Biogenic Magnetite Nanoparticles: Development and Optimization for Potential Applications

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Abstract

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Biogenic Magnetite Nanoparticles: Development and Optimization for Potential Applications

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The bioproduction of magnetite (Fe$_3$O$_4$) nanoparticles was demonstrated through the reduction of amorphous Fe(III)-oxyhydroxide starting materials by the dissimilatory iron reducing bacterium *Geobacter sulfurreducens* in an environmentally benign method. Magnetite nanoparticles have magnetic characteristics coupled with a high surface area to volume ratio and biogenically produced magnetite often has a highly reactive surface Fe(II) layer.

Through the work described in this thesis, the properties of magnetite nanoparticles were manipulated in several different ways. The control of particle size was achieved through the adjustment of the total amount of bacteria (biomass) introduced at the start of the Fe(III)-oxyhydroxide reduction process. High concentrations of bacteria led to the formation of small (∼10 nm) nanoparticles whereas low concentrations led to larger (∼50 nm) particle formation. Additional mineral phases were formed, with goethite and siderite observed for very low and very high bacterial concentrations respectively. The change in particle size and additional mineral phases formed were attributed to the rate and extent of Fe(II) formation, linked to changes in biomass loadings, with high biomass releasing high concentrations of Fe(II) and low biomass releasing low concentrations of Fe(II).

The control of magnetic properties was achieved by the incorporation of transition metal dopants including zinc and cobalt into the crystal structure of the magnetite, producing nanoparticles of the form $M_xFe_{3-x}O_4$, ($M=Zn$ or Co). The different dopants substitute into the crystal structure in different locations (as determined through X-ray absorption and Mössbauer spectroscopies). Zinc has a preference for replacing Fe(III) in tetrahedral coordination, resulting in a decrease in the antiferromagnetic component between octahedral and tetrahedral lattice sites, leading to an increase in saturation magnetization of the material (>50 %) compared to stoichiometric magnetite. Cobalt has a strong affinity to replace Fe(II) in octahedral coordination which results in an increase in the measured coercivity without significantly decreasing the saturation magnetization.

The biotechnological potential of biogenic magnetite was also investigated through an appraisal of Fe(II)-mediated chromate (Cr(VI)) remediation. Decreasing particle size (i.e. increasing surface area to volume ratio) led to an enhanced ability to reduce highly toxic Cr(VI) to non-toxic Cr(III). In separate experiments, cobalt doping in magnetite also significantly increased the effectiveness of the nanomaterial for use in magnetic hyperthermia treatments, which could ultimately be used for cancer therapy.

Finally, the scalability of biogenic magnetite production was shown. *Geobacter sulfurreducens* growth in batch culture and subsequent iron transformation stages were significantly increased in scale by factors of 500× and 1000× respectively. This could pave the way for future commercial production of biogenic magnetite for use in many different applications.
Declaration

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The author graduated from the University of St. Andrews, Scotland in 2008 with an undergraduate masters degree in Physics (MPhys). The author first joined the Geomicrobiology group, School of Earth Atmospheric and Environmental Sciences, the University of Manchester in 2005 for a summer internship project. In October 2008, the author returned to pursue a postgraduate degree and has been engaged in the research reported in this thesis since October 2008 under the supervision of Prof Jon Lloyd and Prof Richard Patrick.
Introduction and Thesis Content
1. Introduction and Thesis Content

1.1. Aims and Objectives

The overall aim of this project was to optimize and scale up the formation of magnetite based magnetic nanoparticles produced via the microbial reduction of iron(III)-oxyhydroxides by the subsurface bacteria *Geobacter sulfurreducens* and *Shewanella oneidensis*. This would be carried out with a view to develop and expand on previous work (outlined in Chapter 2) into biogenic magnetite formation and explore the potential uses of the material in different applications. Overall this project can be summarised into three distinct objectives including:

i) Development and optimization of biogenic magnetite based magnetic nanoparticles and investigating how to manipulate their properties including size and magnetization.

ii) Explore the potential uses of biogenic magnetite nanoparticles in different applications.

iii) Design a method through which the formation of magnetite nanoparticles could be scaled up to industrial volumes.

These overlapping aims have been explored in a series of experiments, written up as four distinct first authored research papers and one additional co-authored research paper in this thesis.

1.2. Thesis Layout

This thesis is presented in the alternative format style, with each research chapter presented as an independent body of work. Due to the nature of the alternative style, there is some amount of repetition in each of the chapters in particular in the introduction and methodology sections, however this is to enable the submission of
each chapter for publication in a scientific journal. Several chapters included in this thesis have already been accepted for publication and have been included in the format as presented in their respective journal.

1.3. Thesis Content, Status of Manuscripts and Author Contributions

Chapter 1 – “Introduction and Thesis Content”

J M Byrne.

Chapter 2 – “A Review of the Production of Biogenic Magnetite Nanoparticles and Potential Applications”

J M Byrne.

Chapter 3 – “Methodology”

J M Byrne.

Chapter 4 – “Control of nanoparticle size, reactivity and magnetic properties during the bioproduction of magnetite by Geobacter sulfurreducens”

J M Byrne, N D Telling, V S Coker, R A D Pattrick, G van der Laan, E Arenholz, F Tuna, J R Lloyd.

Status:
Published: Nanotechnology, IOP, 2011, 22, (45), 455709.

Author Contributions:

J M Byrne – Principal author, sample preparation, XAS and XMCD data collection and analysis, TEM analysis, SQUID analysis and chromate remediation experimentation; N D Telling – Manuscript review, assisted with XMCD and SQUID analysis and assisted chromate remediation experimentation; V S Coker – Manuscript review, assisted with XMCD beamtime and assisted chromate remediation experimentation; R A D Pattrick – Manuscript review and co-supervisor; G van der Laan – Provided calculated XMCD spectra used for analytical fitting; E Arenholz – Station scientist for beamline 4.0.2, Advanced Light
Chapter 5 – “Biosynthesis of Zinc Substituted Magnetite Nanoparticles with High Room Temperature Magnetization”

J M Byrne, N D Telling, V S Coker, P L Wincott, D J Vaughan, R A D Pattrick, G van der Laan, E Arenholz, F Tuna, J R Lloyd.

Status:
Manuscript submitted subject to review, Advanced Functional Materials, Wiley.

Author Contributions:
J M Byrne – Principal author, sample preparation, XAS and XMCD data collection and analysis and performed XRD, TEM, SQUID and Mössbauer analysis; N D Telling – Manuscript review and assisted with XMCD and SQUID analysis; V S Coker – Manuscript review and assisted with XMCD beamtime; P L Wincott – Mössbauer data collection; D J Vaughan – Manuscript review and assisted with Mössbauer analysis; R A D Pattrick – Manuscript review and co-supervisor; G van der Laan – Provided calculated XMCD spectra used for analytical fitting; E Arenholz – Station scientist for beamline 4.0.2, Advanced Light Source (ALS), Berkeley, CA, USA; F Tuna – Collected SQUID magnetometry measurements; J R Lloyd – Manuscript review and principal supervisor.

Chapter 6 – “The Controlled Doping of Cobalt into Biogenic Magnetite Nanoparticles”

J M Byrne, N D Telling, V S Coker, S Moise, P L Wincott, D J Vaughan, F Tuna, E Arenholz, G van der Laan, R A D Pattrick, J R Lloyd.

Status:
Manuscript in final preparation for submission to Advanced Functional Materials, Wiley.

Author Contributions:
J M Byrne – Principal author, sample preparation, XAS and XMCD data collection and analysis, XRD, TEM, SQUID and Mössbauer analysis; N D Telling – Manuscript review, assisted with XMCD and SQUID analysis and responsible for magnetic hyperthermia measurements; V S Coker – Manuscript review and assisted with
XMCD beamtime; **S Moise** – Carried out magnetic hyperthermia measurements; **P L Wincott** – Mössbauer data collection; **D J Vaughan** – Manuscript review and assisted with Mössbauer analysis; **F Tuna** – Collected SQUID magnetometry measurements; **E Arenholz** – Station scientist for beamline 4.0.2, Advanced Light Source (ALS), Berkeley, CA, USA; **G van der Laan** – Provided calculated XMCD spectra used for analytical fitting; **R A D Pattrick** – Manuscript review and co-supervisor; **J R Lloyd** – Manuscript review, principal supervisor.

**Chapter 7** – “Scale-up of Biomagnetite Production Using *Geobacter sulfurreducens*”


**Status:**

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**Author Contributions:**

**J M Byrne** – Principal author and data collection; **H Muhamad Ali** – Manuscript review and data collection; **V S Coker** – Manuscript review and data collection; **J R Lloyd** – Manuscript review and principal supervisor.

**Chapter 8** – “Characterisation of the dissimilatory reduction of Fe(III)-oxyhydroxide at the microbe - mineral interface: The application of STXM-XMCD”


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**Author Contributions:**

**V S Coker** – Principal author, STXM data collection and analysis and XRD data analysis; **J M Byrne** – Preparation of bacteria, STXM data collection and analysis; **N D Telling** – Manuscript review; **G van der Laan** – Provided calculated XMCD spectra used for analytical fitting; **J R Lloyd** – Manuscript review; **A Hitchcock** – Manuscript review, assisted with STXM data analysis; **J Wang** – Beamline scientist, BL 10ID1 of the Canadian Light Source (CLS), Saskatoon, Canada; **R A D Pattrick** – Manuscript review.
Chapter 9 – “Biotechnological synthesis of functional nanomaterials”

J R Lloyd, J M Byrne, V S Coker

Status:

Author Contributions:
J R Lloyd – Principal author; J M Byrne – Author of subsection entitled “Biomineralization of magnetic iron-based nanoparticles”; V S Coker – contributed to review of Au, Te and Ce interactions with nanomaterials.

Chapter 10 – “Conclusions and Future Aims”

J M Byrne
A Review of the Production of Biogenic Magnetite Nanoparticles and Potential Applications
2. A Review of the Production of Biogenic Magnetite Nanoparticles and Potential Applications

2.1. Introduction

Iron is the fourth most abundant element in the earth’s crust and is found in almost all aquatic and terrestrial environments. At circumneutral pH the element exists in a number of different oxidation states and minerals including goethite, hematite, magnetite, maghemite and siderite which have existed in the environment for billions of years as a result of a mixture of geological, biological and chemical conditions. A number of these different mineral products and the biological routes through which they are produced are currently undergoing intense research for many different applications; in particular magnetite nanoparticles are the subject of much review for their potential in the rapidly emerging field of nanotechnology.

Nanotechnology is the science of the very small and operates on the order of billionths of a metre with the properties of many materials changing from that of their macro or microscopic counterparts. The increasing push for environmental and economically more efficient technologies is in part fuelling the drive towards functional nanomaterials as well as for their potential use as materials for medical, computational and industrial process. One of the most promising of these nanomaterials is the iron based mineral magnetite (Fe$_3$O$_4$). There are several different chemical and mechanical synthetic methods of producing nanoscale magnetite, however these often require high temperatures and the use of toxic organic solvents that are both environmentally and economically undesirable. An alternative approach, and one that will be further investigated throughout this thesis, utilises the reductive capabilities of bacteria, which can use naturally occurring iron(III) for energy conservation and navigational purposes. These biological methods of nano magnetite formation provide routes through which this important mineral can be produced at ambient temperatures and pressures whilst also maintaining the ability to
manipulate the properties of the nanoparticles (a vital requirement for use in different applications).

The following chapter outlines some of the current research into magnetite based magnetic nanoparticles (MNP’s) and serves as a review, covering material which ranges from the fundamental basics on the crystal structure and magnetism of magnetite, to a comparison between several different methods of synthesis, and the potential applications for which they may ultimately be applied.

2.2. Magnetite structure and properties

2.2.1. Structure

Magnetite ($\text{Fe}_3\text{O}_4$) is a member of the spinel class of minerals (named after spinel; $\text{MgAl}_2\text{O}_4$) which have an approximately close packed face-centred cubic array crystal structure with mixed anions and cations. The group of oxide spinels is described by $(\text{A})[\text{B}_2]\text{O}_4$ where A and B denote divalent and trivalent cations respectively. A ‘normal’ spinel structure consists of all A atoms in tetrahedral coordination, surrounded by four oxygen atoms and all B atoms in octahedral coordination surrounded by six oxygen atoms. In an ‘inverse’ spinel structure, the A atoms occupy half of the B sites. Spinels are also often found with distribution of cations that is neither inverse or normal and may be termed ‘partially inverted’ with the formula $(\text{A}_{1-c}\text{B}_c)[\text{A}_c\text{B}_{2-c}]\text{O}_4$ where c is the inversion parameter (1≤c≤0).

Magnetite has an inverse spinel structure, hence it can be represented by the structural formula $(\text{Fe}^{3+})_n[\text{Fe}^{2+}\text{Fe}^{3+}]_8\text{O}_4^{2-}$ with each unit cell consisting of eight $\text{Fe}^{3+}$ atoms in tetrahedral coordination (A), eight $\text{Fe}^{2+}$ in octahedral sites [B] and eight $\text{Fe}^{3+}$ also in octahedral sites (figure 1). The substitution of transition metal cations including Cr, Mn, Fe, Co, Ni and Zn into the structure of the magnetite spinel in place of either $\text{Fe}^{2+}$ or $\text{Fe}^{3+}$ can have a profound impact on the properties of the ferrites including magnetism, reactivity and size.
2.2.2. Magnetism

All materials exhibit some form of magnetism, however the magnitude of the effect varies hugely between different substances. The magnetism of a solid is dependent upon the behaviour of electrons within, including the spin state (which can be either up or down), and the orbital movement of the electron around the atom. Materials can be classified by the type of magnetism they exhibit as either diamagnetic, paramagnetic, ferromagnetic, anti-ferromagnetic, ferrimagnetic or superparamagnetic.

Figure 1 – Crystal structure of magnetite $\text{Fe}_3\text{O}_4$, adapted from Cornell and Schwertmann\textsuperscript{1}. (a) Polyhedral model representing alternating octahedral and tetrahedral layers; (b) Ball and stick model with unit cell represented by dashed line box; (c) More detailed representation of octahedral and tetrahedral arrangement.
The magnetization $\mathbf{M}$ exhibited by a material under the influence of an applied magnetic field $\mathbf{H}$ can be described by the equation $\mathbf{M} = \chi \mathbf{H}$, where $\chi$ (dimensionless) is defined as a measure of the magnetic susceptibility of a material, i.e. the higher the value, the more magnetic the material. In materials which are diamagnetic or paramagnetic, the only magnetization exhibited occurs under the influence of an applied external field (figure 2), and the effect is only very small with $\chi$ ranging from $-10^{-6}$ to $-10^{-3}$ in diamagnetic materials (e.g. water) and $\chi$ between $10^{-6}$ to $10^{-1}$ in paramagnetic materials (e.g. aluminium). In such materials, the internal magnetizations (spin states) of the atoms are randomly orientated due to thermal motion and thus require an applied field to polarise them sufficiently enough to be detected. Ferromagnetic, ferrimagnetic and anti-ferromagnetic materials exhibit magnetism in the absence of an applied magnetic field due to the orientation of the spin states within. Ferromagnetic materials contain atoms which have all magnetic moments orientated parallel to each other whereas anti-ferromagnets contain lattice sites such that layers of anti-parallel magnetic orientations are formed. Ferrimagnetic materials can be considered to be a combination of the two.

Figure 2 – Types of magnetism, adapted from Pankhurst et. al. (a) Diamagnetic materials are weakly repelled by an applied magnetic field $\mathbf{H}$. (b) Paramagnetic materials are weakly attracted by an applied magnetic field. (c) Ferro and ferrimagnetic materials retain magnetization in the absence of an applied field and exhibit coercivity $H_c$. (d) Superparamagnetic materials exhibit no coercivity and retain zero magnetization in the absence of an applied field.

The $M-H$ curves corresponding to ferromagnetic and ferrimagnetic materials (figure 2c) indicate that a saturation magnetization ($M_s$) is reached above an applied field $H$, which corresponds to the point at which all of the magnetic moments within the material are aligned in the same direction. A hysteresis is often present which is
related to the effect of magnetic domains (i.e. localised islands of magnetic moment) within the material. As a result, an applied field \( H \) will be sufficient to orientate some of the domains parallel to the field, however other domains will still not be fully aligned and require a larger field. This effect gives rise to the open loop hysteresis visible in figure 2c. The applied field required to change the direction of the magnetization is denoted the coercivity \( H_c \) of a material. In superparamagnetic materials, the particles are so small (of the order of tens of nm or less\(^\text{10}\)) that they no longer have individual magnetic domains and as a result have no hysteresis, i.e. \( H_c = 0 \) (figure 2d). In such cases, thermal energy is sufficient to randomly orientate the magnetic moment of the particles in the absence of a magnetic field. The temperature above which this takes place is called the blocking temperature \( (T_B) \).

Magnetite nanoparticles exhibit ferrimagnetic behaviour, with particles smaller than \( \sim 30 \) nm in diameter exhibiting superparamagnetism\(^\text{11}\). The magnetism of magnetite and the associated iron oxides is due to the contribution from all of the iron sites within the crystal governed by a combination of antiferromagnetic superexchange (SE) and ferromagnetic double exchange (DE) interactions. There are three antiferromagnetic SE interactions (A-O-A, B-O-B, and A-O-B) between the Fe\(^{3+}\) ions on the (A) and [B] sites, mediated by the oxygen (O) ions. Ferrimagnetism in Fe\(_3\)O\(_4\) is obtained without any DE interaction\(^\text{12}\), resulting in the antiparallel alignment of moments on (A) and [B] sites, i.e. (A) sites have spin down, [B] sites have spin up orientation. Since the antiparallel Fe\(^{3+}(A)\) and Fe\(^{3+}[B]\) moments compensate each other, a saturation magnetization of 4 \( \mu_B / \text{f.u.} \) (formula unit) is expected from the remaining Fe\(^{2+}[B]\) moments. This simple Néel model has been extended by Yafet and Kittel\(^\text{13}\), who proposed a more elaborate model in which the B sublattice is subdivided into two [Fe\(^{2+}\) and Fe\(^{3+}\)]\(_B\) sublattices. It was shown that on weakening the A-O-B and strengthening B-O-B, the B site magnetic moments are no longer rigidly parallel to the A site moments. The stronger B-O-B SE interaction results in spin canting thus a reduction in the saturation magnetization. This is the reason why the introduction of dopants (Co, Zn, Ni etc.) into the structure of the crystal has such a profound effect on the magnetic properties of magnetite.
2.3. **Synthesis of magnetite**

Several methods currently exist through which magnetite nanoparticles can be produced, either in the environment during biogenic processes (which have been replicated in controlled laboratory processes over the last few decades), or through synthetic approaches such as chemical or mechanical means. The following section outlines some of these methods.

2.3.1. **Biological reduction of Fe(III)**

The formation of magnetite by microorganisms has been known for several decades however the existence of microbial reduction for energy conservation or navigation has been present in life for millions of years. Biogenic magnetite was first discovered in the teeth of marine molluscs (chitons)\(^1\). In that example, the purpose of the mineral was thought to be for grazing, however since then magnetite has also been found in honey bees, homing pigeons, algae and magnetotactic bacteria\(^1\). There are two recognized methods through which magnetite nanoparticles are formed by bacteria, either externally in a biologically induced reaction through which Fe(III) compounds are reduced outside of the cell wall, or intracellular production in a biologically controlled mechanism which occurs within the bacteria.

2.3.1.1. **Extracellular production**

Many soils and sediments contain 50-200 mM of ferric iron minerals per kg dry matter\(^15\) and as a result, microorganisms have adapted a variety of methods of energy generation which utilise the abundance of iron within their ecosystem where oxygen is depleted including Fe(III) reduction and Fe(II) oxidation. The formation of biogenic Fe minerals thorough these mechanisms is regarded as a key component in the global iron cycle\(^15-17\).

The production of magnetite by Fe(III) reducing bacteria was only discovered relatively recently in the 1970’s\(^18\) and is now commonly observed in a variety of environments such as fresh and marine waters\(^19-21\), thermal springs and leachate
ponds\textsuperscript{22}, and in particular regions which are deficient in oxygen or at oxic-anoxic interfaces. The mechanism involved combines the oxidation of an organic compound or hydrogen (electron donor), coupled with the reduction of ferric iron (electron acceptor), leading to the release of Fe(II). This leads to the formation of iron oxides including magnetite (Fe\textsubscript{3}O\textsubscript{4}), goethite (FeOOH), vivianite (Fe\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}·8H\textsubscript{2}O), siderite (FeCO\textsubscript{3}) and hematite (Fe\textsubscript{2}O\textsubscript{3}) depending upon the conditions of formation (i.e. pH and temperature)\textsuperscript{23-24}. The biomineralization of magnetite has been studied in a number of different microorganisms that are able to utilize Fe(III) as an electron acceptor for growth in particular \textit{Shewanella oneidensis}\textsuperscript{9,25} and \textit{Geobacter spp.}\textsuperscript{26-28}.

The source of the electron acceptor is often observed to be amorphous ferric oxyhydroxide (also known as 2-line ferrihydrite). Ferrihydrite is a poorly crystalline material with the approximate chemical formula 5Fe\textsubscript{2}O\textsubscript{3}·9H\textsubscript{2}O\textsuperscript{29}, which is widespread in many natural environments and has a high surface area, consisting of nanometre sized crystals. The structure of ferrihydrite has previously been solved with it observed to be non-amorphous\textsuperscript{30} and containing short range order, although some debate still remains\textsuperscript{31}. The crystallinity of the ferrihydrite depends upon the conditions under which it is formed such as rate, pH and temperature\textsuperscript{1}. Many other organisms have since been isolated that can also reduce Fe(III) and lead to the formation of magnetite by biologically induced mineralisation including \textit{Geobacter metallireducens}\textsuperscript{16}, \textit{Geobacter sulfurreducens}\textsuperscript{32}, \textit{Shewanella oneidensis} MR-1\textsuperscript{33} and \textit{Thermoanaerobacter ethanolicus}\textsuperscript{34}.

\textit{Geobacter} species metabolize organics as electron donors within the cell via acetate and the tricarboxylic acid cycle (TCA)\textsuperscript{35-36}. The mechanism of electron transfer to the surrounding Fe(III) compound is still not fully understood, however it appears that Fe(III) reduction predominantly takes place on the surface of the outer membrane of the cell, with electron transfer facilitated by periplasmic and \textit{c}-type cytochromes\textsuperscript{37-38}. Other mechanisms however do exist through which electron transfer between bacteria and Fe(III) minerals can take place away from the cell, such as the use of electron shuttling compounds (e.g. flavins or quinones). Alternatively, chelating compounds increase the solubility of Fe(III), and thus increase the bioavailability of the electron acceptor. In general, the methods through which Fe(III) reduction is considered to take place include:

- Direct contact between the Fe(III) containing mineral and the bacteria.
• Use of an electron shuttling compound (e.g. flavins, quinones and humics) that can transfer electrons between cell surface and mineral.
• Release of chelating compounds to increase the solubility of the Fe(III)-bearing mineral to increase bioavailability.
• Pilli (nanowires) which are produced by the bacteria and form a bridge through which the electrons can flow.

The different pathways to reduction are outlined in Figure 3. Some organisms are thought to apply more than just one of the above methods for the production of energy such as *Shewanella species* and *Geothrix fermentans*\textsuperscript{39,40}. It has also been seen in some *Geobacter* species that pilli are produced when grown on insoluble ferric iron, but not on ferric citrate\textsuperscript{41}. Pilli could ultimately have potential applications as biological nanowires in the development of the computer industry\textsuperscript{42}.

![Figure 3 – Potential mechanisms of iron(III) reduction.](image)

**2.3.1.2. Intracellular production**

Another method of producing biogenic magnetite nanoparticles is using bacteria which are able to perform biomineralization within the cytoplasm of the cell. The formation of magnetic nanoparticles within organisms was first observed in magnetotactic bacteria by Blakemore in a marine environment in 1975\textsuperscript{8}. These bacteria contained
small crystals of magnetite which respond to the application of a magnetic field. Subsequent experimentation confirmed that magnetotactic bacteria are influenced by the earth’s magnetic field (~0.5 Gauss) and it is thought they use this for navigational purposes in a process called magnetotaxis. Since the initial discovery, magnetotactic bacteria have been found in many different environments including freshwater lakes and in anaerobic soils.

The magnetic behaviour of these organisms is attributed to the presence of internal crystals of MNP’s which form with sizes ranging between 11-120 nm within intra-cytoplasmic membranes described as magnetosomes. These internal vesicles provide a permanent magnetic dipole moment of around 6 x 10^{-17} J T^{-1} per crystal and are often found in chains aligned along the axis of motility to maximise the magnetic moment. The magnetosome membrane is about 8-12 nm thick and determines the size that the magnetite crystal can reach.

Biomineralization by such organisms has been observed in environments with only very low concentrations of iron (0.01 – 1 mg L^{-1}) and it is thought that within the cell, iron which has passed through the cell wall in a soluble form produces highly reactive Fe(III) oxides such as ferrihydrite which reacts with Fe(II) that is formed and re-crystallizes into magnetite. Evidence of the formation of ferrihydrite associated with magnetite has been found in *Magnetospirillum magnetotacticum MS-1* which supports this formation mechanism. Several specific proteins have been identified in the magnetosome membrane which are involved in the uptake of iron, crystallization of magnetite and are responsible for the assembly of magnetosome chains. However, there is still relatively little known about the molecular mechanism of magnetite and magnetosome membrane formation.

One significant drawback of producing magnetic nanoparticles through this approach is that the culturing process only yields relatively low quantities of
nanoparticles in comparison to the extracellular alternatives, and the microaerophilic organisms are quite difficult to cultivate.

### 2.3.2. Synthetic approaches

Many synthetic approaches to magnetite formation exist including chemical and mechanical techniques which often require high temperatures and toxic reagents, leading to high costs and potentially undesirable environmental consequences. The ability to manipulate the properties of the particles produced often require the use of organic reagents used as solvents,

leading to their unsuitability for delivery into the human body which is a major drawback for their potential use in targeted cancer therapies (see section 5.3.1 for details). The following section briefly outlines the methods used by several of these procedures ranging from mechanical ball milling and laser pyrolysis to chemical methods including co-precipitation, thermal decomposition, microemulsion and hydrothermal synthesis.

#### 2.3.2.1. Mechanical

Ball milling is a process in which high energy and low energy mills grind down coarse grained powders of iron ferrites to produce nanoparticles. Particles with sizes ranging from 20 to 30 nm can be produced using the low energy approach with 22 mm steel balls used to mill ferrites in the presence of water in an aerobic atmosphere at different intervals of 40, 50, 80 and 120 hr.

High energy milling required the ferrites to be sealed under an argon atmosphere with steel balls of different sizes (12.7 mm and 6.4 mm) used to grind the samples between 2 and 12 hr resulting in the formation of 12 nm diameter nanoparticles.

Laser pyrolysis induces chemical reactions in a flow of a mixture of different gases through heating by a continuous flow carbon dioxide laser. The nucleation of nanoparticles occurs above certain threshold pressures and laser power resulting in the formation of particles with small size, narrow size distribution and limit aggregation. Maghemite \((\text{Fe}_2\text{O}_3)\) nanoparticles have been synthesised using this technique by the laser pyrolysis of \(\text{Fe(CO)}_5\) vapours, resulting in the formation of 5 nm diameter particles which have the potential to be used as magnetic resonance imaging (MRI) contrast agents.
2.3.2.2. Chemical

Co-precipitation is one of the most commonly used chemical synthesis methods due to the simplistic nature; however there is limited ability to control the shape and particle size. The technique synthesizes iron oxides including magnetite and maghemite through the precipitation of Fe$^{2+}$/Fe$^{3+}$ salt solutions at room or elevated temperatures, at ambient pressure. The ratio of salts, pH and temperature of the media have a significant impact on the size, shape and composition of the products. The poor control over size distributions yields challenges for use in many applications, however recent developments have suggested the use of organic additives such as polyvinylalcohol (PVA)$^{62}$ or oleic acid to stabilise the formation of magnetite nanoparticles$^{63-64}$.

In thermal decomposition, organometallic compounds are thermally decomposed in high boiling point organic solvents and stabilised with other surfactants to produce magnetic nanoparticles. The process uses many different organometallic precursors including metal acetylacetonates, metal cupferronates ($M$-$\text{NH}_4[C_6\text{H}_5\text{N(O)NO]}$ where $M$ is a metal cation) or carbonyls. Oleic acid, hexadecylamine and fatty acids are often used as surfactants. The technique can be used to produce different magnetic nanocrystals including Fe$_3$O$_4$, Cr$_2$O$_3$, MnO, Co$_3$O$_4$ and NiO with size and shape controlled by variation of the reactivity conditions. The time taken for the procedure can range from hours to days, with the potential for high yields, however the synthesis can be complicated and uses high temperatures.

Hydrothermal synthesis is a technique based on a general phase transfer and separation mechanism which occurs at solid, liquid and solution interface phases$^{65}$. Monodisperse, hydrophilic, ferrite microspheres were prepared by the vigorous stirring of a mixture of FeCl$_3$, ethylene glycol, sodium acetate and polyethylene glycol and heated to 200 °C for between 8 and 72 hr. The mechanism is unclear, however, it enables the formation of desired materials using a simple approach with disadvantages including high pressures, high temperatures and a long reaction time.
2.4. Manipulating properties of magnetite produced by microbial Fe(III) reduction

The incorporation of dopants into the structure of magnetite can have profound impacts on the size and magnetic properties of the nanoparticles. Other factors have also been shown to have an impact on the properties of magnetic nanoparticles as well as on the mineral phases that are produced through microbial reduction of Fe(III) compounds.

2.4.1. Effect of dopants

By considering the structural formula of magnetite (Fe$_3$O$_4$) and the associated ferrites ($M_x$Fe$_{3-x}$O$_4$, where $M$ is a metal dopant) we can begin to understand the dramatic effects that the addition of transition metals or lanthanides can have, in particular on magnetic properties. As already discussed in section 2.1, the structure of stoichiometric magnetite contains Fe$^{3+}$ in tetrahedral coordination matched by equal numbers of Fe$^{2+}$ and Fe$^{3+}$ cations in octahedral coordination. The magnetic nature of the stoichiometric magnetite nanoparticles is due to the anti-ferromagnetic interactions between (A) and [B] lattice sites (A-O-B superexchange), resulting in zero net moment from Fe$^{3+}$ cations, and the magnetism due to the remaining Fe$^{2+}$ cations. The substitution of one or more of these atoms in a unit cell can thus lead to changes in the overall saturation magnetization (increase or decrease) or by altering the superparamagnetic state by changing the coercivity.

Several studies using conventional synthetic approaches have demonstrated the ability to incorporate dopants into magnetite such as chromium$^{66}$, cobalt, manganese and zinc$^{67}$. There have also been several studies that have demonstrated the ability to incorporate dopants through biogenically generated nanoparticles. Moon et. al.$^{68}$ have shown that a number of different transition metals and lanthanides can be incorporated into magnetite through the reduction of Fe(III)-(transition metal/lanthanide) oxide precursors by thermoanaerobic bacteria $T$. ethanolicus TOR-39 and psychrotolerant Shewanella spp PV-4$^{68}$. The dopants included Cr, Mn, Co, Ni, Zn with cationic molar fractions (CMF) between 0.02-0.3, and lanthanides including Nd,
Gd, Tb, Ho, Er with CMF 0.01 – 0.02. Several of the dopants (Mn and Zn) exhibited enhanced magnetic saturation measurements compared to biogenic magnetite. Lanthanides are used to reduce the material’s Curie Temperature ($T_C$ i.e. temperature above which a ferromagnet or ferrimagnet becomes paramagnetic). Experiments using *Shewanella* spp PV-4 lead to higher values of $M_s$ in all samples than those produced using TOR-39. This is attributed to difference in reaction temperature (PV-4 was incubated at 20 °C, TOR -39 incubated at 65 °C). Other studies using organisms producing extracellular magnetite include *G. sulfurreducens*, which has been used to incorporate cobalt by as much as 23 atom% into the magnetite crystal structure in place of Fe$^{2+}$ cations, leading to a significant increase in the coercivity (1400% at 5 K) without significantly reducing the overall saturation magnetization of the nanoparticles. Cobalt doping was also achieved in magnetite produced in the magnetosomes of magnetotactic bacteria *Magnetospirillum*, however this was only to a relatively low concentration of ~1% and the increase of coercivity was found to be 36-45% (300 K). Studies have also shown that the structure of the magnetite formed within magnetosomes can be changed through the controlled addition of other metal dopants such as nickel or manganese.

The incorporation of dopants, however, is also seen to have an impact on the size of the crystalline phases produced. The size of magnetosomes produced in the magnetotactic bacterium *Magnetospirillum magnetotacticum* (MS-1) was studied to see the effect of substitution of zinc and nickel into the crystal structure. Zinc and nickel doping was seen to result in larger particles than stoichiometric magnetite. With 23 ± 3 nm (zinc), and 25 ± 5 nm (nickel) particles produced compared to 15 ± 3 nm in the control group containing no additional transition metal. An interesting effect in this study was the change in the size of the bacterium depending upon the metal dopants, with zinc and nickel producing longer and shorter cells respectively than bacteria grown on iron alone. Conversely, doping with transition metal elements in experiments using extracellular Fe(III)-reducing bacteria often demonstrate that the particle size of the magnetic nanoparticles decreases with the effect enhanced with increased dopant concentration.
2.4.2. Additional factors affecting magnetite mineralization

A change in the starting conditions of magnetite mineral has a profound effect on the type of mineral phases produced. Under abiotic conditions goethite/lepidocrocite and goethite/magnetite are formed in conditions containing low and high concentrations of Fe(II) respectively. Alternatively, controlling the concentration of Fe(III)-oxyhydroxide (electron acceptor) at the start of microbial reduction can lead to the formation of Fe(II)-bearing minerals such as siderite as well as magnetite. This was observed with Fe(III) reduction by *Shewanella oneidensis*.

The reduction of Fe(III) minerals by anaerobic respiration by dissimilatory iron reducing bacteria (DIRB’s) is coupled with the oxidation of organic carbon, with the product of reduction changing depending on the pathway utilised by the bacterium. For example, the influence of the carbon source on the products of anaerobic iron(III) reduction by *Shewanella putrefaciens* W3-18-1 was investigated by Salas et. al. This study used pyruvate, uridine and lactate which lead to the formation of siderite with green rust and magnetite with green rust respectively. The formation of the different mineral products was thought to be due to the rate at which the Fe(II) was produced depending upon the starting carbon source.

Other factors may also change the products of mineralization by iron reduction. These may include the presence of different buffer systems (vivianite is formed in the presence of phosphate buffer rather than carbonate buffer) or the variations in anaerobic conditions under which reduction takes place (siderite is seen as the end product rather than magnetite in cultures of *S. oneidensis* when CO$_2$-H$_2$ is chosen as the headspace for the anaerobic reduction in place of N$_2$). Ultimately though, Fe(II) concentration is the dominant factor which affects the final mineralogical endpoint of Fe(III) oxide bioreduction.

2.4.3. Scale up

Many of the potential applications for biogenic magnetic nanoparticles are highlighted below, however, one of the major pitfalls to using such a technology is the ability to produce the required material reproducibly and at scale. The ultimate goals of the scale-up work described in this thesis, is to define and propose methods for with
biogenic magnetite can be generated at kg or even tonne quantities at commercially competitive prices compared to the environmentally less benign routes. Some work has already begun into finding solutions to this problem including work carried out by Moon et. al. 2010\textsuperscript{80}. This work centred around the use of \textit{Thermoanaerobacter} \textit{spp.} TOR-39 at 65 °C to generate various doped magnetite nanoparticles. After over three weeks of incubation 1 kg wet weight was generated from a 35 L reactor.

Other studies have demonstrated the ability to grow \textit{Geobacter sulfurreducens} under continuous growth conditions in chemostats\textsuperscript{81-83}. Chemostats provide a system in which input and output flow rates can be controlled provide steady state conditions which keep process variables constant including volume, biomass, product and substrate. They provide one of the best opportunities for studying microbial physiology under carefully controlled and often environmentally relevant conditions\textsuperscript{84}. The study by Esteve-Núñez et al.\textsuperscript{81} was designed to study the physiology of \textit{G. sulfurreducens} under conditions more closely matching those found in the subsurface than in batch cultures.
2.5. Applications

There are potentially huge commercial applications of magnetic nanoparticles which could have major impacts in the field of biomedicine and for environmental purposes. As mentioned previously, the expected uses of such particles include the remediation of contaminated waste waters and land, targeted cancer therapies, catalysis and in magnetic data storage devices. The following section outlines some of the main areas for which magnetic nanoparticles could be applied and highlights some recent research into each field.

2.5.1. Remediation

The widespread use of heavy metals such as chromium\textsuperscript{85} and zinc\textsuperscript{86} in industrial processes and the presence of arsenic in aquifers used for drinking water and irrigation in areas of Bangladesh and south east Asia highlights the global problem of contaminated land and water. Whilst there are many potential methods of contaminant clean up, the use of magnetic nanoparticles has the potential to gain considerable momentum due to their reactive nature, large surface area to volume ratio and magnetic recoverability. Much research is focused on the potential use of magnetite in the clean up of these toxic environments, either by the sequestration of the toxic element within the cubic spinel structure of the nanoparticles, or by reduction of the toxic contaminant to a less mobile, and less toxic form\textsuperscript{87}.

Chromium contamination has been caused by the range of commercial applications that use the heavy metal including wood treatment, electro plating, leather tanning and pulp production\textsuperscript{85}. Chromium usually occurs in two stable oxidation states, trivalent Cr(III) and hexavalent Cr(VI). Cr(VI) is a strong oxidising agent which is toxic to plants, animals and humans, carcinogenic\textsuperscript{88} and highly mobile in the environment. Cr(III) however has a lower solubility and adsorbs strongly to the surface of soil minerals, thus limiting its mobility and inhibiting its biological uptake, hence is considered less toxic. Studies performed by Cutting et al\textsuperscript{88}, have focused on the potential for using biogenically produced magnetite for chromium remediation. Through batch studies, it was found that magnetite derived by the reduction of both
schwertmannite (Fe(III) oxyhydroxyl-sulphate) and ferrihydrite (Fe(III) oxyhydroxide) by *Geobacter sulfurreducens* were significantly more efficient at Cr(VI) removal than commercially available chemically synthesised magnetite, despite similar particle sizes. Reaction of the magnetite with a 1mM Cr(VI) solution showed 58%, 70% and 95% removal of Cr(VI) for synthetic magnetite, ferrihydrite derived magnetite and schwertmannite derived magnetite respectively. The increased reactivity of schwertmannite magnetite was attributed to an increased availability of Fe(II) at the surface of the particles, which was able to reduce the Cr(VI) to Cr(III). Studies into the nature of the chromium remediation by x-ray absorption (XAS) and x-ray magnetic circular dichroism (XMCD) 88-89 indicated that whilst the Cr(VI) was predominantly reduced to Cr(III), (with the trivalent cation incorporated into the crystal spinel), a surface layer of Cr(III)/Cr(VI) was formed which was thicker in schwertmannite derived magnetite than that which was from ferrihydrite. Column studies confirmed the order of effectiveness of the different biogenic magnetite samples and also showed the effectiveness of remediation of the redox active fission product Tc(VII). The results indicated that the use of schwertmannite derived biomagnetite resulted in 97.8% cleanup of Tc(VII) compared to ferrihydrite derived biomagnetite which achieved 77.9% Tc(VII) clean up. These studies indicate the importance of selection of starting Fe(III) mineral, and demonstrates the increased abilities of biogenically derived magnetite over synthetic counterparts.

Contaminated ground water is a problem of enormous magnitude in regions all over the world, and is affecting tens of millions of people. Most notable effects can be seen in parts of South East Asia such as Bangladesh and West Bengal where high levels of arsenic is present in aquifers used for drinking and irrigation water 90. Several studies have focused on the potential application of biogenic nanomaterials on the clean up of Arsenic based contaminants found in oxidation states of As(V) and As(III). The ability of surface modified iron nanoparticles to remediate arsenite As(III) has been demonstrated by Kanel et al. 91 who showed that MNP’s held in sand columns (10 cm, containing 2 g MNP with size distributions of 2-10 nm) can remove up to 100% As(III) in varying concentrations of 0.2, 0.5 and 1.0 mg L \(^{-1}\) in 9, 7 and 4 days respectively at a flow rate of 1.8 ml min \(^{-1}\). A recent study by Coker et al. 92 investigated the structural interaction of arsenic compounds with biogenic magnetite. Based on initial work by Islam et. al. 78, 93, two separate Fe(III) oxyhydroxide (10 mM Fe(III)) slurries containing
sodium arsenite (100 μm As(III)) and sodium arsenate (100 μm As(V)) respectively were reduced by *Geobacter sulfurreducens*, yielding As-incorporated magnetite. Standards were made by reacting solutions of arsenite and arsenate with biogenic magnetite. The studies indicated that the As(III) appears to be mostly adsorbed to the surface of the nanoparticles during the process of magnetite formation, with little change in the structure of the materials. As(V) however appeared to become incorporated within the magnetite structure itself in tetrahedral coordination when compared to the surface-absorbed As(V)-magnetite standard. Incorporation of As(V) into magnetite is only possible provided that the adsorption of As(V) onto the surface of the Fe(III)-bearing compound occurs before the reduction of As(V) to As(III). In Bengal, arsenic is found adsorbed to different minerals that include amorphous ferric oxides\(^9\), which implies that there is potential for an active use of metal-reducing bacteria in the environment.

Other potential contaminants that could be remediated by MNP’s include hexachlorocyclohexans\(^9\), with more than 95% removed from solution containing 2-30 g L\(^{-1}\) biogenic MNP’s. The same study also used zero valent iron nanoparticles to remediate organic pollutants such as PAH’s, PCB’s and pesticides. Also, other studies into pollutants demonstrated the abilities of magnetite Fe\(_3\)O\(_4\) in dehalogenation reactions (dechlorination of carbon tetrachloride CCl\(_4\))\(^7, 96\) or reduction of hexachloroethane and 4-chloronitrobenzene\(^97\).

Thus there is a significant potential for the use of magnetic nanoparticles in the remediation of contaminated sites with the prospect of applying biogenically produced magnetite leading to even more efficient and effective clean up than synthetically generated counterparts.

**2.5.2. Catalysis**

Catalysis provides a route to convert raw materials into valuable chemicals and fuels and is increasingly used in a number of chemical reactions to increase efficiency to ultimately reduce costs and decrease environmental impact. The use of nanosized catalysts has many benefits including the high surface area/volume ratios, however their removal for reuse can prove problematic and time-consuming\(^6\). Magnetic nanoparticles, however, could be magnetically recovered. This could also lead to the increased reusability of expensive catalysts.
Several experiments have focussed on the loading of catalysts onto the surface of magnetite nanoparticles to ensure large surface areas and that magnetically removable catalysts can be reused. These include the loading of ruthenium (II) complexes onto chemically synthesized magnetite in a reaction aimed towards the hydrogenation of aromatic ketones, with the material reused up to 14 times\textsuperscript{98}. Methods of generating biogenic magnetite with zero valent palladium Pd(0) loaded onto the mineral surface has also been demonstrated by Coker et. al. 2010\textsuperscript{99} (Figure 5). Heck reactions indicated the equal or superior catalytic activities of the heterogeneous catalyst compared to equimolar colloidal palladium catalysts with the additional benefit of reusability, with the catalysts removed and re-used up to four times.

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{false_colour TEM image of palladium (blue) loaded biomagnetite (red). (From Coker et. al.\textsuperscript{99}).}
\caption{False colour TEM image of palladium (blue) loaded biomagnetite (red). (From Coker et. al.\textsuperscript{99}).}
\end{figure}

\textbf{2.5.3. Biomedical Applications}

The application of the small size and magnetic behaviour of MNP’s for a range of other applications has been demonstrated in medical treatments including cancer therapy, drug targeting, magnetic resonance imaging (MRI) and biosensors\textsuperscript{100-102}.

\textbf{2.5.3.1. Induced Hyperthermia}

The principle of the technique of induced hyperthermia lies in the idea of heating a tumour to a temperature above a thermal threshold and holding it for a certain time frame at which point the cancerous tissue is destroyed. One of the main drawbacks to this method is the unspecific nature and unacceptable levels of heating of healthy tissue surrounding the tumour. Research into the use of magnetic nanoparticles for induced hyperthermia is showing increasing potential to overcome this problem.
Magnetic nanoparticles resonantly respond to a time varying magnetic field (i.e. AC magnetic field), resulting in the transfer of energy from the excitation field to the nanoparticles. This transfer of energy can be used as a hyperthermia agent for the enhancement of chemotherapy or radiotherapy treatments. The process involves the dispersion of magnetic nanoparticles throughout a target tissue with an AC magnetic field of sufficient frequency and strength to cause the particles to heat the surrounding tissue to a temperature between 40 and 45 °C in order to destroy the cancerous cells\textsuperscript{103}. The principle behind the technique lies behind the injection of a tumour with magnetic nanoparticles. The patient is then passed through an alternating magnetic field that is operating at high frequency. Through a combination of Néel relaxation and Brownian relaxation, the nano-magnets heat up, resulting in a heating effect on the surrounding area. Cancerous cells tend to be more sensitive to heat than normal cells\textsuperscript{5} and so just a minor increase in temperature can be applied to eradicate the targeted area whilst leaving healthy tissue relatively unharmed.

Due to the nature of the therapy, specific parameters including the heating power, magnetic hardness and physical dimensions of the nanoparticles must be modified and tailored to ensure the maximum treatment with the least amount of material using AC magnetic field conditions that are acceptable for clinical use. The use of externally applied magnetic fields has previously been shown to lead to physiological effects\textsuperscript{104-105} including stimulation of muscular tissue and potential cardiac complications. The range of frequencies (\(f\)) currently regarded as safe are limited to between \(f = 0.05 – 1.2\) MHz with amplitudes of magnetic field \(H = 0 – 15\) kA m\(^{-1}\).\textsuperscript{10} Heat transfer within the tissue is also regarded as a problem, with successful treatment requiring the maintenance of a tissue temperature at 42 °C for over 30 minutes, hence sufficient power is required to overcome the cooling effects of the surrounding blood flow and tissues. The current widely held view is that a heat deposition rate of 100 mW cm\(^{-3}\) of tissue provides sufficient heating which will overcome cooling effects\textsuperscript{10}.

Several studies have demonstrated the therapeutic effects of the method in animal studies\textsuperscript{106} and more recently in human clinical trials. A study by the Jordan group at Berlin’s Charité Hospital reported the first human study into the feasibility of using magnetic nanoparticles for hyperthermia treatment of cancer (thermotherapy)\textsuperscript{107}. The study was an early phase experiment investigating the effects
of insertion of aminosilane coated iron oxide nanoparticles into 14 patients suffering from a severe type of brain cancer. The experiment worked in combination with radiotherapy and successfully demonstrated that hyperthermic temperatures could be achieved in the treatment of brain tumours. This was only an initial test to determine the feasibility of using magnetic nanoparticles for thermotherapy however more rigorous testing has been subsequently tried on 66 patients suffering from recurrent glioblastoma multiforme brain cancer\textsuperscript{108}. Other clinical studies by the same group have focused on the treatment of prostate cancer using thermotherapy\textsuperscript{109-110}.

Biological approaches have also been applied to produce magnetic nanoparticles suitable for use in hyperthermia treatments. This includes a recent study in which magnetosomes were produced that were able to generate a thermal heating of 960 W g\textsuperscript{-1} at 410 kHz and 10 kA m\textsuperscript{-1} \textsuperscript{111}. The magnetosomes used were \approx 40 nm in diameter with a narrow size distribution and whilst the frequency used is outside the considered safe limit for human treatments, the study has demonstrated the potential for using biogenically synthesised nanoparticles in this treatment.

Ultimately, significant research is still required to demonstrate the full abilities of using magnetic nanoparticles for thermotherapy, either as lone treatments or in combination with radiotherapy or chemotherapy; however there are some exciting prospects.

\subsection*{2.5.3.2. Targeted therapy}

One of the current goals of medicine is the ability to administer intravenous drugs to a specific location in low enough concentrations to treat the diseased area without leading to any adverse damage or side affects to the surrounding healthy tissue\textsuperscript{112}. One such method is the idea of targeted magnetic drug delivery, which was first proposed as a potential treatment in the 1970s\textsuperscript{113}. The technique enables the specific delivery of pharmacological agents such as chemotherapeutic agents which have been coated around magnetic nanoparticles by the application of an external magnetic field. The small size of the materials allows their transport through narrow blood vessels\textsuperscript{100} in order to become concentrated at the specific site to minimize the risk to other organs. Animal studies have shown that the increase in concentration allows for a significant reduction in the total amount of the agent administered to as little as 20\% of the regular dosage\textsuperscript{114}, with the specificity removing an aspect of dilution.
2.5.3.3. Molecular imaging

Magnetic resonance imaging (MRI) is an imaging technique which is used to visualise detailed internal structures inside the human body. MRI relies on exploiting the huge numbers of protons present in biological tissue \((6.6 \times 10^{19} \text{ mm}^{-3} \text{H}_2\text{O})\) which have very small magnetic moments in order to observe a measurable effect under the application of a large magnetic field\(^{10}\). Under the influence of an applied magnetic field of 1 T, an effective signal of \(2 \times 10^{14}\) proton moments \text{mm}^{-3} is observable. MRI machines use the magnetic signal to magnetize the protons and use resonant radio frequency fields to alter the alignment of magnetization which is then detected.

When the resonant frequency is removed, the relaxation time of different tissues will be different, thus enabling the contrast between different organs. Contrast agents are used to change the relaxation time of a tissue, with agents usually based on gadolinium ion complexes. Superparamagnetic iron oxide (SPIO) agents have been used as contrast agents, with size dependent effects. Nanoparticles around 30 nm are more rapidly collected by the liver and spleen than 10 nm particles which are more likely to remain in the blood stream before collecting in lymph nodes and bone marrow\(^{115}\). The use of SPIO’s for tumour detection has been demonstrated for several types of cancer including liver, brain and lymphatic\(^{116-118}\).

2.5.4. Data storage

Magnetic data storage devices are ubiquitous in computers with a demand to store ever increasing amounts of information. These storage devices contain two principal components, the disk (on which data are recorded in bits, i.e. 1’s and 0’s) and the read head sensor (for detecting the changing bits). The data storage capacity is dependent upon a balance on the areal size of the bit, and the ability for the sensor to resolve that area sufficiently for detection. Over the last 25 years, developments in read head sensors has led to an increase in areal density in disk drive products by 50-60% annually\(^{119}\) based on the principle of Giant Magneto Resistance (GMR), first discovered independently in 1988 by independent research groups led by Peter Grunenberg\(^{120}\) and Albert Fert\(^{121}\).

GMR is dependent upon the increased electrical resistance of magnetic materials which are polarised with one spin direction compared to if they were the
opposite spin direction. In thin film read head sensors, ferromagnetic layers are separated by a non-magnetic metal. The distance between the ferromagnetic layers (i.e. thickness of the non-magnetic layer) is designed to render the magnetization of ferromagnetic layers anti-parallel in the absence of an applied magnetic field. Based on the GMR effect, one of the films is electrically conductive based on its spin direction to electrons polarized in the same orientation, whereas the other is now an insulator based on its spin direction, hence no current can pass through the layers. When a magnetic field is applied, both ferromagnetic materials align in the direction of the field, and provided they are aligned in the electrically conductive spin direction, and then a current of electrons polarized spin up can pass. In this sense, the ferromagnetic layers can act as gates, allowing current to flow in the presence of a magnetic field, and not in the absence of a field (Figure 6).

Several studies have focussed on increasing GMR effects by modification of the width of the non-magnetic layer and materials used, however half-metals are also considered to be promising materials. First introduced by de Groot et al\textsuperscript{122}, half metals contain an energy gap at the Fermi level $E_F$ for one spin orientation, i.e. the materials are electrically conductive when magnetic moment is in one direction (spin up), and electrically insulating when orientated in the opposite spin direction (spin down)\textsuperscript{123}. Magnetite (Fe$_3$O$_4$) is postulated to be a half-metal and the use of magnetic nanoparticles is considered to be a potential alternative to expensive thin films currently used in read head sensors.

![Figure 6 – Giant magnetoresistance in thin films.](image)

(a) In the absence of a magnetic field, the moment of ferromagnetic materials (FM) separated by a non-magnetic layer (NM) are anti-parallel and no current can pass ($I = 0$). (b) When a magnetic field is applied, the materials moments become aligned parallel and electrons polarized spin up can flow, producing a current ($I > 0$).

Recent research suggests that instead of layers, nanoparticle arrays could be arranged as an alternative method of utilising GMR. Whereas multilayer films have
only one interface between ferromagnetic and non-magnetic layers, granular arrays would have several. An array of magnetite particles placed in an applied magnetic field could act either as a conductor or insulator, depending on the direction of the field due to the half-metallic behaviour of the nanoparticles. Mixtures of particles with different coercivities could be exploited, for example magnetite and cobalt ferrite. Cobalt ferrite has a much larger coercivity than magnetite, hence will more strongly resist changes in its magnetization direction. A mixture of the two nanoparticles could be placed in an applied magnetic field which had been chosen so that the magnetite particles would be magnetized in one direction, whereas the cobalt ferrite particles would be randomly orientated. These configurations result in systems comprised of many conducting islands, in weak electrical contacts which due to the high density of magnetic interfaces could enhance spin-dependent electronic transport\textsuperscript{124}.

An increase in the magnetoresistance effect observed at room temperature would potentially lead to an increase in the volume of data that can be stored on a magnetic hard drive, with a higher sensitivity of the read head sensor. The idea is that as the sensor moves across magnetic ‘bits,’ a current is passed through the sensor, but not when the sensor is over a non-magnetic bit. To distinguish between non-magnetic bits and magnetic bits on a hard drive disk, the sensor needs to be able to switch magnetizations rapidly.
2.6. Conclusions

Biogenic magnetite nanoparticles can be produced by a wide range of extracellular and intracellular Fe(III)-reducing bacteria. The methods through which this is achieved offer promising alternatives to mechanical or chemical synthesis techniques which are commonly used to produce these materials. Recent research has demonstrated the abilities of biogenic magnetite and transition metal doped magnetite to be used in a wide range of applications including the bioremediation of toxic industrial sites, the inducement of hyperthermic temperatures which can destroy cancerous tumours, reusable catalysts and magnetic data storage devices. Some of the major advantages of using biological approaches include the ability to produce magnetite at low temperature and ambient pressures, coupled with the ability to tune the properties of the particles to match the specific needs of the many different applications. Ultimately, with further study it may be possible to produce the material at large scales for commercial availability using environmentally benign routes.

2.7. References


3

METHODS
3. Methods

The focus of this research project has been the development of magnetic nanoparticles based on magnetite iron oxides which have been produced by the reduction of Fe(III) compounds by dissimilatory iron reducing bacteria. This is a multi-disciplinary research area which has encompassed a wide range of experimental and analytical techniques to characterize the materials produced as effectively as possible. This section is intended to provide background insight into the main analytical methods used throughout this thesis to supplement the information presented in each of the research chapters. As such, more specific information related to each of the techniques used is already included in the ‘Experimental Methods’ section of each respective paper.

3.1. X-ray Diffraction

X-ray diffraction (XRD) is an important analytical method for providing information about the mineral phases which are formed as a result of the microbial reduction of iron. The technique is also very useful in the determination of average crystallite particle size. XRD was first developed by William Lawrence Bragg and William Henry Bragg when they discovered the principle of constructive interference of X-rays which have been scattered from a set of parallel lattice planes. This is possible due to the comparable interatomic distance in a crystal to the wavelength of X-rays (~1Å). X-rays produced from a Cu $K_{\alpha}$ source bombard the sample and are diffracted by the crystal planes of the material under investigation at an angle (glancing angle) twice the angle of incidence (Figure 1a).
Figure 1 – X-ray diffraction by a crystal lattice. (a) An incoming X-ray will be scattered by lattice point A at an angle twice the glancing angle (θ). (b) Multiple lattice planes with spacing $d$ will scatter incoming X-rays by an angle θ according to Bragg’s Law (equation 3.1).

Constructive interference occurs between waves scattered at points A and B if the distances AC and DB are equal. Successive planes also scatter in phase (Fig 1b) on the condition that the path difference is an integral number of wavelengths, i.e. obeys Bragg’s law:

$$2d \sin \theta = n\lambda$$  \hspace{1cm} (3.1)

where $d$ is the spacing of the planes, $\theta$ is the glancing angle, $\lambda$ is the wavelength of the scattered X-ray and $n$ is an integer.

Diffraction from any set of lattice planes can only occur at the angles predicted by Bragg’s law. The peaks (referred to as reflections) observed in diffraction patterns are labelled using Miller indices $(h k l)$ with higher order reflections labelled $(nh nk nl)$. Figure 2 illustrates a typical XRD pattern expected for magnetite.

Figure 2 – Typical X-ray diffraction pattern. (a) XRD of magnetite, showing reflections corresponding to Miller indices $(111)$, $(220)$, $(311)$, $(400)$, $(511)$ and $(440)$. (b) Fitting of the reflections with a Lorentzian curve is used to determine average crystallite particle size.
Analysis of the height and broadness of the peaks provides information related to the average crystallite diameter of spherical nanoparticles through fitting of a Lorentzian curve onto the diffraction peaks. This is used to determine the linewidth at full width half maximum (β) and the Bragg angle (θ). These parameters can then be inserted into the Scherrer equation (3.2)\(^1\),\(^2\) to determine particle size:

\[
d = \frac{K\lambda}{\beta \cos \theta}
\]

Where \(d\) is the average crystallite particle size, \(K\) is the shape factor and \(\lambda\) is the X-ray wavelength.

### 3.2. Synchrotron Analysis

Synchrotron based techniques are often used for a variety of different disciplines including condensed matter physics, biology, medicine and materials science. Synchrotron radiation is produced in the form of X-rays which are emitted by a beam of electrons which are constantly accelerating to speeds close to the speed of light in a circular path. X-rays (photons) are directed using strong magnetic fields along a branch line to an end station of which many are situated at angles to the storage ring. The interaction and absorption of the X-rays with a sample can be used to give detailed spectroscopic information such as components of the crystal structure on a nanometre scale, as discussed below.

#### 3.2.1. X-ray Absorption and X-ray Magnetic Circular Dichroism

X-ray absorption (XAS) and X-ray magnetic circular dichroism (XMCD) are synchrotron based techniques which provide the ability to determine the molecular and magnetic structure of magnetic nanoparticles such as magnetite. The techniques were used extensively in all four research chapters in this thesis to determine changes in the cation occupancy of biogenic magnetite. Measurements were carried out on beamlines 4.0.2 and 6.3.1 at the Advanced Light Source (Berkeley, CA, USA). The specific methods of preparation and analysis used for these techniques have been discussed in each of the research papers; an overview of the techniques and derivation of the spectra is provided in this section.
X-ray absorption occurs when an X-ray photon has sufficient energy to excite a core electron (1s, 2s, 2p, etc.) in an atom into a higher energy orbital. These absorption events are detected and recorded, producing an absorption spectrum related to energy (see Figure 4) and these form the basis for the development of an XMCD spectrum. The physical origin of XMCD is defined by the two-step (one electron) model, first proposed by Stöhr and Wu. The focus of this research thesis has been on the \( L_{2,3} \) absorption edges of Fe, Co, Zn and Cr which are a result of the transitions between \( p \) and \( d \) orbitals. The first step of the model concerns the excitation of electrons (of a magnetically polarised material) from the spin-orbit \( 2p_{3/2} \) and \( 2p_{1/2} \) energy levels into empty \( 3d \) valence levels (Figure 3). The excitation process depends upon the polarization of the photon used to excite an electron from one level to another. Due to selection rules, an absorbed photon with circular polarization (helicity) will transfer its angular momentum to the electron which is then transferred to the spin of that electron. Reversing the direction of the circular polarization reverses the sign of the photon angular momentum and hence transfers the opposite amount to the electron spin. The amount of polarisation depends upon the helicity of the photon and the elemental edge under consideration. For an \( L_2 \) edge, left circularly polarised light (LP) will excite 1/4 spin up electrons and 3/4 spin down electrons from the \( 2p \) shell into unoccupied \( 3d \) states. The relative amounts for excitations are reversed for right circularly polarised light (RP), (i.e. 3/4 spin up and 1/4 spin down electrons are excited). For the \( L_3 \) edge, LP light excites 5/8 spin up electrons and 3/8 spin down electrons with the reverse applying for RP.
Figure 3 - Energy diagram outlining the principle of XMCD. (a) Non-magnetically polarized sample, excitation of spin-up and spin-down electrons into equal number of empty valence states. (b) Magnetically polarised sample, imbalance in empty valence states produces different absorption spectra (i.e. XMCD).

The second step of the model is concerned with the excited state. If the number of empty spin up and spin down states in the valence state are identical, LP and RP photons will be absorbed identically. However in magnetic materials, the two states are not the equal and the distribution of empty valence holes is different. Spin flipping is not possible during the excitation of electrons between 2p and 3d levels and consequently spin-up polarised electrons will only fill spin-up empty valence sites. Spin-down polarized electrons will only fill spin-down empty valence sites. The splitting of valence states thus acts as a spin dependent detector resulting in transition intensities (i.e. absorption) which are proportional to the number of empty states of a given spin. From this principle, a material such as magnetite, in which tetrahedral and octahedral lattice sites interact so as so have opposite spin orientations will produce different absorption spectra depending upon the helicity of the incoming photon (Figure 4a). The difference between the two spectra is defined as the XMCD (Figure 4b). The effect is dependent upon the cosine of the angle between photon direction and magnetization direction, hence is at maximum when the magnetization direction is parallel, or anti-parallel to the direction of the photon beam.

Changing the direction of magnetization is directly equivalent to reversing the helicity of the photon\(^4\). This is often a convenient approach and used on synchrotron
beamlines which do not have an undulator that is required to reverse the polarity of the light. This method of producing an XMCD was applied throughout this project with magnetic fields of +/- 0.6 T used to yield the different XAS spectra corresponding to spin-up and spin-down magnetization directions.

Figure 4 - XAS and XMCD Spectra. (a) XAS spectra obtained using left circularly polarised photons (red) and right circularly polarised photons (blue). The effect can also be produced by the application of +0.6 T (red) and -0.6 T (blue) magnetic fields parallel and anti-parallel to the direction of the beam. (b) Difference between two XAS spectra produces XMCD spectra. (c) Fitting of the XMCD provides data corresponding to Fe$^{2+}$[B] (Green), Fe$^{3+}$(A) (Red) and Fe$^{3+}$[B] (Blue).

Quantitative analysis of the iron XMCD spectra can be provided by comparison of collected data to data calculated for cation occupancy in ferrite spinels. For example, in magnetite each cation (Fe$^{2+}$[B], Fe$^{3+}$(A) and Fe$^{3+}$[B]) is fitted to atomic multiplet calculations (Figure 4c) to determine the relative occupancy of each$^{5,6}$. Stoichiometric magnetite contains a distribution of cations in a ratio of 1:1:1 (Fe$^{2+}$[B]:Fe$^{3+}$(A):Fe$^{3+}$[B]), hence variations due to the incorporation of dopants such as cobalt or zinc or vacancies are easily detectable as changes from this ratio.
3.2.2. Scanning Transmission X-ray Microscopy

Scanning Transmission X-ray Microscopy (STXM) is a synchrotron based technique which is used for the chemical analysis of a material at scales down to 20nm. It can be thought of as a combination of transmission electron microscopy (TEM) and chemical spectroscopies such as infrared or nuclear magnetic resonance (NMR). TEM is able to provide excellent spatial resolution images in order to visualise a sample, however chemical sensitivity is limited to elemental level mapping. Chemical spectroscopies however are unable to provide direct imaging of a material, but can provide information on elemental distribution and oxidation state.

STXM usually uses soft X-rays (absorption range 100 – 2000 eV) to map chemical changes in samples in vacuum or at atmospheric pressure and can be used to measure dry samples, hydrated polymers or biological material. X-rays produced in a synchrotron are passed through a monochromator before being passed through a Fresnel zone plate (Figure 5a). The zone plate is used to focus a coherent beam of X-rays on to a spot (pixels) on the sample plane with unwanted diffraction orders filtered out by a pinhole called the order sorting aperture (OSA). The transmitted X-rays from each spot are then detected and collected over an energy range, producing an absorption spectrum. The spatial results are combined to produce a complete picture in which each pixel contains distinct spectrum.

![Figure 5 - STXM experimental arrangement (a) Zone plate filters X-rays onto the sample, with unwanted diffraction orders filtered by the order sorting aperture (OSA). Spectra are collected in pixels (down to 20nm) with multiple pixels measured over a sample. (b) Set up of sample holder with magnets for collection of STXM - XMCD.](image)

Recently this technique has been demonstrated in combination with XMCD to produce spatially resolved images with detailed spectra corresponding to each pixel. This is achieved through fixing a magnetic field and changing the helicity of the
incoming X-rays, rather than changing the applied magnetic field polarization. Scans were measured using left circularly polarised and right circularly polarised X-rays with the sample magnetized with the fixed field. Figure 5b shows the sample holder with fixed magnetic field surrounding the sample grid. This has enabled, for instance, the production of an XMCD spectrum on individual magnetosomes contained within the magnetotactic bacterium marine Vibrio strain MV-1 (Figure 6).

Figure 6 - Example of the use of STXM to investigate changes in the oxidation state of magnetite produced within the magnetosomes of magnetotactic bacterium marine Vibrio strain MV-1. (a) Spatially resolved organic matter measured before the C K-edge 280 eV (red) and just above the C K-edge 288eV (blue). Many curved-rod bacteria are observable (blue), with residual salts from sample preparation also present (red). (b) Enhanced image used to distinguish organic matter (green) from iron magnetosomes (blue). (c) Spectra collected per pixel allow for the formation of spatially specific XAS and XMCD, in this example used confirm the XMCD spectra expected of magnetite.

The application of STXM is described in Chapter 8 where it is used to investigate the changes in oxidation state of iron in magnetite nanoparticles produced by the iron reducing bacterium Shewanella oneidensis. The aim of this work was to combine electron microscopy with XMCD techniques and spectroscopic chemical analysis of the bacteria. The research was carried out on the soft X-ray spectromicroscopy (SM) beamline BL 10ID1 of the Canadian Light Source (CLS), Saskatoon, Canada.
3.3. Mössbauer

Mössbauer spectroscopy is a technique commonly used to identify iron oxide minerals. The principle of the technique relies upon the resonant absorption of gamma rays (γ-rays) produced by the decay of a $^{57}$Co source by an $^{57}$Fe nucleus in a solid iron mineral. The general experimental setup of a Mössbauer spectrometer comprises a source, sample (absorber) and a detector. The source is oscillated about its initial position to produce γ-rays within a narrow range of photon energies by exploitation of the Döppler effect. The total absorbance of the emitted γ-rays by the sample is measured and recorded as a function of the mean velocity (mm s$^{-1}$) of movement of the source.

In the simplest example of a Mössbauer spectrum, γ-rays are absorbed by a sample when the energy of the incoming photons is resonant with the $^{57}$Fe nucleus, resulting in a peak at 0 mm s$^{-1}$. In reality, the electronic and magnetic environments of nuclei are not so simple and the Mössbauer spectra are split into different peaks (Figure 7).

![Mössbauer Spectroscopy Diagram](image)

**Figure 7 – Nuclear energy levels.** (Image from Cornell and Schwertmann – The Iron Oxides)³ (a) Isomer shift (δ) is observed as a deviation of the absorption spectra from 0 mm s$^{-1}$. (b) Quadrupole splitting Δ$E_q$ is the result of non-symmetrical charge distribution around a nucleus. (c) The hyperfine field $B_{hf}$ is observed in samples with magnetic ordering.
The different peaks observable in Mössbauer spectra are dictated by three parameters. These include the isomer shift, electronic quadrupole interaction (quadrupole splitting) and magnetic hyperfine field:

### 3.3.1. Isomer shift (δ)

The isomer shift is an observable shift in the absorption lines of Mössbauer spectra from zero (Figure 8) as a result of the interaction of part of the electronic cloud of an atom with the nucleus. δ provides information about the electron density at the nucleus and chemical changes in the atom, or lattice and can be interpreted mathematically as:

\[
\delta = \left( \frac{2}{3} \right) \pi Z e^2 (\rho_A - \rho_s) (R_e^2 - R_g^2)
\]

where \(\rho_A\) and \(\rho_s\) are the charge densities of the absorber and source respectively. All other terms are constant between samples except for \((R_e^2 - R_g^2)\) which describes the difference between the radius of the excited nucleus \(R_e\) and the nucleus in the ground state \(R_g\). For the example of magnetite, ferrous iron \(\text{Fe}^{2+}\) has six electrons in its valence \(d\) orbital, one more than ferric \(\text{Fe}^{3+}\). This extra \(d\)-electron contributes to the shielding of \(s\)-electrons from the nucleus, hence leads to a decrease in the contribution to the charge density at the nucleus of the sample (absorber) \(\rho_A\). Combined with the fact that the nuclear factor \((R_e^2 - R_g^2)\) is negative for \(^{57}\text{Fe}\), the isomer shift for \(\text{Fe}^{2+}\) is larger than that of \(\text{Fe}^{3+}\). This is an important property for the determination of site location of transition metal dopants such as cobalt as seen in Chapter 6. In that example, the average isomer shift of the octahedral component measured on a series of cobalt doped magnetite samples of the form \(\text{Co}_x\text{Fe}_{3-x}\text{O}_4\) decreases with increasing \(\text{Co}^{2+}\) concentration, indicating that \(\text{Co}^{2+}\) substitutes octahedral \(\text{Fe}^{2+}\) within the magnetite crystal structure.

### 3.3.2. Electric quadrupole interaction (Δ\(E_q\))

The quadrupole interaction is generated when an electric field gradient acts on the nucleus resulting in a non-symmetrical charge distribution. In minerals, the local point symmetry of electrons at a nucleus is rarely cubic and the interaction of nuclear quadrupole moment with the field gradient leads to a split in the nuclear energy levels.
(Figure 7b). This is denoted the quadrupole splitting $\Delta E_q$ and provides information about the site distortion of the nucleus.

### 3.3.3. Magnetic hyperfine field ($B_{hf}$)

In magnetically ordered materials, such as magnetite, a magnetic hyperfine interaction develops which leads to the splitting of the nuclear energy levels into six different levels (Figure 7c). $I=1/2$ splits into two sublevels and $I=3/2$ splits into four sublevels. There are now six possible transitions of electrons from the ground state ($I=1/2$) to excited state ($I=3/2$), corresponding to six different peaks observable on the Mössbauer spectra. The intensities of the peaks are different, with the area ratios of the six lines in proportion to Clebsh-Gordan coupling coefficients $(3:2:1:1:2:3)^9$.

The interpretation of these three parameters is crucial in determining the changes in the iron oxide which may be present in a sample. Figure 8 indicates a typical Mössbauer spectrum observable for a sample of magnetite at room temperature. The material displays hyperfine field structure (sextet) as would be expected from the magnetic material. It is also possible to distinguish two overlapping sextets. These two sextets correspond to the tetrahedral (A) site environment of Fe$^{3+}$(A) cations and octahedral [B] site (Fe$^{2+}$Fe$^{3+}$)[B] cations. Changes in the sample with the introduction of dopants such as cobalt or zinc leads to changes in the spectra and their interpretation is discussed in further detail in Chapters 5 and 6.

Line fitting is carried out using Lagarec/Rancourt Recoil software (Intelligent Scientific Applications Inc.) to determine the parameters. Spectra are fitted using Lorentzian line shape symmetrical doublets/sextets with isomer shift data calibrated with reference to metallic Fe foil.
Figure 8 – Typical room temperature Mössbauer spectra observed for a sample of magnetite. Two distinct sextets can be observed corresponding to tetrahedral [A] site (Red) and octahedral [B] sites (Blue). The sum of the two spectra results in the best fit (Green line) of the data (Circular points).

Fitting is used to determine \( \delta \), \( \Delta E_q \) and \( B_{hf} \) parameters. Iron oxides can be identified using these parameters with significant changes observable for different minerals. Tables such as those indicated in Table 1 are used to identify the iron mineral present based upon the parameters obtained by fitting spectra.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>( B_{hf} )</th>
<th>( \delta/\text{Fe} )</th>
<th>( \Delta )</th>
<th>( B_{hf} )</th>
<th>( \Delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Room temperature</td>
<td>4.2 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematite</td>
<td>51.8</td>
<td>0.37</td>
<td>−0.20</td>
<td>53.5</td>
<td>−0.20</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>or 54.2(^b)</td>
<td></td>
</tr>
<tr>
<td>Magnetite</td>
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<td>0.26</td>
<td>≤0.02</td>
<td>50.6</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>46.1</td>
<td>0.67</td>
<td>≤0.02</td>
<td>36−52(^a)</td>
<td>1.18−(−0.79)</td>
</tr>
<tr>
<td>Maghemite</td>
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<td>0.23</td>
<td>≤0.02</td>
<td>52.0</td>
<td>≤0.02</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.35</td>
<td>≤0.02</td>
<td>53.0</td>
<td>≤0.02</td>
</tr>
<tr>
<td>Goethite</td>
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<td>−0.26</td>
<td>50.6</td>
<td>−0.25</td>
</tr>
<tr>
<td>Akaganèite</td>
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<td>0.55</td>
<td>47.8</td>
<td>48.9</td>
<td>−0.02</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.95</td>
<td>48.9</td>
<td>48.9</td>
<td>−0.02</td>
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<tr>
<td>Lepidocrocite</td>
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<td>0.53</td>
<td>45.8</td>
<td>45.8</td>
<td>0.02</td>
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<tr>
<td>Feroxylyte</td>
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<td>−0.06</td>
<td>53</td>
<td>53</td>
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</tr>
<tr>
<td>Ferrhydrite</td>
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<td>0.62(^a)</td>
<td>59(^a)</td>
<td>−0.07</td>
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<tr>
<td></td>
<td>0.35</td>
<td>0.78(^a)</td>
<td>47(^a)</td>
<td>−0.02</td>
<td></td>
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<tr>
<td>Bematite</td>
<td>41.5</td>
<td>0.38</td>
<td>≤0.01</td>
<td>56.2</td>
<td>≤0.01</td>
</tr>
</tbody>
</table>

Table 1 - Mössbauer parameters of iron oxides and iron oxyhydroxides. \( B_{hf} \) is reported in Tesla T, \( \delta \) and \( \Delta E_q \) are in mm s\(^{-1}\). (Table extracted from Murad 2010)\(^a\).

\(^a\) Value is approximate.
3.4. Chemical Assays

Several assays were used throughout the course of this work which required the use of spectrophotometry to measure the absorbance of UV light (at specified wavelengths) by an aqueous sample in a cuvette. The technique is able to provide information on concentrations of a coloured compound or bacterial biomass by reference to a known standard. The following assays were used:

3.4.1. Ferrozine

Ferrozine assay is used to measure the concentration of aqueous Fe$^{2+}$ in solution. The technique was used in Chapter 7 to determine the rate of Fe$^{3+}$ reduction by the bacterium Geobacter sulfurreducens. Fe$^{2+}$ reacted with Ferrozine solution (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium solution 1g L$^{-1}$; HEPES 11.9 g L$^{-1}$; pH=7) is observable as a purple solution which is quantified by measurement at an absorption wavelength of 562 nm.

Samples are measured by dissolving in 0.5 M HCl (0.1ml sample, 4.9 ml HCl) for 1 hr to release surface Fe$^{2+}$ into solution. 50 μl of this is reacted with Ferrozine solution and the spectrum measured. The absorbance is converted into concentration (mM) by comparison against a standard curve produced from a series of solutions reacted with Ferrozine solution which contain known concentrations of Fe$^{2+}$ in solution, (i.e. 1 mM, 5 mM, 10 mM, 20 mM and 50 mM).

3.4.2. Protein Assay

The amount of bacteria present in solution (biomass) is most easily determined as the optical density (OD$_{600}$) which corresponds to the absorbance of 600 nm light by the sample. This value is then quantified by the amount of protein (mg ml$^{-1}$) present in the solution which corresponds to approximately 50% of the dry weight mass of the bacteria.

A solution of Bicinchoninic acid (BCA) is mixed with Cu(II)SO$_4$ solution in a ratio of 50:1 (BCA:Cu(II)). A standard curve is prepared containing known concentrations of Bovine Serum Albumin (BSA) and reacted with the BCA-Cu(II) solution in a ratio of 1:100 BSA:BCA-Cu(II) with absorbance measured at 562 nm. Samples with a known
OD$_{600}$ are also reacted with BCA-Cu(II), (562 nm; 1:100) to determine amount of protein present in solution. This technique allows for the direct conversion of OD$_{600}$ values into mg protein ml$^{-1}$.

3.4.3. DPC

DPC assay$^{12}$ is used to determine the concentration of chromate (Cr(VI)) present in solution. The technique was used in remediation experiments in Chapter 4 and 7 to determine the amount of reduction/sequestration of a solution of Cr(VI) to Cr(III).

The assay is prepared by dissolving 37.5 mg 1,5-diphenylcarbazide (DPC) in 7.5 ml acetone, 7.5 ml d.H$_2$O and 1.25 ml 3M HCl. This stock solution is diluted 1:25 in d.H$_2$O and is used as the reagent. Samples were extracted from experiments and centrifuged at 14000 rpm to separate solid material from aqueous Cr(VI). The liquid phase was reacted with dilute DPC solution (1:15) and left for 15 min for development of colour. The absorbance was measured at 540 nm and compared against a standard curve prepared which contained known concentrations of Cr(VI), (i.e. 1 μM, 5 μM, 10 μM, 20 μM and 50 μM).

3.5. Superconducting quantum interface device

Superconducting quantum interface device (SQUID) magnetometry is a key component in the understanding of magnetic materials and is able to detect very small magnetic fields, as low as 10$^{-14}$ T. The magnetometer exploits a quantum tunnelling effect which is observed when two superconductors are separated by a thin insulating layer (Josephson junction). In a DC SQUID, two Josephson junctions are aligned parallel in a superconducting loop, with the presence of a magnetic field resulting in the emergence of a detectable voltage across the junction.

Using SQUID, measurements can be made to determine some of the magnetic properties of samples through the formation of a hysteresis loop (Figure 9). These include the coercivity ($H_c$), remanence ($M_r$) and saturation magnetization ($M_s$). $H_c$ describes the magnetic field required to reverse the magnetization of the sample. $M_r$ denotes the residual magnetization present in a sample after an applied field is removed. Saturation magnetization, $M_s$, describes the maximum magnetization
achievable by a sample in which all magnetic moments are aligned in the same direction. Superparamagnetic samples have zero coercivity or remanence and exhibit paramagnetism in the absence of a magnetic field.

The magnetic properties of materials are temperature dependent with some samples that appeared superparamagnetic at room temperature exhibiting coercivity at low temperature. This occurs when a sample is cooled through its blocking temperature ($T_B$) and the internal magnetic moments have insufficient thermal energy to orientate randomly.

![Magnetic hysteresis loops](image)

Figure 9 – Magnetic hysteresis loops corresponding to ferro/ferrimagnetism (red, $H_c>0$) and superparamagnetism (black, $H_c=0$). Superparamagnetic materials exhibit no coercivity.

Samples measured using this technique were prepared by drying in an anoxic cabinet and then constrained in eicosane. Hysteresis loops were obtained at room temperature (300 K) and low temperature (5 K) under the application of a magnetic field. Measurement at these temperatures allowed for hysteresis loops to be collected above and below $T_B$ for many different samples. Further details of the analysis of magnetite nanoparticle samples using SQUID can be read in Chapters 4, 5 and 6.

### 3.6. References


Control of nanoparticle size, reactivity and magnetic properties during the bioproduction of magnetite by *Geobacter sulfurreducens*

Control of nanoparticle size, reactivity and magnetic properties during the bioproduction of magnetite by *Geobacter sulfurreducens*

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Abstract

The bioproduction of nanoscale magnetite by Fe(III)-reducing bacteria offers a potentially tunable, environmentally benign route to magnetic nanoparticle synthesis. Here, we demonstrate that it is possible to control the size of magnetite nanoparticles produced by *Geobacter sulfurreducens* by adjusting the total biomass introduced at the start of the process. The particles have a narrow size distribution and can be controlled within the range of 10–50 nm. X-ray diffraction analysis indicates that controlled production of a number of different biominerals is possible via this method including goethite, magnetite and siderite, but their formation is strongly dependent upon the rate of Fe(III) reduction and total concentration and rate of Fe(II) produced by the bacteria during the reduction process.

Relative cation distributions within the structure of the nanoparticles have been investigated by x-ray magnetic circular dichroism and indicate the presence of a highly reduced surface layer which is not observed when magnetite is produced through abiotic methods. The enhanced Fe(II)-rich surface, combined with small particle size, has important environmental applications such as in the reductive bioremediation of organics, radionuclides and metals. In the case of Cr(VI), as a model high-valence toxic metal, optimized biogenic magnetite is able to reduce and sequester the toxic hexavalent chromium very efficiently to the less harmful trivalent form.

1. Introduction

Nanometre sized particles have unique applications in areas ranging from computer technology, waste water cleanup, catalysis and medical treatments such as cancer therapy and drug delivery [1–6]. Magnetic nanoparticles (MNPs), in particular magnetite (Fe₃O₄), offer some of the most promising alternatives to conventional therapeutic agents due to their small size and intrinsic magnetic properties. The ability to control and manipulate these properties would allow for the tailoring of the particles to specific applications. For example in biomedicine, superparamagnetic iron oxide nanoparticles (SPIONs) with diameters below ~20 nm are best suited to applications such as cancer therapies and magnetic resonance imaging (MRI).

Many synthetic approaches to magnetite formation require high temperatures and toxic reagents, which are both expensive and environmentally undesirable. The control of particle
size using some of these techniques often requires convoluted processes and the use of organic reagents such as solvents [7] which renders them unsuitable for insertion into the human body. This paper presents an alternative approach to the production and precise control of the particle size of magnetic nanoparticles using the subsurface Fe(III)-reducing bacterium *Geobacter sulfurreducens*, which is able to generate large amounts of extracellular magnetite at ambient temperatures.

Metal-reducing bacteria conserve energy for growth in anoxic environments through the oxidation of an electron donor (such as organic matter) coupled with the reduction of oxidized metal cations, for example Fe$^{2+}$ in compounds including Fe(III)-oxyhydroxides. The reduction of solid-phase Fe(III) leads to the release of soluble Fe$^{2+}$ which recrystallizes into new mineral phases ranging from goethite, magnetite, siderite to vivianite depending on the conditions of formation such as buffer, pH and temperature [8, 9].

Magnetite has an inverse spinel structure, with chemical formula (Fe$^{3+}$)$_4$Ti$_2$(Fe$^{3+}$)$_2$Fe$^{2+}$O$_8$ which consists of tetrahedral (T$_d$) and octahedral (O$_h$) sites. In stoichiometric form, Fe$^{3+}$ occupies both sites equally, while Fe$^{2+}$ is only situated in the octahedral sites, resulting in a distribution of 1:1:1 at the Fe$^{3+}$:$\text{Fe}$:$\text{O}_h$ sites. Antiparallel magnetic moments of the Fe$^{3+}$ cations on the T$_d$ and O$_h$ sites cancel out, resulting in zero net magnetization, whereas the Fe$^{2+}$ moments at the O$_h$ sites have no magnetic opponents, resulting in the ferrimagnetism exhibited by magnetite at room temperature.

In addition to the magnetic properties, Fe$_3$O$_4$ MNPs also have important uses due to external reactivity created by a large surface/volume ratio. In this study the variation in the reactivity of the nanoparticles was investigated through reaction with potassium chromate (K$_2$CrO$_4$). The reduction and immobilization of highly soluble, potentially carcinogenic Cr(VI) to less harmful Cr(III) has many important environmental consequences, particularly in ground waters that contain high concentrations of Cr(VI) due to industrial or mining processes. Fe(II)-bearing minerals, such as magnetite, have been shown to offer a potential route for chromium remediation [10] through the reduction of Cr(VI) and incorporation of the Cr(III) cations into octahedral sites of the magnetite spinel structure [11].

This study demonstrates the manipulation of the size of biogenic magnetite particles through the careful control of biomass levels during the conversion of Fe(III)-oxyhydroxides to magnetite by whole cells of *Geobacter sulfurreducens* [12]. Crystallographic properties such as lattice structure and site occupancies of the iron within the biogenic magnetite have been probed using a variety of techniques such as powder x-ray diffraction (PXRD), transmission electron microscopy (TEM), x-ray absorption spectroscopy (XAS) and x-ray magnetic circular dichroism (XMCD). The bulk magnetic signatures of the samples were investigated using superconducting quantum interference device (SQUID) magnetometry. The potential for contaminant reduction was also measured using a Cr(VI) assay supported by inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis.

![Figure 1](image-url) (a) X-ray diffraction patterns observed for samples prepared with AQDS for different optical densities (OD$_{600}$) of initial bacterial biomass. Different mineral phases formed depending on the OD$_{600}$, including goethite, magnetite and siderite. (b) Mean crystallite size for samples produced with and without the mediator AQDS, determined from the (3 1 1), (2 2 0) and (4 4 0) reflections of magnetite (error bars were determined from the standard deviation obtained by fitting the three peaks).

### 2. Results and discussion

PXRD was used to determine the changes in particle size and iron mineral phases that were produced through the reduction of Fe(III)-oxyhydroxide inoculated with biomass between OD$_{600}$ values of 0.015 and 4, corresponding to 0.005 and 1.5 mg ml$^{-1}$ cellular protein, respectively. OD$_{600}$ corresponds to the optical density of the culture in growth medium as measured by the absorption of light by a cell suspension at a wavelength of 600 nm. The conversion of OD$_{600}$ values to protein content (mg ml$^{-1}$) is detailed in section 4 of this paper. These experiments were conducted in the presence of the electron shuttling compound and humic analogue anthraquinone-2,6-disulfonate (AQDS) (figure 1a). Goethite ($\alpha$-FeOOH) was the predominant end product at low concentrations of biomass, with magnetite formed at intermediate concentrations, and evidence of siderite (FeCO$_3$) formation at the highest biomass concentrations. The sample with OD$_{600} = 0.2$ (0.06 mg ml$^{-1}$ protein) displays peaks corresponding to both magnetite and goethite. Magnetite is the only crystalline material observed within the range 0.4 $\leq$ OD$_{600} < 1.0$, with a reflection at $26 \approx 32^\circ$ representing siderite formation at the highest optical densities (OD$_{600} = 2$ and 4). It should be noted that in an analogous experiment, run simultaneously without AQDS, magnetite did not form until higher optical density values (OD$_{600} = 0.4$) and there was no evidence of siderite formation at OD$_{600} = 4$. These results lend support to the suggestion that the mechanism underpinning iron biomineral formation is strongly dependent upon the rate of Fe(III)-reduction as AQDS greatly increases reduction rates [13], as do increased biomass (i.e. biocatalyst) levels. This may also have a bearing on the production of additional mineral phases such as goethite and siderite which are have been shown to be produced in Fe$^{2+}$ poor
Figure 2. Transmission electron microscopy images of magnetite nanoparticles produced by *Geobacter sulfurreducens* with the electron shuttle AQDS: (a) $OD_{600} = 0.4$, (b) $OD_{600} = 1$, (c) $OD_{600} = 2$ and (d) $OD_{600} = 4$. Inset graphs indicate the differences in particle size distributions.

PXRD results reveal that the goethite and magnetite peaks became progressively broader and shorter as the biomass density used to catalyse Fe(III) reduction increased, concomitant with a decrease in the size of the crystallite obtained as an end product. The results of the size analysis using the magnetite (3 1 1), (2 2 0) and (4 4 0) reflections with and without the addition of AQDS in the starting culture are shown in figure 1(b). The crystallite size decreased as the $OD_{600}$ increased both with and without the electron shuttle. For the samples including the electron shuttle AQDS, the change of particle size with respect to optical density appears to be rapid before reaching a minimum size limit of $\sim 10 \text{ nm}$ at $OD_{600} = 2$. In the samples without AQDS, a limit was not reached, although the rate of decrease in crystallite size was decreasing with increasing $OD_{600}$ and extrapolation would suggest a similar lower size limit.

Images produced using TEM for biomass levels corresponding to $OD_{600} = 0.4$, 1, 2 and 4 are presented in figures 2(a)–(d), respectively. These images confirm that the magnetite particles produced by $OD_{600} = 0.4$ are much larger and show more polydispersion of size than the other samples, with a mixture of particle shapes including spherical and square. Samples with higher biomass produced progressively smaller particles which were predominantly spherical and had a narrow size distribution. Goethite was not visible in any of the samples, either by examination of the images or through selective area electron diffraction (SAED), in keeping with the PXRD data. There was also no visible evidence of any siderite in samples $OD_{600} = 2$ and 4, which was visible in the PXRD analysis, suggesting that it is a minor phase.

Size distributions were determined for the $OD_{600} = 1$, 2 and 4 cultures, with 200 measurements applied for each (insets figures 2(b)–(d)). The resulting distributions closely followed a log-normal function, for which a fit was applied to determine mean particle size and most probable particle size. The distributions observed are very narrow, an important property for technological applications, and the range reduces as the mean particle diameter decreases, as indicated by the dispersion index $\sigma_d$ (table 1). The mean diameters, as
Table 1. Structural and magnetic properties of biogenic magnetite produced at different biomass concentrations (OD₆₀₀). Results from TEM obtained using sample size n = 200 (n = number of particles).

<table>
<thead>
<tr>
<th></th>
<th>OD₆₀₀ = 0.4 (0.14 mg ml⁻¹)</th>
<th>OD₆₀₀ = 1 (0.38 mg ml⁻¹)</th>
<th>OD₆₀₀ = 2 (0.76 mg ml⁻¹)</th>
<th>OD₆₀₀ = 4 (1.5 mg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRD Crystallite size (nm)</td>
<td>34.8 ± 5.2</td>
<td>13.7 ± 0.9</td>
<td>13.3 ± 0.6</td>
<td>12.1 ± 1.8</td>
</tr>
<tr>
<td>TEM Mean diameter (d) (nm)</td>
<td>—</td>
<td>13.9 ± 0.3</td>
<td>13.8 ± 0.2</td>
<td>11.5 ± 0.1</td>
</tr>
<tr>
<td>TEM Most probable diameter dₚ (nm)</td>
<td>—</td>
<td>13.1 ± 0.3</td>
<td>13.3 ± 0.2</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>TEM Dispersion index σₜ₀</td>
<td>0.25</td>
<td>0.21</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>SQUID Saturation mag. Mₛ (emu g⁻¹) at 300 K</td>
<td>79.2</td>
<td>69.9</td>
<td>59.6</td>
<td>54.8</td>
</tr>
<tr>
<td>SQUID Saturation mag. Mₛ (emu g⁻¹) at 5 K</td>
<td>87.7</td>
<td>79.3</td>
<td>68.2</td>
<td>62.9</td>
</tr>
<tr>
<td>SQUID Coercivity, H_c (Oe) at 300 K</td>
<td>33</td>
<td>9</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>SQUID Coercivity, H_c (Oe) at 5 K</td>
<td>268</td>
<td>303</td>
<td>281</td>
<td>276</td>
</tr>
<tr>
<td>SQUID Mean particle diameter (d) (nm)</td>
<td>—</td>
<td>9.1</td>
<td>9.6</td>
<td>9.5</td>
</tr>
<tr>
<td>SQUID Dispersion index σₜ₀</td>
<td>—</td>
<td>0.29</td>
<td>0.3</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Figure 3. SQUID magnetometry of samples OD₆₀₀ = 0.4, 1, 2 and 4 providing measurements of (a) saturation magnetization, (b) zero-field cooled (ZFC) and field cooled (FC, at H = 100 Oe) data, (c) hysteresis loops indicating the coercivity H_c of the samples and (d) particle size distributions obtained through fitting by Langevin functions.

In both thin film and nanoparticle formation, the dominant processes controlling grain size and particle size are crystal growth and nucleation rates [15–17]. The mean grain or particle size (D) is related to both of these parameters by $D = 1.203\sqrt{G/N}$, where G describes the growth rate and N the nucleation rate. Assuming a constant crystal growth rate, the mean particle size is inversely proportional to the cube root of the nucleation rate, i.e. the higher the nucleation rate the smaller the particles formed. However, for a constant nucleation rate the particle size varies in proportion to the crystal growth rate. The growth rate of the magnetite is thought to be limited by the thermodynamics of crystal formation, which in these experiments is expected to be relatively constant. In this study, only the biomass levels have been varied, which in turn should lead to an increase in the rate and total amount of Fe(II) produced within the system at higher biomass levels, hence increasing the probability of nucleation sites being formed. An increased nucleation rate caused by the raised biomass concentration would explain the decrease in particle size observed, in accord with the equation given above.

Changes in magnetization across the samples were measured by SQUID magnetometry (figure 3). Ferromagnetic and ferrimagnetic particles become superparamagnetic when their size reaches a critical length, typically below ∼30 nm for magnetite [18], at which point the particle’s magnetic moment direction varies randomly due to thermal fluctuations.
leading to a vanishing magnetic remanence and coercivity. The smaller particles produced in this investigation (OD<sub>600</sub> = 1, 2, 4) all have an average size well below this threshold, and their hysteresis loops (figure 3(a)) display the characteristic features of superparamagnetism, such as a negligible coercivity (<10 Oe). The plots also show that the saturation magnetization increases with average particle size from M<sub>sat</sub> = 62.9 emu g<sup>-1</sup> for OD<sub>600</sub> = 4 at 5 K, to 87.7 emu g<sup>-1</sup> for OD<sub>600</sub> = 0.4. Bulk magnetite is observed to have M<sub>sat</sub> ∼ 92 emu g<sup>-1</sup> [19], which is much larger than that observed for the smallest particles and roughly comparable to the larger particles (OD<sub>600</sub> = 0.4). This effect is dependent upon a number of factors, including changes in particle size, the presence of mineral phases such as goethite and siderite, and changes in stoichiometry (relative ratio between Fe<sup>3+</sup> and Fe<sup>2+</sup> cations) [20, 21].

The temperature above which thermal energy inside a magnetic system is high enough to enable the free alignment of the magnetization in arbitrary directions is called the blocking temperature (T<sub>B</sub>) and can be observed from the zero-field cooled (ZFC) and field cooled (FC) magnetization curves (figure 3(b)). The blocking temperature is graphically determined to be the point at which the gradient of the ZFC curve approaches zero. It can be seen that T<sub>B</sub> decreases with decreasing particle size, ranging from ∼290 K (OD<sub>600</sub> = 1) to ∼260 K (OD<sub>600</sub> = 2 and 4). The largest particles (OD<sub>600</sub> = 0.4) have a higher blocking temperature and T<sub>B</sub> is not well defined but appears to be just above the highest measurement of 300 K. Examination of the TEM image corresponding to this sample (figure 2(a)) reveals a number of different sized particles with several distinct shapes, hence it could be anticipated that some of these different particles will not exhibit superparamagnetism at room temperature, and are in fact above the threshold of ∼30 nm for superparamagnetic behaviour. This also explains why a residual coercivity seen in figure 3(c) is greater for the OD<sub>600</sub> = 0.4 sample than for the other three samples. This indicates that there is an incomplete superparamagnetic state, i.e. some particles will be superparamagnetic whereas others are not, as reflected in the magnetic measurements which are an average over all these particles. The hysteresis loops are not influenced by the presence of siderite in samples OD<sub>600</sub> = 2 and 4 due to the antiferromagnetism [22] of the additional mineral phase. Although the presence of significant antiferromagnetic material would be expected to suppress complete saturation, the minor proportion of siderite present in these samples is insufficient to cause noticeable changes within the field range used here. The Verwey transition observed in bulk magnetite is absent for all of these samples, as would be expected in superparamagnetic Fe<sub>3</sub>O<sub>4</sub> [1].

The form of the magnetization curve for the superparamagnetic particles is dependent on the magnetic particle volume, and can be neatly described by a Langevin function. Applying a mathematical fit to this curve provides a quantitative analysis of the magnetic particle size distribution [23]. The resulting log-normal distribution can be seen in figure 3(d), with the values obtained for mean particle size and most probable particle size listed in table 1. Due to the residual coercivity seen in the largest particles (OD<sub>600</sub> = 0.4), an accurate fit was not possible here, as only superparamagnetic curves may be fitted by this method. Mean particle size is seen to be almost equal for samples OD<sub>600</sub> = 1, 2 and 4 with values 9.1, 9.6 and 9.5 nm, respectively (we estimate the error due to a small residual coercivity to be ∼4%), and all have comparable dispersion index values of close to 0.3, which corresponds to the relative distribution widths. Compared to the results obtained from the TEM images, the mean particle sizes appear to be smaller for the magnetic measurements and also have broader distributions. The reason for this is unclear; however, it could be due to a non-magnetic surface layer that has previously been reported in other investigations of magnetite nanoparticles [24, 25].

XMCD measurements were performed on samples prepared in the presence of the redox mediator AQDS without air exposure in order to preserve the surface oxidation state of the nanoparticles. Biomass concentrations of OD<sub>600</sub> = 0.4, 1 and 4 were used, with spectra and relative site occupancies displayed in figure 4 (inset table). The XMCD signal originates only from the contribution of magnetic material (the ferrimagnetic magnetite), hence there is no involvement of the antiferromagnetic siderite or goethite.

The Fe L<sub>2,3</sub> XMCD spectra are characteristic of those expected for magnetite [26, 27]. The results indicate that as particle size decreases, the leading negative peak in the Fe L<sub>3</sub> edge reduces in magnitude, corresponding to a decline in Fe<sup>3+</sup> occupancy on octahedral sites, implying that the samples are becoming less reduced as particle size decreases. Previous work on biogenically produced magnetite has shown the existence of a reduced Fe<sup>2+</sup> layer around the particles [28], a result that is considered to have an impact on the surface reactivity of the particles, and could make biogenic magnetite more effective for remediation (e.g. reduction of metals such as Cr(VI) or reductive dechlorination of solvents) compared
to inorganically produced equivalents. Quantitative analysis of the site occupancies by fitting with atomic multiplet calculations [29, 30] indicates that the largest particles have a Fe$^{2+}$:Fe$^{3+}$ ratio of 1.18:2, which exceeds the 1:2 ratio expected for stoichiometric magnetite. However, the smaller particles have a ratio below that value, with both OD$_{600}$ = 1 and 4 showing a Fe$^{2+}$:Fe$^{3+}$ ratio of 1.04:2, which is very close to stoichiometric magnetite.

The results of the XMCD analysis suggest that the smallest measured particles (OD$_{600}$ = 1, 4) do not have the Fe$^{2+}$ rich surface (due to occupancy of Fe$^{2+}$O$_6$ ∼ 1) that is observed for OD$_{600}$ = 0.4 and other biogenic magnetite nanoparticles [22]. This apparent difference is due to the surface sensitivity of the technique. In total electron yield (TEY) mode ∼ 60% of the signal originates from the top 2 nm surface layer of the sample, and hence the particle size will affect the relative sampling volume of the particles [31]. As the particle size decreases the effective probing depth is such that almost the entire sample is probed and the measurement is more representative for the bulk. This may explain why the smaller particles appear to be less reduced than the larger particles because the signal from the reduced surface is diluted by that of the bulk material. Shape variations may also have an impact on the XMCD, in relation to the TEY probing depth, as can be observed from TEM images that the larger particles produced at biomass loading equivalent to OD$_{600}$ = 0.4 are made up of square shapes with varying aspect ratios, compared to the more spherical particles produced at higher OD$_{600}$.

The reactivity of the MNPs produced in this study was measured in relation to their effectiveness at reducing/sequestering a 5 mM aqueous solution of potassium chromate (Cr(VI) as K$_2$CrO$_4$). The results of an assay specific to chrome (figure 5(a)) support the supposition that the smaller particles (OD$_{600}$ = 1–4) would contain greater reducing power due to their larger surface-to-bulk ratio. The larger particles (OD$_{600}$ = 0.2, 0.4) removed ∼ 1 mM Cr(VI) from solution after 25 min of reaction time. ICP-AES analysis of the supernatant taken at 42 h (2520 min) indicated 3.7 mM (±0.7%) and 3.4 mM (±1.4%) total Cr remaining in solution prepared with a biomass of OD$_{600}$ = 0.2 and 0.4, respectively, confirming that only the Cr(VI) identified by the wet chemical assay remained in solution and no Cr(III) was present. The three samples with smaller particles initially showed very similar reduction trends to each other, with the removal of more than half (∼2.5 mM) of the Cr(VI) from solution after 25 min. During the 42 h of the reaction, Cr(VI) continued to be reduced/sequestered from solution, but at a less intense rate than was first observed. ICP-AES indicated 0.7 mM (±0.6%) chromium remaining in solution for the sample prepared with a biomass of OD$_{600}$ = 1 after 42 h, and 0.03 mM (±1%) and 0 mM (±6%) left for those prepared with OD$_{600}$ = 2 and 4, respectively, after the same time period. It is possible that a small amount of biomass remained on the magnetite samples after washing, but due to the lack of an electron donor it is unlikely that bacterial action would have contributed to the reduction of the Cr(VI).

XAS and XMCD analysis of the reacted MNPs was used to determine the nature of the chromium oxidation state after removal from solution. The results displayed in figures 5(b) and (c) correspond to measurements on the sample prepared with a biomass of OD$_{600}$ = 0.4. Here the first negative peak, corresponding to Fe$^{2+}$O$_6$, is reduced in intensity after reaction with Cr(VI). Previous studies have shown this result to be due to displacement of Fe$^{2+}$ in the spinel by chromium.

Figure 5. Cr(VI) reduction and sequestration by magnetite nanoparticles. Magnetic nanoparticles of varying size were added to 5 mM potassium chromate solution and left to react for 48 h. (a) A Cr(VI) specific spectrophotometric assay was used to measure the amount of Cr(VI) in solution (error bars determined from standard deviation from the mean absorption reading from samples measured in triplicate). (b) XMCD at the Fe L$_3$ edge was measured to show the crystallographic site location of Cr incorporation in the OD$_{600}$ = 0.4 sample; before addition of chromate (dashed line) and after reaction (solid line). (c) XAS indicated the final valence state of the Cr(III) by comparison to calculated spectra [30].
in the octahedral site [11, 28]. Chromium exists in multiple valence states, most notably Cr(VI) and Cr(III), and the state incorporated into the magnetite can be determined by examination of the XAS at the Cr L$_{2,3}$ edge and comparison to standard compounds (figure 5(c)). The spectrum obtained is very similar to that of the calculated Cr(III) spectrum and shows no similarity to the calculated Cr(VI) spectrum [30]. The calculated spectra show a strong resemblance to the previously measured Cr(III) and Cr(VI) containing compounds chromite and crocoite, respectively [32].

3. Conclusion

This study has demonstrated a method for producing size controlled magnetite nanoparticles through the reduction of Fe(III) minerals by the anaerobic Fe(III)-reducing bacterium Geobacter sulfurreducens. The average particle size and distribution was varied by adjusting the amount of the whole cell biocatalyst introduced at the start of the experiment, with higher concentrations of bacteria resulting in smaller particles and also leading to narrower size distributions. The mechanism behind this change is thought to be related to the rate of nucleation of the magnetite particles, with higher nucleation rates due an increase in Fe$^{2+}$ availability with greater biomass concentrations resulting in the production of smaller nanoparticles. The total Fe$^{2+}$ available also appears to have a bearing on the type of minerals formed in this process, with goethite formed at low biomass (i.e. low Fe$^{2+}$) concentrations, and siderite forming at high biomass (high Fe$^{2+}$) concentrations. A potential application of these different particle sizes has been demonstrated with respect to Cr(VI) remediation. Smaller particles are shown to be more effective at the reduction/sequestration of Cr(VI) into the less toxic form of Cr(III) based on total surface area and for the smallest particles can lead to a complete removal of Cr(VI) from solution. This result can have important implications for applications where particle size and reactivity of Cr(VI) from solution. This result can have important implications for applications where particle size and reactivity of Cr(VI) from solution. This result can have important implications for applications where particle size and reactivity of Cr(VI) from solution.

Late log-phase cultures of Geobacter sulfurreducens were harvested by centrifugation at 4920g and 4°C for 20 min and washed twice in bicarbonate buffer (30 mM; pH 7) under a N$_2$:CO$_2$ (80:20) gas line. Cells were transferred into bicarbonate buffer, forming a total volume of 30 ml bacterial cell suspension. Optical density at 600 nm (OD$_{600}$) was measured using an M501 single beam scanning UV/visible spectrophotometer. The cell suspension was diluted with buffer to achieve an OD$_{600}$ of 0.4 in 10 ml volume (0.2 ml cell suspension, 9.8 ml deionized H$_2$O). Multiples of the 0.2 ml Geobacter sulfurreducens suspension were introduced into each culture depending on the total OD$_{600}$ desired (e.g. OD$_{600}$ = 4 required 2.0 ml, OD$_{600}$ = 2 required 1.0 ml, etc). Further dilution with buffer was necessary to achieve an OD$_{600}$ lower than 0.4. Each biomass concentration was repeated in triplicate.

Final concentrations of bacteria in each experiment were determined by protein assay. A volume of 50 μl of sample was reacted with 950 μl bicinechonic acid (BCA)-CuSO$_4$ solution (50:1 BCA to Cu(II)SO$_4$). Absorption was measured at a wavelength of 562 nm against a reference series of bovine serum albumin standards, also reacted with BCA-CuSO$_4$ after 1 h incubation at 30°C. Optical densities were converted into biomass (mg ml$^{-1}$ cellular protein) according to: OD$_{600}$ = 0.015 = 0.005 mg ml$^{-1}$, OD$_{600}$ = 0.03 = 0.010 mg ml$^{-1}$, OD$_{600}$ = 0.05 = 0.016 mg ml$^{-1}$, OD$_{600}$ = 0.2 = 0.06 mg ml$^{-1}$, OD$_{600}$ = 0.4 = 0.14 mg ml$^{-1}$, OD$_{600}$ = 0.6 = 0.220 mg ml$^{-1}$, OD$_{600}$ = 0.8 = 0.297 mg ml$^{-1}$, OD$_{600}$ = 1.0 = 0.375 mg ml$^{-1}$, OD$_{600}$ = 2.0 = 0.763 mg ml$^{-1}$, OD$_{600}$ = 4.0 = 1.54 mg ml$^{-1}$. Cellular protein makes up 50% of the dry weight mass of the bacteria.

Cultures were incubated in the dark at 30°C for 1 week. The magnetite formed was washed twice with deionized water to remove cells and buffer solution.

Fe(II) concentrations were measured after the completion of magnetite formation using a Ferrozine assay approach [35]. Cultures were shaken to form a homogeneous solution, 0.1 ml was removed and added to 4.9 ml 0.5 N HCl and incubated for 1 h. 0.05 ml of this solution was added to 2.45 ml Ferrozine solution (2 mM) and the Fe$^{2+}$ concentration measured against a standard curve at 562 nm in an M501 spectrophotometer.

Cr(VI) reduction experiments were used to test the reactivity of each biogenic magnetite sample. Bacterial cells and supernatant were removed by magnetic separation and washing, and a solution of Cr(VI) (potassium chromate, K$_2$CrO$_4$, 5 mM) added to make up a total volume of 10 ml. All bottles had an anaerobic headspace and were stored at 20°C in the dark on a continuous roller-mixer at 33 rpm. The Cr(VI)
remaining in solution was measured at frequent intervals using a diphenyl carbazide (DPC) spectrophotometric assay [36]. Each experiment was repeated in triplicate, with the Cr(VI) concentration remaining in solution determined as the mean of the three, with error bars determined as the standard deviation from the mean.

PXRD measurements were carried out using a Bruker D8Advance with Cu Kα1 source. Data were acquired over a 2θ range of 5°–70° with a step size of 0.02°. Where magnetite formation was observed, the average particle size was determined by the mean of fitting a Lorentzian curve to the three most intense reflections ((3 1 1), (2 2 0) and (4 4 0)) and measuring the full width half maximum and Bragg angle; these values were used to determine particle size via input into the Scherrer equation [37, 38]. Error bars were determined as the standard deviation from the mean of the three fits. Fitting of the Lorentzian curve was performed using OriginPro 8.1 SR3 (OriginLab Corporation) software.

XMCD spectra, derived from the XAS spectra at the Fe L2,3-edge, were measured at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, California. The XMCD spectrum is the difference between the absorption of circularly polarized x-rays measured for two opposite magnetization directions (±0.6 T) parallel and antiparallel to the beam direction. The XMCD spectrum changes, depending upon magnetization, site location and valence state (number of d electrons). Atomic multiplet calculations can be applied to XMCAD and XAS spectra to determine site distributions of Fe ions within the structure of the crystalline material [29, 30]. Samples were dried and ground in an anaerobic cabinet and mounted onto carbon tape attached to the sample probe with transportation to the beamline taking place in a sealed anaerobic container to minimize potential exposure to air. Measurements were made in TEY mode with an effective probing depth of ~3–4 nm.

TEM was carried out at the Leeds Electron Microscopy And Spectroscopy (LEMAS) Centre, University of Leeds, UK using a Philips CM 200 electron microscope equipped with a field emission gun (FEG), EDX detector (Oxford Instruments, ISIS software) and Gatan imaging filter (GIF200). All images are obtained using an accelerating voltage of 200 kV. Direct measurements of the images were carried out to determine average particle size using a population size of n = 200 particles per sample.

Magnetic measurements were performed on polycrystalline samples restrained in eicosane, using a Quantum Design MPMS-XL SQUID magnetometer equipped with a 7 T magnet. ZFC and FC magnetization curves were recorded over 5–300 K temperature range with an applied magnetic field of 100 Oe. The diamagnetism of the sample holder and of eicosane was measured and extracted from the raw magnetic data.

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Biosynthesis of Zinc Substituted Magnetite Nanoparticles with High Room Temperature Magnetization

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5. Biosynthesis of Zinc Substituted Magnetite Nanoparticles with High Room Temperature Magnetization

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5.1. Abstract

The magnetic moments of magnetite nanoparticles have been dramatically enhanced through the addition of zinc in a microbiologically driven synthesis procedure. The particles were produced through the reduction of Fe(III)- compounds containing Zn(II) by the iron reducing bacterium Geobacter sulfurreducens, generating zinc ferrites of the form ZnₓFe₃₋ₓO₄. The results presented indicate a significant increase in the saturation magnetization by over 50% compared to magnetite when measured at both room temperature and low temperature (5 K) for relatively minor quantities of zinc incorporated into the crystal structure. In particular, a maximum saturation magnetization of nearly 100 emu g⁻¹ was obtained at room temperature. X-ray absorption spectra of Fe and Zn L₂,₃ edges showed that zinc is incorporated in place of tetrahedral Fe³⁺ as is often observed. However, we also present evidence that Zn²⁺ is also substituted in place of octahedral Fe²⁺, with the effect becoming more apparent with increasing total zinc. This substitution, combined with maintaining an overall neutral charge, led to initial increases in the magnetic moment before ultimately producing an effectively antiferromagnetic material. The synthesis technique applied here involves an environmentally benign route and offers the potential to tune the magnetic properties of these magnetic nanoparticles, with increased overall magnetization desirable for many different commercial applications.
5.2. Introduction

The synthesis of magnetic nanoparticles for use in a number of potential applications has been a focus of recent research. These applications include targeted cancer therapies, drug and gene delivery, MRI contrast agents, ferrofluids, magnetic recording materials and bioremediation technologies\textsuperscript{1-4}. Magnetic nanoparticles such as magnetite (Fe\textsubscript{3}O\textsubscript{4}) and transition metal-doped ferrites of the form $M\textsubscript{x}Fe_{3-x}O\textsubscript{4}$ ($M = \text{Mn, Co, Ni, Zn, etc.}$) offer promising materials due to their intrinsic magnetism and small size, with manipulation of stoichiometry providing a method of tailoring the properties to specific applications. In particular, the ability to increase the magnetic moment has great significance, for example in MRI imaging where it can enhance the effectiveness of magnetic contrast agents, or in hyperthermia cancer therapies, where an increase in magnetization at a given applied field maximises the heating power achievable.

Stoichiometric magnetite (Fe\textsubscript{3}O\textsubscript{4}) is an inverse spinel with the cations Fe\textsuperscript{2+}[B]:Fe\textsuperscript{3+}(A):Fe\textsuperscript{3+}[B] in a ratio of 1:1:1, where (A) and [B] represent tetrahedral and octahedral sites, respectively. The magnetic exchange in magnetite is governed by a combination of antiferromagnetic superexchange (SE) and ferromagnetic double exchange (DE) interactions. There are three antiferromagnetic SE interactions between the Fe\textsuperscript{3+} ions on the (A) and [B] sites, mediated by the oxygen (O) ions which are denoted A-O-A, B-O-B, and A-O-B. As pointed out by Néel\textsuperscript{5}, in the simplest model, ferrimagnetism in Fe\textsubscript{3}O\textsubscript{4} is obtained without any DE interaction, forcing an antiparallel alignment of the moments on the (A) and [B] sites. Since the antiparallel Fe\textsuperscript{3+}(A) and Fe\textsuperscript{3+}[B] moments compensate each other, a saturation magnetization of $4 \mu_B$/ f.u. (formula unit) is expected from the remaining Fe\textsuperscript{2+}[B] moments. This simple Néel model has been extended by Yafet and Kittel\textsuperscript{6}, who proposed a more elaborate model in which the B sublattice is subdivided into two (Fe\textsuperscript{2+}[B] and Fe\textsuperscript{3+}[B]) sublattices. It was shown that on weakening the A-O-B interaction and strengthening the B-O-B interaction, the B site magnetic moments are no longer rigidly parallel to the A site moments. The stronger B-O-B SE interaction results in spin canting, and thus a reduction in the saturation magnetization.

Based on the theory of this model and experimental evidence, the substitution of other metal cations within the structure of magnetite can be used to change the magnetic properties of nanoparticles in different ways, depending upon where the
dopant is incorporated, e.g., Ni$^{2+}$ and Co$^{2+}$ can substitute for Fe$^{2+}$[B], whereas Zn$^{2+}$ has a strong affinity for the tetrahedral (A) site$^{7,8}$. Of particular interest has been the substitution of Fe$^{3+}$(A) by diamagnetic Zn$^{2+}$ which leads to a decrease in the magnetic component that is antiparallel to the Fe$^{3+}$[B] moment, hence would be expected to yield larger total magnetization which increases as zinc concentrations increase. This is seen to be the case only up to a certain percentage of zinc$^9$ because exchange interactions within the octahedral lattice begin to take over, resulting in spin canting.

A number of methods can be used for the synthesis of magnetic nanoparticles, including co-precipitation, hydrothermal approaches and mechanical ball milling, amongst others. These are often both economically and environmentally undesirable, due to the high temperatures and toxic chemicals used. Alternatively, biogenic approaches can also be used to produce magnetic nanoparticles in a method in which the anaerobic respiration of Fe(III) oxides by subsurface bacteria is utilised to produce magnetic nanoparticles. Fe(III) reducing bacteria are able to generate large amounts of extracellular magnetite at ambient temperature through the oxidation of an electron donor (organic matter or hydrogen), coupled with the reduction of metal cations such as Fe(III). Using this approach, Fe(III)-oxyhydroxides (and related phases containing other transition metals) can be reduced, producing soluble Fe(II) which re-crystallises into a new mineral phase such as goethite, magnetite or siderite, depending upon the synthesis conditions (including pH, cell concentration, geochemical matrix and temperature)$^{10-12}$.

There have been many successful attempts to produce magnetic nanoparticles through the microbial reduction of Fe(III) oxides, including several which have demonstrated the ability to incorporate transition metal dopants into the crystal structure$^{13-15}$. Most recently, the high temperature iron reducing bacterium Thermoanaerobacter TOR-39 (incubated at 65 °C) was used to produce zinc doped ferrite nanoparticles$^{16}$ which exhibited higher magnetic moments than stoichiometric magnetite nanoparticles.

The overall aim of this work is to generate nanoparticles with enhanced room temperature magnetic moments compared to those currently achievable by other means$^{17}$ through the microbial reduction of Fe(III) oxyhydroxides containing various concentrations of zinc dopants at ambient temperatures by the iron reducing bacterium Geobacter sulfurreducens. The temperature of synthesis is thought to have a potential impact on the ordering of cations within the crystal structure$^{16}$ which
would have a direct impact on overall magnetic moment of the nanoparticles. It is anticipated that the low temperature iron reduction in this work could ultimately lead to zinc ferrites with higher values of saturation magnetization than counterparts produced using high temperature synthesis.

A number of different techniques have been applied to study the biologically-produced particles in their entirety including powder X-ray diffraction (XRD) and transmission electron microscopy (TEM) to observe changes in the structural properties; superconducting quantum interface device (SQUID) magnetometry to measure changes in magnetization; X-ray absorption spectroscopy (XAS), X-ray magnetic circular dichroism (XMCD) and Mössbauer spectroscopy to measure cation site changes in order to further understand the underlying crystal structure and how zinc incorporation affects iron site occupancies in the crystal structure.

5.3. Experimental

5.3.1. Synthesis of zinc-iron oxyhydroxides

Zn(II)-Fe(III) Zinc-iron oxyhydroxides were synthesized by dissolving varying quantities of ZnCl$_2$ and FeCl$_3$ in aqueous solution, with the amount of ZnCl$_2$ added to each sample designed to produce a molar ratio of 0, 5, 15, 20 and 33% Zn to Fe, respectively. Precipitation of a solid metal cation oxyhydroxide (MCO) was then facilitated through hydrolysis by 10 N sodium hydroxide, which was added until the final solution had a pH of 7.0. Chloride ions that were still present in solution were removed to prevent interference with microbial action by centrifugation of the MCO solution at 17000 g for 20 min with the supernatant being removed and the MCO re-suspended in deionised water. This process was repeated six times to ensure total removal of chloride ions. Total iron concentration was determined by the ferrozine assay.$^{18}$

5.3.2. Bacterial cultures

Starting microbial cultures were prepared in 500 ml bottles containing 50 mM L$^{-1}$ electron acceptor (MCO), 20 mM electron donor (sodium acetate), 30 mM buffer (sodium bicarbonate) and 10 μM of an electron shuttle (anthraquinone 2,6-disulphonate) to accelerate Fe(III) reduction. Cultures were prepared under a gas flow
of \( \text{N}_2 \cdot \text{CO}_2 \) (80:20) and then separated into 100 ml bottles in an anaerobic cabinet, to maintain anoxic conditions. Sterility was ensured by autoclaving the bottles at 121°C for 20 min.

*Geobacter sulfurreducens* was grown at 30°C under anaerobic conditions on modified freshwater medium\(^1\) containing 25 mM sodium acetate and 40 mM sodium fumarate as the electron donor and acceptor respectively. After 24 hours of growth, the late-log phase bacterial cultures were harvested by centrifugation at 5000 \( g \) for 20 min, and washed twice in bicarbonate buffer (30 mM; pH 7). Following the final wash, the cell pellet was resuspended as a slurry with the optical density measured at 600 nm using an M501 single beam scanning UV/visible spectrophotometer. The Zn(II)-Fe(III) oxyhydroxide cultures were then inoculated with *G. sulfurreducens* (~0.2 mg protein ml\(^{-1}\)) and incubated in the dark at 30°C.

### 5.3.3 Nanoparticle characterization

X-ray absorption spectroscopy (XAS) data at the Fe \( L_{2,3} \) and Zn \( L_{2,3} \) edges were acquired at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, Berkeley, California, USA. XAS were collected in total-electron-yield (TEY) mode, using circularly polarized X-rays with the sample in an applied magnetic field of -0.6 T and +0.6 T, i.e., parallel and antiparallel to the direction of the beam respectively. TEY mode has an effective probing depth of ~3-4 nm, with the intensity exponentially falling off with depth. X-ray magnetic circular dichroism (XMCD) data were obtained using the difference between the two XAS spectra collected in opposite applied magnetic fields. XMCD spectra reveal changes in magnetization, site location and valence state (i.e., number of \( d \) electrons) and, for metal oxides, are able to provide information about magnetic cations with different oxidation states at different lattice sites\(^7\). Atomic multiplet calculations were applied to determine site distributions of Fe cations within the structure of the crystalline material\(^20, 21\). The diamagnetism of Zn gives a zero XMCD signal. Dried samples were placed on carbon tape mounted onto a copper sample probe. Sample loading took place in an anoxic glove bag, with the sample probe encased in an airtight container to allow for transportation between glove bag and sample chamber. The container was removed as late as possible in a backflow of nitrogen to ensure as little exposure to air as possible and to maintain the oxidation state of the iron at the surface of the particles.
Mössbauer spectra were recorded with a FAST ComTek 1024-multichannel analyzer system using a constant acceleration drive (RT, γ-ray source ~25 mCi $^{57}$Co/Rh matrix). Measurements at low temperatures were carried out using a liquid nitrogen cryostat. For line fitting, the Lagarec/Rancourt Recoil fitting routine was utilised (Intelligent Scientific Applications Inc.). Spectra were fitted using Lorentzian line shape symmetrical doublets/sextets. Isomer shift data were calibrated with reference to Fe foil spectra recorded at RT. The absorber thickness was <4 mg Fe cm$^{-2}$.

Mineral phases were obtained using powder X-ray diffraction (XRD) carried out using a Bruker D8 Advance instrument with Cu $K_{α1}$ source. Data were acquired over a 2θ range of 5-70° with, step size 0.02°. The average crystallite particle size of the magnetite was determined using the Scherrer equation by fitting a Lorentzian function to the (220), (311), (400), (511), and (440) reflections$^{22,23}$, with errors generated as the standard deviation from the mean.

Transmission electron microscopy (TEM) was carried out using a Philips CM 200 electron microscope at the Leeds Electron Microscopy and Spectroscopy (LEMAS) Centre, University of Leeds, UK. The microscope was equipped with a field emission gun, EDX detector (Oxford Instruments, ISIS software) and Gatan imaging filter (GIF200). All images were obtained using an operating beam voltage of 200 kV. Particle size distributions were determined by measurement of the diameter of a population size of $n$=200 particles per sample.

Superconducting quantum interface device (SQUID) magnetometry was used to determine the magnetic properties of samples constrained in eicosane. Measurements were performed using a Quantum Design MPMS-XL SQUID equipped with a 7 T magnet, with zero-field cooled (ZFC) and field-cooled (FC) magnetization curves recorded between 5 K and 300 K in an applied magnetic field of 100 Oe. Residual diamagnetic signals detected from the sample holder and eicosane were measured and subtracted from the raw data.

The chemical composition of solid particles was determined using electron probe microanalysis (EPMA) with a Cameca SX100 microprobe equipped with wavelength dispersive spectrometer and operating at a voltage of 15 kV with a specimen current of 20 mA. Pure metals were used as standards.
5.4. Results and Discussion

5.4.1. Structural Properties

The ratios of zinc to iron in the substituted magnetite nanoparticles were determined by electron probe microanalysis (EPMA) and showed that a large proportion of the Zn was incorporated into the spinel structure. From the EPMA results, the values of $x$ in the formula unit $\text{Zn}_x\text{Fe}_{3-x}\text{O}_4$ were determined (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starting material</th>
<th>EPMA Final At% Zn</th>
<th>TEM $&lt;d&gt;$</th>
<th>TEM $\sigma$</th>
<th>XRD $&lt;d&gt;$</th>
<th>$M_s$ (emu g$^{-1}$) at 5 K</th>
<th>$M_s$ (emu g$^{-1}$) at 300 K</th>
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<tr>
<td>Zn$_0$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16.1 ± 0.5</td>
<td>0.3</td>
<td>21.3 ± 2.9</td>
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<td>Zn$_{0.16}$</td>
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<td>5.2</td>
<td>0.16</td>
<td>13.9 ± 0.3</td>
<td>0.3</td>
<td>17.6 ± 1.1</td>
<td>112</td>
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<tr>
<td>Zn$_{0.42}$</td>
<td>15</td>
<td>14.1</td>
<td>0.42</td>
<td>10.5 ± 0.3</td>
<td>0.3</td>
<td>11.4 ± 2.9</td>
<td>112</td>
</tr>
<tr>
<td>Zn$_{0.56}$</td>
<td>20</td>
<td>18.6</td>
<td>0.56</td>
<td>10.5 ± 0.1</td>
<td>0.2</td>
<td>9.5 ± 1.2</td>
<td>102</td>
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<tr>
<td>Zn$_{0.92}$</td>
<td>33</td>
<td>30.6</td>
<td>0.92</td>
<td>8.3 ± 0.1</td>
<td>0.2</td>
<td>6.9 ± 1.1</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1 – Structural and magnetic properties of zinc-doped magnetite nanoparticles. $<d>$ is mean particle size, $\sigma$ is dispersion index, $M_s$ is saturation magnetization.

The particles were imaged by TEM (Figure 1) with the images showing mostly spherical particles, some sub-rounded particles and some with an indication of hexagonal facets; the sample without zinc (Zn$_0$) appearing to show some cube-like particles. The size distribution of the particles was determined by measurement of 200 particles per sample on TEM images, resulting in the distribution curves shown in Figure 1f. As more zinc entered the magnetite structure, the size range became narrower, with mean diameter decreasing. Samples Zn$_{0.42}$ and Zn$_{0.56}$ had very similar mean particle sizes and size distributions, and potentially provide the best comparisons between other properties such as magnetism and cation distributions, without particle size effects having to be taken into account explicitly.
Powder XRD data (Figure 2) confirmed the presence of magnetite in all samples, with siderite also detected in all samples, although this secondary phase was not observed using TEM. Crystallite particle size analysis was carried out using the most intense magnetite reflections (2 2 0), (3 1 1), (4 0 0), (5 1 1) and (4 4 0), with results indicating that an increase in the zinc concentration in the starting material led to the production of smaller crystallites, supporting the observations from the TEM images.

Values of the magnetite lattice constant ($a_0$) were determined for each sample by measuring positions of all main magnetite reflections and taking the average; these are plotted against zinc concentration (Figure 2c). The diffraction peaks showed an increase in the lattice constant $a_0$ for the magnetite nanoparticles from 8.37 Å to 8.45 Å as zinc concentrations increased, which is in close agreement with the findings of Tian et al.\textsuperscript{24} Values of $a_0$ were determined for all samples through measuring positions of all main peaks, and with the averages plotted against zinc concentration (Figure 2c). The increase in $a_0$ with increasing zinc can be ascribed to the larger ionic radius of Zn$^{2+}$ compared with Fe$^{3+}$ in tetrahedral coordination (0.06 nm and 0.049 nm, respectively, see Appendix)\textsuperscript{25, 26}. This result shows that the incorporation of Zn$^{2+}$ is not confined to the surface regions of the magnetite particles but involves the bulk structure.
Figure 2 – Powder X-ray diffraction data. (a) XRD patterns displaying magnetite and zinc doped magnetite peaks. Siderite is also present. (b) Mean crystallite particle size from powder XRD compared to TEM determined by fitting a Lorentzian curve to all peaks, then using the Scherrer equation to calculate mean particle size, compared against TEM determined mean size. (c) Lattice parameter for magnetite with error bars determined from the standard deviation from the mean of all fitted powder XRD reflections.

Siderite (FeCO₃) has previously been reported as an impurity in biogenically produced magnetite samples, presumably formed via the interaction between excess Fe(II) formed by the bacteria, and, carbonate in the buffer system used (NaHCO₃). Evidence of this mineral phase was not found using TEM, suggesting that it is a minor mineral phase.

5.4.2. Nanoparticle Magnetism

Changes in the magnetic properties of the particles were determined using SQUID magnetometry, including measurements of saturation magnetization ($M_s$), coercivity ($H_c$, the applied field required to reverse magnetization direction) and blocking temperature ($T_B$, the temperature above which a particle exhibits superparamagnetism). Hysteresis loops collected at room temperature (300 K) and at low temperature (5 K) between -6 T and +6 T (Figure 3a,b) show the maximum magnetization achievable in the nanoparticles in the presence of an applied magnetic field. The results show that as zinc is incorporated into the magnetite structure, $M_s$ initially increases before decreasing again. At low temperatures, $M_s$ (Table 1) for magnetite ($Zn_0$) was 72 emu g⁻¹ and the $M_s$ was seen to increase by 56% reaching a
maximum of 112 emu g$^{-1}$ for Zn$_{0.16}$ and Zn$_{0.42}$. $M_s$ starts to decrease again at Zn$_{0.56}$, although with a value still higher than that of Zn$_{0}$, before reaching a minimum of 40 emu g$^{-1}$ for Zn$_{0.92}$. The trend at room temperature differs slightly, with the maximum $M_s$ reached at Zn$_{0.16}$ (97 emu g$^{-1}$) and decreasing for higher Zn concentration, with Zn$_{0.56}$ (56 emu g$^{-1}$) and Zn$_{0.92}$ (12 emu g$^{-1}$) samples having a smaller saturation magnetization than Zn$_{0}$.

It is expected that Zn$^{2+}$ enters the tetrahedral site, and once there is a sufficient amount, it gives a weakening of the A-O-B interaction as described by the Yafet and Kittel model$^6$. The B-O-B interaction then begins to dominate leading to spin canting which reduces the saturation magnetization as seen in the SQUID measurements. Enhanced $M_s$ in biogenic zinc doped ferrites compared to biogenic magnetite has previously been reported by Love et. al., although with a more modest enhancement 29%$^{27}$ (measured at 5 K), compared to 56% in this study for Zn$_{0.16}$. However, of particular significance is that a much less dramatic temperature dependence of the magnetization is observed here than in previous studies$^{27-30}$. This yields remarkably high magnetic moments in the nanoparticles even at room temperature.

Although powder XRD measurements showed the presence of siderite, there should be no influence on the hysteresis loops from this antiferromagnetic$^{31}$ material, given the low proportion present in the samples.

The superparamagnetic transition was also studied by measuring field cooled (FC) and zero-field cooled curves (ZFC). The spectra show that the blocking temperature $T_B$ of Zn$_{0}$ and Zn$_{0.16}$ must be above room temperature, because the ZFC and FC curves do not intersect at any point, unlike those for Zn$_{0.42}$ where a $T_B$ of ~270 K can be deduced (Figure 3d,e). Similarly, the maxima observed in Figure 3f for Zn$_{0.56}$ (~170 K) and Zn$_{0.92}$ (~20 K) correspond to the blocking temperatures ($T_B$) of those samples.
Figure 3 – SQUID magnetometry. (a) 5 K hysteresis loops, (b) 300 K hysteresis loops, (c) variations in saturation magnetization $M_s$ and coercivity $H_c$ obtained from hysteresis loops. (d-f) FC and ZFC curves highlighting changes in magnetization of nanoparticles as a function of temperature.

5.4.3. Cation Distributions

Figure 4a and 4b show the X-ray absorption spectra (XAS) at the Fe $L_{2,3}$ and Zn $L_3$ edges, respectively, with the maximum of the average Fe spectra normalized to one. It is clear from the Fe $L$ edge spectra that, as the zinc concentration increases through the series, the shoulder feature on the low energy side of the $L_3$ edge becomes increasingly resolved into a separate peak. Such a feature is normally observed in oxidized magnetite samples, and reveals a decrease in the Fe$^{2+}$ concentration in the magnetite relative to the Fe$^{3+}$ concentration. The Zn $L_3$ edges of the doped nanoparticles display
spectra corresponding to those reported for a bulk ZnFe\textsubscript{2}O\textsubscript{4} powder\textsuperscript{32}, thus showing incorporation of the Zn into the iron oxide spinel rather than formation of additional particles at the surface, in agreement with XRD lattice constant measurements. The intensity of the Zn \textit{L\textsubscript{3}} spectra increases with zinc concentration as expected.

Figure 4 – X-ray absorption data. (a) XAS of Fe \textit{L\textsubscript{2,3}} edge, shoulder feature on low energy side of Fe \textit{L\textsubscript{3}} edge indicates increase in Fe\textsuperscript{3+}[B], (b) XAS of Zn \textit{L\textsubscript{2,3}} edge, (c) XMCD of Fe \textit{L\textsubscript{2,3}} edge measured at room temperature. Solid line corresponds to the fit of the data points for each sample. (d) cation distributions determined through fitting of Fe XMCD spectra.

XMCD data for the Zn bioferrites are presented in Figure 4c. The intensity of the XMCD spectrum is a measure of the magnetization of the particles, provided that the average of the two associated XAS spectra has been normalized to one. This is demonstrated by sample Zn\textsubscript{0.16} appearing to be more magnetic than Zn\textsubscript{0} with Zn\textsubscript{0.92} exhibiting only a very small magnetic component. The peaks in the Fe \textit{L\textsubscript{3}} edge XMCD corresponds to the relative amounts of ferrous and ferric iron oxidation states and their coordination environment, with Fe\textsuperscript{2+}[B] matching the first negative peak (lowest energy), Fe\textsuperscript{3+}(A) the positive peak and Fe\textsuperscript{3+}[B] the second negative peak. The spectra show that, as initial zinc concentration increases, there is both a decrease in the intensity of the peaks associated with octahedral site Fe\textsuperscript{2+}[B] and tetrahedral site
Fe$^{3+}$(A), and an increase in octahedral site Fe$^{3+}$(B). Fitting of the data using calculated spectra confirms this interpretation (see Table 2 & Figure 4d).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe$^{2+}$(B)</th>
<th>Fe$^{3+}$(A)</th>
<th>Fe$^{3+}$(B)</th>
<th>Zn$^{2+}$</th>
<th>Fe(A)%</th>
<th>Fe(B)%</th>
<th>Zn$^{2+}$(A)</th>
<th>Zn$^{2+}$(B)</th>
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<tr>
<td>Zn$_0$</td>
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<td>0.92</td>
<td>0.97</td>
<td>0</td>
<td>30.8</td>
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<tr>
<td>Zn$_{0.16}$</td>
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<td>0.85</td>
<td>1.1</td>
<td>0.16</td>
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</tr>
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<td>Zn$_{0.92}$</td>
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<td>1.03</td>
<td>0.92</td>
<td>33.2</td>
<td>66.8</td>
<td>0.17</td>
<td>0.76</td>
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</table>

Table 2 – XMCD fitting results with cation values accurate to ±0.02.

Stoichiometric magnetite has a total electronic charge of zero, with the positive charges on the iron offset by the negative charge on the oxygen, i.e., one Fe$^{2+}$ and two Fe$^{3+}$ ions are neutralised by four O$^{2-}$ ions. If Zn$^{2+}$ displaces Fe$^{3+}$(A) as observed, a charge imbalance is created. However, for overall charge neutrality to be maintained, for every Fe$^{3+}$(A) ion displaced by Zn$^{2+}$, one Fe$^{2+}$(B) ion would need to oxidise to Fe$^{3+}$(B). Figure 4d illustrates the changes in Fe cation site occupancies with respect to zinc substitution as determined by fitting XMCD spectra measurements. The decrease in Fe$^{3+}$(A) tetrahedral mirrors the increase in Fe$^{3+}$(B) octahedral site occupancies as zinc increases, as would be expected if one unit of Fe$^{2+}$(B) was oxidized to Fe$^{3+}$(B) in order to balance the overall charge when Fe$^{3+}$(A) is replaced by Zn$^{2+}$(A). An unexpected observation is the significantly faster decrease in Fe$^{2+}$(B) than Fe$^{3+}$(A), as shown by the more negative gradient of the straight line of best fit through the Fe$^{2+}$(B) site changes than that through the Fe$^{3+}$(A) site changes. If Zn$^{2+}$ was only entering Fe$^{3+}$(A), the decrease in Fe$^{2+}$(B) should exactly compensate the decrease in Fe$^{3+}$(A) in accordance with the formula, $[\text{Zn}^{2+}_{x}\text{Fe}^{3+}_{1-x}][\text{Fe}^{2+}_{1-x}\text{Fe}^{3+}_{1+x}]\text{O}_4$. The result suggests that there is also substitution of Zn$^{2+}$ into the octahedral sites in place of Fe$^{2+}$(B). Previous work using various techniques, including neutron scattering and muon spin rotation/relaxation, shows that zinc has very strong affinity for the tetrahedral sites in the spinel, hence stoichiometric zinc ferrite is expected to be an normal spinel $(\text{Zn}^{2+})_a[\text{Fe}^{3+}_{2}]_b\text{O}_4$. However, the partial inversion of zinc ferrite has been observed previously in studies of nanoscale materials (<50 nm) and this appears to agree with the results of the present study. The substitution of Zn$^{2+}$ into the octahedral site in place of Fe$^{2+}$ would not affect the overall charge balance of the nanoparticles, hence its incorporation would not affect either of the Fe$^{3+}$ cations. From the fitting results of Fe$^{2+}$(B), Fe$^{3+}$(A) and Fe$^{3+}$(B) (Table 2), it is possible to estimate the relative occupancy of zinc in both...
tetrahedral and octahedral coordination by representing the formula for zinc ferrite as 
\[ (\text{Zn}^{2+})_x (\text{Fe}^{3+})_{1-x} (\text{Zn}^{2+})_{2-y} (\text{Fe}^{3+})_{1-y} \]A \ \text{Zn}^{2+} (\text{A}) (\text{Fe}^{2+})_{1-x} (\text{Fe}^{3+})_{1-x} \]B \ \text{O}_4, \]
where \( y \) is the total (A) site zinc, and \( x \) is the total zinc. Using the occupancies of each iron cation, values of \( \text{Zn}^{2+}(\text{A}) \) and \( \text{Zn}^{2+}(\text{B}) \) were determined (Table 2). The results show that for low \( \text{Zn}_x \) samples, whilst there is some incorporation of zinc into \([\text{B}]\) sites, the preference for incorporation is on the (A) site. The values obtained for \( \text{Zn}_{0.42} \) show \( \text{Zn}^{2+}(\text{A}) \) and \( \text{Zn}^{2+}(\text{B}) \) to be roughly equal, however, as there are twice as many \( \text{B} \) sites than \( \text{A} \) sites in the spinel, the preference can still be thought to remain with tetrahedral substitution. It is only in sample \( \text{Zn}_{0.92} \), where \( \text{Zn}^{2+}(\text{B}) \) is more than double \( \text{Zn}^{2+}(\text{A}) \) that one can say the zinc is now preferentially incorporated into an octahedral environment. Interestingly, none of the cases is close to a random distribution of \( \text{Zn} \), which would give the formula 
\[ (\text{Zn}^{2+})_{x/3} (\text{Fe}^{3+})_{1-x/3} (\text{Zn}^{2+})_{2x/3} (\text{Fe}^{2+})_{1-x/3} (\text{Fe}^{3+})_{1-x/3} \]A \ \text{Zn}^{2+} (\text{A}) (\text{Fe}^{2+})_{1-x} (\text{Fe}^{3+})_{1+x/3} \]B \ \text{O}_4 \]
which highlights the superior ordering of substituted cations using biogenic nanoparticle production.

The reason for this change in zinc substitution preference is not known; it could be an effect of particle size (i.e., the larger surface area gives a preference for one particular site). A similar result was reported by Ehrhardt et. al. for \( \text{ZnFe}_2\text{O}_4 \) where the amount of \([\text{B}]\) site zinc increased over \([\text{A}]\) site with decreasing particle size\(^{37}\). However, the occupancy changes could also be attributed to the changing value of zinc content \( (x) \), with additional \( \text{Zn} \) weakening the exchange interactions and changing the partitioning, or even to the new biological methods by which the particles were produced. Further investigation is required to determine the reason behind the inversion of \( \text{Zn} \) occupancy.

Mössbauer spectra (\(^{57}\text{Fe}\)) were collected for all five zinc ferrite samples (Figure 5) at room temperature \((300 \text{ K})\) and low temperature \((\sim 110 \text{ K})\). Parameters including isomer shift (IS), quadrupole splitting \((\Delta E_Q)\) and hyperfine field \((B_{hf})\) were obtained through fitting of the spectra (Table 3). Sample \( \text{Zn}_0 \), containing no zinc, shows spectra at both temperatures that are characteristic of magnetite, with two Zeeman patterns visible due to ferric ions in tetrahedral sites, and ferrous and ferric ions in the octahedral environment. Octahedral ferrous and ferric ions are indistinguishable due to rapid electron hopping at frequencies faster than that of the Larmor precession of the iron nucleus in the hyperfine field\(^{38}\). As zinc incorporation increases, the hyperfine field pattern collapses (samples beyond \( \text{Zn}_{0.42} \) at \( 300 \text{ K} \) and beyond \( \text{Zn}_{0.56} \) at \( 110 \text{ K} \)) until the spectra display only a doublet pattern for \( \text{Zn}_{0.92} \). These changes parallel the results...
obtained from SQUID ZFC/FC curve analysis which show that the blocking temperatures are below room temperature for Zn$_{0.42}$, Zn$_{0.56}$ and Zn$_{0.92}$. The $T_B$ of Zn$_{0.42}$ is ~270 K; however, this sample exhibits a range of sizes and some particles will not be entirely blocked, even at 300 K. This gives rise to a mixture of hyperfine field sextet and paramagnetic doublet spectra as observed. The same applies to Zn$_{0.56}$ which also shows Zeeman splitting and doublets present in measurements at 110 K.

The Mössbauer of Zn$_{0.16}$ (300 K and 110 K) and Zn$_{0.42}$ (110 K) consist of sharp Lorentzian lines corresponding to the two overlapping sextets as observed for Zn$_0$ (Figure 5). The line shape of the [B] site sextet is quite broad suggesting that the octahedral component is made up of several overlapping sextets. Fitting of the individual spectra was not possible; however, such sextets have been suggested before and attributed to nearest neighbour interactions$^{39}$.

In magnetite, each octahedral cation is surrounded by six tetrahedral cations, and the exchange interactions with these neighbouring ions gives rise to the hyperfine field at the nucleus of the [B] site cation. Assuming that there is a random distribution of zinc and ferric ions among the tetrahedral sites, as zinc is introduced, a reduction in the number of cations with a full complement of six surrounding Fe$^{3+}$(A) cations might be expected. This could give rise to further Zeeman patterns, each having different numbers of nearest neighbour exchange interactions. These individual spectra have not been sufficiently resolved here; however, the general changes in (A) and [B] site parameters are reported below. At high zinc content (Zn$_{0.56}$) a significant number of octahedral cations have insufficient nearest neighbour magnetic Fe$^{3+}$(A) cations to have ordered spins, giving rise to a quadrupole split doublet and an incomplete hyperfine field even well below the material’s blocking temperature (170 K). Sample Zn$_{0.92}$ exhibits only quadrupole split patterns because there are insufficient nearest neighbour exchange interactions between ferric and ferrous cations, despite the presence of some Fe$^{2+}$ in the octahedral environment as observed by XMCD.
Figure 5 – Mössbauer spectra collected for five zinc doped samples. Solid lines correspond to the fits to the data points. (a) Room temperature measurements shows clear hyperfine structure for sample Zn0 and Zn_{0.16} and a transition between the superparamagnetic and ferrimagnetic state for Zn_{0.42}. (b) Low temperature measurements at 110 K show some hyperfine field structure for all samples except Zn_{0.92}.

The isomer shift (IS), measured as a displacement of the centre of gravity of a sextet or doublet of peaks from zero velocity, is dependent on the s-electron density at the iron nucleus. It is particularly sensitive to the oxidation state and spin state of the iron. Fe^{2+} has six d-electrons in its valence shell, one more than Fe^{3+}. This extra d-electron increases the shielding of s-electrons from the nucleus, resulting in a decrease in the charge density at the nucleus; the result is a larger isomer shift for Fe^{2+} than Fe^{3+}. Thus, supposing octahedral site Fe^{3+} is kept constant, decreasing the amount of octahedral site Fe^{2+} leads to a decrease in isomer shift in the octahedral site as observed for the low temperature data (Figure 5 and Table 3).
<table>
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<th>Sample</th>
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<th>IS</th>
<th>ΔE_Q</th>
<th>B_HF</th>
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<td>(mm s⁻¹)</td>
<td>(kOe)</td>
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Table 3 – Extracted Mössbauer parameters. IS is isomer shift, ΔE_Q is quadrupole splitting, B_HF is hyperfine field, (A) is tetrahedral site sextet, [B] is octahedral site sextet, and Db is doublet.

Fitting of Zn₀ (biogenic magnetite) shows the sample to have isomer shifts of 0.30 and 0.65 for (A) and [B] sextets, respectively, and 486 and 452 kOe hyperfine fields for (A) and [B], respectively. These values match very closely with those commonly observed for magnetite at room temperature⁴⁰–⁴². The Zn₀₁₆ LT and RT, and Zn₀₄₂ LT spectra cannot be accurately fitted without the addition of a doublet. The IS and ΔE_Q values corresponding to these fits most accurately match those of an Fe(II) mineral (based on Murad)⁴² and is most likely due to the carbonate mineral siderite which was observed in powder XRD measurements.

The general trend in isomer shifts shown by the first three spectra (Table 3) indicate that, as the zinc concentration increases, the [B] site IS decreases as would be expected if Fe²⁺[B] was replaced with Fe³⁺[B] to maintain charge neutrality. IS values of the tetrahedral component remain relatively unchanged, indicating that there is no reduction of Fe³⁺(A) to Fe²⁺(A), or incorporation of Fe²⁺(A) during the formation of the magnetic nanoparticles.
The hyperfine field parameter was also determined through fitting of the first three low temperature spectra ($\text{Zn}_0$, $\text{Zn}_{0.16}$ and $\text{Zn}_{0.42}$). The results (Table 3) show the effective fields at the nuclei of ferric and ferrous cations in both octahedral and tetrahedral sites decrease with increasing zinc content. This is thought to be due to a decrease in exchange interaction between [A] and (B) sublattices as the number of nearest-neighbour magnetic interactions decreases. It is the spontaneous magnetization of the sublattice to which a nucleus belongs that is believed to be responsible for the formation of a hyperfine field\cite{38}. The magnetism in $\text{Fe}_3\text{O}_4$ and $\text{Zn}_x\text{Fe}_{3-x}\text{O}_4$ is the result of interactions between octahedral and tetrahedral sublattices, facilitated by the intervening oxygen atom, which result in antiferromagnetic coupling between $\text{Fe}^{3+}(\text{A})$ and $\text{Fe}^{3+}(\text{B})$ sites, and the unopposed magnetization of $\text{Fe}^{2+}(\text{B})$. Removing $\text{Fe}^{3+}(\text{A})$ cations partially removes the A-O-B interaction; hence, there will be less magnetic coupling across both sublattices leading to a decrease in the hyperfine field for both octahedral and tetrahedral sites.

The doublet parameters for $\text{Zn}_{0.92}$ ($\text{IS}=0.36 \text{ mm s}^{-1}$ and $\Delta E_Q =0.41 \text{ mm s}^{-1}$) closely match values previously observed for stoichiometric zinc ferrite $\text{ZnFe}_2\text{O}_4\text{43-45}$ (albeit with a slightly larger quadrupole splitting), suggesting that all of the starting material has been transformed by the bacteria. The increased quadrupole splitting has been observed previously at room temperature by Ehrhardt et. al.\cite{37} whilst studying size-dependent effects in nanosized $\text{ZnFe}_2\text{O}_4$ with smaller particle size leading to increasing $\Delta E_Q$. In that study, the increase was attributed to the zinc substitution, preference switching from tetrahedral to octahedral sites as particle size decreases. This inversion of site preference was observed here using XMCD, but the results from both techniques are unable to determine any conclusive reason for Zn being incorporated into the octahedral sites. The emergence of a second doublet in $\text{Zn}_{0.92}$ at low temperature points to the possible formation of a Zeeman pattern, as temperature decreases and the sample becomes magnetically ordered as observed in previous studies on zinc ferrite\cite{46,47}.
5.5. Conclusions

In this work, zinc has been incorporated into the crystal structure of magnetite to enhance the magnetic moment of magnetic nanoparticles. A range of zinc-doped magnetic nanoparticles containing varying concentrations of zinc were produced using biogenic reduction of Fe(III)-Zn(II) bearing minerals by the bacterium *Geobacter sulfurreducens*. An increase of 56% and 52% in the saturation magnetization of the particles (at 5 K and 300 K, respectively) was achieved through a loading of only 5% zinc. Lattice parameter changes were determined through XRD analysis, confirming that the zinc was incorporated into the crystal structure of the magnetite rather than forming a surface layer.

Changes in the cation distribution of iron and zinc within the crystal structure were investigated using XMCD and Mössbauer spectroscopy to further understand the interactions which dictate the magnetic properties. The results indicate the substitution of Fe$^{3+}$(A) sites with Zn$^{2+}$(A), combined with the oxidation of Fe$^{2+}$(B) sites to Fe$^{3+}$(B). Additionally, evidence suggests that some zinc may have also entered into the octahedral sites in place of Fe$^{2+}$(B). This effect is observed to increase throughout the series with Zn$^{2+}$ being preferentially incorporated into the [B] site rather than (A) for the highest concentration zinc sample (Zn$_{0.92}$). The location of iron within the lattice has a profound impact on the magnetic properties of the material, with the combined effect of substitution and reduction leading to a decrease in the antiparallel magnetic moments of the Fe$^{3+}$ cations, plus an increase in the total Fe$^{3+}$ present in [B] with respect to Fe$^{2+}$ which have magnetic moments of 5 $\mu_B$ and 4 $\mu_B$, respectively. These factors lead to the increase in $M_s$ that is observable for the low zinc concentration samples in the series.

The decrease in overall magnetization as zinc continues to be incorporated is explained by exchange interactions between the crystal lattice sites. In stoichiometric magnetite, the A-O-B site interactions, mediated via oxygen atoms, dominate so as to align the A and B sites antiparallel. As Fe$^{3+}$(A) is replaced with diamagnetic Zn$^{2+}$, B-O-B site interactions begin to take over, initially resulting in spin canting, before aligning anti-parallel magnetic spins within the B site itself, hence the overall magnetic moment.
decreases and tends towards zero. In normal spinel Zn ferrite, antiferromagnetic superexchange interactions occur between Fe$^{3+}$ ions located in B sites, whereas in a mixed spinel, the interactions occur between Fe$^{3+}$ located in A and B sites$^{48}$.

To conclude, the precise control over the Zn cation substitution is required to reveal enhanced $M_s$. The result presented here demonstrates the significant potential of an environmentally benign biogenic route that could be used to achieve the desired level of control, leading to the production of high moment nanoparticles.

Acknowledgements

This work was carried out with the financial support of a NERC PhD studentship awarded to James Byrne. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No.DE-AC02-05CH11231. We acknowledge NERC Envirosync II for providing support for this work. Additional thanks to Dr. Michael Ward for assistance and the provision of access to Transmission Electron Microscope by Leeds Nanoscience and Nanotechnology Facility (LENNF).

5.6. References


The Controlled Doping of Cobalt into Biogenic Magnetite Nanoparticles
6. The Controlled Doping of Cobalt into Biogenic Magnetite Nanoparticles

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6.1. Abstract

Cobalt doped magnetite (CoₓFe₃₋ₓO₄) nanoparticles have been produced through the microbial reduction of cobalt-iron oxyhydroxide by the bacterium Geobacter sulfurreducens. The materials produced show significant increases in coercivity with increasing cobalt content without a major decrease in the overall saturation magnetization. Structural and phase analyses show considerable decreases in the particle size with cobalt doping and the formation of green rust as an additional mineral phase at the highest Co concentrations. Analysis of the cation properties show that the cobalt is predominantly incorporated within the octahedral environment of the spinel. Cobalt doping is seen to increase the effective anisotropy of the magnetic nanoparticles in addition to altering particle sizes. These two effects are considered to be responsible for an increase in the heating power generated under the application of an AC magnetic field. This has very important applications in potential cancer treatment which operate using magnetic hyperthermia.
6.2. Introduction

Research into magnetic nanoparticles (MNP’s) of the form $M_x\text{Fe}_{3-x}\text{O}_4$ ($M = \text{Co, Zn, Ni, Cr}$ etc.) has become an area of widespread activity in recent years in response to the wide range of potential applications for which they can be applied. These applications include the remediation of contaminated land and water\textsuperscript{1-3}, catalysis\textsuperscript{4, 5}, targeted drug delivery\textsuperscript{6}, magnetic data storage\textsuperscript{7} and magnetic thermotherapy targeting cancerous tumours\textsuperscript{8-10}.

Targeted cancer therapies include magnetic hyperthermia in which a heating effect is induced by MNP’s (localised within a tumour) under the influence of an AC magnetic field. Using this effect, once the temperature at the localised region reaches between 40 and 45 °C\textsuperscript{10}, the cancerous cells will be destroyed without causing extensive damage to healthy tissue (cancerous cells are more sensitive to heat than normal cells\textsuperscript{11}). The heating effect is dominated either by the physical rotation of MNP’s within solution (Brownian relaxation), or the changing direction of magnetization within the particles (Néel relaxation). Both processes lead to a release of heat, however the effect is dependent upon the size and anisotropy of the magnetic nanoparticles\textsuperscript{12}. The overall heating produced by the MNP’s under the influence of an AC field is defined by the specific loss power (SLP).

Several methods have been developed for producing MNP’s including chemical and mechanical methods such as co-precipitation and ball milling\textsuperscript{13}. Many of these processes can be expensive and environmentally damaging due to the use of high temperatures and toxic materials. An alternative way in which to produce MNP’s at ambient temperatures is via the use of subsurface Fe(III)-reducing bacteria such as Geobacter sulfurreducens or Shewanella oneidensis, which can conserve energy through the oxidation of an electron donor such as organic matter, coupled with the reduction of Fe(III)\textsuperscript{14, 15}. The reduction leads to the release of soluble Fe(II) which is able to recrystallize with solid phase Fe(III) to produce magnetite ($\text{Fe}_3\text{O}_4$) and potentially other mineral phases including siderite, goethite, vivianite, hematite and green rusts depending upon the conditions of formation (i.e. pH and temperature)\textsuperscript{16-18}. 
Magnetite has a cubic spinel crystal structure with a unit cell containing 32 oxygen ions, eight Fe$^{3+}$ ions in tetrahedral (A) sites, eight Fe$^{2+}$ and eight Fe$^{3+}$ ions in octahedral [B] sites, (i.e. (Fe$^{3+}$)$_8$[Fe$^{2+}$Fe$^{3+}$]$_8$O$_4$). The magnetism exhibited by magnetite MNP’s is formed as a result of the exchange interactions which occur between the (A) and [B] sites which yield magnetic moments on each cation orientated so that the moments in both lattices are anti-parallel to each other. The distribution of iron within the cubic lattice is such that the magnetic moments from Fe$^{3+}$ (5 $\mu_B$ per atom) on both lattice sites effectively cancel each other out, resulting in a net magnetization due to Fe$^{2+}$ (4 $\mu_B$ per atom). The substitution of Fe$^{2+}$ or Fe$^{3+}$ cations with transition metal dopants such as cobalt, zinc, nickel and chromium in the magnetite serves as a method in which to change the magnetic properties of the MNP’s by changing the magnetic interactions between the different lattice sites$^{19,20}$.

The aim of this work is to further enhance the understanding of the effect of cobalt substitution into the structure of biogenic magnetite and to investigate the potential use of these biologically synthesized cobalt ferrites for hyperthermia cancer treatment. Several techniques have been applied in order to fully characterise the material which was produced through the reduction of Co(II)-Fe(III)-oxyhydroxide starting material by Geobacter sulfurreducens. These include superconducting quantum interface device (SQUID) magnetometry, powdered X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray absorption (XAS), X-ray magnetic circular dichroism (XMCD) and Mössbauer spectroscopy. The heating effect of the nanoparticles under the influence of an AC magnetic field was measured in terms of SLP.

6.3. Experimental Methods

Six different cobalt-iron gel solutions (Co(II)Fe(III)-oxyhydroxide) were prepared through the co-precipitation of a mixture of iron(III) chloride (FeCl$_3$) and cobalt(II) chloride (CoCl$_2$) via the addition of sodium hydroxide (NaOH; 10 M) acting as the hydrolysing agent until the solution reached a pH of 7.0$^{14}$. The starting concentrations of FeCl$_3$ and CoCl$_2$ were varied to produce six different “gels” corresponding to 0%, 5%, 15%, 20%, 33% and 50% Co (by molar percentage) for samples Co0, Co5, Co15, Co20,
Co33 and Co50 respectively. After precipitation, the chloride ions were removed by centrifugation at 17000 \( g \) for 20 minutes and washed with de-ionised water, with this step repeated six times. The total concentration of iron was determined by ferrozine assay\(^{21}\). ICP-AES, after extraction using nitric acid (HNO\(_3\); 3 M) was used to determine the relative amounts of cobalt and iron in the starting oxyhydroxide material.

Cell cultures were prepared in de-ionised water to a total volume of 9.9ml containing an electron donor (sodium acetate; 20mM), an electron acceptor (Co(II)Fe(III)-oxyhydroxide; 50 mM L\(^{-1}\)), sodium bicarbonate buffer (NaHCO\(_3\); 30 mM) and an electron shuttle, anthraquinone 2,6-disulphonate (AQDS; 10\( \mu \)M) to accelerate Fe(III)-reduction. All manipulations were carried out under a N\(_2\):CO\(_2\) (80:20) gas line to ensure anaerobic conditions.

*Geobacter sulfurreducens* was grown in anaerobic conditions in the dark at 30\( ^\circ \)C in NBAF\(^{22}\) (25 mM sodium acetate as electron donor, 40 mM fumicaric acid as electron acceptor). Late log-phase cultures of *G. sulfurreducens* were harvested by centrifugation at 5000 \( g \) and 4 \( ^\circ \)C, for 20 min and washed twice in bicarbonate buffer (30mM; pH=7) under a N\(_2\):CO\(_2\) (80:20) gas line. Cells were transferred into bicarbonate buffer, forming a total volume of 30 ml bacterial suspension. Optical density at a wavelength of 600nm (OD\(_{600}\)) was measured using an M501 Single Beam Scanning UV/Visible Spectrophotometer. The cell suspension was diluted with buffer to achieve an OD\(_{600}\) of 0.4 in 10ml volume, (0.2ml cells, 9.8 ml deionised H\(_2\)O). Each culture was then inoculated with 0.2 ml *G. sulfurreducens* suspension (0.132 mg protein ml\(^{-1}\)) and incubated in the dark at 30\( ^\circ \)C for 1 week. The end product was washed twice with deionised water to remove cells and buffer solution.

The relative chemical composition of the material was measured using electron probe microanalysis (EPMA) with a Cameca SX100 microprobe equipped with wavelength dispersive spectrophotometer. The operating voltage was 15 kV with a specimen current of 20mA. Samples were compared against pure metal standards.

X-ray diffraction (XRD) measurements were carried out using a Bruker D8 Advance with Cu K\(_{\alpha1}\) source. Data were acquired over a 2\( \theta \) range of 10\( ^\circ \) – 70\( ^\circ \) with a step size of 0.02\( ^\circ \). When magnetite formation was observed in the XRD trace, the average particle size was determined by fitting a Lorentzian curve to the most intense reflection (311) in order to determine full width half maximum and Bragg angle. These values were used to determine particle size via input into the Scherrer equation\(^{23, 24}\).
Transmission electron microscopy (TEM) was carried out using a Technai F20 electron microscope equipped with a field emission gun, EDX detector (Oxford Instruments), and Gatan SC600 CCD camera. All images were obtained using an operating beam voltage of 200kV.

Magnetic measurements were performed on polycrystalline samples restrained in eicosane, using a Quantum Design MPMS-XL SQUID magnetometer equipped with a 7 T magnet. Zero-field cooled (ZFC) and field-cooled (FC) magnetization curves were recorded over 5-300 K temperature range with an applied magnetic field of 100 Oe. The diamagnetism of the sample holder and of eicosane was measured and extracted from the raw magnetic data.

For hyperthermia experiments, stable aqueous suspensions of nanoparticles were prepared by coating with citric acid using a procedure similar to that described elsewhere. Briefly, 25 mg of biogenic nanoparticles were added to 5 ml of d.H$_2$O and the pH lowered to <3 using concentrated HCl. The solution was bath sonicated for 2 min before adding dry citric acid to give a concentration in solution of 15 mg ml$^{-1}$, and the pH was subsequently increased to 5.2 using concentrated ammonia. The nanoparticle plus citric acid solution was then heated to >80 °C for 30 min. Excess citric acid was removed by magnetic decantation and the nanoparticles washed with acetone several times. The citric acid coated nanoparticles were then resuspended in d.H$_2$O and subjected to several cycles of vortexing, sonication and centrifugation in order to obtain an optimum stable suspension. The concentration of the final citric acid coated nanoparticle suspension was determined by drying a known volume of suspension.

The solution properties of the nanoparticles were determined using a Malvern Zetasizer 3000. Following citric acid coating, the hydrodynamic cluster (particle) sizes in the aqueous suspensions were determined to be in the range 40-75 nm for the magnetite and cobalt-ferrite nanoparticles. The measured zeta potential was -45 mV at pH 7.0 reducing to zero at pH ~2.5 in agreement with previous studies.

Magnetic hyperthermia measurements were performed using a commercially available unit supplied by nanoTherics Ltd. The heating effects were determined in an AC magnetic field of 17 kA m$^{-1}$ at a frequency of 110 kHz. An equivalent volume of d.H$_2$O was measured to confirm that no heating occurred in the absence of nanoparticles.
X-ray absorption (XAS) spectra at the Fe $L_{2,3}$-edge were measured at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, California. Samples were dried and ground in an anaerobic cabinet and mounted onto carbon tape attached to the sample probe with transportation to the beamline taking place in sealed $N_2$ container. The sample probe was loaded into the chamber in a backflow of $N_2$ to limit potential exposure to oxygen. Measurements were made in total electron yield mode (TEY) with an effective probing depth of $\approx 3-4$ nm, although this value is not linear and the intensity of penetration varies by $e^{-1}$ with increasing depth, hence most of the synchrotron energy is lost within the first few nm. XMCD spectra are obtained by the difference between the two XAS collected under two opposing magnetic field strengths of $+0.6T$ and $-0.6T$ (parallel and anti-parallel to the beam direction). Each XAS is dependent upon the direction of magnetization of the sub-lattice spinels in the magnetite structure, (i.e. spin up and spin down), valence number (i.e. number of $d$ electrons) and site location. The distribution of Fe cations within the magnetite structure can be determined through the application of atomic multiplet calculations$^{26,27}$. Each peak on the $L_3$ edge part of the spectra corresponds to a different component of the magnetite, the primary negative peak corresponds to octahedral Fe$^{2+}[B]$, the second positive peak is Fe$^{3+}(A)$ in the tetrahedral site and the third negative peak Fe$^{3+}[B]^{28}$.

Mössbauer spectra were recorded with a FAST ComTek 1024-multichannel analyzer system using a constant acceleration drive (RT, $\gamma$-source $\sim 25$ mCi $57Co/Rh$ matrix). Samples were sealed in between two layers of kapton tape which were glued together in an anoxic glove bag to prevent oxidation. Measurements at low temperatures were obtained using a liquid nitrogen cryostat. For line fitting, the Lagarec/Rancourt Recoil fitting routine was utilised (Intelligent Scientific Applications Inc.). Spectra were fitted using Lorentzian line shape symmetrical doublets/sextets. Isomer shift data are calibrated with reference to metallic Fe foil recorded at room temperature. The absorber thickness was $<4$ mg Fe cm$^{-2}$. 

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6.4. Results and Discussion

6.4.1. Physical characteristics and crystal structure

Electron probe microanalysis (EPMA) was used to determine the relative ratios of cobalt to iron ($x_{\text{EPMA}}$) in the nanoparticles (Table 1). The results show that there is a loss of approximately 30% cobalt during the preparation process from the designed starting %Co for each sample. This loss occurs during the process of producing cobalt-iron gel and is considered to be due to incomplete precipitation of cobalt into solid phase oxyhydroxide at pH 7\textsuperscript{29}. The amount of cobalt incorporated into the nanoparticles closely matches the amount that is available in the starting gels (%Co\textsubscript{ICP}). EPMA is a bulk measurement and gives measurements to a high degree of accuracy (+/- >0.5%) and from the %Co results, the value of x ($x_{\text{EPMA}}$) according to the formula $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$ was determined.

<table>
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<tr>
<th>Sample</th>
<th>Designed starting material %Co</th>
<th>ICP-AES starting material %Co\textsubscript{ICP}</th>
<th>EPMA final material %Co</th>
<th>$x_{\text{EPMA}}$</th>
<th>XAS final material %Co</th>
<th>$x_{\text{XAS}}$</th>
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Table 1 – The relative amount of Co was measured in starting material using ICP-AES (%Co\textsubscript{ICP}), EPMA and XAS were used to measure percentage of Co in the final material. Values of $x$ for the formula $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$ were also determined from both EMPA ($x_{\text{EPMA}}$) and XAS ($x_{\text{XAS}}$).

Powder X-ray diffraction (XRD) was used to determine the nature of the minerals that were produced by the microbial reduction of the Co(II)Fe(III)-oxyhydroxide gel. The first three samples (Co0, Co5, Co15) clearly show (220), (311), (400), (511) and (440) reflections corresponding to magnetite, with Co20 samples having (311) and (440) peaks. Samples with highest cobalt concentrations (Co33 and Co50) do not exhibit such peaks, however this is attributed to their small particle size resulting in reflections that are indistinguishable from the background noise. At the highest cobalt concentration (Co50) it is possible to see the emergence of an extra peak at $2\theta=11^\circ$ corresponding to green rust which appears to be produced as an additional mineral phase.
Figure 1 – X-ray diffraction $10^\circ \leq \theta \leq 70^\circ$. Low cobalt concentrations exhibit reflections corresponding to magnetite. No magnetite peaks are visible for Co33 and Co50 samples, however a green rust peak is observable at $2\theta=11^\circ$ for sample Co50.

Size analysis of the magnetite (311) reflection which was common to the first four samples enabled the determination of mean crystal diameter for samples Co0 through to Co20. The incorporation of cobalt has a significant effect on particle size, with an immediate drop in mean crystallite size from 36.7 nm (Co0) to 11.3 nm with the addition of just ~5% cobalt (Co5). There is little change in particle size for cobalt concentrations higher than this with values of 13.7 nm and 9.5 nm determined for Co15 and Co20 samples respectively. Cobalt is next to iron in the periodic table and has a roughly comparable size (0.074 nm and 0.077 nm for Co(II) and Fe(II) in octahedral coordination, see Appendix$^{30}$), thus this particle size change is not considered to be due to the substitution of the Co(II) cation.

Transmission electron microscopy (TEM) was performed on the samples to investigate morphology and size changes in the series (Figure 2). The results confirm XRD measurements with size changes varying from between 30 – 40nm (Co0), ~10nm (Co5, Co15 and Co20) and ~2-4 nm (Co33 and Co50). Selective area electron diffraction (SAED) indicated that the first five samples in the series (Co0 – Co20) contained only cobalt doped magnetite nanoparticles. Large plate like and needle features were observed in the highest cobalt doped samples Co33 (not shown in Figure 2) and Co50.
These were attributed to be a green rust ([Fe$^{2+}$$_3$Fe$^{3+}$($\text{OH}$)$_8$]$^+$·[Cl·2H$_2$O]$^-$) which exists as hexagonal plates$^{31}$, although SAED was unable to confirm this.

Figure 2 – TEM images of cobalt doped magnetite. (a) Co0 shows large crystals 30-40nm, (b) Co5, (c) Co15 and (d) Co20 particles have approximately 10nm diameter. (e) Co33 and (f) Co50 are much smaller (~2-4 nm diameter) nanoparticles with needle features visible in Co50 samples.

EDX spectroscopy was used to confirm differences in cobalt concentration throughout the series. The relative amount of cobalt in the nanoparticles was measured at 4.4%, 12.8%, 13.9%, 18.8% and 23.3% for samples Co5, Co15, Co20, Co33, Co50. The values of the first four samples match very closely with EPMA measurements, however Co33 is marginally lower, and Co50 is approximately a third less than expected from electron probe analysis. This variation is potentially due to a
high level of Co incorporation into the plate-like features. The EDX spectra on the large plate-like samples indicated the presence of chlorine, iron and cobalt in ratios of 3.0% Cl, 42.5% Fe, 54.5% Co and 2.2% Cl, 36.0% Fe, 61.8% Co for samples Co33 and Co50 respectively which suggests the plates to be an Fe$^{2+}$Co$^{2+}$ doubled layered mineral with similarity to the green rust I (chloride) group$^{16}$. The source of the chlorine is most likely from a reaction between high concentrations of CoCl$_2$ with respect to FeCl$_3$ during the formation of the Co(II)Fe(III)-oxyhydroxide phase. The incorporation of cobalt into the green rust is considered to be due to the substitution of Fe(II) with Co(II)$^{32,33}$.

6.4.2. Magnetic properties and hyperthermia

Magnetic measurements performed on the nanoparticles indicate very large increases in coercivity ($H_c$) as cobalt concentration increases and importantly only relatively small decreases in overall saturation magnetization ($M_s$). Changes in magnetization with respect to applied magnetic field were carried out at room temperature (300 K) and low temperature (5 K) with results for coercivity $H_c$, saturation magnetization ($M_s$) and remanence ($M_r$), obtained from hysteresis loops presented in Figure 3.

Significant increases in the coercivity of the samples at low temperature (5 K) were noted as cobalt doping increases. The increase in $H_c$ reached a limit for Co20 (11.5 kOe), with Co33 exhibiting the same value, before a small decrease with Co50 (10.2 kOe). $M_s$ decreases at both room and low temperature although the most significant reduction is observed for Co50. An important result that can be taken from these measurements is that at Co20 (low temperature), there is a very significant increase in coercivity compared to Co0 (37× larger), whilst the saturation magnetization only decreases by 6 emu g$^{-1}$ corresponding to an 8% decrease. Maintaining a high $M_s$ is important in order to minimize the amount of material required to achieve the desired magnetic response for different applications, in particular those which are focused on medical therapies. Room temperature measurements also show increases in the coercivity of the samples as cobalt concentration increases although the maximum is reached at Co15 where $H_c$ is 12× larger than that measured for Co0. Changes in saturation magnetization parallel those seen for 5 K samples although approximately 8 emu g$^{-1}$ lower, excluding Co50, for which the difference is 20 emu g$^{-1}$.
Figure 3e shows that $M_r$ initially increases with increasing cobalt for both 5 K and 300 K before starting to decrease from Co20, with a value of zero at 300 K for Co50 corresponding to zero coercivity (i.e. superparamagnetism).

![Figure 3 – SQUID results. (a) Hysteresis loops measured at T=300 K, (b) Hysteresis loops obtained at T=5 K. (c) Coercivity measurements. Note room temperature coercivity measurements shown are x10 more intense than actual results to show changes more effectively on the same scale as low temperature measurements. (d) Saturation magnetization, (e) Remanence, (f-h) field cooled (FC) and zero field cooled (ZFC) curves show changes in blocking temperature. Information about the blocking temperature of the samples is obtained through examination of the field cooled and zero-field cooled (FC/ZFC) curves which were collected at 100 Oe (figure 3f-h). The blocking temperature $T_B$ of a sample is defined as the point above which a particle will exhibit superparamagnetism and the nanoparticles magnetization is free to align in random orientations. This is best interpreted as the peak of the ZFC curve at the point at which it intersects with the FC curve. The data show that at low %Co (Co0, Co5), intermediate %Co (Co15, Co20) and Co33 samples, the blocking temperature is just above room temperature with no discernable peak visible below 300 K. A clear peak is visible for Co50 at 220 K at which point the gradient of the ZFC curve is zero as it meets the FC curve. This point corresponds to the blocking temperature $T_B$, above which sample becomes superparamagnetic. This is confirmed by examination of the $H_c$ values which indicate that Co50 has zero coercivity at room temperature.
The approximate anisotropy of the samples ($K$) can be determined from the expression $H_c = 0.958K/M_s^{34}$ (assuming uniaxial anisotropy), where $H_c$ and $M_s$ correspond to coercivity and saturation magnetization at 5 K. The results presented in Table 2 indicate that the anisotropy significantly increases by an order of magnitude through the addition of minor additions of cobalt (Co5). Thus, the inclusion of Co in the spinel leads to an increase in the magnetic anisotropy of the nanoparticles comparable to previously observed values of first-order anisotropy constants of synthetic bulk Co-ferrites ($\text{CoFe}_2\text{O}_4$; $|K_1|$ ~ $2 \times 10^6$ erg cm$^{-3}$)$^{35}$. The effect appears to be greatest for the Co20 and Co33 samples which exhibit the highest recorded coercivities.

The potential applications of using cobalt doped magnetite nanoparticles was explored using magnetic hyperthermia experiments with results shown in Figure 4. The specific loss power (SLP) of the nanoparticles was determined from the rate of change of temperature of a suspension containing a known concentration of cobalt ferrite nanoparticles under the influence of an AC magnetic field (17 kA m$^{-1}$; 110 MHz), as discussed extensively elsewhere$^{12}$ (i.e. $\text{SLP} \propto \frac{dT}{dt}$). The temperature rise observed (normalised to the mass of nanoparticles measured in mg) is shown together with the determined SLP for the biogenic magnetite and two of the cobalt ferrite nanoparticles (Co20 and Co33), (Figure 4).
Figure 4 – Hyperthermia experiments indicate the rate of temperature change of a solution containing MNP’s Co0, Co20 and Co33 under the influence of an AC magnetic field (110 MHz). The specific loss power (SLP) describes the power achievable per unit gram of material

An enhanced heating effect can be observed clearly for the cobalt ferrite biogenic nanoparticles in comparison to the undoped magnetite nanoparticles. SLP is highly dependent upon the strength and frequency of the applied magnetic field and the effective relaxation time (τ eff) of the nanoparticles in suspension. Put simply, assuming constant frequency and field strength, SLP increases as τ eff increases. τ eff is highly dependent upon the size and anisotropy of the nanoparticles with larger anisotropy leading to longer τ eff for particles with the same size. The increase in K is considered to be the main factor responsible for the results observed in Figure 4 with cobalt doping dramatically increasing anisotropy (Table 2) resulting in the overall increase in SLP. Particle size, however, also contributes to τ eff with smaller diameters exhibiting shorter relaxation times than larger particles. This is thought to be the cause of the lower SLP recorded for Co33 than Co20 despite similar anisotropies. Further study is required to fully understand the temperature dependent processes occurring during this study.
6.4.3. Distribution of cations within the crystal structure

X-ray absorption (XAS) and X-ray magnetic circular dichroism (XMCD) spectra were collected to understand changes in the magnetic structure of the magnetite as cobalt is incorporated (Figure 5). The XAS spectra obtained in total electron yield (TEY) mode for both Fe $L_{2,3}$ and Co $L_{2,3}$ are shown in Figure 5a, with the absorption intensity of the Fe $L_3$ peak normalised to 1 with Co edges scaled accordingly. The intensity of the Co peak with respect to Fe provides the relative concentration of cobalt within the structure of the nanoparticles. The numbers match closely with those found using EPMA (Table 1). Examination of the leading Fe $L_3$ edge also shows the emergence of a shoulder feature (indicated by the dashed line) as more cobalt is incorporated. Such a feature is characteristic of oxidised magnetite (or the relative deficiency of Fe$^{2+}[B]$)$^{2,37}$, a result that would serve to confirm that cobalt was entering the magnetite in place of the Fe$^{2+}$ in the [B] site.

Figure 5 – XAS and XMCD measurements. (a) Both Fe $L_{2,3}$ (700 – 730 eV) and Co $L_{2,3}$ (770 – 810 eV) edges X-ray absorption spectra, (b) Fe $L_{2,3}$ XMCD, (c) Co $L_{2,3}$ XMCD.

Figure 5b and c show the XMCD spectra obtained for both Fe $L_{2,3}$ and Co $L_{2,3}$ edges respectively. The spectra clearly show changing cation distributions within the magnetite as the cobalt concentration increases, with the leading Fe$^{2+}[B]$ edge clearly reducing in magnitude with respect to the other peaks corresponding to Fe$^{3+}$. The
relative intensities of the XMCD spectra provide information related to the magnetizations of the samples relative to each other. It is clear that sample Co50 has the smallest XMCD amplitude in all cation sites, reflecting its lower saturation magnetization as shown in Figure 3d.

The cations which make up stoichiometric magnetite generate unique XMCD signatures determined by site location, valence state and magnetization direction. Atomic multiplet calculations\textsuperscript{26, 27} have been applied to the Fe \textit{L\textsubscript{2,3}} XMCD spectra and have been fitted to find the most accurate cation fit and determine the relative intensity of the Fe cations within the spinel. Stoichiometric magnetite has a total iron occupancy of 3 (Fe\textsuperscript{2+}[B]:Fe\textsuperscript{3+}(A):Fe\textsuperscript{3+}[B] corresponding to 1:1:1), however this decreases as cobalt is introduced, and the overall spinel tends towards total iron occupancy of 2. The results in Table 3 have been normalized to include cobalt concentrations as determined through XAS peak height comparison (EPMA is a bulk technique whereas XAS and XMCD are surface measurements thus the XAS results are more relevant for examination of XMCD data).

<table>
<thead>
<tr>
<th>x\textsubscript{XAS}</th>
<th>Fe\textsuperscript{2+}[B]</th>
<th>Fe\textsuperscript{3+}(A)</th>
<th>Fe\textsuperscript{3+}[B]</th>
<th>%Fe(A)</th>
<th>%Fe[B]</th>
<th>Total +Q</th>
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<td>0.88</td>
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<td>63.33</td>
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</table>

Table 3 – Results of XMCD spectral fitting. Relative ratios normalised to include Co determined from x\textsubscript{XAS}. Total charge +Q determined from the sum of positive iron Fe\textsuperscript{2+}, Fe\textsuperscript{3+} and cobalt Co\textsuperscript{2+} cations.

The results in Table 3 confirm a significant decrease in Fe\textsuperscript{2+}[B] site occupancy with increasing Co, however it also points to a decrease in both Fe\textsuperscript{3+}(A) and Fe\textsuperscript{3+}[B] although not as significantly. This result would appear to suggest that the majority of the cobalt is incorporated into the octahedral site in place of ferrous iron, however there might also be substitution into ferric iron tetrahedral and octahedral sites. The total positive charge due to transition metal ions in the nanoparticles also appears to decrease as cobalt increases.

Further analysis of the cobalt XMCD (Figure 5c) is useful in the determination of where the cobalt is entering the crystal structure. Comparison of the Co XAS and XMCD spectra obtained for all samples shows very little differences between the spectra in structural forms other than in intensity. Highest cobalt containing samples (Co50) have
the most intense XAS spectra, corresponding to higher Co concentrations, however the least intense Co XMCD. The cobalt $L_3$ edge XMCD is strongly negative which is indicative of octahedral site occupancy. Comparison between the data and calculated spectra also provides information about the oxidation state of the cobalt$^{26, 27}$. The spectra observed most closely match that of calculated Co$^{2+}$ octahedral (as identified by Coker et al 2009$^{19}$), a result that strongly confirms the incorporation of cobalt in place of ferrous iron in the octahedral site and does not appear to show any other components corresponding to cobalt in different environments (i.e. tetrahedral Co$^{2+}$, or Co$^{3+}$).

Mössbauer analysis was carried out to characterise bulk materials. Mössbauer spectroscopy is a technique that is used to determine changes in the stoichiometry of minerals such as magnetite through examination of changes in the valence states of the Fe$^{57}$ isotope. Spectra for all six different cobalt concentrations have been obtained at room temperature (R = 300K) and liquid nitrogen temperatures (N = 110 K) (Figure 6). The presence of six main peaks for samples Co0 – Co33 at RT and LT indicate the presence of a magnetic hyperfine field at the nucleus that is not present for sample Co50 at RT, but is observable at LT. This last result confirms the result of the FC/ZFC spectra collected using SQUID which suggests that the blocking temperature of Co50 is at 220K, well below room temperature measurement and results in the superparamagnetic spectra visible in Figure 6.
Figure 6 - Mössbauer spectra obtained for a series of cobalt doped magnetite samples at (a) room temperature (R), (b) low temperature (N). Parameters for each sextet obtained including (c) isomer shift, (d) hyperfine field, (e) quadrupole splitting and (f) relative sextet site population.

Sample Co0 can be fitted accurately with two sextets, corresponding to Fe$^{3+}$ in the tetrahedral environment and Fe$^{2+}$-Fe$^{3+}$ in the octahedral sites. The room
temperature spectra also show the presence of an additional doublet, which can be attributed to the wide size distribution of the nanoparticles, in which some of the particles may be small enough to be superparamagnetic at room temperature. This is confirmed as the doublet is not present at low temperature, (i.e. well below the blocking temperature).

Room and low temperature measurements of Co5 to Co33 show at least 2 distinguishable sextets, however analysis shows that there must be at least one more sextet to produce an accurate fit for all the samples. The third sextet is not necessarily expected, as electron hopping between Fe$^{2+}$ and Fe$^{3+}$ in the B site has previously been shown to be too rapid to resolve into separate spectra using Mössbauer, however, this hopping effect is thought to be due to localised hopping between Fe$^{2+}$-Fe$^{3+}$ octahedral pairs, facilitated by the intervening oxygen anion$^{38}$. A consequence of localized hopping is that the Mössbauer spectra should correspond to one spectrum arising from all the Fe$^{3+}$-Fe$^{2+}$ pairs on the octahedral sites, and another arising from the remaining Fe$^{3+}$ ions on both sites$^{38,39}$. Hence, as cobalt is introduced into the system, the total amount of Fe$^{2+}$ available for localised pairing is expected to decrease and it is reasonable to anticipate an extra sextet being required to accurately match the spectra observed (denoted B2 in Figure 6 and Table 4).

Fitting of the samples was performed to obtain parameters including isomer shift (IS), quadrupole splitting ($\Delta E_Q$) and hyperfine field ($B_{hf}$). The IS is an observable shift in the absorption lines of Mössbauer spectra from zero velocity resulting from the interaction of part of the electronic cloud inside the volume of the nucleus with the nucleus and provides information about chemical changes in the atom such as incorporation of cobalt (figure 5c). Fitting of the [B] spectra at low temperature, show IS 0.73 mm s$^{-1}$ for Co0, falling to 0.44 mm s$^{-1}$ for Co50. There is no observable change in the IS determined for Fe$^{3+}$ in the (A) site with all samples displaying an IS of approximately 0.4 mm s$^{-1}$ ± 0.01 mm s$^{-1}$, indicating that even at the highest cobalt concentrations there is no incorporation of Co into the tetrahedral site, a result that could not be confirmed using XMCD. The third sextet observable has also been fitted and displays no obvious change in isomer shift with increasing cobalt concentration with samples displaying an average IS of 0.56 mm s$^{-1}$ ± 0.02 mm s$^{-1}$.
<table>
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<th>Site</th>
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<th>$\Delta E_Q$ (mm s(^{-1}))</th>
<th>$B_{hf}$ (kOe)</th>
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Table 4 - Mössbauer fitting parameters determined for Co ferrite samples at room temperature (R) and low temperature (N). Parameters include isomer shift (IS), quadrupole splitting ($\Delta E_Q$), hyperfine field ($B_{hf}$), linewidth (w) and population (Pop.).
Room temperature measurements of Co50 indicate the presence of 2 doublets, which based on isomer shift and quadrupole splitting correspond to an Fe\(^{2+}\)-containing mineral determined to have similar parameters to green rust which was determined to be an Fe\(^{2+}\)Co\(^{2+}\) double layered structure from TEM results\(^{40}\) and an unidentified Fe\(^{3+}\)-containing mineral purportedly unreduced Co(II)Fe(III)-oxyhydroxide. The low temperature spectrum is best fitted with five different components which correspond to the (A)-Site, [B] and [B2] Sites, the Fe\(^{2+}\)Co\(^{2+}\) double layered phase and un-reduced iron gel.

The quadrupole splitting observed shows the general trend for the (A) and [B2] sites to remain relatively unchanged throughout the series, however there is seen to be a linear decrease in [B] site splitting until sample Co50, for which the value more closely matches that of Co5.

The magnetic hyperfine field ($B_{hf}$) at the nucleus arises from any unpaired spin of the electrons of the atoms and enables the determination of the effective magnetic field acting at the nucleus, hence can provide information relating to the valence state of the nucleus undergoing gamma ray absorption. Since it is a magnetic component, only unpaired electrons contribute to $B_{hf}$. The $B_{hf}$ determined for the pure magnetite sample at room temperature is calculated at 478 kOe at the tetrahedral (A) site, and 460 kOe at the octahedral [B] site at low temperature, which closely matches established data\(^{41}\). Low temperature measurements of 505 kOe and 480kOe for (A) and [B] sites respectively also match pre-existing data closely\(^{40, 41}\). There is an observable overall decrease in the $B_{hf}$ of the [B] sextets through the series. This would correspond to a drop in unpaired Fe\(^{2+}\)[B] sites hence substitution by Co\(^{2+}\). Changes in the $B_{hf}$ for the sextets (A) and [B2] do not appear to change, which also suggests that Co\(^{2+}\) is not substituted into tetrahedral Fe\(^{3+}\) sites.

In summary, the nature of the techniques that we have used allows studies of the surface layer of the nanoparticles to be compared against the bulk measurements, with differences observable across both. It is difficult to rule out possible incorporation of Co\(^{2+}\) in place of Fe\(^{3+}\)(A) by examination of the Fe edge XMCD, with cation occupancy calculations suggesting that a small amount might be incorporated into both tetrahedral and octahedral environments. The Co edge XMCD shows a spectrum appearing to corresponding to entirely Co\(^{2+}\)[B] thus suggesting that if a small amount of Co\(^{2+}\) is introduced into the tetrahedral site, then it is on the surface only and not in
the spinel and it is not magnetically polarised (hence not observable using XMCD). Mössbauer suggests that in the bulk samples, the only substitution of Co\(^{2+}\) is in place of the octahedral site. This result suggests that whilst a significant portion of cobalt can be incorporated into the whole magnetite crystal, some remains at the surface, without being bound fully to the spinel. The incorporation in tetrahedral environments cannot be totally discounted but the evidence would suggest that this is at most minimal.

### 6.5. Conclusions

This work has explored the magnetic properties of biogenic cobalt doped magnetite nanoparticles formed by the reduction of Co(II)Fe(III)-oxyhydroxide starting material by bacteria at ambient temperature and pressure. The overall coercivity of the particles is seen to increase dramatically with cobalt doping, whilst having a relatively minor decrease in saturation magnetization. We have demonstrated that a biological method can be used to obtain nanoparticles with tailored magnetic anisotropy values by Co doping. For applications in magnetic hyperthermia this ability to control anisotropy, together with the possibility of controlling nanoparticle sizes as demonstrated previously\(^{42}\), could provide a method to optimise the nanoparticle relaxation time for a specific applied field frequency, thus greatly enhancing the heating effect. Large heating effects are essential to realise the therapeutic potential of the technique where the applied field amplitude and frequency are limited by practical and clinical considerations.

### Acknowledgements

This work was carried out with the financial support of a NERC PhD studentship awarded to James Byrne. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No.DE-AC02-05CH11231. We acknowledge NERC Envirosync II for providing support for this work. Additional thanks to Dr. Michael Ward for assistance and the provision of access to Transmission Electron Microscope by Leeds Nanoscience and Nanotechnology Facility (LENNF).
6.6. References


Scale-up of Biomagnetite Production Using *Geobacter sulfurreducens*
7. Scale-up of Biomagnetite Production Using *Geobacter sulfurreducens*

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7.1. Abstract

The production of nano-scale biomagnetite particles by the Fe(III)-reducing bacterium *Geobacter sulfurreducens*, was scaled-up successfully from lab-scale production by a factor of approximately three orders of magnitude, while maintaining the surface reactivities and magnetic properties which make this material well suited to commercial exploitation. The bacterium was grown in a 50 L bioreactor, harvested and then inoculated into a buffer solution containing Fe(III)-oxyhydroxide and an electron donor and mediator, which promoted the formation of magnetite in under 24 hours. The particle size was maintained between 10 and 15 nm during scale-up of this second step from 10 ml to 10 L, with conserved magnetic properties and surface reactivities; the latter demonstrated by the reduction of Cr(VI). The process presented provides an environmentally benign route to magnetite production and serves as an alternative to harsher synthetic techniques, which with relatively minor refinements could be used to produce kg to tonne quantities.

7.2. Introduction

Several studies in recent decades have highlighted the potential uses of magnetic nanoparticles, in particular those based on magnetite Fe\(_3\)O\(_4\), for a wide range of applications which exploit their small surface area to volume ratios and magnetic
properties. These applications include targeted cancer therapies\(^1\), magnetic data storage devices\(^2\) and the remediation of contaminated land\(^3\). A number of methods of manufacturing magnetite nanoparticles currently exist, however, these require the use of energetic ball milling, high temperatures and/or toxic reagents which are expensive and damaging to the environment\(^7\), \(^8\). Biological processes catalysed by dissimilatory iron-reducing bacteria (DIRB’s), such as subsurface bacteria of the genera \textit{Geobacter} and \textit{Shewanella} offer an alternative method for the production of nanoscale “biomagnetite” at ambient temperature and pressure\(^9\)-\(^12\). These organisms are able to conserve energy in the subsurface\(^13\) through the oxidation of organic matter as an electron donor, coupled with the reduction of a poorly crystalline Fe(III) containing compounds (e.g. ferrihydrite) which serves as the electron acceptor\(^14\), with the process accelerated by the presence of electron shuttles such as humics, riboflavin or anthraquinone-2,6-disulphonate (AQDS)\(^15\). Soluble Fe(II) released by the reduction of solid phase Fe(III) causes recrystallization processes and the formation of new mineral phases such as magnetite under optimised conditions, or containing goethite, hematite, siderite or vivianite depending upon the conditions of formation including pH, temperature and the geochemical matrix present\(^16\)-\(^18\). Although biomagnetite particles show considerable commercial potential, there have been few studies on the scale-up of Fe(III)-reduction by mesophilic subsurface Fe(III)-reducing bacteria. Recent work has demonstrated the synthesis of biomagnetite at the 30 litre scale using the thermophilic bacterium \textit{Thermoanaerobacter} sp TOR-39 (at 65 °C), with encouraging results. Here approximately 1 kg of magnetite was produced from 30 L bioreactor over a period of two weeks\(^19\). The purpose of the work described in this manuscript is to demonstrate the successful scale-up of biomagnetite production at mesophilic temperatures (30 °C), with conversion of ferrihydrite over relatively short-time scales (less than 24 hours after inoculation), while maintaining optimal surface area and reactivity of the bionanoparticles for the remediation of a model toxic metal contaminant Cr(VI), via Fe(II)-mediated reduction to Cr(III)\(^20\), \(^21\).

The scale-up processes presented in this paper were separated into several distinct stages; (1) the optimisation of growth medium to produce significant quantities of bacteria while maintaining a high Fe(III)-reducing activities; (2) upscaling growth of the organism \textit{Geobacter sulfurreducens} from 100 ml serum bottle cultures to a 50 L pilot-scale bioreactor; (3) comparison of the biotransformation of Fe(III)-
oxyhydroxide to magnetite using biomass from the 50 L bioreactor in discrete mineral biotransformation step scales of between 10 ml to 10 L; (4) detailed characterization of the biomagnetite produced during scale-up by powdered X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray absorption (XAS) and X-ray magnetic circular dichroism (XMCD), while surface reactivity was tested by quantifying Cr(VI) reduction.

7.3. Experimental Methods

7.3.1. Starting materials

7.3.1.1. Iron oxyhydroxide

Amorphous Fe(III)-oxyhydroxide was precipitated by the reaction of 0.66 M iron(III) chloride (FeCl₃) with sodium hydroxide (NaOH; 10 N) until reaching a final pH of 7. The material was centrifuged at 17 000g for 20 min and washed in deionized water to remove chloride ions, with the washing step repeated six times. The total Fe concentration of the starting material was determined by ferrozine assay after reaction with hydroxylamine (HONH₂·HCl; 6 N).

7.3.1.2. Bacterial growth media

Cultures of Geobacter sulfurreducens were grown in the dark in anaerobic conditions (N₂ headspace) and incubated at 30 °C in a modified freshwater medium. The growth medium contained the electron donor sodium acetate (between 25 - 50 mM), electron acceptor fumaric acid (between 40 - 150 mM) and additional components including: 100X NB mix (10 ml L⁻¹), NB mineral elixir (10 ml L⁻¹), vitamin mix (15 ml L⁻¹), CaCl₂·2H₂O (0.3 mM), MgSO₄·7H₂O (0.4 mM), NaHCO₃ (21.4 mM), Na₂CO₃·H₂O (4.7 mM), Na₂O₄Se (0.001 mM), (Supplementary table ST1 contains full details of 100 NB mix, NB mineral elixir and vitamin mix).
7.3.2. Optimization Experiments

7.3.2.1. Growth medium optimisation

Experiments were prepared containing growth medium (90 ml in 100 ml serum bottle) with electron donor and acceptor concentration ratios varied between 25:40, 50:80, 25:100, 50:100, 25:150, 50:150, 40:25, 80:50, 100:25, 100:50, 150:25 and 150:50 (acceptor mM : donor mM) with each ratio tested in triplicate. Cultures were inoculated with 10% late log-phase *G. sulfurreducens* (grown on 25:40 growth medium) and incubated in the dark at 30 °C with no agitation. Optical density were measured at 600 nm (OD$_{600}$) using an M501 single beam scanning UV/visible spectrophotometer at regular intervals.

Each replicate of each donor: acceptor varied culture was harvested after 27 hr (late log-phase of 50:80 medium) by centrifugation at 4920g and 4 °C for 20 min and washed twice in NaHCO$_3$ buffer (30 mM; pH 7) under a N$_2$:CO$_2$ (80:20) gas line. The bacteria were transferred into a minimal volume of bicarbonate buffer forming separate slurries for each donor: acceptor ratio. The OD$_{600}$ of each suspension was measured at 600 nm, with slurries diluted with buffer to achieve an OD$_{600}$ of 0.4 in 10 ml volume (0.2 ml cell suspension, 9.8 ml deionized H$_2$O). The concentration of bacteria in each growth electron donor: acceptor ratio was measured initially as a function of optical density (OD$_{600}$). The OD$_{600}$ value was then converted into a biomass concentration corresponding to the amount of protein present in mg ml$^{-1}$ by reference to standard curve prepared with bovine serum albumin.

Washed cell suspensions for Fe(III)-oxyhydroxide bioreduction experiments were prepared in advance in volumes of 10 ml, containing an electron donor (sodium acetate 20 mM), electron acceptor (Fe(III) concentration, 50 mM L$^{-1}$), NaHCO$_3$ buffer (30 mM) and electron shuttle (riboflavin; 10μM) to accelerate Fe(III)-reduction. Cultures were prepared under an N$_2$:CO$_2$ (80:20) gas line to ensure anaerobic conditions, and autoclaved at 121 °C for 20 min in a Prestige Medical autoclave. Fe(III)-reducing cultures were inoculated at room temperature with *G. sulfurreducens* (0.132 mg ml$^{-1}$ protein) in triplicate and incubated in the dark at 30 °C with no agitation. The rate of Fe(II) production was measured using a ferrozine assay at regular intervals.
7.3.2.2. Bioreactor

An Applikon 7 L dished bottom bioreactor vessel was filled with 4.5 L growth medium (50 mM acetate, 80 mM fumaric acid; pH=7) and sterilised at 121 °C for 20 min in a Boxer 300/150 LR autoclave. The medium was purged with N₂ gas for 30 min to make the system anoxic, pH was neutralised and all input and output valves were then sealed. The medium was inoculated with a 10 % late log phase culture of *G. sulfurreducens* and then incubated at 30 °C. Redox (mV) and pH were observed in situ with biomass extracted via a sample point and measured as a function of optical density at 600 nm absorbance at regular intervals. Stir speed was varied from 0 rpm to 100 rpm over different experiments.

7.3.3. Pilot plant scale production of bacterial cultures and mineral transformation

7.3.3.1. 50 L bioreactor

A Sartorius 10 L seed bioreactor was prepared with 4.5 litres of the growth medium (50 mM acetate, 80 mM fumaric acid; pH=7) and sterilized in situ at 121 °C for 30 minutes. Following sterilization, the medium and vessel were purged with N₂ gas for 30 minutes. The pH of the starting medium was adjusted to neutral using 1 M MOPS (3-(N-morpholino)propanesulfonic acid) and 10 M sodium hydroxide. The 50 L bioreactor was prepared with 45 litres of the growth medium and sterilized at 121 °C for 30 minutes. The medium was then purged with N₂ gas and adjusted to neutral pH using 1M MOPS and 10M NaOH.

The 10 L seed fermenter was inoculated with a 10 % late log phase culture of *G. sulfurreducens* and incubated at 30 °C, and a gentle stir speed of 100 rpm. There was no pH control after inoculation. After 43 hr growth in the seed fermenter, 5 L *G. sulfurreducens* was transferred to the 50 L reactor (10% inoculum). Again, pH was not controlled and the temperature was kept constant at 30 °C, stir speed of 100 rpm.

7.3.3.2. Harvesting of biomass and Fe(III)-oxyhydroxide bioreduction

Fe(III)-oxyhydroxide bioreduction cultures were prepared in deionized water containing electron donor (20 mM sodium acetate), electron acceptor (33 mM L⁻¹ Fe(III)-oxyhydroxide) and buffer (30 mM NaHCO₃) in volumes of 2 x 10 L, 3 x 1 L, 10 x 100 ml, 10 x 10 ml. The bioreduction medium (pH=9) was autoclaved at 121 °C for 20
minutes. 10 μM riboflavin was added as an electron shuttle to each of the bottles which were then purged with N₂ to drive them anaerobic and pH adjusted (pH=7) using 1 M MOPS and 10 M NaOH. The biomass for these experiments was prepared as follows. After a 48 hr biomass production phase, the entire contents of the 50 L fermentation vessel was centrifuged in a Thermo Scientific Sorval RC 3BP+ centrifuge, at 5000 rpm, 4 °C for 20 minutes to pellet the biomass. The bacterial culture was spun in batches of 6 x 1 L. Following the removal of the supernatant, the cell pellets were re-suspended in NaHCO₃ (30mM; pH 7) and centrifuged again, with this washing process repeated twice. The final cell pellet was re-suspended in a minimal amount (approximately 900 ml) of NaHCO₃ to form the bacterial stock solution. The OD₆₀₀ of the bacterial stock solution was determined by dilution of 0.1 ml in 5 ml deionised water and measured using a Jenway 6305 spectrophotometer. Inocula of 320 ml, 32 ml, 3.2 ml and 0.32 ml G.sulfurreducens concentrated cell suspensions were added to each of the 10 L, 1 L, 100 ml and 10 ml bottles respectively. The iron transformation experiments were incubated in the dark at 30 °C.

7.3.4. End product Characterisation

Aqueous Fe(II) concentration in biomineral slurries were measured using the ferrozine assay²³ at intervals of 0 hr, 4 hr, 24 hr and 72 hr using an M501 single beam scanning UV/visible spectrophotometer at 562 nm. Measurements were repeated in triplicate for the 10 ml, 100 ml and 1 L iron reduction experiments. Triplicate measurements at the 10 L was achieved from a single magnetite production bottle, with vigorous shaking performed between taking each sample.

The mineral phases present in the end product were obtained using powder x-ray diffraction (XRD), measured with a Bruker D8 Advance instrument with Cu Kₐ₁ source. Spectra were collected over a 2θ range of 5-70° with a step size of 0.02°. The average crystallite particle size of magnetite was determined by fitting a Lorentzian function to (220), (311), (400), (511) and (411) reflections with the fitting parameters entered into the Lorentzian function²⁶, ²⁷. Errors were generated as the standard deviation from the mean.

Imaging of the end product was performed using transmission electron microscopy (TEM) carried out using a Phillips/FEI Technai electron microscope
equipped with a field emission gun (FEG) and Gatan image filter (GIF). Images are presented in bright-field with an operating beam voltage of 300 keV.

X-ray absorption spectroscopy (XAS) on the Fe $L_{2,3}$-edge of the samples were measured at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, Berkeley, California, USA. Data were acquired in total-electron yield mode (TEY) using circularly polarized light with XAS collected with the sample in an applied magnetic field of -0.6 T and +0.6 T, parallel and anti-parallel to the direction of the beam respectively. TEY has an effective probing depth of ≈3-4 nm, with signal intensity decreasing exponentially with depth. X-ray magnetic circular dichroism (XMCD) data were derived from the difference between the XAS spectra collected under both magnetic field directions. XMCD spectra are dependent upon magnetization, site location and valence state (i.e. number of d electrons) and are used to determine the oxidation state of cations occupying different lattice sites in metal oxides. Site distributions of Fe cations within the crystalline material were determined through the application of atomic multiplet calculations. Dried samples were loaded on to carbon tape mounted onto a copper sample probe in an anoxic glove bag purged with N$_2$. The sample probe was sealed in an air tight container for transportation to the sample chamber with the container removed as late as possible in a back flow of nitrogen to minimize potential exposure to air to prevent oxidation of the sample.

Experiments to determine the source of hematite in selected experiments (Figure 5) were carried out with starting material designed as in section 2.2.1 but in 100 ml volumes. Four different arrangements were prepared: (i) starting material de-gassed with N$_2$:CO$_2$ (80:20) gas mix, pH=7 adjusted using gas and then autoclaved at 121 °C for 20 mins; (ii) starting material (pH=9) autoclaved at 121 °C for 20 mins, de-gassed with N$_2$:CO$_2$ (80:20) gas mix and pH adjusted to 7 using the gas; (iii) starting material de-gassed with N$_2$:CO$_2$ (80:20) gas mix, pH=7 adjusted using gas (not autoclaved); (iv) starting material (pH=9) autoclaved at 121 °C for 20 mins, de-gassed with N$_2$ gas and pH adjusted to 7 using N$_2$ and 1 M MOPS.

7.3.5. Fe(II)-mediated reduction of Cr(VI)

The surface reactivity of biogenic magnetite produced at the various scales was tested by quantifying the Fe(II)-mediated reduction of Cr(VI). The materials were washed twice in d.H$_2$O, using a bar magnet to separate supernatant from magnetic sample and
resuspended to a final concentration of 50 mM L⁻¹ Fe (by Ferrozine assay) in a 5 mM solution of K₂CrO₄. All bottles were mixed continuously on a roller-mixer at 33 rpm, stored in the dark at 20 °C. Aliquots of 0.2 ml were extracted and centrifuged at 15,000 g for 1 min to separate solid material from solution. A 50 μl aliquot of the supernatant was then diluted 1:100 with d.H₂O and used to measure for aqueous Cr(VI) using diphenyl carbazide (DPC) spectrophotometric assay with absorption measured at 540 nm.

7.4. Results and Discussion

7.4.1. Scale up of Geobacter sulfurreducens production

7.4.1.1. Optimisation of growth medium

The first step in the scale-up of biomagnetite production was to optimise the growth medium used to produce microbial biomass for maximum Fe(III) reduction rates, using small scale (100 ml) serum bottle incubations. Previous studies have focussed on the use of a modified fresh water medium (designated NBF)²⁴, with an electron donor (acetate) and electron acceptor (fumarate) concentration ratio of 25:40 mM. The medium also contains essential trace metals, vitamins and carbonate buffer and yields biomass at approximately 0.2 mg protein ml⁻¹ (equivalent to 0.36 mg biomass dry weight ml⁻¹). Optimisation experiments where the electron donor: electron acceptor ratio was varied between 25:40, 50:80, 25:100, 50:100, 25:150 50:150 mM were performed at 100 ml scale (Figure 1). Increasing the electron acceptor (fumarate) concentration led to significant increases in the total biomass produced compared to the standard 25 mM:40 mM (Figure 1a), with biomass values more than doubling from 0.17 mg ml⁻¹ protein (25:40) to 0.45 mg ml⁻¹ protein (25:150). Increasing the electron donor concentration in these experiments did not impact on the final bacterial density, suggesting that the electron acceptor was the limiting nutrient in the standard growth medium formulation.
Figure 1 – Optimization of growth medium. (a) Growth rate of Geobacter sulfurreducens with varied electron donor: acceptor ratios. (b) Fe(III) reduction rates of bacteria produced on varied electron donor: acceptor ratios. (c) XRD of the end product indicates mostly magnetite (M) formation with goethite (G) present in sample 50 mM acetate : 150 mM fumarate.

The rate of reduction of Fe(III)-oxyhydroxide by cells grown at the various electron donor to acceptor ratios was monitored through the measurement Fe(II) produced in washed cell suspensions containing the Fe mineral. *G. sulfurreducens* grown in medium containing 25 mM acetate and 40 mM fumarate exhibited the fastest reduction rates, with those samples containing higher ratios of electron donor:
acceptor (e.g. 25 or 50 mM acetate : 150 mM fumarate) showing the slowest rate of Fe(III) reduction (Figure 1b). Thus, cultures tending towards electron acceptor limitation would seem to have optimal iron-reducing activities. Doubling the concentrations of both electron donor and acceptor (to 50 mM acetate and 80 mM fumarate) maintained high Fe(III)-reducing activities, while increasing biomass yields.

The end product of ferrihydrite reduction was examined visually for colour and for magnetization using a bar magnet, for each of the donor: acceptor ratios tested. Whilst the colour of 25:40 samples were dark black and strongly magnetic, the 25:150 and 50:150 electron donor : acceptor ratio samples had a dark brown/grey colour and were less strongly attracted to the magnet. Samples with an electron donor: acceptor ratio of 50:80 behaved the most closely to 25:40 samples. Further analysis using powdered x-ray diffraction (Figure 1c) confirmed the presence of magnetite in all samples with the additional mineral phase goethite (α-FeOOH) observed in sample 50:150 which could be attributed to the low rate of reduction observed in Figure 1b\textsuperscript{16}, which results in incomplete recrystallization of the ferrihydrite to magnetite. The average crystallite sizes of the magnetite nanoparticles were determined from the (311) reflection to be 43 nm, 32 nm, 51 nm, 55 nm, 57 nm and 51 nm for samples 25:40, 50:80, 25:100, 50:100, 25:150, 50:150 respectively.

From the results presented in Figure 1, a concentration of 50 mM acetate and 80 mM fumarate was chosen as optimal for the production of *Geobacter sulfurreducens* biomass for magnetite production during subsequent scale-up work, offering a good biomass yield together with fast Fe(III) reduction rates. The benefits of choosing higher concentrations of electron donor and acceptor (at similar ratios) were not considered to be sufficient to justify the additional economic costs of the medium constituents, although there may be further scope for medium optimisation if other limiting nutrients are identified at these higher concentrations of electron donor and acceptor.

### 7.4.1.2. Biomass production in a 7 L bioreactor

The defined medium containing 50 mM acetate and 80 mM fumarate was used in an intermediate scale bioreactor (7 L) for biomass production for magnetite synthesis, prior to transfer to the 50 L pilot plant system. The original growth medium experiments took place in 100 ml bottles that were de-gassed (and pH neutralised)
with an 80:20 N₂ and CO₂ gas mix before being sealed and autoclaved. Sterilisation using this approach was not possible in the bioreactor, as the pressure vessel was not able to withstand high pressures associated with autoclaving when the vessel was sealed completely, but required venting at several ports. These venting ports could also allow the backflow of oxygen into the system post autoclaving. Additionally, only an N₂ gas line was available in the pilot plant for the 50 L fermenter, hence an alternative method other than gassing with 20% CO₂ was required to lower the pH of the bicarbonate-containing growth medium to pH 7. Figure 2 shows the growth of *Geobacter sulfurreducens* measured in the 7 L bioreactor containing 5 L medium and with pH control using a range of approaches and compared to growth in the 100 ml bottles (Figure 2-a).

One benefit of working with a bioreactor rather than sealed bottles is the ability to control closely a range of environmental parameters including the pH during an experiment. When 2M HCl and 2M NaOH were used as acid and base respectively, to control pH (at pH 7), a relatively poor rate of growth was recorded (Figure 2-b). This
was also the case when 1M MOPS and 2M NaOH were used to control pH (Figure 2-c). Alternatively, the buffer system (sodium bicarbonate) already present in the modified growth medium proved sufficient to maintain a circumneutral pH. In the absence of a controlled CO₂, headspace 2M MOPS (3-(N-morpholino)propanesulfonic acid) was also used to initially neutralise the medium following the de-gassing stage prior to addition of the inoculum. Stirring at 100 rpm had a negative impact on growth (Figure 2-f), with a slower growth rate than noted at 0 rpm and 50 rpm (Figure 2- e and d). The absence of stirring (0 rpm; Figure 2e)) however resulted in the formation of a film of bacteria (biofilm) on the inside of the reactor vessel, which can be observed by the decrease in planktonic biomass after ~33 hours of growth, hence a minimal amount of stirring (50 rpm; figure 2d) was considered necessary.

7.4.2. Large scale cultivation of *G. sulfurreducens* and biomagnetite synthesis

Following on from the initial experiments to optimise the production of biogenic magnetite nanoparticles at laboratory scale, and biomass production at an intermediate (5 L) scale, these processes were then transferred to a pilot-plant system for further scale-up work. The procedure was divided into two stages focusing on (a) large scale growth of the bacterium *Geobacter sulfurreducens* in a 50 L bioreactor, and (b) iron biotransformation in reaction vessel volumes ranging from 10 ml to 10 L (Figure 3).
Figure 3 – Large scale production of biogenic magnetite nanoparticles. (a) 50 L bioreactor for batch production of *Geobacter sulfurreducens*. (b) Fe(III)-oxyhydroxide starting material was inoculated with washed cell suspensions and electron donor and an electron shuttle (riboflavin) in bottles of varying volume (10 ml to 10 L).

7.4.2.1. Growth of *G. sulfurreducens* in a 50 L bioreactor

The biomass (OD$_{600}$ converted to mg ml$^{-1}$ protein, see 3.2.1 for details), redox (mV) and pH of a culture of *Geobacter sulfurreducens*, grown anoxically in 5 L and 50 L bioreactors in a fully defined medium containing 50 mM acetate and 80 mM fumarate, were monitored over a period of 48 hr and the results compared to the growth profile obtained in 100 ml bottles (Figure 4). A 10 % inoculum from a late exponential starter culture was used throughout.
Figure 4 - *Geobacter sulfurreducens* growth curves. (a) 100 ml bottles; (b) 5 L bioreactor; (c) 50 L bioreactor.

There was a significant increase in the lag-phase of the cultures with increasing vessel volume, with 100 ml sample exhibiting the shortest lag phase. The doubling time was measured as approximately 5 hr and 4 hr for 100 ml and 50 L cultivations cultures respectively. Final biomass yields were similar for all volumes; 0.36 mg ml\(^{-1}\), 0.34 mg ml\(^{-1}\) and 0.39 mg ml\(^{-1}\) (protein) for 100ml, 5 L and 50 L production runs, respectively.

The pH values in the 5 L and 50 L cultures remained circumneutral, with a minor decrease seen in the 5 L bioreactor and a minor increase seen in 50 L bioreactor. The redox values in both vessels decreased as the cultures grew, with a maximal drop from +180 to -362 mV and from +37 to -440 mV for 5 L and 50 L cultures respectively.
7.4.2.2. Scale up of magnetite formation

*Geobacter sulfurreducens* biomass was harvested from the 50 L bioreactor after 49 hours of batch growth. Following centrifugation and washing with a 30 mM bicarbonate buffer, approximately 900 ml of a bacterial slurry was produced after suspension in a 30 mM NaHCO$_3$ buffer solution to a final biomass concentration of 10.5 mg ml$^{-1}$ protein. Inocula of 0.032 ml of the *G. sulfurreducens* slurry were added per 1 ml of 33 mM L$^{-1}$ Fe(III)-oxyhydroxide suspension, corresponding to a final biomass concentration of 0.336 mg ml$^{-1}$ protein. Based on this inoculum concentration, the 900 ml total volume of bacterial slurry harvested from the 50 L fermenter was sufficient for the inoculation of approximately 28 L of Fe(III)-oxyhydroxide biotransformation medium. To examine the effect of volume increase on the biotransformation of ferrihydrite to magnetite this process was carried out in 10 ml, 100 ml, 1 L and 10 L bottles, each containing the same concentrations per litre of electron donor, electron acceptor, buffer and electron shuttle. Fe(III) reduction rates were monitored over 72 hours using the ferrozine assay$^{23}$ (Figure 5a). The results indicate only relatively minor differences in the rate of reduction of Fe(III) to Fe(II) at all scales. Figure 5a displays the rate of Fe(II) generation relative to the total Fe present at the start of the reaction. If stoichiometric magnetite (Fe$_3$O$_4$) was produced and all the Fe(II) was quantifiable by this assay, then the value would be 0.33 (i.e. one third of the total Fe should be Fe(II) in accordance with the cation ratio Fe$^{2+}$[B]:Fe$^{3+}$(A):Fe$^{3+}$[B] of 1:1:1). Figure 5a shows very little difference between the 100 ml, 1 L and 10 L transformations in terms of Fe(II) generation, with all three curves within error of each other, however each fell below the stoichiometric value of 0.33. The smallest volume vessel (10 ml), exhibited significantly less overall Fe(III) reduction, with a final value of only 0.15 Fe(II). All samples were dark black with magnetic characteristics, consistent with magnetite, after only 4 hours of incubation. The reasons for the lower than expected final concentration of Fe(II) could either be due to the limitation of the assay, which uses a 0.5M HCl digestion step that may not fully dissolve all of the Fe(II) present in the magnetite structure, or could be due to the presence of additional mineral phases which is discussed below in more detail.
Figure 5 - Fe(III) reduction rates and formation of iron oxide minerals. (a) Fe(II)/Fe(Total) results show little variation in the rate of reduction in 100 ml, 1 L and 10 L volume transformations. (b) Powdered XRD indicates all samples contain magnetite (M) and hematite (H) with siderite (S) produced in 1 L and 10 L samples.

The density of the final product was 4.3 g L\(^{-1}\) (± 1.6) dry weight, corresponding to a final yield of 120 g magnetite from the \textit{Geobacter sulfurreducens} cells harvested from the 50 L bioreactor.

Powder X-ray diffraction (XRD) was carried out on the end products to identify potential differences between the mineral phases produced in each volume of the iron transformation vessel tested (Figure 5b). The results indicated the presence of three different mineral phases including magnetite (Fe\(_3\)O\(_4\)) which is identified by the (220), (311), (400), (511) and (440) reflections. All samples also contained the Fe(III) oxide hematite (Fe\(_2\)O\(_3\)) at 33.2°(2θ) (104), with siderite (FeCO\(_3\)) observed at 32°(2θ) (104) in the 1 L and 10 L vessel samples. The presence of hematite may explain why none of the data in Figure 5a reach the optimal ratio of 0.33 Fe(II)/Fe(Total) as this mineral structure contains Fe(III) which is not bioavailable to \textit{G. sulfurreducens} for enzymatic reduction 33.

The average crystalline particle size of the magnetite can be calculated by fitting a Lorentzian curve to one or more of the XRD reflections. Due to presence of the three mineral phases with some overlapping reflections (i.e. hematite (110) at 35.6 °(2θ) and magnetite (311) at 35.4 °(2θ)), it is difficult to calculate accurately the
particle size of the magnetite using the most intense reflection. Fitting of the next most prominent (220) peak was used. The results indicate very little difference in the particle size of the magnetite produced with 12.3 nm, 10.6 nm, 14.4 nm and 15.0 nm diameter particles present in the 10 ml, 100 ml, 1 L and 10 L biomagnetite production steps respectively. From previous work, we know that the size of the nanoparticles is small and related to the concentration of biomass introduced at the start of the biotransformation, with large amounts of bacteria leading to the production of smaller nanoparticles. This is considered to be a rate dependent effect, with large amounts of Fe(II) production leading to an increase in the probability of formation of nucleation sites\textsuperscript{34-36}. The size of the particles produced follows closely the results obtained for magnetite nanoparticles produced with similar starting biomass values (13.7 nm from an inoculation of 0.38 mg ml\textsuperscript{-1} protein)\textsuperscript{25}.

TEM was performed to determine variations in the morphology of the magnetite nanocrystals and other mineral phases present in the samples. Material produced in 10 ml and 10 L volume transformations were imaged (Figures 6), with electron diffraction also performed to determine the mineral phases under observation.
Figure 6 – Transmission electron microscopy imaging of end product of Fe(III)-reduction by *Geobacter sulfurreducens*. (a) 10 ml vessel volume, (a-i) image displays spherical particles, most likely magnetite, (a-ii) electron diffraction indicates presence of magnetite and hematite, (a-iii) hematite crystal clearly distinguishable from spherical magnetite. (b) 10 L vessel volume sample. (b-i) spherical magnetite of similar size to 10 ml samples, (b-ii) electron diffraction indicates presence of hematite, magnetite and siderite; (b-iii) very large crystals most likely siderite.

The results of TEM imaging confirmed the analysis from XRD with hematite mineral phases clearly observable at the same time as magnetite (Fig 6a(iii), b(iii)), confirmed by selected area electron diffraction analyses (Figure 6 a-ii, b-ii). The size of the magnetite nanoparticles was 16.9 ± 2.6 nm and 12.8 ± 2.0 nm for samples from the 10 ml and 10 L magnetite synthesis step respectively, in close agreement with XRD.
average crystallite size data (Figure 5b). The size of the hematite produced was approximately 75 nm and 74 nm for 10 ml and 10 L respectively. Fitting of the siderite peak observed using XRD suggests the siderite had dimensions of approximately 350 nm in the 10 L sample. Figure b-iii contains three spherical particles with average size of 208 ± 24 nm which match the expected morphology of the iron carbonate biominerals. Large particles of this type were not observed in the images obtained from the 10 ml sample.

The presence of siderite has been reported previously when magnetite has been produced by a high density of Geobacter sulfurreducens\textsuperscript{25}. This was attributed to an excess of Fe(II) produced in aqueous solution with insufficient Fe(III) available to form magnetite. The remaining Fe(II) reacted with the carbonate buffer present in solution to produce the Fe(II)-carbonate FeCO\textsubscript{3}. This mineral phase was only observed in samples from the 1 L and 10 L biomagnetite production experiments however, and not 10 ml or 100 ml.

Further experimentation was performed to investigate the source of the hematite which was detected in all of the samples (Figure 7). In these experiments, the Fe(III)-oxyhydroxide starting material was prepared under different conditions, focusing on the impact of autoclaving the starting material at different pH values. Magnetite nanoparticles were produced from Fe(III)-oxyhydroxide which had been (i) degassed using N\textsubscript{2} and CO\textsubscript{2} and then autoclaved at pH 7; (ii) autoclaved at pH 9 and then de-gassed with N\textsubscript{2} and CO\textsubscript{2} to set the pH to 7; (iii) degassed using N\textsubscript{2} and CO\textsubscript{2}, pH adjusted to pH 7 with N\textsubscript{2}:CO\textsubscript{2} gas mix and not autoclaved; (iv) autoclaved at pH 9 and then de-gassed with N\textsubscript{2} and CO\textsubscript{2} with 1 M MOPS used to set pH to 7. The end products and starting materials were analysed using XRD.
Figure 7 – Powdered XRD used to determine the cause of hematite formation (F – ferrihydrite; M – magnetite; H – hematite). Fe-gel – unreduced starting material; iF and iS correspond to final product and starting material autoclaved at pH=7 (pH adjusted to 7 using N₂:CO₂); iiF and iiS autoclaved at pH=9 (pH to adjusted to 7 using N₂:CO₂); iiiF and iiiS, not autoclaved (pH adjusted to 7 using N₂:CO₂); ivF and ivS, autoclaved at pH=9 (pH to adjusted to 7 using N₂, MOPS); 100 ml sample produced in section 4.2.2. Results indicate that hematite is produced as a result of the conditions imposed by autoclaving at high pH.

The results presented in Figure 7 show that the pH at which the starting material is autoclaved plays a crucial role in controlling hematite formation. The initial Fe(III)-oxyhydroxide slurries did not contain any hematite and exhibited two broad peaks corresponding to 2-line ferrihydrite. The sample which was autoclaved at pH=7, (iS) and the sample not autoclaved at all (iiiS), also displayed only the characteristic reflections of 2-line ferrihydrite. However samples (iiS) and (ivS) which were autoclaved at high pH displayed the hematite reflections (104) and (110) at 33.2°(2θ) and 35.6°(2θ) respectively. The end products of all samples match closely to the 100 ml
biotransformation results which were produced from the biomass grown in the 50 L bioreactor, with crystallite particle sizes measured at 12.5 ± 0.7 nm, 13.1 ± 2.7 nm, 12.4 ± 2.9 nm, 10.5 ± 0.6 nm for samples (i), (ii), (iii) and (iv) respectively (measured from reflections (220), (400) and (511)). There is a peak visible at 33.2° for sample (i), corresponding to hematite, albeit less pronounced than that observed in (ii) and (iv), however there is no such peak for (iii). It is clear from this result that the magnetite formation which takes place through the reduction of Fe(III) by \textit{Geobacter sulfurreducens} can still be performed without the harsh sterilisation procedures imposed by autoclaving at high temperature and pressure. Provided that adequate steps are taken to minimise potential contamination, the large amount of biomass which is introduced at the start of an experiment will provide the dominant source of iron reduction which leads to the production of magnetite.

Additional measurements were performed on the samples to determine the stoichiometry of the nanoparticles produced to observe the potential impact of iron transformation vessel volume changes on the ratio of Fe$^{2+}$ to Fe$^{3+}$ in the sample. This was carried out through the application of the synchrotron radiation techniques X-ray absorption (XAS) and X-ray magnetic circular dichroism (XMCD) (Figure 8). Magnetite has a cubic spinel structure with Fe$^{2+}$ and Fe$^{3+}$ cations arranged in in tetrahedral (A) and octahedral [B] coordination according to (Fe$^{3+}$)$_4$(Fe$^{2+}$Fe$^{3+}$)$_4$O$_{12}$. The magnetic characteristics of magnetite are the result of anti-ferromagnetic coupling between the two lattice sites which effectively cancel out magnetic moments due to Fe$^{3+}$ cations, resulting in a net magnetisation due to Fe$^{2+}$.

The XAS of the Fe $L_{2,3}$ edges (formed from the average of ±0.6 T collected spectra) displayed in Figure 8a show very little difference between the four samples, despite the presence of siderite in the 1 L and 10 L samples (hematite was present in all samples), indicating that it is only a minor component. Spectra were compared to a sample of biogenic magnetite from small-scale culture, reported previously\textsuperscript{25}, designated “Bio mag” and known to have a close to stoichiometric cation distribution (Table 1) with a slight excess of octahedral Fe$^{2+}$[B], (Fe$^{2+}$/Fe$^{3+}$ 0.52). This is characterized by a smooth shoulder on the low energy side of the peak intensity (dashed line). The shoulder is more pronounced in the 50 L fermenter derived samples which could be indicative of a less reduced sample (i.e. less Fe$^{2+}$[B] in relation to other
iron cations). However it is more likely to be due to the presence of the Fe(III) oxide hematite which is not present in Bio mag

Figure 8 – (a) X-ray absorption spectra (XAS) of samples obtained through Fe(III) reduction in volumes of 10 ml, 100 ml, 1 L and 10 L. Bio mag corresponds to sample of biogenic magnetite with close to stoichiometric cation distribution. (b) X-ray magnetic circular dichroism (XMCD) derived from difference between two XAS collected with sample under influence of a magnetic field of + and – 0.6 T respectively. 50 L fermenter derived samples compared to XMCD of approximately stoichiometric biogenic magnetite (Bio mag). Spectra display peaks corresponding to Fe$^{2+}$[B], Fe$^{3+}$(A) and Fe$^{3+}$[B] as highlighted by fits blue, red and green respectively.

Further information about the crystal structure of the magnetite was obtained from XMCD data. Both hematite and siderite are antiferromagnetic materials, hence they will not contribute to the XMCD spectra. This allows the technique to be used to provide information on the stoichiometry of magnetite alone. XMCD spectra are derived from the difference between two XAS obtained under two different magnetic polarisations (± 0.6 T) set parallel and anti-parallel to the incoming X-ray beam direction. The intensity of the spectra provides a relative measure of magnetisation of the nanoparticles provided that the average of the two associated XAS spectra has been normalised to 1. Using this approach it is clear that there is no obvious difference between the magnetization of the magnetite nanoparticles produced under any of the
conditions. Further quantitative analysis of the stoichiometry of the magnetic nanoparticles can be obtained through fitting of the XMCD spectra using calculated spectra for each of the Fe sites in magnetite. The XMCD spectra at the Fe $L_3$ edge is attributed to three distinct peaks corresponding to octahedral Fe$^{2+}$[B] (first negative peak), tetrahedral Fe$^{3+}$(A) (positive peak) and octahedral Fe$^{3+}$[B] (second negative peak); thus fitting of the spectra can provide information on the relative occupancy of each of the ferrous and ferric cations (Table 1).

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<th>Sample</th>
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<td>1.01</td>
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<tr>
<td>Bio mag</td>
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<td>1.03</td>
<td>0.96</td>
<td>1.02</td>
<td>0.52</td>
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</table>

Table 1 – Fe Cation distribution within the magnetite nanoparticles, with values determined through fitting of XMCD spectra.

Stoichiometric magnetite has the formula $(\text{Fe}^{3+})_a[\text{Fe}^{2+}\text{Fe}^{3+}]_b\text{O}_4^{2-}$, meaning that for every Fe$^{2+}$ in the octahedral coordination, there is one Fe$^{3+}$ in tetrahedral and one Fe$^{3+}$ in octahedral coordination. This leads to the Fe$^{2+}$/Fe$^{3+}$ ratio of 0.5. As can be seen in Table 1, the results of the 10 ml and 100 ml have almost exact stoichiometric occupancy (close to Bio mag), whilst 1 L and 10 L appear to have a less than stoichiometric Fe$^{2+}$[B] which is balanced by a higher Fe$^{3+}$[B]. The result suggests the materials produced at 1 L and 10 L are slightly oxidised in comparison to those produced at smaller volumes.

### 7.4.2.3. Surface reactivity; Cr(VI) reduction

The surface reactivity of the materials from the scale up experiment was tested against chromate Cr(VI), also demonstrating the effectiveness of the materials for toxic metal remediation through Fe(II)-mediated reduction/sequestration. A particular focus was the effect of slight variations in the Fe$^{2+}$/Fe$^{3+}$ ratios between samples. Material prepared in the biotransformation vessels at 100 ml, 1 L and 10 L was scaled down to 10 ml reaction vessels and the total concentration of Fe was normalised to 50 mM L$^{-1}$. Each of these aliquots from the biotransformation experiments was then reacted with 5 mM $K_2\text{CrO}_4$ (potassium chromate) and monitored over 72 hr (Figure 9). The effectiveness of the magnetic nanoparticles at the removal of Cr(VI) from solution was measured using a colorimetric chemical assay specific to chromate$^{31}$. 

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Figure 9 – Demonstrating the applications of biomagnetite for the remediation of chromate. 100 ml, 1 L and 10 L produced samples exhibit comparable reduction/sequestration rates of Cr(VI), however only 10 L produced samples reached zero. Most removal of aqueous Cr(VI) occurred within the first 30 min of reaction (inset graph).

The results in Figure 9 indicate that after only 6 minutes reaction time 30%, 40% and 39% of the Cr(VI) was removed from solution for samples obtained at the 100 ml, 1 L and 10 L scales tested respectively. During the first 6 hr of measurement, the Cr(VI) remediation curves followed the trend of 100 ml > 10 L > 1 L, which follows the order expected from XMCD analysis of the surface Fe$^{2+}$ available, as from Table 1, it is seen that the amount of Fe$^{2+}$/Fe$^{3+}$ follows the trend of 100 ml > 10 L > 1 L. Post- 6 hr, the 10 L sample continued to reduce/sequester Cr, reaching a final Cr(VI) concentration of 0 mM after 48 hr. 100 ml and 1 L samples did not go to completion, having removed 90% and 86 % Cr(VI) from solution respectively after 72 hr. The implications of this experiment are that despite the presence of additional mineral phases such as hematite and siderite, biogenic magnetic nanoparticles produced at large scale from a batch fermentation of *Geobacter sulfurreducens* are highly capable of the remediation of Cr(VI).
7.5. Conclusions

This study has clearly demonstrated that magnetite nanoparticles can be produced at large scale in batch cultures, with only relatively minor differences observable between the final products of small scale and large scale iron transformations. The method through which this has been achieved requires the use of an industrial scale bioreactor, here trialled at a 50 L volume. The material produced was tested and was shown to be suitable for the remediation of chromate contaminated waste waters, with little impact of scaling on the material’s ability to remove Cr(VI) from solution.

Potential improvements to the biogenic magnetite scale-up process have been highlighted, including those required to limit the formation of additional mineral phases in the product. This would be achieved by eliminating the autoclaving procedure used to initially sterilise the starting Fe(III)-oxyhydroxide cultures. The process requires high temperatures (121 °C) and pressure which appears to generate the conditions which are able to form hematite, especially at scale when longer autoclaving times are required. Siderite formation could be limited by reducing the biomass introduced at the start of the iron transformation experiment, or by the use of an alternative buffer system.

Further experimentation would be useful in attempting to reduce the lag phase during the fermentative growth of *G. sulfurreducens*, however ultimately, a highly reactive magnetite mineral can be produced in environmentally favourable conditions in less than 72 hr.
### 7.6. Supplementary information

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<td></td>
<td>thiocytic acid</td>
<td>5.0 mg L&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Supplementary Table 1 – Trace element components contained within modified freshwater medium (NBAF).*
Acknowledgements

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7.7. References


Characterisation of the dissimilatory reduction of Fe(III)-oxyhydroxide at the microbe - mineral interface: The application of STXM-XMCD

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8. Characterisation of the dissimilatory reduction of Fe(III)-oxyhydroxide at the microbe - mineral interface: The application of STXM-XMCD

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8.1. Abstract

A combination of scanning transmission X-ray microscopy (STXM) and X-ray magnetic circular dichroism (XMCD) was used to spatially resolve the distribution of different carbon and iron species associated with Shewanella oneidensis MR-1 cells. *S. oneidensis* MR-1 couples the reduction of Fe(III)-oxyhydroxides to the oxidation of organic matter in order to conserve energy for growth. Several potential mechanisms may be used by *S. oneidensis* MR-1 in order to facilitate Fe(III)-reduction. These include direct contact between the cell and mineral surface, secretion of either exogenous electron shuttles or Fe-chelating agents and the production of conductive ‘nanowires’.

In this study the protein/lipid signature of the bacterial cells was associated with areas of magnetite (Fe₃O₄), the product of dissimilatory Fe(III) reduction, that was oversaturated with Fe(II) (compared to stoichiometric magnetite). However, areas of the sample rich in polysaccharides, most likely associated with extracellular polymeric matrix and not in direct contact with the cell surface, were undersaturated with Fe(II), forming maghemite-like (γ-Fe₂O₃) phases compared to stoichiometric magnetite. The reduced form of magnetite will be much more effective in environmental remediation such as the immobilisation of toxic metals. These findings suggest a dominant role for surface contact-mediated electron transfer in this study and also the inhomogeneity of magnetite species on the submicron scale present in microbial reactions. This study also illustrates the applicability of this new synchrotron-based technique for high-
resolution characterization of the microbe-mineral interface, which is pivotal in controlling the chemistry of the Earth’s critical zone.

8.2. Introduction

Microbial Fe(III) reduction is widespread in the subsurface and has significant environmental consequences, as it has been shown to control the mobility of radionuclides, toxic metals and organic molecules in many different environments\(^1\). Therefore, it is of great interest to identify the precise mechanisms that dissimilatory Fe(III)-reducing bacteria use to reduce Fe(III)-bearing minerals. The Gram-negative, facultative anaerobe *Shewanella oneidensis* MR-1 is able to reduce solid phase Fe(III) oxyhydroxides by coupling the reaction to the oxidation of organic matter \(^2, \)\(^3\) . This electron transfer process can result in the formation of a range of Fe(II)-rich phases, including magnetite, green rusts, siderite or vivianite \(^4-6\) . As well as direct contact between cell surface and mineral, transferring electrons *via* outer membrane cytochromes\(^7,\)\(^8\) , there are a number of alternative methods that this bacterium can use in order to facilitate the electron transfer process, particularly to reduce less bioavailable solid-phase minerals. These include the secretion of electron shuttling compounds\(^9-11\) , the use of endogenous electron shuttles such as humics\(^11\) , the secretion of an Fe(III)-solubilizing ligand\(^12\) and the production of electrically-conducting pili or ‘nanowires’\(^13,\)\(^14\) . Here we describe the use of the relatively novel and fast developing technique of scanning transmission X-ray microscopy (STXM) combined with X-ray magnetic circular dichroism (XMCD) to evaluate the spatial distribution of Fe(II) and Fe(III), versus carbon signatures specific for a range of cellular macromolecules in Fe(III)-reducing cultures of *S. oneidensis* MR-1. This combination of STXM and XMCD has previously been used to identify the individual magnetic moment in magnetite nanocrystals in a magnetotactic bacterium\(^15\) and a comprehensive bibliography of STXM is available in\(^16\) , with more recent updates available at http://unicorn.mcmaster.ca/xrm-biblio/xrm_bib.html. The direct visualisation of the oxidation state of Fe in both the extracellular and cell surface environments gives a unique insight into the mechanism of Fe(III) reduction in this model organism. The study of this specific system has also added interest given the technological potential
of the nanospheres of biomagnetite produced by this process and the ability to control their magnetic properties by the Fe(III)/Fe(II) behaviour.

8.3. Materials and Methods

*S. oneidensis* MR-1 was grown under strictly anaerobic conditions at 30 °C in modified freshwater medium as described previously. Sodium DL-lactate (100 mM) and fumarate (20 mM) were provided as the electron donor and acceptor, respectively. All manipulations were done under an atmosphere of N₂–CO₂ (80:20). Poorly crystalline Fe(III) oxyhydroxide was produced using the method of, where a 0.4 M solution of FeCl₃ is neutralized by 10 N NaOH to pH 7 and the solid then washed by centrifugation six times until no Cl⁻ ions remain. The mineral was stored in the dark at 4 °C for not more than 6 months before use. Late log-phase cultures of *S. oneidensis* MR-1 were harvested by centrifugation at 5000 G for 20 minutes and washed twice in carbonate buffer (NaHCO₃; 30 mM, pH 7.1) under N₂–CO₂ (80:20) prior to use. Aliquots of the washed cell suspension (0.5 ml) were added to sealed anaerobic bottles containing 9.9 ml bicarbonate buffer, 50 mM L⁻¹ poorly crystalline Fe(III) oxide and 20 mM sodium lactate. The final concentration of bacteria corresponded to approximately 0.2 mg protein per ml. Bottles were incubated in the dark at 20 °C. After one week of incubation an aliquot of the suspension (0.1 ml) was deposited and dried on a formvar coated 3 mm 200 mesh copper grid (Agar Scientific) under anaerobic conditions. The grid was transferred on a sample holder from a glove bag to the STXM in an air-tight jar. The chamber contained an overpressure of N₂. The sample jar was only opened within the chamber and the sample mounting was performed quickly to prevent sample oxidation. A second aliquot of sample was taken for powder X-ray diffraction (XRD) measurements. XRD were obtained with a Bruker D8Advance instrument using Cu Kα₁ radiation. Data were acquired over the range 15° (2θ) –70° (2θ), using a step size of 0.02° (2θ).

Scanning transmission X-ray microscopy (STXM) observations were carried out on the soft X-ray spectromicroscopy (SM) beamline BL 10ID1 of the Canadian Light Source (CLS) in Saskatoon, Canada. Observations were made firstly at the C K-edge followed by the Fe L₂,₃-edge in order to minimise possible beam damage. The beamline
has an energy resolving power $E/\Delta E > 3000$ and an incident flux of $10^8 \text{ ph s}^{-1} \text{ 0.5 A}^{-1}$ at 3000 resolving power. Further details can be found in\textsuperscript{20}. The exit slits were adjusted such that the maximum flux used was 18 MHz. Energy calibration was achieved using the sharp peak for protein at 288.2 eV and the peak maximum for magnetite at 709.5 eV. Elemental maps (also called ‘stack maps’) were generated by taking a background image at 280 eV and 700 eV and subtracting this image from a corresponding image for C at 288.2 eV or Fe at 709.5 eV, respectively. Spectroscopic data were collected by scanning a spatial region of interest in $x$-$y$ sample directions, perpendicular to the beam, at each energy increment over an energy range covering an elemental edge thus creating a spectrum at each pixel; known collectively as a stack\textsuperscript{21}. Care was taken to use as low a dose of X-rays as possible to minimize the impact of radiation damage on these measurements. This involved careful selection of dwell time, number of energy points, and use of an automated fast, in-vacuum shutter so only X-rays hitting the sample were contributing to the measured data. A dwell time of 0.95-1.15 ms was used, which has previously been shown to give minimal beam damage to samples containing a combination of iron minerals and bacteria\textsuperscript{22}. The energy step was 0.15 eV over the main features of each edge and the image pixel size was 0.025 μm. Images at damage sensitive energies (289 eV in the C $K$-edge, and 704 eV in the Fe $L_{2,3}$-edge) were measured after stack acquisitions; visible changes in the sample morphology were not noted.

The source point for the CLS-STXM is an elliptically polarizing undulator (EPU) which provides nearly 100% circularly polarized light at the Fe $L_{2,3}$ edge\textsuperscript{20}. In order to determine the Fe $L_{2,3}$ XMCD, two image stacks (see above) were recorded in transmission mode for the same sample area firstly with right circularly polarized (RCP) and then left circularly polarized (LCP) X-rays. A magnetic field of ~0.1 T was applied to the sample by mounting a strip of the grid between two poles of a magnet and having the sample plate at an angle of 30° to the beam direction, resulting in a component of the magnetic field parallel to the beam. Data processing was carried out using the aXis2000 software package\textsuperscript{23}. For both the C $K$-edge and Fe $L_{2,3}$-edge data transmission signals were converted to optical density (OD) units using the incident flux signals measured in the same stack through regions free of C or Fe adjacent to regions of interest. Alignment of each stack was carried out in aXis2000\textsuperscript{23} using the stack analyze function, which aligns images by cross-correlations using Fourier transforms. For the C
K-edge the image sequence was fitted using the singular value decomposition (SVD) method using standard spectra for expected components. Threshold masking was used to identify areas with similar spectral characteristics to the standards. For the Fe $L_{2,3}$-edge the two stacks, for the LCP and RCP X-rays, are then added and ordered in photon energy and the alignment process repeated on the combined stack. The two well-aligned stacks are then separated again and subtracted in order to give a new stack that yields spectra that are the difference between the X-ray absorption spectra for RCP and LCP X-rays, in a magnetic field set at 30° to the incoming beam direction; XMCD spectra.

To obtain the relative amounts of the three Fe sites, the experimental spectra were fitted by means of a non-linear least-squares analysis, using calculated spectra for each of the Fe sites. In these calculations, as described in 24, the Hartree-Fock-Slater integrals for the 3$d$-3$d$ and 2$p$-3$d$ Coulomb and exchange interactions were scaled to 70 and 80 %, respectively, and the crystal fields for the octahedral and tetrahedral sites were taken to be $10D_{q} = 1.4$ and $-0.6$ eV, respectively. The calculated spectra were convoluted by a Lorentzian of $\Gamma = 0.3$ (0.5) eV for the $L_3$ ($L_2$)-edge to account for intrinsic core-hole lifetime broadening and by a Gaussian of $\sigma = 0.2$ eV to account for instrumental broadening. The experimental spectra were fitted over the $L_3$ main peaks only, which has been previously shown to give meaningful results, although fitting over the $L_2$ peak does give good qualitative agreement 25.

8.4. Results and Discussion

After one week of incubation with S. oneidensis MR-1 the orange Fe(III)-oxyhydroxide in the sample tubes had altered to a black magnetic precipitate, expected to be the Fe(II)-bearing mineral magnetite (Fe$_3$O$_4$) as reported previously 26. This was subsequently confirmed using XRD (Figure 1a), and the crystallite size of the magnetite was determined to be 29.15±2.4 nm through fitting the (311) with a Lorentzian line shape and using the Scherrer equation. C K and Fe $L_{2,3}$-edge STXM analyses were performed on an aliquot of the sample slurry. First, the location of carbon-rich and iron-rich areas was mapped with an image step size of 0.05 μm across the sample in order to identify suitable areas for more in-depth analysis and to provide an overview
of the spatial distribution of each element (Figure 1b). It can be seen from Figure 1b that there are oval/rod-shaped structures \( \sim 1 \times 0.5 \) μm; comparing with previous images \(^{14, 27}\) and noting that they are carbon-rich, indicates they are cells of \( S. \) \textit{oneidensis} MR-1, surrounded by deposits rich in iron, likely to be the magnetic precipitate.

![Figure 1](image)

**Figure 1** - (a) X-ray diffraction trace with the reflections for magnetite. (b) Colour composite of two STXM maps (optical density, OD) measured at the C K-edge (OD\(_{288.2} - \) OD\(_{280}\)) in red and the Fe \( \ell_{2,3} \)-edge (OD\(_{709.5} - \) OD\(_{700}\)) in blue. 1 μm scale bar.

A more detailed examination of the lower portion of the area represented by Figure 1b was carried out by collecting images at regular energy intervals across the carbon K-edge between 280-320 eV, forming a ‘stack’ (see methods, above). Singular value decomposition (SVD) analysis using three standard spectra for protein, alginate and lipid, obtained from\(^{28}\), each normalised to an intensity corresponding to 1 nm thickness of the pure material (OD1), and a constant term was applied to the carbon K-edge stack. The areas of the sample containing high concentrations of protein (red), polysaccharide (green) or lipid (blue) are shown in Figure 2(a-c) with a color composite image in Figure 2(d) and the experimental spectra extracted for each of the three different components in Figure 2(e). The spectrum for areas containing a high concentration of protein gives a good fit to the standard spectrum from bovine serum
albumin (BSA) containing both the $1s \rightarrow \pi^*_C=C$ transition at 285.1 eV and the main transition $1s \rightarrow \pi^*_C=O$ at 288.2 eV associated with the peptide bond. The spectrum for areas of high lipid content also contains a large protein component, indicated by the decrease in the intensity at 290.8 eV, demonstrating the lipid-rich areas are also protein-rich. The protein and lipid signals come primarily from the oval/rod shapes ~1 x 0.5 μm, strongly supporting that these structures are cells of *S. oneidensis* MR-1. The polysaccharide spectrum gives a good agreement with the standard for alginate with the most intense peak associated with carboxyl groups occurring at 288.6 eV, a distinctly higher energy than the $1s \rightarrow \pi^*_C=O$ at 288.2 eV of protein. From the component map [Figure 2(b)] and the colour coded composite [Figure 2(d)] the polysaccharide appears to surround the bacterial cell. This is consistent with a diffuse extracellular matrix of polysaccharide.

**Figure 2** - Individual carbon component maps (a-c) derived by fitting an energy sequence of images (stack) of the C-K-edge (96 images from 280-320 eV; 60 nm pixels, 1 ms/pixel) with a linear combination of albumin (protein) (a), alginate (polysaccharide) (b) and lipid (c). The maximum optical density (OD) values are 110 (a), 25 (b) and 70 (c). (d) Colour-coded composite map derived from images (a-c). Red = protein; green = polysaccharide; blue = lipid. (e) C-K-edge spectra (i) protein, (ii) alginate and (iii) lipid extracted from the image stack (solid lines), compared to the reference standards (iv) using the same colours) used to generate the component maps (dotted lines) (from Lawrence et al., 2003). Scale bar is 500 nm for all images.

The distribution of Fe(II) and Fe(III), over the same region for the C-K-edge, was examined in detail by collecting images and spectral data at energy intervals between 700-730 eV creating a Fe L$_{2,3}$-edge stack. Since magnetite contains both Fe(II) and
Fe(III), in a 1:2 ratio, a linear regression analysis of the data using standard spectra for Fe(II) and Fe(III) was not practical for spatially resolving the differences in amounts and spatial distributions for the two oxidation states [see Figure 3(a)(i,ii) for the X-ray absorption spectral (XAS) shapes] since they are too closely associated within the sample. Therefore, XMCD spectral measurements were made as these have previously been shown to resolve the three Fe environments present in magnetite; the Fe(II) octahedral, Fe(III) tetrahedral and Fe(III) octahedral, where each electronic state of Fe has a distinctly different XMCD spectral signature [see Figure 3(a)(iii,iv) for the XMCD spectral shapes].

Figure 3 - (a) Calculated XAS (i) and XMCD (iii) spectra for the Fe $L_{2,3}$-edge of stoichiometric magnetite and individual components of the XAS (ii) and XMCD (iv) spectral calculations, Fe(II) octahedral (green), Fe(III) tetrahedral (red) and Fe(III) octahedral (blue). (b) Fe $L_{2,3}$-edge spectra for *S. oneidensis* MR-1 nanomagnetite (i) XAS taken using left- (red) and right- (black) circularly polarised X-rays for the area within the yellow contour of Fig. 4 and the corresponding XMCD spectrum x2 (blue); (ii) XAS taken in (red) and + (black) 0.6 T magnetic field (Coker *et al.*, 2007) and the corresponding XMCD spectrum (blue).

The *S. oneidensis* MR-1 biomagnetite sample plate was placed within a ~0.1 T magnetic field, at 30° to the X-ray beam resulting in a component of the magnetic field parallel to the beam direction whilst also allowing the beam to pass through the sample into the detector. Two Fe $L_{2,3}$-edge stacks were collected, one with left- and the other with right-circularly polarised X-rays (see above) thus providing two XAS per image pixel; the XMCD is obtained as the difference between these two spectra. The average XAS and XMCD spectra for the sample area within the yellow contour of Figure
4 are shown in Figure 3(b)(i). The average XAS and XMCD spectra obtained for this sample is compared to Fe $L_{2,3}$ spectra previously taken in total-electron yield (TEY) on beamline 4.0.2 at the Advanced Light Source (ALS) for a similar sample of biomagnetite produced by the same bacterium [Figure 3(b)(ii)], published in$^{26}$. In that study, the two XAS were obtained by switching the magnetic field in parallel and anti-parallel direction along the incident X-ray beam.

It can be seen from Figure 3(b) that the magnitude of the STXM-XMCD signal is smaller than in the TEY-XMCD measurement. This can be ascribed in part to the fact that the sample is mounted at 30° to the X-ray beam so only 50% of the XMCD signal is observed. Also there is a lower magnetisation of the sample in the STXM-XMCD measurement, where a smaller magnetic field had to be applied due to the space constraints of the STXM chamber. However, the smaller magnitude does not affect the overall shape of the XMCD signal, but merely leads to weaker signal and thus relatively larger increased noise in the spectra. The STXM-XMCD was collected in transmission mode giving the bulk mineralogy of the sample, whereas the XMCD taken from $^{26}$ was collected in TEY mode which only probes the top 4-6 nm of the sample surface. Therefore, the two measurements allow us to compare the bulk and surface of the magnetite. The peak fitting results of the XMCD spectra derived from non-linear least squares fitting using calculated spectra for each of the three Fe environments$^{25,32}$, are given in Table 1. These results indicate that the surface and bulk measurements are in reasonably good agreement particularly with respect to the overall percentage of Fe(II) in the magnetite. The surface contribution from 29 nm nanoparticles is a significant proportion [up to 80 % assuming a probing depth of 60 Å –see$^{26}$] of the total signal, therefore this similarity might be expected.
Figure 4 - (a) protein component map and (b) polysaccharide component map (both from Figure 2) overlaid by contours defining areas from which Fe L\textsubscript{2,3}-edge XAS were extracted to generate XMCD spectra (Figure 5), these were for either the whole area (yellow contour), or high concentrations of either (a) protein/lipid (P1-P7) or (b) polysaccharide (A1-A4). Scale bar is 500 nm for both images.

Areas of the Fe L\textsubscript{2,3}-edge map found to be high in either protein/lipid or polysaccharide were chosen and labelled P1-P7 for protein/lipid-rich areas and A1-A4 for polysaccharide-rich areas in Fig. 4. XAS were extracted from the two Fe stacks and the XMCD spectra calculated and then fit in the same way as described above (Figure 5). The results listed in Table 1 indicate that areas of magnetite corresponding to the position of protein/lipid in the sample contain a larger amount of Fe(II) compared to stoichiometric magnetite, whereas areas of magnetite associated with polysaccharides are under-saturated in Fe(II) compared to stoichiometric magnetite. For the areas of biomagnetite closely associated with the bacterium the average Fe(II)/Fe(III) ratio is 0.61 ± 0.03, the most reduced magnetite present, and much more reduced than the average value of 0.50-0.53. This is confirmation that direct contact with the cells is an efficient mechanism for reducing Fe(III) to Fe(II), recrystallising the Fe(III)-oxyhydroxide into an ‘over-reduced’ form of magnetite. The areas rich in polysaccharides give XMCD signals that have an average Fe(II)/Fe(III) value of 0.30 ± 0.03, which is much lower than that for stoichiometric magnetite, tending towards maghemite (γ-Fe₂O₃), and suggests that direct contact is required in these cultures for maximal levels of Fe(III) reduction, although enough Fe(II) is still generated to fully recrystallise the Fe(III)-oxyhydroxide to magnetite.
It has been shown previously that biogenic nanomagnetite is (overall) typically slightly oversaturated with Fe(II) compared to stoichiometric magnetite, and this is thought to be a function of the O$_2$-free environment that these bacteria require in order to use Fe(III) as an electron acceptor.$^{26,33,34}$ However, from the limited observations presented in this paper the situation is seen to be more complex, especially during the reduction process, and the mineralogical status of the magnetite depends on the local (nanoscale) interactions of the microbial cell with the mineral substrates. Although this work is only based upon analyses from one sample area the results can be compared to work on Fe(II)-oxidising bacteria, where a gradient in the oxidation state of Fe was observed using STXM, indicating the progression of Fe(II) oxidation beyond the cell microenvironment.$^{35}$ Here the importance of bacterial cell-mineral contact in the bio-reduction of solid-phase Fe(III) is demonstrated; the precise contribution of surface localized cytochromes$^{36}$, or other mechanisms including

*The table below provides the relative amounts of octahedral Fe(II), tetrahedral Fe(III) and octahedral Fe(III) as obtained by fitting the Fe L$_{2,3}$-edge XMCD spectra shown in Figure 5 collected from the corresponding areas in Figure 4 (whole area (yellow contour) or P = protein/lipid and A = polysaccharide). Errors are indicated in a separate column. *from Coker et al. (2007)$^{26}$. **Errors are ±0.02.*
extracellular appendages\textsuperscript{14}, soluble secreted electron shuttles\textsuperscript{11} such as flavins\textsuperscript{9} or electron flow through mineral assemblages\textsuperscript{37} are controversial and could be active in the process we describe. The changes in Fe(II) concentration across the sample has implications for the reactions and sorption processes in subsurface sediments, and in bioremediation approaches that are heavily dependent on the reducing power of the Fe(II) associated with Fe-biominerals, such as Cr(VI) reduction\textsuperscript{38}. During bioreduction there is a very reduced magnetite component present that will be the major driver of toxic metal cycling and reduction and immobilisation of the metals\textsuperscript{39}. There will also be a maghemitic-like component that is less reactive produced by other means such as electron mediator compounds. Future work could centre around investigating the differences in the Fe(II)/Fe(III) ratio across a sample when an electron mediator compound, such as a secreted flavin or a humic material, is introduced into the system.

The STXM-XMCD technique described here offers a unique combination of nano-scale (25 nm) spatial resolution and spectroscopic probing of the speciation of metals and lighter “biological” elements such as carbon in dry and fully hydrated\textsuperscript{40} samples. STXM provides direct imaging, quantification, and correlation of the amounts (e.g.\textsuperscript{41}) and spatial localization (e.g.\textsuperscript{29, 42-44}) of different components in environmental systems, which facilitates significant advances in our understanding of the mechanisms of many geomicrobiological processes, including, the demonstration here that shows the potential of combining STXM with XMCD to identify the redox cycling of iron in both laboratory and potentially environmental samples.
Figure 5 - Fe $L_{2,3}$-edge XMCD spectra (black) and the fits (red) corresponding to the areas of the carbon component images shown in Figure 4.
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8.5. References

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Biotechnological synthesis of functional nanomaterials

Biotechnological synthesis of functional nanomaterials
Jonathan R Lloyd, James M Byrne and Victoria S Coker

Biological systems, especially those using microorganisms, have the potential to offer cheap, scalable and highly tunable green synthetic routes for the production of the latest generation of nanomaterials. Recent advances in the biotechnological synthesis of functional nano-scale materials are described. These nanomaterials range from catalysts to novel inorganic antimicrobials, nanomagnets, remediation agents and quantum dots for electronic and optical devices. Where possible, the roles of key biological macromolecules in controlling production of the nanomaterials are highlighted, and also technological limitations that must be addressed for widespread implementation are discussed.

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Introduction
Nanotechnology, the engineering of functional systems at the molecular scale, is one of the fastest moving areas of science and technology, underpinned by the synthesis of nanomaterials with unusual and useful properties that differ from bulk materials. These differences range from changes in mechanical, electrical and magnetic behaviour, to chemical properties such as solubility, reactivity and catalytic activity. Although chemical processes are established for the synthesis of the burgeoning portfolio of nanomaterials, there has been a considerable amount of recent interest in using biotechnological approaches to achieve scaleable, cost-effective bioproduction options. A particular advantage for these alternative processes is the gentler and more environmentally benign conditions that can be used, which generally lack toxic organic and inorganic reagents and processing extremes of, for example, pressure or temperature. The aim of this review is to describe recent publications in this new area of research to complement recent overviews including [1] and more specific reviews that have focused on bionanomaterials in advanced water treatment [2] and functional biominal synthesis from waste materials [3]. These reviews give coverage of earlier research, from outside the approximately 18-month horizon covered here, many of which underpin the papers described in this review in key areas including catalysis, novel antimicrobial nanoparticles, magnetic and photonic applications (summarized in Figure 1). A particular focus is on the use of microbial cells to synthesize functional nanomaterials, although there is also a limited coverage of plant-based systems.

Precious metal bionanocatalysts
The reviews mentioned above describe early work on the use of *Desalflavobacterium* species followed by studies on *Escherichia coli* and *Shewanella* species to recover Pd(II) from solution and make highly reactive nano-scale Pd(0) particles for use in chemical catalysis and fuel cells. Most of the pioneering work in this area (starting in the late 1990s) falls outside the time period covered by this review, but more recent studies have focused on the use of these Pd-based materials for hydrogenation, reduction and selective dehalogenation in non-aqueous solvents [4] and also the dehalogenation of flame retardant materials [5]. There is also a developing interest in other precious metal bionanocatalysts, especially featuring platinum and gold, with the latter element attracting most interest due to its role in reactions such as the water-gas shift reaction and selective oxidation of CO, and also in photonics, electronics, optics and biomedicine applications. Key to the properties that underpin this wide range of uses are the shape, size and mono-dispersion of the nanoparticles, all of which can be potentially controlled by biological templates.

A recent review of plant-based Au nanoparticle formation by [6] shows the wide range of plants that can be used to produce extracellular Au nanoparticles at ambient temperatures, giving nanomaterials with shapes ranging in morphology from platelets to rods and triangles. A paper by [7] examines the reductive ability of the extract of the *Piper betle* vine. Reduction of Au(III) was complete by 120 hours when stable nano-platelets, 50–500 nm in size, were produced. Changes in temperature allowed the shape of the particles to be manipulated with higher yields also found at higher temperatures. Halides and other counter ions were investigated as these would be present in industrial effluent containing Au, and the halides were also found to control shape. The formation of Au nanoparticles by bacteria has also been studied. For example [8] investigated the reduction of chloroaurate...
using *Bacillus subtilis* chosen as this bacterium produces a cyclic lipopeptide (surfactin) which acts as a ready-made stabilizing agent for the nanoparticles. Spherical gold particles formed both intra-cellularly and extra-cellularly after 72 hours and were approximately 7 nm in size. The particles were well separated from each other, attributed to the lipopeptide coating the particles during formation. New biomolecules were expressed by the bacterium when the gold was present, and it was proposed that proteins between 25 and 66 kDa were key to efficient gold reduction. This organism also produced AgNPs via reductive mechanisms, and proteins of mass 66 and 116 kDa were implicated in this example. A thermophilic bacterial strain has also been used to form Au nanoparticles from Au(III) solutions, aerobically at 50°C [9]. After 5 days of incubation, the average particle size was in the 30–60 nm range; however, particles were not stable beyond one week without the addition of glutaraldehyde as a protective agent to prevent aggregation.

Algal cells of *Klebsormidium flaccidum* encapsulated in silica gels were also able to remove Au from solution and produce both intracellular and extracellular nanoparticles [10]. The silica encapsulation partly prevented the lethal effects of the nano-Au, protecting the living cells. Raman spectroscopy imaging was used to image the algae and monitor the influence of the Au nanoparticles on their photosynthetic system. This biohybrid material was proposed as a significant step towards photosynthesis-based biosensors.

**Biological synthesis of antimicrobial silver nanoparticles**

The potential toxicity of nanoparticles has led to much well-publicized concern about their fate in the environment [11]. However, the biosynthesis of highly toxic nano-scale antimicrobials represents perhaps the most intensively studied research area covered by this review, with the majority of work on the production of silver-based nanoparticles (AgNPs). The development of new antimicrobials has been driven by the proliferation of pathogens such as MRSA (or more fully Methicillin Resistant *Staphylococcus aureus*), which are resistant to conventional antibiotics, and chemically synthesized AgNPs have been finding a role in the clinical setting for more than a decade. Multiple recent studies have focused on the use of fungal cultures to synthesize such particles as an alternative to chemical approaches. For example, Fayaz and colleagues have used *Trichoderma viride* for the extracellular biosynthesis of AgNPs from...
silver nitrate solutions [12]. The particles were produced via naturally occurring (but poorly defined) reductants in culture filtrates, to produce polydispersed Ag nanoparticles of 5–40 nm. These were evaluated against Gram-positive and Gram-negative bacteria, and increased the potency of conventional antibiotics such as ampicillin when applied in combination. In other studies, the same team also incorporated Trichoderma derived AgNPs into sodium alginate films for vegetable and fruit preservation. This study suggested that the application of the films can increase the shelf-life of carrot and pear [13], although one should clearly be cautious of the widespread non-medical use of such materials if they are bound for front-line medical use. It is possible to control the physical characteristics of the Trichoderma AgNPs for example, size and monodispersivity, by simple manipulations such as controlling temperature during biosynthesis [14]. The intense light emission properties of noble metals have also led to uses in biological labelling applications for gold and silver nanoparticles, and this concept has been extended to the Trichoderma AgNPs. Maximum absorbance was noted at 405 nm with blue colour emission along with very narrow range of particle distribution, proposed to be stabilized by fungal proteins [15]. Other fungal systems shown to synthesize AgNPs recently include Alternaria alternata [16], here enhancing the anti-fungal properties of flucanazole against other pathogenic fungi (including Candida albicans and also Aspergillus niger) [17], and also Aspergillus clavatus [18,19] which synthesized AgNPs with antimicrobial activities against a range of fungi and bacteria including MRSA. Again, extracellular mechanisms were central in all of these studies, often with a common theme of stabilization and capping by biological macromolecules, especially secreted proteins, exerting control on particle size. Finally, Aspergillus ochraceus has been used to synthesize intracellular AgNPs which were embedded in carbonaceous supports to enhance performance against bacteria and viruses. The immobilized AgNPs exhibited sustained activity, even after washing and repeated exposure to fresh bacterial cultures [20].

The biosynthesis of silver nanoparticles has also been studied recently in bacterial systems. The subsurface iron-reducing bacterium Geobacter sulfurreducens, which is able to conserve energy for growth via the anoxic reduction of a wide range of high valence metals, has been shown to reductively precipitate extracellular nanoscale AgNPs [21]. The surface localization of several c-type cytochromes, implicated in playing a role in the reduction of Ag(I) to Ag(0) in this organism, may explain the extracellular precipitation noted, and also the ability of this bacterium to reduce either insoluble extracellular AgCl or soluble Ag+ ions. Another well-studied subsurface metal-reducing bacterium, Shewanella oneidensis, is also capable of synthesizing extracellular biogenic AgNPs, which shared enhanced antimicrobial activities reported in the fungal-derived products described above; demonstrated against three bacterial model organisms in this study (E. coli, B. subtilis and S. oneidensis) through comparisons with chemically synthesized silver nanoparticles [22]. Given that these metal-reducing bacteria are capable of synthesizing potentially toxic AgNPs, there has been interest in understanding how these organisms tolerate both Ag+ ions and the AgNPs that they synthesize. In a metabolomic study, S. oneidensis was able to tolerate up to 50 μM Ag(I) added to cultures, but at higher concentrations the metal impacted on both the doubling time and biomass yields, caused a drop in phospholipid fatty acids, membrane damage and the penetration of the cell by Ag(I), and ultimately intracellular precipitation of the toxic metal [23]. Other bacterial systems shown to reduce Ag(I) to make toxic AgNPs with antimicrobial activities include culture supernatants of Staphylococcus aureus; AgNPs active against MRSA and other pathogens including Streptococcus pneumoniae, but with more limited activity against Salmonella typhi and Klebsiella pneumoniae [24]. Extracellular reductive synthesis of AgNPs by Lactobacillus fermentum has also been used to supply materials for the continuous disinfection of viruses in water, via incorporation in a NanoCeram cartridge filter system [25]. Another interesting paper from this group focused on a hybrid microbial/inorganic antibacterial for water disinfection, on the basis of toxic aqueous Ce(III) attached to the surface of cells of Leptothrix discophora and Pseudomonas putida MnB29 [26].

Biomineralization of magnetic iron-based nanoparticles

Microbial routes for magnetic nanoparticle (MNP) synthesis, principally magnetite (Fe₃O₄), have attracted recent interest for potential applications such as cancer therapies, whereby tumours that are hard to reach using conventional surgery (e.g. pancreas and brain), are thermally destroyed by the heat generated by magnetic nanoparticles in the presence of an alternating magnetic field. Data storage devices, such as magnetic hard drives can also take advantage of magnetic properties of MNPs, while large-scale industrial processes including catalysis and bioremediation of contaminated land and water can take advantage of the large surface area, chemical reactivity and magnetic recoverability (hence reusability) of these nanomaterials. There are several methodologies for the synthesis of magnetite, including those involving chemical co-precipitation and ball milling; however, these are often costly and potentially damaging to the environment. Microbiological alternatives for magnetite-based MNP production include extracellular reduction of poorly soluble Fe(III) minerals by subsurface bacteria such as Shewanella spp. and Geobacter spp., that use a respiratory process which produces copious quantities of biominerial, or intracellular production of MNPs by magnetotactic bacteria such as Magnetospirillum spp., which use magnetite, bound in a ‘magnetosome’ membrane, to respond to the magnetic field.

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The ability to manipulate the properties such as the size, saturation magnetization, Curie temperature and coercivity of MNPs is crucial in order to tailor the properties of materials to suit a given application. Such modifications can be achieved through the inclusion of metal dopants including Zn, Co, Ni, Mn and Cr in place of some of the iron cations within the magnetite. In vivo cobalt doping in magnetosomes has recently been demonstrated using three strains of the magnetotactic bacterium *Magnetospirillum* [27], however to only a relatively low concentration (~1%) of the overall cation content. The increase in coercivity compared with non-doped magnetite was found to be 36–45% (measured at 300 K), with little impact on the overall saturation magnetization of the particles, another important feature. *G. sulfurreducens* in contrast, produces very high yields of extracellular MNPs and has been shown to be able to incorporate cobalt at much larger concentrations (23 at%) [28] with greater impact on coercivity (1400% increase at 5 K). Other dopants such as manganese are also incorporated into magnetite by magnetotactic bacteria, for example (up to 2.8 at%) in a mixed population of uncultured bacteria [29]. The addition of dopants also contributes to changes in the structure of the organism and MNPs, with zinc leading to larger particles within larger cells of *Magnetospirillum magnetotacticum* (MS-1), and nickel forming smaller particles within this organism [30]. Such changes in properties of the crystals also occurred when using Fe(III)-reducing bacteria that synthesizes extracellular magnetite, such as *Shewanella* PV-4 [31].

Several studies have looked at the use of biomagnetite for bioremediation applications, linked to the high loading of highly reactive Fe(II) on and within the magnetite structure. For example, the reduction of toxic, highly mobile Cr(VI) to the immobile and less toxic Cr(III), some of which is incorporated into the magnetite ‘spinel’ structure [32,33]. Very efficient reduction and immobilization of the nuclear contaminant pertechnetate (Tc(VII)) has also been demonstrated [32].

Reactions between surface Fe(II) on *Geobacter*-derived biomagnetite and soluble Pd(II), facilitated by a naturally occurring organic coating, have led to the reductive precipitation of nanoparticles of Pd(0) on the MNP [34]. The result is a magnetically recoverable nano-scale precious metal catalyst of wide applicability. Of particular note are the high activities recorded, for example, in Heck coupling reactions, and the minimal loss of expensive Pd catalyst between rounds of catalysis. Non-iron based oxide nanoparticles have also been generated through microbial activities, with CoO4 synthesized by marine bacteria obtained from the Arabian sea coast [35]. Cobalt oxide is antiferromagnetic in bulk, but exhibits ferromagnetism when produced in the nano-scale due to uncoupled surface spins in the material.

Finally, given the commercial promise of these materials, large-scale production of biogenic MNPs is an aim of several groups world-wide. For example, Moon *et al.* [36] were able to up-scale production of zinc-doped magnetite in 30 l fermentations using a *Thermoanaerobacter* sp. TOR-39.

**Nano-scale bio-chalcogenides; S, Se and Te-based quantum dots**

Semiconductor nanocrystals known as quantum dots (QDs) possess unique electronic and optical properties due to quantum confinement effects. Over the past two decades QDs of different sizes, shapes and compositions have been synthesized chemically and developed for practical applications ranging from photovoltaic systems to low energy LEDs, optoelectronics, security tagging, biological imaging and gas sensors devices. Recent studies have shown that these materials can also be synthesized via microbial metabolism, through the reduction of oxo-niums of the group 16 metalloids selenium, tellurium, or sulfur to produce, in the presence of cationic metals, metal selenides, tellurides, sulfides.

The microbial synthesis of metal sulfides has been studied in the context of anoxic metal cycling for more than a century, here coupled to the dissimilatory reduction of sulfate to sulfide by sulfate-reducing bacteria, and the subsequent formation of iron sulfide. More recent work on cysteine-mediated synthesis of CdS by bacteria including *Clostridium* and *Klebsiella* species falls outside the time period covered by this review, as do similar studies with various yeast cultures (*Candida* *glabrata* and *Schizosaccharomyces pombe*) but more recent studies [37] have expanded the range of organisms known to synthesize these materials to include the photosynthetic bacterium *Rhodopseudomonas palustris*. Here stable cadmium sulfide nanoparticles with an average size of 8.01 ± 0.25 nm and a maximum absorbance peak at 425 nm were reported. Recent studies have also taken this concept a further step forward by using microbial cells as a template to form a range of nanostructures using essentially abiotic mechanisms. For example, PbS and ZnS hollow nanostructures have been synthesized chemically using lactic acid bacteria as templates, with shape, size and shell-thickness all tunable. The light-harvesting properties of PbS and ZnS hollow spheres that were produced were superior to those of solid counterparts, and photocatalytic degradation of acid fuchsin by the ZnS nanostructures was also considered superior [38]. Nanoporous hollow structures of various morphologies of semiconducting CdS have also been synthesized using *E. coli* cells as a template. Permeabilization of the cells by ethanol preceded the addition of cadmium acetate and thioacetamide as reactants. The synthesis of CdS nanorod antennas was also reported by utilizing extracellular pili as templates. The structures of the materials could be varied by controlling the S: Cd ratios, and enhanced photocatalytic hydrogen
production rates were reported (versus materials synthesized without the E. coli templates) [39].

In addition to nanoparticles made from sulfur-based materials, other chalcogenides based on selenium and tellurium offer potentially useful properties. For example, the metal-reducing bacterium *Veillonella atypica* can reduce selenium oxanions to form nanospheres of elemental selenium. These selenium nanospheres are then further bioreduced to form reactive selenide, which can be precipitated with a suitable metal cation to produce nano-scale precipitates, such as zinc selenide, for use in optoelectronic devices or biological labelling [40].

This synthesis route is again considered a ‘green’ alternative to conventional organometallic synthesis, using more environmentally benign precursors some of which could even be sourced from industrial wastestreams. In a more recent study, the mechanisms of selenite reduction to elemental selenium nanospheres and then selenide were compared between *V. atypica* and the subsurface metal-reducing bacteria *G. sulfurreducens* and *S. oneidensis*. In this study the potentially important role of extracellular proteins in stabilizing the Se-based nanoparticles was addressed [41], a topic that has also been studied in *E. coli* in a very recent study [42] where four proteins (AdhP, Idh, OmpC and AceA) were shown to play a critical role in controlling particle size and morphology. *E. coli* has also been used to synthesize cadmium telluride (CdTe) QDs with tunable fluorescence emission using a hybrid chemical/microbial system [43]. Here the precursors for CdTe nanocrystals; cadmium chloride (CdCl₂), trisodium citrate, Na₂TeO₃ mercaptosuccinic acid (MSA) and sodium borohydride (NaBH₄), were mixed with *E. coli* cells. As the CdTe crystals formed from chemical reduction of the tellurite by the borohydride, extracellular proteins from the microbial cultures were thought to play a critical role in Cd crystal growth, ultimately forming a biocompatible capping layer around the nanoparticles. After functionalization with folic acid, the QDs were used to image cultured cervical cancer cells in vitro.

Elemental tellurium and selenium, formed via bioreduction as precursors to the more reduced telluride or selenide, can also form nanorod materials. Te(0) nanorods, formed with a chemical host such as poly(m-phenylenevinylene-α,2,5-dioctoxy-phenylenevinylene) (PmPV) have nonlinear photonic behaviour with excellent broadband optical limiting at 532 and 1064 nm, and could be used for nanoelectronic and nanophotonic applications [44]. For Se(0), a selenium-tolerant, aerobic *Bacillus* species isolated from a seleniferous rhizosphere soil in India produced amorphous α-Se(0) nanospheres, which could be converted to hexagonal Se(0) nano-rods by post bioproduction washing steps [45]. The first applications for Se(0) semiconductor mononanocrystals, again synthesized by a *Bacillus* species and here exhibiting diameters ranging from 50 to 400 nm, have also been reported. They were used to enhance the sensitivity and affinity for H₂O₂ in a biosensor [46].

**Discussion and future directions**

The vast and relatively underutilized gene pool of microorganisms has the potential to encode enzymatic systems that, when coupled with biological templating, can result in the synthesis of many potentially new nano-scale materials. Future priorities will include extending the range of materials produced, providing completely new products including more complex designs such as core-shell structures. As we understand the underlying biochemistry and genetics of these systems, such biofabrication techniques will become part of the synthetic biology toolbox currently being developed for the next generation of biotechnology applications. Alongside these developments, it is paramount that new studies are initiated on the scale-up of these bioprocesses, addressing economic considerations (including cost–benefit analyses versus more conventional synthesis routes) and also the impact of scale-up parameters on the structure and properties on bionanomaterials produced. The environmental impact of these materials, in common with those synthesized by more traditional chemical routes, will also have to be addressed. In addition, their unusual properties may also prove useful for the remediation of land and water contaminated with metals and organics, and here appropriate delivery or *in situ* synthesis mechanisms must also be developed.

**Acknowledgements**

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


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27. Staniland S, Williams W, Telling ND, van de Laan G, Harrison A, Ward B: Controlled cobalt doping of magnetosomes in vivo. Nat Nanotechnol 2008, 3:158-162. The incorporation of cobalt within the crystal structure of the magnetite produced within magnetosomes was shown to increase coercivity by up to 45% without a significant impact on the saturation magnetization of the nanoparticles. This is an important quality required in technologies such as magnetic recording devices which require magnetization to remain in place without the need for an external field applied.


Palladium mounted magnetic catalysts were produced by the immersion of biogenic magnetic nanoparticles in NaPdCl$_4$. Reactivity was measured in the Heck reaction, coupling iodobenzene to ethyl acrylate or styrene. Results showed superior performance to a commercial colloidal palladium catalyst, with the additional benefit of magnetic removal allowing the catalysts to be re-used for multiple rounds of Heck coupling.


Thermoanaerobacter sp. TOR-39 was used to generate large quantities of biogenic magnetite (1 kg wet weight). This demonstration serves as a starting point for establishing a process that could have important technological implications for the up-scaling of nanoparticle production crucial for the widespread commercial use of biogenically formed nanomaterials.


Selenide nanoparticles were synthesized by whole cells of an anaerobic bacterium Veillonella atypica supplied with hydrogen as electron donor and the redox mediator AQDS. Manipulation of pH, temperature and use of a thiol-based stabilizing agent was required in a subsequent step to produce luminescent, water-soluble ZnSe and CdSe quantum dot bio-nanocrystals. The evolution of these materials was followed in situ using synchrotron radiation.


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Conclusions and Future Development
10. Conclusions and Future Development

10.1. Conclusions

Throughout the course of this thesis, dissimilatory iron-reducing bacteria have been used successfully to produce magnetic nanoparticles. By variation of key parameters it has been shown that the properties of the nanoparticles can be manipulated to control particle size and magnetization. Five research papers have been produced as a result of this work, of which one has been published (Chapter 4), one has been accepted and awaiting publication (Chapter 8), and another has been submitted for publication and awaiting peer review (Chapter 5). Additionally, a review article into the current advances of the synthesis of functional nano-scale materials by biotechnological approaches has been published.

In summary, three distinct objectives were outlined for this body of work including: i) Size and magnetic property manipulation of magnetite; (ii) Potential use of magnetic nanoparticles in different applications; (iii) Ability to scale up biogenic nanoparticle production. Due to the nature of this research project there was a significant amount of overlap between the different aims.

i) Size and magnetic property manipulation of magnetite

The small size and magnetic properties of magnetite nanoparticles make them potentially very suitable for a wide variety of applications. The ability to optimize these properties is crucial to ensure that they could be used for different applications which each may have specific requirements.

During this thesis it was discovered that the amount of Fe(III)-reducing bacteria (biomass) introduced into Fe(III)-oxyhydroxide starting cultures had a direct impact on the final size of magnetite nanoparticle produced (Chapter 4). This was considered to be a rate dependent effect with increased concentrations of bacteria leading to an increase in the amount of Fe(II) produced. The faster that the Fe(II) was produced (i.e. using more bacteria), the smaller the magnetite nanoparticles produced.
Other experiments aimed at changing the magnetic properties of magnetite nanoparticles focused on the incorporation of transition metal dopants into the crystal structure. This included zinc (Chapter 5) and cobalt (Chapter 6) to produce particles of the form $M_xFe_{3-x}O_4$ (where $M=\text{Co, Zn}$). Substitution into the magnetite structure was characterised using XMCD and Mössbauer techniques. It was discovered that using these dopants, materials could be produced with enhanced magnetic properties including higher saturation magnetization (zinc doping) or larger coercivity (cobalt doping).

ii) Potential use of magnetic nanoparticles in different applications

The second principal aim of this project was to investigate the potential use of biogenically produced magnetite nanoparticles in different applications. This included the reduction of chromate (Cr(VI)) solution as an analogue to chromium remediation (Chapters 4 and 7), and the use of cobalt doped magnetite in magnetic hyperthermia experiments which could serve as a basis for future cancer therapies (Chapter 6).

Chromate based experiments utilised the highly reactive Fe(II) present at the surface of biogenic magnetite coupled with the high surface area to volume ratio of the magnetic nanoparticles. It was observed that by decreasing the particle size (thus increasing surface area to volume ratio) using the methods outlined in Chapter 4, the reactivity of the magnetite nanoparticles with chromate is significantly increased, leading to complete reduction of Cr(VI) to Cr(III) observed for the smallest samples produced. This experiment was replicated using material produced in large volumes (Chapter 7) with results demonstrating that there is little impact of scale up on the end products of biogenic Fe(III) reduction.

Magnetic hyperthermia based experiments investigated the application of cobalt doped magnetite (Chapter 6) for targeted cancer therapy. It was shown that increases in cobalt concentration in nanometre particles of magnetite lead to an increase in the anisotropy of the material. The nanoparticles were suspended in solution and placed under the influence of an AC magnetic field which resulted in a heating effect in the solution. These results indicated that the increase in anisotropy leads to an increase in the measured heating effect, however particle size effects are also thought to have an impact.
iii) Ability to scale up biogenic nanoparticle production

For biogenic magnetite nanoparticles to be useable in different applications, the material needs to be produced in large quantities (i.e. kg to tonne). The scale up of the growth of *Geobacter sulfurreducens* and scale up of iron(III) transformation experiments has been reported in Chapter 7. In laboratory experiments, bacterial growth was carried out at 100 ml scales, which were scaled up to 50 L at an industrial facility. Iron(III) transformation which is usually performed at small volumes of 10 ml was scaled up to 10 L in the same experiment. The results showed that magnetite nanoparticle formation can be successfully carried out at large scale. The only significant difference from 100 ml to 50 L observed in bacterial growth visible was a lengthening of the overall lag phase by several hours before the exponential growth of the organism. The reduction of iron(III) also did not appear to yield significant differences aside from the formation of the siderite mineral phase at high volumes. Hematite was also observed as an additional iron oxide produced at all volumes although subsequent experimentation determined a method of avoiding this product by eliminating the autoclaving stage used to sterilize the media prior to inoculation. Ultimately, this piece of work successfully demonstrated that it is feasible to commercially produce magnetite through biological approaches.

10.2. Future work

This thesis has attempted to cover several different aspects of biogenic nanoparticle production, however there are still many different areas which could be further developed. These are discussed below in terms of the original aims for which this thesis initially set out to achieve:

10.2.1. Controlled manipulation of properties

Different methods of control of properties have been demonstrated including size manipulation and incorporation of dopants. During experiments focused on incorporating dopants into magnetite it was seen that the addition of adding a transition metal such as zinc or cobalt not only led to changes in the magnetic
properties, it also resulted in the decrease in the size of the nanoparticles produced. The effect is seen to be approximately linear with zinc concentration however the effect is much more dramatic for cobalt doping. Particle size can have a significant impact on the magnetic properties of a material. Larger nanoparticles exhibit higher saturation magnetization than smaller particles and could have enhanced results for some applications including magnetic resonance imaging (MRI). Coercivity is also a size dependent effect, with larger particles having increased resistance to applied magnetic fields (i.e. higher coercivity) than smaller particles and could be used in data storage devices. Thus it would be beneficial if marginally larger particles of zinc and cobalt doped magnetite could be produced. Based on experimental results of Chapter 4, size appears to be a rate dependent effect. Slowing down the rate of production of Fe(II) could be a potential route of investigation for producing larger particles of zinc and cobalt doped magnetite. This could be achieved through changes to the electron shuttles which are used to speed up the rate of reaction, either by lowering concentration or exploring different shuttle compounds. Alternatively temperature could be explored as a variable with lower temperatures expected to yield slower rate of reduction and consequently larger particle size.

10.2.2. Use of material for different applications

Biogenic magnetite nanoparticles were demonstrated in chromate remediation and magnetic hyperthermia experiments. Further research could be used to demonstrate the application of nanomaterials in other areas such as magnetic drug delivery, MRI and data storage devices.

Magnetic hyperthermia experiments were performed as part of a larger study into the properties of cobalt doped magnetite nanoparticles. A full study focusing more specifically on hyperthermia measurements could look into a wider range of cobalt doped samples in order to quantify the effect with respect to anisotropy changes. Such a study would require the elimination of particle size as a variable as this would have an impact on the potential heating of the nanoparticles in an AC magnetic field. Another potential hurdle for the use of cobalt doped magnetite is the toxic nature of cobalt. If the material was to be used in human trials, it would be imperative that no leaching of the toxic cation into the body was possible perhaps through the use
of capping agents. Such a coating could also have an impact on the effective heating power of the magnetic nanoparticles and would need to be investigated.

10.2.3. Commercial development

The materials produced by bacterial reduction have been shown to be able to be used in different applications. Chapter 7 highlighted the ability to scale-up the production of magnetite nanoparticles in an industrial bioreactor. Through production of 50 L of *Geobacter sulfurreducens* culture, up to 120 g of product was formed. Through some minor adjustments to the experimental procedures, the total mass of product could be significantly improved. This could be achieved by decreasing the concentration of biomass used in the Fe(III)-transformation experiments and increasing the concentration of Fe(III)-oxyhydroxide in the starting material.

Other areas which could be the focus of improvement include decreasing the lag phase of the bacteria growth curve. Compared to 100 ml growth, the lag phase was significantly longer for 50 L fermentation. The cause of this was not explored during the project, however shortening the length of time required to produce *Geobacter sulfurreducens* at scale could lead to significant improvements in the overall cost incurred during production processes. Initially, changes to the stirring mechanism could be explored as this was observed to result in lag phase increases.

The methods reported in Chapter 7 describe batch growth of bacteria. Continuous culturing techniques could be developed to enable an almost constant supply of bacteria. Such an approach would significantly reduce the length of time required to set up a fermentation run and consequently produce much larger quantities of iron reducing bacteria over the same time frame as batch culturing.

10.3. References


## Appendix

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Table 1 – Effective Radii (Å) of selected transition metal cations extracted from Shannon 1969$^1$ (CN – Coordination number, LS - low spin, HS - high spin, CR – Crystal Radius, IR – Ionic Radius).

## References