The Synthesis and Application of Iron Oxide Nanoparticles for Targeted Imaging using MRI

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Doctor of Philosophy

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School of Medical Sciences

RMIT University
Abstract

The work presented in this thesis describes the use of highly magnetic iron oxide silica nanoparticles conjugated to herceptin for the targeted detection of human epidermal growth factor receptor 2 (HER2) positive breast cancer using magnetic resonance imaging (MRI). The main finding of this study was that iron oxide nanoparticles coated in silica can be used to detect HER2 positive breast cancers in vivo using MRI. The iron oxide silica nanoparticles produced presented with highly magnetic properties and were able to produce MRI contrast. The iron oxide silica nanoparticles were also found to be non-toxic, biocompatible, stable and were easily conjugated to a biomarker, herceptin. The results from the in vitro and in vivo assessment of these iron oxide silica herceptin nanoparticles, demonstrated that significant MRI contrast was visualised relative to HER2 expression levels in three human breast cancer cell lines and HER2 positive breast tumours.

A promising use of nanoparticles is in medical imaging where iron oxide nanoparticles are increasingly being utilised in MRI as contrast agents for targeted imaging. Iron oxide particles have previously been used as contrast agents for imaging the liver, spleen and gastrointestinal lumen in MRI. With advancements in nanoparticle technology, iron oxide nanoparticles that are less than 100 nm in diameter have been able to be synthesised which are capable of imaging specific diseases targeted with a biomarker. This advancement is a crucial development for medical imaging as it allows targeted imaging to be possible with MRI which, unlike nuclear imaging methods, currently does not have the required sensitivity. The improvement in nanoparticle synthesis processes have also enabled the production of iron oxide nanoparticles with higher magnetic properties, less toxicity, improved stability and solubility and surface layer suitable for the attachment of a variety of molecular markers. These properties are important characteristics for iron oxide nanoparticles as they influence their ability to produce contrast at the targeted site.
An application of iron oxide nanoparticles for targeted imaging in MRI is in breast cancers that overexpress HER2 receptors which are associated with poor prognosis. A treatment option for HER2 positive tumours is Herceptin® (herceptin) therapy, which is known to cause down regulation of HER2 expression eventually causing cell death and a reduction in the size of the tumour. Although the well reported response rates to herceptin therapy regimes are high, it is documented that patients acquire resistance to herceptin based therapy regimes after several months. The primary mechanism of herceptin resistance is unknown and there are varieties of pathways that have been proposed. Although there are research efforts to understand the myriad of molecular mechanisms related to herceptin resistance, it is also equally important to identify the patients who may be resistant to herceptin based therapy.

A tool that can be used to image HER2 positive breast tumours and potentially identify these patients is in MRI using iron oxide nanoparticles conjugated to herceptin. Previous studies demonstrate that HER2 positive breast tumours can be targeted using iron oxide nanoparticles in MRI. Unfortunately, these studies do not address all of the characteristics described above that are required to achieve targeted imaging in MRI. Due to this, iron oxide nanoparticles for targeted imaging in MRI have been limited in their translational capability to the clinic.

Therefore, the aim of this study was to synthesise iron oxide nanoparticles addressing all of the characteristics required for targeting HER2 positive tumours \textit{in vitro} and \textit{in vivo}. To confirm that iron oxide silica herceptin nanoparticles possessed the basic characteristics needed for targeted imaging, a series of characterisation techniques were conducted. It was clearly shown that the synthesised iron oxide silica nanoparticles had the major
characteristics needed to be used as targeted contrast and were superior to commercially available iron oxide nanoparticles and previously reported iron oxide nanoparticles. Furthermore, the conjugation between iron oxide silica and herceptin was shown to be stable after exposure to a range of conditions, mimicking different physiological conditions.

*In vitro* studies examined cellular uptake and contrast enhancement capability of the iron oxide silica herceptin nanoparticles. The studies confirmed the uptake into HER2 expressing human breast cancer cells possibly mediated by the herceptin, which was consistent with receptor expression and results from previous studies. The *in vivo* investigation demonstrated uptake of the iron oxide silica herceptin nanoparticles at the tumour site, producing signal enhancements larger than previously reported. Histopathological analysis indicated that there was no toxicity to the liver, lungs, heart and kidneys. Overall, the findings of this study demonstrated that the iron oxide silica herceptin nanoparticles targeted HER2 positive tumours with nanoparticles that are highly magnetic, biocompatible, small in size and size distribution and stable. The results suggest that the iron oxide silica herceptin nanoparticles have all of the basic characteristics required for potential translation into the clinical setting.
Declaration

This is to certify that

i. the thesis comprises of only my original work towards the PhD except where indicated,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices.

Jyoti Arora
October 2011
Preface

The work presented in this thesis was performed at the School of Medical Sciences at RMIT University, under the supervision of Associate Professor Simon Cowell, Dr Dodie Pouniotis and in the Diagnostic Imaging Department at the Peter MacCallum Cancer Center under the supervision of Mr Peter Eu. Some components of this work was also done under the guidance of Dr Vipul Bansal in the School of Applied Sciences.

Some components of this work were performed by other researchers and due acknowledgement is given throughout the thesis. Iron oxide nanoparticle production was performed jointly by me and Jos Campbell from the School of Applied Sciences in Dr Vipul Bansal’s laboratory. The TEM analysis was conducted in the Core Microscopy Facility in the School of Applied Sciences and was performed jointly by me and Jos Campbell. SQUID measurements and analysis were conducted by Dr. Ashish Garg from the Indian Institute of Technology in Kanpur, Uttar Pradesh, India, in the Department of Materials Science and Engineering. Blood samples from the mice were sent to Animal Pathology Laboratory PTY LTD for processing and analysis. The frozen samples of mouse organs for the in vivo toxicity assay of iron oxide silica herceptin nanoparticles were analysed by laboratory technicians and a veterinary pathologist from the Australia Phenomics Network at the University of Melbourne, Australia.

Most of the work presented in the thesis has been presented at scientific conferences and some part of the work has been published in peer-reviewed journals.

Publications submitted:

Arora J, Campbell J, Pouniotis D, Cowell SF, Eu P, Bansal V (Submitted) The application of highly magnetic iron oxide nanoparticles for cancer detection using MRI. PLoS ONE.
Publications:


Conference Presentations:


Whitehorn N, Thompson M, Eu P, Lodhia J, Cowell S (2009) Can iron oxide nanoparticles be used as an MRI contrast agent to track the location of blood cells as an alternative to radiolabelled blood cells. 3rd Annual Nuclear Medicine Research Symposium, RMIT University, Melbourne. P15-16.

Acknowledgement

This thesis is dedicated to Uma Lodhia who died in November 2004 from cancer.

I would like to thank my supervisors, Simon Cowell, Peter Eu and Dodie Pouniotis, for their guidance and input to my PhD project. I appreciate their support and patience given to me throughout the entire candidature. Particularly, Dodie who has given me a lot of opportunities in training to develop my expertise in cancer research; and gave me affirmations and confidence during tough times. I also want to thank Vipul Bansal and Jos Campbell for their valuable advices and mentorship.

I gratefully acknowledge the funding sources that made my PhD work possible. My scholarship and project funding were both sponsored by RMIT University. I had the great pleasure of working alongside Vipul’s research group at the School of Applied Sciences. I also want to thank the staff at Cancer Imaging at Peter MacCallum Cancer Centre who without Peter would not have provided me with the support and access to technical equipment that I needed over the four years. In particular, I want to thank the MRI department especially Noelene Bergen. Noelene has been a source of friendship as well as good advice and collaboration. I want to acknowledge all her overtime in MRI scanning that was required for the project. I want to a say thankyou for all the listening ears and for sharing the joys and tears of research.

Away from the lab I have been supported by an amazing group of family and friends. I want to thank my sister and brother, sister-in-law and their two beautiful children for their support in all my pursuits. Most of all, to my loving, encouraging and patient husband Gaurav, thank you for your faithful support during my PhD. A special thank you to my Bishop Pita and Mareya for their prayers, wisdom and friendship.
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<th>Symbol</th>
<th>Abbreviation</th>
<th>Definition</th>
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<td>(^{111})In</td>
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<tr>
<td>(^{67})Ga</td>
<td>(^{67})gallium</td>
<td></td>
</tr>
<tr>
<td>(^{99m})Tc</td>
<td>(^{99m})technetium</td>
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<tr>
<td>(\alpha)-Fe(_2)O(_3)</td>
<td>iron oxide haematite</td>
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<td>AAS</td>
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<tr>
<td>CEA</td>
<td>anti-carcinoembryonic antigen</td>
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<tr>
<td>CEST</td>
<td>chemical exchange saturation transfer</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CT</td>
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<tr>
<td>DAB</td>
<td>3, 3'-diaminobenzidine</td>
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<tr>
<td>DMSA</td>
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<tr>
<td>emu/g</td>
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<td>EGFR</td>
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</tr>
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</tr>
<tr>
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<tr>
<td>FITC</td>
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<tr>
<td>H&amp;E</td>
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<td>human epidermal growth factor receptor 2</td>
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<td>human umbilical mesenchymal stromal cell Schwann cells</td>
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<tr>
<td>LD(_{50})</td>
<td>median lethal dose</td>
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<tr>
<td>Mg</td>
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<td>MRI</td>
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<td>NIR</td>
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<tr>
<td>PARACEST</td>
<td>paramagnetic chemical exchange saturation transfer</td>
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</tr>
<tr>
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</tr>
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<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>PEG</td>
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<tr>
<td>PFC</td>
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<td>poly (D, L-lactic acid)</td>
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<td>PLGA</td>
<td>poly (D,L-lactic-co-glycolic acid)</td>
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<tr>
<td>QCM</td>
<td>quartz crystal microbalance</td>
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<tr>
<td>RES</td>
<td>reticulo-endothelial system</td>
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<tr>
<td>RF</td>
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</tr>
<tr>
<td>ROI</td>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
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</tr>
<tr>
<td>SE</td>
<td>single echo</td>
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</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>-----------</td>
<td>-----------------------------------------------------------</td>
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</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computerised tomography</td>
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<tr>
<td>SPIONS</td>
<td>superparamagnetic iron oxide nanoparticles</td>
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</tr>
<tr>
<td>SQUID</td>
<td>superconducting quantum interference device</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>spin lattice relaxation</td>
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</tr>
<tr>
<td>T2</td>
<td>spin-spin relaxation</td>
<td></td>
</tr>
<tr>
<td>T2*</td>
<td>T2 star</td>
<td></td>
</tr>
<tr>
<td>TE</td>
<td>time echo</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
<td></td>
</tr>
<tr>
<td>TEOS</td>
<td>tetraethyl orthosilicate</td>
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<tr>
<td>TR</td>
<td>time repetition</td>
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<tr>
<td>uMUC-1</td>
<td>underglycosylated mucin-1 antigen</td>
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<td>ultrasound</td>
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<tr>
<td>UVB</td>
<td>ultraviolet B</td>
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</tr>
<tr>
<td>WHO</td>
<td>world health organisation</td>
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<tr>
<td>XRD</td>
<td>x-ray diffraction</td>
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Chapter 1 Introduction

1.1 Nanotechnology and nanoparticles

The idea of nanotechnology was introduced in 1959 when Richard Feynman, a physicist from the Californian Institute of Technology, gave the revolutionary speech, “There’s plenty of room at the bottom” (Feynman, 1960). Feynman described the idea of building miniature factories with nanoscale machines developed by atoms and molecules that could be precisely manipulated. He suggested that the laws of physics should make possible the creation of objects at a very small scale to carry out tasks with an enormous range of technical applications. He explained that there was not just room at the bottom but plenty of room at the bottom to achieve anything miniature. In his revolutionary speech he gave examples of miniature creations such as building miniature computers with wires with a maximum diameter of 100 atoms to store as much information as possible. In addition, he explained that encyclopaedias could eventually be written on the head of a pin because, there was plenty of room. He envisioned and questioned: if biological systems had microscopic cells that move around with multiple functions and store information in DNA in such a small scale, then why couldn’t tiny objects that have the potential to track biological functions be possible? This vision has developed into what is termed nanotechnology and is defined as the engineering of functional systems at the molecular scale.

Nanotechnology operates at the first level of organisation of atoms and molecules, where the properties and functions are defined, in all systems. Nanotechnology has a wide variety of applications and is not limited to areas such as electronics, space, food, cosmetics and medicine (Sozer & Kokini, 2009). One of the most important and widely researched areas of nanotechnology is the development and use of nanoscale materials such as
Chapter 1 - Introduction

such as nanoparticles.

According to the most widely accepted definition, nanoparticles are defined as particles in the nanoscale that have at least one dimension less than 100 nm. Over the past decade, scientists and engineers have been mastering the intricacies of nanoparticles including their development, properties and characteristics, opening up vast areas of nanoparticle research some of which are highlighted in Table 1.1.

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<tr>
<th>Applications of Nanoparticles</th>
<th>Uses</th>
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<td>Cosmetics</td>
<td>Sunscreens</td>
<td>(Katz Linda, 2007; Kokura et al., 2010; Mu &amp; Feng, 2003; Mu &amp; Sprando, 2010; Souto &amp; Müller, 2008)</td>
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<td>Hyperthermia</td>
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Table 1.1 Applications of nanoparticles
There are many applications of nanoparticles that have evolved over the last decade. This table highlights the different areas where nanoparticles are used, which include but are not limited to industries such as cosmetics, computers, medicine and food.

In computing science, nanoparticles have been utilised in developing mini transistors leading to the production of smaller and more powerful computers and laptops (Matsui, 2005). In the food sector, nanoparticles have improved techniques such as dry milling, where it is now possible to obtain very small particle sizes which are useful in extracting
fine wheat flour (Degant & Schwechten, 2001). Nanoparticles have also been utilised in the development of biodegradable packaging materials for food items. Traditionally, most food packaging materials are made from non-degradable materials, which increase environmental pollution as well as increasing the use of fossil fuels (Sozer & Kokini, 2009; Tharanathan, 2003). The production of alternative packaging materials has been made possible by nanoparticles, which have improved their biodegradability (Sorrentino, Gorrasi, & Vittoria, 2007; Suyatma, Copinet, Tighzert, & Coma, 2004; Tharanathan, 2003). In cosmetics, an area where nanoparticles play a crucial role is in preventing sunburn. In sunscreens, the properties of titanium dioxide nanoparticles reflect harmful sunrays giving maximum ultraviolet A (UVA) and ultraviolet B (UVB) protection (Mu & Sprando, 2010). Another field that has seen significant development by utilising nanoparticles is medicine. In the last decade, research into nanoparticles has made it possible to adjust functional, physical and chemical properties so that they can be tailored and applied to almost any biomedical application.

1.1.1 Nanoparticles in medicine

Over the past decade a great variety of nanoparticles have been synthesised for medical applications. These include but are not limited to substances such as silver, gold, iron, fluorescent particles, polymers and lipids, which have been widely exploited as nanoparticles for a vast range of biomedical applications (De, Ghosh, & Rotello, 2008). For example, silver nanoparticles have attracted interest due to their potent antibacterial, antimicrobial activity and anti-inflammatory properties (JoseRuben, et al., 2005; Nadworny, Wang, Tredget, & Burrell, 2008; Sibbald, et al., 2007). The unique properties of silver nanoparticles make them useful in areas such as wound healing (Chaloupka, Malam, & Seifalian, 2010), where silver nanoparticle dressings are used to treat minor
cuts and grazes, burns and ulcers (Chen, Han, Lin, Tang, & Su, 2006; Huang, et al., 2007; Sibbald, et al., 2007).

Nanoparticles are one billionth of a metre in size, which is smaller than micro sized cells and some cellular components. The small size of nanoparticles makes them useful as carriers for the delivery of drugs and vaccines to targeted sites (Singh & Lillard, 2009). Nanoparticles that are used to deliver drugs vary in their properties depending on their method of synthesis and the release characteristics needed for optimal delivery. In some cases, hollow nanoparticles, acting as vesicular systems, can be loaded with a drug and released \textit{in vivo} at a targeted site. Polymeric nanoparticles made from poly (D, L-lactic acid) (PLA), poly (D,L-lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol) (PEG) have been extensively researched for the loading and delivery of drugs because of their biodegradability \textit{in vivo} (Hans & Lowman, 2002; Kumari, Yadav, & Yadav, 2010). PLGA nanoparticles are among the most successful biodegradable nanoparticle drug delivery systems and currently have FDA approval for human use. PLGA nanoparticles have been successfully used to deliver various anti-cancer drugs such as the chemotherapeutic agents cisplatin, paclitaxel and 9-nitrocamptotecin to tumour sites, reducing overall systemic toxicity (Avgoustakis. et al., 2002; Dadashzadeh, Derakhshandeh, & Shirazi, 2008; Derakhshandeh, Erfan, & Dadashzadeh, 2007; Fonseca, Simoes, & Gaspar, 2002; Gryparis, Hatziapostolou, Papadimitriou, & Avgoustakis, 2007; Mu & Feng, 2003). Apart from chemotherapy drugs, PLGA nanoparticles have also been used to load antipsychotic drugs, insulin, hormones, proteins, restenosis drugs and anti-inflammatories, for delivery to targeted sites (Hans & Lowman, 2002; Kumari, et al., 2010).

Apart from wound healing and drug delivery, nanoparticles in medicine are also utilised in the development and delivery of vaccines, particularly for treating and preventing cancer.
In some vaccines, nanoparticles are used as carriers to deliver the active components to stimulate an immune response such as non-specific immune activation or tumour-specific immune activation (Sheng & Huang, 2011). These immune responses work together with the vaccine by multiple mechanisms to identify and eliminate any cancerous cells that may be present or have the potential to invade the immune system. The components of vaccines can vary from tumour-specific antigens to DNA and proteins/peptides, and their carriers must be appropriate to ensure delivery. Nanoparticles have been shown to be excellent carriers, particularly because of easy synthesis and the ability to produce many different sizes (Kreuter, 1996; Sheng & Huang, 2011). Larger sized nanoparticles up to 1 μm in diameter are ideal for the induction of systemic immunity due to effective internalisation by macrophages and dendritic cells. However, smaller nanoparticles, <100 nm in diameter, have been shown to provide better immunogenicity compared to larger particles. For example, studies by Reddy et al. 2006 and 2007 demonstrated better circulation through the lymphatics by smaller nanoparticles (Reddy, Rehor, Schmoekel, Hubbell, & Swartz, 2006; Reddy et al., 2007). For vaccine development, nanoparticles that are biodegradable polymers, such as PLGA, have shown promising results in the delivery of antigens as vaccines (Eldridge, Staas, Meulbroek, Tice, & Gilley, 1991; Singh, et al., 1997). Similarly, liposomal nanoparticles have shown advantages in attaching to the outer surface or encapsulating and delivering DNA and protein vaccines in vitro and in vivo (Sheng & Huang, 2011).

Over the last decade, the use of nanoparticles in medicine has also been incorporated in medical imaging. In medical imaging, nanoparticles, together with imaging technologies, promise to deliver more specific and sensitive detection of diseases for diagnostic and therapeutic purposes (Bulte & Modo, 2007). Moreover, as imaging modalities continue to
advance, there is also the need to develop and improve tracers or contrast agents that are needed for improved resolution and sensitivity. Advances in the development of nanoparticles in the field of medicine have produced nanoparticles with improved properties and characteristics, which in turn have provided medical imaging with new platforms of contrast agents and tracers to complement currently available imaging technologies and new imaging technologies (Bulte & Modo, 2007). Nanoparticles can be synthesised to display unique chemical properties that can dramatically amplify signal changes, making the nanoparticles visible on images, or they can be given physical properties that allow selective uptake in vitro and in vivo (Bulte & Modo, 2007; Rudin & Weissleder, 2003). As nanoparticles can be utilised to serve many different imaging modalities, a great variety of nanoparticles are under investigation for development and application in medical imaging, as discussed in greater detail in this chapter.

1.1.2 Summary of main themes

Nanoparticles are used in a wide variety of applications such as food, cosmetics and medicine, providing insight into some of these applications. The use of nanoparticles in medicine is emerging, particularly in medical imaging. Over the last decade the use of nanoparticles in medical imaging, together with developments in imaging technology, began to deliver more specific and sensitive detection of diseases for diagnostic and therapeutic purposes. Together with the improvements in medical imaging modalities and the discovery of a range of novel nanoparticles, a new platform of contrast agents and tracers to complement imaging technologies became possible.

1.2 Imaging technologies
1.2.1 Introduction

As discussed in the previous section, nanoparticles can be utilised to serve many different purposes including different imaging modalities. Nanoparticles can be synthesised to possess unique characteristics that can amplify signals and generate contrast for better visualisation. These can be magnetic properties or optical properties that further enhance the imaging modality’s signals to provide contrast for better visualisation of anatomy and pathology. Moreover, the small size (<100 nm) of nanoparticles allows for improved cellular uptake, via passive or active pathways, into various sub-cellular compartments, depending on their composition and whether they are conjugated to biomarkers. These properties of nanoparticles, along with improved synthesising methods, have opened the possibility for specific targeting of molecular and cellular processes using nanoparticles in medical imaging.

For targeted imaging to be possible, two prerequisites must be met: firstly, the imaging device must have high sensitivity and resolution to be able to image molecular interactions, and secondly, there must be target-specific molecular probes. The use of nanoparticles in most cases provides the target-specific probes for imaging modalities and can help some imaging detectors achieve higher sensitivities. The next section describes how nanoparticles are utilised for different imaging modalities, followed by a discussion of the ability of nanoparticles to help achieve targeted imaging.

1.2.2 Imaging technologies and nanoparticles

Nanoparticles can be synthesised to possess unique characteristics that can amplify signals and generate contrast for better visualisation (Bulte & Modo, 2007; Rudin & Weissleder, 2003). Magnetic resonance imaging (MRI) uses magnetism to produce images, and the use
of magnetic nanoparticles or magnetic compounds can serve to amplify the MRI signal to produce contrast for improved visualisation. Magnetic substances used to synthesise nanoparticles have either superparamagnetic or paramagnetic properties (Huber, 2005). These magnetic properties cause alterations in the MRI signal either by decreasing the magnetic resonance signal intensity, producing a dark contrast (superparamagnetic), or by increasing the magnetic resonance signal intensity, producing bright contrast (paramagnetic) (Huber, 2005). Among the most widely used paramagnetic substances are gadolinium compounds, which have been used in MRI to produce contrast in applications such as diagnosing pathologies, pre- and post-operation, infection, infarction and inflammation (Westbrook, Roth, & Talbot, 2005). Several gadolinium products, such as Magnevist®, Optimark™, Prohance® and MultiHance®, are commercially available (Aime & Caravan, 2009). Although gadolinium agents are frequently used and provide signal enhancement, there have been concerns over their safety. Issues such as the possible dissociation of gadolinium from its chelating agents in vivo have been suggested, and deaths due to nephrogenic systemic fibrosis have been reported in patients (Ergun, et al., 2006; Erley, et al., 2004; Grobner, 2006; Perazella, 2009; Sam, et al., 2003). As a result, a research focus into gadolinium agents is on improving their in vivo stability using nanoparticles, and research has also considered the use of compounds other than gadolinium for achieving contrast (Perazella, 2009).

Among the superparamagnetic agents, iron-based particles have been the most widely used and researched (Gupta & Gupta, 2005). Like gadolinium compounds, there are also commercially available iron-based particles, such as Revosist®, Feridex®, Gastromark® and Abdoscan® (Lodhia, Mandarano, Ferris, Eu, & Cowell, 2010). These commercial iron-based nanoparticles are most useful in imaging the liver, spleen and gastrointestinal
lumen. In the pre-clinical setting, iron-based nanoparticles are under development for other MRI applications including the lymphatic system, breast, the musculoskeletal system and various cancers.

Nanoparticles in medical imaging can also be synthesised with electrical and chemical properties that allow them to be visible by modalities such as optical imaging. In optical imaging, nanoparticles are utilised in the form of quantum dots, which are made from semiconductor materials such as cadmium selenide or zinc selenide. Quantum dots have unique optical and electronic properties, such as the emission of photons under excitation that are visible to the human eye; moreover, the wavelengths of photon emissions are related to the size of the quantum dot, allowing their emission to be tuned to any wavelength (Alivisatos, 1996; Qu & Peng, 2002). Quantum dots can also be easily tuned beyond visible light to the infra-red or into ultra-violet light (Alivisatos, 1996). The easy synthesis of quantum dots of various sizes allows linking of multiple agents such as proteins, peptides, and antibodies, and even conjugation of therapeutic agents such as anti-cancer drugs (Wang, Gao, & Su, 2010). Quantum dots can be easily attached to other nanoparticle materials such as magnetic nanoparticles used in MRI or radioisotopes used in nuclear medicine, allowing for dual optical MRI imaging or dual optical nuclear medicine imaging (Bulte & Modo, 2007).

Another primary aspect of nanoparticles and their application in medical imaging is the ability to mediate cellular uptake. The small size (<100 nm) of nanoparticles allows cellular uptake, via passive or active pathways, into various sub-cellular compartments depending on their composition and conjugated materials. This feature of nanoparticles allows imaging modalities such as MRI and optical imaging to utilise their respective
nanoparticles (magnetic nanoparticles and quantum dots) to image molecular and cellular processes. For example, iron-based nanoparticles can be synthesised to be <100 nm in size, highly magnetic in order to amplify the MRI signal and to be conjugated to biomarkers. The small size of the iron nanoparticles mediates cellular uptake depending on the properties of the biomarker, and the magnetic properties of the iron will allow visualisation on the MRI image of the targeted site. Similarly, optical imaging probes can also use the same technique to allow imaging of molecular and cellular processes, utilising different sized quantum dots to view various wavelengths of different colours.

The ability to image molecular and cellular processes is not a new technology established by the development of nanoparticles for medical imaging. In fact, it has been available for decades and used by modalities such as nuclear medicine even before the term ‘nanoparticles’ was defined. As early as the 1970s, nanoparticles such as sulphur colloids were labelled to $^{99m}$Tc for imaging lymph nodes in lymphoscintigraphy (Ashburn, Braunwald, Simon, Peterson, & Gault, 1971; Fee, et al., 1978). Magnetic nanoparticles were also reported to have the ability to enhance MRI signals in the late 1970s (Ohgushi, Nagayama, & Wada, 1978), but their use as contrast agents was not explored until the mid-1980s (Mendonca Dias & Lauterbur, 1986; Renshaw, Owen, McLaughlin, Frey, & Leigh, 1986). During this time, iodinated contrast agents were also used for computed tomography (CT) (Havron, Seltzer, Davis, & Shulkin, 1981). Despite early discoveries of what are now termed nanoparticles, the last decade has seen significant advancement in nanoparticle technology, particularly in relation to synthesis methods and conjugation methods available for the attachment of biomarkers. These advances have allowed successful utilisation of nanoparticles for imaging molecular and cellular processes, particularly for modalities such as MRI and optical imaging
The use of nanoparticles has helped change the focus of medical imaging from anatomical imaging and low-resolution nuclear medicine functional imaging to more sophisticated targeted and functional imaging with improved sensitivity and specificity (Bulte & Modo, 2007; Weissleder, 1999).

The next section details the concept of targeted imaging in medical technologies and describes how nanoparticles have helped play a role in achieving targeted imaging.

1.2.3 Targeted imaging

As early as 1999, Weissleder reported that medical imaging was expanding its focus from anatomical and structural imaging towards targeted molecular and cellular imaging. Weissleder believed that significant advances had been made in genomic research, with further understanding of the molecular mechanisms of disease processes; thereby, discovery of many disease biomarkers was conceivable (Herz, Thomsen, & Yarbrough, 1997; Pardee, 1997; Weissleder, 1999). In parallel, medical imaging had undergone remarkable advances in improving resolution and sensitivity in some modalities (Rudin & Weissleder, 2003). With both areas experiencing significant advances, Weissleder highlighted that researchers had been able to apply the new discoveries in both fields to successfully image new disease markers in the pre-clinical setting (Weissleder, 1999).

Traditionally, in vivo imaging modalities such as MRI, CT and ultrasound (US) have been termed anatomical imaging techniques and have relied on imaging gross anatomy, physical (absorption, scatting, proton density, relaxation) changes and physiologic (blood flow) properties as the main source of contrast for the detection and characterisation of diseases (Weissleder, 1999). Nuclear medicine and optical imaging have been termed
functional imaging methods and have traditionally relied on low resolution detectors to characterise diseases that have been detected by MRI, CT and US. The discovery of new biomarkers and molecular mechanisms has made it possible to consider imaging specific molecules and molecular processes, utilising new biomarkers, as stated earlier in the chapter. For molecular imaging to be possible, it is important to meet two prerequisites: first, the detectors must have high sensitivity and resolution to be able to image molecular interactions, and second, there must be target-specific molecular probes (Bulte & Modo, 2007; Rudin & Weissleder, 2003; Weissleder, 1999).

Nuclear medicine, single photon emission computerised tomography (SPECT) and positron emission tomography (PET) and optical imaging enable molecular imaging. These modalities have high sensitivity compared to modalities such as MRI and CT (Rudin & Weissleder, 2003), and it would therefore be useful if molecular imaging could be conducted with these modalities only. However, the inherent poor spatial resolution of nuclear imaging techniques limits the ability of these modalities to be used routinely in targeted imaging (Rudin & Weissleder, 2003). An advance in medical imaging has been the introduction of dual imaging modalities by fusing SPECT and or PET with CT or MRI to overcome this problem (Beyer, Townsend, Brun, et al., 2000). The introduction of PET/CT has markedly improved the effectiveness of imaging with PET, as CT is known to have high resolution for identifying anatomical detail (Beyer, et al., 2000). The more recent development of PET/MRI also creates a complementary imaging modality, with high resolution and high sensitivity, which could be clinically available in coming years. This is desirable because MRI does not use ionising radiation and is known to have higher resolution and better differentiation of soft tissues compared to CT (Jacobs, & Cherry, 2001). Another limitation in nuclear medicine and PET is the exposure to ionising
radiation, not just of patients but also of sensitive molecular probes, as well as the issue of radionuclide decay, which places limitations on the length of time biological processes can be imaged. In targeted molecular imaging, longer scanning times may be necessary to ensure delivery of the molecular probe to the target. Although nuclear medicine and PET have some limitations, the availability of dual modality imaging, combined with the discovery of new biomarkers, should in the near future be able to minimise or eliminate some of these limitations. The development of nanoparticles has shown promise for addressing these issues, providing the possibility of molecular and cellular imaging (Bulte & Modo, 2007; Weissleder, 1999).

The next section considers the application of nanoparticles as targeted imaging agents for molecular and cellular imaging in modalities such as optical imaging, CT, nuclear medicine and PET and MRI.

1.2.4 Targeted imaging using nanoparticles

1.2.4.1 Optical imaging

As discussed previously, optical imaging inherently provides information about molecular and cellular processes. In optical imaging, the use of traditional organic fluorophores such as free emissive molecules like indocyanine green (ICG) or conjugates of single fluorescent dyes to biological targets such as antibodies, proteins, peptides, small molecules, nucleic acids, is limited in targeted imaging (Wang, et al., 2010). This is primarily due to the small number of biological targets, short circulation half-lives, low photo-bleaching thresholds and lack of strong near-infrared (NIR) absorption and emission (Ghoroghchian, Therien, & Hammer, 2009; Murray, Kagan, & Bawendi, 2000; Wang, et al., 2010). For optical imaging, organic fluorophores are also inherently limited in their
ability to generate fluorescent signals at deep tissue depths greater than 1-2cm (Ghoroghchian, et al., 2009; Wang, et al., 2010). Semiconductor nanoparticles that are quantum dots classify as an important class of inorganic fluorophores, possessing characteristics vastly superior to those of organic fluorophores, making them suitable for targeted imaging (Wang, et al., 2010).

Quantum dots are nanocrystals made from crystalline clusters of a few hundred to a few thousand atoms and are usually composed of atoms from groups II-IV, III-V or IV-VI of the periodic table of elements (Alivisatos, 1996; Henglein, 1989). The fluorescence of quantum dots is intrinsic, bright, and prolonged, surpassing the resolution and sensitivity capability of organic fluorophores and fluorescent proteins. Their small size endows high surface-to-volume ratios, making many of their chemical and physical properties dominated by the surface. One extraordinary property is that the size of the quantum dot depends on the fluorescent emission and wavelength. This enables continuous tuning of fluorescent emission from the NIR to the near-ultraviolet region through the visible light region by changing the size of the quantum dot (Dabbousi, et al., 1997; Murray, et al., 2000). In targeted imaging, this property provides significant advantages over other nanoparticle imaging systems, as the ‘size effect’ of quantum dots provides the ability to utilise a range of quantum dot nanoparticles for simultaneous detection of multiple biomarkers. The large surface area of quantum dots allows not only the conjugation of almost any biomarker but also the conjugation of multiple diagnostic (such as MRI magnetic nanoparticles or radioisotopes) or therapeutic agents (Biju, Itoh, & Ishikawa, 2010; Bulte & Modo, 2007; Medintz, Uyeda, Goldman, & Mattoussi, 2005; Wang, et al., 2010). This affords the opportunity for a range of research in targeted imaging and allows
the development of multi-functional nanoparticles for multi-modality imaging and integrated imaging and therapy.

Despite the immense potential of quantum dots in targeted imaging, one of the drawbacks of the semiconductor nanocrystal is its potential toxicity. Most quantum dots are made from semiconductor materials such as cadmium, selenide andtellurium, all of which are toxic in their elemental state (Bertin & Averbeck, 2006; Taylor, 1996). As a result of potential toxicity, there are currently no clinically available NIR-emissive agents for optical imaging with ideal properties for human use (Ghoroghchian, et al., 2009). Nevertheless, optical imaging probes are available in the preclinical setting, using preclinical optical imaging scanners, and have advanced our understanding of molecular and cellular processes (Ghoroghchian, et al., 2009). To address safety concerns, research efforts have been reported focusing on producing quantum dots with less toxic materials such as zinc selenide, zinc sulphide, indium arsenide and indium phosphide, without compromising conjugation or optical properties (Xie, Chen, Chen, & Peng, 2008). Research efforts have also been directed towards using appropriate ligands, surface shell coatings and quantification of dosages for toxic materials like cadmium, selenide and tellurium, to reduce or even eliminate toxicity (Xie, et al., 2008).

Quantum dot nanoparticles have applications as fluorescent labels for a range of bioanalytical purposes such as detection of DNA, proteins, molecules and cellular labelling, but their most widely used application is in fluorescent imaging. For example, quantum dots have been utilised for targeted imaging using fluorescent imaging techniques for cellular tracking in vitro (Jaiswal, Matoussi, Mauro, & Simon, 2003; Wu, et al., 2003), identifying cancerous cells (Ko, et al., 2009; Orndorff & Rosenthal, 2009;
Yezhelyev, et al., 2007), locating infection (Li, et al., 2009), and nicotinic receptors (Orndorff, Warnement, Mason, Blakely, & Rosenthal, 2008). The use of quantum dots for in vivo targeted imaging is related to four main aspects: biodistribution imaging, vascular imaging, cell tracking and tumour imaging (Smith, Duan, Mohs, & Nie, 2009; Wang, et al., 2010). For most in vivo applications of targeted imaging, nanoparticles are administered intravenously, and many studies have therefore focused on investigating the interaction of a variety of quantum dots with blood, biodistribution and excretion by the body (Ballou, Lagerholm, Ernst, Bruchez, & Waggoner, 2003; Duconge, et al., 2008; Fischer, Liu, Pang, & Chan, 2006; Liu, et al., 2007; Sandros, Behrendt, Maysinger, & Tabrizian, 2007). Studies have also investigated the use of quantum dots for imaging the lymphatic (Kim, et al., 2004) and blood circulatory systems (Khullar, Frangioni, Grinstaff, & Colson, 2009; Larson, et al., 2003; Smith, Duan, Mohs, & Nie, 2009). Quantum dots are advantageous for tumour-targeted imaging because of their excellent fluorescent properties and their small size. Akerman and colleagues (2002) initiated the application of quantum dots in tumour imaging in vivo (Akerman, Chan, Laakkonen, Bhatia, & Ruoslahti, 2002). In that study, quantum dots were coated with peptides and injected intravenously into tumour-bearing mice. The vasculature of the tumour sections was imaged with fluorescence microscopy, showing that the quantum dots localised specifically within the tumour vasculature. Other studies have subsequently shown uptake and localisation of conjugated-quantum dots in various tumours expressing particular biomarkers (Gao, Cui, Levenson, Chung, & Nie, 2004; Papagiannaros, et al., 2010; Yang, et al., 2009; Yong, Roy, Law, & Hu, 2010; Yu, et al., 2007; Zheng, Chen, DeLouise, & Lou, 2010).
Targeted imaging with quantum dots holds great promise for translation into the clinical setting, but further research is needed to address the toxicity component of quantum dots and to ensure that optimal fluorescence can be detected within *in vivo* systems.

### 1.2.4.2 Computed tomography (CT)

CT has primarily been regarded as a structural imaging modality because it can identify anatomical patterns and provide basic information regarding the size, location and spread of certain diseases based on endogenous contrast (Bulte & Modo, 2007). CT also uses non-specific, externally administered contrast agents to enhance structures between anatomical compartments. Most of the contrast agents for CT are iodine based, being very effective in attenuating and absorbing x-rays. The conjugation of iodine-based contrast agents to antibodies is limited due to the lack of surface functional groups available for stable conjugation (Bulte & Modo, 2007; Tilcock, 1999). This also results in short imaging times due to rapid clearance by the kidneys, and further suggests that targeted imaging is limited (Bulte & Modo, 2007; Tilcock, 1999).

Liposomal nanoparticles in medical imaging were introduced in the 1990s, and for CT contrast agents, new methods of liposomal production were discovered that could entrap iodine (Bulte & Modo, 2007). These particles were termed immunoliposomes. This technique introduced major advantages, as the liposomal coating allowed longer blood circulating times so that the contrast agents could be useful as blood pool agents, and permitted the attachment of antibodies to the surface, suggesting that targeted imaging for CT could be possible (Allen, 1994; Leander, Höglund, Kloster, & Børseth, 1998; Tilcock, 1999). At the preclinical level, some studies have used immunoliposomes as CT contrast media to image cells of interest (Torchilin, 2005). One example is the work of Johnston, Lee, and Hawthorne (2003), who studied tissue-specific enhancement in CT using a small
(approximately 100 nm) unmodified iohexol carrying CT immunoliposomes. They examined the targeting ability of the immunoliposomes to three different targeting antibodies (anti-FasL, anti-CD5, anti-FcR) to cell surface proteins. The results demonstrated CT contrast enhancement only in the targeted areas delivered by the conjugated liposomes.

Gold nanoparticles have also been used for targeted imaging in CT. Gold as a metal induces stronger x-ray attenuation than iodine and contains unique physical, chemical and biological properties which make it a better candidate for generating CT contrast (Kim, Park, Lee, Jeong, & Jon, 2007). Moreover, gold nanoparticles have flexibility in activation of available functional groups for conjugating biomarkers and have demonstrated biocompatibility in vivo (Connor, Mwamuka, Gole, Murphy, & Wyatt, 2005; Hauck, Ghazani, & Chan, 2008). Various studies have shown biomarkers conjugated to the surface of gold nanoparticles and demonstrated targeted imaging (Eck, Nicholson, Zentgraf, Semmler, & Bartling, 2010; Ji, 2010; Popovtzer et al., 2008; Wang, et al., 2011). Popovtzer et al. (2008) used gold nanorods conjugated to the UM-A9 antibody which is specific for squamous cell carcinoma (SCC) of the head and neck. After incubation of conjugated gold nanorods with SCC positive cells, distinguishable x-ray attenuation could be seen on the targeted SCC cancer cells in the head and neck regions. Similarly, Eck et al. (2010) conjugated anti-CD34 antibodies to gold nanoparticles to visualise CT contrast in lymph nodes in live mice. They successfully demonstrated in vivo contrast enhancement of the peripheral lymph nodes. Like the use of quantum dots for optical imaging, the use of CT contrast agents such as gold in molecular imaging is still at the preclinical level, with the potential for clinical applications.
1.2.4.3 Nuclear medicine (PET and SPECT)

The use of nanoparticles in nuclear medicine imaging techniques was utilised as early as the 1970s, when sulphur colloids were tagged to $^{99m}$Tc for lymph node imaging in cancer staging (Fee, et al., 1978). Nuclear medicine imaging techniques such as planar scintigraphy, SPECT and PET use the tracer technique to provide information on function and the physiology of diseases, with very high sensitivity. The use of nanoparticles enhanced nuclear medicine imaging techniques by allowing easy attachment of biomarkers to radionuclides for specific imaging of certain diseases. One example is the study by Hu et al. (2007), who compared the use of targeted perfluorocarbon (PFC) nanoparticles loaded with $10^{111}$Indium ($^{111}$In) atoms to one targeted atom of $^{111}$In. In this study, integrin $\alpha$V$\beta$3-targeted $^{111}$In-labeled PFC nanoparticles were reported for the detection of tumour angiogenesis in New Zealand white rabbits implanted with Vx-2 tumours. Hu and colleagues found that targeted nanoparticles bearing approximately $10^{111}$In atoms per particle had better tumour-to-muscle ratio than those with approximately one $^{111}$In atom per particle. These results suggested that the use of the PFC nanoparticles allowed better tumour-to-muscle ratios due to the greater loading of radionuclides compared to conventional loading of only one radionuclide bound to a biomarker.

Similarly, liposomal nanoparticles have been developed to load or surface label radionuclides for applications in areas such as tumour targeting, lymph node tracking, location of infection and inflammation (Bulte & Modo, 2007). Liposomal nanoparticles have great advantages over other nanoparticles as they are completely biocompatible, they can entrap any drug or diagnostic agent either inside or on their surface, are biologically stable, and can be used to deliver drugs into cells. In targeting tumours, current formulations of liposomal nanoparticles have lipid derivatives of the hydrophilic polymer
PEG. PEG prolongs the peripheral blood circulating times of nanoparticles *in vivo*, allowing localisation at tumour sites (Gabizon, Price, Huberty, Bresalier, & Papahadjopoulos, 1990; Goins, Klipper, Rudolph, & Phillips, 1994; Huang, et al., 1992). PEG-liposomal nanoparticles have successfully been labelled with radionuclides such as $^{67}$Gallium ($^{67}$Ga), $^{111}$In and $^{99m}$Tc (Belhaj-Tayeb et al., 2003; Goins, et al., 1994; Goins & Phillips, 2003; Zheng & Tan, 2004). Furthermore, numerous detection studies at the preclinical level have reported accumulation of PEG-liposomes labelled with $^{111}$In for targeting lung cancer (Harrington, et al., 2001), head and neck cancers (Harrington, et al., 2001) and metastatic brain cancer (Koukourakis, et al., 2000).

The use of nanoparticles in nuclear medicine facilitates the development of dual imaging contrast agents for dual imaging modalities like PET/CT, PET/MRI and nuclear medicine SPECT or PET/optical imaging. For targeted imaging, the use of dual modalities is valuable as two imaging methods can provide a range of diagnostic information (Jennings & Long, 2009). For example, it is difficult to accurately quantify a fluorescence signal in living subjects with fluorescence imaging alone, particularly in deep tissues. MRI has high resolution and good soft-tissue contrast but very low sensitivity, whereas radionuclide-based imaging techniques are very sensitive but have relatively poor spatial resolution (Massoud & Gambhir, 2003). A combination of multiple molecular imaging modalities can offer synergistic advantages over one modality alone (Jennings & Long, 2009). Liang et al. (2010) used streptavidin nanoparticles linked to biotinylated anti-HER2 herceptin antibody as a marker of breast cancer, using $^{111}$In and a fluorophore for targeted imaging by dual optical and SPECT imaging. The results showed high accumulation of the anti-Her-indium-fluorophore-streptavidin nanoparticles at the tumour site in nude mice bearing SUM190+ breast tumours in both the optical and SPECT imaging modalities. Similarly,
U87MG human glioblastoma cells and mice bearing U87MG tumours were targeted using an integrin $\alpha v \beta 3$-conjugated quantum dot probe conjugated to $^{64}$Copper ($^{64}$Cu) to permit both optical and PET imaging (Cai, Chen, Li, Gambhir, & Chen, 2007). The results confirmed the localisation of the probe within the vasculature of the tumour, which was indicated by the overlay of the quantum dot signals. Moreover, quantitative probe uptake analysis was possible by the PET probe component.

PET and MRI agents have been conjugated to produce images that have the high resolution of MRI and the high sensitivity of PET. One example of these bifunctional nanoparticle probes was demonstrated in a study conducted by Lee et al. (2008). The purpose of the study was to conjugate RGD peptides to iron oxide nanoparticles and $^{64}$Cu to image tumour integrin $\alpha v \beta 3$ expression. The results indicated that both PET and MRI scanners could detect the conjugated-nanoparticle-probes, which could allow earlier detection of tumours with both a higher degree of accuracy and further insight into the molecular mechanisms.

Nanoparticles have been used in nuclear medicine imaging techniques for many years. The results from preclinical studies show promise in their applications, particularly for tumour-targeted applications, but there are fundamental issues related to radiation dose, toxicity, availability and reproducibility that have limited translation to the clinical setting (Hahn, Singh, Sharma, Brown, & Moudgil, 2011; Jennings & Long, 2009). Nanoparticle technology advances have provided dual imaging probes, which are particularly useful with dual modality imaging. There has been increased research for almost a decade now into the development of dual imaging scanners, but progress in this area has been greatly hampered by technical problems and cost issues (Jennings & Long, 2009). However, the
recent availability of dual imaging scanners affords the possibility of imaging and validation of dual imaging probes.

1.2.4.4 Magnetic resonance imaging (MRI)

MRI has significantly benefited from the application of nanoparticles, particularly metal-based nanoparticles. The properties of metal-based nanoparticles have provided a platform for the development and application of nano-targeting agents that be used in a variety of MRI applications. Magnetic nanoparticles that have been developed can permit contrast-enhanced MRI, targeted imaging and dual imaging and therapy (Bulte & Kraitchman, 2004; Mulder, Strijkers, vanTilborg, Griffioen, & Nicolay, 2006). The advantage of MRI compared to other imaging modalities lies in its inherent high spatial resolution which makes it easier to visualise molecular and cellular targets. Other advantages include the use of non-ionising radiation and the ability to conduct longitudinal imaging. As with other imaging techniques, the use of nanoparticles in MRI was developed to achieve high tissue contrast and to improve imaging sensitivity. Some of the commonly used nanoparticles for targeted MRI are based on metal nanoparticles such as iron oxides and gadolinium, and newer agents are based on chemical exchange saturation transfer (CEST) and paramagnetic chemical exchange saturation transfer (PARACEST) imaging techniques (Hahn, et al., 2011).

Larger iron oxide particles, the first generation of nanoparticles, have been used in MRI for almost 40 years (Berry & Curtis, 2003; Gilchrist, et al., 1957). Some of these first generation nanoparticles like iron-based particles have been commercialised for non-targeted imaging to provide contrast enhancement in the liver and spleen and the gastrointestinal lumen (Bonnemain, 1998; Reimer & Balzer, 2003). Over the last decade there have been major advances in the field of nanotechnology, such as the synthesis of
smaller sized nanoparticles not only with iron oxide but with other organic and inorganic compounds (Huber, 2005). As a result, there has been a shift towards the synthesis and application of smaller (<100 nm) nanoparticles in MRI. Initial applications of smaller nanoparticles in MRI utilised iron oxides and it was reported that a decrease in size prolonged the blood half-life of the iron oxide nanoparticles (Weissleder, et al., 1990). This discovery brought the realisation that targeted imaging could be possible with MRI, as longer blood circulating times could allow for localisation in targeted structures (Weissleder, et al., 1990). It was also recognised that smaller iron oxide nanoparticles demonstrated increased uptake by macrophages in lymph nodes, suggesting that non-specific targeting of the lymph nodes was occurring due to a change in the physical properties of the nanoparticles (Harisinghani, et al., 2003; Sigal, et al., 2002). These results fuelled further research into the use of small iron oxide nanoparticles in lymph node tracking without the need for specific conjugated biomarkers (Harisinghani, et al., 2003; Sigal, et al., 2002; Weissleder, et al., 1990).

Nanoparticles with incorporated gadolinium have also been developed for MRI. These nanoparticles are based on chelated-gadolinium agents that are currently used in routine clinical practice as extracellular, blood-pool or hepatobiliary agents to achieve tissue contrast (Burtea, Laurent, Elst, & Muller, 2008). Chelated-gadolinium agents have been widely used due their favourable magnetic properties that provide a ‘bright’ or positive contrast, which is easily distinguished on the MRI image. Although gadolinium-based contrast agents for MRI are chelated to ligands and may not display toxicity caused by the heavy metal, there are still concerns over its use, as possible in vivo decomplexation and transmetallation by endogenous ions can occur (Ersoy & Rybicki, 2007). Besides the potential toxicity, gadolinium-based contrast agents cannot be used for targeted imaging.
because they have rapid clearance times. Gadolinium contrast agents have reported peripheral blood clearance times of approximately 15-90 minutes, which for targeted imaging may not give sufficient localisation and contrast enhancement at the preferred site (Na, Song, & Hyeon, 2009). Gadolinium as a metal has therefore been incorporated into a variety of nanoparticles for targeted imaging. Carbon nanotubes, mesoporous silica, polymers and perfluorocarbon nanoparticles all have the ability to load large amounts of gadolinium within the nanoparticles, giving the possibility of targeted imaging with high levels of MRI contrast (Hahn, et al., 2011; Shao, et al., 2010; Taylor, et al., 2008).

Another recent development of nanoparticles in MRI is as contrast agents for CEST imaging techniques. CEST agents can image metabolites in biological tissues as well as anatomy. Upon application of a suitable RF pulse, CEST agents reduce the intensity of the bulk water signal by saturation transfer through their chemical exchange sites (Terreno, Castelli, Viale, & Aime, 2010; Viswanathan, Kovacs, Green, Ratnakar, & Sherry, 2010). CEST contrast can originate endogenously from sugars, amino acids, nucleosides and other diamagnetic molecules (DIACEST), thereby making it possible to image molecular changes (Terreno, et al., 2010; Viswanathan, et al., 2010). CEST contrast depends on the frequency difference ($\Delta \omega$) between the protons associated with the contrast agent and water (Terreno, et al., 2010). As there is only a small frequency difference (<5 ppm) between exchangeable protons of the contrast agent and water (Na, et al., 2009), it is difficult to visualise contrast on the MRI image. One approach to achieve more contrast would be to use stronger magnetic fields to increase $\Delta \omega$. Unfortunately, high field magnetic imaging is currently suitable for preclinical studies only, making it difficult to employ CEST contrast agents in clinical settings. Consequently there has been research
into the generation of more efficient contrast agents, such as PARACEST (Na, et al., 2009).

Paramagnetic complexes like PARACEST agents are lanthanide-based and therefore have larger $\Delta \omega$ than CEST molecules (Na, et al., 2009; Terreno, et al., 2010). Like gadolinium, PARACEST agents can be easily loaded within various nanoparticles such as liposomes (Aime, Castelli, & Terreno, 2005), perfluorocarbon and europium ions, which can produce large chemical shifts and allow for targeted imaging (Na, et al., 2009). The use of CEST and PARACEST imaging techniques seems to be promising, particularly in the area of targeted imaging. However, before CEST and PARACEST imaging techniques can be developed in the preclinical setting and translated to the clinical setting a series of technical challenges need to be overcome. These challenges include the development of new imaging sequences that permit CEST imaging (Terreno, et al., 2010) and more sensitive and specific CEST imaging agents.

New generation iron oxide nanoparticles that are less than 100nm in diameter have been the most widely researched in the preclinical setting, particularly for targeted imaging. This is primarily due to their proven biocompatibility (Schwertmann & Cornell, 1991), magnetic properties such as superparamagnetism (Huber, 2005), variety of synthesis processes (Laurent, et al., 2008), versatility in their potential application to targeted imaging, and ability to provide negative contrast. Iron oxide nanoparticles have been used in MRI for applications such as non-specific contrast, some targeted imaging in oncology, drug delivery for dual imaging and therapy, and in the facilitation of inducing hyperthermia for ablation of tumours (Gupta & Gupta, 2005; Hahn, et al., 2011; Huber, 2005; Shubayev, Pisanic, & Jin, 2009).
As mentioned earlier in this section, iron oxide nanoparticles have been used as non-specific contrast agents since the early 1970s (Bonnemain, 1998; Reimer & Balzer, 2003). The development of smaller iron oxide nanoparticles and their applications suggested long blood circulating times, indicating that targeted imaging could be possible (Weissleder, et al., 1990). Along with the initial reports of smaller iron oxide nanoparticles, it was also noted that there was increased uptake by macrophages in the lymph nodes, fuelling further research and eventually clinical trials of the use of iron oxide nanoparticles for lymph node imaging (Harisinghani, et al., 2003; Sigal, et al., 2002). Further research into smaller iron oxide nanoparticles demonstrated targeting imaging for a range of pathologies such as central nervous system (CNS) diseases where nanoparticles offered a variety of transportation mechanisms to deliver CNS-based drugs as well utilisation for MRI (Tysiak, et al., 2009; Ulbrich, Hekmatara, Herbert, & Kreuter, 2009; Veiseh, et al., 2009; Zhang & Pardridge, 2001).

In targeted tumour imaging there have been reports of iron oxide nanoparticles coated in dextran and conjugated to an anti-human E-selectin antibody (Kang, Josephson, Petrovsky, Weissleder, & Bogdanov, 2001; Reynolds, et al., 2006). Kang et al. (2001) demonstrated that E-selectin positive cells, which are found in vascular rich areas such as inflamed tissue and tumours, could be targeted. Another antigen that has been targeted for tumour imaging using iron oxide nanoparticles in MRI is underglycosylated mucin-1 antigen (uMUC-1) (Moore, Medarova, Potthast, & Dai, 2004). uMUC-1 is one of the early hallmarks of tumourigenesis and is over-expressed and underglycosylated on almost all human epithelial cell adenocarcinomas, non-epithelial cancer cell lines, as well as haematological malignancies such as multiple myeloma and some B-cell non-Hodgkin’s
lymphomas (Avichezer, Taylor-Papadimitriou, & Arnon, 1998; Burdick, Harris, Reid, Iwamura, & Hollingsworth, 1997; Dyomin, et al., 2000; Nacht, et al., 1999; Oosterkamp, Scheiner, Stefanova, Lloyd, & Finstad, 1997; Treon, et al., 1999). The ability to target uMUC-1 expression could potentially aid in screening prospective patients for early cancer detection and in monitoring the efficacy of drug therapy. Other carcinomas targeted by iron oxide nanoparticles have included but are not limited to human epidermal growth factor receptor 2 (HER2) positive markers for colon (Toma, et al., 2005) and breast cancers (Chen, et al., 2009), hepsin for prostate cancer (Kelly, et al., 2008), bombesin peptide conjugate for pancreatic cancer (Montet, Weissleder, & Josephson, 2006) and 183B2 monoclonal antibody for ovarian cancer (Quan, et al., 2010).

Another application for MRI iron oxide nanoparticles is in stem cell tracking. Stem cells have been utilised in many clinical trials around the world for the potential treatment of blood disorders, tissue regeneration, stroke and myocardial infarction (Fermand, et al., 1998; Wollert, et al., 2004). Generally, stem cells are tracked using invasive immunohistochemical analysis methods from tissues in small animals (Villa, et al., 2010). For pre-clinical and clinical trials, however, it is necessary for non-invasive techniques to evaluate the therapeutic effect and grafting location to rule out any serious side effects. Due to the inherent high resolution and superb soft tissue differentiation of MRI, tracking the fate of stem cells is potentially feasible without the use of ionising radiation and toxic materials (Villa, et al., 2010). Together with the proven biocompatibility of iron oxide nanoparticles, the use of MRI and iron oxide nanoparticles could provide a likely platform for labelling stem cells and then tracking their fate in vivo. Some applications have been in tracking iron oxide labelled human umbilical mesenchymal stromal cell-derived Schwann
cells (HUMSC-SC) in the brain and spinal cord (Xu, et al., 2010) and mesenchymal stem cells (MSCs) for tracking hepatocyte liver transplantation (Shi, et al., 2010).

1.2.5 Summary of preceding themes

So far this thesis has presented the theme that nanoparticles are used in medical imaging modalities due to their physical properties which give them the potential to assist in the diagnosis and treatment of disease.

One property of nanoparticles examined is the capacity for signal amplification and image contrast in various imaging modalities. Nanoparticles can be synthesised to contain electrical and chemical properties that allow them to be visible by modalities such as optical imaging. In optical imaging, nanoparticles are used in the form of quantum dots that have unique optical and electronic properties, allowing their emissions to be tuned to any wavelength. Similarly in MRI, signals are produced using magnetism, and the use of magnetic nanoparticles amplifies the MRI signal, producing image contrast.

Another property of nanoparticles discussed is the possibility of synthesising various sizes. For targeted imaging, sizes less than 100 nm in diameter are of particular interest for cellular targeting. Sizes less than 100 nm allow cellular uptake, via passive or active pathways, into various subcellular compartments depending on their composition and conjugated materials.

Finally, the use of nanoparticles in targeted molecular and cellular imaging was discussed. With the small size of nanoparticles and their various electrical, chemical and
magnetic properties, cellular targeting or targeted imaging by imaging modalities such as MRI, CT, optical imaging and nuclear medicine was reviewed.

Comparison of the uses of nanoparticles for targeted imaging within the different imaging modalities suggests that MRI offers the greatest potential. This is because quantum dots in optical imaging, liposomal or gold nanoparticles in CT are still at the pre-clinical level. Nanoparticles in nuclear medicine have contributed to more sophisticated and new probes, which however currently lack appropriate spatial resolution capabilities. In MRI, nanoparticles, particularly iron oxide based nanoparticles, have demonstrated potential in targeted imaging, particularly for applications in stem cell tracking and tumour targeting.

Consequently, the next theme in this thesis relates to the evidence regarding the use of iron oxide nanoparticles in MRI and their optimal characteristics needed to produce contrast and to allow targeted imaging, and their \textit{in vivo} biodistribution.

\section*{1.3 Iron oxide nanoparticles and magnetic resonance imaging}

\subsection*{1.3.1 Introduction}

To ensure that contrast is produced and that targeted imaging can occur with the use of iron oxide nanoparticles, it is essential that the nanoparticles display a range of characteristics (Gupta & Gupta, 2005). The next section examines the required characteristics of iron oxide nanoparticles to generate contrast and be utilised for targeted molecular and cellular imaging. It also discusses the principles of MRI and iron oxide nanoparticle contrast agents.
1.3.2 Generating contrast in MRI

1.3.2.1 Basic Principles of MRI

The principles of MRI signals rely on the spinning motion of hydrogen nuclei present in biological tissues (Weishaupt, Köchli, & Marincek, 2003). Hydrogen nuclei are used in MRI due to their abundance in the body and because the solitary proton of the hydrogen nucleus gives it a relatively large magnetic moment. This means that the positively charged proton spins around the hydrogen (Figure 1.1) and therefore the hydrogen nucleus has a small magnetic field around it with a north and south pole of equal strength, represented by a magnetic moment (Weishaupt, et al., 2003; Westbrook, et al., 2005). In the absence of a magnetic field, the magnetic moments of the hydrogen nuclei are randomly orientated (Figure 1.2 a). When they are in an external magnetic field, some of the magnetic moments of the hydrogen nuclei align parallel with the magnetic field (in the same direction) and some align anti-parallel (in the opposite direction) (Figure 1.2 b) (Weishaupt, et al., 2003; Westbrook, et al., 2005). This orientation of the magnetic moments of the hydrogen nuclei is governed by the quantum theory described by Max Planck in the 1900s, and is related to low and high energy nuclei (Westbrook, et al., 2005). The factors that affect the alignment, parallel or anti-parallel, of hydrogen nuclei are related to the strength of the external magnetic field and the thermal energy level of the nuclei (Brown & Richard Semelka, 2005; Westbrook, et al., 2005). Due to the lack of control over thermal energy nuclei in clinical applications, this is not discussed further in this thesis.
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**Figure 1.1** A spinning charged particle hydrogen nucleus generating a magnetic field. The positively charged proton around the hydrogen spins, generating a small magnetic field. This figure is extracted from Hashemi et al. (2004).

**Figure 1.2** Alignment of hydrogen nuclei in the absence and presence of a magnetic field. This figure illustrates (a) the random orientation of hydrogen nuclei in the absence of a magnetic field, and (b) the alignment of hydrogen nuclei in the presence of an external magnetic field. This figure is extracted from Westbrook et al. (2005).

The net magnetic moments of the hydrogen nuclei are relatively low, and a radiofrequency (RF) pulse applied at the Larmor frequency produces a net magnetisation which can be imaged on MRI (Weishaupt, et al., 2003). The external magnetic field causes the magnetic moment of the hydrogen nuclei to spin around the magnetic field at a speed
of the precessional frequency which is determined by the Larmor equation (Brown & Richard Semelka, 2005; Weishaupt, et al., 2003). When RF pulses are applied to the nuclei at the Larmor frequency, these nuclei gain energy by absorbing the energy from the pulses (Brown & Richard Semelka, 2005; Weishaupt, et al., 2003). This action is termed resonance, and is a phenomenon that occurs when an object is exposed to an oscillating perturbation that has a frequency close to its own natural frequency, which causes the nucleus to gain energy from the external source (Weishaupt, et al., 2003; Westbrook, et al., 2005). The resonance that occurs is excitation and the magnetic resonance signal is produced when the RF signal is turned off. When this happens the hydrogen nuclei lose the energy given to them by relaxation and they try to realign with the external magnetic field. During relaxation the hydrogen nuclei give up absorbed RF energy, and concurrently the magnetic moments lose their coherency due to de-phasing. This gives rise to T1 recovery, which is caused by the nuclei giving up their energy to the surrounding environment or lattice, and is termed spin lattice relaxation (Westbrook, et al., 2005). The rate of recovery of the nuclei is exponential and is called the T2 relaxation time. T2 decay occurs when nuclei exchange energy with neighbouring nuclei, and is termed spin-spin relaxation or T2 relaxation (Westbrook, et al., 2005).

The RF pulse is controlled by parameters of the pulse sequences. Factors such as repetition time (TR) control the time and relaxation from the application of one RF pulse to the next RF pulse for each slice and is measured in milliseconds (ms) (Westbrook, et al., 2005). The TR determines the amount of T1 relaxation that occurs. The echo time (TE) is the time from the application of the RF pulse to the peak of the signal induced. The TE determines how much decay of transverse magnetisation occurs, and therefore controls the amount of T2 relaxation that occurs, in ms. Overall, the magnitude and timing of the RF
RF pulse along with the external magnetic field strength form part of the MRI signal and its generation.

1.3.2.2 Contrast

The term ‘contrast’ refers to image contrast and is related to the relaxation rates that produce different levels of black, white and grey on an MRI image. Contrast is essential in separating and differentiating between anatomies as well as identifying pathologies. The ability to produce contrast depends on differences in the relaxation rates in various tissues in vivo, and external agents are administered to affect the MRI signals such as T1 and T2 rates (Westbrook, et al., 2005). In various tissues, MRI pulse sequences such as T1 and T2 weighted imaging can be created which alter the way particular organs are imaged. There are also other sequences such as T2 star (T2*) and proton density contrast, which are not discussed here. In a T1 weighted image, fat has a high MRI signal and therefore appears bright, whereas water has a low signal and appears dark (see Figure 1.3 a). Conversely, on a T2 weighted image, water has a high signal and appears bright and fat has a low signal and appears dark (see Figure 1.3 b). Therefore, the differences in appearance of organs and tissues are due to T1 and T2 weighted sequences that can provide contrast for detecting certain pathologies. In tumour imaging, tumours have low water content and therefore a T2 weighted image would show an area of high signal, appearing bright, allowing the tumour to appear different from normal structures on the MRI image (Westbrook, et al., 2005). However, some pathologies may be difficult to differentiate and characterise from normal structures, therefore external enhancement agents can be used to selectively affect either T1 or T2 relaxation times in tissues (Westbrook, et al., 2005).
Figure 1.3 Examples of T1 and T2 weighted images
This figure illustrates (a) a T1 weighted axial image of the brain, where fat has a high MRI signal appearing bright and water has a low signal appearing dark, and (b) a T2 weighted image of the brain, where water has a high signal and appears bright and fat has a low signal and so appears dark. This figure is extracted from Westbrook et al. (2005).

Some contrast agents shorten T1 relaxation times and appear bright on T1 weighted images; other contrast agents shorten T2 relaxation times and appear dark on T2 weighted images. The mechanism of action of contrast agents is primarily related to dipole-dipole interactions and magnetic susceptibility (Weishaupt, et al., 2003; Westbrook, et al., 2005). Contrast enhancement agents have large magnetic moments and in the presence of water protons cause local magnetic field fluctuations (Weishaupt, et al., 2003; Westbrook, et al., 2005). In the case of T1 weighted imaging, the molecular movement reduces the T1 relaxation time, appearing bright on the MRI image. Magnetic susceptibility is a property of matter and is defined as the ability of the external magnetic field to affect the nucleus of an atom and magnetise it (Weishaupt, et al., 2003; Westbrook, et al., 2005). A substance with high positive magnetic susceptibility can affect the T2 decay by shortening the relaxation time, causing a darkening effect on T2 weighted...
images. Examples of magnetic susceptibility effects include diamagnetism, paramagnetism, superparamagnetism and ferromagnetism (Weishaupt, et al., 2003; Westbrook, et al., 2005). For this thesis, only paramagnetism and superparamagnetism are briefly discussed in the following section.

Paramagnetic substances are known to have positive magnetic susceptibilities and are suitable as MRI contrast agents. Gadolinium (Gd) is an ideal element as it has seven unpaired electrons and a positive magnetic moment causing any water protons in its presence to reduce its T1 and T2 relaxation (Na, et al., 2009). Therefore, on a T1 weighted image, water causes increases in the signal intensity, appearing bright. As mentioned previously in this chapter, gadolinium in its elemental state is highly toxic and for in vivo applications it is usually chelated to compounds that potentially inhibit its toxic effects. Although some chelated-gadolinium agents do not show toxicity, there are still concerns over its safety, due to reported deaths and nephrogenic systemic fibrosis after its administration (Ersoy & Rybicki, 2007). Gadolinium-chelates are currently the most commonly used contrast agents in MRI, but due to safety concerns significant efforts are being made to produce more stable products (Hahn, et al., 2011; Perazella, 2009). Some of these, mentioned earlier in this chapter, include the incorporation of gadolinium into nanoparticles and the development of other contrast agents like T2 weighted iron oxide nanoparticle contrast agents (Hahn, et al., 2011; Perazella, 2009).

Superparamagnetic agents have large magnetic moments, and in the presence of nearby hydrogen atoms shorten their relaxation times or reduce the signal intensity, producing a darkening contrast effect on the MRI image (Na, et al., 2009). The most commonly used superparamagnetic agent is iron oxide. As mentioned previously, iron
oxides are commercially available and are used in imaging the liver, spleen and gastrointestinal lumen. Unlike gadolinium, iron is found naturally in the body and the breakdown of iron oxide contrast agents follows the biological iron excretion pathway (Pouliquen, Le Jeune, Perdrisot, Ermias, & Jallet, 1991). Compared to chelated-gadolinium contrast agents, iron oxide contrast agents are reported to be less toxic in vivo (Weissleder, et al., 1990). Unfortunately, the use of iron oxides has been limited to applications such as imaging the liver, spleen and gastrointestinal lumen, due to large particle sizes that cause rapid elimination by the reticulo-endothelial system (RES) (Chouly, Pouliquen, Lucet, Jeune, & Jallet, 1996). However, due to improvement in nanoparticle technology, iron oxide nanoparticles are increasingly overcoming these limitations. Iron oxide nanoparticles can be created with even better magnetic properties, variable sizing and with appropriate surface coatings to attach molecular markers, opening the area of targeted molecular and cellular imaging for MRI (Gupta & Gupta, 2005). As mentioned previously, MRI has the highest spatial resolution compared to other imaging modalities but lacks sensitivity in differentiation and characterisation of pathologies. The use of iron oxide nanoparticles can improve the sensitivity of MRI because the nanoparticles can be designed to target specific pathologies such as cancer and can be developed to deliver drugs for dual imaging and therapy in MRI (Mulder, Strijkers, van Tilborg, et al., 2006; Na, et al., 2009).

There are several types of iron oxide nanoparticles, namely maghemite, $\gamma$-$\text{Fe}_2\text{O}_3$, magnetite, $\text{Fe}_3\text{O}_4$, and haematite, $\alpha$-$\text{Fe}_2\text{O}_3$, among which magnetite is very promising as a MRI contrast agent because of its highly magnetic properties (Laurent, et al., 2008). When magnetite or iron oxide is synthesised in a nano (<100 nm) size, its structure can change, affecting its magnetic properties. Due to this effect, it is essential to analyse its magnetic
properties after synthesis, in particular the magnetic moment. The magnetic moment is an essential magnetic property as it determines the ability of the nanoparticles to produce MRI contrast enhancement. Another property that iron oxide nanoparticles need is the ability to be utilised in targeted molecular and cellular imaging.

The next section discusses characteristics of iron oxide nanoparticles which ensure that they can be utilised for targeted molecular and cellular applications.

1.3.3 Characteristics of iron nanoparticles needed for targeted MRI

As explained in the previous section, for targeted molecular and cellular imaging purposes, iron oxide nanoparticles need superparamagnetic and high magnetic properties such as a large magnetic moment to ensure that contrast is generated. They also need to possess other characteristics to further utilise them in targeted molecular and cellular imaging. These properties are low toxicity, high biocompatibility and an appropriate surface layer to bind to a range of biological targets (Gupta & Gupta, 2005).

It is important to ensure that iron oxide nanoparticles possess all these properties because each method for producing iron oxide nanoparticles results in different characteristics (Jun, Lee, & Cheon, 2007). For targeted molecular and cellular imaging, the iron oxide nanoparticles need to be coated with a surface or monolayer, usually of organic material, that provides an interface between the core and the surrounding environment (Rochelle, Arvizo, De, & Rotello, 2007). This interface serves many purposes such as biological stability and the facility for attachment of biomarkers for targeting. Iron oxide nanoparticles also need to have a uniform particle size (Laurent, et
The way iron oxide nanoparticles are synthesised has an influence on all of the above properties. For MRI, these properties are important as they determine the overall effectiveness of the contrast agent. For example, an essential characteristic of an effective MRI contrast agent is a high magnetic moment (Jun, et al., 2007; Laurent, et al., 2008; Lawaczeck, et al., 2004). A measure of the magnetic moment is the saturation magnetisation value: the higher the saturation magnetisation value, the higher the magnetic moment, inducing more contrast (Wang, Hussain, & Krestin, 2001). The saturation magnetisation value is expressed in electromagnetic units per gram (emu/g) and affects the relaxation rates, T1 and T2 of the surrounding water protons. The influence of the large magnetic moment and susceptibility causes a reduction in the T1 and T2 relaxation rate, so that in a T2 weighted scan there is more contrast. Therefore, the relaxation rates are an indirect measure of the ability to generate contrast.

Typically, saturation magnetisation values for iron oxide nanoparticles range from 30-50 emu/g, and higher values such as 90 emu/g have been observed for bulk material (Lu, Salabas, & Schuth, 2007; Lu, Wang, Ye, Vaidya, & Jeong, 2007). Factors contributing to the saturation magnetisation value of iron oxide nanoparticles include the size of the particles (with the highest emu/g to volume ratio occurring in the 6-20 nm particle size range (Jun, et al., 2005), spacing between the nanoparticles (where coatings separate magnetic domains allowing each individual magnetite particle to act independently, thereby enhancing the net magnetism per gram) and the crystalline structure of the iron oxide (Goya, Berquo, & Fonseca, 2003; Gupta & Gupta, 2005). It is
therefore essential to use a method of iron oxide nanoparticle production that generates particles with one or more of the above characteristics.

The overall size and size distribution of superparamagnetic iron oxide nanoparticles (SPIONS) is an important consideration. For applications in targeted molecular and cellular imaging, the size can affect the biodistribution in vivo and the localisation to a targeted area. It is well known that particles above 60 nm in diameter are eliminated by the RES, so the use of iron oxide nanoparticles greater than 60 nm in diameter is limited to liver/spleen imaging (Na, et al., 2009; Weissleder, et al., 1990). Similarly, the size distribution can limit the number of particles that arrive at the targeted area, increasing non-specific uptake, so all the iron oxide nanoparticles within the sample must be of similar size. Other properties such as high colloidal stability and low toxicity are important because they increase the chances of translating developmental contrast agents into the clinical setting.

There are many production methods for iron oxide nanoparticles, each method producing nanoparticles with different properties. The following section briefly discusses the basic methods of iron oxide nanoparticle production and their resulting characteristics.

### 1.3.3 Synthesis methods of iron oxide nanoparticles

Iron oxide nanoparticles are crystalline structures that are governed by the principles of crystal formation and growth. A crucial characteristic of iron oxide nanoparticles for targeted molecular and cellular MRI is uniform particle size. Uniform particles are usually prepared via homogeneous precipitation reactions which involve two processes, nucleation
and growth (Laurent, et al., 2008). Generally, for precipitation to occur there must be a saturated solution, in which addition of any excess solute will cause precipitation and start the nucleation and growth phases for the formation of nanocrystals (Burda, Chen, Narayanan, & El-Sayed, 2005).

1.3.4.1 Nucleation and Particle Growth

For nucleation to occur, the solution must be supersaturated leading to a short single burst of nucleation (Jun, et al., 2007; Wang, et al., 2001). Supersaturation can be achieved by dissolving the solute at a high temperature or by adding reactants to produce supersaturation (Peng, Wickham, & Alivisatos, 1998). After the short burst of nucleation the concentration usually drops, stopping the nucleation process. The nuclei then grow by diffusion of solutes from the solution onto the surfaces, until an equilibrium concentration is achieved. To achieve monodispersed particles, the two phases of nucleation and growth need to be separated (Boistelle & Astier, 1988; Burda, et al., 2005; Laurent, et al., 2008; Tartaj, Morales, Veintemillas-Verdaguer, Gonzalez-Carreno, & Serina Carlos, 2006). There are many different mechanisms which can explain this process, such as the classical theory method of the formation of sulphur colloids, which was proposed by LaMer and Dinegar (LaMer & Dinegar, 1950), and Ostwald ripening growth as explained by Den Ouden and Thompson (Den Ouden & Thompson, 1991; Ng et al., 1996). Size control is ultimately achieved by artificially separating nucleation and growth, which would occur before the solution reaches critical supersaturation, or by the end of nucleation.

Several different factors can be adjusted to promote separation of the two processes to control for size, to obtain superior results to old synthesis methods. Adjusting the factors that control size is known to affect the magnetic, biocompatibility and surface
properties of iron oxide nanoparticles, suggesting that alterations in synthesis methods can produce iron oxide nanoparticles with different particular characteristics.

1.3.4.2 Methods of superparamagnetic iron oxide nanoparticle synthesis

Many methods of iron oxide nanoparticle synthesis have evolved for applications to MRI. This is a result of controlling the nucleation and particle growth phases to achieve uniform size, high magnetic properties and biocompatibility (Laurent, et al., 2008). Some of these synthesis methods are hydrolytic methods, chemical precipitation, constrained environments, and non-hydrolytic methods such as high temperature reactions. In keeping with the scope of this thesis, only these selected methods are discussed here, in relation to achieving the major characteristics needed for iron oxide nanoparticles applicable for targeted molecular and cellular imaging in MRI.

1.3.4.3 Hydrolytic methods – chemical precipitation

The precipitation method is the simplest chemical pathway to obtain iron oxide nanoparticles. Magnetite (Fe$_3$O$_4$), the most common iron oxide nanoparticle, is prepared by co-precipitating a stoichiometric mixture of ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) salts in an aqueous medium. The thermodynamics of the reaction require a ratio of 2:1 for Fe$^{2+}$/Fe$^{3+}$, and a pH between 8 and 14 (Laurent, et al., 2008). The overall reaction can be written as:

$$\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- = \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \quad (1)$$

The reaction must be carried out under a nitrogen environment to eliminate oxidation of the magnetite (Kim, Zhang, Voit, Rao, & Muhammed, 2001). If the reaction is not carried out under a nitrogen environment, the ions can become oxidised before
precipitation, leading to the formation of maghemite, Fe$_2$O$_3$ (Kim, Zhang, Voit, Rao, & Muhammed, 2001). Maghemite is an oxidised form of magnetite and is known to have inferior magnetic properties; thus oxidisation of magnetite can critically affect the physical properties of the iron oxide nanoparticles (Corot, Robert, Idée, & Port, 2006). The two differ from each other in the spinel structure; one occupies positions in the octahedral and tetrahedral sites and the other, maghemite, has cationic vacancies in the octahedral position (Laurent, et al., 2008). This crystal structure results in a different saturation magnetisation value (or emu/g) of the iron particles: at 300°K, 92 emu/g$^{-1}$ for magnetite, and 78 emu/g$^{-1}$ for maghemite (Corot, et al., 2006). The use of x-ray diffraction (XRD) can help in differentiating between the formation of magnetite and maghemite, but most of the time it is difficult to separate magnetite from maghemite given that their diffraction spectra are very similar (Lawaczeck, et al., 2004). It has been suggested that iron oxide nanoparticle synthesis always results in the presence of both magnetite and maghemite (Thünemann, Schütt, Kaufner, Pison, & Möhwald, 2006).

Compared to other hydrolytic methods and non-hydrolytic methods, the precipitation method can produce large quantities of iron oxide nanoparticles. This is an advantage for MRI applications because large quantities of iron oxide nanoparticles are needed for administration into humans. There are, however, limitations in the co-precipitation method, such as wide particle size distribution and limited ability to control the size of the nanoparticles (Gupta & Gupta, 2005). For applications in MRI, particularly targeted molecular and cellular imaging, small size of the iron oxide nanoparticles is needed to ensure that targeted imaging can occur, and a small size distribution is needed to decrease non-specific uptake. To control the size and size distribution in the hydrolytic co-precipitation method it is essential to adjust factors that determine the precipitation process
for nanoparticles (Sjogren, Briley-Saebø, Hanson, & Johansson, 1994). Factors such as pH, ionic strength, temperature, nature of salts, $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio, and addition of chelating agents have been shown to change the size and size distribution as well as the magnetic, biocompatibility and surface properties of iron oxide nanoparticles (Sjogren, et al., 1994).

One of the most popular hydrolytic methods of iron oxide nanoparticle production is a co-precipitation reaction by the Massart process. In the Massart process there is co-precipitation of ferrous and ferric chlorides and hydroxides in an alkaline solution (Massart, 1981). To understand factors controlling the size, Massart evaluated parameters such as the strength of the base (e.g. ammonia or sodium hydroxide), the pH value, added cations and the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio, noting the effect on the yield of the co-precipitation reaction and on particle sizes. Massart concluded that the size of iron oxide nanoparticles could be decreased by increasing the pH and/or increasing the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio. Similarly, Jolivet and co-workers conducted a comprehensive study evaluating the effects on size, morphology and magnetic characteristics of adjusting the ratio of $\text{Fe}^{2+}/\text{Fe}^{3+}$ (Jolivet, Belleville, Tronc, & Livage, 1992). Small $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratios (<0.3) were shown to form goethite, and for ratios below 0.5 but above 0.3 there were two phases, consisting of smaller (4 nm) and larger iron oxide nanoparticles. It was concluded that using a ratio of 0.5 corresponded to magnetite stochiometry and the particles were homogeneous in size and composition. Other studies have also shown similar results by adjusting factors such as ionic strength, size and size distribution (Babes, Denizot, Tanguy, Jeune, & Jallet, 1999; Jiang, et al., 2004).

Apart from modulating the parameters of the reaction to achieve monodisperse iron oxide nanoparticles, the addition, either in combination or individually of chelating
organic anions like citric acid (Bee, Massart, & Neveu, 1995), amino acids and dimercaptosuccinic acid (DMSA) (Fauconnier, Pons, Roger, & Bee, 1997) can also decrease the particle size by inhibiting the growth of the crystal nuclei. Polymer surface complexing agents which form monolayers on the surface of the iron oxide nanoparticles, such as dextran (Lee, Kim, Kim, & Kim, 2002), carbodextran and silica (Stöber, Fink, & Bohn, 1968), can also be added, instead of varying the reaction parameters. Some polymer complexing agents such as dextran, carboxydextran and silica are used in iron oxide nanoparticles that are commercially available or are currently in clinical trials. Examples are silica-coated magnetite, AMI-121 (Lumirem®- US) dextran-coated magnetite, Ferumoxides (Endorem® – Europe, Feridex® in the USA and Japan) and carboxydextran coated magnetite, Ferucarbotran (Resovist® – Europe and Japan). It should be noted that these polymer complexing agents can be used for any method of iron oxide production and do not only contribute in reducing the size and size distribution of the iron oxide nanoparticles. These agents also provide water solubility, the facility for attachment of various functional probes, and stabilisation of the magnetite core from any oxidation (Jun, et al., 2007; Rochelle, et al., 2007).

Magnetic properties such as the saturation magnetisation value of iron oxide nanoparticles vary according to the method of synthesis. This difference is primarily attributed to changes in the structural order of the iron oxide nanoparticles (Taylor, Barry, & Webb, 2001), the creation of anti-phase boundaries (Zhou, Wang, O’Connor, & Tang, 2001) and the existence of a magnetically dead layer on the surface (Kim, et al., 2001). In the case of the co-precipitation method to produce iron oxide nanoparticles, the frequent transformation from magnetite to maghemite causes a reduction in the saturation magnetisation values (Corot, et al., 2006). This is due to the changes in the crystalline
structural orientation when magnetite is oxidised to maghemite (Corot, et al., 2006). The co-precipitation method therefore produces iron oxide nanoparticles that are not highly crystalline, resulting in iron oxide nanoparticles with lower magnetisation values (Laurent, et al., 2008). The size of the iron oxide nanoparticles is also known to affect the magnetisation values. That phenomenon was first reported in 1971 by Coey et al. and then by Varanda et al. in 2002, where a reduction in the size of iron oxide nanoparticles was related to reduction in the magnetic properties. It was reported in these studies that the curvature of smaller sized particles was much greater, encouraging disordered crystal orientation on the surface, leading to lower magnetisation values. Jun et al. (2005) reported on the effect of iron oxide nanoparticle size and the resulting contrast enhancement demonstrated by decreasing signal intensity. The iron oxide nanoparticles were highly crystalline, monodisperse, and ranged in size from 4nm to 12nm in diameter. The general trend suggested that as the nanoparticles increased in size, so did the magnetisation values along with the T2-weighted MR signal intensity, showing more contrast on T2-weighted images. High magnetisation values are important for achieving optimal contrast for any MRI application, and it is therefore crucial for iron oxide nanoparticles to have a maximum magnetisation value.

Although the co-precipitation method is the simplest and most efficient chemical pathway to obtain magnetic nanoparticles, it has disadvantages such as high particle size variation, aggregation and poor crystallinity, resulting in low saturation magnetisation values. These disadvantages have led to the development of advanced methods of magnetite synthesis that have better size control, crystallinity and higher magnetisation values (Gupta & Gupta, 2005).
1.3.4.4 Hydrolytic method – reactions in constrained environments

Synthesis reactions in constrained environments are microemulsions and sol-gel processes, which are based on the co-precipitation method but make use of lipid-based structures such as amphiphiles (Dresco, Zaitsev, Gambino, & Chu, 1999; Gobe, Kon-No, Kandori, & Kitahara, 1983; Lee, Sorensen, Klabunde, & Hadjipanayis, 1992; Lopez-Perez, Lopez-Quintela, Mira, & Rivas, 1997; Nassar & Husein, 2006; Santra, et al., 2001) and dendrimers (Strable, et al., 2001) on the surface of the iron oxide nanoparticles during the synthesis process.

Lipid-based structures or colloidal aggregates such as liposomes and micelles are composed of lipids and/or other amphiphilic molecules. Amphiphiles (sometimes referred to as surfactants) are molecules with both hydrophilic (polar head) and hydrophobic (non-polar tail) parts that spontaneously assemble into aggregates in an aqueous solution (Degiorgio, 1985). The hydrophobic tails can vary in length, affecting the ratio between hydrophilic and hydrophobic parts, and the hydrophilic heads can also vary in charge and size, affecting the overall curvature of the aggregate. Due to these properties, various geometries and sizes of iron oxide nanoparticles can be formed, such as cylindrical, spherical and bilayered structures (Degiorgio, 1985; Mulder, Strijkers, ilborg, Griffioen, & Nicolay, 2006).

Micelles for MRI imaging usually contain a hydrophobic core, where the iron oxide core is stabilised by the surfactant, limiting particle nucleation and growth (Burda, et al., 2005). Compared to traditional co-precipitation methods, micelles can control the particle size and size distribution, making nanoparticles suitable for targeted MRI. The first magnetic nanoparticles formed in micelles were produced by oxidation of Fe$^{2+}$ salts,
where the sizes of the magnetite particles were controlled by varying the temperature and the surfactant concentration (Inouye, et al., 1982; Lee et al., 2005). Similar to micelles are reverse micelles. In reverse micelles, the hydrophilic head groups are towards the core of the micelle and the hydrophobic groups are directed outwards. Compared to micelles, reverse micelles can solubilise relatively large amounts of water, suggesting easier size control of the iron oxide nanoparticles. Reverse micelles have also been found to produce better crystalline particles and large amounts of iron oxide nanoparticles, making them a better choice for production of iron oxide nanoparticles (Lee, et al., 2005).

Other lipids such as PEG can also be used for reducing the size of the iron oxide nanoparticles and also in stabilising the iron oxide core, preventing further oxidisation (Wan, et al., 2007). The main advantages of using PEG stabilised lipids is that the resulting iron oxide nanoparticles have a PEG coating that helps to prolong blood circulation times; however, the feasibility of clinical use of these iron oxide nanoparticles is low, because of disadvantages associated with difficult preparation methods and excessive size separation processes (Acar, Garaas, Syud, Bonitatebus, & Kulkarni, 2005).

Bi-layer forming lipids are used to create liposomes; they usually have a polar head group and two fatty acid chains. Iron oxide nanoparticles can be placed inside the liposomal lumen to create magnetoliposomes (Bulte, DeCuyper, & Nejat, 2003). Besides better magnetic properties and controllability of size and size distribution, liposomes have other advantages. These are the potential to coat the iron oxide surface with repellent molecules (to avoid RES clearance), the availability of groups for the easy attachment of specific ligands for targeted molecular and cellular imaging, and the ability to load particular drugs on their surface (Laurent, et al., 2008). There are two types of
magnetoliposomes. The first consists of water-soluble iron oxide nanoparticles within an aqueous lumen and the second contains iron oxide nanoparticles of approximately 15 nm covered with a lipid bi-layer (Cuyper & Joniau, 1988). Both types have been used for MRI applications such as targeting tumours and bone marrow, and have demonstrated excellent size and distribution properties, better magnetism values than traditional co-precipitation methods, long blood circulating times, and the ability to attach biomarkers for targeted imaging (Fortin-Ripoche, et al., 2006; Sabaté, Barnadas-Rodríguez, Callejas-Fernández, Hidalgo-Alvarez, & Estelrich, 2008; Soenen, et al., 2011; Soenen, Velde, Ketkar-Atre, Himmelreich, & DeCuyper, 2011).

Dendrimers are a class of transfection agents that contain three components: core, branches and end-groups. When dendrimers are attached to iron oxides they are termed magnetodendrimers. Generally, during the synthesis process dendrimers are added to the solution, resulting in the formation of highly stable and water soluble iron oxide nanoparticles coated with dendrimers (Strable, et al., 2001). They have a size of 20-30 nm and have been shown to have high T2 relaxivities for generating high contrast in MRI (Strable, et al., 2001). Magnetodendrimers are well suited for the imaging of cell trafficking and migration using MRI (Bulte, et al., 2003; Bulte, et al., 2001; Lee, et al., 2004; Walter, et al., 2004). This is due to the charge on the polymer, which promotes a high non-specific affinity for cellular membranes, resulting in cellular internalisation (Bulte & Kraitchman, 2004). Cells from different origins can be easily labelled to magnetodendrimers and then imaged on MRI, by introducing the magnetodendrimers to the cell culture for 1-2 days at low concentrations (Bulte, Ben-Hur, et al., 2003).
Although magnetoliposomes and magnetodendrimers have shown promising results in the reducing the size and size distribution of iron oxide nanoparticles and producing good magnetisation values during the synthesis process, their use remains in the pre-clinical setting. This is most likely due to the difficult synthesis methods required and the inability to make the large batches that could be needed for human administration.

1.3.4.5 Non-hydrothermal methods – high temperature

Unlike co-precipitation methods and their derivatives, high temperature methods can produce monodisperse particles with significant size control, higher crystallinity and therefore higher magnetic properties, without the use of any size separation processes or adjusting any reaction parameters (Laurent, et al., 2008). In high temperature methods, iron complexes are decomposed using very high operating temperatures in the presence of surfactants and organic solvents. Because of the very high temperatures and the nature of the solvents used in these synthesis methods, there is relatively good control in the nucleation and growth phases of the iron oxide nanoparticle synthesis process (Laurent, et al., 2008). The resulting iron oxide nanoparticles can therefore be controlled to produce a variety of sizes with narrow size distributions and high crystallinity and magnetisation values (Sun & Zeng, 2002).

One method using high temperatures to produce iron oxide nanoparticles is based on seed-mediated growth. Sun and Zeng. (2002) prepared iron oxide nanoparticles of different sizes (3 nm to 20 nm) by controlling the quantity of seeds added to obtain various sizes. In this reaction, iron (III) acetylacetonate was decomposed by heating at 265°C in phenyl ether, alcohol, oleic acid, and oleylamine, to produce iron oxide nanoparticles with an initial diameter of 4 nm. To make particles above 4 nm, a seed-mediated growth was
used producing monodisperse particles. All the iron oxide nanoparticles produced were reported to have very high magnetisation values compared to any of the reported values of iron oxide nanoparticles produced by co-precipitation methods. Similarly Hyeon, Lee, Park, Chung, and Na. (2001) formed an iron oleate complex from the decomposition of iron pentacarbonyl in the presence of octyl ether and oleic acid at 100°C. They reported that the resulting iron oxide nanoparticles could be tailored to any size, depending on the molar ratios of iron pentacarbonyl and oleic acid, without size separation processes and without compromising on size distribution. Their iron oxide nanoparticles also demonstrated very high crystallinity compared to co-precipitation methods and yields of up to 80% or more.

In a pioneering report by Park et al. (2004), iron oxide nanoparticles were produced using a simple high temperature method. Iron oxide nanoparticles were produced in an ultra-large scale synthesis process from non-toxic and environmentally friendly compounds. In that study, instead of using compounds such as iron pentacarbonyl that are potentially toxic in vivo (Brief, Ajemian, & Confer, 1967), the iron oxide nanoparticles were prepared by a metal–oleate complex by reacting environmentally friendly compounds, namely metal chlorides and sodium oleate. Iron salts are more suited for contrast agent research and applications in MRI because they are less toxic and have more potential to be translated clinically (Park, et al., 2004). In the study an iron-oleic complex was formed using iron chlorides (FeCl₃·6H₂O) and sodium oleate, which was slowly heated to 320°C in 1-octadecene. The solution was aged at this temperature for 30 minutes, generating monodisperse iron oxide crystals. Various temperatures and solvents were tried, which produced large amounts (up to 40 g) of iron oxide nanoparticles of different sizes, high magnetisation values and dispersion. It was suggested that
monodisperse particles could be attributed to separation of the growth and nucleation phases, which occurred at different temperatures, nucleation at 200-240°C and growth at 300°C. The authors concluded that their method had several advantages over other iron oxide nanoparticles synthesis methods: large amounts of nanoparticles produced, high magnetisation values, no need for size sorting, non-toxic particles, environmentally friendly compounds, inexpensive products and many sizes of iron oxide nanoparticles.

Of all the synthesis methods of iron oxide nanoparticles, the high temperature method by Park et al. (2004) has demonstrated the best features for MRI applications. This is due to the ability to easily make large amounts of iron oxide nanoparticles in one synthesis process without compromising other properties, the ability to produce different sizes of iron oxide nanoparticles, the high crystallinity and magnetisation values for producing maximum contrast, and the non-toxic nature of iron oxide nanoparticles.

1.3.5 Surface coatings and functionalisation of iron oxide nanoparticles

Surface coatings were discussed in the section dealing with production of iron oxide nanoparticles using the co-precipitation method and its derivatives, because they assist in the synthesis of monodisperse particles by preventing nucleation and growth, and producing superior magnetic nanoparticles by inhibiting oxidation. These were dendrimers, lipids, PEG and micelles. Apart from being used in the synthesis process, these and other surface coatings are used on the surface of naked iron oxide nanoparticles to provide water solubility, reduced aggregation and the facility for attachment of biomarkers (Gupta & Gupta, 2005; Laurent, et al., 2008). Surface coatings thus serve
multiple purposes on the surface of iron oxide nanoparticles for MRI applications, particularly for targeted molecular and cellular imaging.

In the absence of any surface coating, magnetic iron oxide nanoparticles have hydrophobic surfaces, with a large surface area to volume ratio (Hamley, 2003). Due to hydrophobic interactions between the particles, large clusters and agglomerates form, resulting in increased particle size (Hamley, 2003). These clusters exhibit strong magnetic dipole-dipole attractions between them, showing ferromagnetic behaviour, and interfere with the magnetic field of the neighbouring particles (Hamley, 2003). This results in the formation of attractive forces between the particles, further magnetisation and the loss of single magnetic domains, which can affect the magnetic properties (Mendenhall, Geng, & Hwang, 1996). The particles are then attracted magnetically, in addition to Van der Waals forces. To prevent this from occurring there is a need to add a surface layer (Mendenhall, et al., 1996). Surface layers can be dendrimers and lipids and can be added during the synthesis process or afterwards. Other surface coatings on naked iron oxide nanoparticles are dense substances like carboxylates, inorganic materials like silica, and polymers such as dextran.

In targeted molecular and cellular MRI a surface layer is essential, as it allows the attachment of biomarkers on iron oxide nanoparticles. This ensures that the iron oxide nanoparticles can localise to a specific area. For a stable conjugation between a biomarker and the iron oxide nanoparticles, the surface layer must be able to provide a variety of available binding sites for attachment.
The next section discusses three surface coatings, carboxylates, dextran and silica, and their use as surface coatings for targeted MRI.

### 1.3.5.1 Carboxylates

Citric acid is an example of a carboxylate as a surface coating for iron oxide nanoparticles. The surface of the magnetite nanoparticles can be stabilised in an aqueous dispersion by the absorption of citric acid (Sahoo, et al., 2005). This process, as described by Sahoo et al. (2005), occurs by the citric acid being coordinated via one or two of the carboxylate functionalities, depending on steric necessity and the curvature of the surface. As a result, one carboxylic acid group conjugates onto the iron oxide core and another carboxylic group faces the solvent, creating a surface charge and allowing the iron oxide nanoparticles to be hydrophilic. The presence of the terminal carboxylic group provides an avenue for the stable attachment of multiple molecules such as fluorescent dyes, proteins, hormone linkers, and antibodies, so that specific targeting can occur.

Molecules such as DMSA can also be used as a surface coating to achieve water solubility and allow conjugation of molecular precursors (Huh, et al., 2005). DMSA is introduced to the iron oxide nanoparticles in excess after the synthesis, through simple mixing (Huh, et al., 2005). The DMSA binds to the magnetite surface through its carboxylate bonding, and the intermolecular disulfide cross-linking between surface-bound DMSA ligands increases the stability (Fauconnier, et al., 1997). The remaining free carboxylic acid and thiol groups promote the iron oxide nanoparticles to be hydrophilic and these remaining free groups can be used for conjugation of a variety of biomarkers (Fauconnier, et al., 1997).
1.3.5.2 Dextran

The use of dextran on the surface of iron oxide nanoparticles is common. Dextran is a polysaccharide polymer composed of α-D-glucopyranosyl units, and can vary in length (1000 to 2,000,000 Da) and branching. Dextran offers a suitable surface layer on iron oxide nanoparticles because of its proven biocompatibility and long blood circulating times (Berry, Wells, Charles, & Curtis, 2003). Dextran can coat iron oxide nanoparticles by reacting a mixture of ferrous chloride and ferric chloride with the dextran polymers, under alkaline conditions (Molday & Mackenzie, 1982). It has been reported that reducing the size of dextran has an effect on the size and stability of the dextran-coated iron oxide nanoparticles (Kim, Zhang, Voit, Rao, & Muhammed, 2001; Lawaczeck, et al., 2004; Paul, Frigo, Groman, & Groman, 2004; Thomas & Hütten, 1997). It has also been reported that larger molecular weight dextran has significant effects on particle size, coating stability and magnetic properties; on the other hand, iron oxide nanoparticles coated with a lower molecular weight dextran have been reported to be more stable (Pardoe, Chua-anusorn, St. Pierre, & Dobson, 2001; Paul, et al., 2004). Dextran attaches onto the surface of the iron oxide nanoparticles via polar interactions, chelation and hydrogen bonding, and its hydroxyl groups on the surface allow for further surface modification to attach biomarkers (Laurent, et al., 2008). Dextran is used as a surface coating for some iron oxide nanoparticles that are preclinically and clinically available, namely Feridex® and Resovist®.

1.3.5.3 Silica

Another surface coating for iron oxide nanoparticles is silica (Sun, et al., 2005). Silica is an inert molecule that coats the surface of the iron oxide nanoparticles, serves to prevent aggregation of the nanoparticles in solution, and provides stability (Sun, et al., 2004). This
is achieved by two processes: (1) sheltering of the magnetic dipole interaction by the silica shell and (2) charging the magnetic nanoparticles, as silica is negatively charged (Stober, et al., 1968). The silica surface therefore offers high stability for iron oxide nanoparticles in suspensions at high volume fractions, changes in pH or electrolyte concentration (Mulvaney, Liz-Marzan, Giersig, & Ung, 2000). The advantage of silica on the surface of the iron oxide nanoparticles is associated with the availability of silanol groups that easily react with alcohols and silane coupling agents to covalently attach to various biomarkers, making desorption difficult (Ulman, 1996). Like dextran, silica also has proven biocompatibility, and there are commercially available silica coated iron oxide nanoparticles, namely Gastromark® (Hahn, et al., 1990; Trewyn, Nieweg, Zhao, & Lin, 2008; van Schooneveld, et al., 2008).

There are two widely used methods to produce silica-coated iron oxide nanoparticles, the Stober process and the microemulsion process (Thorek, Chen, Czupryna, & Tsourkas, 2006). The Stober process, comprises the hydrolysis and condensation of a sol-gel precursor such as tetraethyl orthosilicate (TEOS) (Stöber, et al., 1968). Although this method has produced very large silica shells, there have been several modifications that have resulted in excellent size control of the silica shell, allowing sizes between 2 nm and 100 nm without the use of surfactants (Fang, 2008; Lu, Yin, Mayers, & Xia, 2002). The limited or non-use of surfactants means that there is no need to separate the produced iron oxide silica nanoparticles from the solution, and simple centrifugation can remove particles from solution (Lu, et al., 2002).

The second most common method of generating iron oxide-coated silica nanoparticles is via the microemulsion process, where micelles or inverse micelles are
used to confine and control the silica coating. Although this method produces smaller sized particles, a disadvantage is the considerable effort required to separate the iron oxide coated silica nanoparticles from the numerous surfactants associated with the microemulsion system (Philipse, van Bruggen, & Pathmamanoharan, 1994; Tartaj & Serna, 2002, 2003).

The use of silica as a coating on iron oxide nanoparticles for molecular and cellular MRI has not been investigated. There have been many applications of iron oxide silica nanoparticles in other areas of medicine, such as controlled drug release and gene and protein delivery (Chowdhury & Akaike, 2005; Lu, Liong, Zink, & Tamanoi, 2007; Trewyn, Giri, Slowing, & Lin, 2007). The advantages of silica as a surface coating on iron oxide nanoparticles make it a promising material for applications in targeted MRI.

1.3.6 Biodistribution of iron oxide nanoparticles

The biodistribution of iron oxide nanoparticles for MRI applications is an important consideration, particularly for molecular and cellular targeted imaging. The distribution of the iron oxide nanoparticles determines whether localisation can occur at the targeted site. Intravenously administered iron oxide nanoparticles are recognised as ‘foreign bodies’ and are taken up primarily by the RES cells or macrophages (Bradfield, 1984). Since the liver has 90% of the total phagocytic capacity of the mononuclear phagocytic system (MPS), the iron oxide nanoparticles distribute mainly to this organ, followed by the spleen, lymph nodes and bone marrow (Bradfield, 1984). The removal of the iron oxide nanoparticles from the bloodstream and distribution into various organs depends primarily on the size of
the iron oxide nanoparticles, followed by the charge and surface nature (Chouly, et al., 1996).

Earlier studies of large iron oxide nanoparticles showed a trend that larger iron oxide nanoparticles have a shorter blood plasma life (Pouliquen, et al., 1991; Weissleder, et al., 1989). For larger iron oxide nanoparticles, approximately >60 nm in diameter, 80-90% of the injected dose can be taken up by the RES within 30 minutes of intravenous administration and localised within the liver, showing a maximum uptake by 2 hours (Pouliquen, et al., 1991; Weissleder, et al., 1989). Other organs such as the kidneys, lungs and brain show minimal or non-detectable amounts of iron oxide nanoparticles (Weissleder, et al., 1989). Iron oxide nanoparticles that are less than 60 nm in diameter have shown significant changes in biodistribution, such as longer peripheral blood circulating times, in the presence of surface coatings (Chouly, et al., 1996; Weissleder, et al., 1990). In this case, it has been reported that the initial liver and spleen uptake is low and the particles remain in the bloodstream for several hours, suggesting that the iron oxide nanoparticles can be available for localisation at a targeted area (Islam & Wolf, 2009; Moore, Marecos, Bogdanov, & Weissleder, 2000; Weissleder, et al., 1990). After this time, iron levels increase in organs such as the liver, spleen, lungs, kidneys and heart (Jain, Reddy, Morales, Leslie-Pelecky, & Labhasetwar, 2008; Weissleder, et al., 1990). For the excretion and breakdown of the iron oxide nanoparticles, a half-life of approximately 3 days in the liver and 4 days in the spleen has been reported (Pouliquen, et al., 1991; Weissleder, et al., 1990). The iron oxide nanoparticles are known to be stored in lysosomes where they are ultimately degraded, and enter the haemoglobin of the erythrocytes and enter the normal body iron metabolism cycle (Pouliquen, et al., 1991; Weissleder, et al., 1990).
1.3.7 Summary of main themes

The themes discussed in this section of the thesis related to principles of MRI and the characteristics of nanoparticles required to achieve targeted imaging,

The first section, on the principles of MRI, introduced the basics of MRI signal generation and the role of contrast agents. In summary, this section presented the fundamentals of MRI signals that rely on the spinning motion of hydrogen nuclei present in biological tissues because of its relatively large magnetic moment. In the absence of a magnetic field the magnetic moments of the hydrogen nuclei are randomly oriented, but when the hydrogen nuclei are placed in an external magnetic field their magnetic moments align with the magnetic field. The net magnetic moments of the hydrogen nuclei are relatively low and a RF pulse applied at the Larmor frequency produces a net magnetisation that is termed resonance and causes the hydrogen nuclei to gain energy. When the RF signal is turned off the hydrogen nuclei lose the energy given to them by relaxation, giving rise to T1 and T2 relaxation that can be imaged on MRI. The ability of the MRI image to produce contrast depends on differences in the relaxation rates in various tissues, and external agents can be administered to affect the MRI signals such as T1 and T2 relaxation. In a T1 weighted image, fat has a high MRI signal and appears bright whereas water has a low signal and appears dark. Conversely, on a T2 weighted image, water has a high signal and appears bright and fat has a small signal so it appears dark.
The section reviewed contrast agents and their properties such as large magnetic moments that in the presence of water protons cause local magnetic field fluctuations. The types of contrast agents examined were those that affect T1 and T2 signals. Paramagnetic contrast agents such as gadolinium shorten T1 relaxation times and appear bright on T1 weighted images. On the other hand, superparamagnetic contrast agents such as iron oxide nanoparticles shorten T2 relaxation times, appearing dark on T2 weighted images. Furthermore, gadolinium in its elemental state is highly toxic. It is chelated and although some chelated-gadolinium agents do not show toxicity there are still concerns over its safety due to reported deaths and nephrogenic systemic fibrosis. Due to these concerns research has been directed at examining other agents that can be used as contrast agents. Studies using traditional iron oxide contrast agents for MRI contrast have had limited success. However, recent developments in nanoparticle technology have resulted in iron oxide nanoparticles overcoming these limitations.

Accordingly, the application of iron oxides has the potential to complement the high resolution of MRI and improve its sensitivity. This is because these biocompatible iron oxide nanoparticles have the ability to target specific pathologies such as cancer and/or to deliver drugs for dual imaging and therapy in MRI. Of the range of iron oxide compounds discussed, magnetite appeared to show most promise because of its high magnetic properties.

Another theme was the range of characteristics necessary for iron oxide nanoparticles to provide targeted MRI contrast. These characteristics include superparamagnetism or high magnetic moment, uniform size distribution, small sizes less than 60 nm, stability and a facility for the attachment of biomarkers.
The section also reviewed the various methods of iron oxide nanoparticle synthesis and their resulting influences on the characteristics. In summary, hydrolytic methods such as co-precipitation and reactions in constrained environments were shown to produce iron oxide nanoparticles with lower magnetisation values, higher size distributions and less control over particle size. Although some hydrolytic methods have been developed to control these parameters, the excessive size separation processes limit their clinical use. Non-hydrolytic methods such as high temperature methods are superior in producing large quantities of iron oxide nanoparticles with monodispersity and higher magnetisation values without the need of size separation processes.

The last theme in the production of iron oxide nanoparticles was a comparison of surface coatings such as lipids, carboxylates, dextran and silica. The use of surface coatings on iron oxide nanoparticles is essential as they permit water solubility, prevent aggregation and allow the attachment of biomarkers. The latter is crucial for iron oxide nanoparticles to be utilised for targeted molecular and cellular MRI. Surface coatings can be conjugated on the surface of iron oxide nanoparticles either during the synthesis process or immediately after. The comparison of the different surface coatings suggested dextran was the most common due to its biocompatibility. However, a surface coating with immense potential for targeted MRI is silica.

1.4 Breast Cancer

1.4.1 Introduction
Breast cancer is classified according to multiple parameters including histopathological type, grade and stage, and the expression of proteins and genes, all of which help determine the diagnosis, prognosis and treatment options for patients (Ellis, et al., 2003). The receptor status is critical for breast cancer diagnosis, prognosis and treatment as it determines the ability to tailor therapy regimes such as chemotherapy, immunotherapy and adjuvant therapy (van’t Veer, et al., 2002). The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors has been widely studied because of its role in many cancers (Schlessinger, 2000; Yarden & Sliwkowski, 2001). The EGFR family of receptors includes four distinct types, namely human epidermal growth factor 1 (EGFR/HER1), HER2, HER3 and HER4, and is expressed in many human cancers (Yarden & Sliwkowski, 2001). In breast cancer, HER2 has been widely studied due to its presence being associated with aggressive behaviour of the tumour and poor prognosis (Slamon et al., 1987). Fortunately, overexpression of HER2 on breast tumours has opened up the ability to target tumour-specific immune therapies, such as Herceptin® (herceptin), which is used in adjuvant and neoadjuvant therapies (Carter, et al., 1992). Despite promising results for herceptin, there has been reported resistance to herceptin-based therapies and there is a need to identify such patients (Slamon, et al., 2001; Vogel, et al., 2002). The use of imaging technologies, particularly targeted imaging with MRI, can play an important role in selecting patients who have stopped responding to these therapies. This next section discusses the different types of breast cancers with a focus on HER2 positive breast cancers, herceptin treatments, and the potential role of iron oxide nanoparticles in MRI in identifying and monitoring these patients.

1.4.2 Types of breast cancer
Breast cancer is classified according to a number of different schemes such as histopathological type, grade and stage, and the expression of proteins and genes, to help determine the diagnosis, prognosis and treatment options (Ellis, et al., 2003). The grade of the cancer refers to the degree of differentiation of the tumour, and reflects its aggressiveness (Elston & Ellis, 2002). The grading system compares normal breast tissue to breast cancer cells and classifies the tumour cells according to poor, intermediate and low differentiation (Elston & Ellis, 2002). The Bloom-Richardson grade is the most commonly used scoring system used by the World Health Organization (WHO), but other schemes such as the Nottingham Score have also been used (Elston & Ellis, 2002). The histopathological type of breast tumour refers to the growth pattern and to morphological and cytological patterns identified in tumour biopsies (Ellis et al., 1992). There is a wide range of classifications, from benign to malignant breast cancers, available from the WHO classification of tumours of breast cancers, the most common being invasive ductal carcinoma, ductal carcinoma *in situ* and invasive lobular carcinoma (Ellis, et al., 2003). The histopathological types of breast cancers can be further divided into classifications according molecular subtypes and the expression of proteins and genes such as oestrogen receptor (ER) positive, ER negative and HER2 receptor positive (Perou, et al., 2000). The subtype and receptor status are critical for breast cancer diagnosis, prognosis and treatment as they determines the ability to tailor therapy regimes such as chemotherapy, immunotherapy and adjuvant therapy (van de Vijver, et al., 2002; van’t Veer, et al., 2002). The expression of receptors is generally associated with poor prognosis, shorter time to progression and low survival rates (Natrajan, Lambros, & Rodriguez-Pinilla, 2009; Natrajan, Weigelt, & Mackay, 2010). Recent studies have classified breast tumours into ER positive tumours and ER negative categories, where ER positive tumours have low grade and high grade categories (Lopez-Garcia, Geyer, Lacroix-Triki, Marchio, & Reis-
Filho, 2010; van’t Veer, et al., 2002). ER negative tumours have distinct molecular features and include HER2 positive, basal-like, luminal B, claudin-low and molecular apocrine tumours (Lopez-Garcia, et al., 2010). It has been suggested that ER negative tumours, which include HER2 positive tumours, fall into the category of more aggressive tumours due to distinct patterns of genetic aberrations caused by the presence of respective molecular entities (Natrajan, et al., 2009; Natrajan, et al., 2010).

1.4.3 HER2 positive breast cancer and herceptin

HER2 positive tumours are of interest because they are one of the most aggressive forms of breast cancer, associated with poor prognosis, lower survival rates and a faster time to disease progression (Slamon, et al., 1987; Slamon, Godolphin, & Jones, 1989). HER2 is part of the tyrosine kinase family and is a growth factor receptor gene (Coussens, Yang-Feng, & Liao, 1985) that is amplified in approximately 25-30% of breast cancers (Slamon, et al., 1987). Furthermore, in vitro studies have shown that HER2 amplification is present in almost 92% of breast cancers (Pauletti, Godolphin, Press, & Slamon, 1996). The function of the HER2 receptor is to mediate growth, differentiation and survival of cells, and its expression produces very aggressive tumours (Slamon, et al., 1987). Studies have shown that HER2 positive breast cancers are associated with poor prognosis compared to HER2 negative breast cancers (Slamon, et al., 1987). Moreover, it has been reported that HER2 positive breast cancers are also associated with frequent relapses and reduced overall survival (Slamon, et al., 1987; Slamon, Godolphin, & Jones, 1989). The identification of the HER receptor on breast cancers presents the opportunity for tumour-specific therapies and has had significant implications in the diagnosis, management and outcomes of patients with HER2 positive breast cancers.
In 1998, the United States Federal Drug Administration (FDA) approved the use of a humanised monoclonal antibody, trastuzumab or herceptin, for the treatment of HER2 positive breast cancers. Trastuzumab or herceptin is a humanised monoclonal antibody that targets the extracellular domain of the protein encoded by HER2 (Carter, et al., 1992). Herceptin was initially developed from a mouse monoclonal antibody which reacts with HER2, and was later humanised (Carter, et al., 1992). The HER2 receptor is not a tumour-specific antigen because HER2 is expressed by many cells (Schlessinger, 2000). However, when HER2 receptors are expressed 10–100 times above normal levels, it becomes a target for herceptin therapy (Schlessinger, 2000). This attribute of HER2 receptors is useful when the purpose is to target only cancer cells, and this discrimination may contribute to the success of herceptin in breast cancer treatment. For patients to qualify for herceptin treatment they must show high levels of HER2 receptor expression as determined by immunohistochemistry (IHC) (Hudis, 2007). At the molecular level, the primary effect of herceptin is to induce regression of tumours that are positive for HER2. Although the mechanism of action is not fully understood, a variety of mechanisms have been proposed (Hudziak, et al., 1989; Shepard, et al., 1991). Herceptin on its own selectively binds to HER2 receptors due to antibody-antigen-specific binding, as illustrated in Figure 1.4. This is followed by internalisation of the herceptin via receptor-mediated endocytosis. It has been suggested that upon internalisation various intra-cellular and extra-cellular events occur, such as cell cycle arrest which is induced due to down-regulation of HER2, eventually leading to a reduction in cell proliferation, suppression of angiogenesis, antibody-dependant cell-mediated toxicity and finally cell death (Baselga, Albanell, & Molina, 2001; Izumi, Xu, & Tomaso, 2002; Roberto, Menard, & Fagnoni, 2004). Further studies have reported that herceptin also causes increased cellular
sensitivity, and therefore when used in combination with chemotherapy therapy can produce synergistic effects (Konecny, Pegram, & Beryt, 1999; Pegram, et al., 1999; Pietras, et al., 1994).

Figure 1.4 The proposed mechanism of action of herceptin. A variety of mechanisms have been proposed for the action of herceptin. Herceptin on its own selectively binds to HER2 receptors present on the surface of breast cancer cells, due to antibody-antigen-specific binding. When herceptin is internalised, cell cycle arrest is induced due to down-regulation of HER2 eventually leading to a reduction in cell proliferation, suppression of angiogenesis, antibody-dependant cell-mediated toxicity, and finally cell death. This figure is reproduced from Burstein et al. (2005).

Initial clinical trials of herceptin in patients with HER2 positive metastatic breast cancer, who had relapsed after chemotherapy, demonstrated that herceptin was active and well tolerated (Baselga, et al., 1996; Cobleigh, et al., 1999). Phase 2 clinical trials also investigated the effects of herceptin when used in combination with chemotherapy drugs, such as cisplatin (Pegram, et al., 1998). This multi-centre trial reported objective clinical
response rates higher than previously reported for cisplatin or herceptin alone, confirming the success of herceptin in pre-clinical studies when used in combination with chemotherapy agents. The use of herceptin was then trialled in Phase 3 clinical trials, as a first-line treatment in combination with chemotherapy agent, paclitaxel, in patients with metastatic breast cancer (Slamon, et al., 2001). The aim of this trial by was to investigate the time to disease progression and the incidence of adverse effects in patients receiving chemotherapy alone or in combination with herceptin. The most troubling adverse effect of herceptin reported in this trial was associated cardiac dysfunction, which had not been reported in previous studies. Patients in this trial who received concurrent treatment with herceptin, anthracycline and cyclophosphamide significantly increased the risk of cardiac dysfunction. Nonetheless, outweighing the poor prognosis of HER2 positive breast tumours with cardiac toxicity, the results showed both increased survival times and increased time to disease progression in patients who received concurrent treatment with herceptin and first-line chemotherapy (Slamon, et al., 2001).

Since the results of this Phase 3 clinical trial, multiple trials have been conducted to evaluate herceptin with various chemotherapy regimes and different modes of administration and durations (Joensuu, et al., 2009; Perez, et al., 2007; Slamon, et al., 2009; Spielmann, et al., 2009). Herceptin has also been trialled in the neoadjuvant setting, where multiple Phase 2 and 3 trials have been conducted in early and advanced stages, which have shown complete responses in some cases and better overall response rates compared to adjuvant settings (Buzdar, et al., 2005; Buzdar, et al., 2007; Gianni, et al., 2010).
Despite the positive results of herceptin therapy, primary and secondary resistance to herceptin in both early and advanced disease has been shown (Goel, et al., 2010; Nahta & Esteva, 2006; Slamon, et al., 2001; Vogel, et al., 2002). Clinical evidence from Phase 2 trials have shown response rates to herceptin alone ranging from 11% to 26%, suggesting that the majority of patients with HER2 expressing tumours demonstrate intrinsic resistance to herceptin alone (Baselga, et al., 1996; Cobleigh, et al., 1999; Vogel, et al., 2002). Furthermore, although adjuvant therapy results in higher response rates, it has been reported that the duration of response to herceptin therapy either alone or in combination with chemotherapy was 5-9 months (Seidman, et al., 2001; Slamon, et al., 2001). The mechanism of acquired resistance is not fully understood and a number of mechanisms have been proposed (Nahta & Esteva, 2006). Price-Schiavi et al. (2002) and Nagy et al. (2005) proposed a mechanism involving disruption of the binding of herceptin to the target receptor protein. According to these studies, the disruption could be caused by a membrane associated glycoprotein mucin-4 (MUC-4) via multiple processes (Nagy, et al., 2005; Price-Schiavi, et al., 2002). Furthermore, the function of MUC4 has been suggested to contribute to cancer progression because of its ability to inhibit immune recognition of cancer cells, promote tumour progression and metastasis, suppress apoptosis and activate HER2 (Carraway, et al., 2001). Another proposed mechanism of herceptin resistance is the signalling of other HER receptors that could contribute to the growth of HER2 positive tumours during herceptin treatment (Diermeier, et al., 2005). Herceptin causes the down-signalling of HER2 by binding to HER2 receptors, but the function of herceptin does not reduce the action of other receptors that are part of the human epidermal growth factor receptor family, affecting the sensitivity of HER2 receptors to herceptin (Wick, Bürger, Funk, & Müller, 1995). Other proposed mechanisms of herceptin resistance include the
activation of pathways such as insulin-like-growth-factor-1 (Lu, Zi, & Pollak, 2004; Lu, Zi, Zhao, Mascarenhas, & Pollak, 2001).

There is no doubt that elucidating the molecular mechanisms by which tumours escape herceptin-directed cytotoxicity is critical to improving the prognosis of patients with HER2 positive breast cancers. Combining herceptin with other novel biologic agents and therapeutic strategies for targeting HER2 receptors may increase the magnitude and duration of the clinical response (Nahta & Esteva, 2006). Many new agents are currently being tested, such as vaccines and novel HER-targeting agents, in combination with herceptin to target the epidermal growth factor receptor family in HER2 positive tumours (Nahta & Esteva, 2006). Equally important is the identification of those patients who could stop responding to herceptin and/or herceptin-therapy regimes. Accordingly there is a clinical need to select the best therapies for clinical indications, based on predictors of response, to identify cost-effective therapies for patients needing treatment.

One aspect which is currently increasingly explored in the evaluation of patients receiving herceptin-based therapy is the use of nanoparticles in medical imaging. Nanoparticles such as iron oxides can be easily conjugated to herceptin and then imaged by imaging technologies such as MRI. This targeted imaging could potentially assist in the selection of patients who have stopped responding to herceptin-based treatment. The advantages of MRI over other imaging modalities lies primarily in its superior resolution, non-ionising radiation and longitudinal imaging capability (Kuhl, 2007). Results from preclinical studies have shown that iron oxide nanoparticles conjugated to herceptin can be used to image HER2 positive breast tumours (Chen, et al., 2009; Hilger, et al., 2007). Imaging the progress of herceptin treatment using iron oxide nanoparticles in MRI has the
potential to provide critical information about the efficacy of herceptin concurrently with treatment. Eventually, this technology could be used to develop a diagnostic and therapeutic evaluation tool for patients receiving herceptin-based treatment.

1.4.4 Herceptin conjugated iron oxide nanoparticles in MRI

The use of iron oxide nanoparticles for targeted imaging in MRI is currently at the preclinical stage. This is due to the inability of currently available iron oxide nanoparticles to be utilised in targeted imaging because of their large size and rapid clearance from the body. Furthermore, most iron oxide nanoparticles currently used in the preclinical setting cannot be used for routine clinical use because of several limitations such as the need for large dosages, the low magnetisation values, small scale synthesis capabilities and large particle size distribution (Budde & Frank, 2009; Corot, et al., 2006; Long & Bulte, 2009). A number of studies using iron oxide nanoparticles conjugated to herceptin have achieved successful targeting of HER2 positive breast tumours \textit{in vitro} and \textit{in vivo}, but each of these studies has particular limitations such as low magnetisation values, reduced contrast enhancement and small-scale production capabilities (Chen, et al., 2009). For example, a study by Hilger et al. (2007) showed less than 20% MRI signal enhancement at the breast tumour site using a minimum dosage of 20 mg/kg of iron oxide nanoparticles. Similarly, Chen et al. (2009) demonstrated low magnetic properties for their iron oxide nanoparticles conjugated to herceptin. In both these studies, higher contrast enhancement could have been observed if iron oxide nanoparticles with high magnetisation properties had been used. Interestingly, Huh et al. (2005) reported high magnetisation values and successful targeting of HER2 breast positive tumours \textit{in vitro} and \textit{in vivo}. However, the method used to produce their iron oxide nanoparticles conjugated to herceptin produced only small
amounts of iron oxide nanoparticles in one synthesis process and required several size separation processes.

The principle of using iron oxide nanoparticles for targeted imaging of HER2 positive breast tumours has been observed in previous studies (Chen, et al., 2009; Hilger, et al., 2007; Huh et al., 2005). However, these studies have not demonstrated that their iron oxide nanoparticles had all the desired characteristics such as high magnetic properties, high contrast enhancement, biocompatibility and a large-scale synthesis method. As discussed in the previous sections, these factors can greatly influence the translation of iron oxide nanoparticles to the clinical setting. Translation to a clinical application requires the synthesis of iron oxide nanoparticles that have the necessary characteristics for targeted molecular and cellular imaging such as high magnetisation values, low toxicity, biocompatibility, small size and low size distribution. Also desirable are an easy synthesis process and the ability to produce large amounts of iron oxide nanoparticles.

To this end, the iron oxide nanoparticles produced in this study will be synthesised and tested for use in targeted imaging, as they will need to possess all the characteristics discussed above and be successfully conjugated to a tumour seeking agent such as herceptin.

**1.4.5 Summary of main themes**

This section of thesis introduced the different types of breast cancer that are categorised according to various schemes such as histopathological type, grade, stage, and the expression of proteins and genes, which determine diagnosis, prognosis and treatment
options. The classification according to expression of proteins and genes of breast tumours is important because it helps identify the prognostic and treatment options for the patient. One of the themes presented in this section related to HER2 positive breast cancers, which are characterised by an over-expression of HER2 receptors. Patients who present with HER2 positive breast tumours have a poor prognosis and decreased overall survival compared to patients with HER2 negative breast tumours. It has been proposed that identification of HER2 receptors on breast cancers will allow the use of more targeted tumour specific therapies using herceptin in suitable patients. Herceptin-based therapies have significant implications in the diagnosis, management and outcomes for patients with HER2 positive breast cancers. In this section, the assessment of herceptin therapy highlighted that patients eventually acquire resistance to herceptin, which could be due to multiple factors. As a result, many new agents are currently being tested including vaccines, novel HER2 receptor targeting agents and agents that target the epidermal growth factor receptor family in combination with herceptin. Concurrently, it is also proposed that before other treatment options become available and even after new treatment options are available it will be equally important to identify patients who could stop responding to HER2 based therapies. Overall, there is a clinical need to select the best therapies for particular clinical indications based on predictors of response. Equally important is the identification of cost effective therapies for patients needing treatment of HER2 positive tumours.

An area of promise is the use nanotechnology in MRI for patients receiving herceptin-based therapies. Iron oxide nanoparticles conjugated to herceptin could be used to select herceptin resistance and improve treatment outcomes in patients using MRI. It was emphasised that the ability to image the progress of herceptin treatment using iron
oxide nanoparticles in MRI could provide critical information about the efficacy of herceptin concurrently with treatment. The principle of targeting HER2 positive tumours using MRI was shown to be well documented, but the inability to translate to the clinical setting lies in poor magnetisation properties, small signal enhancement, large size distribution and difficult synthesis processes. The aim therefore for future studies is to synthesise iron oxide nanoparticles that have the all the characteristics required for targeted molecular and cellular imaging, such as high magnetisation values, low toxicity, biocompatibility, small size and size distribution, and an easy synthesis process for producing large amounts of iron oxide nanoparticles. To demonstrate that the iron oxide nanoparticles produced in this study can be used for targeted imaging with all the characteristics discussed above, they will need to be conjugated to a tumour targeting agent such as herceptin in order to investigate the possibility for translation to clinical applications.

1.5 Main aims of the thesis

The main aims of this thesis are:

1. To create a targeted contrast agent for MRI,
2. To synthesise iron oxide silica nanoparticles addressing the five major characteristics of contrast agents for MRI,
3. To create a targeted iron oxide silica nanoparticle via conjugation to herceptin,
4. To evaluate the ability of the iron oxide silica herceptin nanoparticles to target HER2 positive breast cancer cells and demonstrate contrast enhancement in vitro, and
5. To evaluate the ability of the iron oxide silica herceptin nanoparticles to target HER2 positive tumours and demonstrate contrast enhancement in vivo.
Chapter 2 Materials and Methods

2.1 General chemicals and reagents

Table 2.1 General chemicals and reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-octadecene</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Carbon coated copper grids</td>
<td>ProSciTech (Thuringowa, QLD, Australia)</td>
</tr>
<tr>
<td>Cystamine</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Cystine</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Formalin</td>
<td>Amber Scientific (Midvale, WV, Australia)</td>
</tr>
<tr>
<td>Hexane</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Iron chloride</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Matrigel</td>
<td>BD Biosciences (Bedford, MA, USA)</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Sephadex G-25M column</td>
<td>Pharmacia (Rydalmere, NSW, Australia)</td>
</tr>
<tr>
<td>Sodium oleate</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Tetraethyl orthosilicate (TEOS)</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
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2.2 Kits

Table 2.2 Kits

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
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<tr>
<td>CellTiter 96 Aqueous One Solution Cell</td>
<td>Promega (Madison, WI, USA)</td>
</tr>
<tr>
<td>Proliferation kit</td>
<td></td>
</tr>
<tr>
<td>FITC conjugation kit</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
</tbody>
</table>
### 2.3 Antibodies and conjugates

#### Table 2.3 Antibodies and conjugates

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>FITC</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>FITC-anti-human IgG</td>
<td>Pharmingen (BD Biosciences, Bedford, MA, USA)</td>
</tr>
<tr>
<td>Herceptin*</td>
<td>Roche Diagnostics (Castle Hill, NSW, Australia)</td>
</tr>
<tr>
<td>Hoechst nucleus stain</td>
<td>Molecular Probes (Invitrogen, Carlsbad, CA, USA)</td>
</tr>
<tr>
<td>Novacastra antibody **</td>
<td>Novacastra (Leica Microsystems, GmbH, Germany)</td>
</tr>
<tr>
<td>anti-Her2neu antibody</td>
<td>Novacastra (Leica Microsystems, GmbH, Germany)</td>
</tr>
<tr>
<td>Secondary antibody Dako Dual Envision anti-mouse and anti-rabbit HRP</td>
<td>Dako (Glostrup, Germany)</td>
</tr>
</tbody>
</table>

*Herceptin was kindly provided by Mr Peter Eu from the Peter MacCallum Cancer Centre.
** Novacastra antibody was kindly provided by Dr Janine Danks from RMIT University

### 2.4 Tissue culture reagents, drugs and equipment

#### Table 2.4 Tissue culture reagents, drugs and equipment

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro BCA protein assay kit</td>
<td>Invitrogen (Carlsbad, CA, USA)</td>
</tr>
</tbody>
</table>
### Materials and methods

#### Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
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<tr>
<td>24-well plates and 96-well plates</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>Cell culture flasks</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>Eppendorf Tubes</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>Fetal bovine serum 10%</td>
<td>Bovogen Biologicals (Victoria, Australia)</td>
</tr>
<tr>
<td>Glass chamber slide 6-well</td>
<td>Nunc Lab-Tek II (Roskilde, Denmark)</td>
</tr>
<tr>
<td>Haemocytometer</td>
<td>Hirschmann EM Techcolor (Heilbronn, Germany)</td>
</tr>
<tr>
<td>L-glutamine 1mM</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>RPMI 1640 medium</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>Streptomycin/penicillin 1%</td>
<td>Greiner Bio-one (Frickenhausen, Germany)</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma-Aldrich (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA)</td>
<td>Invitrogen (Carlsbad, CA, USA)</td>
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</table>

#### Cell lines

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
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</table>

2.5 Cell lines

**Table 2.5 Cell lines**

| Reagent                                      | Supplier                                      |
Chapter 2 – Materials and methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
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<tr>
<td>0.01M citrate buffer</td>
<td>0.01M, pH 6</td>
</tr>
<tr>
<td>FACS Fix buffer</td>
<td>1% paraformaldehyde</td>
</tr>
<tr>
<td></td>
<td>2% glucose</td>
</tr>
<tr>
<td></td>
<td>0.02% azide</td>
</tr>
<tr>
<td></td>
<td>96.98% PBS</td>
</tr>
<tr>
<td>PBS</td>
<td>pH 7.4</td>
</tr>
</tbody>
</table>

2.6 Animals

Female BALB/c nude mice 4-6 weeks of age were sourced from the Animal Resource Centre (ARC; Canning Vale, WA, Australia). BALB/c nude mice were used for studying the uptake and contrast enhancement of the iron oxide silica herceptin nanoparticles after intravenous injection for MRI in HER2 positive tumours. SKBR3 tumours were grown by subcutaneously injected 3x10^6 SKBR3 cells in BALB/c nude mice. All animal experiments were performed as approved by RMIT Animal Ethics Committee (AEC approval number 0906).

2.7 Common buffers and solutions

2.8 Equipment

Eppendorf Mini Spin Plus Centrifuge (Eppendorf, Hamburg, Germany)

Beckman Coulter Allegra X-22 Centrifuge (Becman Coulter, CA USA)
Chapter 2 – Materials and methods

Unisonics Sonicator Model FXP08M (Unisonics, NSW, Australia)
Harvard Apparatus PHD 22/2000 Syringe Pump (Harvard Apparatus, MA, USA)
IKA RW 16 basic overhead stirrer (IKA Works Asia, Petaling Jaya, Malaysia)
JEOL 1010 electron microscope (Jeol Korea, Korea)
Bruker D8 ADVANCE X-ray diffractometer (MBraun, NH, USA)
SQUID Quantum Design MPMS-XL5 (CA, USA)
Atomic absorption spectrometer (AAS) Varian AA280FS Fast Sequential (CA, USA)
3.0 Telsa Siemens Trio Magnetom Whole Body Clinical MRI Scanner (Siemens, Medical Solutions, PA, USA)
Q- Sense E4 QCM-D (ATA Scientific, NSW, Australia)
Becton Dickinson FACSCalibur Flow Cytometer (BD Biosciences, Bedford, MA, USA)
UV/Visible Spectrophotometer Cintra 5 (Victoria, Australia)
Zeiss Confocal microscopy

2.9 Synthesis of iron oxide nanoparticles

The iron oxide nanoparticles were synthesised jointly by Jos Campbell and me at the School of Applied Sciences. The iron oxide nanoparticles were synthesised using a two-step process modified from Park et al. (2004). An iron oleate complex was first formed by dissolving 5.4 g of iron chloride and 18.25 g of sodium oleate in a solution comprised of 40 mL ethanol, 30 mL distilled water and 70 mL hexane. Once homogenised, the solution was refluxed at 70°C for 4 hours, followed by separation of the upper organic layer using a separatory funnel, washing and evaporating off hexane, thereby leaving a waxy iron oleate complex. The iron oxide nanocrystals were formed by dissolving 9.0 g of the iron
oleate complex in 1.425 g of oleic acid and 63.3 mL of 1-octadecene, followed by reflux under nitrogen until it reached 320°C, at which point the temperature was held for 30 minutes and the solution was then allowed to cool to room temperature. 250 mL of ethanol was added to the solution and the magnetite particles were separated via centrifugation, followed by three wash cycles with ethanol. At least 40 g magnetic nanoparticles per reaction could be achieved under these laboratory conditions.

2.10 Synthesis of iron oxide silica nanoparticles

Silica-coated iron oxide nanoparticles were prepared using a method modified from Fang et al. (2008). 1 mg of iron oxide nanoparticles from the previous step were sonicated in a solution consisting of 15 mL ethanol and 2 mL deionised water. 1 mL of ammonia (25% solution) was added to the above solution while it was immersed in a sonicator programmed to switch on for 1 in every 10 min. As well, an overhead stirrer was used to mix the solution while 4 mL of 1:60 (tetraethyl orthosilicate:ethanol, TEOS) was added at the rate of 0.4 mL per hour using a syringe pump, and the solution was stirred at room temperature for 12 hours. The silica coated iron oxide nanoparticles were then centrifuged and washed three times with ethanol and redispersed in milli-Q water.

2.11 Synthesis of iron oxide silica herceptin nanoparticles

Iron oxide silica nanoparticles from the previous method were resuspended in milli-Q water and then conjugated to herceptin using a cystamine linker. 2 mg of iron oxide silica nanoparticles and 0.04 mg of cystamine were incubated for 2 hours at room temperature and then washed several times in milli-Q water using a centrifuge. 6 mg of herceptin (1:3
ratio, with herceptin in excess) was added and incubated at room temperature for another 2 hours and then washed several times in milli-Q water by centrifugation.

2.12 Characterisation of iron oxide silica nanoparticles

2.12.1 TEM

Transmission electron microscopy (TEM) analysis was performed jointly by Jos Campbell and me using a JEOL 1010 electron microscope (Jeol Korea, Korea) operating at an accelerating voltage of 100 KeV. The resulting iron oxide silica herceptin nanoparticles were drop cast on strong carbon-coated copper grids and allowed to dry for one hour prior to scanning. The micrographs were enlarged at magnification of 400,000. The size of nanoparticles were assessed manually using the scale bar and generating an average particle size after each synthesis process (n = 20).

2.12.2 XRD

XRD spectra were collected at room temperature on a Bruker D8 ADVANCE X-ray diffractometer, fitted with a graphite-monochromated copper tube source and a scintillation counter detector (MBraun, USA). The samples were drop cast onto glass sample holders to form a thick layer before undergoing XRD analysis. The samples were then placed inside the XRD and irradiated with copper per Kα radiation (wavelength 1.5406 Å). The diffraction patterns were measured with 0.020° step size, 2.0 seconds time/step at 40 kV and 35 mA. The peak areas were determined from computer integration of the diffraction patterns.

2.12.3 SQUID

The synthesised iron oxide nanoparticles (50 g) were dried overnight to form a fine powder. The particles were then posted the Indian Technology Institute in Kanpur, Uttar
Pradesh, India. The particles were prepared and analysed using a superconducting quantum interference device (SQUID) by Dr. Ashish Garg. Specifically, the magnetisation measurements were obtained at ambient temperatures and nonstoichiometric magnetite samples were measured as control.

2.12.4 T2 Relaxation

The iron oxide silica nanoparticles 10 μg, 50 μg and 100 μg, were suspended in milli-Q water, in a volume of 1 ml, in triplicates. A 3.0 Tesla Siemens Trio MRI scanner with a 12-channel head coil was used to scan the samples (Siemens, USA). T2 relaxation times were determined by using a single echo sequence (SE) with a constant repetition time (TR) of 2000 ms and multiple echo times (TE) ranging from 0.99 to 100 ms. The signal was plotted as a function of TE and fitted to the established mono-exponential expression. The T2 values were determined by plotting the relaxation time (i.e. reciprocal of the relaxation rates) at a TE of 10.86 ms, as a function of molar iron concentration in respective samples (determined by AAS), and extracting the T2 value from the slope by linear regression. The mean was calculated from triplicate measurements and the error was calculated as the standard error of the mean.

2.12.5 Micro BCA protein assay and QCM

The amount of herceptin on the surface of the iron oxide-silica nanoparticles was quantified using a BCA protein assay kit following the manufacturer’s instructions (Invitrogen, USA). Triplicate measurements were taken where samples were prepared in 96-well plates and read using a plate reader at absorbance 562 nm.
The quartz crystal microbalance (QCM) flow was controlled by an ISMATEC pump IPC (high precision multichannel dispenser). The QCM was run using gold coated quartz microbalance chips that were new before use. The chips were washed as per manufacturer’s instructions and during the experimental process iron oxide silica nanoparticles, herceptin, cystine and cystamine were suspended in PBS solution at pH 7. Triplicate experiments were carried out in duplicates, using identical cells running in parallel on the same instrument. Each component was added in sequence and allowed to bind to the surface of the chip for 30 minutes before washing with PBS. First cystine was added, followed by cystamine, iron silica nanoparticles and then herceptin. The data was analysed visually by the amount of frequency drop generated by the addition of each component. The measurements were recorded for each run and then an average was calculated.

2.12.6 Stability of iron oxide silica herceptin nanoparticles

The stability of the iron oxide silica herceptin nanoparticles was assessed by conjugating a fluorescein isothiocyanate (FITC) molecule to the herceptin antibody on the iron oxide silica nanoparticles in triplicates. The FITC conjugation was performed using a FITC conjugation kit according to the manufacturer’s protocol for a small-scale conjugation procedure. The iron oxide silica herceptin-FITC nanoparticles were exposed to pH buffers (5, 7.14, 10) and human serum concentrations (25%, 50%, 100%) over a 24 hour time course (1 h, 3 h, 5 h, 8 h and 24 h). The degradation of the particles was assessed by loss of protein in the supernatant indicated by an increase in fluorescence in the supernatant based on a standard concentration series of herceptin-FITC molecules. The iron oxide silica nanoparticles were added to various pH and human serum conditions at a concentration of 2.067 mg and incubated at 370°C for the time points listed above. The
nanoparticles were then centrifuged and the supernatant was transferred to 96-well plates and read using a micro plate reader. The amount of herceptin-FITC in the supernatant was calculated and compared to a standard curve of a known amount of herceptin. The results were analysed according to the amount of herceptin retained on the iron oxide silica nanoparticle sample. The mean value was calculated from triplicate samples and the error was calculated as standard error of the mean.

2.13 In vitro studies

2.13.1 Preparation of the fluorescently labelled iron oxide silica herceptin nanoparticles

To conduct flow cytometry cell uptake studies, fluorescently labelled iron oxide silica herceptin nanoparticles were made. The herceptin was conjugated to FITC using a FITC conjugation kit following the small-scale conjugation procedure according to the manufacturer’s recommended protocol. 6 mg of herceptin was first purified using a sephadex G-25M column and then FITC was added three-times in excess and incubated for 2 hours at room temperature. Un-bound FITC was removed using a sephadex-G25M column following the manufacturer’s protocol of the FITC-conjugation kit. Excess herceptin was then removed by washing several times via centrifugation. The amount of herceptin on FITC was calculated by absorption readings from a UV/visible spectrophotometer for FITC-IgG conjugates according to the following equations:
(1) Molar F/P = \frac{2.77 \times A_{495}}{A_{280} - (0.35 \times A_{495})}

(2) Protein concentration IgG (mg/ml) = \frac{A_{280} - (0.35 \times A_{495})}{1.4}

The FITC-herceptin complex was then added to the iron-silica via the cystamine linker and after 2 hours incubation at room temperature was washed several times with milli-Q water.

2.13.2 Human breast cancer cell lines

Three breast cancer cell lines (SKBR3, BT474 and MCF7) were routinely cultured at 37°C in a humidified atmosphere with 5% CO₂ using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin/penicillin and 1mM L-glutamine. For subculturing, the breast cancer cells were detached by washing with PBS and incubating with trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) for 5 min at 37°C, followed by washing and incubation with supplemented RPMI 1640 medium.

2.13.3 Quantification of HER2 surface expression

Quantification of HER2 expression on SKBR3, BT474 and MCF7 cells was assessed by flow cytometry. Cells were cultured using the previously described method, seeded onto 24-well plates and grown to 90% confluency. Cells were detached using trypsin and media was removed by centrifugation. Cells were quantitated by trypan blue stain and then resuspended in PBS. 10mg of herceptin was added to the cells for 30 minutes at 4°C followed by two PBS washes and then incubated with anti-human IgG-FITC for 30
minutes at 4°C. The removal of the primary antibody from these treatments was used as a measurement of non-specific binding of the secondary antibody. The cells were washed and then analysed by flow cytometry (FACSCanto, BD Biosciences; RMIT Flow Cytometry Facility) where a population of 10,000 live cells was collected and the mean fluorescence intensity was analysed. The stimulation index of mean fluorescence intensity was calculated by dividing the mean fluorescence intensity value of the treated cells by the mean fluorescence intensity value of the controls. The stimulation index of staining above the control was calculated for triplicate studies and then plotted as the stimulation index mean ± standard deviation.

2.13.4 Dose response of iron oxide silica herceptin nanoparticles in vitro

SKBR3, BT474 and MCF7 cells were cultured until 90% confluency, detached with trypsin-EDTA solution for 4 min at 37°C. The cells were centrifuged to remove media with serum and live cells were counted using trypan blue staining. The cells were then seeded on 24-well plates and resuspended in 1 ml of serum-free media. 10 µg, 50 µg and 100 µg of iron oxide silica herceptin nanoparticles were added to each cell line and incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO₂. Cells were detached as described above, washed by centrifugation as described above, and resuspended in PBS and analysed by flow cytometry as previously described. 10,000 viable cells were analysed on the basis of forward versus side scatter and the stimulation index of the mean fluorescence intensity was calculated as previously described. An average of the mean fluorescence intensity was calculated for the triplicate data. The stimulation index of staining above the control was calculated as described above and then plotted as the stimulation index mean ± standard deviation.
2.13.5 Time course of iron oxide silica herceptin nanoparticles uptake in vitro

For the time course study, the same procedure was conducted as with the dose response study, in triplicates. The iron oxide-silica herceptin nanoparticles (50 µg) conjugated to FITC were incubated at various time points (0.5, 1, 3, 5 and 24 hours) at 37ºC in a humidified atmosphere with 5% CO₂. Cellular uptake was assessed by flow cytometry and analysis as previously described.

2.13.6 Uptake of iron oxide silica herceptin nanoparticles by confocal microscopy

*In vitro* visualisation of iron oxide silica herceptin nanoparticles was assessed by confocal microscopy. SKBR3 cells were cultured until 60% confluency, detached, centrifuged and then seeded onto a 6-well slide glass chamber. The cells were treated with 50 mg of iron oxide silica herceptin-FITC nanoparticles for 4 hours at 37ºC in a humidified atmosphere with 5% CO₂ for 30 minutes before imaging, a Hoechst nuclear stain was added and cells were washed several times with PBS before imaging by confocal microscopy. The uptake of the particles were assessed visually by identifying FITC and the nucleus stain.

2.13.7 *In vitro* toxicity of iron oxide silica herceptin nanoparticles

The viability of SKBR3 cancer cells exposed to iron oxide silica herceptin nanoparticles in the absence of a cell growth medium was determined by a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit containing the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the manufacturer’s recommended protocol (Promega, USA). SKBR3 cells were cultured until 90% confluency, detached using tryspin/EDTA, and viable cells
were counted using trypan blue, all as described above. The cells were seeded into 96-well plates in triplicates and resuspended in serum-free media and incubated with 1, 3, 5, 10, 30, 50, 100 µg/ml of iron oxide silica herceptin nanoparticles for 24 and 48 hours at 37°C in a humidified atmosphere with 5% CO₂. MTS colour change was monitored using a plate reader at 490 nm, and cell viability data was plotted by considering the viability of untreated cells as 100%. The mean was calculated from triplicate samples and error was calculated as the standard error of the mean. Statistical analysis was performed by one-way ANOVA and Dunnett’s test. Toxicity was considered significant for p < 0.05.

2.14 *In vivo* animal studies

2.14.1 HER2 positive tumours

HER2 tumours were grown in the right flank of BALB/c nude mice by subcutaneous injection. 2.5-3 x10⁶ SKBR3 breast cancer cells were suspended in an equal volume of Matrigel (BD Biosciences, USA) and made up with PBS to a total volume of 200 µL. A pilot study monitoring tumour growth was conducted prior to iron oxide silica herceptin nanoparticle injection to monitor and confirm the growth pattern of SKBR3 tumours in BALB/c nude mice (n= 3) over a 2 week period. The mean was calculated from the triplicate tumour measurements. The error bars were calculated by standard deviation of the mean.

2.14.2 HER2 receptor expression of SBKR3 tumours grown *in vivo*

SBKR3 tumours were confirmed for their HER2 expression by dissecting SKBR3 grown tumours in BALB/c nude mice by immunohistochemical analysis. Dissected tumours were fixed in 10% neural buffered formalin for 24 hours, processed and embedded in paraffin wax and cut onto microscopic slides at 4 µM thickness. Immunohistochemical staining of
Chapter 2 – Materials and methods

HER2 was performed according to standard protocols with antigen retrieval performed using heat and citrate buffer at pH 6. Sections were stained with an anti-HER2 antibody (Novacastra, Germany), followed by a pre-diluted dual link anti-mouse/anti-rabbit polymer conjugated to horseradish peroxidase (HRP) which was used as secondary antibody (DAKO, Glostrup, Germany), and the chromagen 3, 3’-diaminobenzidine (DAB) was used to visualise the localisation of probed antigens through HRP catalysed reaction. Images of stains were taken using DMD108 micro-imager (Leica Microsystems, Germany).

2.14.3 Toxicity analysis of iron oxide silica herceptin nanoparticles

To evaluate the toxic effect of iron oxide silica herceptin nanoparticles in vivo, acute toxicity by histopathological analysis was performed. Three experimental groups of BALB/c nude mice (n=3) were established. The first group was injected intravenously with iron oxide silica herceptin nanoparticles at 20 mg/kg in a maximum volume of 200 µl, a second group injected intravenously with iron oxide silica nanoparticles at 20 mg/kg and a third group injected intravenously with PBS. Any change in behaviour, health and weight was closely monitored and recorded daily for 7 days post-injection. After 7 days, the mice were anaesthetised with ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight). Blood was collected from each mouse by cardiac puncture and samples were sent to Animal Pathology Laboratory PTY LTD for full biochemistry, liver function and kidney function analysis. Mice were sacrificed and liver, lungs, kidneys and heart were dissected and embedded in OCT medium and snap frozen in liquid nitrogen for cryosectioning. Frozen samples were cut, stained and analysed by the Australian Phenomics Network (APN) at the University of Melbourne. Cryosections were cut at 5 mm thickness and stained for hematoxylin and eosin (H&E). The tissues were analysed and a report prepared by a veterinary pathologist.
2.15 MRI studies

2.15.1 Phantom contrast enhancement

The phantoms for MRI were prepared with iron oxide silica nanoparticles 10 µg, 50 µg, 100 µg were suspended in 1 ml of milli-Q water and a control was prepared containing milli-Q water without any nanoparticles. The MRI of the phantoms were performed in triplicates on a 3.0 Tesla Siemens MRI scanner (Siemens, USA) using a 12-channel head coil and the following parameters: T2-weighted imaging, gradient echo sequence, multiple TE ranging from 0.99-100 ms, TR= 2000 ms, matrix 128x128, slice thickness of 3 mm. Data analysis was performed manually by placing regions of interest (ROIs) around each sample concentration on the MRI image. The signal enhancement of each of the phantom concentrations was calculated relative to the control using the formula: \[ \frac{R2}{R2_{control}} \times 100 \]. The mean was calculated from triplicate measurements and the error was calculated as the standard deviation of the mean.

2.15.2 In vitro contrast enhancement

Iron oxide nanoparticles were prepared and conjugated to herceptin via the previously discussed protocols. SKBR3, BT474 and MCF7 cells were cultured, detached with trypsin-EDTA as previously described, washed and seeded onto 6-well plates and incubated with supplemented RPMI 1641 medium without serum as previously described. The experiment was conducted in triplicates. Cells were treated with 50 µg of iron oxide silica herceptin nanoparticles and incubated for 5 hours at 37°C. Before imaging on MRI the cells were washed three times with PBS and resuspended in 1 ml of milli-Q water. A T2-weighted gradient echo sequence was used with the same parameters as in the phantom studies. Signal enhancement of each of the cell lines was calculated relative to the control,
using $\Delta R_2/R_2^{\text{control}} \times 100$. The mean was calculated using triplicate measurements and the error was calculated as the standard deviation of the mean.

2.15.3 In vivo contrast enhancement

Nude mice bearing subcutaneously grown SKBR3 xenografts were studied by MRI when tumours reached a diameter of approximately 8 mm in diameter. The first group of mice ($n=5$) were injected intravenously with iron oxide silica herceptin nanoparticles (400 µg in 200 µl), the second group of mice ($n=5$) were injected intravenously with iron oxide silica nanoparticles (400 µg) and the third group of mice ($n=5$) were injected with PBS. Before MRI scanning mice were anaesthetised with ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight). The MRI scan was performed at 4 hrs and 24 hours post-injection of contrast agents or saline using a 3.0 Tesla magnetic resonance scanner and a head coil (Siemens, USA). All animals were measured using a T2-weighted sequence with the following parameters: TE 76 ms, TR 2000 ms, FOV 100x100 mm, slice thickness 1.7 mm. Data analysis was performed manually by placing ROIs around the tumour and tissue areas on the images and signal enhancement of the tumours was calculated relative to the control using the formula: $\Delta R_2/R_2^{\text{control}} \times 100$. The mean was calculated using the data from each group of five mice. The error bars represent standard deviation of the mean.
Chapter 3 Synthesis of Iron Oxide Nanoparticles and their Conjugation to Herceptin

3.1 Introduction

There is an increasing demand to develop iron oxide nanoparticles as targeted contrast agents for use in MRI. For targeted imaging, iron oxide nanoparticles are precisely conjugated to molecular or cellular biomarkers which act as vehicles driving the nanoparticles to a targeted area, allowing precise detection of particular pathologies. Newly developed iron oxide nanoparticles allow the attachment of tumour-associated markers. One example is the attachment of the anti-carcinoembryonic antigen (CEA) monoclonal antibody rch 24 (Hu, et al., 2006). The CEA rch 24 antibody is specifically expressed by human colon carcinoma cells, allowing direct in vitro and in vivo imaging of the cancer cells using MRI (Hu, et al., 2006). The benefit of using MRI for targeted imaging over other imaging modalities such as nuclear medicine and PET arises because of its superb spatial resolution (Mitchell & Cohen, 2003). The iron oxide nanoparticles are utilised in MRI to provide contrast enhancement between anatomical structures, improving the specificity of MRI. This enhanced specificity, combined with the inherent high spatial resolution of MRI, together provide a powerful tool for targeted imaging.

For iron oxide nanoparticles to be utilised as targeted imaging contrast agents, a number of characteristics to facilitate optimal targeted imaging need to be considered (Gupta & Gupta, 2005; Jun, Lee, & Cheon, 2007). These characteristics include high magnetic properties, biocompatibility and low toxicity, ease of conjugation to biomarkers, and size and size distribution of the nanoparticles. Currently commercially available iron oxide nanoparticles are not suitable for targeted imaging because they do not possess all the
characteristics required for optimal targeted imaging. The most important characteristic of iron oxide nanoparticles for targeted imaging in MRI is their magnetic property, as it determines their ability to be used as a contrast agent (Gupta & Gupta, 2005). High magnetic properties such as large mass magnetisation values and T2 relaxation values are required so that the maximum possible contrast can be visualised in MRI both \textit{in vitro} and \textit{in vivo} (Gupta & Gupta, 2005; Lee, et al., 2007). Commercially available contrast agents such as Resovist® are primarily made via the common co-precipitation method (see Chapter 1 Section 1.3.3.1) which produces large amounts of nanoparticles at the expense of low mass magnetisation values and lower T2 relaxation values.

Another important characteristic for iron oxide nanoparticles for targeted imaging is the size of the contrast agent. This factor is important as the hydrodynamic diameter of the nanoparticle can affect its biodistribution \textit{in vivo} (Berry & Curtis, 2003). The biodistribution and excretion pathway of iron oxide nanoparticles was discussed in detail in Chapter 1 Section 1.3.5. Iron oxide nanoparticles are eventually taken up by the RES where they are cleared by the liver and spleen. In general, particles larger than 60 nm are taken up by the RES upon intravenous injection (within 1 hour) and accumulate in the liver and spleen (Qiao, Yang, & Gao, 2009). Particles smaller than 60 nm, do not accumulate in the RES as quickly, and their presence for longer periods within the circulation allows particles to localise at a targeted area more effectively. For targeted imaging of iron oxide nanoparticles, therefore, one of the main requirements is a size smaller than 60 nm to ensure uptake at the targeted area before clearance by the RES. Commercially available Feridex® and Resovist® are iron oxide nanoparticles with size ranges of 80-180 nm and 60 nm respectively, and are used only for imaging of the liver and spleen (Josephson, 2006; Qiao, et al., 2009). Because the size of Feridex® is greater
than 60 nm these iron oxide nanoparticles would be unsuitable for targeted imaging; moreover, Feridex® has a large range of particle size distribution. Other iron oxide nanoparticles such as GastroMARK™ and Abdoscan® are specifically designed for imaging the gastrointestinal lumen (Josephson, 2006; Qiao, et al., 2009), but their size is no less than 300 nm and the only administration route is oral. These properties also make both GastroMARK™ and Abdoscan® unsuitable for targeted imaging.

Another important factor is the size distribution of iron oxide nanoparticles, as different sizes of iron oxide nanoparticles will distribute differently in vivo. For targeted imaging it would be optimal to ensure that most of the administered iron oxide nanoparticles localise first at the targeted area, with minimal non-specific uptake. In a sample with large particle size distribution, there would be non-specific uptake of larger nanoparticles and reduction in the amount of iron oxide nanoparticles available to reach the targeted area. Commercially available Feridex® has a size distribution of 80-180 nm, making it unsuitable for targeted imaging (Advanced-Magnetics, 2007). Thus currently approved iron oxide nanoparticles are not suitable for use in targeted imaging, because they do not possess the most important characteristics required for targeted contrast agents, namely magnetic properties, size and particle size homogeneity. Therefore, study is needed of the development of iron oxide nanoparticles that address these characteristics (Jun, et al., 2007; Qiao, et al., 2009). This study addresses all of the characteristics required to achieve targeted imaging using iron oxide nanoparticles in MRI.

As discussed in Chapter 1 (Section 1.3.3.1), there are many methods of iron oxide nanoparticle synthesis, each having a significant affect on the overall characteristics of the iron oxide nanoparticles produced. The chemical methods of iron oxide nanoparticle
Chapter 3 – Nanoparticle synthesis and conjugation to herceptin

production that have been explored so far include hydrothermal and non-hydrothermal methods. It has been highlighted in this thesis that the non-hydrothermal method produces iron oxide nanoparticles which are more suitable for use as MRI contrast agents because they have higher magnetic properties and better control over individual size and size distribution of the nanoparticles (Gupta & Gupta, 2005). The thermal decomposition method developed by Park et al. (2004) is the only reported method to produce iron oxide nanoparticles that were biocompatible, non-toxic, easily reproduced in large batches, and display a good particle size distribution. Park et al. (2004) utilised iron oleate as a precursor and then thermally decomposed the nanoparticles at temperatures up to 320°C, and were able to produce large-scale amounts (>10 g) of nanoparticles compared to other non-hydrothermal production methods. The authors suggested that the high temperatures were an effective tool for achieving iron oxide nanoparticles of different sizes, and the key to monodispersity was in separating nucleation and growth processes. This thermal decomposition method has significant advantages over hydrothermal methods as the particles produced have smaller size distributions (<5%), better control over hydrodynamic diameter, higher crystallinity and therefore highly magnetic particles. The method also has an advantage over other non-hydrothermal methods as the process is relatively easy to reproduce and bulk masses of iron oxide nanoparticles can be created in one synthesis process (up to 10 g).

Despite these advantages of the thermal decomposition method, one of the disadvantages is the inability to produce water soluble particles. Different approaches have been developed for achieving increased water solubility, including modifying the surface of the iron oxide particles by ligands such as DMSA (Huh, et al., 2005) and polymers such as PEG and dextran (Berry, et al., 2003; Kim, et al., 2001; Paul, Frigo, Groman & Groman,
Attachment to the surface layer is advantageous because a selection of biomarkers can be attached depending on the purpose of the study. Having stated this, it is important to note that in any iron oxide nanoparticle production there is an inherent need for surface coating in the iron oxide nanoparticle synthesis process. Not only do these surface coatings serve to increase water solubility, but the hydrothermal methods also utilise surface coatings for the attachment of various biomarkers onto the surface. Another reason for the use of a surface coating is to reduce aggregation of the particles \textit{in vivo} due to protein adsorption and to bypass the RES, increasing blood circulating time (Berry & Curtis, 2003; Gupta & Gupta, 2005; Laurent, et al., 2008). Overall, the thermal decomposition method is suggested to be a superior method of producing iron oxide nanoparticles, and the need for a surface layer only helps to improve its suitability for use as a targeted iron oxide contrast agent.

One of the most widely used surface coatings for iron oxide nanoparticles is dextran because of its proven inert nature and biocompatibility (Bonnemain, 1998; Jung & Jacobs, 1995; Qiao, et al., 2009). Dextran is used with approved contrast agents such as Feridex® and Resovist® (Bonnemain, 1998; Jung & Jacobs, 1995; Qiao, et al., 2009). Other surface coatings have also been investigated, including carbohydrates and small molecules such as DMSA (Fauconnier, Pons, Roger, & Bee, 1997) and citric acid (Bee, Massart, & Neveu, 1995) and results appear promising, although they remain in a preclinical setting. Silica is another promising surface coating because there are approved silica coated iron oxide nanoparticles which are used as oral contrast agents imaging for the gastrointestinal lumen (GastroMARK™ and Abdoscan®) (Bonnemain, 1998; Hahn, et al., 1990; William, Christophoros, Gladys, Eugene, & Pablo, 1996). The approved contrast agents, GastroMARK™ and Abdoscan®, are large iron oxide particles, approximately 300 nm in
diameter and are administered orally (Advanced-Magnetics, 2007); for other applications the size would need to be reduced. The use of silica coated iron oxide nanoparticles in targeted imaging has not been widely investigated because there has been difficulty in controlling the size of the silica shell on the surface of the iron oxide nanoparticle.

In the last few years interest in silica coatings has increased, and many studies have shown the ability to control the size of the silica shell, thereby increasing the potential for silica coated iron oxide nanoparticles to be used in targeted imaging (Deng, Wang, Hu, Yang, & Fu, 2005; Fang, et al., 2008; Im, Herricks, Lee, & Xia, 2005; Liu, Xing, Guan, Shan, & Liu, 2004; Morel, et al., 2008). Several modifications have resulted in excellent size control of the silica shell, allowing sizes between 2 nm and 100 nm (Fang, 2008; Lu, et al., 2002). It has been shown that silica is an inert material and as a surface coating can prevent aggregation of iron oxide nanoparticles in liquids, thereby improving stability (Deng, et al., 2005; Laurent, et al., 2008). Another advantage of silica is that it can bind to a range of biomarkers due to its terminal available silanol groups (Ulman, 1996). These advantages increase interest in designing and synthesising magnetic iron oxide carriers that can be used to deliver specific ligands to target organs.

An important and widely investigated biomarker is HER2, which is overexpressed in many cancers. In breast cancer, HER2 receptors are associated with increased disease recurrence and poor prognosis in patients (Slamon, et al., 1987, 1989). Approximately 20-25% of breast cancers overexpress HER2 (Slamon, et al., 1987), and for this reason the HER2 receptor has been used as a target for immunotherapy agents such as herceptin. Herceptin works by binding to the HER2 receptor on the surface of the tumour cell, causing downregulation of HER2 production, eventually causing cell death and reduction
Chapter 3 – Nanoparticle synthesis and conjugation to herceptin

in the size of the tumour (Nahta & Esteva, 2006). Herceptin has also been shown to increase the sensitivity of the tumour cells to chemotherapeutic agents (Pegram, et al., 2004), therefore its use in conjunction with a variety of chemotherapy agents has synergistic effects, improving overall survival rates and time to disease progression (Slamon, et al., 2001). Despite the positive effects of herceptin on HER2 expressing breast cancers, herceptin-based therapies eventually acquire resistance (Esteva, et al., 2002; Nahta & Esteva, 2006; Slamon, et al., 2001; Vogel, et al., 2002). The mechanisms of acquired resistance and low response rates are not fully understood but a range of possibilities has been suggested (as discussed in Chapter 1). Since it is difficult to predict which patients will respond and stop responding to herceptin therapy regimes, an area of increasing research is using imaging technologies such as MRI to predict treatment responses. The ability to image the progress of herceptin treatment using iron oxide nanoparticles as contrast agents in MRI might be useful in providing critical information about the efficacy of herceptin concurrently with other treatment.

Many studies have not used clinically available iron oxide nanoparticles but have prepared newly synthesised iron oxide nanoparticles to successfully target HER2 receptors (Artemov, Mori, Okollie, & Bhujwalla, 2003; Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005). There have been limitations, however, in the newly developed iron oxide nanoparticles in the translation from pre-clinical to clinical settings. This is primarily because those nanoparticles have the same deficiencies as commercially available iron oxide nanoparticles; that is, they do not address important characteristics such as high magnetism, size and size distribution. Hilger et al. (2007) produced iron oxide nanoparticles coated with dextran via a hydrothermal method, conjugated to herceptin. The iron oxide dextran-herceptin nanoparticles localised within HER2 positive breast
cancer cells and tumours, but there was poor signal enhancement (<20%). The low signal enhancement could be related to the hydrothermal method of iron oxide nanoparticle production, which is known to produce low magnetic properties (Laurent, et al., 2008). Similarly, other studies using dextran coated cross-linked iron oxide nanoparticles (Chen, et al., 2009) and iron oxide microbeads (Artemov, et al., 2003) targeting HER2 receptors have reported problems of low magnetisation values. A study was conducted using iron oxide nanoparticles produced via the thermal decomposition method to produce highly magnetic iron oxide nanoparticles (Huh, et al., 2005). These particles were then conjugated to herceptin via DMSA and tested in vitro and in vivo, showing that the thermal decomposition method provided highly magnetic particles for targeted MRI. Despite the advantages of this method, one of its disadvantages is the low numbers of particles produced per synthesis. Currently, the only published method that is reported to produce large amounts of iron oxide nanoparticles and not compromise the magnetic properties is that of Park et al. (2004). Park and co-authors pioneered the novel high temperature method which is based on the thermal decomposition of an iron oleate complex and is known to produce an ultra-large-scale amount of iron oxide nanoparticles (up to 40g per synthesis). Interestingly, the iron oxide nanoparticles produced by this novel production method have not been investigated for targeted MRI. Also, studies targeting HER2 receptors have used dextran or DMSA as their surface coatings; no reported studies have used silica. As a result of this preliminary work, this chapter aims to investigate whether highly magnetic iron oxide nanoparticles produced via the large scale thermal decomposition method could be utilised as a targeted contrast agent for MRI.

The specific aims of this study were to:
1. Synthesise iron oxide nanoparticles via the non-hydrothermal decomposition method and modify the surface with a conformal silica shell,

2. Confirm that the iron oxide silica nanoparticles possess the major characteristics (size, size distribution, high magnetism, biocompatibility, bonds available for conjugation to biomarkers) for use as targeted contrast agents for MRI, and

3. Conjugate the surface of the silica coated particles to herceptin for eventual targeting of HER2 receptors on breast cancer cells.
3.2 Results

3.2.1 Transmission electron microscopy (TEM)

To demonstrate the size of the magnetite nanoparticles, TEM and high resolution TEM were conducted. Figure 3.1 A shows that the iron oxide nanoparticles with no silica coating had an approximate diameter of $25 \pm 2$nm. The morphology of the core particle appeared to be spherical in nature. The many particles imaged in the solution appeared to be of the same size, indicating that the iron oxide silica nanoparticles were monodisperse. Figure 3.1 B shows the iron oxide nanoparticles coated in silica having a total diameter of $35 \pm 3$ nm. The sample distribution shows one iron oxide covered in a silica shell, again having a spherical morphology.
Figure 3.1 TEM and HR-TEM of the synthesised iron oxide silica nanoparticles. Images (A) and (B) of iron oxide nanoparticles coated in silica demonstrating their size and distribution are shown. The insets show the respective high resolution TEM images.
3.2.2 X-ray diffraction (XRD)

The iron oxide nanoparticles coated in silica were analysed for their chemical composition to confirm the formation of iron oxide via XRD. The observed spectra from the iron oxide nanoparticles is shown in Figure 3.2. This diffraction pattern is characteristic of spinel phases for iron oxide, magnetite Fe₃O₄. The pattern in Figure 3.2 marked Mag was matched to the known standard diffraction spectra pattern (JCPDS file No 75-0449) that typically arises from iron oxide, magnetite. The major peaks indexed in the known diffraction spectra were the same as the diffraction spectra seen from the iron oxide nanoparticles on their own (Figure 3.2 marked Mag). This confirmed the possibility that iron oxide nanoparticles were formed using the synthesis process in the form of magnetite, Fe₃O₄ or maghemite, Fe₂O₃. Although it is difficult to rule out the presence of maghemite, the diffraction did not indicate the presence of a large 221, 203 and 116 peak, suggesting that maghemite was not present in these samples. After the iron oxide nanoparticles were coated in silica, the diffraction spectra appeared similar (Figure 3.2 marked SiO₂@Mag) to the diffraction spectra for iron oxide (Figure 3.2 marked Mag) with the addition of distinct peak (marked *). This additional peak at about 30.5° 20 could be assigned to a mixed iron-silica phase. The overall diffraction spectra suggests that the presence of the silica has not diminished the iron oxide in any way and that most of the peaks from the magnetite nanoparticles are still visible, with multiple low angle peaks which correspond to the silica surface coating.
Figure 3.2 XRD of synthesised iron oxide silica nanoparticles

XRD patterns obtained from the iron oxide nanoparticles and iron oxide nanoparticles coated in silica are shown. The XRD peaks with corresponding to Bragg’s reflections (numbers in parentheses) of iron oxide are shown. The peak marked with * could indicate a mixed iron-silica phase.
3.2.3 Superconducting quantum interference device (SQUID)

The saturation magnetisation is the magnetic moment per unit mass. The saturation magnetisation value shows the overall magnetisation of the nanoparticles with respect to the weight of iron (emu/g). The saturation magnetisation value for the silica coated iron oxide nanoparticles was calculated using SQUID. A maximum value of 74.4 emu g\(^{-1}\) was obtained (Figure 3.3). The curve from the SQUID data shows a lack of hysteresis and remanence at ambient temperatures and suggests that the silica coated iron oxide nanoparticles were characteristic of superparamagnetic materials with magnetic domains (Figure 3.3).
Figure 3.3 SQUID measurements of synthesised iron oxide silica nanoparticles
This figure shows the saturation magnetisation value of the iron oxide silica nanoparticles measured on SQUID in emu/g calculated at ambient temperature.
3.2.4 T2 relaxation

To assess the capability of the silica coated iron oxide nanoparticles to act as a T2 contrast agent in MRI, phantom studies were conducted in triplicates of 5 μg, 10 μg, 50 μg, 100 μg of iron oxide silica nanoparticles to determine the T2 relaxation. Figure 3.4 shows the relaxation rate of the iron oxide silica nanoparticles on a 3 Tesla Clinical MRI scanner at a TE of 10.86 ms, plotted as a function of iron concentration. It can be deduced from Figure 3.4 that the T2 relaxation was 263.23 mM.s.
Figure 3.4 T2 relaxation of synthesised iron oxide silica nanoparticles

The T2 relaxation values calculated in a 3T Clinical MRI scanner are shown. T2 values are shown from various concentrations of iron oxide silica nanoparticles within aqueous phantom solutions and calculated as a function of iron concentration and relaxation rates, extracting the slope by regression. Error bars represent standard error of the mean from triplicate measurements.
3.2.5 MRI contrast enhancement

The magnetic performance of the iron oxide silica nanoparticles was known from the SQUID measurements as 74.4 emu/g and the T2 relaxation measurements as 263.23 mM.s. These measurements provided valuable information about how the iron oxide silica nanoparticles would perform as contrast agents. To measure the contrast capability of the iron oxide silica nanoparticles and put into context the SQUID and T2 relaxation measurements, phantom studies of aqueous iron oxide silica nanoparticles were conducted to evaluate the degree of negative contrast. This was measured by the signal change or T2 shortening of iron oxide silica nanoparticles (10 µg, 50 µg and 100 µg) suspended in phantoms. From Figure 3.5 it is visually evident from the respective MRI phantom images that as the concentration increases from 0 µg to 100 µg, the image progressively appears hypointense. This is confirmed by the signal enhancement data relative to the control phantom as demonstrated by drop in T2 signal intensity with increasing iron concentration. The signal change relative to the control (0 µg) confirms the negative contrast, where at 100 µg of iron oxide silica concentration a signal enhancement of approximately 90% is evident in comparison to 70% signal intensity at a concentration of 10 µg. The large MRI signal enhancement is expected from the iron oxide silica nanoparticles because of their relatively high T2 values and saturation magnetisation values.
**Figure 3.5** MRI phantom and T2 signal change of synthesised iron oxide silica nanoparticles
The changes in relative T2 MRI signal to the control of iron oxide silica nanoparticle suspended in phantoms are shown. Signal changes are shown respective to the corresponding T2 MRI images.
3.2.6 Conjugation of iron oxide silica nanoparticles to herceptin

Figure 3.6A illustrates the iron oxide silica nanoparticles conjugated to herceptin via a cystamine linker. The surface of the silica shell contains OH⁻, which attaches to the cystamine due to its available NH²⁺ group. The herceptin antibody, which has a negative charge, is then adsorbed onto the silica shell via the other available NH²⁺ group, attaching at the fragment crystallisable (Fc) region of the herceptin antibody. To confirm the adsorption and characterise the binding of herceptin onto the iron oxide silica nanoparticles, quartz crystal microbalance (QCM) experiments were performed to obtain real-time kinetic information.

The iron oxide silica nanoparticles were deposited onto the crystal where the mass change was monitored using QCM. Figure 3.6B. i shows an immediate decrease in frequency (∼28 Hz) when the iron oxide silica nanoparticles were added, with no decrease in frequency after washing. Figure 3.6B. ii shows an immediate decrease in frequency (∼10 Hz) when the cystamine was added to the iron oxide silica nanoparticles. This indicates rapid binding of the cystamine to the surface of the silica shell, of which a large proportion remained after washing. Similarly, when the herceptin antibody was deposited on the iron oxide silica nanoparticles with cystamine, a sharp decrease in its frequency (∼30Hz) was observed, indicating binding of the antibody to the iron oxide silica nanoparticles via the cystamine linker (Figure 3.6B. iii). In both cases conjugation of the respective molecules to the surface of the iron oxide silica nanoparticles was confirmed by increases in mass on the QCM which remained after successive washing steps.

The amount of protein on the particle surface was quantified using the BCA protein assay. This colorimetric assay determines the amount of protein on the particle surface, based on
a standard concentration series of the protein. Approximately, 0.66 μg of herceptin was calculated to be present on 1 μg of iron oxide nanoparticles of 35 nm in diameter.
Figure 3.6 Formation of iron oxide silica herceptin nanoparticles via a cystamine linker
(A) Shows a schematic illustration (not to scale) of the formation of iron oxide silica herceptin nanoparticles using a cystamine linker. (B) QCM data showing a characteristic representation of the physical adsorption of cystamine and herceptin to the surface of iron oxide coated silica nanoparticles. (i) iron oxide silica deposition onto the crystal, (ii) adsorption of the cystamine on the surface and (iii) adsorption of the herceptin onto the iron oxide silica via cystamine.
3.2.7 Stability of iron oxide silica herceptin nanoparticles

The stability of the iron oxide silica herceptin nanoparticles was assessed (n=3) by conjugating a FITC molecule to the herceptin antibody and exposing iron oxide silica herceptin-FITC nanoparticles to pH of 5, 7.4, 10 (3.7A) and human serum (3.7B) at various concentrations (25%, 50%, 100%) over a 24-hour period at 37°C. The degradation of the particles was calculated by an increase in fluorescence in the supernatant based on a standard concentration series of the herceptin-FITC molecules. Figure 3.7A shows the amount of protein retained across pH ranges of 5, 7.4, and 10, and Figure 3.7B shows the amount of protein retained after exposure to human serum at concentrations of 25%, 50%, and 100%. Figure 3.7A and 3.7B both show that less than 5% of herceptin-FITC could be detected within the supernatant at the different pH and human serum concentrations.
The stability of the iron oxide silica herceptin nanoparticles-FITC was assessed after incubation over a 24 hour period in various conditions and analysing unbound FITC in the supernatant based on a standard concentration series of the herceptin-FITC molecules. (A) Shows the percent of protein retained after exposure to pH of 5, 7.4 and 10. (B) Shows the percent of protein retained after exposure to human serum concentrations of 25%, 50% and 100% at 37° C. Error bars represent standard error of the mean calculated from triplicate measurements.
3.2.8 Iron oxide silica-herceptin nanoparticle cytotoxicity

Previous studies indicate that iron oxide nanoparticles are mildly toxic at high concentrations. To explore the biocompatibility profile of the iron oxide silica-herceptin nanoparticles, MTS-based in vitro cytotoxicity experiments were performed on SKBR3 breast cancer cells in vitro (n=3). Figure 3.8 shows the results of MTS-based cytotoxicity testing performed on SKBR3 cells after 24 and 48 hour incubation with iron oxide silica-herceptin nanoparticles. It is evident that the iron oxide silica-herceptin nanoparticles at 24 hours did not significantly affect SKBR3 cell viability up to 50 µg/ml (P>0.05) of iron oxide silica herceptin nanoparticles, where a cell viability of 75% can be measured. However, as the concentration of iron oxide silica nanoparticles increased to 100µg/ml (P<0.001) a significant toxic effect can be seen when the viability of the SKBR3 decreases to 65%. After 48 hours incubation of the iron oxide silica-herceptin nanoparticles, the viability of SKBR3 cells is significantly affected where, a concentration of 50 µg/ml (p<0.01) of iron oxide silica herceptin nanoparticles to reduces the viability to 60%. Similarly, at a concentration of 100 µg/ml (p<0.001) of iron oxide silica herceptin nanoparticles a significant toxic effect can be seen, where a viability of only 50% was calculated.
Figure 3.8 MTS cell viability of iron oxide silica-herceptin nanoparticles
Viability of human breast cancer cells SKBR3 after incubation with iron oxide silica-
herceptin nanoparticles assessed at 24 and 48 hours (shown as % viability) by MTS assay. 
Error bars represent standard error of the mean from triplicate measurements. * = p<0.05, 
** = p<0.01, *** = p<0.001 one-way ANOVA and Dunnett’s.
3.3 Discussion

The prerequisite to enable iron oxide nanoparticles to be used as molecular and cellular targeted contrast agents is the fabrication of materials with the following properties: high magnetism, a size less than 60 nm, small size distribution and biocompatibility. These characteristics are all important as they can influence the MRI signals and cellular uptake of the contrast agent (Gupta & Gupta, 2005; Laurent, et al., 2008). Hydrothermal methods produce particles with low crystallinity, low magnetisation values and limited control over size and size distribution, and therefore the non-hydrothermal high temperature method outlined by Park et al. (2004) was used in this study to synthesise iron oxide nanoparticles.

For this method, the iron oxide nanoparticles were synthesised by thermally decomposing an iron oleate complex using a high boiling solvent i.e. sodium oleate and metal chlorides. The iron oxide nanoparticles produced appeared to be spherical, monodisperse, and contained a core size of approximately 25 ± 2 nm in diameter as shown in TEM data (Figure 3.1A). The iron oxide nanoparticles at this point were coated with oleic acid and therefore were insoluble in water. To make them water soluble and biocompatible and to provide an attachment for biomarkers, the iron oxide nanoparticles were coated in silica. As a coating for iron oxide nanoparticles, silica has previously been demonstrated to provide biocompatibility and particle stability as well as a facile surface for further bio-functionalisation in different nanomaterials (Mulvaney, Liz-Marzan, Giersige, & Ung, 2000; Sun, et al., 2005). The silica coating of the iron oxide nanoparticles formed around the core iron oxide shell and the iron oxide silica nanoparticle with a total diameter approximately 35 ± 3 nm silica (Figure 3.1B and inset). The small size of the nanoparticles was important, as nanoparticles larger than 60 nm can be taken up by the RES, making it
impossible for the them to localise at the targeted site. The iron oxide nanoparticles that were synthesised in this study were less than 60 nm in size, making them suitable to be utilised for targeted imaging in MRI.

The silica shell on the surface of the iron oxide nanoparticles was formed by magnetite particles acting as nucleation sites during the hydrolysis of TEOS via the alkaline solution produced by the addition of ammonia within the ethanol and water solvent. From the TEM image in Figure 3.1B, the silica shell appeared to be thin compared to other studies where diameters of up to 100 nm were produced (Fang, 2008; Lu, et al., 2002). The advantage of this method was that it allowed control of the shell thickness by virtue of the continuous addition of silica to the solution by simply changing the hydrolysis duration. The addition of a coating material can sometimes affect the shape of iron oxide core particles, but in this process the iron oxide nanoparticles retained their original morphology and were individually coated with a silica shell. Only a small number of the nanoparticles in the sample were found to contain two or three iron oxide nanoparticles within a silica coating. This issue is inherent in a typical chemical synthesis route of coating nanoparticles with a surface layer and is not always acknowledged in the literature. However, it can be controlled easily by limiting the concentration of iron oxide nanoparticle seeds during the coating process.

Observations were made throughout the study that the iron oxide nanoparticles remained stable without producing any aggregates either in solution (phosphate buffered saline, PBS) or as a powder for up to 6 months. TEM images in Figure 3.1 show that the morphology of the iron oxide nanoparticles after 6 months was similar to that immediately after synthesis. This data supports the literature which suggests that a silica coating over
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Iron oxide nanoparticles can significantly improve long-term stability, in particular reducing aggregation of the nanoparticles (Mulvaney, et al., 2000; Sun, et al., 2005). Long-term particle stability is a crucial factor for iron oxide nanoparticles, particularly for use in the clinical setting. Another notable factor in the synthesis method was the ability to produce more than 10g of iron oxide nanoparticles within one synthesis, as earlier reported Park et al. (2004). The ability to produce ultra-large scale batches of iron oxide nanoparticles is of particular advantage, especially in translating iron oxides to the clinical setting where an average patient could require up to 20 mg/kg of iron oxides. Most synthesis methods cannot produce significant amounts of monodisperse particles without tedious size separation processes which compromise the shape and magnetism of the nanoparticles (Gupta & Gupta, 2005).

The XRD patterns (Figure 3.2) of the iron oxide nanoparticles indicated that the core particles were characteristic of spinel phases of iron oxides. The pattern could also be indexed based on the standard diffraction pattern typically arising from magnetite with the major peaks indexed (JCPDS file No 75-0449). Six crystal planes took on reflection, 220, 311, 400, 422, 511 and 440 (Figure 3.2). It is well known that in all preparations of iron oxide nanoparticles, maghemite, a lower form of iron oxide, is commonly present among magnetite, the preferred form of iron oxide (Jung & Jacobs, 1995). Both forms of iron oxide have comparable line positions and intensities on XRD and it can therefore be difficult to preclude the presence of maghemite unless the minor differences between the XRD patterns of maghemite and magnetite are closely investigated. In the iron oxide nanoparticles produced in this study, a minor detail such as the prominence of the 211 iron oxide magnetite peak in the XRD pattern (Figure 3.2) could be indicative that a separate cubic maghemite is not present. In further support of this statement, maghemite is known
to have two reflections at 23.9 (203) and 26.1 (116) (Jung & Jacobs, 1995). In the XRD pattern of the iron oxide nanoparticles produced these weak maghemite lines did not appear, thus suggesting that maghemite may not be present. However, an expanded diffraction pattern would be needed to confirm this. After the iron oxide nanoparticles were coated in silica, the diffraction pattern peaks arising from the iron oxide nanoparticles could still be detected, confirming that the addition of the silica layer did not affect the iron oxide core. An interesting observation in the XRD pattern after the addition of silica is the presence of an additional peak at ca. 29.5°. This additional peak could be assigned to a mixed iron oxide-silica phase (111 silica phase) possibly formed at the interface of silica and magnetite during core-shell synthesis of the iron oxide silica nanoparticles. There is no reported literature on this additional peak and therefore the origin of this mixed iron oxide-silica phase is not clear at this stage. Subsequent investigation to analyse the additional peak within this study was not conducted, as it was beyond the scope of this project. Subsequent analysis of this peak is warranted to assess its significance.

With respect to the magnetisation properties of the iron oxide silica nanoparticles, the specific magnetisation and the T2 values determine how the iron oxide silica nanoparticles would perform as contrast agents. In Figure 3.3, the SQUID data shows a lack of hysteresis and remanence and suggests that the silica coated iron oxide nanoparticles are characteristic of superparamagnetic material with magnetic domains. The superparamagnetic performance of these particles is similar to that in other studies that have reported superparamagnetic properties of iron oxide nanoparticles (Jung & Jacobs, 1995; Zhang et al., 2009). From the SQUID data (Figure 3.3), the magnetite silica nanoparticles have a saturation magnetisation value of 74.4 emu g⁻¹. Compared to the
value in currently available iron oxide nanoparticles such as commercial Resovist®, this value is slightly higher than Resovist’s® reported mass magnetisation value of 72.9 emu g⁻¹. Other studies that have prepared iron oxide nanoparticles via thermal decomposition, using DMSA as a surface coating, have reported saturation magnetisation values of up to 80 emu/g (Huh, et al., 2005). The higher saturation magnetisation value of the iron oxide silica nanoparticles compared to Resovist® could be attributed to the method of production, in that the non-hydrolytic thermal decomposition method is known to produce particles with higher crystallinity and small size distributions, therefore superior magnetic properties (Laurent, et al., 2008). The reason for the reported higher saturation magnetisation value of the DMSA coated iron oxide nanoparticles (Huh, et al., 2005) compared to the iron oxide nanoparticles prepared in this study via the thermal decomposition method is at this stage unknown. It was expected that because both methods involve thermal decomposition the values would be similar; differences could be related to the surface coating, variations in the method of production, and the use of different surfactants.

It is essential, especially in translational research, for contrast agents to possess high magnetism, especially high saturation magnetisation values or a high magnetic moment. This is because the contrast enhancement effects are directly related to the saturation magnetisation values (Jang, et al., 2009; Sun, et al., 2005). The transverse relaxation, T2 or spin-spin relaxation of the protons is roughly proportional to the square of the saturation magnetisation value; therefore higher saturation magnetisation values will cause faster T2 relaxation and generate a reduction in the signal intensity (Jang, et al., 2009). A high MRI contrast is also significant for clinical purposes, as the probe dosage levels can be reduced when using highly magnetic nanoparticles.
The T2 relaxivity value of the iron oxide silica nanoparticles was reported to be 263.23 mMs (Figure 3.4). A high T2 relaxation was expected due to the high saturation magnetisation values seen in Figure 3.3. In MRI, the relaxation rate is a measure of the efficiency of the contrast agent to enhance the proton relaxation and increase image contrast. Therefore, a high T2 relaxation would cause surrounding water protons to relax more quickly, generating maximum possible contrast. When compared to other iron oxide nanoparticles, the T2 relaxation of the iron oxide silica nanoparticles synthesised in this study was shown to be higher. The commercially available Resovist® nanoparticles have reported T2 values of 151.0 mM.s, and other iron oxide studies have reported values of 200 mM.s even with a saturation magnetisation value of 80 emu/g (Chen, et al., 2009). In that case, the low T2 value could be associated with many factors, one being the influence of surface coatings possibly causing mild aggregation of the nanoparticles in solution, *in vitro* and/or *in vivo*. For the iron oxide silica nanoparticles produced in this thesis, it is known that silica is an inert material and as a surface coating can prevent aggregation of the iron oxide nanoparticles in liquids, allowing them to act as single magnetic domains (Gupta & Gupta, 2005; Laurent, et al., 2008). Another suggested reason for the low T2 values seen in previous studies could also be that the hydrothermal method of iron oxide production, as previously discussed, produced particles with less strong magnetic properties. No T2 values have been reported by other studies that have prepared iron oxide nanoparticles via the thermal decomposition method, but Jun et al. (2007) and Huh et al. (2005) have provided the saturation magnetisation values discussed above. The higher T2 values of the iron oxide silica nanoparticles reported in this study can provide a significant advantage over commercial MRI contrast agents and over synthesised iron oxide...
nanoparticles in previous studies, facilitating faster relaxation of surrounding water protons, along with an expected significant enhancement in MRI image contrast.

A large MRI signal enhancement is expected from the iron oxide silica nanoparticles, because of the relatively high T2 values and saturation magnetisation values. To measure the performance of the iron oxide silica nanoparticles in generating contrast, phantom studies were conducted to evaluate the degree of signal intensity change or negative contrast relative to the control. This was conducted by measuring the T2 shortening at 10, 50 and 100 µg of iron oxide silica nanoparticles. From Figure 3.5 it was evident that as the amount of iron oxide silica nanoparticles increased from 0 µg to 100 µg, the MR signal change relative to the control progressively appeared hypointense. The signal change confirmed the negative contrast, where at 100 µg of iron oxide silica concentration a signal enhancement of approximately 90% was shown compared to 70% signal intensity at a concentration of 10 µg. As there are no previous studies that show signal enhancement data for phantom studies, it is difficult to compare the performance of our iron oxide silica nanoparticles to other iron oxide nanoparticles. However, previous researchers have reported that their iron oxide nanoparticles provided increased negative contrast as indicated by a drop in the T2 value (Hilger, et al., 2007). Indirectly, the drop in the T2 value is related to a darkening effect or contrast enhancement on MRI. So, although the signal enhancement values were not calculated, the relative T2 value drop can be used as an indication to compare performance. The pattern of signal intensity changes seen with the increase in concentration of the iron oxide silica nanoparticles in this study is similar to the pattern of T2 signal reduction in previous studies (Hilger, et al., 2007).
Since the iron oxide silica nanoparticles possessed high magnetic properties, good contrast capability, small size distribution, water-solubility and biocompatibility, the claim as to their suitability for targeted contrast imaging was valid and was therefore examined. This was achieved by conjugation of the iron oxide silica nanoparticles to herceptin, which binds specifically to HER2 receptors. HER2 receptors are found on many cells, but they are overexpressed in HER2 positive breast tumours. The schematic illustration in Figure 3.6A demonstrates the formation of iron oxide silica nanoparticles conjugated to herceptin.

The hydroxyl groups present on the surface of the silica confer a net negative charge on the overall nanoparticle, thereby allowing one of the positive amine groups on the cystamine linker to electrostatically bind. The remaining free amine group of the cystamine linker was used to adsorb the herceptin antibody, as confirmed by QCM (Figure 3.6A). From the rapid decrease in frequency in Figure 3.6A it could be deduced that the iron oxide silica nanoparticles were conjugated to the cystamine linker even after several washing cycles (Figure 3.6B i). Subsequently, Figure 3.6B ii also showed an immediate decrease in frequency upon deposition of cystamine on the iron oxide silica nanoparticles, and a decrease in frequency upon deposition of herceptin, which indicates binding of the antibody to the silica shell via cystamine. The binding of herceptin to the silica shell is most likely due to electrostatic forces between positive charges on the remaining amine group of the cystamine and the negatively charged Fc terminal of herceptin. In the literature it is suggested that the electrostatic binding can also occur at the isoelectric point of the antibody, as the pH of the incubation medium (pH 7.4) is close to the isoelectric point of the herceptin, which is known to favour adsorption onto the surface of the nanoparticles (Tsai, Mehta, & DeLuca, 1996). The results from this study are similar to other studies that have shown irreversible binding of nanoparticles to antibodies via physical adsorption, quantified by QCM (Cortez, et al., 2006).
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It is known that optimal bioconjugation should involve the covalent attachment of the antibody only through the Fc region, leaving Fab sites available for receptor recognition (Arruebo, Valladares, & González-Fernández, 2009). The advantage in using covalent linkages compared to adsorption lies in avoiding possible competitive displacement of the adsorbed antibodies by blood components (Arruebo, et al., 2009; Kocbek, Obermajer, Cegnar, Kos, & Kristl, 2007). Several studies have investigated whether displacement of the antibodies does occur, and have looked at the feasibility of antibodies being absorbed onto nanoparticles, with differing results (Cortez, et al., 2006; Illum, Jones, Baldwin, & Davis, 1984; Illum, Jones, Kreuter, Baldwin, & Davis, 1983; Kocbek, et al., 2007; Tengvall, Lundström, & Liedberg, 1998). Further investigations need to be carried out to confirm whether the adsorption of antibodies onto the surface of nanoparticles is competitively displaced by blood components in vitro and in vivo. Despite this, some researchers favour the adsorption of antibodies onto the surfaces of nanoparticles over covalent linkages. They indicate that covalent binding occurs randomly at multiple sites and can possibly impair the Fab binding sites (Cortez, et al., 2006; Kocbek, et al., 2007; Nobs, Buchegger, Gurny, & Allémann, 2004). Thus when biomarkers covalently attached to nanoparticles are utilised for targeting, small amounts of the nanoparticle complex could arrive at the targeted site. Kocbek et al. (2007) reported that covalent binding using EDC directly onto antibodies adversely affected the recognition properties of antibodies for the target antigen compared to adsorption, occurring at the Fc region. This could be due to proteins having numerous functional groups, as several side reactions could take place in the presence of EDC. They concluded that adsorption of antibodies was preferable to covalent bonding. Similarly, Cortez et al. (2006) reported successful in vitro targeting of human colorectal cancer cells using PAH/PSS nanoparticles electrostatically bound to
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huA33 antibodies. Their study demonstrated the irreversible binding of huA33 antibodies on PAH/PSS nanoparticles using QCM, and they concluded that their conjugated nanoparticles could be used as effective drug delivery molecules.

For in vitro and in vivo studies, stability of the iron oxide silica herceptin nanoparticles is a priority that needs to be addressed particularly for adsorbed complexes. To address stability concerns, the iron oxide silica herceptin nanoparticles were incubated over a 48 hour period at various pH and human serum values (Figure 3.7). From the stability data it was demonstrated that the particles remained bound to the herceptin antibody over a 48-hour period in various pH strengths together with different concentrations of human serum. Stability testing (Figure 3.7) along with the QCM data (Figure 3.6) indicated that the iron oxide silica herceptin nanoparticles were bound through electrostatic interaction. The stability results for the iron oxide silica nanoparticles conjugated to herceptin were similar to those of other studies that have reported stable adsorbed nanoparticles conjugated in various pH levels, indicating that competitive displacement in vivo was unlikely but could not be ruled out (Cortez, et al., 2006).

Apart from magnetic properties and size considerations, iron oxide silica herceptin nanoparticles need to be biocompatible to be used at the preclinical and clinical level. The biocompatibility of iron oxide and silica has already been proven and considerable evidence exists showing that iron oxide nanoparticles are non-toxic at lower concentrations and can be mildly toxic at higher concentrations (Gupta & Gupta, 2005; Laurent, et al., 2008). To explore the biocompatibility profile of the iron oxide silica herceptin nanoparticles, cytotoxicity assays were performed on SKBR3 cancer cells in vitro (Figure 3.8). Results showed that the iron oxide silica herceptin nanoparticles did not
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affect SKBR3 cell viability up to 50 µg of iron, where more than 75% cell viability was demonstrated. However, further increases in iron oxide silica herceptin nanoparticles equivalent to 100 µg resulted in a cell viability loss of 35%. The loss in cell viability could be an indication of the action of herceptin inhibiting the growth of SKBR3 cells that are overexpressing HER2 receptors. Other studies that have assessed iron oxide nanoparticles conjugated to herceptin have reported similar results, with higher concentrations that were potentially toxic to the cells being consistent with the function of herceptin (Chen, et al., 2009).

3.4 Conclusion

The aim of this chapter was to produce iron oxide nanoparticles coated in silica via the thermal decomposition method and to investigate their characteristics to warrant their use as a targeted contrast agent for MRI. Investigations confirmed that the iron oxide silica nanoparticles had the major characteristics necessary for use as a targeted contrast. These characteristics were high magnetic properties, small size and size distribution, biocompatibility, and the ability to be conjugated to a biomarker. The study also compared literature on commercial iron oxide nanoparticles and synthesis methods and the findings suggested that the iron oxide silica nanoparticles prepared via this method had superior properties and therefore were potentially more suitable for targeted contrast applications. In conclusion, these results support the use of iron oxide silica herceptin nanoparticles for targeted MRI.
Chapter 4 Assessment of the Iron Oxide Silica Herceptin Nanoparticles

*in vitro*

4.1 Introduction

The experimental results in Chapter 3 suggested that the iron oxide nanoparticles produced exhibited the major characteristics for use as targeted contrast agents for MRI. These characteristics are size, size distribution, high magnetism, biocompatibility, bonds available for conjugation to biomarkers, and stability. These nanoparticles were coated in a silica layer to improve water solubility, particle stability and also to present a facility for attachment of biomarkers. Furthermore, the iron oxide silica nanoparticles were conjugated to a biomarker called herceptin, which is specific for targeting HER2 receptors, present in human breast cancer. The findings of Chapter 3 suggested that the synthesised iron oxide silica herceptin nanoparticles had the potential to be utilised as targeted imaging agents for MRI. These results from the assessment of the physical characteristics warranted further investigation in a biological system. The aim of this chapter was to investigate the ability of iron oxide silica herceptin nanoparticles to target HER2 expressing human breast cancer cell lines *in vitro*.

Approximately 20-30% of breast cancers overexpress HER2, which is associated with increased disease recurrence and poor prognosis (Slamon, et al., 1987). Herceptin is a well-known humanised monoclonal antibody directed at the epidermal growth factor receptor HER2, which is found in HER2 positive breast cancers (Slamon, et al., 1987). Herceptin is one of the few targeted immunotherapies currently used in breast cancer patients in adjuvant and neoadjuvant settings. The mechanism of action of herceptin is thought to be mediated by antibody-antigen specific binding to the HER2 receptors followed by internalisation via receptor mediated endocytosis (Baselga, et al., 2001). It has
also been reported that herceptin induces cell cycle arrest due to downregulation of HER2 leading to decreased cell proliferation, inhibition of angiogenesis, increased cytotoxicity and eventual cell death (Carter, et al., 1992; Hudziak, et al., 1989; Shepard, et al., 1991). The action of herceptin has been reported to cause increased cellular sensitivity, and when used in combination with chemotherapy therapy it can produce synergistic effects (Konecny, et al., 1999). Clinical trial data has shown positive responses to herceptin in HER2 positive breast cancer patients in combination with various chemotherapy drugs in early and late stages (Pegram, et al., 1999; Slamon, et al., 2001; Vogel, et al., 2002). However, evidence from phase 2 clinical trials has also shown response rates to herceptin alone ranging from 11% to 26%, suggesting that the majority of patients with HER2 expressing tumours demonstrate intrinsic resistance to herceptin alone (Baselga, et al., 1996; Cobleigh, et al., 1999; Vogel, et al., 2002). Furthermore, while adjuvant and neoadjuvant therapy result in higher response rates, it has been reported that the duration of response to herceptin therapy either alone or in combination with chemotherapy ranges from 5 to 9 months (Seidman, et al., 2001; Slamon, et al., 2001). The mechanism of acquired resistance is not fully understood and there are several mechanisms that could contribute to this process (Nahta & Esteva, 2006). While many new agents are being developed and tested, identification of patients who could stop or have stopped responding to herceptin and/or herceptin-regimes is important (Nahta & Esteva, 2006). The use of MRI and iron oxide silica nanoparticles conjugated to herceptin could potentially help identify these patients.

From the QCM data in Chapter 3, it can be assumed that the herceptin antibody is attached to the surface of the iron oxide silica nanoparticles. As herceptin is specific for HER2 receptors, it is possible that the iron oxide silica herceptin nanoparticles can target HER2
positive receptors in the same way that herceptin individually interacts with the receptor. Previous studies conducted with iron oxide nanoparticles but with different surface layers conjugated to herceptin have indicated that the nanoparticles were able to target the HER2 receptors \textit{in vitro} and \textit{in vivo} (Artemov, et al., 2003; Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005). These previous studies observed the uptake of the nanoparticles conjugated to herceptin within different cell lines of varying levels of HER2 expression and suggest that the nanoparticles were taken up relative to the HER2 expression levels (Artemov, et al., 2003; Chen, et al., 2009; Huh, et al., 2005). Targeting cells with different levels of HER2 expression provides a good indication of the sensitivity of the nanoparticle-herceptin detection system as well as of whether the nanoparticles are taken up specifically at HER2 receptor sites. A range of other studies that have not used iron oxide nanoparticles but have used other nanoparticles such as manganese doped (Lee, et al., 2007), human serum albumin (HSA) (Steinhauser, Spänkuch, Strebhardt, & Langer, 2006), poly(lactic-co-glycolic acid) (PLGA) (Sun & Feng, 2009) and fluorescent probes labelled to herceptin (Liang, et al., 2010) have demonstrated uptake at HER2 receptor sites. Based on these studies, the uptake of herceptin conjugated nanoparticles by HER2 expressing cells is well known. To confirm whether the nanoparticles synthesised in the previous chapter performed similarly, the current study investigated uptake of the iron oxide silica herceptin nanoparticles in HER2 expressing cells. This chapter also aimed to measure the uptake of the iron oxide silica herceptin nanoparticles in cells with varying levels of HER2 expression.

Once the nanoparticles were localised within the cells it was not necessary to characterise the mechanism of their uptake, which was detailed earlier within this chapter. It was important, however, to determine the impact of dosage and time on the uptake of the
nanoparticles within the various cell lines. Investigations of the effect of different dosages and time points of herceptin-nanoparticles were designed to identify whether the uptake was related to dose, time and/or HER2 expression levels of the cells. It was anticipated that this would provide evidence that the conjugation of the nanoparticles to herceptin could specifically target HER2 positive cells. This was particularly important for the potential translation of the nanoparticles conjugated to herceptin to the clinical setting. Few previous dose and time course studies have involved iron oxide nanoparticles conjugated to herceptin for MRI. Wartlick et al. (2004) utilised HSA nanoparticles conjugated to herceptin to demonstrate that the binding was related to HER2 expression levels as well as dosage. Several time course studies have shown that 30 minutes after incubation of conjugated nanoparticles, specific uptake was seen, between 30 minutes and 5 hours (Steinhauser, et al., 2006; Wartlick et al., 2004). These uptake studies have also suggested that non-specific uptake could be occurring after 24 hours (Steinhauser, et al., 2006; Wartlick, et al., 2004). To ensure maximum uptake of the iron oxide silica herceptin nanoparticles within HER2 positive breast cancer cells, this study sought to investigate dose response and time course phenomena.

Assessment of the nanoparticles \textit{in vitro} plays an important role in estimating contrast enhancement biologically. In phantom studies the iron oxide nanoparticles might demonstrate good T2 relaxivity and produce contrast enhancement, but \textit{in vitro} and \textit{in vivo} these particles can have reduced contrast enhancement and lower T2 values. The mechanism of this phenomenon is unknown. Some theories suggest the confinement of nanoparticles within the endosome of the target cells as the reason (Billotey, et al., 2003). After subcellular compartmentalisation of the nanoparticles there can be a build-up of magnetic field inhomogeneities (Billotey, et al., 2003; Kato et al., 1999). This was found
to be absent in phantoms where the nanoparticles were uniformly distributed (Billotey, et al., 2003; Kato, et al., 1999). In particular, the geometrical arrangement of the nanoparticles as a result of clustering within the cells is expected to make an important contribution to this effect. Despite the different sizes of nanoparticles and cell systems used, several groups have shown that the relaxivities of native iron oxide nanoparticles in phantoms were higher than those after accumulation in cells (Billotey, et al., 2003; Hilger, et al., 2007; Simon, et al., 2006). The iron oxide silica nanoparticle phantoms in Chapter 3 were found to produce significant contrast enhancement. To ensure that high levels of contrast were still evident in an *in vitro* system, contrast enhancement within cells needed to be investigated to demonstrate whether this had any clinical utility.

To assess the *in vitro* performance of the synthesised nanoparticles, a comprehensive cell uptake study was designed and conducted on HER2 positive breast cancer cells. The aim of this study was to viewing the uptake of the herceptin-nanoparticles, including identifying the effect of dosage and time. Furthermore, the contrast enhancement effect of the iron oxide silica herceptin nanoparticles after localisation within HER2 positive cells needed to be addressed, due to previous reported problems related to reducing contrast. As a result, the study aimed to view the uptake of the synthesised iron oxide silica herceptin nanoparticles in various breast cancer cells lines of different HER2 expression.

The specific aims of this chapter were to:

1. Confirm and compare the uptake of iron oxide silica herceptin nanoparticles by breast cancer cells lines (SKBR3, BT474 and MCF7) *in vitro*;
2. Quantify the uptake of iron oxide silica herceptin nanoparticles by dose and time course studies using three breast cancer cell lines (SKBR3, BT474 and MCF7) \textit{in vitro}; and

3. Observe the ability of the synthesised nanoparticles to generate MRI contrast enhancement from within breast cancer cell lines expressing different levels of HER2 (SKBR3, BT474, MCF7).
4.2 Results

4.2.1 HER2 expression levels in different breast cancer cells

Before the dose- and time-dependent uptake of iron oxide silica herceptin nanoparticles was assessed in human breast cancer cells lines, it was important to assess the expression levels of HER2 on each cell line, as long-term culturing can affect surface receptor expression. Three human breast cancer cell lines, SKBR3, BT474 and MCF7, were used. Among these it has been documented that SKBR3 has the highest level of HER2 expression, followed by BT474 and MCF7 (Chen, Yeung, & Wang, 2000). Characterisation of HER2 expression via flow cytometry surface expression staining showed that the SKBR3 cell line had the highest level of HER2 expression, with a simulation index of staining above 7. The BT474 cell line had a stimulation index of staining above 4 and the MCF7 cell line had the lowest stimulation index of staining, of 2 (Figure 4.1).
Figure 4.1 Stimulation index of HER2
Flow cytometry results represent the HER2 surface expression levels via a stimulation index. The results show the surface expression of the three breast cancer cell lines, SKBR3, BT474, and MCF7. Stimulation index was calculated by mean fluorescence intensity of HER2 expression/mean fluorescence intensity isotype control. Error bars represent standard error of the mean calculated from triplicate measurements.
4.2.2 Dose-dependent uptake of iron oxide silica herceptin nanoparticles by human breast cancer cells in vitro

Herceptin-mediated cellular binding and uptake of the iron oxide silica herceptin nanoparticles were investigated by flow cytometry using 5, 10, 50 and 100 μg of nanoparticles after a 24 hour incubation time. SKBR3, BT474 and MCF7 cells were used to test the binding and uptake efficacy of the iron oxide silica herceptin nanoparticles relative to HER2 expression levels. The binding of iron oxide silica herceptin nanoparticles appeared to be related to the HER2 cell expression levels (Figure 4.2). SKBR3 cells had the highest iron oxide silica herceptin nanoparticle uptake, the stimulation index indicating that uptake was approximately 150 at 5 μg and increased to 200 at 100 μg (Figure 4.2). As indicated by the stimulation index the uptake of the BT474 and MCF7 cell lines was 15 and 10 respectively, much less than that of the SKBR3 cell line (Figure 4.2). This result correlated with the surface expression data in Figure 4.1 that showed the HER2 surface expression levels by a stimulation index of staining. In Figure 4.1, the SKBR3 cell presented with a level 1.5 times higher than the BT474 cells and approximately 3 times higher than the MCF7 cells. This suggested that uptake levels should be higher for SKBR3 cells than for BT474 and MCF7 cells. Comparing the BT474 and MCF7 cell lines, the uptake appeared to increase gradually as the amount of nanoparticles increased from 5 to 100 μg. Uptake in BT474 cells appeared to be higher, with a stimulation index of 10 at 10 μg and 25 at 100 μg, compared to 5 at 10 μg and 18 at 100 μg for the MCF7 cells. Figure 4.1 also showed maximal uptake of iron silica oxide herceptin nanoparticles at 5 μg, and there was no further increase in uptake as the concentration increased. This representation could possibly indicate that the iron oxide silica herceptin nanoparticles were attached to most of the cells at 5 μg, although
saturation might not have been reached as there was a continued increase in uptake as the concentration increased.
Figure 4.2 Dose-dependent uptake of iron oxide silica herceptin nanoparticles by human breast cancer cell lines

The amount of uptake of iron oxide silica herceptin nanoparticles calculated as stimulation index of staining above the control for herceptin + αhuman FITC. The graph shows the dose-dependent uptake after an incubation time of 24 hours of the iron oxide silica herceptin nanoparticles in each of the cell lines, SKBR3, BT474 and MCF7. Error bars represent standard error of the mean calculated from triplicate measurements.
4.2.3 Time-dependent uptake of iron oxide silica herceptin nanoparticles by human breast cancer cells *in vitro*

To assess the uptake kinetics of the iron oxide silica herceptin nanoparticles by HER2 positive breast cancer cells, time-dependant uptake was investigated using flow cytometry. 50 μg of iron oxide silica herceptin nanoparticles was incubated for various time points. Figure 4.3 showed that the binding of the iron oxide silica herceptin nanoparticles to the breast cancer cell lines was related to the HER2 cell expression levels. The SKBR3 cells took up more of the iron oxide silica herceptin nanoparticles than either the BT474 or MCF7. This was in accordance with the surface expression data presented in Figure 4.1 and also the dose response data in Figure 4.2. The SKBR3 stimulation index of staining was 80 at 0.5 hours and increased to 100 at 24 hours (Figure 4.3). The uptake of BT474 cells was higher than that of MCF7 cells, where at 0.5 hours uptake was similar but at 3 hours and 24 hours, the stimulation index of staining was 15 and 10 at 3 hours and 25 and 15 at 24 hours, respectively. Uptake of iron oxide silica herceptin nanoparticles by all the breast cancer cells was evident as early as 30 minutes and continued to increase up to 5 hours (Figure 4.3). Uptake was monitored to 24 hours, and the kinetics of uptake increased significantly between 5 and 24 hours, consistent with non-specific passive uptake of the nanoparticles as suggested by other time course studies (Steinhauser, et al., 2006).
Figure 4.3 Time-dependent uptake of iron oxide silica herceptin nanoparticles by breast cancer cells
The amount of uptake of iron oxide silica herceptin nanoparticles calculated stimulation index of staining above the control for herceptin + αhuman FITC. The graph shows time-depandant uptake of the iron oxide silica-herceptin nanoparticles incubated at a concentration of 50 μg for each of the cell lines, SKBR3, BT474 and MCF7. Error bars represent standard error of the mean calculated from triplicate measurements.
4.2.4 Cellular uptake via confocal microscopy

For iron oxide silica herceptin nanoparticles to be imaged on MRI, the imaging agent must not only bind to the HER2 receptors present on the cell membrane but must also gain entry into cells via active endocytosis so that it is internalised. Cellular internalisation of the iron oxide silica herceptin nanoparticles was investigated using SKBR3 cells using FITC-herceptin conjugated iron oxide silica nanoparticles at a concentration of 50 μg for 3 hours. SKBR3 cells were imaged using confocal microscopy to visualise green fluorescence to indicate cellular internalisation. A Hoechst nuclear stain was also added to the cells to indicate the presence of live cells. Uptake of iron oxide silica herceptin nanoparticles was observed within the cells as shown in Figure 4.4. In all cells imaged, a bright green rim was seen around the SBKR3 cells, demonstrating binding to the cell surface. These appeared as spots or clusters on or just beneath the surface membrane (Figure 4.4B), indicating the binding of the FITC-herceptin iron oxide silica nanoparticles to HER2 receptors. The green fluorescence was further seen throughout the cell membrane for more of the cells shown in Figure 4.4B. It is well known that HER2 receptors are on the surface of SKBR3 cells as well as extending through the cell membrane (Cho, et al., 2003). The FITC-herceptin iron oxide silica nanoparticles appeared to be confined to the cytoplasm and did not localise in the nucleus, as seen in Figure 4.4A and B. Furthermore, the presence of the FITC-herceptin iron oxide silica nanoparticles could be located in the phase-contrast images (Figure 4.4C), supporting the theory that the iron oxide silica nanoparticles were taken up by the cells and did not just remain on the surface of the cells. The data generated here supports the dose and time course uptake studies suggesting that HER2 expressing breast cancer cell lines were able to take up and internalise the iron oxide silica herceptin nanoparticles which were specific to the HER2 surface expression levels.
Figure 4.4 Cellular internalisation of the FITC conjugated iron oxide silica herceptin nanoparticles by SKBR3 cells
Cellular internalisation of the herceptin-FITC iron oxide silica nanoparticles at a concentration of 50μg for a period of 3 hours in SKBR3 cells is illustrated by confocal microscopy. (A) Hoechst (nuclear) staining of the SKBR3 cells; (B) FITC-herceptin conjugated iron oxide silica nanoparticle uptake in SKBR3 cells; and (C) phase contrast images of iron oxide silica herceptin nanoparticles.
4.2.5 MRI contrast enhancement by human breast cancer cells

The uptake of iron oxide silica herceptin nanoparticles by human breast cancer cells was confirmed in in vitro studies as shown in Figures 4.2, 4.3 and 4.4. For these nanoparticles to be used as MRI contrast agents, the particles must be able to demonstrate signal enhancement related to the uptake of the nanoparticles. Since SKBR3, BT474 and MCF7 have different levels of HER2 surface expression, it was anticipated that the iron oxide silica herceptin nanoparticles would be taken up relative to these expression levels and on MRI display respective contrast enhancement. SKBR3, BT474 and MCF7 cells were incubated with 50 μg of iron oxide silica herceptin nanoparticles for a period of 3 hours and controls consisting of the respective breast cancer cell line were incubated with water. MRI images were acquired in live cells after washing each cell line several times with water. MRI images of the three cell lines are shown in Figure 4.5. Figure 4.5A is the control image for each cell line and Figure 4.5B is the cell line incubated with the nanoparticles. When the control images for each cell line (4.5A) are compared with the images of the cell incubated with nanoparticles (4.5B), contrast enhancement can be seen relative to their respective control. Comparing each of the cell lines to their controls, the MRI image appears hypointense. Comparing the three cell lines in Figure 4.5B, the SKBR3 cell line appears to be more hypointense than the BT474 and MCF7 cell lines. To confirm this, a signal enhancement relative to control graph was generated using the T2 signals from the control and nanoparticle treated cells to indicate the drop in the T2 weighted signal intensity (amount of darkening) for the three cell lines (Figure 4.5C). It can be observed from the signal enhancement graph that the SKBR3 cell lines had the lowest signal intensity (50%) compared to the BT474 (65%) and MCF7 (85%) cell lines. This suggests that the SKBR3 cell line had greater signal enhancement than the BT474
and MCF7 cell lines. The signal enhancement graph confirms Figures 4.5A and 4.5B, where visually a more hypointense image is seen for the SKBR3 cell line than for the BT474 and MCF7 cell lines. The results from Figure 4.5 also confirm the uptake of the iron oxide silica herceptin nanoparticles and also confirm the results seen in Figures 4.2, 4.3 and 4.4.
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Figure 4.5 MRI images and signal enhancements showing contrast generated from SKBR3, BT474 and MCF7 cell lines

MRI signal enhancement of SKBR3, BT474 and MCF-7 cell lines after 3 hours incubation with iron oxide silica herceptin nanoparticles at a concentration of 50 μg. (A) MRI contrast image of SKBR3, BT474 and MCF7 cells with no nanoparticles, (B) MRI contrast image of SKBR3, BT474 and MCF7 cells with iron oxide silica herceptin nanoparticles, and (C) percentage (%) of signal enhancement of SKBR3, BT474 and MCF7.
MCF7 cells relative to their control MRI T2 signal values. The error bars represent standard error of the mean calculated from triplicate measurements.
4.3 Discussion

Iron oxide silica nanoparticles synthesised via a high temperature method and demonstrating crystalline structure, monodispersity, biocompatibility, high T2 relaxation and phantom contrast enhancement were used in an in vitro system to test their ability to be used in targeted imaging for MRI. The humanised monoclonal antibody herceptin is a well characterised biomarker against HER2 receptors. HER2 is overexpressed in 20-30% of breast cancers, and is known to have poor prognosis and limited responses to treatment (Nahta & Esteva, 2006; Slamon, et al., 1987). Therefore, the attachment of herceptin on the surface of iron oxide silica nanoparticles potentially provides the ability to target HER2 receptors, which could provide valuable information about the efficacy of herceptin and concurrent treatments. In targeted imaging, it is essential that the nanoparticles which are tagged to a biomarker can localise at the area of interest, particularly if the nanoparticles are designed to provide visual contrast. In this study, to ensure that the herceptin drove the iron oxide silica nanoparticles to HER2 positive tumours, in vitro studies were performed to demonstrate the uptake of the iron oxide silica nanoparticles via herceptin by HER2 expressing cells.

The in vitro targeting ability of the synthesised iron oxide silica herceptin nanoparticles was investigated using flow cytometry, where a dose response and time course response uptake study was conducted to test the ability of the nanoparticles to bind specifically to HER2 receptors. Initially, a surface stain experiment was conducted to quantify the level of HER2 expression of SKBR3, BT474 and MCF7 breast cancer cell lines. Flow cytometry results demonstrated that SKBR3 cells had the highest level of HER2 expression followed by BT474 and then MCF7 cell lines (Figure 4.1). The results of this study are consistent with the literature, which indicates that of the three breast cancer cell
lines, SKBR3 has the highest HER2 expression levels (Chen, et al., 2000; Steinhauser, et al., 2006). Although the expression levels of these cell lines are well known and widely reported, it was important to validate the cells used in this study, as factors such as passage number, freezing, thawing and cell culture techniques can affect surface receptor expression levels.

As herceptin is attached to the surface of the iron oxide silica nanoparticles, it is known from other studies using nanoparticle-herceptin systems, that the delivery is due to the herceptin acting as the vehicle to drive the nanoparticles to HER2 expressing cells (Artemov, et al., 2003; Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005). To confirm that the uptake of the iron oxide silica herceptin nanoparticles was mediated via the herceptin, a dose response and time course study was conducted to ascertain the kinetics of uptake using the three breast cancer cell lines, SKBR3, BT474 and MCF7. The dose response study showed that as the amount of herceptin-FITC iron oxide silica nanoparticles increased, uptake of iron oxide silica herceptin nanoparticles also increased (Figure 4.2). Furthermore, the level of herceptin-FITC iron oxide silica nanoparticles uptake seen in this study correlated with expression levels of HER2, suggesting that uptake of the nanoparticles was likely to be mediated by presence of herceptin (Steinhauser, et al., 2006; Wartlick, et al., 2004).

As well as dose, the time required for uptake of nanoparticles is another important consideration when assessing uptake kinetics. A study quantifying the uptake of HSA nanoparticles conjugated to herceptin in SBKR3, BT474 and MCF7 cells over a period of 24 hours suggested that time-dependant uptake of the nanoparticles occurred related to HER2 expression levels over a 5-hour period, and after this time point uptake could be
related to non-specific mechanisms (Steinhauser, et al., 2006; Wartlick, et al., 2004). In this study, Figure 4.3 demonstrated that the nanoparticle uptake increased over a 5-hour time period and that the amount of uptake correlated with HER2 expression levels. Furthermore, a large increase in uptake kinetics of the iron oxide silica herceptin nanoparticles occurred between 5 and 24 hours. Although no measurements were taken during 5 and 24 hours, the large increase in uptake kinetics could be indicative of a passive non-specific uptake mechanism, similar to the study using HSA nanoparticles conjugated to herceptin (Steinhauser, et al., 2006).

It is interesting to note that most of the uptake studies that used nanoparticles conjugated to herceptin have not shown dose response curves, instead reporting only the differences in uptake using MRI. For example, Chen et al. (2009) investigated the use of herceptin conjugated dextran-coated iron oxide nanoparticles in four human breast cancer cells lines in vitro. They demonstrated uptake of the nanoparticles relative to HER2 expression as indicated by the different signal enhancements seen in MRI. Similarly, other studies (Lee, et al., 2007; Huh, et al., 2005; Artemov, et al., 2003) used cell lines with different levels of HER2 expression (characterised by western blot analysis and/or flow cytometry) and showed specific binding of the herceptin-nanoparticle conjugates using MRI signal enhancement change. This method is essential in understanding the MRI contrast enhancement behaviour between phantoms and in vitro systems, but it is not useful in understanding the behaviour of the herceptin-nanoparticles in in vitro systems. The disadvantage in not accurately characterising uptake of the nanoparticles is in targeting efficiency. For targeted imaging in MRI, it is important to ensure that the uptake of the nanoparticles seen is related to the targeting mechanisms of the biomarker and not just non-specific uptake of the nanoparticles.
To achieve targeted imaging with the iron oxide silica herceptin nanoparticles, the nanoparticles must bind to HER2 receptors on the membrane of cells and mediate cellular internalisation. This cellular internalisation was viewed by confocal microscopy after incubating the iron oxide silica herceptin-FITC nanoparticles with SKBR3 cells. Intracellular fluorescence was observed within the cell membrane and cytoplasm with 50 μg of iron oxide silica herceptin nanoparticles over a 3-hour time period (Figure 4.4). The results seen were similar to those of previous studies investigating the uptake of herceptin conjugated nanoparticles by SKBR3 cells (Chen, et al., 2009; Huh, et al., 2005). It is well known that herceptin binds to HER2 receptors that are located on the surface of the cell membrane and extend throughout the whole membrane (Cho, et al., 2003). Herceptin is known to be internalised within HER2 positive cells via receptor mediated endocytosis and signals a variety of pathways to exhibit its effect, resulting in the herceptin accumulating within the cytoplasm.

For iron oxide silica herceptin nanoparticles to be utilised in targeted imaging, a maximum amount of nanoparticles must arrive at the targeted area so that the highest contrast can be visualised on the MRI image. In studies that do not report data on dose response and time course response studies of the herceptin conjugated nanoparticles it is impossible to accurately determine the concentration of nanoparticles required to produce contrast prior to imaging.

For targeted imaging, the ability to view contrast in vitro at a similar level to phantom studies is necessary for research that is intended to be translated from a preclinical to clinical settings. It is well known that phantom studies can represent better contrast
enhancement of the nanoparticles than *in vitro* cell studies, where the nanoparticles can present lower signal enhancement. This phenomenon is not related to the amount of nanoparticles within cells but is related to theories of nanoparticles being confined within the endosome of target cells creating a build-up of magnetic field inhomogeneities, which are absent in phantoms where the nanoparticles are uniformly distributed (Billotey, et al., 2003; Kato, et al., 1999). In particular, the geometrical arrangement of the nanoparticles as a result of clustering within the cells is expected to be an important mechanism contributing to the effect of lower signal intensities *in vitro*.

Studies have used different mechanisms to overcome this phenomenon, such as the determination of T2 values in phantoms and within cells to confirm that the signal reduction *in vitro* is not significant (Hilger, et al., 2007). Other studies have not included phantoms but simply showed *in vitro* cell phantoms and calculated signal enhancements relative to a control (Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005; Lee, et al., 2007). In the current study, phantom experiments were conducted which showed signal enhancements of up to 80% for an iron oxide silica concentration of 50 μg (Chapter 3 Figure 3.5). In this chapter, *in vitro* localisation of the iron oxide silica herceptin nanoparticles at a concentration of 50 μg for 3 hours demonstrated a maximum signal enhancement of 50% relative to the control (Figure 4.5). The results suggest that although there was a reduction in the contrast enhancement from phantoms, the level of signal enhancement seen *in vitro* was still sufficient for *in vitro* and *in vivo* targeting MRI studies. The results from this study are comparable to studies that have used high performance nanoparticles such as metal-doped iron oxides which were superior to iron oxides alone, showing signal enhancements greater than 50% *in vitro* (Lee, et al., 2007). Although metal-doped iron oxides are ideal as contrast enhancements due to their high
magnetic capabilities, translation to the clinical settings could be difficult due to the biological toxicity of metals such as manganese. Nevertheless, the results from this study showed contrast enhancement at the same level as reported for metal-doped iron oxides.

Maintaining the ability to provide contrast in phantoms and then in \textit{in vitro} assays using MRI is fundamental in developing targeted imaging conjugates. Another important aspect is the ability for conjugated nanoparticles to demonstrate selective binding to target cells. In this case, the iron oxide silica nanoparticles conjugated to herceptin demonstrated selective binding, as indicated by the dose and time course studies (Figures 4.2 and 4.3) and also by the MRI contrast enhancement studies (Figure 4.5). The data indicated that the SKBR3 cells, which had the highest level of HER2 expression (Figure 4.1), demonstrated the highest level uptake (Figures 4.2 and 4.3) and highest level of contrast (Figure 4.5) compared to BT474 and MCF7 cells. A similar trend of selective binding of iron oxide nanoparticles conjugated to herceptin has also been reported in other studies (Chen, et al., 2009; Huh, et al., 2005).

\textbf{4.4 Conclusion}

This chapter sought to confirm whether synthesised iron oxide silica nanoparticles conjugated to herceptin could potentially be used for targeted imaging \textit{in vitro}. The iron oxide silica herceptin nanoparticles were tested for their targeting ability using three breast cancer cell lines with different levels of HER2 expression in a dose and time course response uptake study. The breast cancer cell lines were then scanned on MRI to see whether MRI contrast could also be detected. These investigations confirmed that the iron oxide silica herceptin nanoparticles were able to target breast cancer cells based on HER2
expression, and uptake was both dose- and time-dependent. The data also suggested that the iron oxide silica herceptin nanoparticles localised within the cells, and demonstrated MRI contrast enhancement \textit{in vitro} relative to controls. The level of contrast or signal enhancement was related to the HER2 expression levels, indicating specific uptake of the iron oxide silica herceptin nanoparticles by breast cancer cell lines. The results showed a potential use for the iron oxide silica herceptin nanoparticles in an \textit{in vitro} biological system, warranting the use of iron oxide silica herceptin in an \textit{in vivo} system.
Chapter 5 Assessment of the Iron Oxide Silica Herceptin Nanoparticles

in vivo

5.1 Introduction

The previous chapter analysed the in vitro performance of the synthesised iron oxide silica herceptin nanoparticles and concluded, based on dose and time evidence, that breast cancer cells of various HER2 expression levels could be targeted. The investigation also demonstrated that the iron oxide silica herceptin nanoparticles localised within breast cancer cells and produced significant MRI contrast. This data suggested successful targeted imaging in vitro and that the synthesised iron oxide silica herceptin nanoparticles might be utilised for in vivo imaging. This chapter therefore investigated the in vivo performance of the synthesised iron oxide silica herceptin nanoparticles.

As discussed in previous chapters of this thesis, herceptin is currently a targeted therapy against HER2 proteins, which are overexpressed in 20-30% of breast cancers (Slamon, et al., 1987). The association of HER2 positive expression in these breast cancers is linked with increased disease recurrence and poor prognosis (Slamon, et al., 1987). At the molecular level, herceptin induces regression of HER2 expressing tumours (Carter, et al., 1992; Hudziak, et al., 1989; Shepard, et al., 1991). Although the mechanism of this action of herceptin has not yet been fully elucidated, several mechanisms have been reported (Nahta & Esteva, 2006). Herceptin selectively binds to HER2 receptors, followed by internalisation via receptor-mediated endocytosis (Nahta & Esteva, 2006). It has been suggested that herceptin induces cell cycle arrest due to down regulation of HER2, eventually leading to reduced cell proliferation, suppression of angiogenesis, antibody-dependent cell-mediated toxicity, and finally cell death (Nahta & Esteva, 2006). Herceptin
was initially tested as a first-line therapy for its safety and efficacy in Phase II clinical trials for metastatic breast cancer (Vogel, et al., 2002). While it was found that the therapy was well tolerated and active in these patients, the overall response rate for herceptin on its own was less than 25% (Vogel, et al., 2002).

One of the early findings relating to herceptin therapy concerned the synergistic effects that occurred when it was used in combination with a variety of chemotherapy agents (Pietras, Pegram, Finn, Maneval, & Slamon, 1998; Pegram, et al., 1999; Pegram, et al., 2004; Slamon, et al., 2001). As a result, the effects of herceptin in combination with chemotherapy agents were investigated further. The findings from a landmark randomised phase III clinical trial indicated that herceptin significantly enhanced the activity of first-line chemotherapy (Slamon, et al., 2001). The outcome of this trial triggered various investigations using several chemotherapy agents in combination with herceptin for further evaluation of adjuvant therapy in clinical trials (Slamon, et al., 2001). It was shown that adding various combinations of chemotherapy agents including single (Gasparini, et al., 2007; Marty, et al., 2005; Slamon, et al., 2001), double or triple combinations (Pegram, et al., 2007; Robert, et al., 2006) could improve median survival rates, reduce disease progression and yield higher response rates. The use of herceptin was also investigated in patients with disease progression after treatment and in locally advanced breast cancer, where results showed improved response rates, overall survival and time to disease progression in these patients (Bontenbal, et al., 2008; O'Shaughnessy et al., 2008; von Minckwitz, et al., 2009).

Despite the anti-tumour effects of herceptin on HER2 positive breast cancers, there were reports of primary and secondary resistance to herceptin-based therapies (Goel, et al.,
2010; Slamon, et al., 2001; Vogel, et al., 2002). The mechanisms of acquired resistance and low response rates are not fully understood. The range of possible contributing factors includes the quantification and levels of HER2 expression, altered receptor-antibody interaction, increased cell signalling, and over-expression of the insulin-like growth factor-I receptor, which were discussed in detail in Chapter 1 Section 1.4.3 of this thesis. There is no doubt that elucidating the molecular mechanisms by which tumours escape herceptin-based cytotoxicity is critical to improving the prognosis of breast cancer patients whose tumours overexpress HER2. Equally important is identification of those patients who could stop responding to herceptin-based therapy regimes. A large body of evidence suggests that herceptin resistance is acquired (Slamon, et al., 2001; Vogel, et al., 2002) and because various mechanisms are possible it is difficult to predict which outcome a patient will exhibit. Therefore a clinical need exists to select the best therapies for clinical indications based on predictors of response which will identify effective therapies for patients needing treatment (Goel, et al., 2010).

A critical area of development involving a potential predictor of therapy is the use of iron oxide nanoparticles in MRI. Compared to other imaging methods MRI is the preferred complementary imaging tool for targeted imaging. This is because MRI offers superb resolution without the use of ionising radiation that can potentially be harmful to biomarkers used for targeting (Long & Bulte, 2009). The ability to image the progress of herceptin treatment using iron oxide nanoparticles with MRI presents the possibility of providing critical information about the efficacy of herceptin concurrently with treatment.

At the present time, all iron oxide nanoparticles for targeted imaging using MRI in oncology are at the preclinical stage where there is research and development into
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applications such as in cell tracking (Budde & Frank, 2009; Long & Bulte, 2009). The slow development of iron oxide nanoparticles for targeted imaging using MRI is largely attributable to the lack of studies that translate to the clinical setting. This is due to many areas of difficulty, such as quantifying dosages to ensure maximum contrast, particles with low magnetisation values, manufacturing large amounts of nanoparticles without compromising quality, and the ability to target nanoparticles to the area of interest *in vivo* (Corot, et al., 2006; Gupta & Gupta, 2005; Laurent, et al., 2008; Peng et al., 2008).

The iron oxide nanoparticles that have been developed for targeting HER2 positive breast cancers also present some of these issues. For example, Chen et al. (2009) successfully targeted HER2 positive tumours using dextran coated iron oxide nanoparticles, but their particles had low magnetisation values. Similarly, Hilger et al. (2007) also used dextran coated iron oxide nanoparticles with low magnetic properties and reported signal enhancement of the targeted area to be less than 20% in an *in vivo* model of breast cancer. On the other hand Huh et al. (2005) successfully targeted HER2 positive tumours using highly magnetic cross-linked iron oxide nanoparticles conjugated to herceptin. Although their study showed signal enhancement at the targeted area to be higher than other studies, the process to reproduce cross-linked iron oxide nanoparticles would require a number of complicated steps. Nevertheless, all these studies provide valuable insight into the use of iron oxide nanoparticles for targeting HER2 positive breast cancers and areas of possible improvement.

The iron oxide silica herceptin nanoparticles that were investigated in Chapters 3 and 4 of this thesis have been shown to overcome some of these limitations. For example, they can be produced in large quantities, they have high magnetisation values, and they produce
significant MRI contrast in phantoms and in vitro. The aim, therefore, of experiments in this chapter was to utilise them for in vivo imaging of HER2 positive breast cancers.

Iron oxide nanoparticles are well known to be phagocytosed by the RES cells of the spleen, lymph nodes, bone marrow, and liver when administered intravenously (Pouliquen, et al., 1991; Weissleder, et al., 1989). They are stored in lysosomes where the iron oxide nanoparticles are ultimately degraded and enter the biological iron metabolism cycle (Pouliquen, et al., 1991; Weissleder, et al., 1989). For larger iron oxide nanoparticles, above approximately 60 nm in diameter, 80-90% of the injected dose can be taken up by the RES within 30 minutes of intravenous administration and then eliminated (Pouliquen, et al., 1991; Weissleder, et al., 1989). This means that if the iron oxide nanoparticles need to be targeted to an area of interest there would not be enough of them available for localisation. Thus large iron oxide nanoparticles are unsuitable for targeted imaging. It is well known that reducing the size of iron oxide nanoparticles and adding a surface coating increases their half-life in the systemic blood pool circulation in vivo(Gupta & Gupta, 2005; Laurent, et al., 2008; Weissleder, et al., 1990). Studies using smaller iron oxide nanoparticles with a surface coating have shown that the initial liver and spleen uptake is low and that the particles remain in the bloodstream for several hours, suggesting that they can be available for localisation at a targeted area (Chouly, et al., 1996; Islam & Wolf, 2009; Moore, et al., 2000; Weissleder, et al., 1990). After this period, iron levels have been shown to increase in organs such as the liver, spleen, lungs, kidneys and heart (Jain, et al., 2008; Weissleder, et al., 1990). This is followed by the breakdown and elimination of iron from the system, which is reported to take up to 3 weeks, following the normal biodistribution and elimination patterns of biological iron (Islam & Wolf, 2009; Jain, et al., 2008; Weissleder, et al., 1989).
The biodistribution of iron oxide nanoparticles coated in silica has not been widely investigated, primarily due to the recent emergence of the use of silica in the nanoscale as nanoparticles or coatings on nanoparticles. As stated in Chapter 3, silica is known to possess extraordinary properties such as easy synthesis, low cost, high hydrophilicity and facile surface modification, with the result that silica is increasingly used for biomedical applications (Rieter, et al., 2007). The biodistribution of the commercially available large iron oxide silica nanoparticles such as GastroMARK® and Abdoscan® is well known. Both these contrast agents are administered orally, the iron oxide nanoparticles coated in silica are minimally absorbed through the bowel, and within several hours the compound is excreted primarily through the bowel (Advanced-Magnetics, 2007). There are a few reports of the biodistribution in vivo of smaller iron oxide nanoparticles coated with silica. Kim et al. (2006) studied 50 nm iron oxide nanoparticles coated with silica and conjugated to a fluorescent marker. The biodistribution was noted in organs such as the liver, spleen, heart, lungs, kidneys, brain, testes and uterus. The maximum uptake, which occurred in the liver and spleen, was negligible immediately after injection but increased over a 4-week period, where minimum uptake was seen in the lungs (Kim, et al., 2006). The biodistribution of lipid-coated silica nanoparticles conjugated to gadolinium metal has showed long circulating blood times (approximately 164 mins) (van Schooneveld, et al., 2008). These nanoparticles remained in the circulation for up to 4 hours and then increasingly accumulated within the liver and spleen, where they were cleared from the blood (van Schooneveld, et al., 2008). Another investigation of the biodistribution of silica nanoparticles approximately 45 nm in diameter showed similar distribution patterns, where the silica nanoparticles remained in the blood circulation for 3 hours, after which they increased in uptake in the liver and were then excreted via the urinary system (He, et
al., 2008). All these studies suggest that silica on the surface of the iron oxide nanoparticles can be beneficial for targeted imaging, based on the reported biodistribution patterns and size parameters.

The uptake of nanoparticles into tumours has been described as related to the high vasculature nature of the tumours. Studies have shown that nanoparticles are taken up in the peripheries of tumours and then heterogeneously accumulate throughout the tumour site (Huh, et al., 2005; Moore, et al., 2000). This heterogeneous uptake corresponds to the presence of blood vessels in the tumour periphery and is related to natural tumour vasculature (Moore, et al., 2000). With the attachment of an antibody such as herceptin to the surface of the iron oxide nanoparticle, the internalisation of the nanoparticles in breast tumours would occur by the antibody-antigen recognition action at the surface of the tumours. Studies using iron oxide nanoparticles for targeting HER2 positive breast cancers in vivo have not shown the mechanism of uptake of the nanoparticles to the tumour site. Specific imaging of a target site, such as a solid HER2 overexpressing breast tumour, requires several steps: long circulation of the conjugated nanoparticles to ensure availability, permeation of the conjugated nanoparticles into tissues, recognition of the conjugated nanoparticles at the antigen-antibody receptor site, and receptor-mediated endocytosis of the conjugated nanoparticles into the tumour site. On the evidence from previous studies, iron oxide nanoparticles and iron oxide silica nanoparticles with a smaller size can remain in the circulation for a minimum of 3 hours (Chen, et al., 2009; He, et al., 2008; Kim, et al., 2006). It can then be assumed that iron oxide silica herceptin nanoparticles can localise at the tumour site, most likely due to the receptor mediated action of the herceptin, as in the in vitro studies detailed in Chapter 4. Other studies have shown that uptake at the tumour site after intravenous injection occurs as early as 3 hours.
and up to 24 hours (Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005), when the uptake begins to clear.

Apart from localisation of the iron oxide silica herceptin nanoparticles, no toxicity is one of the fundamental requirements for the use of iron oxide silica herceptin nanoparticles in vivo and is one of the key factors in the determination of clinical use. The in vitro toxicity of the iron oxide silica herceptin nanoparticles was assessed in Chapter 4, and toxic effects were consistent with the literature and the action of herceptin was consistent with previous studies. The earliest reports of iron oxide nanoparticles demonstrated no acute or sub-acute toxic effects in vivo, as the excretion of iron oxide nanoparticles followed the natural channels of iron metabolism (Weissleder, et al., 1989). Although the toxic effects of iron oxide nanoparticles coated in silica have not been widely investigated, silica on its own has demonstrated toxic effects that caused DNA cleavage (Chen & von Mikecz, 2005) especially when used in large concentrations (Chang, Chang, Hwang, & Kong, 2007; Jin, Kannan, Wu, & Zhao, 2007). Most of the earlier studies assessing silica have shown toxic effects caused by silica in the lungs (Lin, Huang, Zhou, & Ma, 2006), primarily due to the inhalation of large amounts of silica (Castranova & Vallyathan, 2000). However, in vivo toxicity studies assessing nanoparticles with silica have shown almost little to no localisation of the silica-based nanoparticles within the lungs (Kim, et al., 2006). The studies utilising silica nanoparticles have reported no biological toxicity, suggesting that silica is safe to use in minimal amounts (Gregersen, Lopez, & York, 2003; He, et al., 2008; Kim, et al., 2006). On the basis of earlier studies it was expected that the iron oxide silica nanoparticles would have minimal or negligible toxic effects in vivo.
The previous chapters showed that iron oxide silica herceptin nanoparticles could be produced on a large scale, had significant magnetic properties, were biocompatible and stable. Furthermore, Chapter 4 found that these nanoparticles could be applied as contrast agents to target HER2 receptors in vitro and that they provided significant MRI contrast enhancement. Based on the investigations of these two experimental chapters, the experiments in this chapter aimed to investigate the in vivo potential of the synthesised iron oxide silica herceptin nanoparticles.

On the basis of these previous studies, the aims of this chapter were to:

1. Grow HER2-expressing SKBR3 xenograft tumours in BALB/c nude mice;
2. Image SKBR3 xenografts in BALB/c nude mice at 4 hours and 24 hours post-injection with iron oxide silica herceptin nanoparticles and observe signal enhancement relative to the control; and
3. Investigate acute toxicity of the iron oxide silica herceptin nanoparticles post-injection in BALB/c nude mice in vivo.
5.2 Results

5.2.1 Growth of HER2 positive tumours in BALB/c nude mice

The growth of SKBR3 tumour cells in BALB/c nude mice was confirmed after conducting a xenograft tumour growth curve analysis study. The results indicated that SKBR3 cells formed tumours as early as 4 days post-injection in the right flank of BALB/c nude mice. By day 5, the SKBR3 tumours had reached approximately 3.5mm$^2$ and by day 12 had reached approximately 8mm$^2$ (Figure 5.1).
**Figure 5.1** Tumour growth curves of SKBR3 xenograft tumours in BALB/c nude mice. $3 \times 10^6$ SKBR3 cells were injected subcutaneously into the right flank of BALB/c nude mice ($n=3$) and tumour growth was monitored daily; measurements (length x width) were recorded as tumour size (mm). Error bars were calculated by standard deviation of the mean.
5.2.2 HER2 expression in SKBR3 xenograft tumours

Immunohistochemical analysis of SKBR3 xenograft tumours was performed using immunohistochemistry to confirm expression of HER2 receptors. Following the growth of xenograft SKBR3 tumours in BALB/c nude mice, to a maximum size of 8 mm² over a 12-day period, the mice were killed and tumours were dissected and processed for histological analysis. Figure 5.2 shows the staining pattern of HER2 in SKBR3 xenograft tumours grown in BALB/c nude mice. The inset shows clear membranous (M) and cytoplasmic (C) staining within the tumours cells. This pattern of staining was consistently seen with all the SKBR3 xenograft tumours.
Figure 5.2 Immunohistochemical analysis of HER2 expression in SKBR3 xenograft tumours using HRP
Dissected SKBR3 tumours were processed for histological analysis. The samples were stained with an anti-HER2 antibody and the inset shows membranous (M) and cytoplasmic (C) staining of the SKBR3 tumour clusters grown in BALB/c nude mice at the time of animal sacrifice (magnification x20).
5.2.3 Intratumoral MRI contrast enhancement of SKBR3 xenografts

To assess optimal MRI contrast enhancement within SKBR3 xenograft tumours, 50 µg of iron oxide silica herceptin nanoparticles was injected intratumorally and then assessed for contrast enhancement. After injection at the tumour site, significant enhancement could be seen visually as indicated by the hypointense signal (Figure 5.3). Figure 5.3A (i) shows the sagittal slice of a SKBR3 xenograft tumour that was injected with saline (control group), demonstrating no contrast as indicated by the white arrow. Figure 5.3A (ii) indicates contrast enhancement demonstrated by a large hypointense signal after intratumoral injection of 50 µg of iron oxide silica herceptin nanoparticles (as indicated by the white arrow). The calculated signal enhancement relative to the control mouse was presented and a maximum signal enhancement of 80% was calculated (Figure 5.3B).
Figure 5.3 MRI Signal enhancement of SKBR3 tumours after intratumoral injection of iron oxide silica herceptin nanoparticles
(A) (i) MRI image of a control mouse injected intratumorally with saline and (ii) MRI image of mouse injected intratumorally with 50 μg of iron oxide silica herceptin nanoparticles. (B) Signal enhancement of the mouse injected with saline and 50 μg of iron oxide silica herceptin nanoparticles. Error bars represent standard deviation of the mean calculated from triplicate measurements.
5.2.4  *In vivo* toxicity of iron oxide silica herceptin nanoparticles

The *in vivo* acute toxicity of the iron oxide silica herceptin nanoparticles was assessed in three groups of BALB/c nude mice (n=3) each with a separate experimental condition. The first group of mice received an intravenous injection of saline. The second group of mice received an intravenous injection of iron oxide silica nanoparticles at a maximum dose of 20 mg/kg. The third group of mice received an intravenous injection of iron oxide silica herceptin nanoparticles at a maximum dose of 20 mg/kg. The mice were monitored over a 7-day period after which organs including liver, lungs, heart and kidneys were collected for histopathological analysis to assess acute toxicity. The results indicate that injection of iron oxide silica herceptin nanoparticles had no effect on mortality, morbidity, body weight or food consumption in the BALB/c nude mice. Laboratory values of blood biochemistry, liver and kidney function were all within normal limits (data not shown). Histologic studies of liver, lungs, kidneys and heart demonstrated no necrosis, no loss of nuclei, no inflammation or any other pathological processes. The conjugated nanoparticles were not identifiable, possibly due to insufficient resolution.
Figure 5.4 Histological analysis of in vivo toxicity in BALB/c nude mice after intravenous injection of iron oxide silica herceptin nanoparticles. (I) Group 1 shows histological analysis showing liver, kidney, lung and heart of control mice injected with saline. (II) Group 2 shows histological analysis showing liver, kidney, lung and heart of mice injected with iron oxide silica nanoparticles (20 mg/kg). (III) Group 3 shows histological analysis showing liver, lung and heart of mice injected with iron oxide silica herceptin nanoparticles (20 mg/kg). Magnification at 10X.
5.2.5 MRI targeting and contrast enhancement of SKBR3 xenograft breast tumours in vivo

To evaluate the targeting ability and MRI contrast enhancement of iron oxide silica herceptin nanoparticles in vivo, SKBR3 xenograft breast tumours were grown in BALB/c nude mice (n=5) and injected intravenously with iron nanoparticles at a dose of 400 µg. To demonstrate targeted imaging by contrast enhancement, iron oxide silica herceptin nanoparticles were intravenously injected and imaged at 4 hours and 24 hours post-injection. To complement the control mice, iron oxide silica nanoparticles with no herceptin were also injected and imaged at 4 and 24 hours post-injection. Figure 5.5 shows the T2 weighted images of a tumour-bearing mouse with (A) no contrast agent, (B) 4 hours post-injection with iron oxide silica nanoparticles, (C) 24 hours post-injection with iron oxide silica nanoparticles, (D) 4 hours post-injection with iron oxide silica herceptin nanoparticles and (E) 24 hours post-injection with iron oxide silica herceptin nanoparticles. The data suggests that mice with SKBR3 tumours injected with iron oxide silica herceptin nanoparticles (Figure 5.5D-E) had the most significant contrast enhancement, as indicated by a hypointense signal compared to the control mice who received iron oxide silica nanoparticles without herceptin (Figure 5.5B-C) and saline with no contrast agent (Figure 5.5A). The appearance of the hypointense signal in mice who received iron oxide silica herceptin nanoparticles would be seen only if the iron oxide silica herceptin nanoparticles were present. To confirm this, signal enhancement analysis calculation was conducted by placing a region of interest over the entire tumour. These calculations suggest that the average enhancement was approximately 50% for iron oxide silica herceptin nanoparticles at 4 hours and less than 8% for iron oxide silica
nanoparticles and controls at 4 and 24 hours (Figure 5.5F). This suggests that the mice who received iron oxide silica herceptin nanoparticles demonstrated signal enhancement at the tumour site relative to the controls. Comparison of the iron oxide silica herceptin nanoparticles contrast at 4 and 24 hours indicated that uptake at both these time points was similar, suggesting that localisation of the iron oxide silica herceptin nanoparticles remained within the tumour at 24 hours post-injection.
Chapter 5 – *in vivo* assessment of Herceptin-nanoparticles

SKBR3 tumours were grown in BALB/c nude mice and the uptake of iron oxide silica and iron oxide silica Herceptin nanoparticles (400 μg) after intravenous injection was observed (n = 5). T2 weighted images show (A) control (saline), (B) iron oxide silica nanoparticles with no Herceptin at 4 hours, (C) iron oxide silica with no Herceptin at 24 hours, (D) iron oxide silica Herceptin nanoparticles at 4 hours and (E) iron oxide silica Herceptin nanoparticles at 24 hours. The calculated % MRI signal enhancement analysis relative to the control for each conjugate is shown in (F). Error bars represent standard deviation of the mean.
5.3 Discussion

One of the promising areas of targeted imaging is the use of iron oxide nanoparticles in detecting HER2 positive receptors in breast tumours. Previous studies have used iron oxides for \textit{in vitro} and \textit{in vivo} targeting of HER2 positive breast cancers, but nanoparticles have presented critical problems which have limited their development to the clinical setting (Chen, et al., 2009; Hilger, et al., 2007; Huh, et al., 2005). Issues such as low magnetisation values, low signal enhancement and high amounts of iron required are important as they impair the basic fundamental properties for successful \textit{in vivo} targeted imaging. In the current study, iron oxide silica herceptin nanoparticles with high magnetisation values and that had shown high contrast enhancement \textit{in vitro} were tested in an \textit{in vivo} setting. The results indicated that significant contrast enhancement could be visualised at the tumour site in breast tumour bearing BALB/c nude mice, with approximately 50\% contrast enhancement relative to the control.

Before the iron oxide silica herceptin nanoparticles were analysed for their targeting ability, the growth of the HER2 positive tumours was first monitored in BALB/c nude mice. The tumour growth study indicated that SKBR3 tumours could be grown successfully in BALB/c nude mice using Matrigel™ over a 12-day period where an average maximum diameter of 8 mm$^2$ was noted (Figure 5.2.1). That initial step in assessing the growth of tumours using SKBR3 cells was important as variations in cell culture passages and the origin of the nude mice could affect growth patterns of xenograft tumours. Analysis of HER2 receptors on the SKBR3 xenograft tumours grown in BALB/c mice was shown by immunohistochemistry where the tumours retained their HER2 receptor expression, a finding that was similar to previous studies which have retained
HER2 expression in SKBR3 tumour xenografts (Chen, et al., 2009; Hilger, et al., 2007). This result therefore warranted the use of the SKBR3 cells in developing HER2 positive tumours in nude mice as the animal tumour model for this study.

It is well known that for any nanoparticle design for applications in MRI, iron oxide nanoparticles with the highest T2 relaxation or contrast enhancement in phantoms need to be considered, followed by assessment of their efficiency in \textit{in vitro} and \textit{in vivo} studies. Thus the iron oxide silica herceptin nanoparticles were initially assessed for their intratumoral contrast before investigating contrast \textit{in vivo}. From the phantom studies, a dosage above 50 $\mu$g of iron oxide silica nanoparticles was sufficient to generate 80% of signal enhancement (Chapter 3, Figure 3.5) and for \textit{in vitro} studies a dosage of 50 $\mu$g of iron oxide silica herceptin was sufficient to generate 55% signal enhancement (Chapter 4, Figure 4.5). Furthermore, a dosage of 50 $\mu$g demonstrated lower cytotoxic effects than the higher dosage of 100 $\mu$g (Chapter 3, Figure 3.8). On the basis of these studies, a dosage of 50 $\mu$g of iron oxide silica herceptin nanoparticles was injected intratumorally into BALB/c nude mice bearing HER2 positive tumours to investigate the amount of MRI signal enhancement at the tumour site. The results indicated an MRI signal enhancement of approximately 80%, similar to the phantom studies, suggesting that there was no loss of contrast enhancement due to body mass or tumour tissue. This suggests that the iron oxide silica herceptin nanoparticles were highly magnetic and that body fat present within the mice had minimal or no effect of reducing the signal enhancement. There are no previous studies with which to compare these results, and it can not be ruled out that body mass and/or tumour tissue contributed to the effect of lower signal enhancement. Overall, the results suggest that the contrast seen after intratumoral injection of nanoparticles was similar to that in the phantom studies. The fact that the maximum contrast was seen after
intratumoral injection could suggest that the nanoparticles were not compartmentalised sub-cellularly and the nanoparticles were uniformly distributed, as in the phantom studies. This was discussed in detail in Chapter 3, where one of the factors contributing to the phenomenon of reduced contrast \textit{in vitro} was the build-up of magnetic field inhomogeneities after the nanoparticles were in subcellular compartments of the cells.

Acute toxicity of the iron oxide silica herceptin nanoparticles was investigated and was not evident at a dose of 20 mg/kg body weight. The median lethal dose (LD$_{50}$) of iron is known to be 30 mg/kg (Weissleder, et al, 1989) a dosage of iron oxide silica herceptin nanoparticles (20mg/kg) is well below this number. It is known that cirrhosis and hepatocellular carcinoma can develop if iron levels in the liver exceed 4000 μg/g wet weight (normal, 200 μg/g wet weight) (Bassett, Halliday, & Powell, 1986; Weir, Gibson, & Peters, 1984). Furthermore, the amount of iron used in this study was very small compared to normal liver iron stores. If these iron oxide nanoparticles were used in humans, an average 70 kg adult would receive a dosage of 1400 mg. It has been reported that a dose of up to 80 mg of iron is known to increase liver iron from 200 to 212 μg/g wet tissue, suggesting that the dosage used in this study was well below the limits reported to induce any hepatotoxic changes (Weissleder, et al, 1989). The assessment of the hepatic parenchyma and hepatocytes showed no toxicity as there were no histopathological changes detected including inflammation, necrosis or loss of cellular nuclei. Iron oxide nanoparticles have previously demonstrated localisation in other organs such as kidneys and lungs (Weissleder, et al, 1989). Histopathological analysis demonstrated that there were no toxic effects in the kidneys and lungs of mice that received iron oxide silica herceptin nanoparticles compared to control groups. Therefore, there was no evidence of acute toxicity caused by the iron oxide silica herceptin nanoparticles used here. These
results are consistent with other studies that have investigated the acute toxicity of iron oxide nanoparticles in mice (Weissleder, et al., 1989). The administration of herceptin is also known to cause toxic effects on the myocardium (Chien, 2006; Slamon, et al., 2001), and therefore histopathological assessment of the heart tissue of the mice was undertaken. As with the other organs, there was no evidence of acute toxicity. The cause of cardiotoxicity induced by administration of herceptin is uncertain, and it has been reported that in approximately 10% of patients who receive herceptin treatment there is a decrease in cardiac function (Chien, 2006). The possible reason for the lack of toxic effects in this study could be the low amount of our labelled iron oxide silica herceptin nanoparticles used.

In vivo imaging showed that after intravenous administration of the iron oxide silica herceptin nanoparticles a maximum signal enhancement of approximately 50% at 4 hours and at 24 hours was calculated. The results suggested that the iron oxide silica herceptin nanoparticles successfully localised and bound to the HER2 positive tumour, producing significant MRI contrast enhancement at the tumour site. Compared to previous studies, the iron oxide silica herceptin nanoparticles used in this study for in vivo targeting appear to have achieved better performance in generating contrast enhancement at the targeted area. A study that used iron oxide nanoparticles coated with dextran demonstrated maximum in vivo contrast enhancement of up to 20% (Hilger, et al., 2007). The most likely explanation for the difference in contrast enhancement could be that the iron oxide nanoparticles used in that study had limited magnetic properties and therefore produced lower amounts of MRI contrast enhancement. As discussed in Chapter 3, the iron oxide nanoparticles used by Hilger et al. (2007) were produced via a co-precipitation method, which is known to produce large amounts of particles at the expense of lower
magnetisation values. Furthermore, a study that produced iron oxide nanoparticles via a thermal decomposition method, similar to method used in this study, coated their particles in DMSA and observed a maximum drop in T2 intensity of only 20% (Huh, et al., 2005). Although the authors did not calculate the signal enhancement value, a small drop in the T2 intensity suggests a possible low signal enhancement. The difference in the results could be due to a range of factors such as T2 values, which were not stated, size of the nanoparticles, and the influence of the surface coating. A study by Chen et al. (2009) used a novel iron oxide dextran herceptin nanoparticle in a cross-linked pattern and demonstrated a 45% enhancement at the tumour site. This large value demonstrates the effect of the well-known high magnetisation value capability of cross-linked iron oxide nanoparticles (Högemann, Josephson, Weissleder, & Basilion, 2000). The disadvantage in the usage of cross-linked iron oxide nanoparticles is the low production yield and limited surface coatings that can be used. The iron oxide silica herceptin nanoparticles in this study were not cross-linked but still provided greater than 50% signal enhancement after intravenous injection, higher than the cross-linked iron oxide nanoparticles previously reported. The results from this study can also be compared to dual doped metals used in MRI targeting studies (Lee, et al., 2007). Dual doped metal nanoparticles have been developed to produce more MRI contrast than iron oxide nanoparticles on their own (Lee, et al., 2007). The theory behind dual doped metal particles is that a high magnetic moment performance metal such as manganese (Mg) II ions can be impregnated within iron oxides to enhance the contrast (Lee, et al., 2007). The enhancement seen in SKBR3 tumours by iron oxide doped with Mg (II) conjugated to herceptin was under 40% at 8 hours post-intravenous injection, which is significantly lower than the contrast seen in this study.
The signal enhancements of the SKBR3 tumours at 4 and 24 hours post-injection with iron oxide silica herceptin nanoparticles were similar, with enhancements of 50% and 52%, respectively. Firstly, the uptake of the iron oxide silica herceptin nanoparticles was seen as early as 4 hours, suggesting that the nanoparticles could be visualised within the tumour as early as 4 hours. Secondly, the calculated signal enhancement at 4 and 24 hour time points showed similar values of 50% and 52%, respectively. This data suggests that the iron oxide silica herceptin nanoparticles could remain within the tumour for up to 24 hours post-intravenous injection, potentially allowing longitudinal studies. The overall uptake pattern of the nanoparticles was consistent with other studies that have used iron oxide nanoparticles with different surface coatings such as DMSA and dextran (Chen, et al., 2009). For example, in their time-dependant study, Huh et al. (2005) observed contrast enhancement as early as 1 hour as well as 4 and 12 hours post-intravenous injection, where the uptake at 12 hours was similar or unchanged to that at 4 hours. Similarly, Chen et al. (2009) reported MRI contrast enhancement as early as 3 hours post-injection.

If the signal enhancement data is compared between the iron oxide silica herceptin time points (Figure 5.2.5D and 5.2.5E) and iron oxide silica time points (Figure 5.2.5B and 5.2.5C), it can be observed that there was little to no signal enhancement by the iron oxide silica nanoparticles at 4 and 24 hours within the SKBR3 tumours. The aim of intravenously injecting iron oxide silica nanoparticles was to demonstrate any non-specific tumour uptake caused by the iron oxide silica nanoparticles. From the results shown, it can be deduced that the iron oxide silica nanoparticles did localise within the SBKR3 tumours and did not induce any non-specific tumour uptake. This suggests that only the nanoparticles conjugated to herceptin localised within the SKBR3 tumours and produced contrast. Therefore, it is suggested that the iron oxide silica herceptin nanoparticles
localised within the tumour due to the action of the herceptin conjugated on the surface of the iron oxide silica nanoparticles. As far as we know, no other reported studies have compared conjugated and non-conjugated nanoparticles in producing MRI contrast.

Another observation of interest is the similar uptake pattern of the nanoparticles in this study and in other studies. The uptake of the nanoparticles within the tumour occurred towards the bottom region (Figure 5.2.5E and 5.2.5F) and then gradually built up to the central and upper regions of the tumour (Huh, et al., 2005; Moore, et al., 2000). It is presumed that this time-dependant MRI signal change and nanoparticle uptake could be related to the heterogeneous pattern of the intratumoral vasculature, where the iron oxide nanoparticles were easily permeable in the highly vasculature region of the tumour and afterward gradually reached the less vascular region as time progressed (Huh, et al., 2005; Moore, et al., 2000; Weidner, Semple, Welch, & Folkman, 1991). The unbound nanoparticles recirculate continuously in the blood and if they are not taken up by the RES to be eliminated, continue to target the HER2 expressing tumour cells. Liver uptake of the iron oxide silica nanoparticles is inevitable as this is the primary excretion pathway, particularly after intravenous injection. However, as the in vivo results from this study show, it appears that the amount of iron oxide silica herceptin nanoparticles reaching the tumour washigh. Future investigators should consider conducting a longitudinal study evaluating the clearance of the iron oxide silica herceptin nanoparticles from the tumour, to investigate whether longitudinal scanning could be considered for these nanoparticles in tumour tracking or turnover kinetics. Such investigation is particularly useful, especially in tracking the progression of tumours, identifying sentinel nodes, and also in monitoring responses to treatment such as herceptin. Other future areas of investigation should include a biodistribution study and using these iron oxide silica nanoparticles to image tumours of
tumours of different sizes, which would provide valuable information in relation to further optimising targeted HER2 imaging with iron oxides.

### 5.4 Conclusion

The aim of this chapter was to confirm whether the iron oxide silica herceptin nanoparticles could be utilised for *in vivo* tracking of HER2 positive breast cancers. SKBR3 tumour cells were used to produce subcutaneous xenografts in BALB/c nude mice to a maximum diameter of 8mm$^2$, and the histopathological staining analysis confirmed the expression of HER2 positive tumours. MRI studies showed that after intravenous injection of the iron oxide silica herceptin nanoparticles significant targeted uptake was present in the SKBR3 tumours, as indicated by the contrast enhancement in comparison to the control injected with saline and the control injected with iron oxide silica nanoparticles only. The results further showed that the tumour site continued to produce contrast enhancement even 24 hours post-intravenous injection of the iron oxide silica herceptin nanoparticles. The *in vivo* investigations therefore confirmed that the iron oxide silica herceptin nanoparticles could target HER2 positive breast tumours *in vivo* and could potentially be used with MRI in the clinical setting.
Chapter 6 Conclusions and Future Directions

6.1 Summary

The work presented in this thesis describes the use of highly magnetic iron oxide silica nanoparticles conjugated to herceptin for the targeted detection of HER2 positive breast cancer using MRI. The main finding of this study was that iron oxide nanoparticles coated in silica could be used to detect HER2 positive breast cancers in vivo using MRI. The iron oxide silica nanoparticles produced presented with highly magnetic properties and were able to produce MRI contrast. The nanoparticles were also found to be non-toxic, biocompatible and easily conjugated to a biomarker, herceptin. The conjugation of herceptin to the iron oxide silica nanoparticles was shown to be stable in various conditions. Furthermore, the results from the in vitro assessment of these nanoparticles demonstrated that significant MRI contrast was visualised relative to HER2 expression levels in three human breast cancer cell lines. Other findings of this study arose from the in vivo assessment of the iron oxide silica herceptin nanoparticles, which showed successful localisation to the HER2 positive tumour site, demonstrated by contrast enhancement seen at various time points after injection on the MRI images.

6.2 Overview

It is well known that HER2 positive breast cancers are associated with poor prognosis, increased disease progression and low responses to herceptin therapy even when treated with adjuvant and neoadjuvant therapies. The use of iron oxide nanoparticles in MRI is an
area of interest, which can potentially be used to help identify patients who have stopped responding to herceptin-based therapies.

6.2.1 Synthesis of iron oxide silica herceptin nanoparticles

The principles in manufacturing and utilising iron oxide nanoparticles for targeted imaging of HER2 positive tumours in breast cancer were described in Chapter 1. For the synthesis of iron oxide nanoparticles, many previous studies had not addressed essential characteristics such as high magnetic properties, high contrast enhancement, biocompatibility and a simple large-scale synthesis method for iron-based nanoparticles. These properties are important as they form the basic requirements and key features to ensure that successful targeted imaging with MRI can occur and can be translated to the clinical setting.

Therefore, the first aim of this study, therefore, was to synthesise iron oxide nanoparticles conjugated to herceptin that had the necessary characteristics for targeted imaging, including high magnetisation values, low toxicity, biocompatibility, small size and size distribution, and a simple large-scale production method.

Previous studies related to the synthesis of iron oxide nanoparticles were based on either hydrolytic or non-hydrolytic methods. It was identified that hydrolytic methods produced lower performing iron oxide nanoparticles, had poor control over size and size distribution, and lower magnetic properties. Non-hydrolytic methods, particularly the ultra-large-scale synthesis process described by Park et al. (2004), demonstrated superior iron oxide nanoparticles, with better control over size, smaller size distributions, higher
magnetic properties and an easier production process with larger quantities of nanoparticles. The authors describing this method also indicated that iron oxide nanoparticles with these properties had not been previously reported for use in MRI.

To ensure targeted imaging in MRI, another essential component of these nanoparticles that was discussed was the surface layer properties. Surface layers are necessary on iron oxide nanoparticles for a variety of reasons: they stabilise the iron oxide core to prevent further oxidation, allow the iron oxide nanoparticles to be soluble in water, prevent aggregation of the iron oxide nanoparticles, and provide a surface for the attachment of biomarkers. One of the most widely documented surface coatings on nanoparticles discussed was dextran, primarily due to its high biocompatibility. However, another surface coating that has also shown biocompatibility and suitability for use in MRI is silica. Silica as a surface layer had not previously been investigated for targeted MRI, although silica had shown promising results for targeted drug delivery.

The conjugation of the iron oxide silica nanoparticles to a biomarker is mandatory for targeted imaging. In this study the iron oxide silica nanoparticles were conjugated to herceptin. The conjugation of herceptin to the iron oxide silica nanoparticles was shown to be stable in various conditions. To develop iron oxide silica nanoparticles conjugated to herceptin with the major characteristics needed for targeted MRI, iron oxide nanoparticles were developed using the non-hydrolytic synthesis method of Park et al. (2004), and coated with silica. The results of this assessment were presented in Chapter 3.

6.2.2 In vitro assessment of the iron oxide silica herceptin nanoparticles
Previous studies had demonstrated that iron oxide nanoparticles conjugated to herceptin were non-toxic, were able to target HER2 positive receptors in breast cancer, and demonstrated MRI contrast enhancement relative to HER2 expression levels of breast cancer cells. *In vitro* uptake studies had also shown localisation of the nanoparticles to the tumour site over time. To our knowledge, however, there have been no reported studies for the uptake of various dosages of herceptin-conjugated nanoparticles within HER2 positive cells. To investigate the toxicity and uptake of the iron oxide silica herceptin nanoparticles synthesised in this study, a thorough cell uptake study was conducted to investigate the effect of dose and time on HER2 positive breast cancer cells. MRI signal enhancement of the breast cancer cell lines was also quantified. The results of these studies were presented in Chapter 4.

### 6.2.3 In vivo assessment of the iron oxide silica herceptin nanoparticles

The biocompatibility of iron oxide nanoparticles has been described in earlier reports and the principle of targeted imaging using iron oxide nanoparticles has also been reported by other studies targeting HER2 tumours. To prove that the iron oxide silica herceptin nanoparticles synthesised in this study were biocompatible, highly magnetic and capable of producing MRI contrast enhancement *in vivo*, an *in vivo* investigation was conducted in BALB/c nude mice bearing HER2 positive xenograft tumours. The results from this study were presented in Chapter 5.

### 6.3 Key findings of this study
Chapter 6 – Conclusions and future directions

The aim of this study was to synthesise highly magnetic iron oxide nanoparticles for targeted MRI of HER2 positive tumours to be tested \textit{in vitro} and \textit{in vivo} in animal models. To confirm that the synthesised nanoparticles possessed the basic characteristics needed for targeted imaging, a series of characterisation techniques were conducted. It was clearly shown that the synthesised iron oxide silica nanoparticles had the major characteristics required for use as a targeted contrast for MRI and were superior to commercially available iron oxide nanoparticles and previously reported iron oxide nanoparticles. These characteristics included high magnetic properties (74.4 emu/g), high T2 relaxation (T2 263.23 mMs.) small size and size distribution (<35 ± 3 nm), low toxicity, and easy conjugation to herceptin. Furthermore, the conjugation between iron oxide silica and herceptin was shown to be stable after exposure to a range of conditions, mimicking different physiological conditions.

\textit{In vitro} studies examined cellular uptake and contrast enhancement capability of the iron oxide silica herceptin nanoparticles. The studies confirmed the uptake into HER2 expressing human breast cancer cells possibly mediated by the herceptin, which was consistent with previous studies. The uptake of the iron oxide silica herceptin nanoparticles was shown to be relative to the HER2 expression levels of the breast cancer cells as well as to dose and incubation times. The MRI signal enhancement produced by the iron oxide silica herceptin nanoparticles in human breast cancer cell lines was also analysed, and it was demonstrated that there was higher contrast enhancement than reported in previous studies. It was concluded that the iron oxide silica herceptin nanoparticles could detect HER2 positive cells under \textit{in vitro} conditions and could produce significant amounts of MRI contrast.
This study also investigated the *in vivo* targeting capability of the iron oxide silica herceptin nanoparticles. SKBR3 breast cancer cells were injected subcutaneously into BALB/c nude mice, allowed to grow, and histopathological staining confirmed the presence of HER2 positive cells, consistent with previous studies. The MRI study demonstrated uptake of the iron oxide silica herceptin nanoparticles at the tumour site, producing signal enhancements greater than previously reported. The tumour uptake pattern was consistent with other studies, indicating localisation of herceptinnanoparticles at the tumour site 4-24 hours post-injection. To address the biological safety of the iron oxide silica herceptin nanoparticles, an *in vivo* toxicity analysis was also conducted. The results showed no toxicity to the liver, lungs, heart and kidneys, as indicated by histopathological analysis. *In vivo* studies confirmed that the iron oxide silica herceptin nanoparticles were safe and produced high amounts of MRI contrast at the tumour site as early as 4 hours and up to 24 hours post-injection.

Overall, the findings of this study demonstrated that the iron oxide silica herceptin nanoparticles targeted HER2 positive tumours with nanoparticles that were highly magnetic, biocompatible, small in size and size distribution, and stable. Furthermore, the results suggested that the nanoparticles had all the basic characteristics required for translation into the clinical setting.

### 6.4 Future directions

This study has presented preliminary data on the use of iron oxide silica herceptin nanoparticles for targeted imaging with MRI. The iron oxide silica nanoparticles synthesised demonstrated all the characteristics necessary for translation of MRI contrast
agents into the clinical setting. The next step for these nanoparticles is to further address the safety component. The results from this study showed that no acute toxicity was caused by the iron oxide silica herceptin nanoparticles, but for translation into clinical trials a full toxicity analysis investigating chronic toxicity needs to be undertaken.

The next stage of further investigations should be related to the sensitivity of the iron oxide silica herceptin nanoparticles in relation to detecting HER2 positive breast tumours. Studies are needed that image HER2 positive tumours of different sizes, to demonstrate the ability of the iron oxide silica herceptin nanoparticles to target and localise within tumours of different levels of HER2 expression. In vitro cell uptake studies from this study have shown that these nanoparticles can detect varying levels of HER2 expression, and these findings provide a good basis for investigation in in vivo settings.

These nanoparticles have all the basic requirements for targeted imaging, are superior to nanoparticles used in previously reported studies, and have the potential to be translated clinically. However, for the iron oxide silica herceptin nanoparticles to be used in patients who are being treated with herceptin-based therapies, further studies need to focus on the ability of these nanoparticles to detect and provide diagnostic and prognostic information regarding response to therapy. Another avenue for further investigation is the ability of these iron oxide silica nanoparticles to be conjugated to other biomarkers and to be used in MRI for targeted imaging. The results from this thesis show that targeted imaging is possible. Yet its progress in medical imaging has been greatly hindered by the inability to generate iron oxide nanoparticles that meet all the requirements for targeted imaging agents. The results from this thesis have demonstrated a superior contender for targeted
imaging and, based on these results, these iron oxide silica nanoparticles should be investigated for other targeted imaging applications.


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Quasi-Cubic Magnetite/Silica Core-Shell Nanoparticles as Enhanced MRI Contrast Agents for Cancer Imaging

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Abstract

Development of magnetic resonance imaging (MRI) contrast agents that can be readily applied for imaging of biological tissues under clinical settings is a challenging task. This is predominantly due to the expectation of an ideal MR agent being able to be synthesized in large quantities, possessing longer shelf life, reasonable biocompatibility, tolerance against its aggregation in biological fluids, and high relaxivity, resulting in better contrast during biological imaging. Although a repertoire of reports address various aforementioned issues, the previously reported results are far from optimal, which necessitates further efforts in this area. In this study, we demonstrate facile large-scale synthesis of sub-100 nm quasi-cubic magnetite and magnetite/silica core-shell (Mag@SiO2) nanoparticles and their applicability as a biocompatible T2 contrast agent for MRI of biological tissues. Our study suggests that silica-coated magnetite nanoparticles reported in this study can potentially act as improved MR contrast agents by addressing a number of aforementioned issues, including longer shelf life and stability in biological fluids. Additionally, our in vitro and in vivo studies clearly demonstrate the importance of silica coating towards improved applicability of T2 contrast agents for cancer imaging.
INTRODUCTION

Interest in magnetic nanomaterials has persisted over the last few decades primarily due to their applications across many fields such as magnetic data recording, sensing, catalysis and biomedicine [1]-[5]. Magnetic nanomaterials have attracted particular attention in biomedicine due to their great potential in improving the currently available disease diagnostics, prevention, and therapeutic approaches [6]. For instance, the potential of magnetic nanoparticles to precisely deliver highly biotoxic drugs to specific locations in the body [6], as well as their use as highly specialized bio-probes for diagnostic imaging has been demonstrated by attaching biomolecular markers to their surface [1], [7]. With these developments, there is an increasing demand to develop biocompatible magnetic nanomaterials with ultra-sensitive imaging capabilities in order that they can be used for a wide range of in vivo medical imaging applications.
Magnetic resonance imaging (MRI) is regarded as a powerful imaging tool because of its high spatial resolution capability, non-invasive nature and its capability to avoid ionizing radiation in contrast to nuclear imaging techniques such as positron emission tomography (PET) [8]-[10]. Briefly, MRI operates by taking advantage of the exceptionally small magnetic moment inherent on each proton that, under the presence of a large magnetic field, produces an effect measurable as a signal on the MR image. The signals produced via T1 relaxation (spin-lattice relaxation) or T2 relaxation (spin-spin relaxation) depends on the sequence parameters programmed to acquire the MR image. Overall, T1 weighted and T2 weighted imaging provide different contrasting effects between fluid and body tissue. For instance T1 weighted images show fluid as dark, water-based tissues as grey and fat-based tissues as bright, thereby very clearly showing the boundaries between different tissues. Conversely, on T2 weighted images, fluid appears bright and water- and fat-based tissues appear grey. The use of contrast agents greatly improves the specificity and sensitivity of MRI by shortening either T1 or T2 relaxation of the water protons adjacent to them, thus providing more detailed information about pathology. Gadolinium-based T1 contrast agents are most commonly used in MRI, however growing concerns over the safety of gadolinium-based contrasts have lead to a major shift towards iron oxide based T2 contrast agents that are deemed to be relatively biologically safe [11]-[13].

Although, iron oxide based contrast agents have been clinically approved for MRI, their use has been predominantly restricted to liver/spleen imaging (AMI-25 Feridex® - not in use anymore) and the gastrointestinal lumen imaging (Lumirem®/Gastromark®). This limitation is primarily due to the larger size of the iron oxide particles involved in these agents, which are either taken up immediately by the reticuloendothelium system after intravenous administration (Feridex®), or are administered orally (Lumirem®/Gastromark®). Therefore, there is a clinical urgency to develop commercially viable and biologically safe contrast agents that can be used for MR imaging of a wide range of body tissues [14]-[16]. Moreover, there have been numerous reports on different synthesis routes to magnetic nanoparticles-based contrast agents, including biologically synthesized magnetic nanoparticles [17]-[18], magnetic nanoparticles with dendrimer cores [19], superparamagnetic liposomes [20], lipid-based MR contrast agents [21], metal-doped magnetic nanoparticles [22]-[25], CoFe2O4@SiO2 particles with fluorescent dyes incorporated [26], and magnetic nanoparticles for both imaging and
therapeutic applications [27]. Additionally, in the pre-clinical setting, the trend over the last few years has been towards the development of small (sub-100 nm) iron oxide nanoparticles [24], [28]–[31]. The previous studies suggest that to shift from sub-micron iron oxide particles to their nanoparticulate form in the clinical environment, the challenges that need to be overcome include their low chemical and biological stability, small shelf life, inherent low-to-high cytotoxicity, and low magnetization associated with the iron oxide nanoparticles, which has although been addressed by few recent studies to some extent, it still requires additional efforts in this area [32]–[34]. This is predominantly because the aforementioned properties of MR contrast agents can strongly depend on their synthesis route.

In this manuscript, we address most of the aforementioned issues by demonstrating the development of a T2-weighted, iron oxide-based MRI contrast agent with reasonably low cytotoxicity, high relaxivity, and particularly notable high stability that can be stored at room temperature for more than 6 months without any visible aggregation. The chemical stability of these nanoparticles is achieved by coating them with an inorganic silica (SiO2) layer, leading to Mag@SiO2 core-shell nanoparticles. The resulting nanoparticles were analyzed by a superconducting quantum interference measurement device (SQUID), high resolution transmission electron microscopy (HRTEM), X-ray diffraction (XRD) and a 3 Tesla clinical MRI scanner. Our in vitro studies indicate that coating with SiO2 renders these nanoparticles biocompatible and they are actively taken up by prostate cancer cells under in vitro conditions. Our preliminary in vivo studies with a breast tumor animal model further suggests their potential utility as good MRI contrast agents for tumor imaging.

RESULTS AND DISCUSSION

Figure 1A shows the TEM image of the magnetic (Mag) nanoparticles, which indicates that the as-synthesized Mag nanoparticles prepared by our synthesis route were quasi-cubic in morphology with good monodispersity and an average size of 40±5 nm. Notably, using our approach, large scale synthesis of Mag nanoparticles could be achieved (at least up to 10 g particles per batch) without compromising the nanoparticle shape or monodispersity. From the higher magnification TEM image, these Mag nanoparticles were found to have spherical edges, and it appears as if these nanoparticles consist of several smaller spherical particles that assemble together giving rise to quasi-cubic structures (inset Figure 1A). It is important to note
that under room temperature storage conditions, pristine Mag nanoparticles lose their quasi-cubic morphology and turn spherical after two weeks of synthesis. The shelf life of commercially available MRI contrast agents is in fact one of the major limitations associated with clinical applicability of such materials. SiO₂ shell coating has been previously demonstrated to provide biocompatibility, particle stability as well as a facile surface for further biofunctionalisation in different nanomaterials [27]-[29]. Therefore, to provide chemical stability to magnetic nanoparticles, a silica shell was grown around quasi-cubic Mag particles (within 3 days of their synthesis), thereby producing Mag@SiO₂ core-shell nanoparticles (Figure 1B). The controlled silica coating of Mag nanoparticles led to formation of Mag@SiO₂ core-shell structures with a ca. 20±2 nm silica shell around 40±5 nm quasi-cubic Mag nanoparticles (Figure 1B and inset). Large area TEM analysis of Mag@SiO₂ core-shell structures indicated that most of the Mag nanoparticles retained their quasi-cubic morphology after silica coating, and more than ca. 75% of particles in the sample were found to be individually coated with a silica shell. However, less than ca. 25% of structures consisted of either two or three or no Mag particles within the silica shell. Notably, this type of particle distribution is typical of a chemical synthesis route, which is not necessarily always explicitly acknowledged in the prevailing literature. Additionally, we observed that after coating Mag nanoparticles with silica, the Mag@SiO₂ particles remain stable in phosphate buffer saline (PBS) solution for at least up to 1 mg/mL concentration, as well as in the readily-dispersible powder form for at least up to 6 months. The TEM image shown in Figure 1B was acquired after 6 months of storage of Mag@SiO₂ nanoparticles at room temperature and was similar to those imaged immediately after synthesis. This suggests that a silica coating over Mag nanoparticles can significantly improve their stability for long-term storage conditions, thus retaining their magnetic properties by improving their shelf life. This is one of the crucial parameters for developing MRI-based contrast agents for clinical and commercial applications.

**Figure 1.** TEM images of (A) Mag and (B) Mag@SiO₂ core-shell nanoparticles.

Insets show the respective higher resolution TEM images.

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Figure 2 shows the XRD patterns of Mag and Mag@SiO2 nanoparticles. The XRD pattern obtained from quasi-cubic Mag nanoparticles (curve 1) could be indexed based on standard diffraction pattern typically arising from magnetite (Fe₃O₄) with major peaks indexed (JCPDS file No 75–0449). After silica coating, most of the diffraction peaks arising from Mag nanoparticles could still be detected. However interestingly, after silica coating, an additional peak at ca. 29.3° 2θ was observed that could be assigned to the (220) plane of a FeSi₂ phase (curve 2) (JSPDS file no. 73-0963). The mixed Fe-Si phase is most likely formed at the interface of silica and magnetite during core-shell synthesis of Mag@SiO2 nanoparticles.

![Figure 2: XRD patterns obtained from Mag and Mag@SiO2 nanoparticles.](image)

XRD peaks with corresponding Bragg reflections of magnetite have been indicated. (*) corresponds to the XRD peak arising from a mixed Fe-Si phase.

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High saturation magnetization of MR contrast agents is an important requirement for the magnetic nanoparticles to be used for MRI application. The magnetic hysteresis curve of Mag@SiO2 nanoparticles obtained by SQUID measurement is shown in Figure 3, which was found to have no coercive fields, thus confirming their superparamagnetic nature. Mag@SiO2 nanoparticles were found to possess a relatively high mass magnetization value of 74.4 emu/g, which is comparable to the previously reported mass magnetisation values of 72.9 emu/g for commercially available Resovist iron oxide particles [35].

![Figure 3: Magnetic hysteresis curve of Mag@SiO2 nanoparticles used for MR imaging of tumor cells and tissues.](image)

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The Mag and Mag@SiO2 synthesized in this study were further tested for their ability to be internalised by human prostate cancer PC3 cells (Figure 4). When subjected to cell uptake studies for 24 h, 50 µg/mL Mag@SiO2 nanoparticles were found to be uptaken by PC3 prostate
cancer cells more efficiently than similar a concentration of bare Mag nanoparticles (compare Figures 4B and C). When PC3 cancer cells were exposed to Mag nanoparticles, we observed that bare Mag nanoparticles without any SiO2 coating tended to form large aggregates (of dimensions similar to cell size) in the solution over a 24 h exposure period, which restricted their ability to be uptaken by PC3 cells (Figure 4B). As can be inferred from Figure 4B, these large clusters of bare Mag nanoparticles predominantly attach to the exterior of the cells, and are difficult to be internalized by PC3 prostate cancer cells. Conversely, after SiO2 coating, Mag@SiO2 nanoparticles remain well-dispersed in the solution even after 24 h, which facilitates their efficient uptake by PC3 cells, as can be seen from a higher density of Mag@SiO2 nanoparticles inside PC3 prostate cancer cells (Figure 4C). Our group and others have previously demonstrated that nanoparticle size and aggregation in biological media can play a crucial role in cellular uptake processes, as non-specific uptake of sub-100 nm nanoparticles is generally observed via endocytosis mechanism of the cells [36]–[39]. Aggregation of bare (pristine) Mag nanoparticles in biological media, and avoidance of their aggregation after silica coating clearly suggests the important role of SiO2 coating, and advantage of Mag@SiO2 core-shell nanoparticles over bare Mag nanoparticles for biological applications. Based on results from cell uptake studies, pristine Mag nanoparticles were found to be unsuitable for biological applications, and therefore only Mag@SiO2 nanoparticles were chosen for further studies regarding their suitability for MRI applications.

From the cell uptake studies, it is also evident that Mag@SiO2 nanoparticles do not cause any significant change to the morphology of PC3 prostate cancer cells. Previous studies indicate that iron oxide nanoparticles are non-toxic at lower concentration, but can be mildly toxic at higher concentrations [40]–[41]. Before exploring Mag@SiO2 nanoparticles for MRI application, biocompatibility profile of these particles was assessed by performing MTS-based in vitro cytotoxicity experiments on PC3 prostate cancer cells, which is one of the measures of
biocompatibility (Figure 5). It is evident from Figure 5 that Mag@SiO2 nanoparticles did not significantly affect PC3 cell viability for at least up to 50 µg mL−1 Fe concentrations, at which more than 85% PC3 cells viability was maintained. However further increase in Mag@SiO2 nanoparticles concentration equivalent to 100 µg mL−1 Fe resulted in a cell viability loss of ca. 30%. This suggests that Mag@SiO2 nanoparticles reported in this study may be suitable for MRI applications within 50 µg mL−1 Fe concentration range. However, this aspect may require further detailed investigation, wherein effect of Mag@SiO2 nanoparticles on cytokine production profile of cells will need to be investigated.

Since magnetic nanomaterials can modulate MR signal enhancement effects, the capability of Mag@SiO2 nanoparticles as T2 MR contrast agent was further assessed in terms of their relaxivity (R2 or relaxation rate, which equals 1/T2 where T2 is spin-spin relaxation time) on a 3 Tesla clinical MRI scanner at an echo time (TE) of 10.86 ms. Relaxivity is a measure of the efficiency of a MR contrast agent to enhance the proton relaxation and increase the efficiency to which image contrast is produced during MRI [42]. The relaxivity measurements were performed both on nanoparticles as suspension in phantoms as well as after being uptaken by PC3 prostate cancer cells. Mag@SiO2 nanoparticles were found to have a high relaxivity value of 263.23 l/mmol/s in cell free suspensions, and 230.90 l/mmol/s for Mag@SiO2 nanoparticles within the PC3 cells. High relaxivity value (that is, better MR contrast) along with high mass magnetisation value for MRI are important considerations when developing T2 contrast agents, as the spin-spin relaxation process of protons in water molecules surrounding the nanoparticles is facilitated by the large magnitude of magnetic spins in nanoparticles [43]–[44]. Mag@SiO2 nanoparticles with high mass magnetization and high relaxivity values may therefore result in strong T2-weighted MR signal intensity decrease as measured by MRI [45]. This is critical in allowing nanomolar activity of contrast agents, which will facilitate in reducing the overall contrast agent dose to the patients.
The relaxivity data also suggests a reduction in the relaxivity value of Mag@SiO2 nanoparticles in PC3 cells after cellular uptake compared with that in suspension. This finding corroborates well with previous studies, which showed that the relaxivities of native iron oxide nanoparticles were higher compared to those after accumulation in the cells [46]–[47]. The mechanisms responsible for this effect have not yet been fully understood, however it can possibly be attributed to the confinement of nanoparticles within endosomes of the target cells, which might cause a build-up of magnetic field inhomogeneities after sub-cellular compartmentalization, which would conversely be absent in uniformly distributed nanoparticles in suspensions [48]. Additionally, the different geometrical arrangement of nanoparticles in suspensions and in cells, and possibly antiferromagnetic coupling as a result of clustering within the sub-cellular compartments may play some role in reducing relaxivity values after cellular uptake [28], [48]. Notably, in contrast to relaxivity values of 230–269 l/mmol/s observed for Mag@SiO2 nanoparticles in this study, commercial Resovist based nanoparticles have been reported with lower values of 151 l/mmol/s [35]. The observed relaxivity value of Mag@SiO2 nanoparticles prepared in this study is also relatively higher than those reported for undoped magnetite particles (218 l/mmol/s) in recent detailed studies [24]. For doped magnetic particles, it has been reported that high relaxivities of up to 358 l/mmol/s can be achieved by doping magnetite with Mn (MnFe2O4) [24]. However, potential leaching of Mn during administration of these MR contrast agents in the body might pose cytotoxicity issues, and to the best of authors' knowledge, undoped Mag@SiO2 nanoparticles with such high relaxivity values have not hitherto been reported.

Furthermore, relaxivity studies as a function of different concentrations of Fe in Mag@SiO2 nanoparticles, both as a nanoparticle suspension in phantoms (Figure 6A), and after 24 h of nanoparticle uptake by PC3 prostate cancer cells (Figure 6B) revealed that Mag@SiO2 nanoparticles act as outstanding T2-weighted contrast agents. This is shown by an image darkening effect, demonstrated by drop in R2 (ΔR2/R2control) signal intensity with increasing Fe concentrations. For instance, at 100 µg/mL Fe concentration, Mag@SiO2 nanoparticles provide a signal enhancement of ~90% in comparison to more than 70% signal enhancement during imaging of PC3 prostate cancer cells. This is a significant signal enhancement in comparison to most of the previously reported materials, in which generally only 15–20% signal enhancement
has been observed [28]. Such strong MR signal enhancement is expected from Mag@SiO2 nanoparticles because of their relatively high relaxivity and saturation magnetization values.

**Figure 6.** Evaluation of Mag@SiO2 nanoparticles as a T2 MR contrast agent is shown in the form of % signal enhancement with increasing concentration of Fe using a 3 Tesla MR scanner.

Panel A shows the studies performed in phantoms for particles in suspension, while panel B shows the similar studies in PC3 human prostate cancer cells after nanoparticles uptake for 24 h. Corresponding T2-weighted MR images of different samples, showing the image darkening effect with increasing Fe concentration are also shown under each bar.

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*In vivo* MRI studies in a breast tumor mouse model also demonstrated T2 signal enhancement at the tumor site by Mag@SiO2 nanoparticles (**Figure 7**). The images following *in vivo* administration of 10 µg dose of Mag@SiO2 nanoparticles demonstrate its ability to produce MR enhancement of the tumor site relative to the body. T2-weighted signal enhancement effects by the Mag@SiO2 nanoparticles on an MR image are visualised as darkening or contrast between areas infiltrated with Mag@SiO2 nanoparticle and those without nanoparticles. Future studies on Mag@SiO2 can be tailored for targeted MRI, utilising its superior magnetic characteristics in the diagnosis of pathologies.

**Figure 7.** T2-weighted MR images of nude mice with breast tumor obtained (A) before and (B) after injection of MR contrast agent, obtained using a 3 Tesla MR scanner.

Mouse 2 was injected with Mag@SiO2 nanoparticles as T2 contrast agent, while Mouse 1 was injected with saline as a control. Tumor sites in the control (mouse 1) and in the treated mouse (mouse 2) have been labelled as blue and red circles respectively. Panels C and D show the higher magnification transverse section images of tumor site corresponding to Panels A and B respectively, wherein tumor region injected with MR contrast agent has been highlighted using white circles.

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In summary, important considerations for an efficient MRI contrast agent include smaller particle size, their efficient uptake by cells, reduced aggregation in biological fluids, improved shelf life, and improved biocompatibility. A control over all these parameters will provide an ability to target a range of molecular/cellular imaging applications without causing acute toxicity to the normal cells. Particularly for tumor imaging applications, sub-100 nm particles can provide significant an advantage, as the cut-off diameter of tumor vessel pores is 400–600 nm [41]-[43], [49]-[51].

In this study, we have demonstrated a facile, large-scale synthesis of quasi-cubic magnetite and Mag@SiO2 nanoparticles of sub-100 nm size. The Mag@SiO2 nanoparticles reported here have a shelf life of more than 6 months, and they are efficiently uptaken by the cells without causing significant aggregation or cellular toxicity. The biological half-life of smaller and silica-coated iron oxide nanoparticles is expected to be further increased due to their reduced interaction with the body fluids. This study therefore clearly underlines the importance of SiO2 coating towards improving the uptake of Mag@SiO2 nanoparticles by PC3 prostate cancer cells, and improving the shelf life of MR contrast agents. The magnetic-silica composite nanoparticles act as promising T2 contrast agents offering a potentially viable option as a commercial MR contrast agent. This is attributable to their small size, high MR signal enhancement, relative biocompatibility, longer shelf life, and highly modifiable silica surface chemistry which will allow the adhesion of multiple molecular markers for targeted MRI in the future. These characteristics of a T2 contrast agent are highly desirable for magnetic resonance imaging applications at the pre-clinical level and for later use clinically.

MATERIALS AND METHODS

Ethics Statement

The breast tumor mice model was developed in-house, and all the studies involving animals were pre-approved by institutional animal ethics committee.

Materials

All chemicals were purchased from Sigma-Aldrich and used as received without further modification. The prostate cancer cells (PC3 cell line) were purchased from American Type
Culture Collection (ATCC). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) kit was purchased from Promega Corporation.

**Synthesis of iron oxide nanoparticles**

Quasi-cubic iron oxide nanoparticles (referred as ‘Mag’) were synthesized using a two step process significantly modified from Park *et al.*, thus leading to controlled large-scale synthesis \[52\]. During synthesis, an iron oleate complex was first formed by dissolving 5.4 g of iron chloride and 18.25 g of sodium oleate in a solution comprised of 40 mL ethanol, 30 mL distilled water and 70 mL hexane. Once homogenized, the solution was refluxed at 70°C for 4 h, followed by separation of the upper organic layer using a separatory funnel, washing and evaporating off hexane, thereby leaving a waxy iron oleate complex. The iron oxide nanocrystals were formed by dissolving 9.0 g of the iron oleate complex in 1.425 g of oleic acid and 63.3 mL of 1-octadecene, followed by reflux under nitrogen until it reached 320°C, at which point the temperature was held for 30 min and then allowed to cool to room temperature. 250 mL of ethanol was then added to the solution and the magnetite particles were separated via centrifugation, followed by three washing cycles with ethanol. Notably, by designing this protocol, scale up of at least up to 10 g magnetic nanoparticles per reaction could be easily achieved under laboratory conditions.

**Synthesis of silica-coated iron oxide (Mag@SiO2) nanoparticles**

Silica-coated iron oxide nanoparticles (Mag@SiO2) were prepared using a method significantly modified from Fang *et al* and Morel *et al* \[53\]-\[54\], wherein controlled hydrolysis of silica precursor in the presence of magnetite nanoparticles was performed. In our approach, pre-formed magnetic particles were used as nucleating sites for subsequent hydrolysis of silica precursor around them. Briefly, 1 mg of iron oxide nanoparticles prepared in the previous step were sonicated in a solution consisting 15 mL ethanol and 2 mL deionized water (MilliQ). 1 mL of ammonia (25% solution) was added to the above solution while immersed in a sonicator programmed to switch on for 1 min in every 10 min. Further, an overhead stirrer was additionally used to mix the solution while 4 mL of 1:60 (tetraethyl orthosilicate:ethanol) was added at the rate of 0.4 mL/h using a syringe pump, and the solution was allowed to stir at
room temperature for 12 h. The silica coated iron oxide nanoparticles were centrifuged, washed three times with ethanol and redispersed in MilliQ water.

**Materials characterisation**

The morphology and size of Mag and Mag@SiO2 nanoparticles was characterized using JEOL 2010 high resolution transmission electron (HRTEM) microscope operated at an accelerating voltage of 200 kV. Samples for HRTEM measurements were prepared by drop casting particles on to a carbon-coated copper grid, followed by air drying. The crystallography of the nanomaterial powders was obtained on a Bruker D8 ADVANCE X-ray diffractometer using Cu Kα radiation. For magnetic measurements, a superconducting quantum interface device based magnetometer (Quantum Design MPMS-XL5) was used. The iron content of the nanoparticle solutions used for *in vitro* and *in vivo* studies was ascertained on a Varian AA280FS Fast Sequential Atomic Absorption Spectrometer (AAS) after digestion of particles overnight in nitric acid.

**In vitro cell studies and cytotoxicity assays**

Human prostate cancer cells (PC3 cell line) were routinely cultured at 37°C in a humidified atmosphere with 5% CO2 using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin/penicillin and 1 mM L-glutamine. For sub-culturing, PC3 prostate cancer cells were detached by washing with phosphate buffered saline (PBS) and incubating with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) for 5 min at 37°C, followed by washing and incubation with supplemented RPMI 1641 medium. For cell uptake, the cells were first seeded in 24-well polystyrene dishes for 24 h, followed by incubation with Mag and Mag@SiO2 nanoparticles for 24 h at 37°C in complete cell media, and subsequent three times washing of cells with PBS, before imaging under an inverted microscope. For cytotoxicity assays, the viability of PC3 prostate cancer cells exposed to Mag@SiO2 nanoparticles in the absence of cell growth medium was determined. A CellTiter 96 AQeuous One Solution Cell Proliferation Assay (Promega) kit containing the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS), was used to monitor cell viability according to the manufacturer's protocols. MTS color change was monitored using a plate reader at 490 nm, and cell viability data was
plotted by considering the viability for the untreated cells as 100%. Experiments were performed in triplicates, and error bars represent standard experimental errors.

**Magnetic resonance imaging (MRI) studies**

MRI studies were performed for nanoparticle solutions stored in phantoms, in PC3 prostate cancer cells after nanoparticle uptake, and in a mouse model with breast cancer. For phantom MRI studies, phantoms were prepared in Eppendorf tubes with Mag@SiO2 nanoparticles at three different Fe concentrations (0.18 mM, 0.9 mM, 1.79 mM) and a saline solution without any nanoparticles was used as a control. For *in vitro* MRI studies, PC3 cancer cells were cultured using the above protocol in 24 well polystyrene plates, and incubated for 24 h with Mag and Mag@SiO2 nanoparticles at three different concentrations (0.18 mM, 0.9 mM, 1.79 mM) and a control with cells but no nanoparticles. MRI measurements for phantoms and PC3 cells were performed with a clinical 3.0 Tesla Clinical Siemens Trio MRI scanner using a 12-channel head coil and the following parameters: T2-weighted imaging, gradient echo sequence, multiple echo time (TE) ranging from 0.99–100 ms, repetition time (TR) = 2000 ms, matrix 128×128, slice thickness of 3 mm. Relaxation rates (R2) were determined by using a single echo sequence (SE) with a constant TR of 2000 ms and multiple TE ranging from 0.99–100 ms. The signal was plotted as a function of echo time and fitted to obtain the R2 values. The R2 values of the Mag@SiO2 in phantoms and PC3 cells were determined by plotting the relaxivity at a TE of 10.86 ms, as a function of molar iron concentration in respective samples, and extracting the T2 value from the slope by linear regression of data points obtained at lower Fe concentration values. Only lower Fe concentrations were used to determine the T2 values, predominantly because with increasing Fe concentrations above a particular threshold, the MR signals tend to lose their linearity. For the *in vitro* MRI measurements in phantoms and PC3 cells, enhancement of the R2 signal within the PC3 cells was calculated by: ΔR2/R2control*100. For *in vivo* MRI experiments, breast tumor bearing mice were developed in-house, anaesthetised with ketamine (80 mg per kg body weight) and xylazine (5 mg per kg body weight), and placed within the 12-channel head coil. Images were acquired before and after injection of 100 µL of Mag@SiO2 particles suspension of 100 µg/mL concentration in saline locally at the tumor site. A T2-weighted spin echo sequence was acquired with TE/TR of
60/2000 ms, a slice thickness of 3 mm and a 128x128 matrix. Data analysis was performed manually by placing ROIs in tumor and tissue areas on the images.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JLC JA SFC PE SKB VB. Performed the experiments: JLC JA. Analyzed the data: JLC JA SFC AG PE SKB VB. Contributed reagents/materials/analysis tools: VB PE. Wrote the paper: JLC JA VB.

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Herceptin-functionalized Magnetite/ Silica Core-Shell Nanoparticles as MRI Contrast Agents for Targeted Cancer Imaging

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Introduction

Iron oxide nanoparticles have been widely researched in the pre-clinical setting for various applications in magnetic resonance imaging (MRI) such as enhanced image contrast in the liver (Reimer & Balzer, 2003) and gastro-intestinal lumen (MacVicar, Jacobsen, Guy, & Husband, 1993; Vlahos et al., 1994), magnetic cell labelling (Bulte, 2009) and local therapy in the form of hyperthermia in cells (Hadjipanayis et al., 2008). Despite years of pre-clinical research, iron oxide nanoparticles have not been successfully translated to the clinical setting primarily due to non-optimal properties such as limited magnetisation values, low contrast enhancement, difficulties in producing large amounts of particles and large intravenous dosages; therefore there is continued interest in their development. One of the areas of interest is applications of iron oxide nanoparticles in cellular and molecular imaging, where biomarkers are attached to the iron surface which drives the particles to an area of interest and as a result, the nanoparticles provide MRI contrast enhancement at the targeted area providing valuable diagnostic and therapeutic information. The aim therefore has been to produce iron oxide nanoparticles that have high magnetisation values, small size, biocompatibility and a particle surface that is easily functionalised by various biomarkers (Jun, Lee, & Cheon, 2007; Laurent et al., 2008; Lawaczeck, Menzel, & Pietsch, 2004).

Magnetisation values are important for MRI applications of nanoparticles because they influence the ability to provide enhanced contrast on the magnetic resonance image (Lu, Wang, Ye, Vaidya, & Jeong, 2007). Values such as the electromagnetic unit per gram (emu/g) and the T2 relaxation (spin-spin relaxation) determine the degree of T2 relaxation shortening that occurs in the surrounding water protons. Shorter T2 relaxation times produce image contrast appearing as dark on a magnetic resonance image relative to longer T2 relaxation times that appear brighter.
In addition to magnetic characteristics, MRI applications of iron oxide nanoparticles also require a small nanoparticle size and size distribution, as larger particles limit in-vivo applications. It is well known that particles larger than 50nm in diameter are eliminated by the reticulo-endothelial system (RES) therefore these particles are only useful for imaging the liver and spleen. An example of this is the commercially available Resovist which has a hydrodynamic diameter of 60nm and upon intravenous injection, is immediately taken up by the liver therefore allowing imaging of liver lesions. Non-commercially, numerous studies have been conducted over the years adjusting factors such as pH, ionic strength, temperature, nature of salts, Fe$^{3+}$/Fe$^{2+}$ ratio, and addition of chelating agents, which improve the size and size distribution of the iron oxide nanoparticles produced (Babes, Denizot, Tanguy, Jeune, & Jallet, 1999; Jiang et al., 2004; JOLIVET, BELLEVILLE, TRONC, & LIVAGE, 1992; Massart, 1981). Recent studies have looked at high temperature methods of iron oxide nanoparticle production to control the size and produce particles with exceptional magnetic characteristics (Hyeon, Lee, Park, Chung, & Na, 2001; PARK et al., 2004). Other methods that have been investigated include the addition of surface coatings such as dextran(Jarrett, Frendo, Vogan, & Louie, 2007; Moore, Marecos, Bogdanov, & Weissleder, 2000), monolayer ligands (Yee et al., 1999) and polymers (Harris et al., 2003). These coatings not only help reduce the size of the iron oxide nanoparticles but also play a role in achieving biocompatibility and acting as a surface for the attachment of various biomarkers.

Biocompatibility is particularly important for iron oxide nanoparticles used in molecular and cellular imaging as water-soluble iron oxide nanoparticles can not withstand harsh biological conditions such as changes in pH and inability to escape uptake by the RES. The later is a fundamental consideration, because applications in molecular and cellular imaging need iron oxide nanoparticles to have long blood circulating times to
ensure that they localise to an area of interest. Biocompatibility is therefore ensured by the addition of lipids (Y. Lee et al., 2005; Tartaj & Serna, 2002) or polymers on the surface of the iron oxides such polyethylene glycol (PEG) (Acar, Garaas, Syud, Bonitatebus, & Kulkarni, 2005; Wan et al., 2007). Other ways biocompatibility has been achieved is by the addition of chelating agents such as dimercaptosuccinic acid (DMSA) on the surface of the iron oxides (Fauconnier, Pons, Roger, & Bee, 1997; Huh et al., 2005b) or transfection agents such as dendrimers (I. H. Lee et al., 2004; Walter et al., 2004).

For molecular and cellular applications of iron oxide nanoparticles need to be conjugated to a particular biomarker. Herceptin or trastuzumab is a monoclonal antibody that targets the epidermal growth factor receptor HER2, which is overexpressed in HER2/neu positive metastatic breast cancers (Valabrega, Montemurro, & Aglietta, 2007). It is well known that these breast cancers are expressed in 25-30% of all breast cancers and are usually diagnosed at a late stage, this combined with the aggressive nature of the cancer generally lead to an overall poor prognosis (Borg et al., 1990; SLAMON et al., 1987). Trastuzumab was developed to target HER2/neu receptors via an antibody-receptor complex and undergoes internalisation via receptor-mediated endocytosis at the HER2/neu receptor site. Trastuzumab then causes down regulation of HER2/neu production eventually causing cell death (Valabrega, et al., 2007). Unfortunately, despite the positive effects Trastuzumab has on HER2/neu positive breast cancers, the overall response rates are low. The low response rates are due to many factors, such as the development of resistance to Herceptin and the level of HER2/neu protein and gene expression within the tumour. The ability to image the progress of trastuzumab treatment can be useful, providing critical information about the efficacy of trastuzumab concurrently with treatment.
A range of studies have already reported in-vitro and in-vivo detection using trastuzumab conjugated to a variety of iron oxide based nanoparticles. Studies have looked at in-vitro models using a biotinylated antibody conjugated to streptavidin microbeads (Dmitri, Noriko, Baasil, & Zaver, 2003) and other studies have looked at dextran coated iron oxide nanoparticles with low T2 characteristics (T. Chen et al., 2009). More recent studies have looked at highly magnetic DMSA coated (Jun et al., 2005) and metal doped (J.-H. Lee et al., 2007) iron oxides made via a novel high temperature method, conjugated to herceptin. Recently, we reported quasi-cubic iron oxide nanoparticles coated with a silica shell of 60nm in hydrodynamic diameter having superb magnetic characteristics and significant MRI signal enhancement capabilities (add our ref). Our previous study showed low cellular toxicity of the silica coated quasi-cubic particles to PC3 prostate cancer cells, and in-vitro studies with up to 90% MRI signal enhancement. In this report, we now present the successful synthesis of 25nm iron oxide silica nanoparticles conjugated to herceptin. In this investigation we present the in vitro diagnosis of breast cancer in various cell lines (SKBR-3, BT474 and MCF-7) and the in vivo diagnosis of breast cancer within HER2/neu overexpressing tumours.

MATERIALS AND METHODS

Reagents, chemicals and assay kits

All chemicals were purchased from Sigma Aldrich Pty LTD and used as received without further modification. Cell lines (SKBR3, BT474) were purchased from American Type Culture Association (ATCC). CellTiter 96 Aqueous One Solution Cell Proliferation Assay
(Promega) kit was purchased from Promega Corporation. BCA protein assay was purchased from Invitrogen. Herceptin was a generous gift from the Pharmacy Department at The Peter MacCallum Cancer Centre.

**Synthesis of silica coated iron oxide nanoparticles**

The iron oxide nanoparticles were fabricated using a high temperature method previously described by Park *et al.* In brief, an iron oleate complex was refluxed with oleic acid in 1-octadecene at 320°C. The resulting magnetite nanoparticles were centrifuged from the solution and washed. The magnetite was then treated with nitric acid to remove any surface contaminants, washed and surface treated with citric acid to aid suspension and stabilisation of the particles during the silica addition process. Using a modified method of silica coating previously presented by Fang *et al.* the magnetite particles were coated with a thin layer of silica by slow addition of tetraethylorthosilane (TEOS) to a solution of ethanol, water ammonia and highly dispersed magnetite nanoparticles over several hours.

**Conjugation of iron oxide nanoparticles to Herceptin**

Iron oxide-silica nanoparticles were resuspended in milli-Q water and then conjugated to herceptin using 5mg/ml of cysteamine linker. The iron-silica and herceptin were incubated at room temperature for 2 hours and then washed several times in milli-Q-water by centrifugation. The amount of herceptin on the surface of the iron oxide-silica nanoparticles was quantified via a BCA protein assay kit according to the manufacturer’s instructions.

**Assessment of nanoparticle-herceptin stability**

The stability of the iron oxide silica nanoparticles tagged to herceptin was assessed by conjugating a FITC molecule to the herceptin antibody using a FITC conjugation kit (Sigma, USA) according to the manufacturer’s protocol small scale conjugation procedure. The iron oxide silica herceptin-FITC nanoparticles were exposed to a range of
pH (5, 7.14, 10) and human serum concentrations (25%, 50%, 100%). The degradation of the particles was assessed by loss of protein in the supernatant indicated by an increase in fluorescence in the supernatant based on a standard concentration series of the herceptin-FITC molecules.

**In vitro studies**

**Cell culture**

Breast cancer cell lines, SKBR-3, BT474 and MCF-7 cells were routinely cultured at 37°C in a humidified atmosphere with 5% CO₂ using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin/penicillin and 1mM L-glutamine (manufacturer, country). For sub-culturing, cells were detached by washing with phosphate-buffered saline (PBS) and incubating with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) for 4 mins at 37 °C, followed by washing and incubation with supplemented RPMI 1641 medium.

**Cell cytotoxicity studies**

To assess cytotoxicity of the iron oxide-silica herceptin nanoparticles on breast cancer cells, the viability of SKBR-3 cells incubated with the herceptin labeled particles was assessed after 24 and 48 hours *in vitro*. SKBR-3 cells were seeded into 24-well plates for 24 hours, after which medium without serum was added with iron oxide-silica nanoparticles at concentrations ranging between 1 μg/ml to 100 μg/ml. A CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, USA) containing the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to monitor cell viability according to the manufacturer’s instructions and the MTS colour change was measured at 490 nm using a microplate reader (manufacturer, country) and cell viability was plotted against 100% viability for untreated cells.
Conjugation of iron-oxide herceptin nanoparticles to FITC

Herceptin was conjugated to FITC using a FluoroTag FITC conjugation kit (Sigma, USA). The herceptin was first purified using a Sephadex G-25M column (Pharmacia, Sweden) and then added to the FITC using the small scale conjugation procedure following the manufacturer's protocol. The FITC-Herceptin complex was then added to the iron-silica via the cysteamine linker and after 2 hours incubation at room temperature, washed several times with milli-Q water.

Cellular uptake

Confocal microscopy

SKBR3, BT474 and MCF-7 were cultured and then seeded onto a 6-well slide glass chamber. All cell lines were treated with 50 μg/ml of iron oxide-silica herceptin-FITC nanoparticles for 4 hours at 37°C and cellular uptake was assessed by confocal microscopy (Zeiss, model, country). Thirty minutes before imaging, a Hoechst nuclear stain was added to the cells and then washed several times with PBS to aid in the identification of live cells within the culture.

Flow cytometry

To quantify the uptake of the iron oxide-silica herceptin nanoparticles by SKBR3, BT474 and MCF7, dose response and time course studies were performed using flow cytometry. Cells were grown to 90% confluence in 24-well plates and for the dose-response study, 10 μg/ml, 50 μg/ml and 100 μg/ml of iron oxide-silica herceptin nanoparticles were added to cells and incubated for 24 hours. For the time-course studies, the iron oxide-silica herceptin nanoparticles conjugated to FITC were incubated at varying time points of 30 min, 1, 3, 5 and 24 hours and at a constant concentration of 50 μg/ml. After incubation for both dose-response and time-course studies, the cells were detached from the plates with trypsin-EDTA and washed twice in PBS and resuspended in FACS-fix buffer for analysis.
by flow cytometry and a minimum of 8,000-10,000 viable cells were assayed (FACSCanto, BD Biosciences, RMIT Flow Cytometry Facility, Bundoora, Australia).

**Quantification of Her-2 neu expression**

Quantification of HER-2 expression of SKBR-3, BT474 and MCF-7 was assessed by flow cytometry. Cells were cultured using the previously mentioned method, seeded onto 24-well plates and grown to 90% confluency. Herceptin was added to the cells at a concentration of $10\mu$g/ml for 30 min at 4°C followed by two washes and then incubation with FITC-anti-human IgG secondary antibody (company, country) for 30 min at 4°C. The removal of the primary antibody from these treatments was used as a measurement of non-specific binding of the secondary antibody. The cells were washed and then analysed by flow cytometer as previously described. The mean fluorescence was determined by subtracting the mean channel number for the background from the mean channel number for the primary antibody treated cells.

**MR imaging**

**MR Imaging in vitro**

Iron oxide nanoparticles were prepared and conjugated to herceptin using the described protocols. SKBR-3, BT474 and MCF-7 cells were cultured, detached using trypsin-EDTA solution, washed and seeded onto 6-well plates and incubated with supplemented RPMI 1641 medium without serum. Each cell line was treated with $50\mu$g/ml of iron oxide silica-herceptin nanoparticles and incubated for 5 hours at 37 °C. Before imaging on MRI, the cells were washed 3 times with PBS and then fixed with glutaraldehyde. A T2-weighted gradient echo sequence was used with the same parameters as the phantom studies. Signal enhancement of each of the cell lines was calculated relative to the control using: $\Delta R2/R2\text{control} *100$.

**Mouse Experiments**
We obtained ~6 week old female BALB/c nude mice (ARC) in accordance with the guidelines and under approval of the Animal Ethics Committee of RMIT University. The nude mice were injected subcutaneously with equal volume of SKBR3 cells (2.5 x 10^6) and Matrigel (BD, USA) into the right flank. A full tumour growth analysis was conducted to ensure growth of the tumours as well as the average time to reach a maximum diameter of approximately 1cm.

**MR imaging *in vivo***

Nude mice (n= 15) bearing SBKR3 tumours were studied by MRI when the subcutaneous tumours reached a diameter of approximately 1cm. A solution of iron oxide silica herceptin nanoparticles (400ug) was infused via the tail vein of a group of mice and a solution of iron oxide silica nanoparticles (400ug) was infused via the tail vein of another group of mice. The MRI of ketamine-xyalazine-anesthetised mice was performed at 4 hrs and 24 hours on a 3.0 Tesla clinical Siemens Trio MRI scanner using a 12-channel head coil and the following parameters: T2-weighted imaging, spin echo sequence, transverse orientation, echo time= 76 ms(TE), repetition time (TR)= 2000 ms, matrix 320x320, slice thickness= 1.70mm, FoV= ?, number of averages=?

**Results and Discussion**

The *in vitro* and *in vivo* studies demonstrate that we were able to specifically apply iron oxide nanoparticles to molecular/cellular imaging using MRI. Our study shows that iron oxide nanoparticles conjugated to Herceptin can target breast cancers that overexpress HER2/neu receptors and provide optimal MRI contrast enhancement.

The iron oxide nanoparticles in this study were synthesised via a high temperature method outlined above to maintain high crystalinity and then surface modified with a thin silica coating to infer higher biocompatibility and provide a surface compatible for
molecular labelling. Before utilising iron oxide nanoparticles as contrast agents for MRI, it is important to accurately characterise the particles in terms of size, solubility, biocompatibility, targeting ability and image contrast capabilities, because the MRI signal can be affected by any one of these characteristics.

Figure 1 shows the HRTEM image of the iron oxide nanoparticles, where Figure 1B illustrates the synthesised iron oxide nanoparticles with no coating and suggests that they are approximately X nm in diameter. It is well known that iron oxide nanoparticles in their pure chemical form are unstable therefore need to be stabilised by the addition of a surface layer. Furthermore, iron oxide nanoparticles are insoluble in water restricting their use in a biological system. To stabilise the iron oxide core and provide aqueous solubility a silica layer was added to the surface of the iron oxide (Figure 1A). The HRTEM images illustrate that they are approximately X nm in diameter. Will have to get new images before we can quote these figures.

For successful MRI applications in vivo, the iron oxide nanoparticles need to retain a size smaller than 50 nm in diameter (Jun, et al., 2007). This consideration arises because studies have reported that particles larger than this size limit cell targeting capability and reduce uptake efficiency. Another problem that occurs due to larger particle size is non-specific uptake in the RES as well as biological instability (Jun, et al., 2007). In our previous investigation we reported iron oxide silica nanoparticles with a hydrodynamic diameter of 60 nm, making these particles unsuitable for in vivo targeted MRI. Therefore the method of synthesis was modified to reduce the size of the particles making them suitable to in vivo use (already have, I don’t know how much materials stuff you want to add directly to this paper).

The iron oxide nanoparticles demonstrated a crystalline structure, monodispersity, high T2 relaxation and excellent contrast enhancement and we went further to investigate
the ability of our particles to be surface functionalised with the well-known trastuzumab and then test its *in vitro* and *in vivo* detection and MRI contrast capabilities.

The iron oxide silica nanoparticles were conjugated to herceptin by modifying the surface of the silica layer with a cysteamine linker. The chemistry of the nanoparticles-cystamine-herceptin indicates the particles are bound electrostatically. The formation of the iron oxide silica herceptin nanoparticles was confirmed and quantitated by a bicinchoninic acid protein assay kit. Under these experimental conditions the herceptin concentration was 0.66 µg of herceptin per 1 µg of iron oxide silica particles. For *in vitro* and *in vivo* studies, stability is a concern that needs to be addressed particularly for adsorbed complexes. To address stability concerns, the iron oxide silica herceptin nanoparticles were incubated over a 24 hour period at a range of pH and human serum and the iron oxide silica nanoparticles remained bound to the herceptin antibody over a 24-hour period in various pH strengths and human serum (data not shown). The stability results are similar to other studies that have reported stable particles in various pH indicating that competitive displacement *in vivo* is unlikely (Cortez et al., 2006).

It is well known that low toxicity is associated with lower iron oxide nanoparticle concentrations (Weissleder et al., 1989) and silica shells have also previously demonstrated to provide biocompatibility as well as particle stability (Ohmori & Matijevi, 1993; Ohmori & Matijevic, 1992). It is also well known that herceptin is cytotoxic to tumour cells as it undergoes internalisation via receptor-mediated endocytosis at the HER2/neu receptor site and then causes down regulation of HER2/neu production eventually causing cell death (Valabrega, et al., 2007). To investigate the cytotoxic potential of the iron oxide silica herceptin nanoparticles, MTS based assays were conducted on SKBR3 cells (Figure 2). SKBR-3 cells were incubated with iron oxide silica herceptin nanoparticles for 24 and 48 hours at varying concentrations ranging from
1-100 μg/ml. Figure 2 shows that lower concentrations (1-30 μg/ml) have no significant toxicity on SKBR-3 cells however the toxic effect increases between 50-100 μg/ml. These results are consistent with the function of herceptin causing down regulation of HER2/neu production and eventual cell death. The degree of toxicity is similar to previous studies that have used iron oxide conjugated herceptin to target SKBR3 cells (T. Chen, et al., 2009).

In order to achieve targeted imaging, the iron oxide silica-herceptin nanoparticles must be able to specifically bind the Her2/neu receptors on the surface of breast cancer cells and be taken up by trigger active endocytosis. To show cellular uptake and internalisation into SKBR-3 cells, FITC-labeled iron oxide silica-herceptin nanoparticles were analysed by confocal microscopy. 50 μg/ml of iron-oxide silica-herceptin nanoparticles were added to SKBR-3 cells in vitro for 3 hours at 37°C showed significant uptake (Fig. 3A). Uptake was also confirmed by phase contrast whereby the iron oxide silica-herceptin nanoparticles can clearly be seen within the cells (Fig. 3B). The images in this study are similar to that of previous studies that have looked at the uptake of herceptin conjugated nanoparticles by SKBR-3 cells (T. Chen, et al., 2009; Huh et al., 2005a). It is well known that HER2/neu undergoes receptor mediated endocytosis, therefore resulting in the herceptin-tagged nanoparticles being accumulated within the cells.

We then examined the in vitro binding specificity and efficiency by dose response and time course of iron oxide silica-herceptin nanoparticles by breast cancer cell lines that express different amounts of HER2/neu and analysed by flow cytometry. Initially, expression of HER2/neu was assessed in SKBR3, BT474 and MCF-7 cell lines. Analysis by flow cytometry showed that SKBR3 showed the highest HER2/neu expression followed by BT474 and then MCF-7 (Table 1). The results of this study are consistent with the literature which indicates that amongst SKBR3, BT474 and MCF-7, SKBR3 cells
have the highest HER2/neu expression (add more appropriate reference) (X. Chen, Yeung, & Wang, 2000; Steinhauser, Spänkuch, Strebhardt, & Langer, 2006). Although the expression levels of these cell lines are well known and widely reported, it was important to confirm expression levels as factors such as passage number, freezing, thawing and cell culture techniques can decrease expression levels.

The mechanism of cellular uptake by the iron oxide silica-herceptin nanoparticles should mediated by herceptin. To confirm this a dose response and time course response of iron oxide silica-herceptin nanoparticles by SKBR3, BT474 and MCF7 was conducted. For the dose response study, the iron oxide silica-herceptin FITC nanoparticles were incubated at different concentrations (5, 10, 50, 100 μg/ml) for 24 hours with the SKBR3, BT474 and MCF7 and then by flow cytometry. The results show that level of FITC fluorescence increases as the concentration of the iron oxide silica-herceptin nanoparticles increases in all cell lines suggesting, that more nanoparticles are being uptaken (Fig. 4A). Furthermore, the level of iron oxide silica herceptin FITC nanoparticle uptake is consistent with the amount of HER2.neu expression shown in Table 1 for each of the cells lines, indicating specific HER2/neu mediated uptake. The results from this study are in accordance with cellular uptake studies of human serum albumin nanoparticles conjugated to Herceptin which also demonstrate linear uptake of nanoparticles at varying concentrations. It is interesting to note that most of the studies that use nanoparticles conjugated to Herceptin do not show dose response uptake studies and instead only show the differences in uptake via MRI. For example the study by Chen et al (T. Chen, et al., 2009) investigated the use of Herceptin conjugated dextran-coated iron oxide nanoparticles in vitro in 4 human breast cancer cells lines and in vivo. This method is essential in understanding the MRI contrast enhancement behaviour between phantoms.
and *in vitro* systems however is not useful in understanding the behaviour of the tagged nanoparticles in *in vitro* biological systems.

In addition to dose response, the time required for uptake of nanoparticles is another important consideration. Time-dependent cellular uptake of iron oxide silica-herceptin nanoparticles used a constant concentration of 50 μg/ml and were incubated with SKBR3, BT474 and MCF7 for 30 mins, 1hr, 3hr, 5hr and 24hr. Similarly, the results show that as the time increases, nanoparticle uptake also increases and the level of uptake appears to be consistent with the surface expression of the cell lines as shown in Table 1 (Fig. 4B). These findings are concurrent with previous studies suggesting uptake to be associated with HER2/neu receptors (Steinhauser, et al., 2006; Wartlick et al., 2004). As with the dose response studies, there is limited data available on time course response studies of nanoparticles conjugated to herceptin except for one study showing uptake of HSA conjugated to Herceptin in SBKR3, BT474 and MCF-7 cells over a 24 hour time period (Steinhauser, et al., 2006). The results showed time dependant uptake of the nanoparticles and the influence of incubation times greater than 5 hours to be associated with non-specific uptake.

The ability to provide contrast in phantoms and then *in vitro* using MRI is one fundamental aspect in targeted imaging. Another important aspect is the ability for the targeted nanoparticles to demonstrate selective binding at the target cells. In this case, the iron oxide nanoparticles conjugated to Herceptin show selective binding as indicated by the dose and time course response studies (Figures) but also by the MRI contrast enhancement study (Figure). This can be visualised in Figure 6A where SKBR3 cells appear to be dark relative to BT474 and MCF-7 cell lines which is also confirmed by a plot of the drop in T2 values against the cell lines (Figure). The signal enhancement relative to the control of SKBR-3, BT474 and MCF-7 cells were calculated to be 52.3%,
34.7% and 12.5% respectively, and no enhancement was observed for the control cells. These results are consistent with the surface expression data from Table 1 suggesting that as the expression level of HER2/neu increases more particles are taken up thus producing a drop in T2 signals or higher signal enhancement.

On the basis of our successful verification of iron oxide silica herceptin nanoparticles for in vitro imaging, we further tested their capability for in vivo use. Nude mice (n=15) were subcutaneously injected with SKBR3 cell lines which overexpress HER2/neu receptors. MR imaging of the mice were performed at 4 hours and 24 hours after intravenous tail injection. The study consisted of two control groups (saline and iron silica with no herceptin) and one experimental group (iron silica herceptin). The first set of control subjects received only saline and the second set received 400ug of iron oxide silica nanoparticles. The experimental group received 400ug iron oxide silica herceptin nanoparticles. Figure (X) shows the MR images at 4 hours and 24 hours post injection of the three groups. The results indicate that by 4 hours post injection uptake of the iron oxide silica herceptin nanoparticles can be seen as indicated by the signal enhancement relative to the control. To further confirm the specific binding, the signal enhancement was also calculated against the second control group which received iron oxide silica nanoparticles. The results suggest that the iron oxide silica herceptin nanoparticles have specifically targeted the HER2/neu receptor expressing tumours.

Conclusion
Extra
IHC using immunoperoxidase was done to confirm expression of her2/neu in
tumours from mice. Staining patterns show membranous cytoplasmic staining of
her2/neu indicated by dark blue (cell clusters) and dark brown (her2/neu). Fc
receptors were blocked to inhibit non specific uptake.
References


Appendix C. A review publication on the synthesis of iron oxide nanoparticles for MRI

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Development and use of iron oxide nanoparticles (Part 1):
Synthesis of iron oxide nanoparticles for MRI
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Abstract
INTRODUCTION
SYNTHESIS OF IRON OXIDE NANOPARTICLES
REFERENCES

Contrast agents, such as iron oxide, enhance MR images by altering the relaxation times of tissues in which the agent is present. They can also be used to label targeted molecular imaging probes. Unfortunately, no molecular imaging probe is currently available on the clinical MRI market. A promising platform for MRI contrast agent development is nanotechnology, where superparamagnetic iron oxide nanoparticles (SPIONS) are tailored for MR contrast enhancement, and/or for molecular imaging. SPIONs can be produced using a range of methods and the choice of method will be influenced by the characteristics most important for a particular application. In addition, the ability to attach molecular markers to SPIONS heralds their application in molecular imaging.

There are many reviews on SPION synthesis for MRI; however, these tend to be targeted to a chemistry audience. The development of MRI contrast agents attracts experienced researchers from many fields including some researchers with little knowledge of medical imaging or MRI. This situation presents medical radiation practitioners with opportunities for involvement, collaboration or leadership in research depending on their level of commitment and their ability to learn. Medical radiation practitioners already possess a large portion of the understanding, knowledge and skills necessary for involvement in MRI development and molecular imaging. Their expertise in imaging technology, patient
care and radiation safety provides them with skills that are directly applicable to research on the development and application of SPIONs and MRI. In this paper we argue that MRI SPIONs, currently limited to major research centres, will have widespread clinical use in the future. We believe that knowledge about this growing area of research provides an opportunity for medical radiation practitioners to enhance their specialised expertise to ensure best practice in a truly multi-disciplinary environment. This review outlines how and why SPIONs can be synthesised and examines their characteristics and limitations in the context of MR imaging.

**Keywords:** Magnetic resonance imaging (MRI), iron oxide, nanoparticles, synthesis

Abstract INTRODUCTION SYNTHESIS OF IRON OXIDE NANOPARTICLES REFERENCES INTRODUCTION

Nanotechnology has evolved into a multidisciplinary field, revolutionising industries such as applied physics, mechanical, chemical, electrical and biological engineering, machine design, robotics, and medicine [1]. In medical imaging, the development of nanoparticles has attracted a phenomenal amount of research, particularly for applications in molecular imaging.

The nano size (<100nm) of these particles enables conjugation with many molecular markers, which can interact at molecular and cellular levels, thereby offering an ever increasing range of disease targets for molecular imaging.

Nanoparticles also have the potential to revolutionise conventional imaging techniques [2]. Conventional imaging modalities lack the combination of high sensitivity and high spatial resolution required for molecular imaging. MRI has high resolution, but lacks sensitivity to molecular signals, while high sensitivity nuclear medicine modalities such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) provide superb sensitivity, at the cost of reduced spatial resolution [2-4].

The use of nanoparticles in modalities like MRI can greatly increase sensitivity, presenting the potential for high-resolution molecular imaging. MRI has high spatial resolution [2, 5], is non-invasive in nature, uses non-ionising radiation, and offers multi-planar tomographic capabilities [2]. Nanoparticles can be engineered to have magnetic characteristics that can be detected by MRI at low concentrations, and at the same time contain ligands which target specific molecules [2].
Iron oxide nanoparticles have been widely researched for MRI, as they are mainly superparamagnetic. There are several types of iron oxide nanoparticles, namely maghemite, $\gamma$-Fe$_2$O$_3$, magnetite, Fe$_3$O$_4$, and haematite, $\alpha$-Fe$_2$O$_3$, among which magnetite, Fe$_3$O$_4$, is very promising, because of its proven biocompatibility [1]. For molecular imaging purposes, superparamagnetic iron oxide nanoparticles (SPIONS) need to be biocompatible, non-toxic and magnetic. They also need to bind to a range of drugs, proteins, enzymes, antibodies, or other molecular targets.

There have been a number of approaches to the production of SPIONS for use as MRI contrast agents, and each method produces particles with different sizes and magnetisation parameters. The iron oxide nanoparticles can also be coated with a surface layer, usually of organic material, that provides an interface between the core and the surrounding environment [6]. This surface layer can be used to direct the particles to a target site. In this review, we summarise some of the chemical routes for the synthesis of SPIONS, such as classical synthesis, reactions in constrained environments, and high temperature reactions. It will also discuss some of the major methods for structural and physicochemical characterisation of the SPIONS, such as x-ray powdered diffraction (XRD), transmission electron microscopy (TEM), dynamic light scattering (DLS), nuclear magnetic resonance spectroscopy (NMR), and atomic absorption spectroscopy (AAS).

Abstract
The choice of core and monolayer material is critical to the design of specialised contrast agents as each layer dictates a specific function. The composition of the core material dictates the primary physical and chemical properties of the nanoparticle, which in turn determine how it can be imaged. Iron particles, for example, are potentially very useful as MRI contrast agents because they are magnetic and behave as single magnetic domains when exposed to an external magnetic field. On the other hand, CdSe nanoparticles or ‘quantum dots’ can be used as optical probes for fluorescent imaging.

The monolayer provides the interface between the core and the surrounding environment [6] and can serve two purposes. Firstly, to act as a barrier between the nanoparticle core and the environment, to protect and stabilise the core [6]. Some materials used for the core such as iron oxides, on their own, are not stable, and are readily oxidised, changing valuable properties of the nanoparticle. Secondly, the chemical nature of monolayers dictate the reactivity, solubility and interfacial interactions [6], and may also determine the biological handling, of the nanoparticle. Most of the inorganic cores are not soluble in aqueous environments, and monolayer designs serve to overcome this problem, particularly for in-vivo applications. The inorganic core, when used alone, does not have a specific target, however if the monolayer is a particular molecular precursor or is conjugated to a specific molecule, it can direct the particle to an area of interest.

Nanoparticle design for MRI

As well as having a suitable iron core and monolayer, SPIONS, need to possess a range of other properties to ensure they are useful as MRI contrast agents. These are:

- uniform particle size [20, 21]
- a uniform and high superparamagnetic moment [2, 20, 21]
- high colloidal stability [2]
- low toxicity and high biocompatibility [2]

The way SPIONs are produced has an influence on all of the above properties [2]. For MRI, these properties are important as they determine the overall effectiveness of the contrast agent. For example, an essential characteristic of an effective MRI contrast agent is a high saturation magnetisation value, (expressed in electromagnetic unit/gram, [emu/g]). Saturation magnetisation values are a measure of the magnetic moment, so higher values produce more magnetic susceptibility, and therefore stronger MRI signals [22].
Relaxation rates are a measure of the ability of a contrast agent to enhance the relaxation rate of water protons, i.e. increase the efficiency with which image contrast is produced [23]. SPIONS with high T2 values have faster relaxation with surrounding water protons, and therefore faster relaxation rates (1/T1 and 1/T2).

Typically, magnetisation values for SPIONS range from 30-50emu/g, while higher values such as 90emu/g have been observed for bulk material [24, 25]. Factors contributing to the magnetisation value of SPIONS include; the size of the particles (with the highest emu/g to volume ratio occurring in the 6-20nm particle size range [26]), spacing between the nanoparticles (where coatings such as silica separate the magnetic domains, allowing each individual magnetite particle to act independently and thus enhancing the net magnetism per gram) and the crystalline structure of the iron oxide. It is therefore essential to use a method of SPION production that generates particles with one or more of the above characteristics.

The overall size and size distribution of the SPIONS is an important consideration as it can affect the biocompatibility and biodistribution in-vivo. It is well known that particles above 50nm in diameter are eliminated by the reticulo-endothelial system (RES) so SPIONs greater than 50nm in diameter are limited to liver/spleen imaging. A range of synthesis methods have been developed to produce SPIONs with varying sizes and this relationship between size and biocompatibility will be discussed in the following section. Other properties, such as high colloidal stability and low toxicity, are important, because they increase the chances of translating developmental contrast agents into the clinical setting.

The following sections will briefly discuss the basic method of SPION growth, and then discuss the different methods of SPION production and their respective properties for MRI.

Nucleation and particle growth

In making iron oxide nanoparticles for MRI, the particles need to be of uniform size. Uniform particles are usually prepared via homogeneous precipitation reactions [2], which involve two processes, nucleation and growth. This is because iron oxide nanoparticles are crystalline structures that are governed by the principles of crystal formation and growth. Generally, for precipitation to occur, there must be a saturated solution, in which addition of any excess solute will cause precipitation, and the formation of nanocrystals [8]. For nucleation to occur, the solution must be supersaturated [2], leading to a short single burst of nucleation [27]. Supersaturation can be achieved by dissolving the solute at a high...
temperature, or by adding reactants to produce supersaturation [28]. After the short burst in nucleation, the concentration drops and nucleation stops. The nuclei then grow, by diffusion of solutes from the solution onto the nuclear surfaces, until an equilibrium concentration is achieved.

In order to achieve monodisperse particles, the two phases of nucleation and growth need to be separated [8, 20, 27, 29]. There are many different mechanisms which can explain this process, however we refer the reader to LaMer and Dinegar [30], who proposed the classical theory method of the formation of sulphur colloids, Den Ouden and Thompson who explained ‘Ostwald ripening growth’ [31, 32] and other mechanisms proposed by Morales et al. [33], and Ocana et al. [34].

Size control is ultimately achieved by artificially separating nucleation and growth. This would occur before the solution reaches critical supersaturation, or by the end of nucleation [20]. A wide variety of factors have been adjusted in many ways to promote separation of the two processes to control size, magnetic characteristics, or surface properties. Some of the factors have contributed to the development of new synthesis methods, and some have just improved classical methods. A few of these factors will be discussed below.

Methods of superparamagnetic iron oxide nanoparticle synthesis

There are numerous methods of iron oxide nanoparticle synthesis for applications to MRI [20], for example; chemical precipitation, constrained environments and high temperature reactions. In keeping with the scope of this paper, only these selected methods will be discussed.

Chemical precipitation

The precipitation method is the simplest chemical pathway to obtain SPIONS [8, 20]. The SPIONS, either magnetite (Fe₃O₄), or maghemite (γFe₂O₃), are prepared by co-precipitating a stoichiometric mixture of ferrous and ferric salts in an aqueous medium. The thermodynamics of the reaction require a ratio of 2:1 for Fe²⁺/ Fe³⁺ and a pH between 8 and 14. The precipitated magnetite is black in colour. The overall reaction can be written as [1, 20]:

\[ \text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \]  (1)

The ions can become oxidised before precipitation, critically affecting the physical and chemical properties of the SPIONS. For iron oxide, or magnetite, oxidation usually means
the formation of maghemite. The reaction must therefore be carried out under a nitrogen environment to eliminate oxidation.

The transformation from magnetite to maghemite can pose a serious problem for the production of contrast agents. The two differ from each other in the spinel structure; one occupies positions in the octahedral and tetrahedral sites, and the other, maghemite, has cationic vacancies in the octahedral position. This crystal structure results in a different net spontaneous magnetisation (or emu/g) of the iron particles: at 300⁰K, 92 emu/g⁻¹ for magnetite, and 78 emu/g⁻¹ for maghemite [35].

Most of the time it is difficult to separate magnetite from maghemite [36], given that their diffraction spectra are very similar [21]. Some synthesis methods suggest the presence of both magnetite and maghemite in the resulting preparations [37].

In the co-precipitation process there are two main processes involved. The first is a short single burst of nucleation, followed by growth of the nuclei, as discussed in the previous section. The precipitation method provides an advantage because large quantities can be synthesised; however, problems arise from the wide particle size distribution.

As mentioned above, size affects the magnetisation values as well as the biodistribution in-vivo. Factors that influence the biodistribution of a particle are important, as they also determine the possible MRI applications. To control the size, and size distribution, it is essential to adjust factors that determine the precipitation process. Numerous studies have been conducted adjusting factors such as pH, ionic strength, temperature, nature of salts, Fe³⁺/Fe²⁺ ratio, and addition of chelating agents, which improve the size and size distribution of the SPIONS produced.

The Massart process describes the co-precipitation of ferrous and ferric chlorides, and hydroxides in an alkaline solution [38]. Parameters such as strength of the base (eg ammonia or NaOH), the pH value, added cations, and the Fe³⁺/Fe²⁺ ratio were evaluated, noting the effect on yield of the co-precipitation reaction and particle sizes. It was concluded that the size decreases as the pH, and/or Fe³⁺/Fe²⁺ ratio, increase, and as ionic strength in the medium increases.

A comprehensive study on the ratio of Fe²⁺/Fe³⁺ was conducted by Jolivet et al. in 1992 [39] and 1994, illustrating the effects on size, morphology and magnetic characteristics. Small values of the Fe²⁺/Fe³⁺ ratio (<0.3) were known to form goethite. For ratios less than 0.5, but greater than 0.3, there were two phases, consisting of smaller (4nm) and larger nanoparticles. However, a ratio of 0.5 corresponded to magnetite stoichiometry, and the particles were homogenous in size and composition.
In 1999, Babes et al. [40] investigated different properties such as iron concentration, temperature and oxygen. It was highlighted that one of the most important parameters was the $\text{Fe}^{2+}/\text{Fe}^{3+}$ molar ratio. A high ratio produced larger particles, which is consistent with the literature [39, 41], suggesting that only ratios between 0.4 and 0.6 produce monodisperse particles, suitable for use as contrast agents in MRI [20].

It is reported that the higher the pH and the ionic strength, the smaller the particle size and size distribution [41, 42]. Vayssières et al. [42] observed that for a higher pH and ionic strength, the particles were smaller due to the thermodynamics of the solution. At a lower pH and ionic strength, the particles continued to grow during the ageing phase associated with Ostwald ripening, thus forming larger particles.

A recent study on the size of the SPIONS, and its effect on magnetisation and MR signal, was conducted by Young-wook Jun et al. [26]. The SPIONS were highly crystalline, monodisperse, and stoichiometric for magnetite, and ranged in size from 4nm to 12nm in diameter. The general trend suggested that as the nanoparticles increased in size, the T2-weighted MR signal intensity decreased, the particles therefore appearing hypointense on T2-weighted images.

Apart from modulating the parameters of the reaction to achieve monodisperse particles, the addition, either in combination or individually, of chelating organic anions like citric acid [43, 44], amino acids, and dimercaptosuccinic acid (DMSA) [45], can also decrease the particle size by inhibiting the growth of the crystal nuclei. Polymer surface complexing agents, which form monolayers on the surface of the iron oxide, such as dextran [46], carbodextran, and silica [47] can also be added, instead of varying the reaction parameters. Some polymer complexing agents such as dextran, carbodextran and silica are commercially available, and are currently used in iron oxide-based MRI contrast agents. Examples are: silica-coated magnetite, AMI-121 (Lumirem®- US) dextran-coated magnetite, Ferumoxides (Endorem® – Europe, Feridex® in the USA and Japan) and carboxydextran coated magnetite, Ferucarbotran (Resovist® – Europe and Japan).

It should be noted that these agents can be used for any method of iron oxide production. The coatings often serve multiple purposes; they allow for water solubility [2], the attachment of various functional probes [2, 48], promote the formation of monodisperse particles [20, 45] and stabilise the magnetite core [6].

Although the co-precipitation method is the simplest and most efficient chemical pathway to obtain magnetic particles, it has disadvantages such as large particle size distribution, aggregation and poor crystallinity, resulting in low saturation magnetisation values. These
disadvantages have led to the development of advanced methods of magnetite synthesis [1].

**Reactions in constrained environments**

Synthesis reactions in constrained environments have made use of lipid-based structures with amphiphiles [12, 49-53] and dendrimers [54]. Lipid-based nanoparticles, or colloidal aggregates such as liposomes, micelles or microemulsions, are composed of lipids and/or other amphiphilic molecules. Amphiphiles (sometimes referred to as surfactants) are molecules with both hydrophilic (polar head) and hydrophobic (non-polar tail) parts that spontaneously assemble into aggregates in an aqueous solution [55]. Because of these properties, there are various geometries and sizes that can be formed due to unfavourable interactions between the hydrophobic tails and water [55], such as cylindrical, spherical, and bilayered. The hydrophobic tails can vary in length, affecting the ratio between hydrophilic and hydrophobic parts, and the hydrophilic heads can also vary in charge and size, affecting the overall curvature of the aggregate. Other factors, such as pH, temperature and concentration, can also affect the end-product.

Mulder et al. [14] illustrate the various geometries that can be formed.

In micelle-forming lipids, the hydrophobic chains are oriented toward the inside of the micelle, and the hydrophilic chains outward. Micelles for MR imaging contain a hydrophobic core, where the iron oxide core is stabilised by the surfactant, which limits particle nucleation and growth [8]. The first magnetic nanoparticles formed in micelles were produced by oxidation of Fe^{2+} salts [56]. The size of the magnetite particles were controlled by varying the temperature and the surfactant concentration [57]. Micelles give control to the particle size formed, however reverse micelles are of importance for applications to MRI.

In reverse micelles, the hydrophilic head groups are towards the core of the micelle and the hydrophobic groups are directed outwards. Reverse micelles can solubilise relatively large amounts of water, which can be controlled, to make them suitable for the synthesis of nanoparticles. A diverse range of nanoparticles can be obtained by varying the nature and amount of surfactant, co-surfactant, and solvent.

Reverse micelles are essentially formed by aqueous iron salt solutions, encapsulated by a surfactant that separates them from the surrounding organic solution. Publications have suggested that iron oxide nanoparticles synthesised via the reverse micelle process can be used for MRI applications [58]. For example Lee et al. [57], investigated an inexpensive,
large-scale, and highly crystalline method of magnetite production. The synthesis was carried out at high temperatures whilst varying the relative proportion of iron salts, surfactant and solvents. It was suggested that the particle size could be controlled to produce monodisperse particles in one sample.

Poly(ethylene glycol) (PEG) stabilised lipids can also be used for targeting and stabilising the iron oxide core [59]. The advantages of using PEG stabilised lipids are long blood circulation times, and water solubility, while the disadvantages are associated with difficult preparation methods, and excessive size separation processes [60].

Bi-layer forming lipids are used to create liposomes; they usually have a polar head group and two fatty acid chains. Iron oxides can be placed inside the liposomal lumen to create magnetoliposomes [61]. There are two types of magnetoliposomes; the first consists of water-soluble iron oxide particles within an aqueous lumen [62]. The second contains iron oxide particles of approximately 15nm, covered with a lipid bi-layer [63]. The second type, developed by De Cuyper and Joniau [63], has been used in-vivo for MRI as a bone marrow contrast agent [15]. The magnetoliposomes are produced by first synthesising iron oxides in solution. The particles are then solubilised and stabilised by the addition of laurate, which acts as a surfactant. A solution with excess phospholipids is then added to the particles and undergoes dialysis for a number of days. The surfactant molecules on the iron oxide surface exchange with the phospholipid molecules which, over time, cause the formation of a lipid bi-layer on the iron oxides nanoparticles. Furthermore, molecules such as PEG can also be added to the lipid bi-layer, increasing the half life in blood [64] and therefore increasing the number of applications for MRI contrast.

Dendrimers are a class of transfection agents that contain three components: core, branches and end-groups. When dendrimers are coated to iron oxides they are termed magnetodendrimers. Carboxylated polyamidoamine dendrimers have been used to coat and stabilise the iron oxide nanoparticles [54, 65]. More importantly, magnetodendrimers are well suited for the imaging of cell trafficking and migration using MRI [66-68]. This is due to the charge on the polymer, which promotes a high non-specific affinity for cellular membranes, resulting in cellular internalisation [65, 67].

Generally, the oxidation of Fe(II) at an elevated temperature and pH, in the presence of dendrimers, results in the formation of highly stable and soluble SPIONS with dendrimers [54]. They have an approximate size of 20-30nm, and high T2 relaxivities [54]. Cells from different origins: mouse, rat or human, can then be easily labelled to the
magnetodendrimers, by introducing the magnetodendrimers to the cell culture for 1-2 days at low concentrations [66].

**High temperature methods**

Monodisperse particles with significant size control, and high crystallinity, can be achieved using high temperature methods. In this method, iron complexes are decomposed in the presence of surfactants and organic solvents. The high temperatures used in this method, and the nature of the solvent, result in the SPIONS having suitable size, and size distribution, with high crystallinity [69].

There are many studies on the synthesis of SPIONS using the high temperature method, for example Sun and Zeng [70] prepared iron oxide nanoparticles of different sizes, 3nm to 20nm. In this reaction, iron(III) acetylacetonate was decomposed by heating at 265°C in phenyl ether, alcohol, oleic acid, and oleylamine, to produce SPIONS 4nm in diameter. To make larger particles, a seed-mediated growth was used, controlling the quantity of seeds added to obtain various sizes.

Similarly, Hyeon et al. [71] formed an iron oleate complex from the decomposition of iron pentacarbonyl in the presence of octyl ether and oleic acid at 100°C. After cooling to room temperature, (CH3)3NO was added, and then the SPIONS were obtained by heating, followed by refluxing. When the molar ratios of iron pentacarbonyl and oleic acid were changed from 1:2 to 1:4, the particle size increased from 7nm to 11nm.

In another study by Park et al. [72], iron salts were used instead of toxic organometallic compounds such as iron carbonyl. Iron salts are more suited for contrast agent research and applications in MRI because they are less toxic. An iron-oleic complex was formed using iron chlorides, (FeCl3·6H2O) and sodium oleate, which was slowly heated to 320°C in 1-octadecene. The solution was aged at this temperature for 30 minutes, generating monodisperse iron oxide crystals. Various temperatures and solvents were also tried, which produced particles of different sizes and dispersity. It was concluded that monodisperse particles could be attributed to the separation of growth and nucleation phases, which occurred at different temperatures; nucleation at 200-240°C, and growth at 300°C.

**Monolayers for superparamagnetic iron oxide nanoparticles**

On their own, iron oxides are not very stable, and are not soluble in water. Stabilisation of SPIONS is essential to prevent against aggregation and oxidisation. Furthermore, for use as MRI contrast agents in-vivo, the SPIONS need to be soluble in water and be easily conjugated to molecular and cellular markers.
As discussed briefly in the previous sections, there are numerous ways for SPIONS to achieve water solubility and stability. Some of these methods include coating with carboxylates (such as citric acid), inorganic materials such as silica, and polymers such as dextran and PEG. These compounds protect the iron core, and also provide an avenue for conjugation of molecular precursors, therefore providing a biocompatible functional component for the SPIONS.

**Carboxylates**

The surface of the magnetite nanoparticles can be stabilised in an aqueous dispersion by the absorption of citric acid [72]. This process, as described in Sahoo et al. [45], occurs by the citric acid being coordinated via one or two of the carboxylate functionalities, depending on steric necessity, and the curvature of the surface. As a result, at least one carboxylic acid group is exposed to the solvent, and this group is responsible for making the surface charged and hydrophilic. The presence of the terminal carboxylic group provides an avenue to extended bond formation with fluorescent dyes, proteins, hormone linkers, and other molecules, so that specific targeting within biological systems can be facilitated.

Molecules such as DMSA can also be used to stabilise the SPIONS, achieve water solubility and allow conjugation of molecular precursors [73]. DMSA has successfully been used as a monolayer [74], where the DMSA is introduced to the SPIONS, in excess, through simple mixing. The DMSA binds to the magnetite surface through its carboxylate bonding, and the intermolecular disulfide cross-linking between surface-bound DMSA ligands strengthens the stability. The remaining free carboxylic acid and thiol groups make the SPIONS hydrophilic, and can be used for further conjugation of target-specific antibodies.

**Silica**

Iron oxide nanoparticles can also be coated with silica [74]. Silica is an inert molecule that coats the surface of the iron oxide nanoparticle, and, as a result, prevents aggregation of the SPIONS, and provides stability [75]. This is achieved by two processes: (1) sheltering of the magnetic dipole interaction by the silica shell; and (2) charging the magnetic nanoparticles, as silica is negatively charged [47]. These two features are essential, particularly for applications in MRI, as aggregation of the magnetite particles can reduce or diminish their ability to be superparamagnetic [76].

There are two widely used methods to produce silica-coated iron oxide nanoparticles. The first method is based on the Stober process [76], which comprises the hydrolysis and
condensation of a sol-gel precursor such as tetraethyl orthosilicate (TEOS). There have been numerous studies conducted on the formation of iron oxides coated with silica using the Stober process \[46, 77\].

The second most common method of generating iron oxide-coated silica nanoparticles is via the microemulsion process, where reverse micelles are used to confine and control the silica coating. In this method, non-ionic surfactants are used to form inverse microemulsions for preparation or suspension of magnetic nanoparticles \[78\]. The silica is formed around the magnetic nanoparticles by hydrolysis and condensation of TEOS \[79\].

**Dextran**

Dextran is a polysaccharide polymer that is composed of $\alpha$-D-glucopyranosyl units and can vary in length (1000 to 2,000,000 Da) and branching. Dextran offers a suitable monolayer for SPIONS because of its biocompatibility \[80\]. The formation of iron oxide coated by dextran was first documented by Molday and Mackenzie \[80\]. In this study, dextran 40,000 was coated to the iron oxide nanoparticles by reacting a mixture of ferrous chloride and ferric chloride with the dextran polymers, under alkaline conditions. Other studies have looked at smaller dextran coatings such as dextran 10,000 \[21, 81, 82\]. Reducing the size of dextran has an effect on the formation and stability of the dextran-coated iron oxide nanoparticles \[83, 84\]. Paul et al. \[85\] describe that the smaller dextran has significant effects on particle size, coating stability, and magnetic properties. It was concluded that SPIONS coated with a reduced dextran were more stable than those coated with a larger molecular weight dextran. Higher molecular weight dextran produced larger particles, and only the 10,000 Da dextran gave a particle with high magnetic properties.

**Characterisation of superparamagnetic iron oxide nanoparticles**

There is a wide variety of analysis tools to characterise SPIONS. It is important to define the exact characteristics of SPIONS, as these characteristics can influence the application of SPIONS in MRI.

For any biological application, a range of tests such as biocompatibility, toxicity and efficacy, needs to be considered. However, within the scope of this paper, and for preliminary development of SPIONS in MRI, the most general properties that need to be analysed are the physical (size, shape, chemical phases) and magnetic (MR properties, magnetic saturation values (emu/g)) properties.

**Physical properties**

When analysing the size of the SPIONS, we are in fact measuring a range of dimensions. This includes different parts of the nanoparticle: size of the iron oxide core, size of the
monolayer e.g. silica or DMSA, size of the iron oxide core and monolayer, e.g. silica or DMSA, combined. It also includes the size range of the particles present in the sample. The size of the iron oxide core can be determined by transmission electron microscopy (TEM) [83-85]. TEM gives the total particle size, core and monolayer, and also provides details on the size distribution and the shape of the SPIONS. There are two different types of TEM; low resolution and high resolution. With the high resolution TEM, the atomic arrangement of the SPIONS can be deduced. It also allows better characterisation, or separation, between the core and monolayer. The lattice arrangement and the surface atomic arrangement of the crystals can also be studied, by the use of diffraction patterns. Generally for TEM, a small portion of the sample is placed on a coated copper grid and then imaged. Although it provides precise direct information about size, size distribution and shape of the particles, it has several disadvantages such as operator bias, a risk of change in particle properties as the sample dries and contrasting of the sample [21].

Dynamic light scattering (DLS) is also a useful technique in particle size characterisation, and holds some advantages over TEM. DLS can obtain information about the size and size distribution in solutions generally at a lower cost and with less time. In DLS, the distribution of diffusion coefficients are calculated which are transformed into measurements of the hydrodynamic or total diameter of the particles [21, 86]. Like all modalities, DLS also has disadvantages such as contamination by dust or small amounts of aggregates in the sample; these can create misleading results [21].

X-ray powdered diffraction (XRD) can also be used to estimate the size of the particles, and the crystalline structure. XRD gives a diffraction pattern of the sample, and this is compared to a reference peak or pattern. Line broadening from the XRD pattern is used to calculate the crystal sizes [87], using the Scherrer formula, and these results can indeed be compared to the TEM results.

Mossbauer spectroscopy is another method that can be used to approximate the size of the SPIONS [87] to complement DLS and TEM results. A resonant absorption of nuclear gamma radiation, e.g. of the non-radioactive $^{57}$Fe isotopes, gives information on the magnetic coupling of the sample, the valence state of the iron ions, and also on the size of the core.

**Magnetic properties**

Measurements of the magnetisation as a function of the applied magnetic field allow the determination of magnetic properties: magnetic susceptibility, saturation magnetisation, and $r_1$ and $r_2$ relaxivities.
A vibrating sample magnetometer (VSM) or Superconducting Quantum Interference Device (SQUID) magnetometer can be used to analyse the magnetic properties. Parameters such as magnetic moment and hysteresis loop measurements can be measured. The necessary equipment is scarce, and although these parameters are useful, for the requirements of MRI, other analytical tools can be used.

Nuclear magnetic resonance spectroscopy (NMR) can be used to analyse properties such as the $T_1$ and $T_2$ relaxivities. After analysing the iron concentrations (see below), the $r_1$ and $r_2$ relaxivities can be calculated by plotting the $T_1$ and $T_2$ over the iron concentration \cite{88}. Alternatively, MRI can be used, using the same process for analysis.

**Iron concentration**

The iron concentration of the sample is generally measured using atomic absorption spectroscopy (AAS), or inductively coupled plasma mass spectrometry (ICPMS). AAS compares light absorbance of the unknown sample with light absorbance from known calibrated standards. It is relatively easy to use and well understood; however some limitations are that only one sample can be measured at a time and there can be interference with some elements. On the other hand, ICPMS is a form of mass spectrometry that is highly sensitive and can determine a range of metals and non-metals at very low concentrations.

**MRI with SPIONS: current status and future directions**

The previous sections discuss many studies that have researched and developed SPIONS. Some research has improved magnetic characteristics, while other studies have developed novel methods for reducing the size of the SPIONS, as well as producing monodisperse particles. Despite research over many years, SPIONS as MRI contrast agents, particularly for molecular imaging, are still not available clinically. The problems in the translation of SPION research for MRI to the clinic lie primarily in particle size: larger particles limit applications; dosage: large amounts of SPIONS are needed to produce adequate contrast; and production: the ability to adapt synthesis methods to industrial levels of production. SPIONS that are larger than 50nm are eliminated by the RES, therefore they are mainly useful for liver/spleen imaging applications. Smaller SPIONS are also taken up by the RES; however, because of their smaller size, their blood circulation time is longer, providing greater opportunity for specific localisation. SPIONS produced commercially generally have had narrow applications and have been withdrawn from some markets due to low demand. An example is SHU-55A, Resovist. Resovist has an iron oxide core of 4.3nm and is coated with carbodextran to a total diameter of 60nm. Resovist also has
excellent T2 relaxivities of 151.0 mmol$^{-1}$sec$^{-1}$, and was used only for liver/spleen imaging. Products like OMP (Abdoscan) and AMI-121 (Lumirem, Gastromark) have total diameters of approximately 300nm and are coated with polystyrene and silica. They are administered orally and used for gastro-intestinal contrast. Unfortunately the prices of these products are high, and they are only available on the European and US markets. Smaller-sized SPIONS such as AMI-227 (Sinerem, Combidex) and SHU-55C (Supravist an optimisation of Resovist, SHU-55a) are ultra-small iron oxide particles coated with dextran and carboxy-dextran respectively. Both products yielded a total diameter of approximately 20nm. They have been proposed for applications in lymph node and bone marrow imaging, as well as the imaging of inflammatory processes. These products are not yet approved and are still undergoing development and/or clinical trials. Another product, NC100150 (Clariscan) for perfusion/MR angiography was discontinued. The iron core was 5-7nm, and it was coated with PEG, having a total diameter of 20nm.

There are other SPION contrast agents that are available at a pre-clinical imaging level. Monocrystalline iron oxide particles (MION) are used for angiography, lymphography, tumour detection, and infarction. Companies such as BioPhysics Assay Laboratory (BioPAL) Inc. provide products such as Molday ION and FerroTrack that can be used for molecular and cellular imaging at the pre-clinical level. See Table 1 for a summary of SPION contrast agents.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of SPION Contrast Agents</th>
</tr>
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<tbody>
<tr>
<td>Name</td>
<td>Status</td>
</tr>
<tr>
<td>AMI-21</td>
<td>Europe/ US</td>
</tr>
<tr>
<td>OMP</td>
<td>Abdoscan</td>
</tr>
<tr>
<td>AMI-227</td>
<td>Sinerem</td>
</tr>
<tr>
<td>SHU-55C</td>
<td>Resovist</td>
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<tr>
<td>SHU-55A</td>
<td>SHU-55a</td>
</tr>
</tbody>
</table>

For SPIONS to be used for more than one application, they need to be coated with a monolayer that can promote the attachment of molecular and cellular probes. An example is a study by Hilger et al. [88], in which iron oxide nanoparticles were coated with dextran, and then attached to anti-Her2/neu antibodies via the carboxyl groups on the dextran surface, for breast cancer imaging. Most of the SPIONS available or undergoing developments (see Table 1) have suitable surface coatings for molecular/cellular imaging; however, their clinical and/or proposed uses have been restricted to relatively narrow generic roles.

Another drawback in the translation of contrast agent research to the clinic has been the large amount of iron needed to produce adequate contrast. The challenge is to develop
highly magnetic particles that can produce the strong signal enhancement, allowing low
doses of SPION to be administered without compromising the MR signal.
Other problems are in translating the synthesis of SPIONS, easily made in the lab, to
industrial processes able to produce large quantities on a consistent basis.
With the wide range of SPIONS that are currently being developed for single MR
applications there are possibilities that in the future these SPIONS will be available for use
as MR contrast agents. As for molecular and cellular imaging with MRI, the current
research sets a platform for the further development of SPIONS. If SPIONS as MR
contrast agents for single applications can be utilised, then the next step in SPION
development would be towards molecular imaging. Although molecular imaging with
MRI will not likely replace nuclear medicine and PET, it may play a useful
complementary role. The current decade has seen extensive progress in SPION design,
utilisation and characteristics, and we expect that the future will see highly magnetic
SPIONS available for molecular and cellular imaging in MRI.

Abstract


Appendix D. A review publication on the application of iron oxide nanoparticles

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Development and use of iron oxide nanoparticles (Part 2): The application of iron oxide contrast agents in MRI

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ABSTRACT

Magnetic resonance imaging (MRI) is a medical imaging tool that can incorporate contrast agents to enhance its ability to identify and characterise pathologies. MRI contrast agents can be paramagnetic such as gadolinium, or superparamagnetic such as iron oxide. Significant concerns of Nephrogenic Systemic Fibrosis (NSF) have arisen involving gadolinium-based contrast media.

Recent research has focused on iron oxide nanoparticles because their sizes are more comparable to biological units. These can give MRI the potential to detect a broader range of pathology, while also track and observe biological processes.

This is the second article of a two-part series and will review iron oxide nanoparticles as a MRI contrast agent, and the potential applications of iron oxide nanoparticles to a range of pathologies and processes involving MRI. © 2010 Biomedical Imaging and Intervention Journal. All rights reserved.

Keywords: Iron oxide nanoparticles, MRI

INTRODUCTION

Since its clinical introduction, Magnetic Resonance Imaging (MRI) has been viewed as a highly advanced imaging modality. In particular, over the past decade, MRI has demonstrated its capability to generate images of anatomy and pathology with excellent contrast and spatial resolution. Also noteworthy has been MRI’s capability of imaging physiological processes with functional MRI (fMRI), Diffusion Weighted Imaging (DWI) and Perfusion Weighted Imaging (PWI). This of course has coincided with improved hardware and software developments. Overall, this has resulted in the medical community having a greater understanding and awareness of biological processes. Therefore, the clinical role and utility of MRI has evolved and is continually expanding.

Broadly defined, a contrast agent or medium is any substance that can be used together with an imaging technique to provide additional and useful information. Contrast media can be either exogenous or endogenous. Endogenous material which can be used include water molecules inherent within the blood stream when performing Arterial Spin Labelling (ASL) or Tagging (AST). Exogenous substances include the already well-known paramagnetic gadolinium-based agents and now emerging from literature, we are noting...
an increasing experimental usage of superparamagnetic iron oxide nanoparticles.

This is the second publication in a two-part series reviewing the potential use of iron oxide nanoparticles. Specifically, we will discuss the characteristics essential in iron oxide nanoparticles for MR imaging (as a contrast agent) as well as their potential clinical applications for a range of pathologies and physiological processes.

CONTRAST MEDIA IN RADIOLOGY AND THE CURRENT INTRAVASCULAR MRI CONTRAST MEDIA

Intravascular contrast agents have been continually used since the early 1900’s across all imaging modalities in the field of radiology [1]. Over the past century, there has been a continual evolution of contrast media in medical imaging. These changes have been based on safety concerns (adverse reactions by patients), improved chemical technology (oil-based, water-soluble, ionic, non-ionic) and designing contrast agents dedicated to a particular imaging modality or technique [1, 2]. Additional concerns surrounding their implementation and use include the cost of contrast media, the need for certain contrast media preparations to be warmed to body temperature (viscosity, minimise adverse reactions) and also the need to have a recent reading of a patient’s renal function (prior to administration of contrast media). There are also concerns surrounding compatibility, whereby a patient having an intravascular contrast media administered for a computed tomography (CT) study will be unable to have a nuclear medicine thyroid examination for several months; furthermore, this same contrast media is incompatible with metformin-based diabetic medication.

When MRI first became a clinical reality, it was thought that no contrast media would ever be needed because of MRI’s superior contrast and spatial (isotropic voxels) resolution compared with other modalities [3]. It was soon realised that a contrast media was needed to improve the specifity of MRI [4]. In 1988 the first MRI-specific contrast media preparation was approved by the Food and Drug Administration (FDA) for intravascular administration in clinical use [3, 5]. This was needed to define a pattern of contrast enhancement so that a characteristic enhancement pattern of a particular disease process could be recognised and also to narrow the differential diagnosis. This contrast media preparation contained gadolinium as its base. However, there have always been some concerns in relation to this preparation. These include its expense (cost per millilitre and patents are strongly held and continually renewed) and its degradation with exposure to ambient light.

The way that such gadolinium-based contrast media is chemically altered and eliminated by the body is not entirely understood [6]. For some time now, a condition known as “cross reactivity” has existed. It cannot be entirely explained, however, it is thought to result either from the chelated molecules or elements, or the chelated structure themselves [7].

Within the last several years, a more serious condition has been attributed to gadolinium-based preparations. This condition is referred to as nephrogenic systemic fibrosis (NSF) and can lead to eventual death. This condition is almost always seen in patients with reduced renal function (less than normal glomeruli filtration rate, GFR) and there have been a number of deaths recorded and attributed to NSF. In early 2008, the Royal Australian and New Zealand College of Radiologists (RANZCR) has recommended that all clinical centres offering gadolinium-based contrast media for MRI scanning examinations to establish a new policy concerning intravascular administration and, in particular, with respect to NSF and patients with impaired renal function [6]. Due to the above trends, anecdotal reports suggest more caution and less reliance upon gadolinium-based contrast media even though no alternative currently exists in Australia.

The current commercially available contrast media is gadolinium-based and also referred to as para-magnetic and only benefits T1-weighted MR imaging. No mainstream commercially available contrast media for T2-weighted imaging is currently available in Australia. There are benefits attributed to contrast media if it can be prepared for T2-weighted MR imaging [6]. Coincidently, an increasing trend is underway towards clinical MRI scanners with higher field strengths such as 3 Tesla (6). The main drivers are improved capital and running costs and increased signal-to-noise (SNR) ratio. However, at higher field strengths such as 3 Tesla, T1 and T2 relaxation times of human tissue are altered compared to 1.5 Tesla. Gadolinium-based contrast media “works” by shortening T1 tissue relaxivity values and therefore only T1 optimised sequences can be used (T2 relaxivity values are not altered significantly for MR imaging practicality).

It is proposed that contrast media preparations based on nanoparticles can overcome all of the abovementioned challenges related to MRI scanning while simultaneously addressing the current medical
safety concerns [6, 9]. More specifically, iron oxide-based nanoparticles have the following attractive attributes:

- It can offer T2-weighted imaging opportunities
- It has a well-recognised and understood pathway for breakdown and excretion from the human body
  - Degradation causes iron to enter plasma, where it is processed by the body
  - Risk of iron overload is minimal
  - Average dose of iron in contrast agent is comparable to iron contained in less than one unit of blood
- It provides “negative” enhancement

Depending on the physio-chemical property of the coating (surrounding the iron oxide nanoparticle), both generalised and specific contrast media can be created [9, 10]. The term “specific” means that contrast media preparations can be targeted to a particular organ within the body or a particular disease process. If this can be achieved, then it follows that not only can diagnostic imaging be successful, but also therapeutic drugs/medication can be tagged to the preparation so that it can reach and work on the target tissue.

At one point, it was estimated that 30% to 40% of MR examinations were performed with intravascular contrast media [7]. With the awareness of NSF, no hard data currently exists to determine if the use of intravascular contrast media has decreased or remains at the same level.

The most commonly available intravenous contrast media contains gadolinium. A gadolinium ion has seven unpaired electrons in its outer shell [11] and is considered a paramagnetic substance because it has an overall positive effect on the local magnetic field [3]. In brief, when placed within a magnetic field, the negatively charged gadolinium ion demonstrates characteristics such as a magnetic moment, producing a large time-varying magnetic field in its vicinity, allowing rapid exchange of bulk water, altering the relaxation rates (both T1 and T2, or longitudinal and horizontal) of adjacent water protons [7, 11, 12]. Gadolinium is referred to as a T1 enhancement contrast agent as it affects T1 to a greater extent than it does T2. The act of molecular tumbling and local magnetic field alterations occur near the Larmor frequency value. This leads to a reduction of the T1 relaxation value of adjacent water protons, which in turn, leads to an increased signal strength on T1 weighted images [11]. This is due to an increased rate of longitudinal magnetisation recovery [7]. For acceptable biocompatibility, gadolinium is chelated to other molecules. This reduces any acute toxicity effects, and also allows the gadolinium-based agent to remain circulating within the body for a relatively longer period (than without chelation) [12] with an elimination half life of 1 hour to 2 hours.

Currently available paramagnetic contrast agents are commonly administered intravenously. Its biodistribution is into the blood stream and then into the extra cellular space. It is therefore not taken up by any specific body organ, tissue type or pathologic lesion. Hence, gadolinium compounds are also regarded as non-specific contrast agents [12]. However, enhancement patterns are known to be characteristic of certain pathology groups. For example, a hyperintense circular rim with a hypointense centre may be representative of a cystic lesion.

CURRENT CONCERNS WITH NEPHROGENIC SYSTEMIC FIBROSIS AND GADOLINIUM

Up until about ten years ago, gadolinium-based contrast agents have been regarded as having a relatively excellent safety record [13]. NSF was originally referred to as nephrogenic fibrosing dermopathy by Cowper et al. in 2000 [14]. It was described as being scleromyxoedema-like cutaneous disorder and thought to only affect the skin or dermis. It was noted in patients undergoing renal dialysis [14, 15]. As additional cases became recorded and further understanding of the pathology grew, the currently used term of NSF has become accepted. This is due to the now recognised systemic nature of the pathology [16, 17]. Commonly, NSF commences with swelling at the distal aspects of the extremeties. This may then resolve, however, leaving behind thick, firm plaques over the affected skin.

In the majority of patients, initial skin lesions appear on the legs, then the arms and lastly on the trunk of the
body. It has also been reported that the skin lesions are often symmetrical and bilateral. It may then progress to a point where the patient has significantly reduced range of motion of their extremities and joints [18]. In addition to the flexion and joint contractures accompanying extremity skin lesions [19, 20], fibrotic effects may also be widespread and penetrating; involving organs including the liver, lungs and heart, among others [20, 21].

Today, the only successful approach in treating NSF is to restore the normal renal function. This can only be achieved by renal transplant surgery [18, 20]. NSF is almost always seen in patients with less than normal renal function or patients requiring ongoing renal dialysis. Therefore, NSF may be a resulting consequence in patients with renal impairment because the contrast media excretion half life is markedly increased [22]. This situation then permits disassociated or de-chelated gadolinium ions an increased circulation time. Some authors consider NSF to be an, “adverse reaction to gadolinium contrast agents in particular the less stable gadodiamide” [23].

Even though all gadolinium-based preparations carry a level of risk, there is published evidence to suggest that some gadolinium-based contrast agents offer a greater risk than others in inducing NSF [23]. This variation in risk is linked to the overall molecular structure of the gadolinium-based contrast agent and in particular, its level of chemical stability within the human body [24]. Gadodiamide has been associated with the greatest incidence of induced NSF [23, 24]. On its own, gadolinium is highly toxic. Not only can it cause injury to the liver and spleen, but it can also inhibit secretions of certain enzymes and it can induce haematological ailments [24-26]. To minimise such toxic consequences, the gadolinium ion is chelated to other chemical elements and compounds [24]. This improves its biocompatibility. The molecule or atom that is bonded to the gadolinium can be referred to as a ligand. The gadolinium ion is chelated in either a linear or macrocyclic fashion and prepared as either ionic or non-ionic formulations [7, 24]. Published data reflect that the least stable preparations are non-ionic linear chelate formulations such as gadodiamide (Omniscan, GE healthcare, Chalfont, ST Giles, UK) and Gadoversetamide (OptiMark, Coviden, St Louis, USA). Gadodiamide has been reported to have a kinetic stability of 35 seconds. That is, at a pH of 1.0, half of the preparation will shed the linearly chelated material; thus leaving free gadolinium ions to search for other metals (body cations) to bind to within the body. These would include iron, copper, zinc and calcium. This process is referred to as transmetallation. Of the above body cations, zinc has the highest relative concentration (55-125 micromole per litre) within the blood stream [24].

The most stable gadolinium-based preparation is the ionic, macrocyclic chelate formulation; namely, gadoterate. As of April 2009, no cases of NSF linked to any macrocyclic formulation has been reported [24] or confirmed by the International Centre For Nephrogenic Fibrosing Dermopathy Research [27].

This is because a macrocyclic structure provides relatively superior protection of the gadolinium ion. That is, the gadolinium ion is caged by the chelating agent [7, 24, 28, 29]. Conversely, a linear chelate is referred to as being a flexible open chain and thus not providing a strong bond to the gadolinium ion. Gadoterate is documented to have kinetic stability of greater than one month [24].

High et al. [13] obtained paraffin embedded tissue samples from the NSF registry (the International Centre for Nephrogenic Fibrosing Dermopathy Research). These tissue samples had histopathologic diagnosis of NSF. This research group demonstrated with energy dispersive spectroscopy (EDS), a device used to characterise chemical elements, that in four of seven patients, gadolinium was able to be identified and all detectable gadolinium particles were less than 1 micrometre in size. Further analysis with field emission scanning electron microscope (FESEM) demonstrated that in all of the positive tissue samples, gadolinium particles were present within the intracellular space and most probably located within, or adjacent to, the lysosome structures. Also noteworthy was an excessive amount of iron deposition within the tissue samples.

While the exact cause of NSF has not been conclusively established [20, 21, 29-33] and precise pathologic pathways are yet to be determined [15], there is however, convincing evidence that gadolinium may be responsible somehow [32, 34]. The most probable theory is that de-chelation occurs [35], resulting in the release of free gadolinium ions, which in turn may or may not lead to transmetallation [36, 37].

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**IRON OXIDE NANOPARTICLES AS MRI CONTRAST AGENTS**

The most common form of iron oxides used in nanoparticle preparations are magnetite (Fe₃O₄) and maghemite (γFe₂O₃) [38, 39], and research with these has been intensive for about a decade now. They are both insoluble in water and because of their size, these superparamagnetic substances only exhibit their
magnetic properties when placed within a magnetic field \cite{12, 40}. The hydrodynamic size of a nanoparticle preparation is the term used to describe its overall size, that is, the iron oxide core plus the coating plus any additional ligand attachments (see Figure 1). If the overall hydrodynamic size is greater than 50nm, then the preparation is referred to as a superparamagnetic iron oxide nanoparticle (SPION). If the hydrodynamic size is less than this, then the preparation is termed ultra small superparamagnetic iron oxide (USPION). For the purpose of this manuscript, the authors will use the term iron oxide nanoparticle (IONP) as a generic term to refer to nanosystems containing a core of iron oxide.

Iron oxide nanoparticle preparations are highly complex. There are numerous production methods which can be used to generate them. Each method can result in iron oxide nanoparticles having specific dimensions, as well as unique imaging and therapeutic characteristics. Each manufacturing method is undertaken in a strict controlled environment to ensure consistency of dimensions, characteristics and biostability.

The physiochemical properties of iron oxide nanoparticles are determined by the size of the iron oxide core, its overall charge and the zeta charges between coatings and the overall hydrodynamic size. With respect to magnetic resonance imaging, the above factors also play a fundamental role in determining their efficacy (or imaging efficacy), stability within the body's environment, biodistribution, opsonisation, metabolism, clearance from vascular system and then excretion from the body \cite{41}.

Part one of this journal article series discussed the variety of production methods. The advantages and disadvantages of this method were also presented and therefore that information will not be presented here.

The coating

An understanding of the bonding and the geometry of the coating will help us appreciate the pharmokinetic pathways and biodistribution of contrast agents composed with iron oxide nanoparticles \cite{40, 42-44}. From a chemical perspective, the iron oxide core is coated for four main reasons. Firstly, to prevent destabilisation; secondly, to prevent agglomeration (aggregation or sedimentation) as it will be a colloidal suspension; thirdly, it allows for the iron oxide nanoparticle formulation to be soluble in an aqueous solution or a biological medium; fourthly, it determines either the role it performs within the body (diagnostic magnetic resonance imaging, cell tracking or therapeutic purposes such as tailored drug delivery) or the ligand that can be bonded to it to support the imaging, tracking or therapeutic roles.

The coating used can also facilitate the method of endocytosis \cite{45}. For example, it has been shown that IONP coated with monomer citrate (overall hydrodynamic size of 8nm) demonstrated cell entry via phagocytosis. When the same iron oxide nanoparticles were coated with polymer carboxydextran (overall hydrodynamic size of 31nm), cell entry or penetration was demonstrated by pinocytosis. In both examples, the same cell line was used.

Stabilisation

In the literature, high density coatings have been reported to be effective in stabilising iron oxide nanoparticles \cite{40, 46-48}. Such high density coatings are commonly polymeric and monomeric materials or species. Polymeric coatings include dextran, carboxymethylated dextran, carboxy dextran and starch. Whereas monomeric coatings include dimercapsoucic acid (DMSA), amino acids and \(\alpha\)-hydroxamates (such as citric, tartaric or gluconic acids).

The Hydrodynamic Size

There is evidence to suggest that USPION is less prone to phagocytosis by the liver; whereas SPION greater than 50nm are rapidly phagocytosed \cite{40}. Therefore, hydrodynamic size can affect biodistribution and blood half life in a time dependent manner \cite{49}. Eventually, USPIO will actually be processed by the liver.

Biodistribution

Iron oxide nanoparticles greater than 50nm are readily macrophaged by the reticuloendothelial system (RES). This namely refers to the Kupffer cells of the liver, the spleen and bone marrow \cite{40}. Iron oxide nanoparticles less than 50nm have been used to demonstrate uptake by lymph nodes \cite{40, 41, 50-53}. Of the available iron oxide-based contrast agents which are currently on the market and also undergoing clinical trials, the blood half life values can vary considerably from 40 mins to up to 36 hours. There is a link between the hydrodynamic size and the biodistribution and blood half life.
As has been established, particles greater than 50nm are readily taken up by the liver in a matter of minutes. USPION are not readily phagocytosed by the liver and can have a longer blood half life and thus reach other structures within the body [40]. It must also be emphasised that the coating itself can be responsible for aspects of biodistribution as well as ligands; for example the targeting of specific cells or organs [40, 41, 51].

**Metabolism and excretion**

The manner in which the body metabolises iron oxide nanoparticles is determined by their overall chemical composition. In particular the immediate coating and any ligands are strong determinants as to the site (that is, which particular organ or body system) of metabolism and thus also the rate of metabolism and excretory pathways.

The commonly used dextran coating should ideally be of low molecular weight. This is vital as higher molecular weight dextrans, such as those used as plasma substitutes, have a reported association with adverse reactions. Immunoglobulin G (IgG) antibodies have been reported to be reactive to such high molecular weight dextrans [54]. Low molecular weight dextrans will initially undergo dextranases which is an intracellular level breakdown process. The majority of the breakdown components are excreted with urine over a period of nearly 2 months [40]. Once the low molecular dextran has metabolised in this manner, the iron oxide core has been found to enter the normal iron store of the body. Such iron elements can also be found as haemoglobin with the body’s red blood cells [55]. This iron then follows the same excretory pathway as endogenous iron. That is, approximately one-fifth is eliminated mostly with the faeces and over a three-month period. Therefore, as both dextran and iron from iron oxide nanoparticles are incorporated into the body’s normal metabolic pathways, without raising these levels noticeably, and with the evidence available today, it can thus be stated that these substances do not trigger any long-term toxicity.

In the average healthy adult, normal total human iron stores is about 3500mg with the liver containing an average of 0.2mg of iron per gram [55]. From the currently approved iron oxide nanoparticles for diagnostic MR imaging, a regular adult dose can contain 50-200mg of iron. This value can be considered relatively small compared to the human body’s iron store. Chronic iron toxicity is known to occur when the concentration of iron within the liver reaches a level of 4mg of iron per gram of liver [38].

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**MRI IMAGING WITH IRON OXIDE NANOPARTICLES**

**Phenomenon of superparamagnetism**

Iron oxide nanoparticles, as discussed here, are referred to as being superparamagnetic. The superparamagnetic phenomenon is observed when the thermal energy of the medium is sufficient to alter the crystallite or nanoparticles’s magnetisation direction by overcoming coupling forces. When crystallite or nanoparticles are placed within an external magnetic field, its magnetic moment will align with the externally applied magnetic field.

At least two points distinguish superparamagnetism from paramagnetism. Firstly, with paramagnetism, it is each individual atom or ion that becomes aligned with an externally applied magnetic field. Secondly, superparamagnetism will occur when the crystal or hydrodynamic size is less than its ferromagnetic domain. Authors report this size to be less than 30nm [40], with the “critical size” being about 15nm [44, 56, 57].

When not in the presence of an externally applied magnetic field, superparamagnetic particles are not magnetised, nor do they demonstrate any remnants of magnetism once removed from the magnetic field. When the crystals or particles are under the influence of an applied magnetic field, their magnetic spins are considered to be in perfect alignment and very high local magnetic field gradients are generated. These gradients then cause spin dephasing of the surrounding water protons; thus reducing their T1 and T2 relaxivity [12, 56, 58].

It must also be noted that there is a relationship between the iron oxide nanoparticle size and the level of superparamagnetic saturation. As the particle size decreases, so too does the superparamagnetic saturation. This then has an effect on reducing the observed relaxivity or further reducing T1 and T2 relaxation.

**IONP Influence on magnetic resonance image characteristics**
Compared to a paramagnetic material such as gadolinium, the relatively high magnetic moment of superparamagnetic species, such as iron oxides, are sometimes referred to as super spins [40, 59]. The dipolar interactions between the super spins and adjacent water protons result in both high longitudinal ($r_1$) and transverse ($r_2$) relaxation values. IONPs therefore increase $T_2^*$ relaxation rates through the susceptibility effect and thus have their greatest visual impact on $T_2^*$-weighted images produced with gradient echo-based pulsed sequences [48, 60-62]. The accelerated phase loss due to local field gradients generated by super spins, all stem from the (induced magnetisation) high susceptibility level of iron oxide.

At the common clinically available field strengths of 1.5T and 3.0T, published data [40] indicate that any aggregation of SPION will only slightly decrease $r_1$ (longitudinal relaxation) and dramatically increase $r_2$ (horizontal relaxation).

**Magnetic resonance imaging with iron oxide nanoparticles**

Even though images composed with gradient echo pulse sequences possess lower signal-to-noise ratio and spatial resolution compared to spin echo pulse sequences, they are currently the most appropriate imaging sequence to use with SPIONs. This is often termed magnetic susceptibility imaging [4, 12, 48, 63]. The contrast enhancement captured on an MR image is dependant upon a number of factors. Most notable are the biodistribution and opsonisation of SPIONs.

**Iron oxide nanoparticles as contrast agents for MRI**

In addition to their superior biocompatibility, IONP MRI contrast agents have been documented to increase diagnostic sensitivity and specificity [41, 45, 64-66] in both animal model experimentation and in human trials.

This improved accuracy has been attributed to their superparamagnetic effects and relaxation times [41, 64, 66]. Efficacy of IONPs as MRI dedicated contrast media also largely depends upon their physiochemical properties [41]. Such attributes include: size (both of the iron oxide core and the overall hydrodynamic dimensions); coating (dextran derivative or other); and the zeta surface charges. Their efficacy can be further increased with complex surface modifications. This is achieved by bonding or attaching active material such as monoclonal antibodies, receptor ligands and also proteins [41].

For intravascular administration, the hydrodynamic diameter of IONPs are very rarely greater than 150nm. The iron oxide core itself is usually no more than about 15nm [67]. The coating itself is preferably composed of dextran, or a derivative, of a low molecular weight. These are positive properties, as the dextran is biodegradable and its low molecular weight minimises possibilities of adverse reactions [54]. IONPs have also been incorporated into oral contrast media [41].

**Imaging challenges to consider and overcome**

There are a few imaging challenges with the use of IONPs [68]. They are in relation to commonly encountered artefacts in MRI imaging. On their own, they can be frustrating to deal with in everyday imaging. However, when IONPs are included in the imaging regime, a further layer of complication is added.

The first criticism is that it is difficult to determine or differentiate a signal void induced by IONPs compared to signal voids generated as artefacts by materials such as metal (susceptibility artefact). The second artefact is that of partial volume averaging. IONPs are capable of being involved in processes occurring at a cellular level. Hence, signal voids induced by IONPs that are smaller than the spatial resolution of the MRI image will not be represented as distinct and within a voxel; as individual signal intensities within a voxel are averaged together [4, 11, 12, 68].

**EMERGING TRENDS: APPLICATIONS OF IRON OXIDE NANOPARTICLES AND THE ROLE OF MRI**

Where possible and practicable, medical investigators and clinicians would prefer an investigation means that is non-invasive or minimally invasive. This approach is safer for patients, it expedites the medical management of patients, and can negate morbidity and mortality consequences. IONP preparations, combined with MRI, have the potential to revolutionise a number of investigative and treatment procedures.
This would be achieved by combining the advantages provided by IONP together with MRI, leading to safer and superior imaging alternatives. IONPs as MRI contrast agents have already been discussed in this manuscript. To re-iterate, they promise improved levels of toxicity and increased diagnostic sensitivity and specificity. These are achieved through careful chemical preparations, leading to IONPs having the required characteristics for biocompatibility and MRI image enhancement. It is recognised that further research is required to overcome the challenges mentioned.

We now follow with a discussion on the innovative uses of IONPs combined with MR techniques. These promise to revolutionise clinical therapies and improve patient outcomes.

Molecular imaging is a broad term concerning the imaging of biological events at the cellular or molecular level. It should also be non-invasive and the imaging characteristics representing the biological activity should be quantifiable [69]. MRI is seen as having an emerging and innovative role. Molecular imaging can be possible with MRI when IONPs are conjugated with biologically active materials such as antibodies.

The near future looks promising for MRI, together with IONPs, to have a positive impact in leading non-invasive imaging of biological and biochemical processes. Not only can such processes be diagnosed; but also progression and treatment can be imaged over time.

**Angiogenesis**

Angiogenesis, the growth of new blood vessels (for development, wound repair or tissue reproduction), is related to tumour growth and progression [70]. There are several known molecular markers associated with angiogenesis. That is, endothelial cells active in angiogenesis express known surface receptors compared to endothelial cells not partaking in angiogenesis [71]. The commonly occurring receptors include integrins and vascular endothelial growth factor receptors. Antibodies or drugs to seek out angiogenic markers, can be conjugated to IONPs and imaged with MRI. Thus, the angiogenic process can be identified and any success in treatment can be accurately monitored. This can be achieved by exploiting the increased permeability of newly formed tumour vessels compared to normal healthy vessels [72].

**Apoptosis**

Apoptosis is the self destruction of cells. When determined by cell age or cell health status, the nucleus triggers this process. It requires metabolic activity by the dying cell and is commonly characterised by a redistribution of phosphatidylserine in the cell membrane [70]. It can even be associated with tissue development and homeostasis [71].

The degree of apoptosis can determine how successful chemotherapy and radiation therapy can be. Identifying apoptotic events in vivo would hence further evaluate treatment regimes and progression of pathology [71]. It is known that apoptotic cells express lipid phosphatidylserine (a phospholipid) on their cell membrane. Synaptotagmin I is a protein that is used to detect this phospholipid. When this protein was conjugated to IONPs, apoptosis was demonstrated in vivo with mice [73].

The capability to image apoptosis can allow for almost real-time monitoring of efficacy of drug therapies [56].

**Targeted drug delivery with IONP and MRI**

Many therapeutic drugs that are available, can be considered non-specific in nature. By non-specific, it is meant that such drugs are administered intravascularly and are thus distributed randomly. This can lead to unwanted effects on healthy tissue [74]. Specificity for target tissue or cells can be achieved by conjugating IONPs with ligands [71]. Such ligands include antibodies (in particular for targeting cancerous tissue or cells), proteins, peptides and other biological markers.

Targeted drug delivery, as provided by superparamagnetic colloid suspensions, can be guided by an external magnetic field to the site of interest [41], thus, having the capability to minimise both side effects and required dose [57, 74, 75]. Therefore, pharmaceutical drugs can be binded to IONPs designed to reach a specific, or target, organ, and then be released there [41, 44]. The emerging breakthroughs that make magnetic drug targeting possible and promising are the new classes of IONPs less than 50nm. This allows for improved circulation time, thereby permitting delivery to the target site without the likelihood of being sequestered by the RES before this can occur [41]. With the original classes of IONPs that became commercially available over ten years ago, RES uptake occurred within a few minutes following intravascular administration (hence, their original application as dedicated contrast agents for the liver) [41].
Drug targeting can be achieved by passive, active or physical means [41]. Magnetic drug targeting falls into the category of physical means; as the pharmaceutical is attached to a carrier system (the IONP) and its distribution is facilitated with an external influence (the magnetic field).

Not all therapeutic drugs can be conjugated to one single variety of IONP. Characteristics of IONPs that can determine attachment of therapeutic drugs include their size, surface charges (zeta charges) and capacity for protein absorption [41]. The process of cell uptake is determined by the overall size of the nano-system (IONP, surface coating/s and pharmaceutical); phagocytosis or pinocytosis. Pinocytosis occurs for items less than 150nm [41, 74]. The condition under which a cell finds itself in, may alter its susceptibility to a nano-system. For example, under normal conditions, walls of endothelial cells are permeable to objects 10nm or smaller. However, when involved in pathologic processes such as tumour infiltration and inflammation, the endothelial wall can be permeable to objects up to as large as 700nm [41]. Zeta charges need to be carefully managed. They determine whether or not nanoparticles aggregate or if they remain suspended in its medium. More importantly, they also play a part in endocytosis. It is noted that the likelihood of phagocytosis increases with a higher zeta charge [41, 76], while time spent within the circulatory system is reduced. There is an electrostatic process involved when particles and substances are absorbed by a cell’s outer membrane [77].

Understanding nanosystem interaction with proteins is vital, as when they are injected into the vascular system, their first interaction is with the plasma proteins. Therefore, the manner in which nanosystems are capable of interacting with opsonins (proteins that encourage phagocytosis such as IgG) and dysopsonins (proteins inhibiting phagocytosis) also determine if they are readily phagocytosed by the RES or if they can reach their intended target and release their pharmaceuticals. Hence, protein repulsive molecules such as polyethylene glycol (PEG) can be used to modify the surface of nano-systems to reduce their recognition by the RES [74] and reduce non-specific cellular uptake [78].

A phase I/II clinical trial of IONPs combined with epirubicin designed to image and treat solid tumours (such as sarcomas), showed that these nanosystems were reasonably well tolerated by the fourteen patients involved. No organ toxicity attributable to iron oxide was noted. However, toxicity responses to epirubicin were recorded at doses greater than 50 mg/m^3 [79].

**Thermal applications for cancer cells: magnetocytolysis and hyperthermia with IONP and MRI**

Compared to normal healthy cells, cancer cells are known to be sensitive to temperatures above 42 degrees Celsius. Normal cells can survive at higher temperatures [44]. In cancer cells, at temperatures above 42 degrees Celsius, protein function is disrupted which can lead to apoptosis [80]. Thus, hyperthermia is a proposed treatment regime for certain cancers. Until recently, hyperthermic approaches have included irradiation with radiofrequency, ultrasound and microwaves. One known criticism of these approaches is the likelihood of hyperthermic injury extending to healthy tissue. The term, magnetic induction hyperthermia, now refers to cancer tissue being exposed to an alternating magnetic field [44].

Hyperthermia using IONPs together with MRI has demonstrated positive results in pre-clinical evaluation studies. With this combined approach, magnetic nanoparticles can be either directly injected into a tumour volume or designed to be selectively uptaken by a tumour site. This target-selective capability improves local heating treatment to the tumour while dramatically minimising potential for injury to surrounding healthy tissue [81]. Furthermore, the alternating magnetic field (not absorbed by tissue), together with appropriately prepared IONP, can allow hyperthermia treatment [41] to be applied to areas deep within the body [44]. Radiofrequency pulses provided by MRI can be designed to provide frequencies and amplitudes to increase local cell temperature up to 55 degrees Celsius [80], thereby inducing cytolysis. This process, therefore, can be used to generate heat to target cells [82].

However, for magnetic hyperthermia to be successful, it requires accurate delivery of magnetic nanoparticles to the tumour site.

A number of experiments report successful use of magnetic induction hyperthermia in cancerous cell models and also in animal models [44, 81, 83].

Salado et al. [84] successfully demonstrated in a rat model, with in vivo MRI imaging, that the IONPs which they developed were capable of providing positive contrast enhancement of induced liver tumour and also successfully treated these liver tumours with MRI-induced hyperthermia. Thereby, demonstrating that IONPs can have both a diagnostic and therapeutic use. Analysis of the rats following the experimental study demonstrated no vascular embolisms (the IONP preparation was injected through the ileo-colic vein) and specimens of the liver demonstrated insignificant inflammatory changes.
Xu et al. [85] have produced nanoparticles containing a core composed of iron and cobalt and a gold shell. These nanoparticles demonstrated a specific magnetisation value far greater (226 emu/g) than that achievable with commercially available iron oxides (78.8 emu/g). This higher magnetic moment is claimed to improve heating efficiency in hyperthermia applications. However, their publication did not mention any results or discussion of toxicity or biocompatibility studies.

Initial success of magnetic hyperthermia with small groups of human patients provides a promising outlook for future clinical applications. Plotkin et al. [86] reports a study on eleven consecutive patients (mean age 44 years), each with recurrent supratentorial glioblastoma. All patients had previously undergone surgery, nine patients also had radiation therapy and eight patients also had chemotherapy. Based on the prognosis following these treatments, these eleven patients were eligible as candidates for hyperthermia using nanoparticles and MRI. Nanoparticles were then administered directly into the tumour volume. MRI hyperthermia, or nano cancer therapy, followed and in ten patients, the mean reduction in gross tumour volume (GTV) was 74% as indicated with PET-CT fusion imaging.

Cancer imaging with IONP and MRI

Diagnosing cancer in its early stages significantly improves patient outcomes and survival rates. The initial use for IONPs was directed at imaging liver tumours [87]. This was due to the nanoparticles being greater than 60nm and therefore readily phagocytosed by the liver. This has been occurring for several years now and there are a few commercially available preparations for this specific purpose. The authors will therefore discuss IONPs in relation to other cancers.

Current MRI techniques allow for the detection of tumour sizes in the order of one centimetre cubed. By conjugating known cancer antibodies with IONPs, then MRI can be used to identify cancerous tissue of smaller dimensions through molecular interactions. This is an improved sensitivity for cancer markers, compared with current cancer marker detection probes [89].

IONP can be coated with (DMSA), a bi-functional chelating agent and ligand. Herceptin, a monoclonal antibody, uses elements from within the immune system to stop tumour progression by binding to HER2 receptor and triggering a response by natural killer (NK) cells [89]. With a 1.5 Tesla clinical MRI scanner, Lee et al. [88] successfully demonstrated how to identify cancer sizes as small as 50mg in mice using IONPs conjugated with Herceptin.

The above approach improves patient outcomes compared with just chemotherapy alone.

This principle has also been used to target other tumour antigens. IONPs conjugated with peptide EPPT1 (synthetic peptide EPPT1, also known as alpha-M2 peptide (YCAREPPTRFYWG), derived from the CDR3 V\textsubscript{s} region of a monoclonal antibody, ASM2) are able to target underglycosylated MUC-1 (mucin 1); which is a tumour antigen expressed by many epithelial cell adenocarcinomas such as pancreatic, colorectal, gastric and prostate [56].

The role that lymph nodes play in cancer staging has not gone unnoticed by researchers in this field. IONPs and MRI can be used to image the condition of lymph nodes and more accurately determine the extent of metastatic spread [89]. Oghabian et al. [90] demonstrated 98% detection sensitivity with in vivo imaging of a rat model at 1.5 Tesla. They also concluded that the type of surface coating and its thickness were factors in determining MRI signal intensity.

Today, prostate cancer is still a leading cause of death in men. Current treatment, among others, involves brachytherapy, and as a procedure, it has its own level of invasiveness, morbidity and mortality. Wang et al. [91] have conjugated IONPs with prostate specific membrane antigen (PSMA) and also with doxorubicin, a chemotherapy drug. By performing whole cell assays on human cell lines, Wang et al. demonstrated that their conjugate can detect, with high sensitivity, prostate cancer cells expressing PSMA, thereby, promising the possibility of a multifunctional (diagnostic and therapeutic) nanoparticle system.

IONPs can also be used to better identify tumour boundaries within the brain [69, 87, 92, 93]. This leads to improved quantification of tumour volumes. Compared with gadolinium-based contrast agents for MRI and also taking into consideration oedema surrounding a tumour volume, IONPs can define tumour margins for longer time periods [69, 92, 94]. This is as a result of the IONPs being endocytosed by tumour cells. Thus being internalised, their elimination rate from the tumour is longer compared to extracellular gadolinium-based contrast media.

Cell labelling and tracking; including stem cell therapies
MRI imaging of cells labelled or endocytosed with IONPs is considered an indirect imaging technique. This is because changes in MRI signal intensity is in relation to the amount of IONPs and not due to the number of cells. One concern is the MRI signal characteristic and change over a time period. As stem cells rapidly divide, the fixed amount of IONPs is spread throughout the volume of newly divided daughter cells. This will be a relative decrease in MRI signal not accurately reflecting the activity of cells. The second noteworthy concern is the possibility of false positive signal findings. This is a result of iron presented from cells undergoing apoptosis or lysis. Despite these challenges, possibilities are being created for many biomedical applications.

The possibility of imaging stem cells in therapeutic applications with MRI is becoming an ever increasing area of active research. Published research so far indicates that IONPs combined with peptides or transfection agents can be used for stem cell uptake. A study using stem cells with IONPs injected into the infarcted myocardium of pigs was able to be successfully imaged at 1.5 Tesla in vivo. Following this, histological analysis revealed that the MRI signal appearance attributed to IONPs corresponded with stem cells that had taken up IONPs.

Heymer et al. combined human mesenchymal stem cells (hMSCs) with IONPs; placed these in collagen type I hydrogel (clinically approved for the repair of articular cartilage) and imaged them in a 11.7 Tesla MRI scanner. Iron uptake was confirmed by histological analysis and correlated with hypo-intense regions demonstrated on the MRI images. Their technique offers the possibility to use MRI to track the migration of IONPs loaded with hMSCs following implantation for articular cartilage repair.

Stem cell research is highly regarded as offering possible treatment solutions for patients with neuronal pathologies and injuries. Guzman et al. proved that IONPs themselves do not alter survival rates, migration patterns or differentiation capabilities of stem cells from the human central nervous system (CNS), compared with unlabelled human CNS stem cells. They demonstrated this by administering the combined human CNS stem cells and IONPs into neonatal, adult and injured rodent brains. They used MRI to track the migration of stem cells and confirmed the image findings histologically.

Stem cells labelled with IONPs have been widely used in animal models (mice, rats and pigs) to demonstrate regeneration of the myocardium following infarction. The limitation of this technique is that the conjugation of IONPs and stem cells need to be injected directly into the myocardium. This introduces an element of invasiveness. However, the region of infarct can be clearly delineated. So far, administration of stem cell and IONP conjugations for myocardial regeneration via a vascular route (intravenous or intracoronary), has not been as successful as direct injection into the infarcted myocardium. Furthermore, comparative high volumes are needed.

**Cardiovascular imaging with IONP and MRI**

IONPs have also demonstrated capabilities in imaging cardiovascular pathologies including atherosclerosis, thrombosis and myocardial infarcts.

Atherosclerosis is considered both a progressive disease and chronic inflammation. The endothelial cells of the vascular wall express receptors from their cell membrane to attract monocytes. Monocytes establish themselves in the subendothelial space and then differentiate into macrophages. The macrophages then take up oxidised low density lipoproteins. Therefore, this lipoprotein can be conjugated to IONPs and used to identify active regions of atherosclerosis with MRI. This approach has been often used in studies involving animal models. Furthermore, apoptosis leads to plaque instability and is known to occur before plaque rupture. Identifying apoptic plaques in vivo with MRI is seen as advantageous in improving patient outcomes, as inflammatory activity of atherosclerotic plaques may be associated with an increased risk of rupture.

Aortic valve disease is also an inflammatory response with macrophage involvement. Ruehm et al. successfully used commercially available IONPs to demonstrate (with MR imaging) in hyperlipidemic rabbits that atherosclerotic plaques containing macrophages take up these nanoparticles. This was confirmed with histopathological evaluation techniques on samples removed from the aorta. The MRI appearance demonstrated an aorta with irregular pattern where the signal dropout occurred at plaque sites containing macrophages endocytosing the IONPs.

IONPs can also be used to target fibrin containing thrombi. This approach may be considered a sensitive method of identifying patients at risk of more serious cardiovascular consequences. A study by Winter et al. demonstrated that by using antifibrin antibodies together with nanoparticles, clots in canine plasma in...
vitro could be identified with MRI (at 4.7 Tesla).

**Blood pool contrast agent**

There has been a high level of success in using IONPs as blood pool agents; in both animal models and in human subjects.

One study [106] investigated size and dose of IONPs as a blood pool agent for MR angiography in New Zealand White rabbits. The rabbits were divided into a control group (administered with gadopentate dimeglumine), and three other groups (each receiving a different concentration of IONPs). MR angiography was performed at varying time points; ranging from first pass to 24 hours post-intravascular administration. Assessment included signal enhancement of the abdominal aorta, renal arteries and the iliac arteries. Results demonstrated that the highest level of signal enhancement was identified during the first pass imaging strategy. It was also noted that enhancement in the abdominal aorta was greatest with the smallest nanoparticle size of 21nm. In addition to this, a concentration of 40micromole of iron per kilogram was recognised as the dose providing signal enhancement comparable to gadopentate dimeglumine. The size and dose of IONPs provided sufficient signal changes to allow imaging from first pass time-point up to 25 minutes post-intravascular administration. In this 25-minute window, multiple imaging acquisitions and measurements are possible.

Another study [107] successfully demonstrated the use of IONPs as a blood pool agent in rats with induced myocardial injuries. Here, a commercially available blood pool agent, Clariscan, was used with an MRA technique. The image findings were also compared with post-mortem histochemical stains of the infracted rat myocardium. It was found that the Clariscan-enhanced MR angiogram images over-estimated the ischemic myocardial regions when compared with histology inspection. The use of Clariscan identified the presence and dimensions of both transmural and non transmural microvasculature insults. The peri-infracted zone was seen to be over-estimated to a greater extent in rats with non-transmural ischaemic injuries. Overall, there was an over-estimation of the size of the true infarct but an under-estimation of the region at risk.

Another commercially available IONP preparation (Resovist) has been used to assess the abdominal aorta and the inferior vena cava in a human pilot study [108]. With a 3D MRA (T1-weighted) acquisition technique at 1.5 Tesla, first pass imaging was achievable with results being comparable to those obtained by MRA with conventional Gadolinium based contrast agents.

Ferumoxytol has also been used for first pass enhanced 3D MRA of blood vessels of varying dimensions in 12 human subjects. The following vessels were investigated: the carotid arteries, thoracic aorta, abdominal aorta and peripheral arteries [109]. With delayed MRA imaging it was noted that both arterial and venous structures displayed enhancement characteristics.

**Nanosensor**

A critical and relatively new-found application of IONPs is to include them in preparations to produce what is now termed “nanosensors”. Nanosensors, in their broadest definition, are IONP preparations that have been designed to detect the presence of a specific biological interaction.

Protease specific nanosensors have been developed for in vivo detection of enzyme activity with MRI [110]. In particular, T2* MRI together with nanosensors of 25 nm (hydrodynamic size) have demonstrated the involvement of the metalloproteinase 9 (MMP-9) enzyme in processes including inflammation, atherosclerosis and tumour spread. This opens the potential for early diagnosis of pathologies with altered protease activity and also the monitoring of treatment and therapies that act on protease enzymes.

Protein functionalised nanosensors have also demonstrated capabilities in identifying and measuring the concentration of anti-human serum albumin antibodies [111]. One of the additional benefits of the combined approach of MRI with nanosensors is that the NMR capabilities can allow for detection in natural human substances such as blood and urine.

Nanosensors have also been developed with the capability to detect single human alveolar cancer cells (A549) within 15 mins in blood in vitro [112]. High density folic acid, when conjugated to polyacrylic acid coated IONPs were designed to interact with A549 cancer cells expressing the folate receptor.

**Metal doped IONPs**
Metal doping of IONPs is designed to provide a comparatively higher level of magnetism at the nano scale and also allow successful magnetic tuning [113]. Such metal doped IONPs can increase MRI signal contrast by as much as 14 times compared to conventional IONPs. The implication of this is that a lower dose or lower concentration can be administered to the patient. Their magneto-thermic effects can also increase by a factor of 4.

Lanthanide metals have also been used to dope IONPs [114]. The advantages that lanthanide metals can offer include optical imaging properties, detection by neutron activation, utilised in neutron capture therapy procedures and also being detectable by time resolved fluorescence.

IONPs doped with manganese have shown to improve the quality of contrast-enhanced MR imaging of the liver [115]. These nano systems have a mean diameter of 80nm. The highest level or change in signal intensity occurs at 5 mins post-intravascular administration. However, unlike early IONPs, the imaging window for IONPs doped with manganese can last for approximately 36 hours.

CONCLUSION

In the immediate future, the reviewers see that perhaps the most likely application of IONPs will be as an MRI contrast agent. This is because IONPs offer several important advantages over commonly available gadolinium based contrast agents; including, but not limited to, lower toxicity. They can be used to diagnose a variety of pathologies and biological activities such as inflammation, infarction and tissue repair.

In addition to improved diagnostic outcomes, there can also be tailored therapeutic functions, in particular with tumour treatment. IONPs can be binded with antibodies, drugs, enzymes and proteins. They can be directed to specific organs or tissues and can also be guided with magnetic fields to target tumours and induce hyperthermic effects.

Multifunctional IONPs, together with MRI, offer unique advantages with diagnostic and therapeutic capabilities. In particular, molecular imaging with IONPs and MRI has the potential to heavily impact upon early detection of a variety of diseases and pathologic processes. It can also be used to devise treatment approaches dedicated to individual patient circumstances. This should all lead to improved patient outcomes. These are areas of high research activity and there is no doubt that this will lead to a new frontier in magnetic resonance imaging.

Figure 1 Schematic representation of a basic iron oxide nanoparticle, or nanosystem.

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