T field to steer a suspension of Fe$_3$O$_4$ aggregates of around 11 µm in a y-shaped channel.\textsuperscript{182} The flow rates were carefully controlled in this in vitro experiment and steering ratio of NPs was measured for gradients of 50, 100, 200 and 400 mT m$^{-1}$. It was observed that the steering ratio increases with the amplitude of the magnetic gradient and largest magnetic aggregates required lower magnetic gradient amplitude. P. Pouponneau et al. used MRN to guide and image magnetic FeCo-PLGA (iron-cobalt NPs encapsulated in poly(D,L-lactic-co-glycolic acid)) microparticles (58 µm) through phantom mimicking hepatic arteries.\textsuperscript{183} For steering studies a
1.2 Superparamagnetic iron oxide nanoparticles

1.5 T MRI was used with a gradient magnetic field of 400 mTm\(^{-1}\). The microparticles formed aggregates in MRN which facilitated steering with applied field gradient. Steering efficiency was measured by estimating the amount of iron and cobalt from two channels, using atomic absorption spectroscopy (AAS) and showed successful magnetic steering.

**IONP as susceptibility contrast agent for MRI**

MRI is a powerful non-invasive tool to image and investigate internal anatomy of living subjects and *ex vivo* preparations. MRI is based on the phenomenon of nuclear magnetic resonance where atomic nuclei in the presence of magnetic field, absorb and reemit characteristic resonant-electromagnetic waves (RF). IONPs with biocompatible surface coatings are widely used as susceptibility contrast agents in MRI.\(^{122-125}\) Superparamagnetic IONPs affect the local magnetic susceptibility of nuclei (\(^1\)H in H\(_2\)O) in tissue around them thereby altering the nuclear magnetic resonance (NMR) signals, which is observed as a contrast in MRI. In this section, a brief background on the principles behind MRI necessary to better appreciate one of the key applications of IONPs and the concepts in chapter 3 is presented. There are dedicated books on MRI for further reading.\(^{184,185}\)

**MRI basics**

The spin of a nucleus is described by the nuclear spin quantum number (\(I\)) and can have values of \(I = 0, \frac{1}{2}, 1, \frac{3}{2}, 2, \) etc. MRI is only possible on species that have a non-zero spin (\(I \neq 0\)). The magnitude of the generated dipole due to nuclear spin is known as nuclear magnetic moment. For MRI of biological systems, proton (\(^1\)H) is the most preferred nuclei as they are abundant (natural abundance 99.985 %), possess an \(I\) value of \(\frac{1}{2}\) and a high
gyromagnetic ratio \((\gamma = 2.675 \times 10^8 \, \text{s}^{-1} \text{T}^{-1})\). A proton with spin can be considered as a vector having an axis of rotation with definite orientation and magnitude (Figure 1.15A). In the absence of the magnetic field the protons (e.g., in tissue) have their spin vector oriented randomly and a vector addition of all the spin vectors and hence net magnetization \((M)\) has a value of zero (Figure 1.15B). In the presence of a magnetic field \((B_0)\), the protons will begin to precess parallel to the axis of the magnetic field (Figure 1.15C) which by convention is fixed to be parallel to the \(z\)-axis of a Cartesian coordinate. The rate of precession is proportional to the strength of the magnetic field and is termed as Larmor frequency \((\omega_0)\), given by the larmor equation

\[
\omega_0 = \frac{\gamma B_0}{2\pi}
\]

Eq. 1.7

where \(B_0\) is the magnetic field strength in tesla (T) experienced by the proton and \(\gamma\) is the gyromagnetic constant expressed in \(\text{s}^{-1} \text{T}^{-1}\). Performing a vector addition in the presence of magnetic field will result in a net magnetization \(M_z\) along the \(z\) direction. The protons that are precessing along \(z\) direction will undergo a nonzero interaction with \(B_0\) termed as Zeeman interaction. This interaction will result in a splitting of energy levels between the protons that are aligned parallel and antiparallel to \(B_0\) as shown in Figure 1.15D. The energy difference \(\Delta E\) is proportional to \(B_0\) and is given by

\[
\Delta E = \hbar \frac{\gamma B_0}{2\pi}
\]

Eq. 1.8
where $h$ is the Planck's constant. The protons aligned parallel to $B_0$ occupy the lower energy level and there is a slight excess of proton population in the lower energy level as governed by the Boltzmann distribution ($N_U/N_L = e^{\Delta E/kT}$, where $N_U$ and $N_L$ are the number of protons in the upper and lower energy levels respectively, $T$ is the absolute temperature and $k$ is the Boltzmann constant). A slight excess of protons that are aligned parallel to $B_0$ will result in nonzero vector sum of spins and the system will have a net magnetization ($M_z$) aligned along the magnetic field with no transverse (xy plane) component (Figure 1.15D).

**Figure 1.15.** Schematic illustration of the effect of magnetic field on protons A) a positively charged proton spinning around its own axis produces a magnetic field along the direction of its spinning axis similar to a bar magnet (Magnetic field proceeding from south to north pole). B) Protons (e.g., in tissue) in the absence of a magnetic field are oriented randomly and has no net magnetization C) A proton in the presence of a magnetic field $B_0$ precesses around the direction of magnetic field. And D) There is a slight excess of protons aligned parallel (low energy level) than the ones aligned antiparallel to $B_0$. 
The energy ($\Delta E$) between the upper and lower energy levels is quantized. When a proton is irradiated with energy of the correct frequency ($\omega_0$), the proton will be excited from the lower energy level (spin up) to the higher energy level (spin down). At the same time protons in the higher energy level can release energy (of corresponding frequency) to go to the lower energy level. When the energy supply is halted, it is the tendency of the protons to return to the initial equilibrium distribution. The explanation of the net magnetization ($M_z$) due to precessing protons can be simplified by using a “rotating frame of reference”. In the rotating frame of reference the Cartesian coordinates rotates about z-axis while the x and y-axes rotate at the Larmor frequency ($\omega_0$). In the rotating frame of reference, the precessing proton and the direction of magnetization will appear to be stationary, with a fixed set of x, y and z coordinates (Figure 1.16A).

$T_1$ and $T_2$/$T_2^*$ relaxation:

MRI is generated by the manipulation of the net magnetization ($M_z$) of protons (in tissues) over a region of interest. In an MRI machine, the magnetic field $B_0$ is applied by the main magnet, typically with field strengths ranging from 0.2 to 3.0 Tesla (T). MRI machines meant for preclinical studies, for example to image mice or a small ex vivo preparations, may require higher field strengths of 7 T or more to provide a better signal to noise ratio. During MRI, a short burst or pulse of radiofrequency (RF) containing a range of frequencies is applied and the protons absorb the frequency corresponding to $\Delta E$. This quantized energy absorption is termed as resonance absorption, and the frequency the resonant frequency. For MRI, the effect of energy absorption on net magnetization is more interesting. When the applied electromagnetic RF pulse ($B_1$) is perpen-
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dicular to $B_0$, the resonant absorption will rotate $M_z$ to transverse plane (perpendicular to $B_0$ and $B_1$). An applied RF frequency of high enough amplitude, when applied long enough (90° pulse), will completely rotate the $M_z$ into the transverse (xy) plane (Figure 1.16A). When the RF pulse is turned off, the protons emit the absorbed energy and return to equilibrium distribution by the process known as relaxation and $M_z$ recovers. The spin-lattice or longitudinal relaxation is the process by which protons release the resonant energy and return to their original equilibrium. The spin-spin or transverse relaxation occurs when an excited proton transfers its energy to a neighboring proton and eventually perturbing $\omega_0$ of protons; and the coherence of $M_{xy}$ is eventually lost. Following excitation, the longitudinal magnetization $M_z$ recovers with time constant $T_1$ [$M'_z = M_z(1 - e^{-\tau/T_1})$] and transverse magnetization $M_{xy}$ dephases with time constant $T_2$ [$M'_{xy} = M_{xy}e^{-\tau/T_2}$]. $T_1$ relaxation is the time when $M'_z$ reaches 63% of $M_z$ value (Figure 1.16B). Spin-spin or transverse or $T_2$ relaxation is the time required for $M_{xy}$ to decay to 37% of its initial value (Figure 1.16C). In biological systems, $T_1$ is considerably longer than $T_2$ and both $T_1$ and $T_2$ values differ from one tissue type to other (Table 1.2), which can be observed in an MRI.
Figure 1.16. (A) The net magnetization $M_z$ of protons in a rotating frame of reference. After a 90° RF pulse (field $B_1$) $M_z$ rotates completely into transverse xy plane, denoted as $M_{xy}$. Types of relaxation post 90° perturbation: B) $M_z$ recovers as shown by the $T_1$ relaxation curve and C) $M_{xy}$ dephases as shown by the $T_2$ relaxation curve following 90° RF.

In addition to spin transfers between neighboring protons responsible for the true $T_2$ relaxation, other factors also contribute to the loss of coherence in $M_{xy}$. These additional factors include local magnetic field inhomogeneities due to: a) differences in magnetic susceptibility of different tissues and b) nonuniformity of $B_0$ due to imperfections in magnet. Hence the net transverse relaxation time $T_2^*$ has all three contributions and given by the following relation

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_{2MS}} + \frac{1}{T_{2M}}$$

Eq. 1.9
where $T_{2MS}$ is the dephasing time due to magnetic susceptibility differences and $T_{2M}$ is the dephasing time due $B_0$ inhomogeneity.\textsuperscript{184}

### Table 1.2. $T_1$ and $T_2$ relaxation times of protons in different tissue types.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Relative proton density</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids (Fat, etc.)</td>
<td>0.60</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
<td>White matter</td>
<td>0.75</td>
<td>670</td>
<td>85</td>
</tr>
<tr>
<td>Gray matter</td>
<td>0.85</td>
<td>920</td>
<td>95</td>
</tr>
<tr>
<td>Peripheral muscle</td>
<td>0.80</td>
<td>620</td>
<td>45</td>
</tr>
<tr>
<td>Liver</td>
<td>0.70</td>
<td>570</td>
<td>45</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1.00</td>
<td>2000</td>
<td>1000</td>
</tr>
<tr>
<td>Edema</td>
<td>0.90</td>
<td>1060</td>
<td>150</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>0.90</td>
<td>1410</td>
<td>200</td>
</tr>
<tr>
<td>MS plaque</td>
<td>0.90</td>
<td>1100</td>
<td>150</td>
</tr>
</tbody>
</table>

**Spatial encoding:**

During MRI, the protons in an area are spatially separated by applying three linear gradient magnetic fields along the x, y and z directions (Figure 1.17). These gradients are small perturbations introduced to the main magnetic field $B_0$. As discussed earlier (Eq. 1.7 and 1.8), the magnetic field ($B_0$) is proportional to $\Delta E$ and hence proportional to $\omega_0$. Now, when $B_0$ is distorted by gradient fields the precession frequency of a proton in position $r_i$ is described by a modified Larmor equation:

$$\omega_i = \gamma (B_0 + G \cdot r_i)$$  \text{Eq 1.10}
where \( \omega_i \) is the frequency of proton at position \( r_i \) and \( G \) is a vector representing the total gradient amplitude and direction expressed in mT m\(^{-1}\). After the application of gradient fields, protons in different regions precess at different frequencies and MR image is a spatial mapping of these frequencies.

The first step in MR imaging is selecting a slice of a given tissue by applying a frequency selective RF pulse with a narrow range in the presence of a slice-selection gradient \( G_{SS} \) (z-axis). The duration and amplitude of the RF pulse determines the extent of proton rotation in the selected slice. The second gradient namely the readout or frequency encoding gradient \( G_{FE} \) (x-axis) applied perpendicular to \( G_{SS} \) further differentiates the precession frequencies of protons along its direction and provides one of the two dimensions of the MR image. The third phase encoding gradient \( G_{PE} \) (y-axis) is applied perpendicular to both \( G_{SS} \) and \( G_{FE} \), which introduces a phase variation among the spins and provides the second dimension of the 2D MR image. To obtain position information from the phase the process is repeated several times with incremental variations in \( G_{PE} \) amplitude.
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Figure 1.17. A 3.0 T MRI instrument for humans (Signa HDxt 3.0T, Photograph © 2013 General Electric Company), B) Illustration of the key components of MRI; the main magnet ($B_0$), and gradient coils generating gradient field along x, y and z directions and RF transmitter/receiver. C) The positioning of a patient and the direction of gradient fields.

The MRI image is acquired by orchestrating the time, length and the sequence in which the hardwares connected to RF pulse, $G_{SS}$, $G_{FE}$, $G_{PE}$ and signal collection are switched on and off. These hardware instructions termed as pulse sequence is controlled by a computer program. One of the commonly used pulse sequences is the spin echo sequence. During the sequence, a slice selection $90^\circ$ excitation pulse rotates $M_z$ into transverse plane $M_{xy}$ (Figure 1.18A). After time $\tau$ some of the proton spins go out of phase and $M_{xy}$ decays to $M'_{xy}$. A $180^\circ$ RF pulse causes the spins to refocus and come into phase in what is termed as spin echo. The precessing $M_{xy}$ registers a signal proportional to the magnitude of $M_{xy}$ at the receiver. When $M_{xy}$ decays, the corresponding free induction decay (FID) signal is observed, and after the $180^\circ$ RF pulse the signal strength reaches maximum at echo maximum. In spin echo based sequence, multiple $180^\circ$ pulses may be used
to produce multiple echoes. The echo time (TE) is the time between the excitation pulse and the echo maximum. The time between successive excitation pulses for a given slice is termed as TR. Both $T_1$ weighted and $T_2$ weighted MR images can be obtained using spin echo sequence by varying the TR and TE times. A short TR (400–600 ms, maximizes the differences in longitudinal magnetization) and TE (5–30 ms, minimizes $T_2$ dependency) produces a $T_1$ weighted image where tissues with shortest $T_1$ values produce highest intensity. Longer TR (1,500–3,000 ms) and TE (60–150 ms) will reduce $T_1$ effects and produce a $T_2$ weighted MR image.\(^{184}\)

Figure 1.18B shows the timing diagram for the standard spin echo sequence. Each line corresponds to different hardware component with their activity time and span shown as a deviation from the baseline. The amplitude corresponding to $G_{SS}$ and $G_{FE}$ that does not change from measurement to measurement are shown as a single deviation from baseline whereas the amplitude of $G_{PE}$ that changes incrementally through repetitions is shown as multiple levels of deviations. The MR signal is always recorded in the presence of $G_{FE}$. The three commonly used spin echo (Figure 1.18B) sequences are single echo, standard multi echo and fast spin echo. In the single echo sequence, each echo (after multiple 180° pulses) within the scan is measured at the same TE but with different $G_{PE}$ amplitude. This is used to produce $T_1$-weighted images and the variations in signal intensity from measurement to measurement are solely due to changes in $G_{PE}$. Standard multiecho involves multiple 180° pulses to refocus signals applied at multiple TEs. Multiecho sequences at short TE produce proton-density weighted images and at long TE produces $T_2$-weighted images. The fast spin echo sequence based on the RARE (rapid acquisition with
relaxation enhancement) technique requires a short scan time. This sequence like standard multi echo sequence uses multiple 180° pulses to produce multiple echoes; but as a deviation, each echo signal is acquired with different TE and $G_{PE}$-amplitude.
Figure 1.18. A) spin echo based pulse sequences and the effects on net magnetization ($M_z$). B) The timing diagram for a standard spin echo sequence. Adapted from reference 184.
To generate an image, a slice of tissue is excited repeatedly with $G_{FE}$ amplitude constant and $G_{PE}$ amplitude varying incrementally. The resulting signals are collected as a function of time as a series of lines in 2D array known as k-space (Figure 1.19B). A 2D Fourier transformation (Figure 1.19 C and E) of k-space data produces the 2D MRI image.

**Figure 1.19.** Image generation in MRI: A) Representation of a slice with two voxels of interest shaded in 10 × 10 array. B) After slice selection, $G_{FE}$ and $G_{PE}$ (incremental amplitude) are applied several times and the signals are collected as a function of time in k-space. 2D Fourier transformation along C) $G_{FE}$ and D) $G_{PE}$ directions of k-space data brings out the spatial distribution of the signal and an image is generated. F) the $T_1$ weighted 2D MRI of a slice of the human brain, Reproduced with permission from reference 151, copyright © 2007, Wolters Kluver Health.

Contrast agents that increase the relaxation rate ($R_1$ and $R_2$) of surrounding atoms (e.g. protons) have been used to visualize their area of distribution by the resultant MR signal enhancement. The increase in relaxation rate by a contrast agent is given by
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\[ R_i = \frac{1}{T_{i0}} + r_i C \]

Eq 1.11

where \( R_i \) is the longitudinal \((R_1 = \frac{1}{T_1})\) or transverse \((R_2 = \frac{1}{T_2})\) relaxation rate, \( T_{i0} \) is the longitudinal \((T_1)\) or transverse \((T_2)\) relaxation time in the absence of contrast agent, \( r_i \) is the longitudinal \((r_1)\) or transverse \((r_2)\) relaxivities and \( C \) is the concentration of the contrast agent. Relaxivities \((r_i)\) is defined as the increase in relaxation rates produced by 1 mM of contrast agents and has the unit \( \text{mM}^{-1}\text{s}^{-1} \). Paramagnetic contrast agents such as gadolinium complexes induce an increase in both \( R_1 \) and \( R_2 \). Paramagnetic relaxation is caused by the spin-spin interaction of the nuclei and the fluctuating magnetic field caused by the paramagnetic ions. Superparamagnetic IONPs on the other hand considerably increases \( R_2^* \) due to induced local magnetic field inhomogeneity and magnetic susceptibility differences (Eq. 1.9). IONPs that shorten the \( T_2/T_2^* \) of surrounding nuclei causes signal darkening in MR images and are also termed as negative contrast agents. The clinically approved MRI contrast agent ferumoxide (dextran coated IONP; IONP core diameter \( \approx 5 \) nm and hydrodynamic diameter 120 – 180 nm) has \( r_1 \) and \( r_2 \) values of 19 and 100 \( \text{mM}^{-1}\text{s}^{-1} \), respectively at 0.47 T and Ferumoxtran–10 (dextran coated IONP; core diameter \( \approx 5 \) nm and hydrodynamic diameter 15 – 30 nm) has \( r_1 \) and \( r_2 \) values of 28 and 100 \( \text{mM}^{-1}\text{s}^{-1} \), respectively at 0.47 T.

IONPs accumulation at disease sites through passive or active targeting enable better visualization of affected area and diagnosis using MRI. Combidex® (Ferumoxtran–10) have been successfully used in the diagnostic MRI of metastatic lymph nodes in prostate
cancer patients.\textsuperscript{154} High resolution MRI was recorded 24 hours after the intravenous administration of Ferumoxtran–10 (2.6 mg of Fe per Kg of body weight over 15 to 30 minutes) in saline. In $T_2$ weighted MRI, malignant nodes showed a decrease in signal intensity and metastatic regions within the node exhibited hyperintensity (Figure 1.20).

**Figure 1.20.** A) Illustration of the intravascular administration of long circulating dextran coated IONPs ($D_H = 28$ nm) which later leaks into the interstitial fluid and drain into the lymph node through lymphatic vessels. Metastases in lymph nodes cause disturbances in lymph flow and abnormal distribution of IONPs within. MRI of metastatic lymph node before (B) and 24 hours after (C) the intravenous administration of Ferumoxtran–10. In C, node area with IONP produces hypointense region and metastasis area without IONPs produces a hyperintense region (arrows). Reproduced with permission from reference 154, copyright © 2003, Massachusetts Medical Society.

Recently a clinical micro-NMR device (Figure 1.21A) has been developed for the fast quantitative analysis of several marker proteins of cancer with the aid of IONPs.\textsuperscript{187} This portable device (10 × 10 cm) is easy to operate and the samples can be directly placed on
the fluid channels (Figure 1.21B) or in thin walled glass tubes for analysis. Adding to its portability, the whole system can be operated using a smart phone interface (Figure 1.21C).

![Figure 1.21. A) The micro-NMR device with the mini magnet placed above the NMR electronics/hardware case. B) Illustration of the micro-NMR probe and C) The smart phone interface to operate the system. Reproduced with permission from reference 187, copyright ©2011 American Association for the Advancement of Science.](image)

In the first clinical trial, micro-NMR was used to measure well-known cancer markers including EpCAM (epithelial cell adhesion molecule), MUC-1 (mucin1, cell surface associated), HER2, EGFR (epidermal growth factor receptor), B7-H3, CK18, Ki-67, p53, and vimentin, from the cells obtained from fine-needle aspirate. Monoclonal antibodies for the target marker proteins were modified with trans-cyclooctene (TCO). Later IONPs modified with tetrazine (IONP-Tz) was incubated with cell adhered antibody-TCO to accomplish conjugation through TCO/Tz chemistry. The transverse relaxation rate ($R_2$) of the cell-IONP sample were measured using this micro-NMR operating with Carr-Purcell-
1.2.4 Other applications

Early transition metal (Mn, Fe, Co, Ni, Cu, Zn, etc.) oxide NPs are widely studied for their redox properties and electrochemical energy storage (electrochemical capacitors (ECs) and batteries) applications.\textsuperscript{188-190} Recently much research work has focused on ECs due to their potential applications in high power devices such as lasers, electric vehicles (for acceleration) and also in other variety of energy storage devices.\textsuperscript{191,192} Generally there are two modes of energy storage mechanism operative in ECs. One based on the double layer capacitance arising from the separation of charges (non-faradaic) at the electrode-electrolyte interface and the other based on pseudocapacitance resulting from faradaic reactions occurring at or near the solid electrode surface due to the presence of electro-active materials. The well-known electric double-layer capacitors (EDLCs) are usually based on the high surface area carbon materials namely activated carbon, carbon fiber cloth, carbon areogels and foams having very high specific capacitance values.\textsuperscript{193} Utility of this kind of capacitors is generally limited by the maximum capacitance range (10–40 \(\mu\)F/cm\(^2\)),\textsuperscript{194} electrochemical stability of the electrolyte, the utilization of electrode surface area due to the presence of non-wettable micropores (in terms of wettability and accessibility) and high internal resistance associated with these materials.\textsuperscript{195} On the other hand,
transition metal oxides such as ruthenium oxide (RuO$_2$) and iridium oxide (IrO$_2$) exhibit faradaic pseudocapacitance behavior with the single electrode capacitance values ranging between 720 and 760 F/g$^{196,197}$. In spite of the very high capacitance values and high reversibility, the high cost of ruthenium and iridium restrict their applications. Alternatively, other cheaper electrode materials based on the oxides of iron,$^{198}$ nickel,$^{188}$ cobalt,$^{199}$ and manganese,$^{200}$ which exhibit pseudocapacitance behaviour similar to that of ruthenium oxide were also reported. Iron oxide is particularly interesting as electrode material for electrochemical energy storage applications as they are inexpensive, biocompatible, environment friendly and easy to synthesize. The problem with iron oxide like other transition metal oxides lies in its poor electrical conductivity and is usually fabricated with conductive additives (e.g. conductive carbon, graphene, etc.).$^{198,201–203}$ Specific capacitance value of about 135 F/g has been reported for iron oxide based electrode material.$^{198}$

1.3 Characterization techniques

The chemical and physical properties of IONPs and any surface functional groups may be studied using one or more of the following techniques including powder X-ray diffraction (XRD),$^{204}$ electron microscopy,$^{205,206}$ infrared spectroscopy (FT-IR),$^{207}$ thermo gravimetric analysis (TGA), dynamic light scattering (DLS)$^{208}$. 

Figure 1.22 (top) shows the electromagnetic spectrum displaying wavelength ($\lambda$) ranges of different electromagnetic radiations. The electromagnetic radiation possesses two components, an electric sinusoidal wave-vector and a magnetic sinusoidal wave-
vector intersecting at zero-crossings at a 90° angle (Figure 1.22, bottom) and both wave-vectors are perpendicular to the direction of propagation. The wavelength (λ) of a radiation is the distance between the two consecutive crests (Amplitude; E and M for electric and magnetic components, respectively) or troughs of its sinusoidal wave. The energy associated with a particular radiation is given by $E = h\nu = hc/\lambda$, where $E$ is the energy of radiation (joules), $h$ is the Planck’s constant ($6.626 \times 10^{-34}$ J s), $\nu$ is the frequency (Hz), $c$ is the velocity of light (ms$^{-1}$) and $\lambda$ is the wavelength (m). From the equation, electromagnetic radiation with shorter wavelength (λ), will have a higher frequency (ν) and energy (E). Hence γ-rays and X-rays with short wavelengths are high-energy radiations and pose a greater health hazard on exposure in comparison to other low energy radiations. The wavelength of electromagnetic radiations may be described in meter (m), micrometer (1 μm = 10$^{-6}$ m) or nanometer (1 nm = 10$^{-9}$ m), as per convenience or practices specific to a characterization technique.
Figure 1.22. Top: The electromagnetic spectrum with wavelengths. Bottom: electromagnetic wave with electric field and magnetic field oscillating perpendicular to each other and the direction of propagation. Here, \( \lambda \) is the wavelength, \( E \) and \( M \) are the amplitudes of electric and magnetic fields respectively and \( c \ (2.998 \times 10^8 \text{ ms}^{-1}) \) is the velocity of propagation.

The human eye is sensitive to visible light wavelengths; from violet (\( \lambda = 400 \text{ nm or } 4 \times 10^{-7} \text{ m} \)) through the colors of the rainbow to red light (\( \lambda = 800 \text{ nm or } 8 \times 10^{-7} \text{ m} \)). Under normal daylight conditions, a healthy naked human eye can detect the VIBGYOR (Rainbow color) wavelengths emitted by the surrounding objects. The signals are processed by the visual cortex region of the human brain and the results are interpreted by one’s perception. Material characterization technique that uses electromagnetic radiation follows the same principle, with suitable light source, detector and output devices (computer monitor, image) to obtain direct or indirect structural information of a material for interpretation. Beyond the visible light there is a continuum of shorter and longer wave-
length lights that interacts with matter in unique ways. Following are the few examples of different radiations and their applications in different characterization techniques. Radiofrequency ($\nu = 60$–$600$ MHz, $\lambda = 5.0$–$0.5$ m) induces change in magnetic properties of some atomic nuclei ($^1$H and $^{13}$C) and is used in NMR spectrometer to study the chemical structure of organic molecules and MRI. Microwave ($\nu = 9500$ MHz, $\lambda = 3.1 \times 10^{-2}$ m) induces change in the magnetic properties of unpaired electrons and is used in electron spin resonance, ESR, or electron paramagnetic resonance, EPR, spectrometer to study free radicals. Infrared ($\lambda = 2.5 \times 10^{-6}$ to $2.5 \times 10^{-6}$ m) radiation, induce changes in the vibrational and rotational movements of atoms in a molecule, and is used in FT-IR spectrometer to identify the chemical functional groups in a material. Ultraviolet-Visible (UV, $\lambda = 190$–$400$ nm and visible $\lambda = 400$–$800$ nm) radiation causes transition in electronic energy levels in a molecule and is used in UV-Vis spectrometer for colorimetric analysis of molecules (UV-Vis active – with conjugated unsaturation). X-rays are scattered from crystals in a characteristic way and is used in powder X-ray diffractometers to study crystal structures of materials.

1.3.1 Powder X-ray diffraction (XRD)

Powder X-ray diffraction (XRD) is a powerful technique to characterize the phase composition, crystal structure (positions of atoms) and unit cell parameters of crystalline materials including as-synthesized or surface modified metal/metal oxide NPs. The data obtained from a diffractometer is called a diffraction pattern, which is a plot of user defined diffraction angle range ($2\theta$°) and corresponding diffracted X-ray intensities in counts per
second. The XRD pattern from single-crystalline NPs can be useful to identify their particle size.

Crystalline materials unlike amorphous materials have long-range order (across $\approx 10^3$ to $10^{20}$ atoms) i.e. there is a periodic repetition of atoms across a 3D space. Such an arrangement gives rise to several crystallographic planes (in which atoms are arranged) that intersect all lattice points. Planes of the same types are stacked parallel and equidistant from one another. Crystallographic planes are described using three integer indices $h$, $k$ and $l$ enclosed in brackets ($hkl$) called Miller indices (Figure 1.23) and the same family of crystallographic planes are grouped under same Miller indices. The distance between two adjacent planes is termed as interplanar distance or $d$-spacing ($d_{hkl}$) given by:

$$d_{hkl} = \sqrt{\frac{h^2}{a^2} + \frac{k^2}{b^2} + \frac{l^2}{c^2}}$$

Eq. 1.12

where $a$, $b$ and $c$ are the unit cell dimensions. Since X-rays have their wavelengths in the same order of magnitude as inter-atomic (scattering object) distances (between $\approx$0.5 Å and $\approx$2.5 Å), it is in the favourable range to produce diffraction. Like X-rays, neutrons ($\lambda$ $\approx$1 Å) and electrons ($\lambda$ = 0.02–0.05 Å) can diffract from crystals and are the basis for neutron diffraction and selected area electron diffraction (an option in TEM) experiments.
Figure 1.23. Cubic unit cells (a = b = c and $\alpha = \beta = \gamma = 90^\circ$) showing $hkl = 100$ (Left) and $hkl = 200$ (Right) type crystallographic planes.

The incident X-ray wave front on a crystal scatters from all the atoms (electron density) in the crystal lattice. For scattered intensities that deviate from the incident propagation vector ($2\theta > 0$, $\theta$ – scattering angle), a path difference is introduced between parallel propagating waves. Hence the intensity of diffracted waves is a function of the path difference, phase angle ($\phi$) and $\theta$. When several atoms are involved, the scattered wavefronts from a group of atoms may grow in amplitude or decay (depending on $\phi$ and $\theta$) due to constructive and destructive interferences (Figure 1.24A). Now when we consider crystals, we can replace atomic scattering by unit cell scattering as the latter is now forming a periodic array. The phase angle $\phi$ is the function of lattice spacing, which in turn is a function of Miller indices $h$, $k$ and $l$. The discrete points of intensities ($I$) in a reciprocal space (Diffraction pattern registers the ordered atomic positions into a reciprocal space rather than a direct image) are given by:
$I_{hkl} = K \times G(\theta) \times F_{hkl}^2$ \hspace{1cm} \text{Eq. 1.13}

where $K$ is the scale factor ($K = CU_1^2U_2^2U_3^2$, $U_1$, $U_2$, and $U_3$ are the total numbers of unit cell in corresponding $h$, $k$ and $l$ directions), $G$ is the various functions of diffraction angle $\theta$ and $F$ is the unit cell scattering function.\textsuperscript{204} An important law formulated by W. H. Bragg and W. L. Bragg (father son duo founded X-ray diffraction in 1913-1914 and were awarded Nobel Prize in Physics in 1915) proposed the relation between diffraction angle or Bragg angle, wavelength and interplanar spacing. According to the Braggs, the diffraction can be simply explained as a mirror reflection of incident X-rays by various crystallographic planes (Figure 1.24B). As already discussed, a set of planes of same $hkl$ family will have same interplanar distance $d_{hkl}$ and all planes can be considered as a scattering object. When the incident X-ray wavefront with parallel propagation meets with $hkl$ planes at an angle $\theta$, the diffracted wave (mirror reflection analogy) will also form the same angle $\theta$ with the $hkl$ planes. The path difference ($\Delta$) introduced between a pair of waves (incident and reflected) by adjacent planes is given by $\Delta = d_{hkl} \sin \theta$. The constructive interference is observed when the total path difference $2\Delta = n\lambda$, where $n$ is the integer and $\lambda$ is the wavelength of the incident X-ray wavefront. Simple geometrical derivation gives the Bragg’s law:

$$n\lambda = 2d_{hkl}\sin \theta_{hkl}$$ \hspace{1cm} \text{Eq. 1.14}

where $n$ is the integer known as the order of reflection and often takes the value of 1.\textsuperscript{204}
1.3 Characterization techniques

Figure 1.24. A) Diagrammatic representation of propagating electromagnetic waves and their interactions: in-phase waves give rise to constructive interference and the resulting amplitude is much larger than the amplitude of the individual waves. Out-of-phase waves give rise to destructive interference and the resulting amplitude is much lower than the individual waves. B) X-rays incident on crystal planes at certain angles fulfill Bragg’s law and gives raise to diffracted waves that are in-phase.

In a powder XRD instrument, X-rays are generated by bombarding high-energy electrons against a metal anode (Cr, Fe, Co, Cu and Mo). When copper is used as the anode, the X-ray spectrum obtained has three peaks namely $K\alpha_1$ ($\lambda = 1.540598$ Å), $K\alpha_2$ ($\lambda = 1.544426$ Å) and $K\beta$ ($\lambda = 1.39225$ Å). $K\alpha_1$ is of twice the intensity of $K\alpha_2$ and both of them are of higher intensities than $K\beta$. For XRD measurements only the $K\alpha$ radiations are
used and the $K_\beta$ radiations are removed using a Ni filter. Figure 1.25A shows a typical powder XRD instrument with X-ray source, sample holder and detector positioned in Bragg-Brentano geometry in a goniometer assembly. The whole goniometer assembly is placed inside a radiation enclosure. Figure 1.25B depicts the key components of the goniometer assembly with the X-ray source and the detector sitting at the end of two independent goniometer arms and the sample stage at the centre. The X-rays from the source are collimated by the divergence and soller slits before hitting the sample. The source, sample stage and the detector fulfil the Bragg-Brentano geometry required for recording the characteristic Bragg peaks of a powder sample across desired 2θ range (between 5° and up to 150°).

A good sample, and hence the sample preparation is crucial to obtain a good quality powder XRD. For XRD analysis, the powder sample is placed on a low background quartz (SiO$_2$ single crystal cut at 6° from $hkil = 0001$ plane) or silicon (Si single crystal cut parallel to $hkl = 510$ plane) discs, usually of 25 mm diameter. Ideally a powder sample should have infinite number of individual particles with infinite random orientations. Hence it is necessary that the particle sizes of a powder sample are small enough ($<$ 50 µm) to accommodate maximum number of particles with random orientations, for the given area. Large particles have to be ground using a mortar and pestle (made of agate for grinding hard materials) to reduce their particle size. In addition, the random orientation of the sample particles can be enhanced by spinning the sample during the measurement. In the case of NPs, the individual particle sizes are already small enough for powder
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XRD measurements; any large clumps or preferred alignments may be removed by sonicating or grinding (without exerting too much downward force) a NP dispersion in a suitable volatile solvent like acetone. The NP dispersion can be dropped on the sample disc and instantly dried to form a sample layer of uniform thickness.

Once a good quality sample is prepared it is equally important to select the right parameters to collect a good quality pattern. Instrumental parameters such as power settings, X-ray wavelength, monochromatization (using right divergence slits) of X-rays and goniometer optics and data collection parameters including scanning mode, scan range (2θ range), step size (2θ) and dwell time (in each step) should be set to subjective values to obtain a good quality XRD pattern. After data collection, phase(s) present in the sample is (are) identified by an automated peak search procedure and comparing it to a database (e.g., ICCD – International Centre for Diffraction Data) for a close match. The peak search and matching are done by a computer program and often the best matching profile out of a group of profiles have to be identified manually. Once the phase(s) present is (are) identified, the atomic structure and unit cell parameters can be learnt from the database or literature. Rietveld refinement can further confirm the structure and quantitatively determine the composition if more than one phase is present in the sample.
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Figure 1.25. A) The goniometer assembly of a powder XRD instrument (PANallytical X’Pert Pro, Department of Chemistry, University of Manitoba) inside the radiation enclosure. The goniometer assembly has the X-ray source, sample stage and detector placed in Bragg-Brentano geometry. B) Depiction of a goniometer assembly with goniometer radius $R$. In this particular setup, the X-ray source remains stationary, while the detector and the sample stage revolve around the centre (axis) in a synchronized fashion to attain the required $\theta$-$\theta$ values (C). The incident and diffracted X-rays are collimated by divergence and soller slits. Adapted from reference 204.

Figure 1.26A and C show the powder XRD pattern of magnetite NPs and bulk scale magnetite particles with their characteristic Bragg peaks labeled with their reflecting Miller planes. The Bragg peaks from NPs are broader than the peaks from bulk scale particles due to small size and microstrain. The peak broadening in the XRD pattern of NPs can be employed to determine their sizes using Scherrer equation:

$$D = \frac{0.9\lambda}{B \cos(\theta)} \quad \text{Eq. 1.15}$$
where $D$ is the particle size, $\lambda$ is the wavelength, $B$ is the peak broadening ($B_{\text{ob.}} - B_{\text{instrumental}}$) and $\theta$ is the diffraction angle.

Figure 1.26. A) and B) The powder XRD pattern and HR-TEM image of magnetite nanoparticles ($\approx 5$ nm), respectively. C) and D) The powder XRD pattern and SEM image of bulk-scale magnetite particles, respectively. The characteristic Bragg peaks from different $hkl$ crystallographic planes are labeled; the observed peak broadening in the XRD pattern of magnetite NPs compared to the sharp XRD peaks from bulk-scale magnetite is due to the strain resulting from small crystallite sizes. Bulk magnetite data reproduced with permission from 210, copyright © 2006 Elsevier Ltd.
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1.3.2 Electron microscopy

Perhaps the most important and widely used technique to literally visualize nanoscale dimensions and morphology of NPs is EM. The human eye is sensitive to visible light (wavelength range of 400–700 nm or 0.4–0.7 μm) and depending on one’s eyesight and lighting conditions has a resolving power of around 0.1–0.2 mm. In order to visualize an object or its details that scale below the human resolving power, we need the aid of a microscope. Anton van Leeuwenhoek, considered as “The Father of Microbiology” developed the first microscope in the 17th century. Using his single-lens microscope, he pioneered studies on single-cell organisms and microorganisms. Later advancements led to the development of compound microscopes that had two or more lenses. Most of the earliest microscope used visible light for sample illumination. The wavelength of the illuminating light source determines the resolving power of a microscope, i.e. the larger the wavelength, the lower the resolving power and vice versa. An advanced optical microscope with visible light as the illumination source can have a maximum resolving power of ≈0.2 μm (100× magnification). A visible-light optical microscope is incapable to resolve nanoscale dimensions of a material. In order to probe nanoscale dimensions and atoms, we need a light source with wavelengths comparable to inter-atomic distances. Louis de Broglie (Nobel Prize in Physics, 1929) in 1924 proposed a then controversial theory that matter possess wave nature (de Broglie wavelength $\lambda = h/mv$, where $\lambda$ is the wavelength of a matter, $h$ is the Planck’s constant, $m$ is the mass of the particle and $v$ its velocity; $mv$ is the momentum of the particle). The wave-matter theory was confirmed by
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Clinton J. Davisson and Lester Germer in 1927 when they demonstrated that an electron-beam, like X-rays, produced characteristic electron diffraction pattern from a single crystal of nickel (Clinton J. Davisson and George P. Thomson were jointly awarded The Nobel Prize in Physics in 1937). The wave nature of the electron very soon led to the idea of a microscope with electrons as the illumination source and the first electron microscope was developed by M. Knoll and E. Ruska in 1932. E. Ruska was awarded The Nobel Prize in Physics in 1987 for developing electron optics and designing the first electron microscope. In his Nobel Lecture, E. Ruska recalls how he was “heartened” when he found the wavelength of the electron calculated using de Broglie equation was around five orders of magnitude shorter than that of the visible-light. In addition to wave properties, electrons also behave like negatively charged particles and hence can be deflected and focussed using electric or magnetic fields.

Electron microscopy is a broad term used to describe several microscopy techniques such as TEM, high resolution TEM (HR-TEM), selected area electron diffraction (SAED), analytical EM (AEM) and scanning electron microscope (SEM). The commonality between these microscopy techniques is that they all use electron beams to irradiate, visualize, image and analyze materials. Depending on the energy of the electron beam these microscopes can have nanoscale and/or atomic resolution. Figure 1.27 illustrates the result of an incident high-energy electron beam hitting a crystalline sample. Depending on the sample thickness a portion of the electrons may be transmitted which forms the magnified image (×10³ to ×10⁶) of the sample in TEM and HR-TEM. The elastically
scattered electrons from the sample fulfilling Bragg’s law will form a characteristic electron diffraction pattern (DP) in selected area electron diffraction (SAED) analysis. Chemical information of the sample can be obtained from the characteristic X-rays and secondary electrons using X-ray energy-dispersive spectrometry (EDS) and electron-energy loss spectrometry (EELS), respectively. EDS and EELS fall under the category of analytical EM (AEM). SAED, AEM and STEM are usually available as optional features in many commercial TEMs.

**Figure 1.27.** The effect of a high-energy electron beam, incident on a thin crystalline sample (NP) is shown.

The sample preparation for inorganic metal or metal oxide NPs for TEM analysis is straightforward. The sample is usually placed on a circular grid (copper, molybdenum, etc.) measuring around 3 mm in diameter, with different mesh sizes (200, 400, etc) and with carbon coating. A dilute dispersion of the NPs in a suitable solvent (preferably fast
drying) is dropped on to the carbon coated copper grid and air-dried prior to TEM analysis. The sample grid is placed in a TEM sample holder and introduced into the TEM column (perpendicular to the optical axis) through a side-entry door, equipped with an air-lock chamber. The sample holder allows the sample to be moved horizontally along the xy plane (for surveying different regions) and rotated (for electron tomography) along the long axis during imaging.

Figure 1.28A shows a typical TEM with a cylindrical column enclosing the electron-optical system including electron gun (at the top), magnetic lenses, sample chamber and a viewing chamber with fluorescent screen and a binocular. The electron-optical column is maintained under high vacuum during TEM analysis. Figures 1.28B and C are the representative diagrams of a TEM in imaging and diffraction mode, respectively, with its key inner components and electron beam path shown. The electron gun consists of an electron source (Cathode: W or LaB$_6$) and an electron-accelerating chamber (anode) and produces the high-energy electron beam for sample irradiation. The wavelength of high-energy electron beam generated at 200 kV has a wavelength of 0.0025 nm, short enough to probe atoms. The incident electron beam is focused on the sample using one or more condenser lenses that are electromagnets. The focusing of electron beam can be adjusted by increasing or decreasing the current flowing in the electromagnetic lenses.
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Figure 1.28. A) A transmission electron microscope (JEOL JEM-2100F, The Department of Mechanical and Manufacturing engineering, University of Manitoba). B) The diagram showing the TEM electron beam path in imaging and C) diffraction modes. The image planes formed in the beam path are indicated by arrows (perpendicular to optical axis) and the diffraction planes are indicated by shaded circles.

The objective lens magnifies the diffracted electron beam from the sample and generates a real image of the sample at around 10 cm from the centre of the objective lens. Electrons scattered through large angles are absorbed by the diaphragm of the objective aperture. As a result, areas in the sample that scatter strongly will appear as dark regions in the image. The contrast resulting from scattering is termed as scattering or diffraction contrast. The intermediate lens allows the magnification in TEM over a range of $10^3$ to $10^6$ by changing its focal length in small steps. When a large change in focal length is introduced by reducing the intermediate lens current, the lens produces a diffraction pattern at the projection plane. The projector lens projects the image or a DP across the
whole area of the fluorescent screen. The fluorescent screen emits visible light in the yellow-green region when hit by electrons, which can be observed through the viewing window or optical binoculars. The image on the fluorescent screen is mainly used for manipulations such as finding a sample area, magnifying, fine focusing, selecting an area for diffraction using SAD aperture, etc. Once a satisfactory image is obtained at the fluorescent screen, the image can be recorded using image plates or CCD cameras. All new TEMs are equipped with CCD cameras that allow the user to view a live image in a computer monitor and capture a digital image.

In the case of NPs, the digital TEM image can provide direct information on the appearance of the NPs. The dimensions of NPs can be measured precisely with reference to the scale by using image analysis software like ImageJ. The HR-TEM image reveals the atomic arrangements in a crystal plane and their characteristic $d$-spacing can be measured. The crystal structure of NPs can be studied using the electron DP from SAED analysis. Further analysis such as EDS and EELS can provide information on the elemental composition of the NPs.

TEM is a powerful tool in materials and nano science, but there are few limitations that one should be aware of while making apparent interpretations. In many cases, corrective measures can be taken to overcome these limitations: a) A TEM can only focus on a very small area of the sample at a time at nanoscale resolution. Therefore a single imaged area may not be an absolute representation of the whole sample. To overcome this, any observed trend (size, shape, SAED, EDS or EELS) in TEM should be verified by sampling as many random areas of the sample grid as possible. b) Like any digital photo-
1.3 Characterization techniques

graph, a TEM image is the 2D representation of a 3D subject and is prone to artifacts. It is difficult to identify an artifact especially in a TEM image, due to the lack of point of references, crucial for our brains perception. Hence caution must be taken while visually interpreting a TEM image. The limitation with a 2D image may be overcome by imaging an area of interest at different tilt angles by rotating the sample holder. Images from a sequence of tilt angles can be used to generate a 3D image by the electron tomography technique to obtain a precise representation. c) The TEM does not have the depth perception for SAED, EDS and EELS analysis and the DP or spectra is an average representation of the whole thickness of the sample. d) The high-energy electron beam is strongly ionizing and may damage the sample, especially polymers and organic molecules; hence prolonged exposure of a small area should be avoided.

Figure 1.29. Schematic diagram of a typical scanning electron microscope (SEM). The electron optical column and the sample chamber are kept under vacuum during operation.
Scanning electron microscopy (SEM) is another widely used EM technique in materials science (Figure 1.29). The electron gun in a SEM usually operates at 30 kV and the low kinetic energy electrons require smaller lenses to focus. SEM follows a different imaging principle, which requires fewer lenses than TEM. Hence a typical SEM electro-optical column is considerably smaller than the ones found in TEM. The incident electron beam width at the sample also called the electron probe has to be very small (10 nm to 1 nm) whereas in TEM the beam width while imaging can be around 1 µm. The TEM uses a stationary electron beam whereas the electron probe in SEM scans horizontally across the sample in x and y directions, following a raster scanning format. The scanning path of the electron probe is manipulated by the magnetic field generated by the scanning coil. There are detectors to record secondary electron (ejected atomic electrons by inelastic scattering) and backscattered electron signals from the sample. The secondary electron originates from a very small depth from the surface of the sample (< 2 nm) and provides surface morphology or topographical contrast while imaging using a secondary detector. The number of backscattered electrons depends on the type of the nuclei present in the sample and can provide compositional contrast and details of the sample while using a backscatter detector. The contrast in an SEM image is generated by translating the detector signal at a particular xy coordinate of the sample as the brightness for the corresponding xy coordinates of the screen pixel in the image. The magnification in a SEM is given by: (scan distance in the image)/(scan distance on the sample). Many of the commercial SEM also feature an EDS spectrometer to analyze the elemental composition of a sample.
The sample meant for SEM imaging (except for Environmental SEM) must be conducting to ground the sample current. When the sample is non-conducting, it may build up electrostatic charging from the electron probe. A negatively charged sample will deflect the electron probe and distort the image. A non-conducting sample can be coated with a thin film of conducting metal (gold) or carbon (up to 4 nm thickness) by sputter coating. Before coating the powder sample placed on a sticky conducting tape (carbon) on the SEM sample mount. In coated samples, the image generated from secondary electron signal originates from the coating rather than the actual specimen.

1.3.3 Infrared Spectroscopy

The surface organic coating on magnetic NPs is usually identified using infrared spectroscopy (FT-IR). The primary technique used by Organic Chemists, to study the structure of organic molecules is NMR (¹H and ¹³C NMR) spectroscopy. But it is difficult to investigate organic molecules on IONPs surface as they exert magnetic field inhomogeneity on protons and carbons (in organic surface coating and solvent) at a distance of up to 50 μm, thereby causing peak broadening and resulting in the loss of characteristic peak splitting.²¹³-²¹⁴ FT-IR as a standalone characterization technique for magnetic NP-organic coating is acceptable when complex organic molecules are conjugated and not synthesized on the NP surface.

At ambient conditions, molecules are in constant state of vibration. There are two types of molecular vibrations called stretching and bending. The stretching vibration is the rhythmical motion of two atoms connected by a chemical bond (along the bond axis)
such that they move to-and-fro from one another. Bending vibrations involve change in bond angle between two bonds with one of the atoms as the centre. A molecule has many degrees of freedom depending on the total number of atoms. An atom has three degrees of freedom along x, y and z direction of a Cartesian coordinate. Hence a molecule with \( n \) number of atoms will have \( 3n \) degrees of freedom. In the case of a molecule, three degrees of freedom correspond to its rotation and the other three degrees of freedom correspond to its translational motion; the remaining \( 3n-6 \) degrees of freedom corresponds to its vibrational degrees of freedom. For a linear molecule the vibrational degrees of freedom is given by \( 3n-5 \), since two degrees of freedom can describe rotation. For example, the non-linear \( \text{H}_2\text{O} \) and the linear \( \text{CO}_2 \) molecules will have a total of 3 and 4 modes of vibration respectively. It is the change in charge distribution from oscillating dipole moment that actually interacts with the oscillating electric field of the electromagnetic IR radiation. Hence, only the vibrational modes that produce a rhythmical change in dipole moment of a molecule are observed in an IR spectrum.

IR radiation range is classified as near (\( \lambda = 7.8 \times 10^{-5} - 2.5 \times 10^{-3} \text{ cm} \), \( \bar{\nu} = 14290 - 4000 \text{ cm}^{-1} \)), mid (\( \lambda = 2.5 \times 10^{-4} - 2.5 \times 10^{-3} \text{ cm} \), \( \bar{\nu} = 4000-400 \text{ cm}^{-1} \)) and far (\( \lambda = 2.5 \times 10^{-3} - 5 \times 10^{-2} \text{ cm} \), \( \bar{\nu} = 400-20 \text{ cm}^{-1} \)) regions and among the three, the mid IR range is the most useful for studying organic and inorganic molecules. When infrared radiation (\( 4000-400 \text{ cm}^{-1} \)) passes through sample (e.g., IONPs with organic coating), the radiation, the resonant frequencies (\( \nu = c/\lambda \) in Hz; where \( c \) is the speed of light) are absorbed and the rest transmitted. In IR spectroscopy, the absorption or transmittance peaks or bands are described in wavenumbers: \( \bar{\nu} = 1/\lambda \), where \( \lambda \) is the wavelength in cm and \( \bar{\nu} \) has the unit
cm\(^{-1}\). Transmittance is the ratio of radiant power transmitted to the radiant power incident and absorbance is given by \(\log_{10}\left(\frac{1}{\text{transmittance}}\right)\). The FT-IR spectrum is the plot of transmittance or absorbance against wavenumber. Some older textbooks and literature may use wavelength in \(\mu\text{m}\) instead of wavenumber.

The theoretical approximation of stretching frequencies can be derived using Hooke’s law by comparing two bonded atoms to two masses held together by a spring (a harmonic oscillator). Then according to Hooke’s law, the frequency of oscillation (\(\bar{\nu}\)) of a bond is given by:

\[
\bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{f}{(M_x M_y)(M_x + M_y)}}
\]

Eq. 1.16

where \(\bar{\nu}\) is the vibrational frequency, \(c\) is the velocity of light, \(f\) is the bond force constant \((f = 5 \times 10^5 \text{ dyne/cm for single bonds and around twice and thrice the value for double and triple bonds, respectively}), M_x\) and \(M_y\) are the masses of the \(x\) and \(y\) atoms, respectively. Hence different bond types (\(\text{C}−\text{C}, \text{C}−\text{H}, \text{C}−\text{O}, \text{C}−\text{N}, \text{O}−\text{H}, \text{N}−\text{H}, \text{C}═\text{C}, \text{C}═\text{O}, \text{C}═\text{N}, \text{C}≡\text{C}, \text{C}≡\text{N}, \text{Fe}−\text{O}, \text{etc.})\) possess different stretching frequencies and absorption frequencies. The actual absorption frequency of a functional group in a molecule may vary slightly based on the neighboring atoms, due to factors such as coupling interactions, hydrogen bonding, conjugations, etc. For example the C═O stretching in \(\text{CO}_2\) and acetophenone appears at \(\approx 2350\) and \(\approx 1685\ \text{cm}^{-1}\), respectively. Figure 1.30 shows the various stretching and bending modes for \(\text{CH}_2\) group in an aliphatic organic molecule and their
corresponding vibrational frequencies are given in the figure caption. Here the $3n-6$ rule does not apply as the CH$_2$ group is not a molecule but is the part of a molecule. The FT-IR spectrum usually does not display peaks/bands corresponding to all theoretical number of fundamental vibrations due to one or more of the following reasons: a) frequencies falling outside the measured range (400 – 4000 cm$^{-1}$), b) the bands are too weak to be observed, c) the vibrational frequencies that are very close display convoluted or overlapping bands, d) Absorptions at the same frequency range (e.g., in highly symmetrical molecules) display a single band and d) the vibrational mode doesn’t induce change in the molecular dipole moment.

![Diagram of vibration modes of CH$_2$ group]

**Figure 1.30.** Illustration of the different vibration modes of CH$_2$ group: A) asymmetrical ($\approx 2900$ cm$^{-1}$) and B) symmetrical ($\approx 2800$ cm$^{-1}$) stretching vibrational frequencies. In-plane bending vibrational frequencies: C) scissoring ($\approx 1450$ cm$^{-1}$) and D) rocking ($\approx 720$ cm$^{-1}$). and out-of-plane bending vibrational frequencies: E) twisting ($\approx 1350$–1150 cm$^{-1}$) and F) wagging ($\approx 1350$–1150 cm$^{-1}$).

Modern FT-IR spectrometers allow speedy analysis (few seconds to minutes depending on number scans) on a small ($\approx 1$ mg) amount of sample. A conventional single beam FT-IR spectrometer is shown in Figure 1.31A. Commercial FT-IR spectrometers offer
flexibility in terms of IR sources (near, mid and far IR), sample mounts (KBr pellet, attenuated total reflectance ATR with ZnSe, Ge or diamond crystals; for direct IR on solid and liquid samples, etc.) and detectors (pyroelectric tri-glycine sulfate TGS, photoconductive HgCdTe MCT, etc.) to mention a few. Figure 1.31B is the schematic diagram of inner components of FT-IR spectrometer with IR source, interferometer, sample mount and detector. The IR source emits all the frequency range used to probe molecules. The interferometer assembly consists of a beam splitter (KBr), and two mirrors (one stationary and one movable). The collimated IR beam from the source, with all resonant frequencies (4000–400 cm⁻¹) hits the beam splitter, which divides the incident amplitude into two. One part of the IR radiation hits the fixed mirror and the other part hits the movable mirror. The returning beams from both the mirrors recombine at the beam splitter and passes through the sample and then to the detector. During IR measurement, the motion in movable mirror introduces path difference between the two returning beams and due to constructive and destructive interference of all wavelengths; an interferogram is obtained. The resulting signal from the detector contains the information on all resonant frequencies. The FT-IR spectrum is obtained by the Fourier transformation of the signal from space domain (scan mirror position) to wavenumber domain.
1.3 Characterization techniques

Figure 1.31. A) A single-beam Bruker Tensor-27 FT-IR spectrometer (Department of Geological Sciences, University of Manitoba) with its sample chamber door open. The sample holder attachment (8) is designed for KBr pellet. B) The schematic diagram of a single beam IR spectrometer.

Sample preparation is key for obtaining good quality FT-IR spectra. Techniques including attenuated total reflectance (ATR), photo-acoustic spectroscopy (PAS) and diffused reflectance infrared Fourier transform (DRIFT) spectroscopy involve fewer sample preparation steps. The NP samples in this thesis were all prepared as KBr pellets, which yielded better results than ATR technique. Solid samples can mixed with IR transparent material such as KBr and pressed into pellets for the transmission technique. A good quality KBr pellet with sample should be homogeneous, thin and translucent to IR for effective analysis. During sample preparation, around 150 mg of anhydrous KBr, along with $\approx 1\text{–}3\ \text{mg}$ of the dry NP sample was mixed well in agate mortar and pestle making sure that NP is homogeneously incorporated. The fine powder mixture is placed between
the anvils (two stainless steel cylindrical blocks) of the pellet-making die (13 mm) and pressed in a hydraulic press at 9 ton pressure for one minute. The KBr pellet (diameter 13 mm and thickness \( \approx 1 \) mm) is removed carefully without breaking and used immediately for analysis or stored in a desiccator for later analysis.

When a single-beam IR spectrometer is used, a background scan (without sample) is recorded in order to subtract any signals from the instrument (organic coatings) and atmospheric moisture and CO\(_2\), which strongly absorbs IR radiation. Many modern FT-IR instruments have built in rechargeable desiccants for moisture and CO\(_2\) and provisions to purge the chamber with nitrogen gas. The FT-IR spectrum is collected by selecting desirable parameters such as wavenumber range, number of scans (for better signal to noise ratio), etc. From the FT-IR spectrum of a pure sample with adequate resolution and intensity, different functional groups in the sample can be identified from their characteristic absorption bands. A detailed list of organic and inorganic functional groups and their characteristic absorption frequencies can be found in any good spectroscopy book.\(^{207}\) Figure 1.32 bottom shows the FT-IR spectrum of IONP-Sil-PEG(COOH) (with IONP core, silane shell and conjugated surface PEG acid) and the characteristic bands are labeled in the spectrum. The broad band at 3437 cm\(^{-1}\) can be assigned to –OH stretching vibration of carboxylic acid group and moisture. The two peaks at 2916 and 2850 cm\(^{-1}\) corresponds to the asymmetrical and symmetrical C–H stretching of CH\(_2\) groups, respectively. Peaks at 1720 and 1637 cm\(^{-1}\) correspond to the C═O stretching vibrations in surface carboxylic acids and amide groups, respectively. The N–H stretching vibrations of secondary amide shows a band at around 3330–3060 cm\(^{-1}\) (combined with –OH stretch-
Characterization techniques

ing band) and a bending vibration is observed at around 1570–1515 cm\(^{-1}\) (appears as a shoulder to amide C═O stretching band). The C–O–C stretching vibrations in PEG chain is observed as a band at 1107 cm\(^{-1}\). The Si–O stretching frequencies of the silane shell is observed as a band at 956 cm\(^{-1}\). The peak at 588 cm\(^{-1}\) corresponds to the Fe–O stretching vibrations in the IO core.

**Figure 1.32.** The representative structure of IONP with silane shell and conjugated PEG acid (top) and its FT-IR spectrum (bottom) displaying characteristic peaks from IONP core, silane shell and the conjugated PEG acid. FT-IR spectrum reproduced with permission from reference 215, copyright © 2013 American Chemical Society.

Precise interpretation of absorption bands from complex organic molecules is not always possible using FT-IR. Hence the bands and corresponding functional groups may be
confirmed through direct or indirect means. The functional groups may be directly confirmed by comparing the FT-IR interpretations with the data obtained from other characterization techniques such as mass and NMR spectroscopy. On the other hand the knowledge of the expected product from known reactants can be helpful in identifying the peaks characteristic of the product. In the above example, IONP-Sil-PEG(COOH), is formed by the amidation reaction between well characterized reactants IONP-Sil(NH₂) and PEG diacid (the latter was purchased). Hence, while interpreting the IR spectrum of IONP-Sil-PEG(COOH) the functional groups carried over from the reactants can be easily assigned and any characteristic bands that are supposed to be produced by the newly formed functional groups can be searched for and assigned. The presence of two C═O bands for carboxylic acid and amide groups (newly formed while coupling) and the characteristic peaks corresponding to IO, silane and PEG chain reasonably supports the structure of a conjugated product.

1.3.4 Thermogravimetric analysis (TGA)

The apparent mass of the surface organic coating on IONP can be quantified using thermogravimetric analysis (TGA). A TGA instrument measures the mass of a sample as a function of temperature or time.

Figure 1.33 shows the schematic diagram of a TGA instrument that typically has a sample holder held by a precision microbalance. The sample holder is positioned inside a furnace (high temperature furnaces may have a range of 50 °C to 1500 °C), which is controlled by a heating, cooling and timing program. Sample holders (metallic/ceramic
pans/crucibles) made of materials including platinum, tungsten, alumina, etc. that are inert to samples and can withstand several heating cooling cycles are used. The amount of dry sample required for the measurement depends on the TGA balance and may vary from as low as 2 mg to up to 50 mg. The atmosphere of the sample can be controlled by using different purge gases (e.g. argon or nitrogen for inert atmosphere) depending on the experiment and interest.

The sample when subjected to a programmed heating may lose (e.g. pyrolysis of organic materials) or gain weight (e.g. oxidation of metals) depending on the temperature, nature of the material, atmosphere and the time. To measure the amount of organic coating on IONPs, the TGA chamber is purged with inert nitrogen gas during heating (usually up to 700 °C). At specific temperatures, the organic coating undergoes pyrolysis and registers weight loss in the microbalance as function of temperature. If identifying the temperatures of different weight loss events (loss of water, loss of solvent, pyrolysis, decomposition, oxidation, etc.) is of interest, a better resolution and hence a lower scanning rate

Figure 1.33. Schematic diagram of a typical TGA instrument.
Characterization techniques

(e.g. 10 °C per minute) is desirable. If instrument time is a constraint and the interest is merely to quantify the percentage weight loss, a higher scan rate of 50 °C per minute is sufficient and yields reproducible results. Another useful step is to calculate the first derivative of the weight loss with temperature (mostly available as an option in the instrument’s operating program). The peaks in the first derivative curve indicate the different temperatures at which greater rate of change (weight loss event) has occurred. Figure 1.34 displays the TGA weight loss curve and the derivative weight curve (scan rate 20 °C per minute, N₂ atmosphere) for IONP with silane shell and PEG-acid coating (Figure 1.32, top). The pyrolysis of the organics (propyl group in silane and PEG-acid) registered ≈31% weight loss. The dominant peak at 275 °C in the derivative curve indicates the temperature of major weight loss event.

![TGA and derivative curves](image)

**Figure 1.34.** TGA (blue line) and derivative (maroon line) TGA curves of IONP with silane shell and conjugated PEG acid (Figure 1.32, top). Reproduced with permission from 215, copyright © 2013 American Chemical Society.
TGA is a very sensitive technique and as a result is prone to errors from instrumental and external factors, if proper measures are not taken during experiments. External factors including vibrations, temperature, sample preparation (mass, packing and position in sample holder) may introduce errors during TGA and affect reproducibility. Care must be taken to prevent possible errors due to external factors. Instrumental factors including calibration, furnace cleanliness, temperature range, scanning rate and sample atmosphere may also affect the reproducibility of results. Hence, it is important to calibrate (balance, furnace and temperature) the instrument for specific temperature program before every experiment.

1.3.5 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) or photon correlation spectroscopy is a useful technique to study the size distribution, stability and agglomeration of NPs in solution. NPs in solution undergo Brownian motion i.e. move in random directions due to colliding solvent molecules. The DLS studies the hydrated NPs in motion (Brownian) and estimate the hydrodynamic radius from scattered light intensities.

Commercial DLS instruments use low power laser beams (λ ≈ 780 nm) from diode lasers as the light source to irradiate the sample (colloidal dispersion). Depending on the instrument, the sample may be held in a fixed sample chamber or removable quartz cuvet. The detector to collect scattered intensity may be positioned at different angles (180° or 90°); in the schematic of the DLS instrument shown in Figure 1.35 the photodetector is positioned at a 90° angle. When light is shone on the NPs under Brownian motion, they
scatter light (Rayleigh scattering, when NPs are smaller in size than the wavelength of the light) in all directions and the scattered rays have random phase. The scattered rays from multiple particles in motion experience constructive and destructive interference and produce a time dependent intensity fluctuation. The scattered light is registered as real-time intensity \( I(t) \) at the photodetector that operates as a pulse counter.

![Diagram](image)

**Figure 1.35.** Schematic diagram of a dynamic light scattering instrument.

The intensity time-correlation function \( G(\tau) \) is given by

\[
G(\tau) = \frac{I(t_0)I(t_0+\tau)}{I(t_\infty)^2} = B + \beta e^{-2\Gamma \tau}
\]

Eq. 1.17

where \( B \) is the baseline of the correlation function at infinite delay, \( \beta \) is the correlation function amplitude at zero delay, \( \Gamma \) is the decay rate and \( \tau \) is the lag time.\(^{216}\) For diffusing spherical particles, \( G(\tau) \) decays exponentially with the decay rate given by

\[
\Gamma = 2Dq^2
\]

Eq. 1.18

where \( \Gamma \) is the decay rate, \( D \) is the diffusion coefficient of the particle and \( q \) is the magnitude of the scattering wavevector given by
1.3 Characterization techniques

\[ q = \frac{4\pi n \sin \frac{\theta}{2}}{\lambda} \]  \hspace{1cm} \text{Eq. 1.19}

where \( \theta \) is the scattering angle, \( n \) is the refractive index of the carrier medium (solvent) and \( \lambda \) is the wavelength of the laser beam. The decay rate \( \Gamma \) is obtained from the correlation function Eq 1.17 and the diffusion coefficient is obtained by substituting the value in Eq 1.18. For non interacting particles, the diffusion coefficient \( D \) is related to the hydrodynamic radius as given by the Stoke-Einstein equation

\[ R_H = \frac{kT}{6\pi \eta D} \]  \hspace{1cm} \text{Eq. 1.20}

where \( k \) is the Boltzmann constant, \( T \) is the temperature, \( \eta \) is the viscosity of the solvent. The measured \( R_H \) is the radius of a hypothetical hard sphere which diffuses at the same speed as the particle being examined and includes the effect of shape and solvation. The particles in suspension are hydrated and dynamic and the radius obtained from the diffusional properties of particles using DLS represents the apparent size of the hydrated and dynamic particles (Figure 1.36). The \( R_H \) of NPs is affected by its stability in a solvent and any unfavorable conditions that promote agglomeration (pH, high concentration, etc.). Agglomeration or attached surface molecules will result in a \( R_H \) larger than crystallite sizes obtained from TEM and XRD.
Figure 1.36. Schematic representation of the Hydrodynamic radius ($R_H$) of the hypothetical hard sphere (dotted circle) of A) spherical particle, radius of NP $R_{NP} \approx R_H$, B) agglomerate, $R_{NP} \neq R_H$, C) spherical NP with surface coating, $R_{NP} \neq R_H$, D) rod-shaped particle and E) protein molecule.

1.4 Thesis overview

The work in this thesis focuses on IONPs synthesis, manipulations (surface chemistry, size, shape, etc.) and in vitro pharmacological studies for potential biomedical applications. This set of work presents a significant advancement with the use of a novel reduction-hydrolysis approach for preparing magnetite NPs. Through the chapters, the versatility of the reduction-hydrolysis synthesis is established by adapting it to prepare a robust IONP nanocarrier suitable for a variety of bioconjugations and was further extended to prepare other early transition metal oxide NPs and silica particles. The experimental results are divided into five chapters (2 through 6).

Chapter 2, presents the synthesis of IONPs with biocompatible poly(ethylene glycol) (PEG), dextran and poly(ethyl methacrylate) (PEMA) surface coating, following Mas-
sart’s coprecipitation method. The model drug protein bovine serum albumin (BSA) was physisorbed on all three IO core and polymer shell NPs. As a proof of principle, the magnetically directed convection of model drug BSA is demonstrated in vitro using agarose gel and egg under the gradient magnetic field of a 7T MRI instrument.

Chapter 3 presents the synthesis of pure magnetite NPs by a novel reduction-hydrolysis method. This method follows the same principle of coprecipitation method, except that a single iron precursor [Fe(acac)₃] was used instead of two (FeCl₂ and FeCl₃) used in Massart’s method. The reducing agent NaBH₄ initiates reduction and hydrolysis of Fe(acac)₃ to form Fe³⁺ and Fe²⁺ species, which at suitable concentration nucleate and precipitate into crystalline magnetite NPs. The versatility of this synthesis in terms of IONP size selection, in situ or post synthetic surface coating ability with suitable hydrophilic (PEG, glutamic acid) or hydrophobic (oleic acid) organic molecules are shown. Mössbauer spectroscopy studies on the reaction intermediates assisted in formulating a plausible mechanism of IONP formation. Toxicity studies of the as-synthesized IONPs were carried out in vitro, on cell culture models. This versatile reduction-hydrolysis method addresses the issues of broad size distribution and mixed IO phases associated with the coprecipitation method, IONP hydrophobicity, and the toxic organic stabilizers/solvents associated with high temperature synthesis.

Often the plethora of strategies followed to prepare IONP cores, surface modifications and conjugation chemistries are overwhelming while preparing custom IONP-TNs. This cumbersome process of starting from scratch for different conjugations can be great-
ly simplified by developing a library of versatile general TN precursors (IONP-TN-PCs). In chapter 4, the reduction-hydrolysis method was adapted to prepare IONPs with a robust aminosilane shell [IONP-$Sil$(NH$_2$)]. The robust silane shell protects the IONP core and the surface amino group that is useful for further conjugations. PEG-diacid was conjugated to IONP-$Sil$(NH$_2$) following a simple amidation reaction to form IONP-$Sil$-PEG(COOH) with silane shell, PEG spacer and surface carboxylic acid groups. IONP-$Sil$(NH$_2$) and IONP-$Sil$-PEG(COOH) are presented as general IONP-TN-PCs for TN conjugations, which was demonstrated by conjugating hydrophobic oleylamine and BSA (which was physisorbed in chapter 2) following simple amidation chemistry. Both cell-uptake and toxicity studies of the general IONP-TN-PCs were performed.

In chapter 5, the reduction-hydrolysis synthesis was further extended as a novel general synthesis to prepare early transition metal (Mn, Fe, Co, Ni, Cu and Zn) oxide NPs. In the same chapter the reduction-hydrolysis is presented as a novel method to obtain silica particles. IONPs supported on a silica surface and fluorescent dye encapsulated silica particles were prepared following the reduction-hydrolysis method.

In chapter 6, the reduction-hydrolysis method using iron precursor FeCl$_3$ was carried out in a confined environment formed by liquid crystal template (water/Triton X–100). This soft template synthesis yielded crystalline iron/IO nanosheets with long-range order. The final chapter (7) will present an overall general summary along with a future outlook.
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Chapter 2:

Simultaneous Magnetically Directed Drug Convection and MR Imaging
Simultaneous Magnetically Directed Drug Convection and MR Imaging

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Authors’ Contributions

The idea of magnetically directed drug convection for brain drug delivery was conceptualized by Dr. David F. Moore (DFM), Dr. Torsten Hegmann (TH) and Dr. Johan van Lierop (JvL). Vinith Yathindranath (VY) was responsible for the choice of: IONP synthetic strategy, surface coatings and mode of attachment of BSA. All experiments related to IONP syntheses and characterization were designed and carried out by VY. The electrophoretic and Magnetic Resonance Imaging/Microscopy studies and related experiments were planned and carried out by Dr. K. Potter (KP) and C. B. Fowler and the pertaining data and figures were provided by them. The manuscript with all figures and tables was prepared by VY. VY, TH, JvL, KP and DFM were responsible for the final revisions of the manuscript, prior to its publication in “Nanotechnology”.

Abstract

Superparamagnetic iron oxide nanoparticles (IONPs) are of interest for their usefulness in biomedical applications. In this work, we have synthesized iron oxide nanocomposites surface-modified with different biocompatible polymers. Bovine serum albumin (BSA) was physisorbed onto these IONPs along with an excipient during freeze-drying. The mass transport of the protein attached to the iron oxide core–shell nanoparticles (IO cs-NPs) under a gradient magnetic field of an MRI instrument was observed in vitro and in an egg as a model system for a biological fluid. From the in vitro experiments in agarose gels, it was observed that the protein gets separated from the core during mass transport for some cs-IO, but co-migration was observed for PEG-modified IO cs-NPs. These experiments demonstrated proof-of-concept for the use of IO cs-NPs in magnetically directed drug convection.
2.1 Introduction

Penetrating the blood–brain barrier (BBB), overcoming a lack of specificity and directionality, and reducing significantly the dosage are challenges to delivering high molecular mass drugs to the brain. Current intratumoral, intrathecal and intravenous injection methods used to deliver drugs do not address adequately the above issues. Convection-enhanced delivery (CED) techniques are capable of bypassing the BBB, as shown in animal studies of CED with bulky molecules such as taxol,\textsuperscript{1} gemcitabine\textsuperscript{2} and glucocerebrosidase,\textsuperscript{3} where successful increases of the concentration of the active ingredients at a targeted site was achieved; however, this was at the expense of excess dosages. Uncontrolled infusion of drug molecules inside brain tissue is associated with low tissue resistance and preferential flow along interstitial spaces of fiber tracts.\textsuperscript{4} To address these issues, a versatile nanosized delivery system that can carry a drug and be steered and positioned accurately at a target location may be the basis of future drug delivery systems. Iron oxide core–shell nanoparticles (IO cs-NPs) have the potential to enable a range of new drug carrier options for neurological disorders. IO cs-NPs with core sizes in the tens of nanometers are capable of permeating the BBB and tissue to deliver drugs as well as simultaneously acting as a ($T_2$-weighted) contrast agent for magnetic resonance imaging (MRI).\textsuperscript{5–7} Drugloaded IO cs-NPs combined with CED techniques could be used as a method for drug delivery inside brain tissue for the treatment of illnesses such as tumors and Gaucher disease.\textsuperscript{3} IO cs-NPs also offer a possible way to treat bloodstream conditions such as ischemic stroke by directly delivering thrombolytics into the occluding in-
tra-arterial clot. In addition to in situ MRI, the intrinsic magnetic properties of IO NPs can be exploited by providing thermal mediation for cancer thermotherapy, and perhaps most intriguingly, offer a platform for targeting locations in the body precisely. This is particularly relevant to the brain where therapeutic targeting of specific neuronal nuclei may be highly desirable.

As a potential treatment paradigm, magnetically directed drug convection (MDDC) may be helpful in achieving target specificity during drug delivery, thereby allowing an increased therapeutic window and reducing the risk of drug toxicity while increasing drug tissue selectivity. Along these lines, in vivo sequestration of magnetic carriers in large and smaller arterial branches after arterial upstream and systemic venous injection has been recently demonstrated. Nucleic acids have been delivered into target cells using IONPs and a static magnetic field under well plates as well. Most recently, in vivo MRI detection of gliomas using IONPs in mouse models have been demonstrated. Disease detection using MRI and magnetic nanoparticles has been applied to the imaging of Alzheimer’s amyloid. At present, MDDC is still in a development phase. Biocompatible polymer coated IO cs-NPs may be suitable materials for MDDC and simultaneous MRI. A proper understanding and development of potential drug-carrying IONP-based materials coupled with developing the technology to direct them inside the human body magnetically is crucial before MDDC can be applied medically. To serve as a probe with a useful lifetime in the body during MDDC, the IO cs-NPs should overcome crucial physiological processes in vivo. For example, evading the mononuclear phagocyte system and avoiding any unwanted immune response is a requirement for pharmaceutical particulate
carriers in circulation. Attachment of hydrophilic biocompatible coatings on the surface of IONPs is often necessary to increase stability and allow circulation in the bloodstream. In addition, coatings prevent the IONPs from irreversible aggregation. Current treatments for many medical conditions such as heart attack, stroke and inborn errors of metabolism such as Fabry disease use large-molecule protein drugs such as rt-PA, α-galactosidase A and, in the near future, potential treatments will likely include also stem cells. Hence, for the design of proof-of-principle MDDC carriers, we focused on addressing the challenge of delivering large molecules or structures rather than low molecular mass drug molecules. In this work, we present the synthesis of three types of IONPs with different coatings carrying an immobilized model protein. Key questions that this study set out to answer were (1) what is the role, (2) how important is the structure (e.g. its hydrophilicity or hydrophobicity) and (3) what is the drug carrier potential of different biocompatible coatings? To investigate these problems, IONPs were coated either in situ or post-NP synthesis with three structurally different biocompatible polymers, i.e. poly(ethylene glycol) (PEG), dextran and poly(ethyl methacrylate) (PEMA).

### 2.2 Experimental

Iron(III)chloride hexahydrate (FeCl₃·6H₂O, 98%), 2-bromo-2-methyl propionic acid (BrMPA, 98%), copper(I) bromide (CuBr, 98%), N,N,N′,N′,N″-pentamethyl diethylene-triamine (PMDETA, 99%), ethyl methacrylate (EMA, 98%), bovine serum albumin fraction V (66.43 kDa, 99%) and L-arginine (98%) were purchased from Sigma-Aldrich. Iron(II)chloride tetrahydrate (FeCl₂·4H₂O, 99%), dextran (Mr 1500) and poly(ethylene glycol)
2.2 Experimental

glycol) (PEG 4000) were obtained from Fluka. Ultrapure agarose, tris-acetate EDTA (TAE) buffer and 10X BlueJuice gel loading buffer were purchased from Invitrogen Corp. (Carlsbad, CA). To synthesize bare IONPs, 1.0812 g of FeCl$_3$·6H$_2$O and 0.3976 g of FeCl$_2$·4H$_2$O were dissolved in 200 ml of DI water ($R = 18$ M$\Omega$) that was then deoxygenated with N$_2$ gas. The solution was stirred at 1000 rpm and the temperature was maintained at 80 °C. After 15 min, 12 ml of aqueous NH$_4$OH (14–15%) was added drop-wise to the reaction flask accompanied by stirring. The rise in pH triggered the nucleation and subsequent growth of IONPs, evident from the change in the color of the reaction mixture from orange to black in a matter of a few seconds. The as-synthesized IONPs were subjected to three washings followed by magnetic separation in DI water and ethanol. The remaining solvent was removed under reduced pressure and the IONPs were dried under vacuum overnight at room temperature. PEG or dextran was coated in situ by adding 2 g of PEG or dextran to the solution containing FeCl$_3$·6H$_2$O and FeCl$_2$·4H$_2$O before the addition of NH$_4$OH. To coat NPs with BrMPA, 0.5 g of IONPs was dispersed in 10 ml of hexane under sonication for 15 min. To that, 0.25 g of BrMPA was added and the solution was sonicated for an additional 5 min. The mixture was stirred for 48 h at room temperature. Thereafter, 20 ml of ethanol was added to the mixture. A brown precipitate was obtained, which was then collected by a rare earth magnet. The residue obtained was washed three times using hexane under mild sonication to remove any unattached BrMPA. PEMA was grafted onto BrMPA-functionalized IONPs by atom transfer radical polymerization. To 0.09 g of CuBr, 0.15 g of PMDETA was added and stirred till a clear, dark blue solution was obtained. The copper PMDETA complex was then added to the
freshly distilled EMA (1.5 g) and anisole (4.5 g) contained in a 100 ml round-bottomed flask. Subjecting the contents to three freeze–pump–thaw cycles degassed all contents. Once the mixture attained room temperature, the mixture was placed in an oil bath pre-heated to 85 °C and stirred for 3 h. The reaction mixture was diluted with THF (times 10 by volume). The brown precipitate was centrifuged at 4000 rpm and the residue was washed three times with dichloromethane and ethanol. Finally, the product was dried at room temperature under vacuum. Immobilization of BSA and coating with L-arginine was achieved by sonicating surface-modified IONPs (0.1 g) in DI water (25 ml) until the particles were dispersed homogeneously. To this solution, 0.1 g of BSA was added and sonicated for 15 min. Thereafter, the solution was subjected to mechanical stirring (1000 rpm) for 3 h. The final BSA immobilized IO cs-NP would not settle in solution. Then, the solution was reduced to half its volume under reduced pressure at 40 °C. To the solution, 0.2 g of L-arginine was added and the solution was thoroughly mixed by shaking the flask. Once L-arginine was homogeneously mixed in solution, the remaining water was removed using a freeze dryer. Finally, the IO cs-NPs were stored in a freezer.

All samples were freeze-dried using a Virtis freeze-mobile 5SL freeze dryer. X-ray diffraction (XRD) measurements were carried out on a Philips PW1710 powder X-ray diffraction system with PW3830/40 x-ray generator. The data were processed using Jade data collection/processing software. Crystallite sizes were also determined using a PANalytical XPert Pro Bragg–Brentano powder X-ray diffractometer using a CuKα radiation (λ = 1.540598, 1.544426 Å) equipped with a diffracted beam Ni filter and an XCelerator detector. High-resolution transmission electron microscopy (HR-TEM) was carried out
2.2 Experimental

on a JEOL FEG-T/STEM. The 400-mesh copper grid with carbon support film was used to hold the nanoparticles. The NPs were dispersed in either acetone or dichloromethane. A drop of the clear dispersed solution was dropped onto the copper grid placed on a filter paper and allowed to dry under nitrogen. Scanning electron microscopy was done on a Cambridge Stereoscan 120 scanning electron microscope with backscattered electron detector. The dry samples were fixed onto a carbon tape and were coated with gold–palladium approximately 4 nm in thickness. Magnetometry experiments were performed using a Quantum Design MPMS system. Transmission Mössbauer spectroscopy measurements were made with a Wissel spectrometer in constant acceleration mode calibrated using α-Fe at room temperature with a 1 GBq $^{57}$CoRh source. Spectra were collected in a Janis SHI-850 closed-cycle refrigeration system. Thermogravimetric analysis (TGA) was carried out using a TA Instruments TGA-Q500. Nitrogen was used as a purge gas and the weight loss was measured from room temperature to 600 °C using a heating rate of 20 °C/min. The electrophoretic experiments on the IO cs-NPs were performed on a Horizon 58 horizontal gel electrophoresis apparatus (Invitrogen) using 0.5% agarose gels in 1X TAE buffer. 20 μl aliquots of 1 mg ml$^{-1}$ aqueous solutions of the IO cs-NPs were mixed with 2 μl of gel loading buffer and the samples were electrophoresed at 70 V. The gels were then imaged by magnetic resonance imaging (MRI) and stained for the presence of protein. The mobility of the IO cs-NPs in response to an external magnetic field was measured using gels comprised of 0.4–1.0% agarose in DI water. 15–20 μl of the IO cs-NP solutions were loaded directly into each well and gels were positioned in a magnetic field gradient located 34 cm from the center of a 7 T horizontal bore MRI Bruker magnet
(bore diameter = 20 cm). After 66–72 h, the distribution of the IO cs-NPs within the gels was interrogated by placing the gels in a 72 mm radio-frequency probe and acquiring T2-weighted images on a Bruker Biospec spectrometer (Bruker Biospin Corp., Billerica, MA) coupled to the 7 T magnet (300 MHz for protons). T2-weighted images were acquired with a RARE (rapid acquisition with relaxation enhanced) imaging sequence (TR/TE = 2500/12 ms, RARE = 8). After MR imaging, agarose gels were stained for the presence of iron using the Perls method (Prussian blue staining). The 20% (v/v) HCl and 10% (w/v) K$_3$[Fe(CN)$_6$]·3H$_2$O (Sigma-Aldrich, St Louis, MO) solutions were prepared in DI water immediately prior to use. The gel was incubated in the HCl solution for 10 min and rinsed three times with DI water. The gel was then immersed in a 1:1 mixture of both solutions for approximately 2 h, or until the blue color developed. Finally the gel was rinsed three times with DI water. The gels were stained for the presence of protein after MR imaging using the coomassie-blue-based colloidal staining kit from Invitrogen. After staining, the gel images were documented using a Scanmaker i900 flat-bed scanner (Microtek, Carson, CA) and annotated in Adobe Photoshop (San Jose, CA).

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The general structure of the IO cs-NPs is depicted in Figure 2.1 and the composition is listed in table 1. PEG was chosen as a coating for A since it is a neutral, hydrophilic polyether that is known to form stable protein conjugates. PEG is also nonimmunogenic and nonantigenic with a long in vivo circulation lifetime. These properties mark PEG as a model MDDC candidate. A dextran IONP coating for B was examined since it is a
highly branched polymer comprised of glucose units that is used currently as a hydrophilic coating for IONP-based MRI contrast agents.\(^29\) A PEMA IONP coating for C provided a structural contrast to the PEG coating as it is a hydrophobic acrylate polymer capable of trapping hydrophobic drugs.\(^30,31\)

![Figure 2.1](image)

**Figure 2.1.** Schematic representation of the IO cs-NPs A–D.

**Table 2.1.** Composition of the IO cs-NPs A–D.

<table>
<thead>
<tr>
<th>IO cs-NP</th>
<th>Core</th>
<th>Coating</th>
<th>Protein</th>
<th>Stabilizer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>IO</td>
<td>PEG</td>
<td>BSA</td>
<td>L-arginine</td>
</tr>
<tr>
<td>B</td>
<td>IO</td>
<td>Dextran</td>
<td>BSA</td>
<td>L-arginine</td>
</tr>
<tr>
<td>C</td>
<td>IO</td>
<td>PEMA</td>
<td>BSA</td>
<td>L-arginine</td>
</tr>
<tr>
<td>D</td>
<td>IO</td>
<td>PEG</td>
<td>BSA</td>
<td>–</td>
</tr>
</tbody>
</table>

Bovine serum albumin (BSA), an anionic protein, was used as a model drug protein. For example, properties such as its globular shape and molecular weight are similar to therapeutic protein drugs such as rt-PA used for treatment of heart attack and stroke or α-galactosidase A for Fabry disease (table 2). The ease of handling and much lower cost in
comparison to rt-PA or α-galactosidase A make it an ideal candidate for the study of an IONP protein carrier system. BSA in solution adopts a spheroid-like structure with a diameter of about 8 nm,\(^{32}\) and can withstand reaction conditions like stirring and sonication in aqueous solutions.

Proteins in dry, solid form have a longer shelf life than in solution, and freeze-drying is used widely during the manufacture of protein pharmaceuticals. In principle, freeze-drying IONP cs-NPs with immobilized proteins should prevent solvent-induced degradation of the coating. However, freezedried proteins are altered structurally by freezing (cryo-) and drying (lyo-stresses).\(^{33}\) Arakawa et al.\(^{34}\) have shown that protein dehydration induces conformational changes that often irreversibly alter the useful properties upon rehydration. Fortunately, these effects can be avoided by using sugar, polymer or amino-acid-based stabilizers (or excipients) during lyophilization.

To address the above issues lyophilization of BSA decorated IONP cs-NPs was performed with the stabilizer L-arginine that is used commonly with proteins such as rt-PA. L-arginine interacts with the protein during lyophilisation by forming hydrogen bonds with the polar groups in the protein,\(^{35}\) fulfilling H-bonding requirements, and serving as a water substitute. Protein conformation and bioactivity is protected and L-arginine around the BSA-loaded IONP cs-NPs may increase significantly the aqueous media solubility.

The IONPs were synthesized following Massart’s coprecipitation method\(^ {36, 37}\) and consisted of magnetite (Fe\(_3\)O\(_4\)) and some ferrous hydroxide (see below). PEG and dextran, which are water soluble, were used to coat the IONP in situ during co-precipitation, and the resulting precursor IONP cs-NPs \(A_{pc}\) and \(B_{pc}\) (pc refers to the precursor IONP) were
easily dispersible in water. For IO cs-NP C, PEMA was grafted onto the surface of the as-synthesized IONPs using atom transfer radical polymerization in a similar procedure as reported by Bai et al. BSA was immobilized onto the polymercoated IO cs-NPs by sonication and mechanical stirring, and the BSA-loaded IO cs-NPs were then suspended in a solution of L-arginine. PEG-coated IO cs-NP D was not exposed to L-arginine to examine the effects of the stabilizer.

![X-ray diffraction patterns](image)

**Figure 2.2.** X-ray diffraction patterns of (a) bare IO NP (used for the preparation of the PEMA-coated precursor IO cs-NP Cpc), (b) Apc, (c) Bpc and (d) Cpc.

Bare IONPs (used for the preparation of Cpc) and the precursor IO cs-NPs Apc–Cpc were investigated using powder X-ray diffraction (XRD). The powder XRD patterns obtained were consistent with magnetite (Fe₃O₄) and its diffraction peaks are indexed in Figure 2.2. All diffraction peaks show the characteristic peak broadening due to the small crystallite size of the NPs. Using Scherrer analysis, the average crystallite size for bare IO NP, Apc, Bpc and Cpc were determined to be 11.4 nm, 12.0 nm, 12.6 nm and 12.2
nm, respectively. An additional amount of peak broadening is observed for the polymer-coated \( A_{pc}, B_{pc} \) and \( C_{pc} \) in comparison to the bare IONP.

To quantify the precursor IO cs-NP magnetism and possible effects that coating might induce that would affect MDDC, the average magnetization of the IO cs-NPs was measured at room temperature (Figure 2.3). The magnetic-field-dependent magnetization of the bare IONP and IO cs-NPs \( A_{pc} \) and \( C_{pc} \) (\( A_{pc} \) and \( B_{pc} \) were made with identical IONPs) at room temperature is shown in Figure 2.3(d). The 84 emu g\(^{-1}\) saturation magnetization (\( M_s \)) of the bare IONPs is consistent with the ‘bulk’ value for Fe\(_3\)O\(_4\), while the reduced saturation magnetization of \( A_{pc} \) and \( C_{pc} \) are in agreement with cores consisting of a mixture of Fe\(_3\)O\(_4\) and ferrous hydroxide (as identified by Mössbauer spectroscopy described below), likely on the particle’s surface. Since ferrous hydroxide is an antiferromagnet with a bulk Néel temperature below 35 K,\(^{39}\) the measured magnetization at room temperature will simply be reduced with respect to the total iron content ascertained by inductively coupled plasma for the magnetometry samples. All IO cs-NPs exhibited no magnetization in zero applied field (Figure 2.3(d)), clear evidence that the IONP cores are superparamagnetic at room temperature.\(^{40}\) The magnetic properties indicate that the IO cs-NP coatings have no measurable effect that would alter MDDC capabilities.
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Figure 2.3. Transmission Mössbauer spectra at 10 K of (a) bare IONPs, (b) A\textsubscript{pc} and (c) C\textsubscript{pc}. Solid lines are fits described in the text. (d) Room-temperature magnetization curves of bare IONPs and of the precursor IO cs-NPs A\textsubscript{pc} and C\textsubscript{pc}. (Note: B\textsubscript{pc} and A\textsubscript{pc} have identical IO cores.)

In addition, to identify clearly the composition of the IO for each IO cs-NP system, transmission Mössbauer spectra were collected. All spectra were collected at 10 K, well below the temperature where superparamagnetic fluctuations of the nanoparticle moments need to be accounted from in a spectral lineshape model. Spectra were nonlinear least-squares-fitted with a model based on two sextets of Lorentzians with independent
linewidths that described the nuclear magnetism of the Fe moments (shown in Figure 2.3). Two sextets were required to describe the local Fe environments of the octahedral and tetrahedral sites of the iron oxides. The relative intensity of the spectral lines is proportional to the respective transition probability from the $^{57}\text{Fe}$ exited state to its ground state ($m_e = \pm 3/2, m_g = \pm 1/2$). The bare IONPs were well described by the octahedral site of Fe$_3$O$_4$ having a hyperfine field of $B_{hf} = 51.66 \pm 0.08$ T, a chemical isomer shift of IS = 0.418 ± 0.005 mm s$^{-1}$ and a small quadrupole shift of QS = 0.03 ± 0.01 mm s$^{-1}$. The tetrahedral site had a $B_{hf} = 53.70 \pm 0.06$ T, IS = 0.466 ± 0.004 mm s$^{-1}$ and QS = 0.007 ± 0.008 mm s$^{-1}$. QS = 0 in bulk Fe$_3$O$_4$, however, the site asymmetry inherent in nanoscale-sized Fe$_3$O$_4$ crystallites invariably results in a small electric field gradient being present about the Fe ions. The fitted linewidths of $\Gamma = 0.30\pm0.01$ mm s$^{-1}$ and $\Gamma = 0.25\pm0.01$ mm s$^{-1}$ for the octahedral and tetrahedral sites, respectively, is considerably larger than the source natural linewidth of $\sim 0.13$ mm s$^{-1}$, reflecting the significant amount of chemical disorder about the Fe sites in the IONPs due to their nanoscale size. The fitted values of $B_{hf}$ and IS are in excellent agreement with bulk Fe$_3$O$_4$. Mössbauer spectra of IO cs-NPs A$_{pc}$–D$_{pc}$ were well described by the above hyperfine parameters, with IO cs-NPs A$_{pc}$ and D$_{pc}$ also showing the presence of ferrous hydroxide that occurred during the coating process, as shown by an extra sextet that had $\Gamma = 0.15 \pm 0.06$ mm s$^{-1}$, IS = 0.36 ± 0.04 mm s$^{-1}$, QS = 1.29 ± 0.08 mm s$^{-1}$ and $B_{hf} = 26.4 \pm 0.3$ T.
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Figure 2.4. FT-IR spectra of $A_{pc}$–$C_{pc}$. The inset shows the region between 1400 and 1900 cm$^{-1}$ for $C_{pc}$.

Fourier transform infrared (FT-IR) spectra of the precursor IO cs-NPs $A_{pc}$–$C_{pc}$ clearly show the presence of the polymer coatings (Figure 2.4). Prominent peaks at 572 cm$^{-1}$ correspond to the Fe–O stretching in IO, and symmetric as well as antisymmetric C–H stretching vibrations of the polymers are observed between 2900 and 3000 cm$^{-1}$. The peak at 1726 cm$^{-1}$ in the IR spectra of $C_{pc}$ corresponds to the C=O stretching in PEMA (Figure 2.4(c)). PEG- and dextran-coated IO cs-NPs show prominent OH stretching vibrations around 3400 cm$^{-1}$ (Figure 2.4(c), $A_{pc}$ and $B_{pc}$).
Figure 2.5. HR-TEM image of IO cs-NP $A_{pc}$ (a) and TEM images of $A_{pc}$ (b), $B_{pc}$ (c) and $C_{pc}$ (d). SEM images of (e) bare IONPs, (f) $A_{pc}$, (g) $B_{pc}$ and (h) $C_{pc}$. 
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Figure 2.6. Top: (a) dispersion of the IO cs-NPs A–C in H₂O. (b) A–C attracted to small rare earth magnets. Bottom: size distribution of the IO cs-NPs measured by DLS of: (a) A, (b) B and (c) C.

Figures 2.5(a)–(d) show the transmission electron microscopy (TEM) images of the PEG-, dextran- and PEMA coated precursor IO cs-NPs Aₚc–Cₚc. The high-resolution TEM image for Aₚc in Figure 2.5(a) shows distinct lattice planes in the IONP core, clear evidence of its crystallinity. The NP diameters measured from TEM images are in the
range of 11–13 nm and are in agreement with XRD results. Figures 2.5(e)–(h) also show the SEM images of the bare IONPs (Figure 2.5(e)) and the IO cs-NPs $A_{pc} - C_{pc}$. Samples for SEM imaging were coated with a thin gold/palladium layer (~4 nm) and attached to a carbon tape. SEM revealed a slightly irregular sized, quasi-spherical shapes of all polymer-coated precursor IO cs-NPs, and an average size of $80 \pm 10$ nm for the PEG and dextran-coated precursor IO cs-NPs $A_{pc}$ and $B_{pc}$ (Figures 2.5(f) and (g)) and $100 \pm 12$ nm for the PEMA-coated IO cs-NP $C_{pc}$ (Figure 2.5(h)).

Figure 2.6 shows the final lyophilized IO cs-NPs $A$, $B$ and $C$ after decoration with BSA and stabilization with L-arginine re-dissolved in H$_2$O. Minimal shaking of a vial resulted in a highly dissolved (dispersed) solution of IO cs-NPs in water that was a clear brown color which displayed no NP aggregation over several weeks (as demonstrated also by dynamic light scattering (DLS) shown in Figure 2.6). Figure 2.6(b) (top) shows the same IO cs-NPs attracted to a rare earth magnet. The particles in solution start to accumulate rapidly onto the walls of the sample vial. The picture was taken 2 min after placement of the magnet.

To determine the amount of coating and BSA immobilized onto the different IO cs-NPs (i.e. ‘carrying capacity’), TGA analysis was performed on samples $A$, $B$ and $C$, shown in Figure 2.7. We find that PEG represents $8.0 \pm 0.1\%$, PEMA $11.2 \pm 0.1\%$ and dextran $13.6 \pm 0.1\%$ by weight of the NPs. The amount of BSA immobilized onto IO cs-NP $A$ was $18.0 \pm 0.1\%$, $B$ $8.7 \pm 0.1\%$ and $C$ had $11.3 \pm 0.1\%$ by weight. These results (and the staining work described below) show clearly that the proteins can be adsorbed on the NP surfaces and can withstand the necessary reaction conditions. Further qualita-
tive evidence of the large amount of protein molecules coating the IO cs-NPs is shown by the large radial MRM image about the NP dispersions, shown in Figure 2.8 described below.

Figure 2.7. (a) TGA results and (b) corresponding dTGA of PEG-coated IO cs-NPs with immobilized BSA. (c) TGA results and (d) corresponding dTGA of dextran-coated IO cs-NPs with immobilized BSA. (e) TGA results and (f) corresponding dTGA of PEMA-coated IO cs-NPs with immobilized BSA.

A key aspect of this research is the role of the biocompatible polymer coatings and the effect of the stabilizer L-arginine on the mobility of the IO cs-NPs. For a comparative
run, aqueous solutions of all four IO cs-NPs A–D were pipetted into four equidistant wells of 0.5% aqueous agarose gel. Agarose gels of similar concentrations have been shown to be ideal surrogates (realistic brain tissue phantoms) for in vivo brain in exploratory studies of convection-enhanced delivery. The gel was then placed in the magnetic field gradient of a 7 T horizontal bore MRI magnet. Representative T2-weighted images in Figures 2.8(a) and (b) show the mass transport of the nanocarriers after 66 h in the magnetic field gradient. To verify the location of the BSA-loaded IO cs-NPs, the gel was stained for iron using the Perls method.

The Prussian-blue-stained gel in Figure 2.8(c) shows the distance traveled by the different IO cs-NPs in the magnetic field gradient. Both A and C migrated the furthest distance, closely followed by D while the IONPs of B did not migrate out of the well. Figure 2.8(d) shows an identical gel, stained with coomassie blue, in which the convection of the BSA protein carrier of the IO cs-NPs can be observed. The staining also indicated that a smaller portion of the total amount of BSA immobilized on the surface of the IO cs-NPs A, C, and D co-migrated with the IONP cores (red arrows in Figure 2.8(d)). Interestingly, there was no convection of the BSA on B outside of its well.
Figure 2.8. (a) and (b) T$_2$-weighted MR images of 0.5% agarose gels with IO cs-NPs A–D after 66 h in the magnetic field gradient located 34 cm from the center of a 7 T MRI magnet. (c) Image of the agarose gel “(a)” stained with Prusian blue iron stain and (d) Image of agarose gel “(b)” stained with coomassie blue protein stain. Red arrows in “(d)” indicate the co-migration of some of the immobilized BSA in A, C and D.
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Figure 2.9. (a) MRM image of 0.5% agarose gel with IO cs-NPs A–D after electrophoresis showing the location of the IONPs. (b) Image of the agarose gel in (a) stained with coomassie blue showing the location of BSA.

To confirm that all IO cs-NPs A–D had immobilized BSA on the surface or within their polymer shells, we also performed electrophoresis experiments on the agarose gels. MR microscopy of one of the electrophoretic gels (Figure 2.9(a)) confirmed that the IO cs-NPs A, C and D migrated an equal distance into the gel with the application of an electric current, with the exception of B where there was no evidence of iron oxide below its wells. When the same gel was stained with coomassie blue for BSA (Figure 2.9(b)) it was evident that, under electrophoresis, the protein moved ahead of IO cs-NPs A, C and D but not in the case of B. The BSA in B did not migrate during the electrophoretic experiments, although loading of BSA onto B was clearly established via coomassie staining after the magnetically directed convection experiment (see B in Figure 2.8(d)). These results indicate that in the dense agarose gel the protein shell is first removed because there is no specificity of protein absorption, and the polymer coating dominates the transport process.
Figure 2.10. T_2 relaxivity versus IO cs-NP concentration in agarose for A–D. The solid lines are linear fits described in the legend where y = 1/T_2 and x = concentration. R is the regression factor.

Particularly interesting in both the MRI and electrophoresis experiments was the behavior of B, the dextran-coated IO cs-NP, which showed no mass transport of the IO cs-NPs in response to the magnetic field gradient, absolutely no convection of the immobilized BSA and mass transport under the influence of an electric current. We ascribe the observation of these effects to possible strong H-bonding interactions between the large number of surface OH groups of the dextran coating and the agarose gel matrix as well as similarly significant interactions between dextran and BSA allowing no release of the protein into the surrounding gel as observed for A, C and D. A possible explanation is that the dextran coating of IO cs-NP B is not covered completely with BSA, which permits H-bonding interactions of the dextran with the agarose gels. This effect clearly warrants further investigations of B in agarose gels prepared from saline solutions in the
Results and discussion

The presence of phosphate buffers with and without L-arginine as a stabilizer, particularly as superparamagnetic IONPs with dextran coatings are already used as FDA-approved MRI contrast agents. MRI T2 relaxivity measurements as a function of nanoparticle concentration shown in Figure 2.10 provide a measure of the contrast capability of the IO cs-NPs. These measurements were made under the same experimental conditions as the MR images.

Another key finding is that A, C and D show co-migration of the loaded BSA in the MRI experiments. It appears that A is the best compromise between magnetic convection of the IO cs-NP and co-migration of the protein within the investigated series. D convects completely out of the well but very little co-migration of BSA is observed. Almost all protein radially migrates into the surrounding gel (see Figure 2.8(d)). A very similar behavior was also observed for the PEMA coated IO cs-NP C. Given the density of the agarose gels, this emphasizes that PEG not only forms reasonably stable protein conjugates with BSA for co-convection, but also that L-arginine as a stabilizer for the protein is critical for drug delivery of proteins using such IO cs-NPs. Based on these results, if large macromolecules or drug proteins are to be more efficiently delivered by magnetically directed drug convection, a stronger binding (such as chemical bonding) between the IONP coating and the macromolecule or protein may be needed.
Figure 2.11. T$_2$-weighted MR images of C in gels prepared in DI water with different agarose concentrations (w/v): (a) 0.4%, (b) 0.5%, (c) 0.6%, (d) 0.7%, (e) 0.8% and (f) 1.0%. (g) Plot of movement after three days (mm) versus % agarose gel.
Figure 2.12. Representative T2-weighted MR images of an egg injected with IO cs-NP C acquired at time zero (a) and at three slice positions within the egg (b)–(d) after 60 h of exposure to a magnetic field gradient. The magnetic field was lowest at the injection site, indicated with a white arrow, and highest at the bottom of the images shown. Scale bar represents 1 cm.

Finally, we also performed initial experiments to investigate the effect of the density and complexity of the agarose gel. To simulate different tissues (the 0.6% agarose gel best simulates healthy brain tissue), the magnetic mass transport of one of the IO cs-NP (C) was investigated in gels with agarose concentrations ranging from 0.4 to 1.0% (Fig-
ures 2.11(a)–(f)). The MR images show the mass transport of the IO cs-NPs after 72 h in the gradient magnetic field and demonstrate that the mobility is highest in the least dense gel (0.4% agarose) and lowest in the densest gel (1% agarose). As the viscosity of the gel increases approximately as the reciprocal of the % agarose/H₂O mixture, we expect the IOcs-NP displacement to follow the same essential relationship, which is indeed observed (dashed line in Figure 2.11(g)). Similarly, mass transport of IO cs-NP C was observed over a time span of 60 h in a raw hen’s egg as an in vitro example for a biological fluid (Figure 2.12). The particles behaved in much the same way in an egg white as observed in agarose gels.

2.4 Conclusions

In conclusion, we have shown that IO cs-NPs, in principle, can be used for magnetically directed drug convection and simultaneous MRI contrast enhancement. The gradient field of the MRI was used for mass transport of the IO cs-NPs with PEG and PEMA as biocompatible polymer coatings with and without a stabilizer L-arginine (A, C and D). The dextran-coated IO cs-NPs because of strong intermolecular H-bonding interactions were not suitable for protein drug delivery under the described experimental conditions, and future experiments will focus on understanding this result. The PEG- and PEMA-coated IO cs-NPs with or without L-arginine as stabilizer proved to be suitable protein nanocarriers showing some simultaneous directional convective transport of carrier and protein. Once administered inside a biological system the protein carrier can be used for magnetically directed drug convection and MRI. We envision that the loosely attached protein
will undergo a burst release followed by the slow diffusion of the proteins trapped in the polymer matrix of the nanocarrier. The whole diffusion-controlled release process will be triggered by the drug protein concentration gradient in the surrounding tissue. Ongoing research in our laboratories now focuses on using gels prepared under more physiological conditions (saline, phosphate buffer), other linkages between coating and proteins (e.g. chemical bonding to the nonactive site of proteins) and will then move on to medically relevant protein drugs and in vivo magnetically directed drug convection studies.
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2.5 References


Chapter 3:

A Versatile Method for the Reductive, One-Pot Synthesis of Bare, Hydrophilic and Hydrophobic Magnetite Nanoparticles
A Versatile Method for the Reductive, One-Pot Synthesis of Bare, Hydrophilic and Hydrophobic Magnetite Nanoparticles

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Authors’ Contributions

The novel synthesis of magnetite nanoparticles presented in this chapter was conceptualized by Vinith Yathindranath (VY). All syntheses and characterization related experiments were designed and performed by VY, exceptions follow. Dr. Leila Rebbouh performed the Mössbauer characterization of the as-synthesized magnetite NPs. The manuscript with all figures and tables was prepared by VY. VY, Dr. Torsten Hegmann, Dr. Johan van Lierop, Dr. Donald W. Miller and Dr. David F. Moore were responsible for final revisions before its publication in “Advanced Functional Materials”.
Abstract

A biocompatible, reliable, and particularly versatile synthesis of magnetic iron oxide nanoparticles (IONPs) is presented that uses iron(III) acetylacetonate Fe(acac)_3 as an iron precursor and sodium borohydride as a reducing agent. Both the reaction temperature and the concentration of the reducing agent have considerable effects on the IONP size. These dependencies can be used to prepare IONPs ranging in size from 5 to 8 nm, as determined by transmission electron microscopy (TEM). Synthesis at room temperature or with higher sodium borohydride concentrations always resulted in smaller particle sizes. Powder X-ray diffraction patterns show the presence of an iron oxide phase with a cubic unit cell and allow for the determination of the lattice parameters and average crystallite sizes for all synthesized IONPs. Transmission Mössbauer spectroscopy shows that the as-synthesized IONPs are pure magnetite (Fe_3O_4) and is further used to elucidate the reaction pathway by analyzing iron intermediates formed prior to nanoparticle formation and precipitation. TEM and high-resolution TEM reveal quasi-spherical shapes and lattice fringes for most IONPs. With only minor modifications of the synthesis procedure, this versatile, one-pot synthesis is proven to be suitable for the production of bare (uncoated) IONPs, IONPs with hydrophilic poly(ethylene glycol), L-arginine, and L-glutamic acid coatings, as well as IONPs with hydrophobic coatings such as oleic acid. All coated IONPs were characterized by FT-IR spectroscopy. In addition, the bare IONPs could easily be modified post-synthesis with a suitable capping agent using ultrasonication. To verify the biocompatibility of the IONPs, in vitro cytotoxicity studies were carried out on bare IONPs with intestinal (Caco2) and liver epithelial (HepG2) cell cultures using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Phase contrast mi
crosscopy after hematoxylin-eosin staining showed the intact morphology of the Caco2 and HepG2 cells treated with IONPs.
3.1 Introduction

The synthesis of magnetic iron oxide nanoparticles (IONPs) with excellent size control is both of significant academic and industrial interest. In the recent years, IONPs composed of magnetite ($\text{Fe}_3\text{O}_4$) and maghemite ($\gamma$-$\text{Fe}_2\text{O}_3$) with appropriate surface coatings have been used for an increasing number of applications in clinical medicine. For example, coated IONPs have been used as $T_2/T_2^*$ enhancing contrast agents for magnetic resonance imaging (MRI), as thermal mediators in tumor thermotherapy, and as vectors for targeted drug delivery and imaging.\textsuperscript{1–4} IONPs with suitable biocompatible coatings are being widely studied for their diagnostic and potential therapeutic capabilities in central nervous system (CNS) disorders, such as blood–brain barrier (BBB) dysfunction in brain tumors, stroke, multiple sclerosis, epilepsy, and traumatic brain injury.\textsuperscript{5} When administered intravenously, IONPs, which are intrinsically superparamagnetic at normothermia, can be detected in the CNS; in neurons after BBB disruption\textsuperscript{6} as well as in malignant glial tumors.\textsuperscript{7} Inside the human body, IONPs show a different distribution over the organs compared to larger, mesoscale sized particles and are useful for lymph node or bone marrow MRI.\textsuperscript{8,9} With a longer blood circulation half-life and a $T_1$-shortening effect, IONPs can also be used as a blood-pool contrast agent for MR angiography (MRA).\textsuperscript{10} Additionally, IONPs require magnetic fields with a far lower strength than larger ferromagnetic species to achieve the same\textsuperscript{11} or significantly better\textsuperscript{12} effects in tumor thermotherapy or magnetic hyperthermia.\textsuperscript{11} Previously, we reported on the application of IONPs with different polymer coatings and immobilized proteins for magnetically directed protein con-
vection in vitro, using MRI. The study served as a proof-of-principle for the use of polymer-coated IONPs and an external magnetic field for drug transportation and enhancement of the drug concentration at a defined site. For such biomedical studies and applications, it is of immense interest to develop a simple synthetic method that provides flexibility with regard to surface coatings and avoids toxic reactants as well as solvents.

There are various methods to produce IONPs, including coprecipitation, non-aqueous and aqueous sol-gel, microemulsion, hydrothermal/solvothermal and sonochemical processes, that commonly yield acceptable results for the above biomedical applications. Among the aqueous phase syntheses, Massart’s method of co-precipitation is used widely for its simplicity, with the final product typically comprising both magnetite ($\text{Fe}_3\text{O}_4$) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) iron-oxide phases. In short, ferric and ferrous salts in a 2:1 ratio in an aqueous medium are precipitated as IONPs by carefully adjusting the pH of the reaction mixture to 11 or above, usually with aqueous ammonia ($\text{NH}_3\cdot\text{H}_2\text{O}$) or sodium hydroxide (NaOH). This method provides a mild way of preparing bare IONPs and IONPs with hydrophilic coatings in a one-pot synthesis. The drawback of this aqueous-phase synthesis is that variations in the molar ratio of the iron salts often result in complex changes of the crystal structure of the final IONPs, and the final product is often a mixture of magnetite and maghemite (vide supra). Also, it is problematic to obtain IONPs with a narrow size distribution (an essential requirement for many applications) using Massart’s method. To overcome these problems, a number of high-temperature syntheses were developed over the past decade that are based on the thermal decomposition of an organometallic iron species such as iron(III) acetylacetonate [$\text{Fe}($acac$)_3$] or iron
pentacarbonyl in an organic phase (i.e., solvent).\textsuperscript{20} Sun et al. reported an organic-phase synthesis of monodisperse magnetite nanoparticles starting from Fe(acac)\textsubscript{3}.\textsuperscript{21} The precursor, Fe(acac)\textsubscript{3} in phenyl ether, was refluxed at 265 °C in the presence of 1,2-hexanediol, oleic acid, and oleyl amine to yield magnetite nanoparticles with diameters of \(\approx 4\) nm. Pinna et al. described a high-temperature synthesis for magnetite nanoparticles, also from Fe(acac)\textsubscript{3}, in the absence of specific surfactant molecules where benzyl alcohol served both as a solvent and a protective ligand.\textsuperscript{22} The precursor was decomposed thermally at 200 °C to yield magnetite particles with an average diameter of 20–25 nm. Many reports yielding reasonably monodisperse IONPs follow such high-temperature protocols.\textsuperscript{23–26} In all high-temperature organic-phase syntheses, the as-synthesized IONPs do not form colloidal solutions in aqueous and physiological media, rendering them unsuitable for many biomedical applications unless sophisticated post-preparative procedures are performed to make them “water soluble”.\textsuperscript{27,28} A few methods have been developed that avoid these post-preparative steps. For example, Gao et al. reported the high-temperature synthesis of water-soluble 5 nm IONPs from Fe(acac)\textsubscript{3} and FeCl\textsubscript{3}.6H\textsubscript{2}O by refluxing the precursors in 2-pyrrolidone that served both as a solvent and a ligand.\textsuperscript{29,30} Furthermore, Yonezawa et al. proposed the synthesis of gelatine-stabilized IONPs from a Fe\textsuperscript{3+}-gelatine complex using sodium borohydride (NaBH\textsubscript{4}).\textsuperscript{31} However, the above IONP-synthesis methods fail to overcome one challenge: in spite of their dependence on stabilizing molecules to control particle size, they should simultaneously tolerate the presence of biocompatible or functionalizable surfactant molecules; i.e., a simple, one-pot method under mild conditions has yet to be developed. A variety of inorganic metal nanoparticles can be precipitated
from homogeneous solutions of metal cations using common reducing agents such as gaseous H₂, solvated NaBH₄, hydrazine hydrate (N₂H₄·H₂O) or hydrazine dihydrochloride (N₂H₄·2HCl). Reducing the inorganic and organometallic precursors with NaBH₄ is a widely used method in the synthesis of metal nanoparticles such as gold, silver, iron, cobalt, and other, multicomponent nanoparticles.32–35 Surprisingly, reports describing the use of NaBH₄ as a reducing agent for the synthesis of metal oxide nanoparticles such as iron oxide are relatively rare.31 Taking these factors into consideration, the procedure presented here involves an aqueous phase reduction-hydrolysis of Fe(acac)₃ using NaBH₄ at room temperature (RT). This versatile method is applicable for the preparation of both bare and surface-modified IONPs. With this method, surface modifications of IONPs with a suitable polymer or other functional moieties can not only be carried out post-synthesis but, most importantly, in situ during synthesis. To demonstrate this, we prepared hydrophilic PEG-coated (IONP-PEG₁₀(RT)), L-arginine LA-coated (IONP-LA₁₀(RT)), L-glutamic acid GA-coated IONPs and hydrophobic oleic acid OA-coated (IONP-OA₁₀(RT)) IONPs. We also showed that bare IONPs can be modified post-synthesis by coating bare IONP₁₀(RT) with hydrophobic OA using ultrasonication to yield IONP₁₀(RT)-OA. To examine the effects of elevated temperature (ET) and the borohydride concentration on the nanoparticle size, we also investigated a series of IONPₓ(ET) synthesized at a reaction temperature of 78 °C (EtOH/water at reflux) and with molar ratios of NaBH₄/Fe(acac)₃ of x = 10, 25, and 40 (Table 3.1). In comparison to many of the reported procedures, our method does not require hazardous organic solvents or surfactants, high temperatures, or a sophisticated experimental setup. Furthermore, this method pre-
sents a “greener” and more sustainable way of preparing bare, hydrophilic, or hydrophobic IONPs that can easily be scaled up to produce large, commercially relevant quantities.

3.2 Experimental

*Materials*: All syntheses were carried out using reagents that were commercially available. Iron(III) acetylacetonate (99%) (Fe(acac)$_3$) and poly(ethylene glycol) 4000 (PEG) were purchased from Fluka and used without further purification. Oleic acid (OA), L-arginine (LA), L-glutamic acid (GA), sodium borohydride (98%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich. Millipore deionized (DI) water (R = 18 MΩ) was used throughout the experiments.

*Instrumentation*: Powder X-ray diffraction (XRD) measurements were carried out on a Siemens D5000 powder diffractometer equipped with a scintillation detector or a PANalytical X’Pert Pro Bragg-Brentano powder X-ray diffractometer equipped with a diffracted beam Ni-filter and an X’Celerator detector. Cu K $\alpha_1,2$ radiation ($\lambda = 1.540598, 1.544426$ Å) were used as the X-ray sources. FT-IR for all samples was recorded on a Bruker TENSOR 27, using KBr pellets. Transmission electron microscopy (TEM) was carried out on a JEOL 2010F STEM. The NPs were diluted with methanol and a droplet was placed on a porous carbon film that was plasma cleaned prior to analysis. The particle sizes from TEM images were determined using the public domain image processing and analysis software ImageJ from the National Institutes of Health (NIH) (http://rsb.info.nih.gov/ij/). Radial intensity profiles (Figure A3.2C in Appendix A) for all
selected area electron diffraction (SAED) patterns were obtained using DiffTools\textsuperscript{48} script package for Digital Micrograph (Gatan Inc.). The centre of the SAED patterns was located and a circular average was performed on each pattern to create the radial intensity profile. Transmission Mössbauer spectroscopy measurements were carried out on a Wissel spectrometer in constant acceleration mode calibrated using α-Fe at room temperature with a 1 GBq \textsuperscript{57}CoRh source. Spectra were collected in a Janis SHI-850 closed cycle refrigeration system.

*Synthesis of IONP\textsubscript{10(RT)}:* \(\text{Fe(acac)}_3\) (706.4 mg, 2.0 mmol) was dissolved in a 50/50 water/ethanol mixture (100 mL), and the solution was purged with nitrogen gas for one hour. To this, sodium borohydride (756.6 mg, 20 mmol) was added with mechanical stirring at 1000 RPM under a nitrogen atmosphere. At this stage, evolution of \(\text{H}_2\) gas was observed and the solution changed color rapidly from red to pale orange and then to black, where the black color indicated the formation of nanoparticles. The reaction mixture was stirred at 1000 RPM for an additional hour. The formed IONP\textsubscript{10(RT)} were magnetically separated, repeatedly washed with water and ethanol, and then dried under nitrogen at room temperature. The final product was a sooty-black powder.

*Synthesis of Bare IONP\textsubscript{x(ET)} (x = 10, 25, and 40):* A solution of \(\text{Fe(acac)}_3\) (706.4 mg, 2.0 mmol) in a 50/50 water/ethanol mixture (100 mL) was purged with nitrogen for one hour. The reaction flask was placed in an oil bath and the temperature was adjusted to 78 °C with mechanical stirring (1000 RPM) under a steady nitrogen flow. Once the reaction mixture reached 78 °C, required amounts of sodium borohydride were added according to the ratio of interest. In all cases, the color of the reaction mixture turned black rapidly and
the mixture was refluxed for an additional hour. The final IONP products were separated magnetically, washed with DI water and ethanol several times, and finally dried under a nitrogen atmosphere at room temperature.

*Synthesis of IONP-PEG$_{10(RT)}$:* PEG-4000 (2.0 g, 0.5 mmol) was dissolved in DI water (50 mL), and the solution was purged with nitrogen gas. Fe(acac)$_3$ (706.3 mg, 2.0 mmol) was dissolved in ethanol (50 mL) and also purged with nitrogen gas for 1 h. The Fe(acac)$_3$ solution was then mixed with the aqueous PEG solution and nitrogen gas was bubbled through the resulting solution for an additional 15 min as the solution was stirred mechanically at 1000 RPM. To this, sodium borohydride (756.6 mg, 20 mmol) was added and the reaction mixture changed color rapidly from reddish to black. Thereafter, the reaction mixture was stirred for an additional 6 h. The coated NPs were collected using a rare earth magnet, repeatedly washed by alternating between sonication and magnetic separation in DI water and in ethanol, and then dried under a nitrogen atmosphere at room temperature.

*Synthesis of IONP-OA$_{10(RT)}$:* Oleic acid (2.0 g, 7.0 mmol) was dissolved in deoxygenated ethanol (50 mL) along with Fe(acac)$_3$ (706.3 mg, 2.0 mmol). To this, sodium borohydride (756.6 mg, 20 mmol) was added rapidly, and the mixture was stirred at 1000 RPM for 15 min. Thereafter, DI and deoxygenated water (50 mL) was added and the mixture was stirred for 6 h. The color of the final reaction mixture was blackish green. The crude product was separated magnetically and washed several times with ethanol, followed by hexane to remove any free, non-bound OA. The washed particles were then dried under a steady flow of nitrogen.
3.2 Experimental

Synthesis of IONP-GA$_{10}$ and IONP-LA$_{10}$: L-glutamic acid (GA) (3.0 g, 20 mmol) was added to DI water (50 mL) resulting in pH = 3. The pH was adjusted to 7.2 (using 1 M aqueous sodium hydroxide solution), at which point GA was completely dissolved, and the solution was purged with nitrogen for 1 h. In case of L-arginine (LA), 3.5 g (20 mmol) were dissolved in DI water (50 mL) and purged with nitrogen. For both cases, Fe(acac)$_3$ (706.3 mg, 2.0 mmol) was dissolved in ethanol (50 mL) and also purged with nitrogen for 1 h. The Fe(acac)$_3$ solution was then combined with the aqueous amino acid portion, and nitrogen was bubbled through the mixture for an additional 15 min with stirring (1000 RPM). To this, sodium borohydride (756.6 mg, 20 mmol) was added rapidly, and the reaction mixture changed color from reddish to black. The coated NPs were repeatedly washed by alternating between sonication and magnetic separation in water, followed by ethanol, and then dried under nitrogen at room temperature.

Synthesis of IONP$_{10}$-OA: A dispersion of IONP$_{10}$ (100 mg in 20 mL of DI water) was added to a solution of oleic acid (2.5 g) in hexane (20 mL). Initially, IONP$_{10}$ was dispersed in the aqueous layer and would not disperse into the organic layer. The mixture in a round bottom flask was sonicated for 1 h at 25 °C with occasional shaking. After 10 min of sonication, the NPs had completely migrated from the aqueous phase into the organic phase indicating OA capping of the IONP surface.

Cell Viability and Morphology Studies: Caco2 and HepG2 cells were seeded in 96-well plates (5000 cells/well) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, benzylpenicillin (100 U mL$^{-1}$), and streptomycin (10 μg mL$^{-1}$). Cells were maintained at 37
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°C under a humidified atmosphere in a 5% CO₂ incubator. After one day in culture, cells were exposed to DMEM (200 μL) with varying concentrations of IONP₁₀(RT) (0.01, 0.032, 0.1, 0.32, and 1 mg mL⁻¹). After 24 h exposure to IONP₁₀(RT), the cells were then washed once, replenished with fresh media (200 μL), and incubated for an additional 48 h. A total of 25 μL of MTT reagent (5 mg mL⁻¹ in PBS) was added to each well and the cells were incubated for 2 h in the 5% CO₂ incubator. After 2 h, the purple formazan crystals were dissolved by adding solvent (DMF/H₂O) and incubated for 5 h. The absorbance (A) was monitored at 570 nm using a plate reader (Synergy HT, by Biotek). The percentage cell viability of the wells treated with different concentrations of IONP₁₀(RT) relative to control wells which received DMEM alone was calculated by (A Sample/A control)×100. For hematoxylin-eosin staining, Caco2 and HepG2 cells were seeded onto chamber slides and were treated with IONPs in a similar fashion to that of MTT assay. After 48 h, the cells were washed twice with PBS, and 3% paraformaldehyde was added and incubated for 15 min at room temperature. To this, 0.3% triton-X was added and incubated for an additional 5 min at room temperature. The fixed cells were then washed twice with PBS and stored at 4 °C in PBS till staining studies were performed. Before staining, the cover slips were removed, conventional HE staining was performed, the cover slip was mounted with a drop of permount and allowed to dry before evaluation under an optical microscope.

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Figure 3.1 shows the powder X-ray diffraction (XRD) pattern of the bare IONPs synthesized at RT, IONP\textsubscript{10(RT)}, and the series prepared at 78 °C with NaBH\textsubscript{4}/Fe(acac)\textsubscript{3} molar ratios of \(x = 10, 25,\) and 40, respectively. The diffraction peaks showed Scherrer broadening, as it is expected for nanoparticles. Estimations of the nanoparticle diameters were calculated using the Scherrer equation\textsuperscript{36} assuming spherical nanocrystallites for all samples; the values are provided in Table 1. Rietveld refinements of the powder XRD patterns of IONP\textsubscript{10(RT)}, IONP\textsubscript{10(ET)}, IONP\textsubscript{25(ET)}, and IONP\textsubscript{40(ET)} were indexed on a cubic unit cell with space group \(Fd\bar{3}m\) (#227) (ICDD powder X-ray diffraction reference code 01-088-0315) (Figure A3.1 in Appendix A). The calculated lattice parameters from the XRD patterns of IONP\textsubscript{10(RT)}, IONP\textsubscript{10(ET)}, IONP\textsubscript{25(ET)} and IONP\textsubscript{40(ET)} were 8.365, 8.365, 8.369, 8.369.
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and 8.369 Å, respectively, and in good agreement with those of bulk crystalline magnetite (8.375 Å) and maghemite (8.346 Å) iron-oxide phases. The powder XRD patterns of coated IONP-PEG_{10(RT)}, IONP-OA_{10(RT)} IONP-GA_{10(RT)} and IONP-LA_{10(RT)} (Figure A3.2 in Appendix A) are similar to the patterns of uncoated IONPs, and the diameters calculated using the Scherrer equation were 6.02, 9.08, 5.26, and 4.92 nm, respectively. The average particle sizes for the bare IONPs were also determined by analyzing TEM images (Figure 3.2), and the values are given in Table 1 (for the respective size distributions see Figure A3.3 in Appendix A). The HR-TEM images of the bare and the coated IONPs exhibited clear lattice fringes indicating the existence of some highly crystalline areas on the nanoparticle surface with visible inter-fringe distances of 2.9 and 2.5 Å, consistent with cubic spinel iron oxide (Figures 3.2E–H, K, L, Figure A3.4A–D in Appendix A) (ICDD reference code 01–088-0315). Judging from the TEM images, the bare and most of the coated IONPs appeared to be reasonably spherical in shape, except of the oleic acid coated nanoparticles, IONP-OA_{10(RT)}, where some nanoparticles exhibited a quasihexagonal shape (Figure 3.2L and Figure A3.4B in Appendix A). The SAED patterns obtained for all particles showed the characteristic electron diffraction pattern of magnetite (Figure 3.2I, J and Figure A3.5A, B in Appendix A). The peak positions in the radial intensity profiles from the SAED pattern were essentially identical amongst the bare IONPs, indicating similar compositions for all IONPs synthesized at RT and at 78 °C (Figure A3.5C in Appendix A).
To determine the exact iron oxide phase of the nanoparticle systems, we turned to

**Figure 3.2.** Transmission electron microscopic images of: (A) IONP\(_{10\text{RT}}\), (B) IONP\(_{10\text{ET}}\), (C) IONP\(_{25\text{ET}}\), (D) IONP\(_{40\text{ET}}\); HR-TEM images of (E) IONP\(_{10\text{RT}}\), (F) IONP\(_{10\text{ET}}\), (G) IONP\(_{25\text{ET}}\), and (H) IONP\(_{40\text{ET}}\); representative selected area electron diffraction (SAED) images of: (I) IONP\(_{10\text{RT}}\), (J) IONP\(_{10\text{ET}}\). HR-TEM images of (K) IONP-PEG\(_{10\text{RT}}\) and (L) IONP-OA\(_{10\text{RT}}\)
transmission Mössbauer spectroscopy experiments. For all samples, spectra were collected at 10 K, well below the superparamagnetic regime. At this temperature, the spectra exhibited a broadened sextet, as shown in Figure 3.3. The results of the fits to the spectra, shown by the solid lines in Figure 3.3, are summarized in Table A3.1 (see Appendix A). For the IONP$_{10}$(RT), IONP$_{10}$(ET), IONP$_{25}$(ET), and IONP$_{40}$(ET) systems, the spectra were well fitted with two sextets describing the local Fe environments at the octahedral and tetrahedral sites of an iron oxide. The relative area of each sextet was fitted and provided the crystallographic ratio between the octahedral and tetrahedral Fe sites that, together with the hyperfine field values (≈ 53 T and ≈ 51 T), established the iron oxide phase to be magnetite. The values of the quadrupole splitting (QS) of Fe in octahedral and tetrahedral sites were defined as zero and 0.05 mm s$^{-1}$, respectively, for the fits. Non-zero QS values, which are not present for bulk iron oxides, were here required to characterize the effects of the distorted coordination symmetry at the Fe sites on the surface of the IONPs. Also, the fitted isomer shifts (IS) remained consistent among all nanoparticles. Finally, the spectral linewidths ($\Gamma$) of the IONP$_{10}$(RT), IONP$_{10}$(ET), IONP$_{25}$(ET), and IONP$_{40}$(ET) systems is broadened compared to the natural source linewidth of ≈ 0.13 mm s$^{-1}$, which indicates considerable chemical disorder around the Fe sites, consistent with the nanoscale size of the particles.
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Successful surface coverage for the coated IONPs was monitored using FT-IR spec-

**Figure 3.3.** Transmission Mössbauer spectra at 10 K of: (A) IONP$_{10\text{RT}}$, (B) IONP$_{10\text{ET}}$, (C) IONP$_{25\text{ET}}$, (D) IONP$_{40\text{ET}}$, (E) Fe(acac)$_3$, and (F) the IONP$_{10\text{RT}}$ reaction mixture quenched with formaldehyde 10 s after addition of NaBH$_4$. Solid lines are the fits described in the text.
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troscopy. The transmission FT-IR spectra for IONP-PEG$_{10\text{RT}}$ and IONP-OA$_{10\text{RT}}$ are shown in Figure 3.4 (for FT-IR spectra of IONP-LA$_{10\text{RT}}$ and IONP-GA$_{10\text{RT}}$, see Figure A3.6 in Appendix A). The prominent peaks around 590 cm$^{-1}$ corresponded to the Fe$_{\text{octahedral}}$–O–Fe$_{\text{tetrahedral}}$ stretching vibrations of the spinel iron oxide. The bands at 2854 and 2925 cm$^{-1}$ for IONP-OA$_{10\text{RT}}$ were related to symmetric and antisymmetric C–H stretching vibrations in the OA coating, and the broad band ranging from 1200 to 1500 cm$^{-1}$ in the spectrum of IONP-PEG$_{10\text{RT}}$ indicated C–O stretching in poly(ethylene glycol). These results clearly showed that the surface of IONPs had been modified with the desired hydrophilic or hydrophobic molecules. The bare IONPs can also be modified at a later stage with hydrophobic molecules. As an example, we coated bare IONP$_{10\text{RT}}$ with lipophilic OA post-synthesis in a simple sonication bath. In a water/hexane system (1:1 = $\nu$ : $\nu$), the initially bare nanoparticles (Figure 3.5, vial on the left shows IONP$_{10\text{RT}}$ suspended in the aqueous phase of the binary solvent system water/hexane) readily migrated into the hexane layer in the presence of OA after 10 min of sonication at 25 °C, showing the formation of IONP$_{10\text{RT}}$-OA (Figure 3.5, vial on the right). Similar to IONP-OA$_{10\text{RT}}$, IONP$_{10\text{RT}}$-OA formed stable dispersions in hexane lasting for several months. At this point, it should also be mentioned that the bare IONPs and all IONPs protected with hydrophilic coatings (PEG, GA, and LA) were easily dispersed in water (and re-dispersed after drying), forming suspensions that were stable over several hours to days (Figure A3.7 in Appendix A).
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Figure 3.4. FT-IR spectra of: (A) IONP\textsubscript{10(RT)}, (B) IONP-PEG\textsubscript{10(RT)}, (C) IONP-OA\textsubscript{10(RT)}.

Figure 3.5. IONPs in hexane/water. Left: IONP\textsubscript{10(RT)} (oleic acid in the hexane phase) before sonication; right: oleic acid-coated IONP\textsubscript{10(RT)-OA} obtained after sonication of IONP\textsubscript{10(RT)} with oleic acid in hexane, now suspended in the hexane layer.

The steps involved in the liquid-phase synthesis of metal and metal oxide nanoparticles have, in principle, been studied previously, and an in-depth discussion of the mecha-
nistic pathway can be found in several review articles.\textsuperscript{39-41} In high-temperature synthesis of metal oxide nanoparticles from organometallic precursors such as M(acac)\textsubscript{3}, the oxygen is known to be provided by the acetylacetonate group and by the solvents.\textsuperscript{42a} The reaction pathway for these high-temperature decomposition pathways is still not clearly understood. For the present reaction, which involves aqueous conditions and the use of NaBH\textsubscript{4} to form IONPs, the electrochemical half-reaction of the borohydride ion is shown in Equation 1. It has a standard electrode potential ($E^\circ$) of $-0.48$ V. In an aqueous medium, the borohydride ion can thus reduce the ferric ion to a ferrous ion, which has an $E^\circ$ value of $0.77$ V as shown in Equation 2.

\[
\begin{align*}
B(OH)_3 + 7H^+ + 8e^- & \rightleftharpoons BH_4^- + 3H_2O & E^\circ = -0.48 \text{ V} & \text{Eq(1)} \\
Fe^{3+} (aq) + e^- & \rightleftharpoons Fe^{2+} (aq) & E^\circ = 0.77 \text{ V} & \text{Eq(2)} \\
Fe^{3+} + 3OH^- & \rightarrow Fe(OH)_3 & \text{Eq(3)} \\
Fe(OH)_3 & \rightarrow FeOOH + H_2O & \text{Eq(4)} \\
Fe^{2+} + 2OH^- & \rightarrow Fe(OH)_2 & \text{Eq(5)} \\
2 FeOOH + Fe(OH)_2 & \rightarrow Fe_3O_4 & \text{Eq(6)}
\end{align*}
\]

In an attempt to understand the reaction pathway, we collected the Mössbauer spectrum of a reaction mixture of IONP\textsubscript{10(RT)} that was quenched after 10 s. The reaction was quenched by adding an excess of formaldehyde (that rapidly reacts with NaBH\textsubscript{4} forming methanol) 10 s after the addition of NaBH\textsubscript{4}. An aliquot was drawn into a liquid sample holder, and a transmission Mössbauer spectrum (Figure 3.3F) was recorded at 100 K, below the superparamagnetic regime of the nanoparticles. For comparison, the transmission Mössbauer spectrum of the precursor, Fe(acac)\textsubscript{3}, was collected at 100 K as well (Figure
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3.3E). For Fe(acac)₃, the spectrum was fitted by a characteristic broad singlet with hyperfine parameters shown in Table S1 (see the SI). For the reaction mixture, the spectrum was fitted using the above singlet of the precursor, a broad doublet, and a sextet with a low field. The singlet shows the presence of non-hydrolyzed precursor at 10 s that accounts for ≈ 30% of the iron species in the reaction mixture. The isomer shift, ≈ 0.42 mm s⁻¹, and the quadrupole splitting of the doublet, ≈ 0.72 mm s⁻¹, indicate that ≈ 60% consist of octahedral high spin iron(III) species in a hydroxide form at a paramagnetic and/or superparamagnetic regime. An identical value for the isomer shift, ≈ 0.42 mm s⁻¹, and the value of the hyperfine field, ≈ 28 T, indicate that ≈ 10% consist of the same iron(III) species at a slow magnetic relaxation regime. Hence, the obtained iron(III) signals may be assigned to goethite FeO(OH).

From the absence of an iron(II) species and the presence of unreacted Fe(acac)₃, we assumed that the hydrolysis of Fe(acac)₃, facilitated by the reduction of the acetylacetonate group with NaBH₄, preceded the reduction of iron(III) to iron(II). Adding an excess of the more reactive formaldehyde arrested this process, leaving unreacted Fe(acac)₃ in the reaction mixture. Based on the Mössbauer studies on the reaction mixture, we can now explain the reaction pathway. With the addition of NaBH₄, the pH of the reaction mixture rose uniformly to 11 in every case (This basic pH arises from the formation of NaOH from sodium metaborate NaBO₂, the latter is a hydrolysis byproduct of NaBH₄). This uniform increase in pH is advantageous as we avoid high local supersaturation as it is the case for the addition of base in the coprecipitation method. At higher pH, Fe(acac)₃ is hydrolyzed to ferric hydroxide Fe(OH)₃, which precipitates, and is converted into orange colored goethite, FeO(OH), as shown in
Equations 3.3 and 3.4. From this stage, the formation of IONPs can, in principle, follow a precipitation pathway similar to that of Massart’s method.\textsuperscript{45} Nucleation of iron oxide was negligible until a certain critical supersaturation was achieved, and the critical supersaturation must have resulted from the reaction of goethite with ferrous hydroxide (Equation 6). At the onset of nucleation, a large number of small nuclei or singlets (subnanometer particles) of iron oxide were formed, and the equilibrium critical radius determined their diffusional growth. In general, Ostwald ripening favors the formation of larger, thermodynamically stable particles with a lower surface to volume ratio, and all particles with radii greater than the critical radius will continue to grow, while those with lower values will dissolve.\textsuperscript{39} The formation of uniform, single crystalline particles can be adequately explained by the La Mer’s concept, especially if the particles formed are very small as in our case. La Mer’s concept involves two steps, a short nucleation burst followed by diffusional growth of the nuclei to form uniformly sized spherical particles.\textsuperscript{46} To obtain particles with narrow size distribution, these two steps must be well separated, i.e., nucleation should be avoided during diffusional growth. An excess of sodium borohydride helps in separating these two steps, and a molar ratio of NaBH\textsubscript{4}/Fe(acac)\textsubscript{3} of at least 10:1 favors burst nucleation. IONP\textsubscript{10(RT)} synthesized at RT with a NaBH\textsubscript{4}/Fe(acac)\textsubscript{3} molar ratio of 10:1 had a size of around 5 nm (established by TEM). Upon increasing the reaction temperature from RT to 78 °C (ET), as was done for IONP\textsubscript{10(ET)}, the size of the particles increased to about 8 nm. IONPs synthesized at 78 °C with an increasing molar ratio of NaBH\textsubscript{4}/Fe(acac)\textsubscript{3} of 40:1 (IONP\textsubscript{40(ET)}) yielded IONPs of about 5 nm in size. Exact size distributions are listed in Table 1. Upon reducing the molar ratio of NaBH\textsubscript{4}/Fe(acac)\textsubscript{3}, the
average size of the particles increased (as established by TEM and XRD) for IONP$_{25(ET)}$ and IONP$_{10(ET)}$ in a monotonic fashion (Figure A3.3E in Appendix A). The observed decrease in IONP size with increasing NaBH$_4$ concentrations can be rationalized by an increase in the nucleation burst, at a given point in time, and a slowdown of diffusional growth. Commonly, the synthesis of IONPs is sensitive not only to iron precursor and other reactant concentrations but also to other reaction conditions, and variations in any of the above parameters will result in problems to control the size, structure, and composition of the produced IONPs. With the use of a single iron precursor and less reactants, there are relatively few variables in the reaction presented here, which enables us to prepare IONPs that are single-phased, i.e., pure magnetite. The as-synthesized bare IONPs were dispersible in water, physiological buffer, and cell culture media under mild sonication.

To evaluate the toxicity (or the lack thereof) of the as-synthesized IONPs, in particular any toxicity resulting from the synthetic methodology, bare IONP$_{10(RT)}$ were investigated in vitro using Caco2 and HepG2 cells as model systems for evaluating intestinal and liver epithelial cell viability, respectively. The cell viability was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay based on the cleavage of the yellow MTT salt to purple colored formazan by mitochondrial dehydrogenase in living cells.$^{47}$ Mitochondria play a crucial role in maintaining the cellular structure and function via aerobic ATP production and are vulnerable to toxic materials. Both Caco2 and HepG2 cells were not confluent at the time of treatment with NPs and were confluent prior to the addition of the MTT reagent (Figure A3.8 in
Appendix A). From the percentage of cell viability for various concentrations of IONP_{10(RT)} (Table A3.2, see Appendix A), it was concluded that there was no detectable toxicity (Figures 3.6A and B). After 48 h, cell morphology was analyzed by fixing the cells on chamber slides followed by hematoxylin-eosin (HE) staining. HE staining is useful in identifying pyknotic or fragmented nuclei resulting from apoptosis or cell death and any irregularities in cell shape. The nuclei of both Caco2 and HepG2 cells treated with IONP_{10(RT)} were intact as observed under a phase contrast microscope (Figures 3.6D–F), indicating that the cells were viable and morphologically similar to the control cells.

![Graphs showing MTT cytotoxicity assay](image)

**Figure 3.6.** MTT cytotoxicity assay in: (A) Caco2 and (B) HepG2 cells following 24 hour exposure to various concentrations of IONP_{10(RT)}. Values are expressed as percentage of absorbance observed in control wells receiving culture media alone. Each value represents the mean ± SD of at least eight wells. HE staining was carried out to analyze the cell morphology of: (C) control Caco2, (D) Caco2 treated with 1 mg/ml IONP_{10(RT)},
3.4 Conclusions

Pure magnetite IONPs were synthesized from Fe(acac)$_3$ and NaBH$_4$ with a mild reductive method, resulting in particles of 5–8 nm in diameter with narrow size distributions. We have shown that temperature has an effect on the synthesis outcome, with elevated temperatures ($78 \, ^\circ\text{C}$) resulting in larger IONPs in comparison to particles prepared at room temperature. This provides a simple way of size control. All obtained particles are quasi-spherical except for the oleic acid coated IONPs, of which many particles exhibited a quasi-hexagonal shape. Mössbauer spectroscopy of the iron intermediates in a reaction mixture quenched 10 s after sodium borohydride addition provided insight into the reaction pathway, and we found that goethite is a key intermediate in the reaction. The versatility of this reaction was demonstrated by the preparation of bare (non-coated) IONPs ready for subsequent coating steps as well as IONPs coated in situ with either hydrophilic or hydrophobic coatings. The synthesis of hydrophilic PEG, L-glutamic acid, L-arginine, and hydrophobic OA-coated IONPs can be achieved easily, with only minor modification in the synthesis procedure. Based on an MTT assay, the as-synthesized IONPs showed no toxicity to Caco2 and HepG2 cells and did not affect cell morphology. We believe that this synthesis is a significant step forward towards the manufacturing of IONPs not only with regard to the reaction conditions that avoid toxic capping agents limiting the biomedical applicability but also in terms of the potential for industrial scale-up.
Acknowledgements

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3.5 References


3.5 References


Appendix A

Supplementary Information for Chapter 3

(A)
Figure A3.1. Rietveld plots of Powder XRD of: (A) IONP$_{10(\text{RT})}$, (B) IONP$_{10(\text{ET})}$, (C) IONP$_{25(\text{ET})}$, (D) IONP$_{40(\text{ET})}$. In all plots, the dotted red lines show the observed XRD pattern and solid black line shows the obtained fit. The blue line is the difference spectra between the observed and calculated spectra. The vertical lines represent the calculated Bragg positions for spinel magnetite phase.
Figure A3.2. Powder XRD of: (A) IONP-PEG$_{10$(RT)}$, (B) IONP-OA$_{10$(RT)}$, and (C) IONP-GA$_{10$(RT)}$, IONP-LA$_{10$(RT)}$. 
Figure A3.3. Size distribution from TEM of: (A) IONP\textsubscript{10(\text{RT})}, (B) IONP\textsubscript{10(\text{ET})}, (C) IONP\textsubscript{25(\text{ET})} and (D) IONP\textsubscript{40(\text{ET})}, (E) sizes of bare IONPs represented as mean ± SD of at least 200 individual particles. These size differences are statistically significant with p values well below 0.05 based on a student t-test (equal variances, two-tail).
Figure A3.4. TEM micrographs of: (A) IONP-PEG_{10}(RT), (B) IONP-OA_{10}(RT) (inset: HR-TEM micrograph of quasi-hexagonal shaped IONP-OA_{10}(RT)), HR-TEM micrographs of (C) IONP-LA_{10}(RT) and IONP-GA_{10}(RT).
Figure A3.5. SAED of: (A) IONP$_{25(ET)}$ and (B) IONP$_{40(ET)}$. (C) Radial intensity profile of IONPs from SAED (radial intensity profile for all SAED patterns were obtained using DiffTools$^1$ script package for Digital MicrographTM (Gatan Inc.). The centers of the SAED patterns were located and rotational averaging was performed for each pattern.
Figure A3.6. FT-IR spectra of: (A) L-glutamic acid-coated IONP-GA$_{10}$ (RT) and (B) L-arginine coated IONP-LA$_{10}$ (RT). The strong band at around 2600 cm$^{-1}$ to 3700 cm$^{-1}$ in (A) and (B) encompass O–H stretching of COOH and water of crystallization, N–H stretching of NH$_3^+$ and C–H stretching. The C=O stretching vibrations are revealed as a small peak around 1645 cm$^{-1}$. Fe–O–Fe stretching is evident by peaks around 590 cm$^{-1}$.
Figure A3.7. Dispersions of: (A) IONP$_{10(ET)}$, (B) IONP$_{25(ET)}$, (C) IONP$_{40(ET)}$, (D) IONP-PEG$_{10(RT)}$, (E) IONP-LA$_{10(RT)}$, and (F) IONP-GA$_{10(RT)}$ in water. These suspensions are stable over several hours to days without any observable settling of the IONPs.
Figure A3.8. Optical microscopy of: (A) Caco2 cells treated with culture media, (B) Caco2 cells treated with 1 mg/ml of IONP<sub>10(RT)</sub> in culture media, (C) HepG2 cells treated with culture media, and (D) HepG2 cells treated with IONP<sub>10(RT)</sub> at different stages prior to MTT assay.

Table A3.1. Mössbauer spectral parameters obtained from the fit.

<table>
<thead>
<tr>
<th></th>
<th>B (T)</th>
<th>δ (mm/s)</th>
<th>ΔEQ (mm/s)</th>
<th>Γ (mm/s)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IONP&lt;sub&gt;10(RT)&lt;/sub&gt;</td>
<td>51(1)</td>
<td>0.35(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55(5)</td>
<td>56(1)</td>
</tr>
<tr>
<td></td>
<td>53(1)</td>
<td>0.42(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43(5)</td>
<td>44(1)</td>
</tr>
<tr>
<td>IONP&lt;sub&gt;10(ET)&lt;/sub&gt;</td>
<td>51(1)</td>
<td>0.49(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.83(3)</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>53(1)</td>
<td>0.49(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.61(3)</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IONP&lt;sub&gt;25(ET)&lt;/sub&gt;</td>
<td>51(1)</td>
<td>0.31(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66(2)</td>
<td>60(5)</td>
</tr>
<tr>
<td></td>
<td>53(1)</td>
<td>0.40(1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47(3)</td>
<td>40(5)</td>
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Table A3.2. MTT cell viability assay of Caco2 and HepG2 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caco2 Cell viability (%)</th>
<th>HepG2 Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^{a})</td>
<td>99.97 ± 11.27</td>
<td>99.97 ± 10.17</td>
</tr>
<tr>
<td>0.010(^{b})</td>
<td>79.46 ± 12.54</td>
<td>93.97 ± 9.85</td>
</tr>
<tr>
<td>0.032(^{b})</td>
<td>86.40 ± 9.36</td>
<td>88.88 ± 9.51</td>
</tr>
<tr>
<td>0.100(^{b})</td>
<td>87.32 ± 9.23</td>
<td>86.45 ± 11.70</td>
</tr>
<tr>
<td>0.320(^{b})</td>
<td>85.86 ± 7.18</td>
<td>82.40 ± 12.99</td>
</tr>
<tr>
<td>1.000(^{b})</td>
<td>97.47 ± 5.48</td>
<td>88.60 ± 12.67</td>
</tr>
<tr>
<td>10 % EtOH(^{c})</td>
<td>11.96 ± 1.45</td>
<td>13.50 ± 1.41</td>
</tr>
</tbody>
</table>

\(^{a}\) Cell culture media (DMEM), \(^{b}\) IONP\(_{10(\text{RT})}\) (mg/ml) in DMEM, \(^{c}\) 10 % ethyl alcohol in DMEM.

References

Chapter 4:

One-pot Synthesis of Iron Oxide Nanoparticles with Functional Silane Shells: A Versatile General Precursor for Conjugations and Biomedical Applications
One-pot Synthesis of Iron Oxide Nanoparticles with Functional Silane Shells: A Versatile General Precursor for Conjugations and Biomedical Applications

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Authors’ Contributions

The idea behind this chapter i.e. adapting and extending the reduction-hydrolysis synthesis in chapter 3 to prepare aminosilane coated IONPs and using it as a general precursor for conjugations, was conceptualized by Vinith Yathindranath (VY). All the experiments related to synthesis, conjugation and characterization were designed and carried out by VY. Zhizhi Sun carried out all the pharmacological studies including cell toxicity and uptake studies, and gel retardation studies. Mathew Worden carried out the TGA measurements and Dr. Thliveris carried out the TEM studies on cells presented here. Dr. Lynda Donald carried out the mass spectrometry studies presented here. The manuscript with all figures and tables was prepared by VY. VY, Dr. Torsten Hegmann, Dr. Donald W. Miller, Zhizhi Sun and Mathew Worden were responsible for final revisions of the manuscript before its publication in *Langmuir*. 
Abstract

Iron oxide nanoparticles (IONPs) and their surface modifications with therapeutic or diagnostic (theranostic, TN) agents are of great interest. Here we present a novel one-pot synthesis of a versatile general TN precursor (aminosilane-coated IONPs [IONP-Si(NH$_2$)]) with surface amine groups. Surface functional group conversion to carboxylic acid was accomplished by conjugating poly(ethylene glycol) diacid to IONP-Si(NH$_2$). The NPs were characterized using powder X-ray diffraction (XRD), transmission electron microscopy (TEM), high-resolution TEM, selected area electron diffraction (SAED), X-ray photoelectron spectroscopy (XPS), and FT-IR. Biocompatibility and cell uptake profile of the nanoparticles were evaluated in vitro using cultured liver cells (HepG2). Oleylamine (hydrophobic) and bovine serum albumin (BSA) as model drugs were attached to IONP-Si-PEG(COOH). The ability of IONP-Si(NH$_2$) to bind small interfering RNA (siRNA) is also shown.
4.1 Introduction

Superparamagnetic iron oxide nanoparticles (IONPs) comprised of magnetite (Fe$_3$O$_4$) and maghemite ($\gamma$-Fe$_2$O$_3$) phases are among the most widely studied nanomaterials for biomedical applications.\textsuperscript{1-11} As an FDA approved material, IONPs are used in clinical medicine as magnetic resonance imaging (MRI) contrast agents.\textsuperscript{1,12} In clinical trials, hyperthermia generated by IONPs (in alternating magnetic field) in combination with radiotherapy showed enhanced cytotoxicity in glioblastoma multiforme.\textsuperscript{4} Applications using IONPs to target and treat metastatic cancer have also been reported.\textsuperscript{13-16} Area specific delivery of therapeutic and diagnostic (theranostic, TN) agents using IONPs can be achieved through passive, active or a combination targeting strategy with suitably designed IONP-TNs. Examples of passive targeting approaches include the accumulation of IONPs within tumor sites through an enhanced permeation and retention (EPR) effect\textsuperscript{16} as well as within areas of damaged brain through a disrupted blood-brain barrier (BBB).\textsuperscript{9,17,18} In the near future, magnetically directed delivery (MDD) of IONP-TNs using conventional MRI instruments will be a viable targeting strategy.\textsuperscript{19,20} Active targeting of diseased sites has been achieved by attaching exclusive targeting ligands such as antibodies,\textsuperscript{21-23} peptides,\textsuperscript{24,25} and small molecules\textsuperscript{26} to IONP-TNs. Multiple combinations of strategies are used to prepare many of the custom IONP-TNs, from IONP-core synthesis, choice of surface coating, to bioconjugations. However, it is crucial to streamline these processes by adopting a general method wherever feasible. A versatile, general precursor (IONP-TN-PC) suitable for straightforward conjugations of a variety of TNs would sim-
4.1 Introduction

plify the process to a great extent; especially for a multidisciplinary research community. Hence, developing better ways (reduced steps, simple set-up, mild and economical) of preparing a versatile general IONP-TN-PC, using well established materials is of current interest.

While designing an IONP-TN-PC, the key physical and chemical constraints for IONP-TNs have to be addressed. As a paradigm, IONP-TNs should have a highly crystalline core with a crystallite sizes of less than 20 nm to retain superparamagnetic properties. They should display appropriate hydrophilic-hydrophobic balance and have a hydrodynamic size of less than 100 nm to evade the mononuclear phagocyte system and have long blood circulation half-life. Hence the IONP-TN-PC should comply with all constrains and should present opportunities to accommodate any change resulting from TN conjugations (e.g., hydrophilicity, hydrodynamic size, among others). There are several well established methods to prepare superparamagnetic IONP-core material including microwave, sol-gel, microemulsion, hydrothermal, solvothermal and sonochemical processes. Some of these syntheses have complex schematics, require equipment such as autoclaves, furnaces, sonicators or microwave reactors, and use high temperatures and/or toxic solvents and stabilizers. Massart’s co-precipitation is a widely used method for preparing biocompatible IONP-core. The main limitations to Massart’s co-precipitation is that the resulting IONP-core usually have broad size distribution, and slight variations in Fe(II)/Fe(III) precursor-ratio during synthesis can result in the formation of complex undesired oxide phases. Biocompatible IONP-cores with a narrow size distribution have been prepared following high temperature decomposition of iron
precursors in organic solvents.\textsuperscript{40} The as-synthesized IONP-cores from high temperature syntheses typically carry a residual hydrophobic shell, and additional ligand exchange or addition steps are required to make them hydrophilic and biocompatible.\textsuperscript{41-43} We recently reported a novel room temperature synthesis of magnetite (Fe$_3$O$_4$) NPs, via aqueous reduction-hydrolysis of Fe(acac)$_3$, using sodium borohydride (NaBH$_4$).\textsuperscript{44} This method does not require toxic organic solvents, reagents, stabilizers or high temperatures. The as-synthesized quasi-spherical (≈5 nm diameter) superparamagnetic magnetite NPs were single crystalline and biocompatible. For a versatile IONP-TN-PC, the IONP-core should have a robust functional shell that can withstand additional surface modification steps. A silica-based functional shell will be more advantageous than usual polymer and organic stabilizers. From a drug carrier perspective, silica is hydrophilic, biocompatible and forms a robust polycondensed shell (protects magnetic IONP-core from reaction and in vivo environments) around IONP-core. For multimodal imaging applications, dye molecules attached through a silica shell are known to overcome luminescence quenching (commonly observed when dye molecules are directly attached to IONPs).\textsuperscript{45} 3-Aminopropyltriethoxysilane (APTES) is widely used for forming amine functional silane coating of monolayer thickness on surfaces.\textsuperscript{46-49} An aminosilane shell in addition to protecting IONP-core can allow simple surface conjugations through free surface amino groups. Aminosilane-coated IONPs are typically synthesized in two stages, with synthesis of IONP-cores through any of the above discussed methods and formation of the aminosilane shell by refluxing or sonicating the mixture of IONPs and alkoxysilanes in organic solvents for several hours, in some cases days.\textsuperscript{50,51} Herein we report a novel one-pot
synthesis of aminosilane-coated IONPs [IONP-Sil(NH$_2$)] following our room temperature reduction-hydrolysis method. This mild method does not require toxic organic solvents or stabilizers. The simple set-up can be easily scaled up for bulk production.

For precise conjugation studies, it is essential to estimate the core-shell concentrations of IONP-TN-PC. The core-shell concentrations of IONP-Sil(NH$_2$) were determined following strategies outlined in Table B4.1 (Appendix B). To demonstrate a simple functional group conversion, we conjugated poly(ethylene glycol) diacid (PEG diacid) through the surface amine groups. Poly(ethylene glycol) (PEG) is a nonimmunogenic and nonantigenic polymer known to enhance the blood circulation half-life of NPs.$^{52}$ The resulting IONP-Sil-PEG(COOH) in addition to having biocompatible PEG “brushes”, also has surface carboxylic acid groups, suitable for further conjugations. Through the surface carboxylic acid groups, we linked a small model hydrophobic drug molecule (oleyla-mine) and a large model drug protein (bovine serum albumin, BSA) to represent the two ends of spectrum in hydrophobicity. The complete schematic of the IONP synthesis and subsequent surface conjugations is outlined in Figure 4.1.

The toxicity of IONP-Sil(NH$_2$), if any, resulting from the synthetic methodology was assessed in vitro on a cultured liver cell line (HepG2). The uptake of IONP-Sil(NH$_2$) and IONP-Sil-PEG(COOH) were assessed in vitro in cultured HepG2 cells. Synthetic small interfering RNA (siRNA) is emerging as a potential treatment for various genetic diseases including cancer.$^{13}$ The challenge lies in the effective intracellular delivery of siRNA to the tissue that expresses the target gene. NPs are emerging as non-viral alternatives for
the intracellular delivery of siRNA.\textsuperscript{53,54} In our preliminary study, we looked into the binding ability of IONP-Sil(NH\textsubscript{2}) towards the siRNA.

4.2 Experimental

Iron(III) acetylacetonate [Fe(acac)\textsubscript{3}, 99\%] was purchased from Fluka. Sodium borohydride (NaBH\textsubscript{4}, 98\%), (3-aminopropyl)triethoxysilane (APTES, 99\%), 1-ethyl-3-(3-
dimethylaminopropyl)carbodiimide (EDC), poly(ethylene glycol) diacid 600 (PEG diacid 600), Oleylamine (OAm, 70%) and bovine serum albumin fraction V (BSA, 99%) were obtained from Sigma-Aldrich and N-hydroxysulfosuccinimide sodium salt (NHS, 98%) was purchased from AK Scientific. Millipore deionized (DI) water (R = 18 MΩ) was used in the reaction and purification steps.

Powder X-ray diffraction (XRD) measurements were carried out using a PANalytical X’Pert Pro Bragg-Brentano powder X-ray diffractometer equipped with a diffracted beam Ni-filter and an X’Celerator detector. Cu Kα1,2 radiation (λ = 1.540598, 1.544426 Å) were used as the X-ray sources. Transmission electron microscopy (TEM) was carried out on a Jeol ultra-high resolution FEG-T/STEM operating at an accelerating voltage of 200 kV. A droplet of nanoparticle dispersion in methanol was placed onto a carbon coated copper grid (400 mesh) and air dried prior to analysis. FT-IR of the samples was recorded on Bruker TENSOR 27, using KBr pellets. X-ray photoelectron spectroscopy was carried out on Kartos AXIS ULTRA spectrometer with a 300 W monochromated Al Kα source and a delay line detector. Thermogravimetric analysis (TGA) was carried out in a Perkin-Elmer TGA-7 with nitrogen as purge gas. Hydrodynamic sizes were measured in a Malvern Nano-S Dynamic Light Scattering system. The mass spectrum was acquired on a MALDI QqTOF mass spectrometer built in the Dept. of Physics & Astronomy, University of Manitoba.

Synthesis of IONP-Sil(NH2): Fe(acac)3 (2.83 g, 8 mmol) was dissolved in 400 mL of 60% ethanol in DI water. The content in a 1000 mL flask, fitted with an overhead stirrer assembly, was purged with nitrogen for one hour. To this, NaBH4 (3.03 g, 80.0 mmol) in 40
mL of deoxygenated DI water was rapidly added while stirring (1000 RPM). After 20 minutes the colour of the reaction mixture changed from red, orange, dark brown and finally to black indicating the formation of IONPs. An hour into the addition of NaBH₄, APTES (16 mL) was added rapidly and the reaction mixture was stirred overnight at room temperature. The blackish brown solution was filtered through a Whatman filter paper and the solvent was removed at 50 °C under low pressure. The resulting viscous mixture was dissolved in 200 mL of cold ethanol and left until excess NaBH₄ crystallized and was removed by filtration: this step was repeated until no crystals appeared. Later, ethanol was completely removed and the crude product was dissolved in 50 mL DI water and dialyzed (Spectra/Por Dialysis Membrane MWCO 6–8000) against DI water to remove unreacted APTES. The dialyzed solution was centrifuged at 4000 RPM for 30 minutes and the dark reddish brown supernatant (stock solution) was collected and stored at ambient conditions until further use.

**Synthesis of IONP-Sil-PEG(COOH):** To PEG diacid 600 (2.0 g, 3.3 mmol) dissolved in 20 mL DI water, EDC (0.19 g, 1 mmol) and NHS (0.21 g, 1 mmol) were added. The mixture was stirred for 15 minutes. To this, 40 mL of IONP-Sil(NH₂) solution (∼42.0 mg of aminosilane, ∼0.3 mmol) were added and stirred for three hours. The crude product was dialyzed against DI water. Later, the clear red ferrofluid was centrifuged at 4000 RPM and the resulting supernatant was stored under ambient condition until further use.

**Synthesis of IONP-Sil-PEG-OAm:** To 5 mL of IONP-Sil-PEG(COOH) [≈ 2.23 mg of PEG(COOH), ∼3.7 µmol], EDC (0.38 mg, 2 µmol) and NHS (0.43 mg, 2 µmol) were added and stirred for 15 minutes. 1 µmol of oleylamine (1 mg/mL in ethanol) was added
and stirred for three hours. The crude product was purified by several rounds of alternating centrifugation and washing the precipitate with water and ethanol. The purified product was stored as a colloidal dispersion in DI water.

**Synthesis of IONP-Sil-PEG-BSA:** To 5 mL of IONP-Sil-PEG(COOH) [≈ 2.23 mg of PEG(COOH), ≈ 3.7 µmol], EDC (0.38 mg, 2 µmol) and NHS (0.43 mg, 2 µmol) were added and stirred for 15 minutes. To the solution, BSA (9 mg, 0.14 µmol) in 10 ml PBS was added and stirred for 1 hour. The crude product was dialyzed against PBS and the purified product in PBS was stored at 4 °C.

### 4.3 Results and discussion

In our synthesis, the IONP-core precipitated from an aqueous Fe(acac)₃ solution by a reduction-hydrolysis pathway using NaBH₄. The pH of the reaction medium after the addition of aqueous NaBH₄ was alkaline (pH = 11). The Fe(acac)₃ underwent hydrolysis and reduction to form Fe³⁺ [Fe(OH)₃, FeO(OH)] and Fe²⁺ [Fe(OH)₂] intermediates. Condensation reaction between Fe³⁺ and Fe²⁺ species at a suitable ratio (Fe³⁺: Fe²⁺ of 2:1) resulted in the nucleation of Fe₂O₄. APTES [3-AP–Si(OCH₃)₃] in the basic condition hydrolysed to form trihydroxysilane [3-AP–Si(OH)₃] which in turn underwent an alkaline condensation with the surface hydroxyl groups of IONPs (Figure 4.1). A robust aminosilane shell will then be formed by polycondensation of nearby hydroxysilanes on the IONP surface forming IONP-Sil(NH₂).
4.3 Results and discussion

Figure 4.2. (A) Powder XRD pattern of IONP-Sil(NH$_2$), (B) FT-IR spectra of: (1) IONPs prior silanization, (2) IONP-Sil(NH$_2$), and (3) IONP-Sil-PEG(COOH). TGA and dTGA of (C) IONP-Sil(NH$_2$) and (D) IONP-Sil-PEG(COOH).

Figure 4.2A shows the powder X-ray diffraction (XRD) pattern of IONP-Sil(NH$_2$). The peaks confirmed the presence of a cubic iron oxide phase (Fe$_3$O$_4$, ICDD code: 01-088-0315, space group: Fd$ar{3}$m). The small crystallite sizes of the core and the aminosilane shell caused the broadening of the XRD peaks. Aminosilane coating in IONP-Sil(NH$_2$) and conjugation of PEG diacid in IONP-Sil-PEG(COOH) were assessed using FT-IR spectroscopy (Figure 4.2B-1 and 2). The Fe–O–Fe stretching in IONP-Sil(NH$_2$) (Figure 4.2B-1) was observed at 590 cm$^{-1}$. The aminosilane shell formed around the IO core displayed the Si–O–Si stretching band at 985 cm$^{-1}$, symmetric and asymmetric C–H stretching (from propyl group) bands at 2848 and 2916 cm$^{-1}$, respectively, and N–H bending (from free amine groups) at 1608 cm$^{-1}$. The conjugated PEG acid in IONP-Sil-
PEG(COOH) (Figure 4.2B-2) displayed a strong C−O−C stretching band at 1000-1217 cm\(^{-1}\). The carbonyl stretching bands from the amide link was found at 1637 cm\(^{-1}\), and from the free surface carboxylic acid was at 1720 cm\(^{-1}\). The Fe−O−Fe stretching of the core was observed at 588 cm\(^{-1}\). IONP-Sil(NH\(_2\)) was further characterised using XPS (Figure B4.1 in Appendix B). The Fe 2p\(_{3/2}\) (Fe\(^{2+}\) and Fe\(^{3+}\) at 710.2 eV and 712.1 eV) and Fe 2p\(_{1/2}\) (Fe\(^{2+}\) and Fe\(^{3+}\) at 723.8 eV and 726.1 eV) high-resolution spectrum (Figure B4.1A in Appendix B) had two components from Fe\(^{2+}\) and Fe\(^{3+}\) species of magnetite. The peak at 101.4 eV (Figure B4.1B in Appendix B) corresponded to Si 2p indicating the presence of silane group in IONP-Sil(NH\(_2\)). O 1s (Figure B4.1C in Appendix B) peak had three components; a peak at 530.0 eV (from Fe\(_3\)O\(_4\)), 531.4 eV (−OH species) and 533.4 eV (Si−O). The C 1s peak (Figure B4.1D in Appendix B) comprised of three component peaks at 285.0, 286.7 and 288.9 eV corresponding to C−Si, C−C and C−N, respectively, of the 3-aminopropyl group. Figure B4.1E in Appendix B shows the N 1s high-resolution spectrum, which exhibited two components at binding energies ≈ 399.5 eV and 401.4 eV (after deconvolution) corresponding to the amino and cationic amino groups, respectively.

The amount of aminosilane in IONP-Sil(NH\(_2\)) and PEG acid in IONP-Sil-PEG(COOH) were estimated from results of thermogravimetric (TGA) analyses. The TGA and derivative TGA curves of IONP-Sil(NH\(_2\)) (Figure 4.2C) showed two significant weight-loss events with rising temperature. A drop in weight around 100 °C is believed to be related to the loss of adsorbed water, and the second drop through 200 and 500 °C
suggests the pyrolysis of the aminosilane coating, estimated to be \(\approx 10\%\) by weight. IONP-\(\text{Sil}\)-PEG(COOH) (Figure 4.2D) displayed one major weight-loss of \(\approx 31\%\), most likely resulting from the pyrolysis of the aminosilane and the conjugated PEG acid. The 21\% weight gain in IONP-\(\text{Sil}\)-PEG(COOH) against precursor IONP-\(\text{Sil}\)(NH\(_2\)) was attributed to the conjugated PEG acid (Table S1).

![Figure 4.3](image)

**Figure 4.3.** TEM, HRTEM and SAED of IONP-\(\text{Sil}\)(NH\(_2\)) (A, B and C respectively) and IONP-\(\text{Sil}\)-PEG(COOH) (D, E and F respectively).
IONP-Sil(NH$_2$) was observed using TEM and the micrograph (Figures 4.3A and B4.2A in Appendix B) showed quasi-spherical NPs. High-resolution TEM images (Figures 4.3B and B4.2B in Appendix B) showed clear lattice fringes indicating that the IONP-cores are highly crystalline. The selected area electron diffraction (SAED) from these particles showed the characteristic ring pattern for spinel IONPs (Figure 4.3C). TEM studies on IONP-Sil-PEG(COOH) showed that the individual quasi-spherical NPs were well separated (Figures 4.3D and B4.3A in Appendix B). The cores retained the highly crystalline nature (Figures 4.3E and B4.3B in Appendix B), and the SAED con-

\[\text{Figure 4.4. (A) Size distribution of IONP-Sil(NH}_2\text{) from TEM, (B) dispersion of (1) IONP-Sil(NH}_2\text{) and (2) IONP-Sil-PEG(COOH) in DI water (0.5 mg/mL) and their hydrodynamic size distribution: (C) IONP-Sil(NH}_2\text{) and (D) IONP-Sil-PEG(COOH) from DLS.}\]
firmed the presence of a spinel IO phase. The core size measured for the primary precursor IONP-Sil(NH₂) from TEM images was 4.2 ± 0.7 nm and the size distribution is shown in Figure 4.4A. Both IONP-Sil(NH₂) and IONP-Sil-PEG(COOH) formed stable colloidal dispersions in water (Figure 4.4B) with hydrodynamic diameters of 27.6 and 8.4 nm, respectively. The hydrodynamic size distribution of IONP-Sil(NH₂) and IONP-Sil-PEG(COOH) are shown in Figures 4.4C and 4.4D. The PEG-diacid conjugation helped in overcoming minor agglomeration in IONP-Sil(NH₂), by steric repulsion as evident from the reduced hydrodynamic diameter of the IONP-Sil-PEG(COOH) compositions. The iron concentration in aqueous dispersions was estimated using a ferrozine assay (see supplementary information) and the amount of surface coating was estimated using procedures outlined in Table B4.1. The amount of aminosilane in IONP-Sil(NH₂) and PEG acid in IONP-Sil-PEG(COOH) stock solutions were determined to be ≈ 7.5 and ≈ 0.7 µmol/mL, respectively.

The in vitro cell viability of model liver epithelial cells (HepG2) treated with various concentrations of IONP-Sil(NH₂) (0.625–320 µg/mL of Fe) was estimated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] viability assay. The assay is based on the mitochondrial dehydrogenase enzyme which in living cells cleaves the yellow MTT salt to purple coloured formazan whose formation was monitored colorimetrically using a Synergy plate reader at 550 nm absorbance. Figure 4.5A shows the percentage of viable cells following 48 hour treatment with IONP-Sil(NH₂). No significant decreases in cell viability were observed following IONP-Sil(NH₂) exposure (Figure 4.5A). The accumulation profile of IONP-Sil(NH₂) and IONP-Sil-PEG(COOH) (5, 10 and 20
μg/mL of Fe) was also evaluated in HepG2 cells at 37 °C, with and without magnetic field (Figure 4.5B). The iron concentration in cells was estimated and normalized with cell-protein concentration. Cells treated with IONP-Si/(NH₂) showed greater iron concentration than cells treated with IONP-Si-PEG(COOH). The cell accumulation of IONP-Si/(NH₂) over a 5 hour period was concentration dependent. In contrast, the cellular uptake of IONP-Si-PEG(COOH) was comparatively low and displayed no concentration-
dependency. The resulting iron concentrations measured in the cells reflect those NP loosely adhering to the outer plasma membrane as well as those NP internalized through an endocytotic process. In the case of the IONP-\textit{Sil}(NH\textsubscript{2}) a significant portion of the uptake observed appeared to be intracellular as uptake studies performed at low temperatures (to prevent the endocytotic process) resulted in a significant reduction in cell uptake and a loss of concentration dependency (Figure 4.5B-1'). Thus, uptake of IONP-\textit{Sil}(NH\textsubscript{2}) under these conditions represents IONPs that are loosely adherent to the outer plasma membrane of the HepG2 cells. The difference in cellular iron content in the accumulation studies conducted at 37 °C and 4 °C represent the amount of IONPs that were internalized by cells. This trend with high cellular uptake of aminosilane-coated IONPs is consistent with studies in other mammalian cell lines and is likely the result of the positive surface charge associated with the aminosilane coated IONPs.\textsuperscript{58} Application of a magnetic field enhanced IONP uptake by bringing in more NPs to interact with the cell membrane (Figure 4.5, grey bars). The HepG2 cells treated with IONP-\textit{Sil}(NH\textsubscript{2}) and IONP-\textit{Sil}-\textit{PEG}(COOH) at 37 °C were further visualized using TEM (Figure 4.6). From the electron micrographs, the morphology and shape of IONP-\textit{Sil}-\textit{PEG}(COOH) (Figure 4.6C) and IONP-\textit{Sil}(NH\textsubscript{2}) (Figure 4.6D–F) treated HepG2 cells were similar to the control cells that did not receive any NPs, indicating the biocompatibility of these NPs. In Figure 4.6C, we did not locate any IONP-\textit{Sil}-\textit{PEG}(COOH) in treated cells which was consistent with the cell uptake studies. In Figure 4.6D, some IONP-\textit{Sil}(NH\textsubscript{2}) (highlighted with an arrow) were located at caveolae in the cell membrane suggesting a caveloae mediated endocytosis and Figure 4.6E and 6F show them localized in endosomes inside cells. From
the uptake profile in HepG2 cells, IONP-Si\textsubscript{l}(NH\textsubscript{2}) can be useful in targeting liver cells; and IONP-Si\textsubscript{l}-PEG(COOH) with PEG chain, will evade liver cells and have a longer blood circulation half-life.

![Representative TEM micrographs of HepG2 cells](image)

**Figure 4.6.** Representative TEM micrographs of HepG2 cells: (A and B) cells that did not receive any nanoparticles, (C) cells treated with IONP-Si\textsubscript{l}-PEG(COOH) and (D, E and F) cells treated with IONP-Si\textsubscript{l}(NH\textsubscript{2}). Arrows in D, E and F point to sites where IONP-Si\textsubscript{l}(NH\textsubscript{2}) is present.

Hydrophobic oleylamine was attached to IONP-Si\textsubscript{l}-PEG(COOH) via amidation reaction using EDC/NHS. Depending on the equivalent of OAm used against PEG acid, we were able to change the dispersability of IONP-Si\textsubscript{l}-PEG-OAm from non-aqueous to aqueous media. IONP-Si\textsubscript{l}-PEG-OAm with ratios of OAm/PEG acid of 1.4 was predominantly hydrophobic, 0.5 was amphiphilic, and 0.3 was hydrophilic (Figure 4.7A). To be able to transfer a hydrophobic drug into aqueous phase with the aid of nanocarrier is val-
4.3 Results and discussion

Useful for their targeted delivery. The ability to attach large molecular weight TNs was demonstrated by conjugating a model drug-protein BSA to IONP-Sil-PEG(COOH). The BSA conjugated IONP-Sil-PEG-BSA formed stable colloidal dispersion in physiological buffer (Figure 4.7B inset). The covalent linking of BSA was confirmed using mass spectroscopy performed on a supernatant of trypsin digested and centrifuged IONP-Sil-PEG-BSA. The database search showed BSA as the top match for the peptides in the spectrum, with 25% coverage, and the three sequenced peptides, all from BSA (figure 4.7B). The as-synthesized IONP-Sil(NH)$_2$ with a positive surface charge, should bind to negatively charged moieties like siRNA (Figure 4.8, left) or DNA. In preliminary studies, binding of siRNA to IONP-Sil(NH)$_2$ was examined. Figure 4.8 shows the agarose gel electrophoresis of siRNA (1 equivalent) incubated with different weight equivalent of IONP-Sil(NH)$_2$ (Fe: 5, 10, 15, 20, 25, 30, 35 and 40 equivalents). The complete retarded band was ob-

**Figure 4.7.** (A) IONP-Sil-PEG-OAm dispersed in water/hexane solvent system: (1) 1.4, (2) 0.5 and (3) 0.3 equivalent of oleylamine to PEG(COOH) in IONP-Sil-PEG(COOH) (B) MALDI spectrum from the tryptic digestion of IONP-Sil-PEG-BSA. Major ions with M$^+$H$^+$ values that match BSA peptides are identified as such. Inset (sample vial 4) is the dispersion of IONP-Sil-PEG-BSA in PBS.
served at the ratios of siRNA to Fe of 1:20 or above. In terms of IONP-Sil(NH₂), at least 30 equivalents were required to completely bind one equivalent of siRNA.

![Diagram showing binding of siRNA to IONP-Sil(NH₂)](image)

**Figure 4.8.** Left: Cartoon showing the binding of negatively charged siRNA to cationic IONP-Sil(NH₂) and right: Agarose gel electrophoresis retardation study of siRNA incubated with different equivalents by weight of Fe in IONP-Sil(NH₂).

### 4.4 Conclusions

We have devised a novel one-pot synthesis of IONP-Sil(NH₂) with surface amine groups. This method with mild reaction conditions, minimal number of reagents and simple set-ups can be easily scaled up for cost effective bulk production. PEG diacid was conjugated to IONP-Sil(NH₂) to form IONP-Sil-PEG(COOH) following simple and well known EDC/NHS coupling. The IONP-Sil-PEG(COOH) was conjugated to model drug molecules (hydrophobic oleic acid and large molecular weight BSA) by EDC/NHS coupling.
IONP-Sil(NH$_2$) with its positive surface charge was able to bind siRNA and may be useful for magnetic separation applications. Both biocompatible IONP-Sil(NH$_2$) and IONP-Sil-PEG(COOH) are versatile IONP-TN-PCs suitable for precise conjugations for biomedical applications.
Acknowledgements

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4.5 References


References


Appendix B

Supplementary Information for Chapter 4

Table B4.1. Quantitative estimation of various parameters of IONP-$Sil(NH_2)$ and IONP-$Sil-PEG(COOH)$ stock solution in DI water. Here the IONP phase discussed is magnetite and the core is assumed to be a perfect sphere.

<table>
<thead>
<tr>
<th>Parameter/Unit</th>
<th>Description</th>
<th>Equation /Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_{IONP-Sil(NH_2)}$ (g/mL)</td>
<td>Mass of dried IONP-$Si(NH_2)$</td>
<td>Analytical balance</td>
</tr>
<tr>
<td>$M_{IONP-Sil-PEG(COOH)}$ (g/mL)</td>
<td>Mass of dried IONP-$Sil-PEG(COOH)$</td>
<td>Analytical balance</td>
</tr>
<tr>
<td>$M_{Fe}$ (g/mL)</td>
<td>Mass of Fe in solution</td>
<td>Ferrozine assay</td>
</tr>
<tr>
<td>$M_{IO}$ (g/mL)</td>
<td>Mass of Iron oxide ($Fe_3O_4$) in solution</td>
<td>$M_{Fe} \times 1.38$</td>
</tr>
<tr>
<td>$V_{IONP}$ (cm$^3$)</td>
<td>Volume of an individual IONP core</td>
<td>$\frac{4}{3} \pi r^3$</td>
</tr>
<tr>
<td>$SA_{IONP}$ (cm$^2$)</td>
<td>Surface area of an individual IONP core</td>
<td>$4\pi r^2$</td>
</tr>
<tr>
<td>$D_{IO}$ (g/cm$^3$)</td>
<td>Density of iron oxide</td>
<td>Bulk value</td>
</tr>
<tr>
<td>$M_{IONP}$ (g)</td>
<td>Mass of an individual IONP</td>
<td>$V_{IONP} \times D_{IO}$</td>
</tr>
<tr>
<td>$M_{Sil(NH_2)}$ (g/mL)</td>
<td>Mass of aminosilane coating</td>
<td>$M_{IONP-Sil(NH_2)} - M_{IO}$</td>
</tr>
<tr>
<td>$N_{\text{IONP-Sil(NH}_2)}$ ($#/\text{mL}$)</td>
<td>Number of IONP-Sil(NH$_2$)</td>
<td>$M_{\text{IONP-Sil(NH}<em>2)}$ ($\frac{M</em>{\text{IONP}} + M_{\text{Sil(NH}_2)}}{}$)</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>$M'_{\text{Sil(NH}_2)}$ (g)</td>
<td>Temperature dependent weight loss in $M_{\text{IONP-Sil(NH}_2}}$</td>
<td>TGA</td>
</tr>
<tr>
<td>$M'_{\text{Sil-PEG(COOH)}}$ (g)</td>
<td>Temperature dependent weight loss in $M_{\text{IONP-Sil-PEG(COOH)}}$</td>
<td>TGA</td>
</tr>
<tr>
<td>$M_{\text{PEG(COOH)}}$ (g/mL)</td>
<td>Mass of PEG(COOH)</td>
<td>$M'<em>{\text{Sil-PEG(COOH)}} + M'</em>{\text{Sil(NH}_2)}$</td>
</tr>
</tbody>
</table>

**Cell culture**

All reagents for cell culture were purchased from Invitrogen, and other reagents were obtained from Sigma Aldrich, unless otherwise specified. The IONP stock solutions were filtered through a 0.2 micron filter prior to cell studies.

A human hepatocellular carcinoma epithelial cell line (HepG2; American type tissue culture collection) was used as a cell culture model of liver. The HepG2 cells (passage number 10-30) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, HyClone), 50 U/mL penicillin and streptomycin (MP Biomedicals) at 37 °C and 5% CO$_2$. Cells were expanded in T-75 tissue culture flasks, and then passed and seeded at $2 \times 10^5$ cells per well on 12 well and 6 well plates for cell viability and uptake studies, respectively. Culture medium was changed every two days. All experiments were performed on confluent monolayers at typically day 4 or 5, post seeding.

**Cell viability studies**
Cells grown to confluence in 12 well plates (Costar) were treated with various concentrations of IONPs and incubated for 24 hrs at 37 °C. To each well, 200 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT reagent), 5 mg/mL in Phosphate-Buffered Saline (PBS) was added and incubated for 3hrs at 37 °C. Then the PBS was removed and the purple colored formazan crystals were dissolved by adding 1 mL of dimethylsulfoxide and the absorbance (A) at 567 nm was measured using a plate reader (Synergy HT, BioTek). The percentage cell viability of cells treated with various nanoparticle concentrations compared to control cells that just received DMEM was calculated by (A_sample/A_control) × 100.

Cell uptake studies

To the HepG2 cells grown on 6-well culture plates (Costar, Lowell, MA), DMEM containing various concentrations of IONP (5, 10 and 20 μg/mL of Fe) were added. After IONPs addition, cells were placed in a humidified CO₂ incubator maintained at 37 °C or 4 °C. After 5 hours, the IONP solutions were removed and the cell monolayers were washed three times with ice cold PBS to remove the unbound nanoparticles. Cells were lysed by the addition of 500 μL of 0.2 N NaOH and iron content determined based on the ferrozine assay. Cellular accumulation was examined both in the presence and absence of a static magnetic field. For studies involving a magnetic field, the cells were placed over a platform containing cylindrical rare earth magnets. Under these conditions, the cell monolayer was positioned approximately 1 mm above the magnets. Cells remained in the magnetic field for the duration of the experiment. Samples from the cell lysates were normalized for protein content using BCA protein assay kit (Pierce).
**Ferrozine assay**

To 500 µL stock solution or cell lysate containing IONP, 500 µL of concentrated HCl (≈ 12 M) was added and the mixture was incubated for 1 hour at room temperature with gentle shaking. Later, 500 µL of 12 M NaOH was added to the solution. To the neutralized solution, 120 µL of hydroxylamine hydrochloride (2.8 M) in 4 M HCl was added and the samples were incubated for another 1 hour at room temperature with gentle shaking. Following this, 50 µL of 10 M ammonium acetate solution (pH 9.5) and 300 µL of 10mM ferrozine reagent [3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine- p,p'-disulfonic acid monosodium salt] in 0.1M ammonium acetate solution was added to each sample. Absorbance was measured at 562 nm using a Synergy HT plate reader. Iron concentrations were determined from standard curve prepared using iron standard (1000 ppm Iron AA standard from Fisher Scientific).

**Electron microscopy of HepG2 cells**

HepG2 cells were incubated with IONPs in DMEM (50 µg/mL) for 2 hours. After incubation, cells were washed three times with PBS and collected using 0.25% trypsin EDTA (Hyclone). After centrifugation, the cell pellets where fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.3), followed by post-fixation in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.3). Cells were then dehydrated and embedded in Epon 812 using standard techniques. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed in a Philips CM 10 electron microscope. In order to eliminate observer bias, sections were examined without foreknowledge of their source.

**Mass spectrometry**
1 mL of IONP-Sil-PEG-BSA in DI water was transferred to a dialysis tubing (standard cellulose acetate tubing, 12-14 K MWCO). Dialysis was carried out overnight at 8 °C against 1 L of 50 mM NH₄HCO₃ with gentle stirring. The sample was removed from the dialysis tubing, and then added to 10 µL of TPCK-treated trypsin (1 mg/mL in 50 mM NH₄HCO₃) and incubated for 5 hours at 37 °C. 200 µL of the sample was taken in a Centricon10 ultrafiltration unit, and the rest into an Amicon Ultra-free 50 ultrafiltration unit. These were centrifuged for 30 minutes at 5000 rpm. 1 µL each of filtrate and DHB matrix was taken onto a MALDI target. Data were analysed by submitting the peak list to MASCOT (www.matrixscience.com), with 30 ppm and Mammalia taxonomy. Three ions were chosen for tandem mass spectrometry on the same instrument, in order to confirm the identity which was analyzed manually.

**SiRNA binding**

SiRNA (Life Technologies, Burlington, ON) in PBS (500 ng/mL) was incubated with IONP-Sil(NH₂) at various weight ratio of Fe (5, 10, 15, 20, 25, 30 and 40) for 15 minutes. The resultant polyelectrolyte were loaded on 4% agarose gel and examined by gel electrophoresis at 100 V for 30 min. After staining with ethidium bromide, the gel was imaged under UV. Images were acquired on a Gel Logic 100 Imaging System (Kodak, Rochester, NY).
Figure B4.1. High-resolution (A) Fe 2p$_{3/2}$ and Fe 2p$_{1/2}$, (B) Si 2p, (C) O 1s, (D) C 1s and (E) N 1s.
Figure B4.2. (A) TEM and (B) HR-TEM micrographs of IONP-$Si/(NH_2)$.
Figure B4.3. (A) TEM and (B) HR-TEM micrographs of IONP-Si/PEG(COOH).
References

Chapter 5:
A General Synthesis of Metal (Mn, Fe, Co, Ni, Cu, Zn) Oxide and Silica Nanoparticles Based on a Low Temperature Reduction/Hydrolysis Pathway
A General Synthesis of Metal (Mn, Fe, Co, Ni, Cu, Zn) Oxide and Silica Nanoparticles Based on a Low Temperature Reduction/Hydrolysis Pathway

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Authors’ Contributions

The idea of extending the reduction-hydrolysis synthesis of magnetite nanoparticles given in chapter 3 to prepare other early transition metal oxide and silica nanoparticles was conceptualized by Vinith Yathindranath (VY). All experiments related to syntheses and characterization was designed and carried out by VY, exceptions follow. Matthew Worden carried out the synthesis and characterization of manganese oxide and copper/copper oxide nanoparticles and provided the related data. Zhizhi Sun carried out the in vitro toxicity studies on manganese oxide NPs and fluorescence microscopy studies on FITC-silica particles and provided the related data. The manuscript with all figures and tables was prepared by VY. VY, Dr. Torsten Hegmann, Dr. Donald W. Miller, Mathew Worden and Zhizhi Sun were responsible for final revisions, prior to its publication in RSC Advances.
Abstract

A novel general synthesis of nanocrystalline manganese, cobalt, nickel, copper and zinc oxide as well as silica, in situ fluorescent dye-containing silica, and silica@iron oxide core-shell nanoparticles (NPs) is presented following a reduction-hydrolysis-precipitation pathway. As-synthesized manganese, copper and zinc oxide NPs were crystalline, cobalt and nickel oxide NPs were initially amorphous and required calcination. This general synthetic method was extended to larger silica NPs and silica NPs surface-modified with magnetic iron oxide NPs. All particles were characterized using powder X-ray diffraction, transmission electron microscopy, high-resolution transmission electron microscopy, and selected area electron diffraction. The toxicity of the as-synthesized Mn$_3$O$_4$ NPs, which can easily be surface-modified with an aminosilane (hydrophilic) or oleic acid (hydrophobic), was assessed in vitro using three cell culture models.
5.1 Introduction

Transition metal oxide nanoparticles (NPs) have garnered considerable interest in biomedical,1−3 energy storage,4−8 catalysis,9 sensor10−12 and water treatment13−14 fields. The unique, nanosize-dependent magnetic, optical, electrical, and chemical properties are crucial for many of these applications. Identifying the best combination of physical and chemical parameters for specific application is a critical and on-going research process.7−8, 11, 15 The control over these parameters in metal oxide NPs for many applications is achieved by modifying traditional synthetic procedures (following precipitation or high temperature decomposition pathways) such as varying the temperature, ratio of reactants, used surfactants, pH, or solvent.16−20 Developing novel general synthesis routes covering a wide range of metal/metal oxide phases that can be adapted for custom size and surface modifications are important. A general or universal synthesis for a range of metal oxides is also advantageous from an industrial perspective as a single set-up/facility may produce several metal or metal oxide NPs.

Among the earliest efforts in developing a generalized approach, N. R. Jana et al. synthesized Cr$_2$O$_3$, MnO, Fe$_3$O$_4$, Co$_3$O$_4$, NiO and ZnO nanocrystallites following a high temperature decomposition method.21 Metal fatty acid precursors were obtained by treating metal chlorides with stearic acid (for Cr), oleic acid (for Mn, Fe and Co oxides) and myristic acid (for Ni) under basic conditions. Heating of metal fatty acids in the presence of the corresponding free fatty acids at 300 °C to 380 °C in octadecene yielded fairly monodisperse metal oxide NPs. Size control was achieved by varying the concentration of the free fatty acid in the reaction mixture. R. V. Kumar et al. reported a general syn-
thesis of Fe$_3$O$_4$, Co$_3$O$_4$, CuO and ZnO NPs via sonochemical oxidation and hydrolysis of corresponding metal(II) acetate precursors. The metal(II) acetates in water or water/DMF mixtures were agitated using a high intensity ultrasonic horn to yield metal oxide NPs with sizes in the tens and hundreds of nanometers.\textsuperscript{22} Another general synthesis of Fe$_2$O$_3$, NiO, Co$_3$O$_4$, CeO$_2$, MgO and CuO hollow spheres composed of NPs was proposed by M. M. Titrici et al.\textsuperscript{23} In this synthesis, metal precursors such as [(NH$_3$)$_2$Fe(SO$_4$)$_2$·6H$_2$O], [(NH$_3$)$_2$Ni(SO$_4$)$_2$·6H$_2$O], Ce(NO$_3$)$_3$·6H$_2$O, CoSO$_4$·7H$_2$O, MgCl·6H$_2$O and CuSO$_4$·5H$_2$O along with glucose in water were heated to 180 $^\circ$C in a Teflon®-lined autoclave for 24 hours followed by calcinations at 550 $^\circ$C to yield the corresponding metal oxide hollow spheres. A synthesis of Mn$_3$O$_4$, CoO, Co$_3$O$_4$, NiO, ZnO, and CeO$_2$ NPs with different shapes and morphologies was also achieved by high temperature decomposition (200 $^\circ$C) of metal nitrates in octadecylamine.\textsuperscript{24}

In an earlier report, we described a novel method for preparing magnetite NPs (Fe$_3$O$_4$-NPs) by reduction-hydrolysis of iron(III) acetylacetonate using aqueous NaBH$_4$.\textsuperscript{25} In comparison to some other magnetite NP syntheses, this reduction-hydrolysis method requires mild reaction conditions, non-toxic reagents, no heating, and a simple setup to prepare highly crystalline, pure phase Fe$_3$O$_4$-NPs with a narrow size distributions. This method is highly versatile, allowing size selection by varying the NaBH$_4$ concentration and in situ surface modification with hydrophilic or hydrophobic capping reagents. Here, we have extended this reduction-hydrolysis method to a reliable general synthesis method for other metal oxide and silica NPs. We successfully prepared manganese oxide (Mn$_3$O$_4$-NP), cobalt oxide (Co$_3$O$_4$-NP), nickel-nickel oxide (Ni/NiO-NP), copper-copper
oxide (Cu/Cu$_2$O-NP), and zinc oxide (ZnO-NP) NPs (Table 5.1). Mn$_3$O$_4$-NPs, attractive materials for potential therapeutic and diagnostic uses,$^{1,26−27}$ were chosen to demonstrate the in situ surface modification with desirable molecular functionalities. Specifically, we prepared a hydrophilic aminosilane-coated manganese oxide NPs (Mn$_3$O$_4$-NP-AmS) with surface amino-groups for attaching targeting ligands, therapeutic or diagnostic agents. To mimic the attachment of a small hydrophobic drug molecule, oleic acid (OA)-modified Mn$_3$O$_4$-NP-OA was also prepared. Silica NPs (SiO$_2$-NPs) are an important nanoscale platform for biomedical and nanobiotechnology applications and are usually prepared by the Stöber method.$^{28}$ Hence, we also tested our general approach to prepare SiO$_2$-NPs, which is to the best of our knowledge the first time that silica NPs were prepared in such a way. Following this strategy, we also prepared SiO$_2$-NPs with a magnetite NP corona (useful for magnetic separation applications, SiO$_2$@Fe$_3$O$_4$-NPs) and in situ fluorescent dye-containing SiO$_2$-NPs (demonstrating potential diagnostic and carrier applications).

The NPs were characterized by powder X-ray diffraction (XRD), transmission electron microscopy (TEM), high-resolution TEM (HR-TEM), selected area electron diffraction (SAED), and FT-IR. The biocompatibility of the as-synthesized Mn$_3$O$_4$-NPs was assessed in vitro on cell-culture models for intestinal, brain and liver cells.

**Table 5.1.** Reagents and reaction parameters used for the metal oxide and silica NP synthesis.

<table>
<thead>
<tr>
<th>NP abbreviation</th>
<th>Precursor</th>
<th>Ratio precursor/NaBH$_4$</th>
<th>Reaction $T/˚C$</th>
<th>Calcination $T/˚C$ (duration)</th>
<th>Phase(s) by powder XRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn$_3$O$_4$-NP</td>
<td>Mn(acac)$_3$</td>
<td>1:1</td>
<td>78</td>
<td>–</td>
<td>Mn$_3$O$_4$</td>
</tr>
<tr>
<td>Co$_3$O$_4$-NP</td>
<td>Co(acac)$_3$</td>
<td>1:10</td>
<td>78</td>
<td>500 (3 h)</td>
<td>Co$_3$O$_4$</td>
</tr>
</tbody>
</table>
5.2 Experimental

Manganese(III) acetylacetonate, cobalt(III) acetylacetonate, nickel(II) acetylacetonate, copper(II) acetylacetonate and zinc(II) acetylacetonate, were purchased from Fluka, and used without purification. Sodium borohydride (NaBH₄), iron(III) acetylacetonate, oleic acid (OA), (3-aminopropyl)triethoxysilane (APTES), tetraethylorthosilicate (TEOS), and Flourescein isothiocyanate (FITC) were purchased from Sigma-Aldrich and used as such. Millipore deionized (DI) water (R = 18.2 MΩ) was used throughout the experiments.

Powder X-ray diffraction (XRD) measurements were carried out on a PANalytical X’Pert Pro Bragg-Brentano powder X-ray diffractometer equipped with a Cu Kα₁,₂ (λ = 1.540598, 1.544426 Å) source, diffracted beam Ni-filter and an X’Celerator detector. FT-IR spectra for all samples were recorded on a Bruker TENSOR 27, using KBr pellets. Transmission electron microscopy (TEM) imaging was carried out on a JEOL 2010F STEM and a Hitachi H 7000 transmission electron microscope. High-resolution TEM and selected area electron diffraction (SAED) were obtained on JEOL 2010F STEM. TEM samples were prepared by drop-casting dispersions of the NPS in methanol onto a carbon coated copper grids (400 Mesh), which were dried under ambient conditions overnight.

<table>
<thead>
<tr>
<th>NP</th>
<th>Molecule</th>
<th>Ratio</th>
<th>Temperature</th>
<th>Time (h)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni/NiO-NP</td>
<td>Ni(acac)₂</td>
<td>1:10</td>
<td>RT*</td>
<td>78</td>
<td>Ni and NiO</td>
</tr>
<tr>
<td>Cu/Cu₂O-NP</td>
<td>Cu(acac)₂</td>
<td>1:10</td>
<td>RT*</td>
<td>–</td>
<td>Cu and Cu₂O</td>
</tr>
<tr>
<td>ZnO-NP</td>
<td>Zn(acac)₂</td>
<td>1:10</td>
<td>RT*</td>
<td>–</td>
<td>ZnO</td>
</tr>
<tr>
<td>SiO₂-NP</td>
<td>TEOS®</td>
<td>1:10</td>
<td>RT*</td>
<td>–</td>
<td>SiO₂</td>
</tr>
<tr>
<td>SiO₂@Fe₃O₄-NP</td>
<td>Fe(acac)₃</td>
<td>1:10</td>
<td>RT*</td>
<td>–</td>
<td>SiO₂-Fe₃O₄</td>
</tr>
</tbody>
</table>

* RT = room temperature, ** TEOS = tetraethyl orthosilicate.
prior to TEM imaging. Fluorescence microscopy was carried out on an Olympus IX51 inverted biological microscope.

*Synthesis of Mn$_3$O$_4$-NP, Mn$_3$O$_4$-NP-AmS and Mn$_3$O$_4$-NP-OA:* 704.0 mg of Mn(acac)$_3$ (2 mmol) was dissolved in 50/25 (v/v mL) water/ethanol mixture and purged with nitrogen for one hour. The solution was heated to reflux under the nitrogen atmosphere and mechanical stirring (1000 RPM). To this, 75.7 mg of NaBH$_4$ (2 mmol) in 25 mL of deoxygenated water was added under a steady flow of nitrogen and continued refluxing. Upon addition of NaBH$_4$, the reaction mixture turned from dark brown to clear yellow, and over a period of one hour slowly turned to brown. After one hour the reaction mixture was cooled down to room temperature and the brown precipitate was collected by centrifugation and washed several times with water, ethanol, and then dried under ambient conditions. The uncoated Mn$_3$O$_4$-NP was a black powder.

To prepare Mn$_3$O$_4$-NP-AmS, the reaction mixture was cooled down to room temperature and 0.5 mL of APTES (2 mmol) was added rapidly to the reaction flask. The contents were stirred for 20 hours at room temperature. The crude Mn$_3$O$_4$-NP-AmS was washed several times with water to remove any free APTES, and then re-dispersed in water. To prepare Mn$_3$O$_4$-NP-OA, 2.0 mL of OA (6 mmol) was added to the reaction flask and the contents were stirred for three hours at room temperature. The resulting precipitate was washed with water and ethanol to remove free OA, and re-dispersed in hexane as a stable brown dispersion.

*Synthesis of Co$_3$O$_4$-NP and Ni/NiO-NP:* 712.5 mg of Co(acac)$_3$ (2 mmol) (for Co$_3$O$_4$-NP synthesis) or 513.8 mg of Ni(acac)$_2$ (2 mmol) (for Ni/NiO-NP synthesis) in 40/50 (v/v, mL) water/ethanol mixture was purged with nitrogen. The precursor solution was gradu-
ally heated to reflux temperature (78 °C) and 756.6 mg of NaBH₄ (20 mmol) in 10 mL deoxygenated water was added rapidly under stirring (1,000 RPM) and steady flow of nitrogen. In the case of Co₃O₄-NP_precursor synthesis (precursor = prior to calcination), the color of the reaction mixture rapidly turned from green to pink and finally to black. There is considerable amount of hydrogen evolution during the reaction and a large reaction flask was used to contain the effervescence. In the case of Ni/NiO-NP_precursor synthesis, the color of the reaction mixture turned rapidly to black. One hour after the NaBH₄ addition, the reaction mixture was cooled and the precipitate washed several times with water and ethanol, and then dried under ambient conditions. The as-prepared Co₃O₄-NP_precursor and Ni/NiO-NP_precursor were calcinated at 500 °C for three hours to yield black colored Co₃O₄-NPs and dark gray colored Ni/NiO-NPs.

*Synthesis of Cu/Cu₂O-NP:* 130.0 mg of Cu(acac)₂ (0.5 mmol) in 60 mL of ethanol was heated slightly to dissolve the precursor and the solution was purged with nitrogen. To this, 190.0 mg of NaBH₄ (5 mmol) in 25 mL deoxygenated water was added at room temperature and stirred (1,000 rpm). Upon addition of the NaBH₄, the reaction mixture turned from blue to yellow, to brown, and finally to black. 10 minutes after NaBH₄ addition, the formed particles were washed with water and ethanol, and then dried under ambient conditions. The final product was a fine black powder.

*Synthesis of ZnO-NP:* 527.2 mg of Zn(acac)₂ (2 mmol) in 60/30 (v/v, mL) water/ethanol mixture was purged with nitrogen for 1 hour. To this, 756.6 mg of NaBH₄ (20 mmol) in 10 mL deoxygenated water was added rapidly under mechanical stirring (1,000 RPM) and nitrogen atmosphere. After one hour, the white precipitate was washed with water
and followed by ethanol, and then dried under ambient conditions.

*Synthesis of SiO₂-NP, SiO₂-NP-FITC, SiO₂@Fe₃O₄-NP: 416.7 mg of TEOS (2 mmol) was dissolved in 40/60 (v/v mL) water/ethanol. To this, 756.6 mg of NaBH₄ (20 mmol) in 10 mL water was added rapidly under magnetic stirring and ambient conditions. The reaction mixture was stirred for an additional three hours during which the reaction mixture turned from clear to cloudy white. The white precipitate was washed with water and ethanol and dried under ambient conditions to yield SiO₂-NPs. To prepare silica-NP-FITC, 4.0 mg of Fluorescein isothiocyanate (FITC) (10 µmol) was added to the initial TEOS solution; otherwise the procedure was identical to the SiO₂-NP synthesis.*

To prepare SiO₂@Fe₃O₄-NP, 60.0 mg of as-synthesized SiO₂-NP was dispersed 10 mL (50:50) water/ethanol mixture containing 35.3 mg (0.1 mmol) of Fe(acac)₃ and purged with nitrogen for one hour. Thereafter, 37.8 mg (1 mmol) of NaBH₄ in 1 mL deoxygenated water was added rapidly under mechanical stirring (1,000 RPM) and nitrogen atmosphere. Over a one-hour period, the color of the reaction mixture gradually turned from red to gray. The crude product was washed several times with water and ethanol, and then dried under ambient conditions to yield SiO₂@Fe₃O₄-NP as a fine gray powder.
5.3 Results and discussion

The NPs synthesis described here follows in principle a precipitation method. The formation of the thermodynamically stable NPs from their aqueous precursor solutions involves reactions leading to supersaturation conditions, nucleation and growth. The choice of precursor has an influence on the phase, shape and morphology of the formed NP; this is especially true when additional stabilizing molecules are avoided. In our earli-
er studies, the reduction-hydrolysis of Fe(acac)$_3$ yielded monodisperse, quasi-spherical magnetite NPs (Fe$_3$O$_4$-NPs). When FeCl$_3$ was used as a precursor, aggregated sheets comprising of cubic iron/iron oxide phase were obtained. Metal acetylacetonates are inexpensive, often non-toxic, and commercially available for a wide range of transition metals; key reasons for the choice as metal precursors in this general synthesis study. Metal acetylacetonates are less susceptible to hydrolysis and reduction in comparison to metal halides or acetates. This will affect the timeframe of these key processes, and thus imparts unique chemical and morphological features to the as-synthesized NPs.

Figure 5.2. (A) TEM and (B) HR-TEM images of Mn$_3$O$_4$-NP; (C) TEM and (D) HR-TEM images of Co$_3$O$_4$-NP; (E) TEM and (F) HR-TEM images of Ni/NiONP; (G) TEM and (H) HR-TEM images of Cu/Cu$_2$O-NP; (I) TEM and (J) HR-TEM images of ZnO-NP. SAED of (K) Mn$_3$O$_4$-NP and (L) Co$_3$O$_4$-NP.
To identify the metal oxide phases, the NPs were characterized using powder X-ray diffraction (XRD) (Figure 5.1). The XRD pattern of Mn$_3$O$_4$-NPs (Figure 5.1A) was consistent with a tetragonal Mn$_3$O$_4$ (ICDD powder X-ray diffraction reference code 00-001-1127, space group: I41/amd) phase and the unit cell parameters determined by Rietveld refinement (Figure C5.1A in Appendix C) were $a = b = 5.76686(5)$ and $c = 9.44804(8)$ Å. The as-synthesized Co$_3$O$_4$-NP$_{\text{precursor}}$ did not show any characteristic peaks in XRD indicating its amorphous nature (Figure C5.2A in Appendix C). Calcination of Co$_3$O$_4$-NP$_{\text{precursor}}$ yielded crystalline Co$_3$O$_4$-NP with well-defined XRD peaks (Figure 5.1B). The XRD pattern of Co$_3$O$_4$-NP indicated the presence of a pure cubic Co$_3$O$_4$ (ICDD reference code 00-001-1152, space group: Fd3 m) phase, with unit cell parameter $a = 8.08263(6)$ Å. Ni/NiO-NP$_{\text{precursor}}$ displayed a very broad single peak at around 44.6° (2-theta), most probably from amorphous nickel phase (Figure C5.2B in Appendix C). The calcinated product (Ni/NiO-NP) showed the presence of two phases, a cubic NiO (ICDD reference code 00-047-1049, space group: Fm3 m) and a cubic Ni (ICDD reference code 00-004-0850, space group: Fm3 m) phase. The two low intensity $hkl = 111$ and 200 peaks at 44.5° and 51.8° (2 Theta) corresponding to Ni are indicated on the pattern (Figure 5.1C). The weight fractions of NiO and Ni in Ni/NiO-NP obtained from the two-phase Rietveld refinement were 96.88(3.19) and 3.12(1.20)% respectively. Calcination of the amorphous nickel (Figure C5.2B in Appendix C) in air facilitated the formation of crystalline Ni and oxidized NiO phase. The unit cell parameter, $a = 4.17655(3)$ Å, was in agreement with NiO values. The XRD pattern of Cu/Cu$_2$O-NP showed the presence of a cubic Cu$_2$O (ICDD reference code 00-005-0667, space group: Pn3 m) and Cu (ICDD reference code:
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00-003-1005, space group: Fm3 m) phase with unit cell parameter $a = 4.25734(8)$ Å. The weight fractions of Cu and Cu$_2$O were 42.94(1.90) and 57.06(1.63)%, respectively. XRD analysis of ZnO-NP indicated the presence of a hexagonal ZnO (ICDD reference code 01-079-0208, space group: P63mc) phase with unit cell parameters $a = b = 3.25053(7)$ and $c = 5.20831(8)$ Å.

Figure 5.3. TEM images of (A) and (B) SiO$_2$-NP and (C) and (D) SiO$_2$@Fe$_3$O$_4$-NP. (E) Phase contrast and (F) fluorescent micrograph of SiO$_2$-NP-FITC.

TEM images of Mn$_3$O$_4$-NP (Figure 5.2A and Figure C5.3A, C5.3B, and C5.3C in Appendix C) revealed quasi-spherical shaped NPs of around 12.5 nm in diameter. The crystallite size determined from the XRD peak broadening (due to small crystal size) using Scherrer equation was 31 nm. Co$_3$O$_4$-NP appeared as creased sheets spanning across few hundred nm (Figure 5.2C and Figure C5.4B and C5.4C in Appendix C). TEM images
taken at higher magnification displayed small, quasi-spherical crystals ($\approx 5$ nm) distributed across the sheet. The crystallite size determined from the XRD pattern was 8 nm and is in good agreement with the crystallite domain size from the TEM images. These crystalline domains were absent in the Co$_3$O$_4$-NP$_{\text{precursor}}$ (Figure C5.4A in Appendix C). The TEM images of Ni/NiO-NPs (Figure 5.2E and Figure C5.5B–C5.5D in Appendix C) displayed clusters of connected hollow quasi-spherical structures ($\approx 56$ nm). These hollow structures composed of several smaller crystallites of $\approx 5$ nm in diameter (Figure C5.5E in Appendix C). TEM micrograph of Cu/Cu$_2$O-NPs showed a network of several connected quasi-spherical structures (Figure 5.2G), which are made up of small crystallites ($\approx 5$ nm) (Figure C5.6A in Appendix C). ZnO-NP when observed using TEM displayed quasi-spherical shaped NPs of $\approx 5.5$ nm in diameter. HR-TEM of Mn$_3$O$_4$-NP (Figure 5.2B), Co$_3$O$_4$-NP (Figure 5.2D), Ni/NiO-NP (Figure 5.2F), Cu/Cu$_2$O-NP (Figure 5.2H), and ZnO-NP (Figure 5.2J) displayed clear lattice fringes. The characteristic $d$-spacing and corresponding Miller indices are recorded on the HR-TEM images. The SAED of Mn$_3$O$_4$-NP (Figure 5.2K), Co$_3$O$_4$-NP (Figure 5.2L), Ni/NiO-NP (Figure C5.5F in Appendix C), Cu/Cu$_2$O-NP (Figure C5.6B in Appendix C), and ZnO-NP (Figure C5.7B in Appendix C) gave characteristic diffraction patterns for Mn$_3$O$_4$, Co$_3$O$_4$, Ni/NiO, Cu/Cu$_2$O, and ZnO (labeled Miller indices) nanocrystals. Figure 5.3A and 5.3B are the TEM micrographs of as-synthesized SiO$_2$-NP. The SiO$_2$-NPs have a spherical shape and are comprised of individual and some apparently fused NPs. The sizes of SiO$_2$-NP measured from TEM ranged between 250 and 550 nm (Figure C5.8B in Appendix C).
Figure 5.4. Synthesis of (a) SiO$_2$-NP, (b) SiO$_2$-NP-FITC, and (a) SiO$_2$@Fe$_3$O$_4$-NP in H$_2$O/ethanol in the presence of NaBH$_4$. The dye, FITC, is incorporated in situ in (b) and leaches out when SiO$_2$-NP-FITC is suspended in PBS (phosphate buffered saline). The SiO$_2$-NPs prepared in (a) are used when the synthesis is repeated in the presence of Fe(acac)$_3$ as iron precursor in (c).

The TEM images of SiO$_2$@Fe$_3$O$_4$-NP (Figure 5.3C and 5.3D) showed the Fe$_3$O$_4$-NP supported on the SiO$_2$-NP surface similar to the sun’s corona. The XRD pattern of the SiO$_2$@Fe$_3$O$_4$-NPs was indexed to cubic magnetite (Figure C5.9 in Appendix C). Therefore, the larger silica NPs with a magnetite corona are magnetic, i.e. they are attracted to a rare earth magnet. Figure 5.3E and Figure 5.3F show the phase contrast and fluorescent micrographs from the same region of the SiO$_2$-NP-FITC, prepared in situ in the presence of FITC (Fluorescein isothiocyanate) as fluorophore. By comparing both images, one can see that the majority of the SiO$_2$-NP-FITC fluoresces, which confirms the successful encapsulation of FITC by the SiO$_2$-NP. Figure 5.4 shows the versatility of our synthetic approach to SiO$_2$-NPs. The same synthesis can in fact be used to prepare porous SiO$_2$-NPs.
silica NPs loaded with a dye (payload), SiO$_2$-NP-FITC, and decorated with small magnetite NPs (SiO$_2$@Fe$_3$O$_4$-NPs). The biocompatibility of Mn$_3$O$_4$-NPs was examined using a human liver epithelial cell line (HepG2), a mouse brain endothelial cell line (bEnd.3), and a human intestine epithelial cell line (Caco2). No toxicity was observed up to 320 µg/mL or less, suggesting a safe profile across different tissues (Figure 5.5). Successful surface coating of the Mn$_3$O$_4$-NPs with a hydrophilic aminosilane and hydrophobic oleic acid (Mn$_3$O$_4$-NP-AmS and Mn$_3$O$_4$-NP-OA), respectively, was confirmed by FT-IR spectroscopy (Figure 5.5). The two peaks at 638 and 532 cm$^{-1}$ correspond to Mn$_{tetrahedral}$(O$^-$)Mn$_{octahedral}$ stretching vibrations in Mn$_3$O$_4$. The FT-IR spectrum of Mn$_3$O$_4$-NP-AmS, in addition to characteristic Mn$_3$O$_4$ peaks, shows peaks corresponding to the Si–O–Si (1000-1100 cm$^{-1}$) stretching vibrations confirming the formation of the aminosilane coating. Mn$_3$O$_4$-NP-OA displayed two strong C–H stretching peaks at 2858 and 2929 cm$^{-1}$, and a carbonyl-stretching peak at 1558 cm$^{-1}$ from the oleic acid coating, along with characteristic Mn$_3$O$_4$ peaks. The surface coating on the particles is further demonstrated by their preference towards a polar or non-polar solvent. The hydrophilic Mn$_3$O$_4$-NP-AmS prefer water, whereas Mn$_3$O$_4$-NP-OA with the hydrophobic oleic acid coating prefer $n$-hexane when shaken vigorously in a sample vial containing both water and $n$-hexane (Figure 5.5). In both cases, the particle dispersions were stable for several weeks with minimal settling. Reduction-hydrolysis-precipitation pathways for metal oxide phases in NPs from aqueous or non-aqueous solutions often require the metal to be reduced by a reducing agent. In principle, the desired reduction of a metal ion by a reducing agent may be predicted by knowing the standard electrode potential ($E^\circ$) values of the
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half-reaction of the metal ion and the reducing agent. Aqueous NaBH$_4$ has a standard electrode potential of $-0.48$ V and should be able to reduce any metal ion with a more positive standard electrode potential ($E^\circ > -0.48$ V) value, at room temperature. Naturally, the stability of newly formed ions or metals under a given set of reaction conditions will affect the final outcome, but necessary measures may be taken to force a desired outcome. For example, in our synthesis, oxygen in the reaction medium interferes with the reduction process and hence all syntheses were carried out in an oxygen-free environment. NaBH$_4$ also elevates the pH of the reaction medium to 11, which is crucial for the hydrolysis and condensation reactions responsible for metal oxide formation. In the presence of aqueous NaBH$_4$, the metal acetylacetonates will undergo hydrolysis forming metal hydroxides [Mn(OH)$_3$, Fe(OH)$_3$, Co(OH)$_3$, Ni(OH)$_2$, Cu(OH)$_2$ and Zn(OH)$_2$]. To explain the formation of the metal and/or metal oxide phases in the as-synthesized metal oxide NPs we start with the $E^\circ$ values of the participating metal ions. The half reaction of Mn$^{3+}$ to Mn$^{2+}$ ($E^\circ = +1.51$ V) is highly favorable, however Mn$^{2+}$ to Mn ($E^\circ = -1.18$ V) is not. Hence, Mn(OH)$_2$ is formed, seen as the yellow colored intermediate during the reaction. When the suitable ratio of Mn(OH)$_2$ to Mn(OH)$_3$ of 1:2 is reached, a condensation reaction will form the Mn$_3$O$_4$ phase. The $E^\circ$ values for the half reaction of M$^{3+}$ to M$^{2+}$ for Fe$^{3+}$ and Co$^{3+}$ are $+0.77$ V and $+1.80$ V and for the reduction of M$^{2+}$ to M for Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ are $-0.41$ V, $-0.29$ V, $-0.23$ V, $+0.34$ V and $-0.76$ V, respectively.

During the Co$_3$O$_4$-NP synthesis, the conditions are favorable to reduce Co$^{3+}$ to Co$^{2+}$ [Co(OH)$_2$ caused the pink color change during synthesis] and Co$^{2+}$ to Co, but the condensation between Co$^{3+}$ and Co$^{2+}$ species prevailed, forming a Co$_3$O$_4$ phase. With Mn$^{3+}$ as
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oxidizing species, we were able to prepare Mn$_3$O$_4$-NPs even using a lower concentration of NaBH$_4$ (see Table 1), although the reaction results in the same product for the reaction with tenfold excess of NaBH$_4$ (Figure C5.10 in Appendix C). In the case of the Ni/NiO-NPs and Cu/Cu$_2$O-NPs, as per $E^\circ$ values of Ni$^{2+}$ and Cu$^{2+}$, metallic Ni or Cu did form along with their oxides. Cu$_2$O was formed, as the reduction of Cu$^{2+}$ to Cu$^{+}$ ($E^\circ = +0.16$) is feasible under the given reaction condition. Zn$^{2+}$ species, due to the unfavorable $E^\circ$ value, was not reduced and just underwent the hydrolysis and condensation reactions to form ZnO. Finally, the silica-NPs were formed by the hydrolysis of TEOS [Si(OC$_2$H$_5$)$_4$] to [Si(OH)$_4$], followed by an alkaline condensation to form SiO$_2$.

The formation of a hollow structure in Ni/NiO-NP is known to occur when metal NPs oxidize to form metal oxide NPs through the Kirkendall effect. When exposed to air, Ni NPs will form an outer oxide layer forming a Ni/NiO interface. During calcination, the high temperature favors diffusion of Ni atoms towards the surface and oxidizes, and generates vacancies inside. Atomic diffusion and vacancy clustering over time will lead to hollow nanostructures.
5.4 Conclusions

We have successfully developed a simple and fast general method for the synthesis of Mn$_3$O$_4$, Fe$_3$O$_4$, Co$_3$O$_4$, Ni/NiO, Cu/Cu$_2$O, and ZnO NPs. The as-synthesized manganese, iron, copper and zinc oxide NPs were crystalline right from the reaction, the cobalt and nickel oxide NPs were initially amorphous and required calcination to afford crystalline
NPs. The domain sizes (considering the precursors prior to calcination in the case of 
\( \text{Co}_3\text{O}_4\)-NP and Ni/NiO-NP) and diameters of all other crystalline metal oxide NPs are on 
average \( \approx 5 \) nm. In this work, NaBH\(_4\) was used to prepare the metal oxide phases based on 
its favorable standard electrode potential values and hydrolyzing capability. This general 
synthetic method was also extended to larger silica NPs, in situ fluorescent dye contain-
ing silica NPs, and silica NPs surface-modified with magnetic Fe\(_3\)O\(_4\)-NPs by simply re-
peating the synthesis in the presence of the as-prepared silica-NPs. This method is robust 
ough to accommodate additional organic molecules for in situ surface modifications as 
demonstrated for the Mn\(_3\)O\(_4\)-NPs. The versatility, flexibility, and mildness of this synthe-
sis, comparable to other, versatile non-aqueous metal oxide NP synthesis methods\(^{35}\) tol-
erates reasonable modifications required for preparing a wide range of custom metal ox-
ide NPs for specific applications in medical diagnostics, drug delivery as well as energy 
conversion and storage applications, among others.
Acknowledgement

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5.5 References


5.5 References


Appendix C

Supplementary Information for Chapter 5

Cell culture

All reagents for cell culture were purchased from Invitrogen, and other reagents were obtained from Sigma Aldrich, unless otherwise specified.

A human liver epithelial cell line (HepG2; American type tissue culture collection), a mouse brain endothelial cell line (bEnd.3; American type tissue culture collection); and a human intestine epithelial cell line (Caco2; American type tissue culture collection) were used as cell culture models for liver, brain and intestine, respectively. The HepG2 cells (passage number 10-30) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Hyclone), 50 U/mL penicillin and streptomycin (MP Biomedicals) at 37 °C and 5% CO₂. Cells were expanded in T-75 tissue culture flasks, and then passed and seeded at 2 × 10⁵ cells per well on 12 well and 6 well plates for cell viability and uptake studies, respectively. Culture medium was changed every two days. All experiments were performed on confluent monolayers typically at day 4 or 5, post seeding.
Cell viability studies

Cells grown to confluence in 12 well plates (Costar) were treated with various concentrations of Mn$_3$O$_4$-NPs and incubated for 24 hrs at 37 °C. To each well, 200 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent), 5 mg/mL in Phosphate-Buffered Saline (PBS) was added and incubated for 3hrs at 37 °C. Then the PBS was removed, and the purple colored formazan crystals were dissolved by adding 1 mL of dimethylsulfoxide. The absorbance (A) at 567 nm was measured using a plate reader (Synergy HT, BioTek). The percentage cell viability of cells treated with various NP concentrations compared to control cells that just received DMEM was calculated by $(A_{\text{sample}}/A_{\text{control}}) \times 100$. 

![Graph](image-url)
Figure C5.1. Rietveld plots for (A) Mn₃O₄-NP, (B) Co₃O₄-NP, (C) Ni/NiO-NP, (D) Cu/Cu₂O-NP, and (E) ZnO-NP. The dotted red line is the observed pattern and solid
black line is the obtained fit. The difference spectrum is shown in blue and the calculated Bragg positions are shown as vertical lines.

**Figure C5.2.** XRD pattern of (A) Co$_3$O$_4$-NP$_{\text{precursor}}$ and (B) Ni/NiO-NP$_{\text{precursor}}$. 
Figure C5.3. (A) and (B) TEM and (C) HR-TEM micrograph of Mn$_3$O$_4$-NP.
Figure C5.4. TEM micrographs of (A) Co$_3$O$_4$-NP$_{\text{precursor}}$ (inset: diffuse ring from SAED indicating amorphous nature), (B) and (C) Co$_3$O$_4$-NP from different regions of the grid; (D) HR-TEM of Co$_3$O$_4$-NP showing smaller crystalline domains (with lattice fringes) as a part of a larger sheet.
Figure C5.5. TEM micrographs of (A) Ni/NiO-NP$_{\text{precursor}}$, (B) and (C) Ni/NiO-NP from different regions of the grid. (D) HR-TEM and (F) SAED of Ni/NiO-NP.

Figure C5.6. (A) HR-TEM and (B) SAED of Cu/Cu$_2$O-NP.

Figure C5.7. (A) TEM and (B) SAED of ZnO-NP.
Figure C5.8. (A) TEM micrograph of SiO$_2$-NP and (B) Size distribution of SiO$_2$-NP measured from TEM images.

Figure C5.9. XRD pattern of SiO$_2$@Fe$_3$O$_4$-NP.
Figure C5.10. FT-IR spectra for Mn$_3$O$_4$-NPs. Top spectrum shows results from synthesis using a 1:1 ratio of NaBH$_4$:Mn(acac)$_3$, bottom spectrum shows a 10:1 ratio. Peaks at $\approx$420 cm$^{-1}$, $\approx$530 cm$^{-1}$ and $\approx$640 cm$^{-1}$ indicate the presence of pure Mn$_3$O$_4$ phase in both samples.
Chapter 6:

Highly Crystalline Iron/Iron Oxide Nanosheets via Lyotropic Liquid Crystal Templating
Highly Crystalline Iron/Iron Oxide Nanosheets via Lyotropic Liquid Crystal Templating

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Authors’ Contributions

The idea behind this chapter to extend reduction-hydrolysis synthesis (Chapter 3) and try to obtain shape control on NPs using liquid crystal template was conceptualized by Dr. Torsten Hegmann. Vinith Yathindranath (VY) designed and carried out all synthesis and characterization related experiments. Dr. V. Ganesh provided insights on water/Triton X–100 system and cyclic voltametry experiments. Dr. Makoto Inokuchi carried out the magnetization studies presented here. The manuscript with all figures was prepared by VY. Later VY, Dr. Torsten Hegmann, Dr. V. Ganesh and Mathew Worden were responsible for final revisions before its publication in “RSC Advances”.

Abstract

A simple, room temperature reduction-hydrolysis of FeCl$_3$ in H$_2$O/Triton X-100 serving as a lyotropic hexagonal columnar liquid crystal template is an effective and mild approach for preparing highly crystalline iron/iron oxide nanosheets.
6.1 Introduction

Coalescence of colloidal crystallites in a controlled fashion is a powerful tool for preparing nanoarchitectures of desired size, shape and morphology.\textsuperscript{1–5} The mesoscale assembly (by coalescence and crystallization) of colloidal crystals and hence the shape of the final nanocrystal can be controlled by introducing interfaces in the reaction medium.\textsuperscript{6} Colloidal crystals in water/oil or solvent/surfactant systems accumulate at the fluidic interfaces, thereby lowering the system’s interfacial energy, and move freely to form clusters and coalesce.\textsuperscript{7} The formation of nanocrystals as a result of mesoscale coalescence of tiny crystallites follows a non-classical mechanism.\textsuperscript{8,9} In principle, crystalline 2D nanosheets can be prepared by using a suitable soft template offering fluidic interfaces with favourable dimensions, with bulk lyotropic liquid crystal phases being ideal candidates.

Transition metal oxides with sheet-like morphologies are of considerable interest for electrochemical energy storage applications (as pseudocapacitors and electrode materials for rechargeable lithium ion batteries).\textsuperscript{10–12} Among transition metals, iron and iron oxides (Fe\textsubscript{3}O\textsubscript{4} and γ-Fe\textsubscript{2}O\textsubscript{3}) are advantageous as they are inexpensive, environmentally benign and abundant in the earth’s crust. Naturally there is growing interest on iron based materials for electrochemical energy storage.\textsuperscript{11,13–16} In addition, iron oxides are biocompatible and well established nanomaterials in bionanomedicine as MRI contrast agents and nanocarriers for therapeutic agents.\textsuperscript{17–21} The shape of a nanocarrier is known to influence its blood circulation half-life and biodistribution, hence providing an opportunity for passive targeting of diseased sites.\textsuperscript{20,21} Therefore, iron oxide (IO) with a sheet like morphology will also be of significant importance in biomedical research. Considering the sub-
stantial body of work on iron oxide nanoparticle (IONP) syntheses focusing on size and surface chemistry, comparatively few reports centre on simple shape control, especially for the formation of nanosheets.\textsuperscript{11,12,22–24} Herein, we report a simple template-based room temperature synthesis of highly crystalline 2D iron/iron oxide nanosheets (I-IONS\textsubscript{TX100}), prepared by the room temperature reduction-hydrolysis of FeCl\textsubscript{3} using sodium borohydride in a H\textsubscript{2}O/Triton X-100 (TX-100) soft liquid crystal template\textsuperscript{25–27} (Figure 6.1).

![Diagram of FeCl\textsubscript{3}-H\textsubscript{2}O/TX-100 reduction to I-IONS\textsubscript{TX100}]

**Figure 6.1.** Formation of highly crystalline 2D iron/iron oxide nanosheets (I-IONS\textsubscript{TX100}) in H\textsubscript{2}O/TX-100. Photograph of I-IONS\textsubscript{TX100} in a sample vial attracted to a rare earth magnet.

### 6.2 Experimental

Iron(III)chloride hexahydrate (FeCl\textsubscript{3}-6H\textsubscript{2}O, 98\%), sodium borohydride (NaBH\textsubscript{4}, 98\%), 4-(1,1,3,3-tetramethylbutyl)phenyl-poly(ethylene glycol) (Triton X-100, laboratory grade) were purchased from Sigma-Aldrich and used as such for synthesis. Nickel foil (0.006") was purchased from McMaster Carr and pre-treated for the electrode fabrication.
6.2 Experimental

Poly(vinylidene fluoride) (PVDF, MW 180,000) pellets and 1-methyl-2-pyrrolidinone (99%) were purchased from Sigma-Aldrich. Carbon black (Black Pearls 2000) was purchased from Cabot. Millipore deionized (DI) water (R = 18 MΩ) was used in the reaction and purification steps. Powder X-ray diffraction (XRD) measurements were carried out using a PANalytical X’Pert Pro Bragg-Brentano powder X-ray diffractometer equipped with a diffracted beam Ni-filter and an X’Celerator detector. Cu Kα1,2 radiation (λ = 1.540598, 1.544426 Å) were used as the X-ray sources. Transmission electron microscopy (TEM) was carried out on Jeol ultra-high resolution FEG-T/STEM instrument operating at an accelerating voltage of 200 kV. The nanosheets were dispersed in methanol and a droplet was placed onto a carbon coated copper grid (400 mesh) and air-dried prior to analysis. Small-angle X-ray scattering (SAXS) was measured in a Rigaku SAXS instrument system with Ni-filter and a multi-wire detector. Polarized Optical Microscopy (POM) studies were carried out in an Olympus BX51-P polarizing microscope coupled with a Linkam LS350 heating and cooling stage. Electrochemical studies were performed using CH Instrument electrochemical workstation (CHI 760C) in a three-electrode cell containing 1 M KOH as an electrolyte. I-IONS\textsubscript{TX100}, Ag/AgCl and a platinum wire were used as working, reference and counter electrodes respectively. The working electrode was electrochemically cycled between −1.15 and 0.1 V for several times before recording the cyclic voltammogram.

Synthesis of I-IONS\textsubscript{TX100}: Triton X-100 (48 g) in the reaction flask was degassed under high vacuum for two hours. Separately, FeCl\textsubscript{3}\cdot 6H\textsubscript{2}O (2 mmol) in 52 g of DI water was purged with nitrogen for one hour. FeCl\textsubscript{3} solution was added to Triton X-100 in the reac-
tion flask under steady flow of nitrogen and was maintained till the completion of the reaction. The contents were mixed using a mechanical stirrer at 100 RPM, until the mixture turned to a taffy-like consistency. At this stage, the contents were slowly warmed to 45 °C (until all solids disappeared) and cooled back to ambient temperature to form a clear yellow LC phase. NaBH₄ (20 mmol) in 10 ml deoxygenated DI water was added rapidly to the reaction vessel with occasional mechanical stirring. The yellow gel turned almost immediately to black color along with formation of excess froth that filled the reaction vessel. Although the reaction is complete after approximately two minutes, after an additional 30 minutes from adding NaBH₄, the black colored froth was collected by adding 200 ml DI water in portions leaving the gel part behind. The crude product from the froth was collected using a rare earth magnet and washed several times with DI water and followed by ethanol. The final product after washing was dried under air at room temperature to yield a black powder. The product isolated from the gel portion (I-IONS_TX100gel) after washing with copious amount of water followed by ethanol was a dark red powder.

Electrode fabrication: The working electrode with I-IONS_TX100 was fabricated on a nickel foil that was used as a current collector. Nickel foil were cut into 1 cm wide strips of a desired length and washed thoroughly with soap and water. The dried strips were dipped in dilute aqua regia (50%) for 1 minute and washed under copious amount of distilled water; dried and weighed. The active material was prepared by mixing I-IONS_TX00 or IIONS_water (70 wt.%), Carbon black (15 wt.%), PVDF (15 wt.%) in 1-methyl-2-pyrrolidinone (2 mg/ml) and sonicating the above mixture for a minute and resting it for 12 hours in a tightly sealed vial. A thin coating of the active material was uniformly applied on both sides of the pre-treated half of the nickel strips (2 cm²) and dried at room
temperature for 2 hours and under high vacuum for at least 12 hours before weighing. Later, a wire was attached to the nickel strip using copper tape and the exposed nickel strip was wrapped in Parafilm.

Figure 6.2. (A) XRD pattern of I-IONSTX100, (B) TEM image of I-IONSTX100, (C) HR-TEM image of I-IONSTX100 (D) HR-TEM image of a nanosheet edge, and (E) SAED pattern from the edge in D.
6.3 Results and discussion

TX-100 is a non-ionic amphiphile forming normal micelles in water (i.e. the hydrophobic hydrocarbon groups face inward and the hydrophilic oxyethylene chains outward at the interface with water). A binary system of H₂O/TX-100 (52/48 by weight) forms a lyotropic phase with a hexagonal array of cylindrical micelles surrounded by a continuous domain of water. The H₂O/TX-100 template is easy to prepare, only involves environmentally benign chemicals, and maintains a hexagonal phase at room temperature. The iron precursor, FeCl₃, when dissolved in this liquid crystal (LC) solvent will be exclusively localized in the continuous water domain. The FeCl₃–H₂O/TX-100 mixture when observed in a polarized optical microscope before and after the addition of aqueous NaBH₄ exhibited a spherulitic texture (Figure D6.1A and C in Appendix D), which indicates the presence and persistence of the hexagonal LC phase during the reaction (Figure D6.1D in Appendix D). The small angle X-ray scattering (SAXS) pattern (Figure D6.1B in Appendix D) obtained for FeCl₃–H₂O/TX-100 were in agreement with the theoretically expected values for a hexagonal phase of $q_1/q_1$, $q_2/q_1$ and $q_3/q_1$ of 1, $\sqrt{3}$, and 2, respectively. The layer spacing $d_{10}$ for the hexagonal phase calculated from $hk = 10$ peak was 5.3 nm and the lattice parameter ($a$) calculated from $d_{10}$ was 6.2 nm.
6.3 Results and discussion

Figure 6.3. (A) TEM images of I-IONS$_{\text{TX100}}$ from an area of interest obtained at -15°, 0°, 15° and 30° tilt-angles (by tilting the TEM sample holder).

Figure 6.4. M–H curves of I-IONS$_{\text{TX100}}$ at 10 and 300 K. The magnetization values at 25000 Oe were 29.2 emu/g (at 10 K) and 24.9 emu/g (at 300 K). The insets show a closer view of zero $H$ field region, to better visualize the coercivities at 10 K ($H_c = 40$ Oe) and 300 K ($H_c = 74$ Oe). The hysteresis at 10 and 300 K were distorted (wasp-waisted) which is usually observed when more than one magnetic phases with considerably different coercivities are present in a material.\textsuperscript{29}
6.3 Results and discussion

Figure 6.2A shows the powder XRD pattern of I-IONS\textsubscript{TX100} isolated from the froth that formed when NaBH\textsubscript{4} was introduced to FeCl\textsubscript{3}–H\textsubscript{2}O/TX-100 (Figure D6.2 in Appendix D). The pattern indicated it to be a multiphase system comprised of cubic iron (Fe, ICSD #64999), magnetite iron oxide (Fe\textsubscript{3}O\textsubscript{4}, ICSD #84611), and Wüstite (FeO, ICSD #82233). Elemental iron in the nanosize range is highly reactive and oxidizes when exposed to air. However, the iron phase in I-IONS\textsubscript{TX100} was stable for months under ambient conditions suggesting that the metallic iron phase was protected by the passive iron oxide phase. I-IONS\textsubscript{TX100} when analyzed by transmission electron microscopy (TEM) showed exclusively crystalline nanosheets that were either about 5 or 10 nm thick (matching with once or twice the layer spacing \textit{d}_{10} of the LC template), with some of them extending up to 200 nm across (Figure 6.2B and D6.3 in Appendix D).

High-resolution (HR)-TEM images of the I-IONS\textsubscript{TX100} showed clear lattice fringes indicating the crystalline nature of the nanosheets (Figure 6.2C). The \textit{hkl} = 111 and 220 planes of iron oxide phase with visible grain boundaries are shown. It can be seen that the 220 plane extended over a long range in the image with few out of focus regions possibly formed above the plane (Figure D6.4 in Appendix D). Figure 6.2D shows the HR-TEM image of the edge of a sheet with uniform 311 planes visible through the entire length of the imaged region. The selected area electron diffraction (SAED) pattern (Figure 6.2E) from the edge of one nanosheet displayed well-positioned spots indicating the single crystalline nature of the sheets. The thinnest section of the nanosheets measured from edges for I-IONS\textsubscript{TX100} was around 5 nm and were single-crystal in thickness. To gain a better understanding of the ‘rod-like’ structures in the TEM images of I-IONS\textsubscript{TX100}, we imaged an area of interest at different tilt-angles by tilting the sample holder (Figure 6.3).
From the tilt experiments we found that the nanosheet labelled ‘2’ appeared like a rod at $-15^\circ$ and $0^\circ$ tilt-angles. However at $15^\circ$ and $30^\circ$, tilt-angles revealed its true sheet-like nanomorphology. The magnetization ($M$) versus magnetic field ($H$) curves ($M$–$H$ curve) of I-IONS$_{TX100}$ at 10 and 300 K are shown in Figure 6.4. The $M$–$H$ hysteresis curves of I-IONS$_{TX100}$ displayed ferromagnetic characteristics. Powder XRD pattern of the nanosheets isolated from the gel (IONS$_{TX100}$gel in Figure D6.2D, in Appendix D) showed the presence of Fe$_3$O$_4$ and $\alpha$-FeO(OH) (Figure D6.5 in Appendix D). TEM and HR-TEM analysis of IONS$_{TX100}$gel (i.e. from the residue of the reaction not forming froth) revealed its crystalline nature and also confirmed long-range order of lattices across sheets measuring microns in length (Figure D6.6A–D in Appendix D).

**Figure 6.5.** Mechanism of formation of highly crystalline nanosheets in the froth phase of the lyotropic liquid crystal template. The difference in composition between froth and gel is due to the reductive atmosphere in the froth where H$_2$ gas is enclosed in the froth’s bubbles. In the gel phase, settled nanosheets are largely protected from the reductive atmosphere by a ‘blanket’ of the bulk lyotropic LC phase.
6.3 Results and discussion

We would now like to discuss a possible mechanistic pathway for the formation of I-IONS\textsubscript{TX100}. FeCl\textsubscript{3} in H\textsubscript{2}O/Triton X-100 will undergo a cascade of reduction-hydrolysis reactions in the presence of aqueous NaBH\textsubscript{4}, forming amorphous intermediates such as Fe(OH)\textsubscript{3}, FeO(OH) and Fe(OH)\textsubscript{2}. Magnetite (Fe\textsubscript{3}O\textsubscript{4}) can form as a result of nucleation reactions between Fe(OH)\textsubscript{2} and FeO(OH).\textsuperscript{30} In the case of I-IONS\textsubscript{TX100}, iron was formed as one of the phases along with Wüstite [Fe(II)O]. The standard electrode potential of NaBH\textsubscript{4} (E\textdegree = \(-0.48\) V) is unfavourable for the reduction of Fe(II) to Fe(0) (E\textdegree = \(-0.44\) V) and hence iron must have formed through an indirect pathway. One hypothesis suggests the formation of Fe(BH\textsubscript{4})\textsubscript{2} as an intermediate which readily decomposes to form Fe(0), and another suggests the formation of iron hydride species in a hydrogen rich environment leading to Fe(0).\textsuperscript{31} The presence of Fe(II)O in I-IONS\textsubscript{TX100} suggests a likely pathway involving Wüstite as an intermediate. In a less dynamic LC medium, (in comparison to an isotropic solvent), reduction of Fe(III) species to Fe(OH)\textsubscript{2} will compete with nucleation of Fe\textsubscript{3}O\textsubscript{4}, resulting in an increased Fe(OH)\textsubscript{2} concentration. Fe(OH)\textsubscript{2} in an alkaline and reducing environment will undergo a hydrolytic polymerization reaction (OH to \(-\text{O}–\) species) forming Fe(II)O.\textsuperscript{3} Fe(II)O at room temperature will readily undergo a disproportionation reaction, forming Fe(0) and Fe\textsubscript{3}O\textsubscript{4} accompanied by crystallization.\textsuperscript{32} As per the classical mechanism of crystal growth, single-crystal nanostructures are formed by burst nucleation and growth by replication of the unit-cell without structural changes to the bulk or surface. Grain boundaries displayed by I-IONS\textsubscript{TX100} in the nanosheets (Figure D6.4 in Appendix D) suggests the involvement of a non-classical pathway of aggregation-mediated crystal growth. The nanocrystallites formed in the aqueous domain are free to move between fluidic interfaces of hexagonal cylindrical micelles (with a given layer
6.3 Results and discussion

spacing) across a 2D space to form 2D clusters of mesocrystals. Thermally activated exchanges between adsorbed and incoming particles will facilitate the coalescing of these nano-crystallites resulting in a 2D sheet (see mechanism suggested in Figure 6.5). Lattices with long-range order can be formed through the Grain-Rotation-Induced grain coalescence mechanism.

![Image](image.png)

**Figure 6.6.** (Top) representative working electrode containing I-IONS\textsubscript{TX100} fabricated for electrochemical studies. (Bottom) Cyclic voltammogram recorded at 2 mVs\textsuperscript{-1} scan rate in an aqueous electrolyte (1M KOH) after twenty cycles.

Finally, we also carried out some preliminary electrochemical characterization of I-IONS\textsubscript{TX100}. The active material was prepared by mixing I-IONS\textsubscript{TX100} with carbon black to enhance conductivity and a binder poly(vinylidene fluoride) (PVDF), and was finally applied on a nickel foil (Figure 6.6, top). The cyclic voltammogram of I-IONS\textsubscript{TX100} (Figure 6.6, bottom) displayed a good correlation with redox peaks of iron, which in turn should have formed at the interface at around $E = -1.1$ V during electrochemical sweep cycles.
The anodic peak (A1) at \(-0.61\) V was from the adsorption of hydroxyl species forming Fe(OH)$_{ads}$. The peak at \(-0.44\) V corresponded to the formation of Fe(OH)$_2$ and the peak at \(-0.27\) V was from the formation of Fe(III) species \([\text{Fe(OH)}_3, \text{FeO(OH)}, \text{Fe}_2\text{O}_3 \text{ and Fe}_3\text{O}_4]\)\(^{35}\). The cathodic peaks C1 and C2 can be assigned to the formation of Fe(II) and Fe, respectively, along with evolution of hydrogen gas at lower potentials.

## 6.4 Conclusions

In summary, we have shown a simple and effective way of preparing iron/iron oxide 2D nanosheets in the presence of a water/Triton X-100 hexagonal lyotropic liquid crystal template. Further investigations of the electrochemical properties and biomedical use (for example as carrier for drugs across the blood brain barrier) are currently underway. This versatile and mild process of making iron/iron oxide nanosheets can easily be adapted for other compliant metal oxide systems, and will allow for straightforward adjustments of aspect ratio (and properties) by using other compatible lyotropic LC solvents.
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6.5 References


Appendix D

Supplementary Information for Chapter 6
Figure D6.1. (A) Polarized optical micrograph (crossed polarizers), (B) small angle X-ray scattering (azimuthally averaged intensity of the scattering vector $q$ in Å$^{-1}$ vs. intensity from 2D SAXS pattern) of FeCl$_3$-H$_2$O/ TX-100 (FeCl$_3$: 2 mmol, H$_2$O/ TX-100 52/48 wt. %) both at 20 °C. (C) Polarized optical micrograph (crossed polarizers) of the FeCl$_3$-H$_2$O/ TX-100 mixture after addition of aqueous NaBH$_4$ showing: (i) spherulitic texture
(red arrows indicate some typical textural defects) indicative of Col\textsubscript{h} phase and (ii) the formation of H\textsubscript{2} gas bubbles (indicating the ongoing reaction as in Figure D6.2B, green arrows). (D) Schematic phase diagram of Triton-X 100 in water. The L\textalpha{} phase is only formed at higher concentrations of the non-ionic surfactant TX-100 and at temperatures below 10 °C. Addition of aqueous NaBH\textsubscript{4} positions the reaction further left (lower TX-100 concentration) in the phase diagram. POM images confirm the existence of the normal Col\textsubscript{h} phase in the nanosheet formation step (formation of froth in which the nanosheets form via coalescence of nanocrystallites).

Figure D6.2. (A) FeCl\textsubscript{3} in the lyotropic Col\textsubscript{h} liquid crystal phase of water/TX-100 (52/48 w/w); (B) after addition of aqueous NaBH\textsubscript{4} hydrogen evolved forming froth; (C) I-IONS\textsubscript{TX100} dispersion in water from the froth; after being washed off the gel (D) The gel part of H\textsubscript{2}O/TX-100 after the reaction was black, but turned reddish brown on exposure to air (see powder XRD data in Figure D6.5 confirming the existence of other Fe(III) species).
**Figure D6.3.** TEM image of I-IONS_{TX100}.
Figure D6.4. HR-TEM image of I-IONSTx100 showing long-range order of 220-plane with few out of focus protrusions (enlarged view of Figure 6.2C)
**Figure D6.5.** Powder XRD pattern of IONS$_{TX100}$.gel.
**Figure D6.6.** (A) Representative TEM image of $\text{IONS}_{\text{TX100gel}}$ measuring microns across with visible tear in the sheet. (B and C) HR-TEM images of crystalline $\text{IONS}_{\text{TX100gel}}$ with visible lattice fringes and grain boundaries. (D) SAED pattern (from C) of $\text{IONS}_{\text{TX100gel}}$ with defined spots indicating its single-crystalline nature.

**References**

Chapter 7:

Summary and Future Outlook
7.1 Summary

This thesis presents several original contributions in the synthesis and surface modifications of superparamagnetic iron oxide nanoparticles (IONPs) with particular focus on biomedical applications. Superparamagnetic IONPs are of great interest as diagnostic (MRI contrast agent, multimodal imaging) and therapeutic (tumor hyperthermia, drug carrier) aid. The past decade has seen considerable amount of work focusing on the design and synthesis of IONPs of suitable size and surface functional groups for various therapeutic and diagnostic (theranostic–TN) applications. The primary design strategy for an IONP-TN meant for non-invasive intravascular administration is to incorporate moieties that enhance their blood circulation half-lives. A long circulating IONP-TN will have a favourable time window to localize at the affected tissue for effective diagnosis and therapeutic procedures. Prior knowledge on biocompatible polymers such as poly(ethylene glycol) (PEG), dextran, etc. and their well established non-immunogenic property led to their successful incorporation of such moieties as surface coating for IONPs.

In chapter 2, the scope of superparamagnetic IONPs for magnetically directed drug convection with the aid of a conventional Magnetic Resonance (MR) instrument was demonstrated. The PEG and poly(ethyl methacrylate) (PEMA) coated IONPs (prepared by coprecipitation method), and physisorbed model drug protein bovine serum albumin (BSA) showed some simultaneous convective transport in agarose gel, under the gradient field of a 7 T MRI. The loosely attached BSA underwent a burst-release and tangled BSA-polymer on IONPs was simultaneously transported. To have absolute control on
therapeutic/diagnostic (TN) agents during magnetic targeting and simultaneous MRI, they have to be covalently linked to the IONP core.

IONPs comprising of magnetite (Fe₃O₄)/maghemite (γ-Fe₂O₃) phases are typically synthesized following classical coprecipitation or thermal decomposition methods. These methods have some limitations. The as-synthesized IONPs from coprecipitation method usually have broad size distribution, and slight variation in iron precursors [Fe(II)/Fe(III)] ratio results in unwanted oxide phases. Broad size distribution and impurity phases will affect the magnetic properties (saturation magnetization and relaxation) that are crucial for its MRI contrast enhancement, and magnetic hyperthermia applications. High temperature thermal decomposition methods yield IONPs with narrow size distribution, but usually require toxic organic solvents/stabilizers, which render the as-synthesized IONPs hydrophobic. Further ligand exchange or addition procedures are required to improve their biocompatibility. In comparison to coprecipitation method, the thermal decomposition methods are relatively expensive and an environmental concern for scaled-up production. Chapter 3 presents a novel one-pot synthesis of pure magnetite NPs, which addresses some of the above mentioned shortcomings of the coprecipitation and thermal decomposition methods. The reduction-hydrolysis method involves the transformation of a single iron precursor [Fe(acac)₃] species into IONPs, in aqueous medium, in the presence of the reducing agent NaBH₄. The synthesis at room temperature yielded pure ≈5 nm magnetite NPs and the reaction carried out at an elevated temperature of 78 °C yielded larger ≈8 nm magnetite NPs. At elevated temperature, an increase in NaBH₄ concentration resulted in the reduction of magnetite NP sizes. Reaction temperature and/or reagent ratios provide a
simple size-control for the as-synthesized IONPs. This method requires mild, inexpensive and commercially available reagents, and the obtained IONPs were hydrophilic and biocompatible. Surface modification of magnetite NPs with suitable biocompatible polymers/molecules was accomplished in situ during synthesis. This method involving mild and inexpensive reagents can be easily scaled-up for bulk production.

Chapter 4 presents a simple strategy for covalently linking TNs to IONPs. Literature presents a plethora of strategies to conjugate TNs to IONP surface which sometimes is overwhelming. The motivation here was to develop a versatile IONP-TN precursor that is suitable for a variety of TN conjugations. The novel reduction-hydrolysis method (chapter 3) was adapted to synthesize aminosilane coated IONPs [IONP-Si\text{H}(\text{NH}_2)] with surface amino groups. PEG diacid was conjugated to IONP-Si\text{H}(\text{NH}_2) through simple EDC/NHS coupling to form IONP-Si\text{H}-PEG(COOH). Both IONP-Si\text{H}(\text{NH}_2) and IONP-Si\text{H}-PEG(COOH) formed stable colloidal dispersion in aqueous media and serve are versatile precursors for precise bioconjugations. Model drug protein BSA and small hydrophobic model drug oleylamine were conjugated to IONP-Si\text{H}-PEG(COOH) through simple amidation reaction to demonstrate its usefulness as a precursor. The IONP-Si\text{H}(\text{NH}_2) with positive surface charge readily attached to negatively charged siRNA through electrostatic attraction demonstrating potential magnetic separation applications.

The reduction-hydrolysis method (Chapter 3) was further adapted as a general method for the synthesis of Mn$_3$O$_4$, Co$_3$O$_4$, Ni/NiO, Cu/CuO, and ZnO NPs from respective M(acac)$_x$ (For M = Mn and Co, $x = 3$, and for M = Ni, Cu and Zn, $x = 2$) precursors in chapter 5. These metal oxide NPs exhibited different shapes including quasi-spherical (Mn$_3$O$_4$, ZnO), creased sheets (Co$_3$O$_4$) and connected hollow quasi-spherical structures.
Large silica NPs, fluorescent dye encapsulated silica NPs and silica NPs with surface immobilized IONPs were also synthesized following the reduction-hydrolysis method. In chapter 6, the reduction-hydrolysis method was successfully adapted to prepare well separated iron/iron oxide nanosheets from FeCl₃. The reaction in this case was carried out in the presence of a lyotropic liquid crystal template.

7.2 Future outlook

Superparamagnetic IONPs are already a well-established nanoplatform in biomedicine. Until now, IONPs with biocompatible polymer coating have been used in clinical medicine as MRI contrast agents for diagnosis and in future has a greater potential to be used in drug delivery for therapy.

The reduction-hydrolysis method has been used to prepare magnetite NPs and was successfully adapted to prepare a variety of other early transition MONPs and large silica NPs. The scope of reduction-hydrolysis method in preparing mixed MONPs has to be explored. Mixed MONPs of the general formula MFe₂O₃ (M = Mn²⁺, Co²⁺, Zn²⁺, etc) have been shown to allow fine tuning of the saturation magnetization of the material.¹ Such mixed MONPs, especially with Mn²⁺ have been shown to have enhanced magnetic properties (Saturation magnetization and magnetic anisotropy) compared to Fe₃O₄, for enhanced MR signal.² Though toxicity/reputation of some of the divalent ions that may leach in vivo is a concern, MFe₂O₃ of sizes <10 nm undergoing rapid renal clearance might be a promising option.
At the NP surface modification front, IONPs coated with functional silane (aminosilane) were demonstrated to be a versatile precursor for bioconjugations. The next natural step will be to move towards conjugating bioactive molecules. The reduction-hydrolysis method should offer the opportunity to prepare IONPs with a variety of other custom or commercial functional silane shells for a variety of bioconjugations. Over the past decade there have been considerable advancements in the field of NP-bioconjugates, but still the design and implementation of complex multifunctional IONP-TN capable of effective active targeting, multimodal imaging and triggered\(^3\)\(^-\)\(^^4\) (pH, temperature, photonic) drug delivery is in its infancy and there is plenty of scope for Chemists to advance in this area.

Recently RNA interference (RNAi) and gene silencing using synthetic small interfering RNA (siRNA) has emerged as a promising therapeutic option for diseases including cancer that rely on one or more specific gene functions. However, the biggest challenge with siRNA is its delivery into the target tissue as they have short blood half-life and low permeability in cell membrane. siRNA complexed with poly(ethylenimine) (PEI) have shown enhanced delivery and gene knockdown in tumor cell culture and mice models.\(^5\)\(^,\)\(^6\) It would be interesting to use the general IONP precursors to conjugate PEI and study its efficacy in intracellular siRNA delivery and gene knockdown. The selectivity of siRNA delivery towards specific cells, say tumor cells, can be improved by conjugating targeting ligands. High selectivity towards tumor cells and enhanced uptake have been shown in NPs attached to tumor homing peptide sequences.\(^7\)\(^,\)\(^8\)

IONP based drug delivery systems are of great interest in brain drug delivery. The brain microvascular endothelial cells (BMEC) present a formidable physical (Tight Junc-
7.2 Future outlook

In general, polar molecules that are not substrates of specific transport systems, and lipophilic molecules larger than 400 D do not cross the BBB. \[^{10}^2\] Unfortunately, almost 98% of the small and 100% of the large molecular weight drugs under development for central nervous system (CNS) disorders including brain tumors, Alzheimer’s disease, schizophrenia, depression, stroke and traumatic brain injury do not cross the BBB. \[^{9,10}\] IONP based drug delivery systems are promising candidates for delivering CNS therapeutics across the BBB at therapeutically significant concentrations. In the case of IONP-TNs, their permeation across the BBB can be enhanced with the aid of an applied magnetic field via magnetically directed drug convection (MDDC) and imaged simultaneously using MRI. \[^{11}\] Some preliminary studies on silane coated IONPs with CNS relevant culture models have been promising in identifying the strengths of different surface coatings in terms of cell uptake in CNS relevant cell culture models. \[^{12}\] It that line, it would be interesting to construct custom IONP-TN from presented general precursors and optimize its properties (hydrodynamic diameter, zeta potential) for MDDC across the BBB cell culture models. Such studies are crucial in identifying the best candidates for further animal studies and later clinical studies.
7.3 References


