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MRI Contrast Enhancement and Cell Labeling using Gd_2O_3 Nanoparticles

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Cover: T_1-weighted MRI image of sample tubes with THP-1 cells treated with increasing concentration of Gd_2O_3-DEG nanoparticles. Monocyte samples are undifferentiated cells and macrophage samples are cells differentiated using PMA (Phorbol 12-myristate 13-acetate). Control samples show cells not treated with Gd_2O_3 nanoparticles.

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Research is what I'm doing when

I don't know what I'm doing

Wernher von Braun

The dose makes the poison

Paracelcus

Abstract

There is an increasing interest for nanomaterials in bio-medical applications and in this work, nanoparticles of gadolinium oxide (Gd_2O_3) have been investigated as a novel contrast agent for magnetic resonance imaging (MRI). Relaxation properties have been studied in aqueous solutions as well as in cell culture medium and the nanoparticles have been explored as cell labeling agents. The fluorescent properties of the particles were used to visualize the internalization in cells and doped particles were investigated as a multimodal agent that could work as a fluorescent marker for microscopy and as a contrast enhancer for MRI. Fluorescent studies show that the Gd_2O_3 nanoparticles doped with 5% terbium have interesting fluorescent properties and that these particles could work as such multimodal contrast agent. Relaxivity measurements show that in aqueous solutions, there is a twofold increase in relaxivity for Gd_2O_3 compared to commercial agent Gd-DTPA. In cell culture medium as well as in cells, there is a clear T_1 effect and an increase in signal intensity in T_1-mapped images. The cellular uptake of Gd_2O_3 nanoparticles were increased with the use of transfection agent protamine sulfate. This work shows that Gd_2O_3 nanoparticles possess good relaxation properties that are retained in different biological environments. Gd_2O_3 particles are suitable as a T_1 contrast agent, but seem also be adequate for T_2 enhancement in for instance cell labeling experiments.

Populärvetenskaplig sammanfattning

Vid undersökningar med magnetresonanstomografi (MRT) kan man se bilder av kroppens mjuka vävnader och organ. Man använder bilderna för att t.ex. diagnostisera MS, olika tumörer eller muskel- och senskador. Eftersom ingen röntgenstrålning används kan man följa upp och kontrollera olika behandlingar och göra många undersökningar utan att riskera att patienten skadas. Ibland behöver man använda ett kontrastmedel för att tydliggöra det område av kroppen man vill undersöka. För närvarande finns det två olika typer av kontrastmedel i bruk. Dessa fungerar ganska bra, men rent teoretiskt borde ett kontrastmedel kunna fungera mycket bättre och skulle då innebära att man t.ex. kunde använda lägre doser till patienterna, alternativt få mycket större kontrast i bilderna med samma doser. De kontrastmedel som finns idag skapar antingen utsläckning, d.v.s. svarta ”hål”, eller så lyser de upp bilden där kontrastmedlet finns. Ofta är det lämpligt med kontrast som lyser upp bilden t.ex. nära benvävnad eftersom benvävnad automatiskt blir svart i magnetkamerabilder. Det kan då vara svårt att se skillnad på benvävnaden och kontrastförstärkt närliggande vävnad. I denna avhandling har man studerat små, små partiklar av grundämnet gadolinium, som har goda egenskaper för att ge stark kontrast i MR-bilder och som skapar ljus kontrast i bilderna. Hittills har undersökningar med partiklarna i vattenlösning och i celler visat positiva resultat. En tanke är att utveckla ett kontrastmedel som t.ex. skulle kunna visa hur stamceller letar sig på plats i benmärg som ju finns nära benvävnad. Man skulle även kunna specialdesigna dessa gadoliniumpartiklar så att de ger både signal i MRT, men även i andra bildgivande modaliteter, t.ex. fluorescensmikroskopi.

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List of Papers

Paper 1

High Proton Relaxivity for Gadolinium Oxide Nanoparticles. Engström M, Klasson A, Pedersen H, Vahlberg C, Käll P-O, Uvdal K. *MAGMA,* 2006; 19: 180-186.

Paper 2

Positive MRI Enhancement in THP-1 Cells with Gd_2O_3 Nanoparticles. Klasson A, Ahrén M, Hellqvist E, Söderlind F, Rosén A, Käll P-O, Uvdal K, Engström M. *Contrast Media and Mol Imaging,* 2008; 3: 106-111.

Paper 3

Detection of Gd_2O_3 in Hematopoietic Progenitor Cells for MRI Contrast Enhancement. Hedlund A, Ahrén M, Gustafsson H, Abrikossova N, Warntjes M, Jönsson J-I, Uvdal K, Engström M. *Submitted to Contrast Media and Mol Imaging on Mars 3, 2011.*

Paper 4

Synthesis and Characterization of Tb^{3+} Doped Gd_2O_3 Nanocrystals: A Bifunctional Material with Combined Fluorescent Labeling and MRI Contrast Agent Properties. Petoral RM Jr, Söderlind F, Klasson A, Suska A, Fortin MA, Käll P-O, Engström M, Uvdal K. *J Phys Chem C,* 2009; 113: 6913-6920.

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Functionalized Gd_2O_3 Nanoparticles to be used for MRI Contrast Enhancement. Uvdal K, Ahrén M, Selegård L, Abrikossova N, Klasson A, Söderlind F, Engström M, Käll P-O. AVS, 2008, Boston, USA.

Abbreviations

DEG	Diethylene Glycol
DTPA	Diethylene Triamine Pentaacetic Acid
EDX	Energy Dispersive X-ray
FBS / FCS	Fetal Bovine Serum/Fetal Calf Serum
FOV	Field of View
HREM	High Resolution Electron Microscopy
ICP-SFMS	Inductively Coupled Plasma Sector Field Mass Spectrometry
IR	Inversion Recovery
MRI	Magnetic Resonance Imaging
NMR	Nuclear Magnetic Resonance
PEG	Polyethylene Glycol
PL	Photoluminiscence
PMA	Phorbol 12-Myristate 13-Acetate
RF	Radio Frequency
SE	Spin Echo
SI	Signal Intensity
SPIO	Superparamagnetic Iron Oxide
TE	Echo Time
TEM	Transmission Electron Microscopy
TI	Inversion Time
TR	Repetition Time
XPS	X-ray Photoelectron Spectroscopy

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Chapter 1

Introduction

Nanoscience has been in focus in recent years and a lot of research is performed in many different areas. 'Nanoscience' is referred to the scientific field where materials smaller than 1 micro-meter (approximately 1-100 nanometers) are studied. The word 'nano' is the SI prefix for a billionth, 10^{-9}. This area of research is believed to offer many interesting applications in a wide range of fields, from sensor technology to bio-medicine and energy conversion, *e.g.* solar cells. Nanoscaled materials have existed since the beginning of time, but today's huge interest in nanotechnology can be ascribed modern techniques making it possible to actually observe nanometer sized materials. This has made it possible not only to understand how these materials work in different applications, but also to control their function [1].

1.1 Nanoparticles

Nanoparticles are by definition particles in size-range of 1-100 nm. Nanosized particles have a high surface to volume ratio since a considerable fraction of the atoms is located at the surface of the particles. Small particles become crystalline when the atoms are assembled as a three-dimensional periodic array of atoms. A nanocrystal of 1 nm has approximately 100% of its atoms located at the surface while a 10 nm crystal has only about 15% of the atoms at the surface [2]. Several physical properties will depend on the particle size, such as magnetic, electric and photonic properties as well as chemical reactivity.

1.2 Bio-medical applications

Magnetic nanocrystals are considered for several applications in bio-medicine. Among the most intensely studied are; targeted drug delivery, cancer treatment by hyperthermia and contrast enhancement in magnetic resonance imaging (MRI) [3, 4, 5]. During the last few decades, MRI has become a well established technique for clinical diagnosis and the use of contrast agents to obtain improved images has played a great role for the increased utility of MRI. Paramagnetic or superparamagnetic nanocrystals shorten T_1 and T_2 relaxation times of the ^1H protons in tissues and are therefore considered as MRI contrast enhancers. Today super-paramagnetic iron oxide particles (SPIOs) are routinely used in MRI examinations, but also gadolinium ion chelates.

1.3 Aim

The overall aim of this work was to evaluate different preparations of crystalline nanoparticles of gadolinium oxide, Gd_2O_3, as a contrast enhancing agent for MRI and to explore the possibilities to develop a cell labeling agent that could give positive contrast as a complement to existing iron oxide particulate agents that give negative contrast. One potential application is visualizing stem cell migration using Gd_2O_3 nanoparticles.

Investigations of the Gd_2O_3 nanoparticles were conducted in water, hydroxylamine buffer, agarose gel and cell culture medium as well as in two different cell types, with or without the aid of transfection agent protamine sulfate for cell labeling. The Gd nanoparticles were also investigated as a multimodal agent, doped with terbium for both fluorescent and MRI applications. The idea was to obtain a material that could be more efficient than currently available agents and to investigate the relaxation properties of such materials.

Chapter 2

Magnetic resonance and relaxation

2.1 Magnetism

Magnetic materials are composed of atoms with valence electrons in the d- or f-shells. Elements with these shells half filled possess a net magnetic moment and build magnetic materials. These materials have magnetic moments that are aligned parallel, anti-parallel or are randomly oriented. Paramagnetic substances are those that have magnetic properties only when placed in an external magnetic field. This type of magnetism arises in atoms and molecules that have unpaired electrons. When removing the external field, the magnetic effect is lost due to the atoms return to the random orientation. Ferromagnetic substances are magnetically polarized even without an external magnetic field. There are only a few elements that possess ferromagnetic properties, for example iron, cobalt, and nickel. Superparamagnetic materials have a combination of paramagnetic and ferromagnetic properties. When an external magnetic field is applied, these materials show magnetic properties similar to those of

ferromagnetic materials. However, when there is no external magnetic field present, superparamagnetic materials have zero magnetization [6, 7]. The gadolinium ion, Gd^{3+}, has seven unpaired electrons in the valence shell and is therefore suitable as an MRI contrast agent. Gadolinium can be designed in different constellations such as complexes or oxides. Small nanoparticles of gadolinium oxide are allegedly superparamagnetic [8], but no unanimous information regarding this have been found in the literature.

2.2 Relaxation

The nucleus of an atom consists of protons and neutrons and has a positive charge, compared to electrons, that are located in orbitals surrounding the nucleus and have negative charge. The nucleus can be considered to rotate around an axis at a constant rate. This rotation is called spin. Nuclear magnetic resonance (NMR) is based on the interaction between nuclei that possess spin and an external magnetic field. Nuclear spin is one of several intrinsic characteristics of an atom and depends on the atomic composition. Almost every element in the periodic system has at least one naturally occurring isotope that possesses spin. There are different values for spins, which can be 0, integer or half-integer values. A nucleus has no spin (0) if it has an even atomic weight and an even atomic number. Such nucleus can not interact with magnetic fields and can therefore not be studied using NMR. If a nucleus has an even atomic weight and an odd atomic number the spin is said to have an integer value (1,2,3 and so on) and if it has an odd atomic number, the spin is said to be of half-integer

value. Nuclei with these values for the spin do interact with an external magnetic field and can be studied using NMR techniques [9].

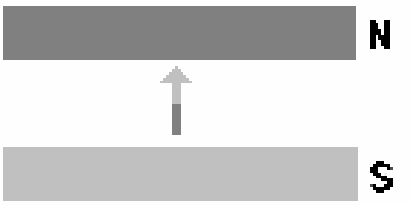

Figure 1. *When placed in an external magnetic field, protons act like bar magnets, lining up to the external field.*

A charged spinning particle creates an electric current, which, in turn generates a magnetic field. Such nucleus can be considered to be a small bar magnet with magnetic dipoles when the nucleus is placed in a magnetic field (figure 1). Hydrogen has only one proton in the nucleus. The hydrogen nucleus, ^{1}H, has spin components of $\pm^{1}/_{2}$ and is the most abundant isotope for hydrogen. Hydrogen is present in many tissues and the water content in the body is close to 80%. Each water molecule spends less than 2% of its time in contact with other molecules in the tissues, which make water molecules free and mobile molecules and thus, ^{1}H is suitable for probing the body in MRI [10, 11]. When placed in an external magnetic field, hydrogen nuclei either align themselves in the direction of the field, usually referred to as the z-direction, or counteralign themselves in the field depending on which spin components they have ($^{1}/_{2}$ or $^{-1}/_{2}$). Thus, the nuclei attain one of two energy states, a lower energy state for aligned nuclei and a higher energy state for counter aligned nuclei. The nuclei are in a state of equilibrium with a vector for the net magnetization pointing in the z-direction (figure 2). In order to get any information out of this system, the vector needs to be

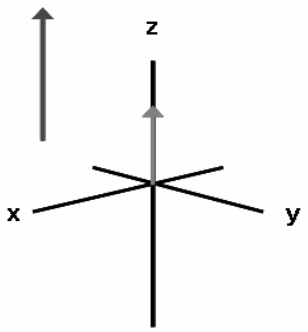

Figure 2. *Net magnetization vector of protons placed in a magnetic field.*

moved away from its equilibrium state. This is achieved by applying a second magnetic field, a radio frequency (RF) pulse that tilts the net magnetization vector away from the z-direction. This RF-pulse is then turned off, whereas the nuclei return to equilibrium (figure 3). The time it takes for the nuclei to return to the z-direction is governed by an exponential time constant called relaxation time and this is a magnetic resonance phenomenon that is detectable since the tilted vector is still spinning and therefore still induces an electric current. The current from the tilted vector is detected by an RF-receiver that is placed around the examined object. Both the transmitting and receiving of RF-pulses are most often performed by a combined transmit/receive coil that is placed around the object in the MRI-scanner. RF-pulses are repeatedly applied in different sequences and data are recorded, analyzed and transformed into images of the examined tissue [12, 13, 14].

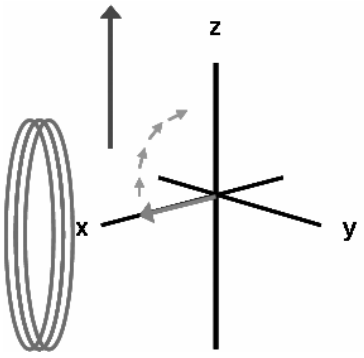

Figure 3. *Longitudinal relaxation. Net magnetization vector returns to the equilibrium state and signal decays in the receiver coil when RF-pulse is turned off.*

The main relaxation processes that influence the magnetic resonance signal are T_1 and T_2 relaxation. T_1 relaxation, or longitudinal relaxation, is the process that occurs when the spin system is returning to the equilibrium state in the z-direction following an excitation pulse. The protons give up their achieved energy and relax to their original orientation. The return of the net magnetization in the z-direction follows an exponential growth process and T_1 is the time constant that describes this process [10, 12, 15].

When exciting a spin system it initially behaves coherent, which means that the magnetization of all the components in the system are in phase in the same direction. There is also a magnetization in the x-y plane of the spin system. Directly after an excitation pulse, all the protons precess in phase and their individual magnetic moments will collectively contribute to the transverse magnetization vector. The magnetization will dephase in the x-y plane when the externally applied RF-pulse is turned off. This transverse relaxation, or T_2 relaxation, is described as the time required for the transverse components to decay (figure 4).

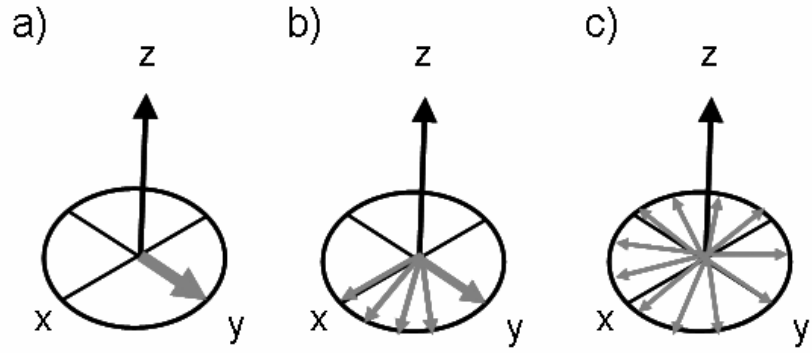

Figure 4. *T_2-relaxation process in the x-y plane of the spin system. a) shows net magnetization vector directly after RF-pulse, b) shows the transverse relaxation starting after RF-pulse is turned of and c) shows the relaxed transverse magnetization.*

When the longitudinal magnetization has reached its equilibrium, there can be no transverse magnetization and the decay of the signal in the x-y plane is therefore faster than the decay of the longitudinal magnetization along the z-axis. This means that T_2 relaxation time is always less than or equal to the corresponding T_1 time [10, 12, 13, 15]. Different tissues have characteristic T_2 relaxation times that are not directly dependent on the magnetic field strength. Due to properties of

the tissues, T_2 is approximately equal to the time at which signal decays by two-thirds. However, the actual signal decay rate depending on the pulse sequence is often faster than predicted based on T_2. This actual observed transverse relaxation time is called T_2* and is affected by other factors including magnetic field inhomogenity and motions [15].

As described above the magnetization of the protons changes directly following an RF-pulse. This causes a change in signal intensity of the examined tissue. After an inversion pulse, the longitudinal magnetization is turned to the z-axis and this can be observed in the T_1 relaxation curve of figure 5. After an excitation pulse a transverse magnetization is created. When the excitation pulse is turned off, the magnetization dephases to equilibrium and the signal detected by the receiver coil decays.

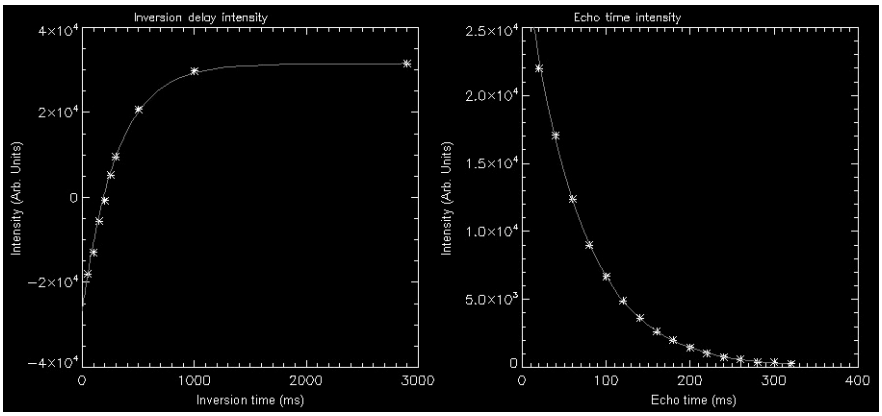

Figure 5. *T_1 and T_2 behavior can be described as relaxation curves. The left curve shows T_1 relaxation after inversion pulse and the right curve shows T_2 relaxation after excitation pulse.*

2.3 T_1-weighted pulse sequences

2.3.1 Spin echo

Conventional spin echo techniques have been the most common method for acquiring T_1-weighted images. Using optimized spin echo sequences, high quality images with T_1-weighting can be obtained within a few minutes. A spin echo is formed by an excitation pulse and one or more refocusing pulses. Usually the excitation pulse is 90˚ and the refocusing pulse 180˚. Refocusing pulses is used to rotate the dispersing excited spins in the transverse plane so that the magnetization vectors will rephase. T_1-weighted spin echo imaging is perhaps the most robust technique in MRI. Generally the repetition time (TR) is 600 ms or less for T_1-weighted images, but shorter TRs are needed with decreasing field strengths. One limitation is that only a few slices can be acquired per TR, but with modern software this limitation is not important because several sets of images can be programmed simultaneously and acquired sequentially. The echo time (TE) is at least as critical as the TR for obtaining optimal contrast in T_1 weighted spin echo images. During the TE, transverse magnetization decays depending on the T_2, reducing the signal-to-noise ratio and introducing T_2 contrast to the images. T_2 contrast works in opposition to T_1 contrast for most tissue comparisons and is therefore generally undesirable on T_1-weighted images. T_2 contrast can be minimized using the shortest possible TE [15].

2.3.2 Inversion recovery

Inversion recovery (IR) pulse sequences can be used to achieve stronger, more flexible T_1 contrast on, for example, spin echo images. Spin echo inversion recovery images are acquired by preceding each excitation pulse with an inversion pulse. When imaging tissues with variations in T_1 relaxation times, this can be manipulated using such preceding inversion recovery pulse prior to the excitation pulse since the inversion pulse will flip the longitudinal magnetization from the +z axis to the –z axis. Before the excitation pulse is applied, a time delay is provided to allow the inverted magnetization to recover towards its equilibrium value. This time between an inversion recovery pulse and the excitation pulse is called inversion time (TI). Tissues with different T_1 values recover at different rates, creating a T_1 contrast among them. The excitation pulse then converts the differences in the longitudinal magnetization into differences in the transverse magnetization, which produces signal that form an image with T_1-weighted contrast. Most IR pulse sequences require a relatively long TR (e.g. TR = 2-11 s) to preserve the contrast established by the IR module and therefore the acquisition time can become long. Inversion recovery pulse sequences have many applications and are widely used in clinical practice and in addition to producing images, IR can also be used to generate T_1 maps [16].

2.4 T_2-weighted pulse sequences

2.4.1 Spin echo

Like T_1-weighted imaging techniques, T_2-weighted images can be obtained via the spin echo (SE) technique, which was common in the first decade of clinical MRI. This technique provides acceptable T_2 contrast for most tissues. However, the number of images that can be acquired per TR decreases with long TE and therefore there is a practical limit to the TE that can be used in clinical practice before the acquisition times are unacceptably long. The long survival of the relatively inefficient T_2-weighted SE images in clinical practice has been due to the lack of acceptable alternatives. However, in recent years, other techniques have been developed for T_2-weighted imaging.

2.4.2 Gradient echo

Gradient echo pulse sequences are faster than T_2-weighted SE sequences and consist of an excitation pulse followed by measurement of a gradient echo. Many gradient echo techniques achieve fast imaging by using short repetition times (TR) and TR is often comparable to or shorter than the T_2-relaxation time of the tissues in the region of interest. Gradient echo pulse sequences do not have 180˚ refocusing RF pulses that are used to form an RF spin echo. Instead, a gradient reversal on the frequency-encoded axis is forming the echo. In addition, gradient echo sequences can be fast due to the use of a flip angle of the excitation pulse that is less than 90˚ [15, 16].

Chapter 3

Contrast agents in MRI

Although the inherent soft tissue contrast of MRI is excellent, the application of contrast agents in clinical imaging was beginning to arise in the 80´s with the first agent being approved in 1988 [17, 18]. Contrast agents in MRI can for example be administered to further enhance tissue contrast, to characterize lesions and to evaluate perfusion and blood flow abnormalities [19, 20]. In addition, there is the field of molecular imaging (visualization of specific molecules) that have developed during recent years and which benefits from the development of cell labeling agents or specific targeting agents, which enable visualization on molecular levels [21]. Combining MRI and molecular imaging can allow the visualization of drug distribution and target binding or visualizing of the target itself, like receptor expression and modulation [22]. Molecular imaging with contrast agents could also be helpful for monitoring certain tissues or cell types in cellular trafficking, cell differentiation or transplant rejection [23]. This could help researchers understand more about for instance stem cell migration that in the future could come to be useful knowledge or

important tools in the treatment of several diseases where patients can be helped by stem cell transplantation.

3.1 Contrast mechanisms and relaxivity

3.1.1 Contrast mechanisms

Contrast mechanisms in MRI are effects of the contrast agent's ability to alter the relaxation times, T_1 and/or T_2, of hydrogen in the tissue, thereby influencing the signal intensity and the image contrast. One requirement for an efficient MRI contrast agent is therefore the ability to interact with hydrogen nuclei that come into close contact with the contrast agent. Single, unpaired electrons have magnetic dipole moments that are over 650 times stronger than that of single, unpaired protons. Because of this, the magnetic effects of unpaired electrons dominate the magnetic effects of the atom. The number of unpaired electrons in the outer shell of an atom is therefore important for determining the effect the particular atom has as a contrast agent. Elements with the highest numbers of unpaired electrons and also the longest electron spin relaxation times will have the strongest magnetic relaxation effects on hydrogen. The effect of paramagnetic contrast agents on tissue relaxation rates is in direct proportion to the concentration of the agent as shown in the formula

$$1/T_{i(observed)} = 1/T_{i(inherent)} + r_i * C \qquad i=1, 2$$

where inherent stands for the tissue relaxation properties without contrast agent and observed stands for the relaxation properties with

contrast agent, r_i are the relaxivities and C is the concentration of the agent [11].

3.1.2 Relaxivity

Relaxivity is a parameter that allows ranking of different contrast agents and it is a measure of how efficient the contrast agent enhances the proton relaxation rates of water. In practice, it is the slope of $1/T_i$ as function of the concentration of the contrast agent [24] (see equation above), usually in units of s^{-1} mM^{-1}. There are different contributions to relaxation rates and relaxivity and paramagnetic relaxation enhancement generally consists of two components; inner-sphere relaxation and outer-sphere relaxation [17].

Inner-sphere relaxation is described as relaxation enhancement of a solvent molecule directly coordinated to the paramagnetic compound, which means chemical exchange of water molecules between the primary coordination sphere of for instance a paramagnetic metal ion and the bulk solvent. Certain parameters affect the water exchange and one important such parameter is rotation. Rotation correlation time of the paramagnetic compound is a major contributor to the relaxivity and strategies to slow down rotation in order to optimize relaxivity have been widely studied with various results [17]. Motions of water molecules normally cause magnetic fluctuations that are much faster than the Larmor frequency of protons and this is also true for contrast agent molecules. Slowing down the molecular motion so that it is more likely to fit the Larmor frequency yields greater relaxation rates and is one way to optimize the relaxivity [25]. This is normally achieved by increasing the molecular weight and altering the

structure of the contrast agent molecules, for example creating macromolecules with complex binding to larger molecules like albumin or working with different dendrimers on the surface of the contrast agent molecules [26]. When slowing down the rotational motion, water exchange becomes more important [27]. Water exchange can be optimized by for instance increasing the steric crowding of the molecule or altering the charge of the complex. More negative charge means faster water exchange [28, 29].

Outer-sphere relaxation is described as relaxation enhancement of solvent molecules in outer coordination spheres of the paramagnetic compound. Coordinatively saturated gadolinium complexes increases relaxivity due to these mechanisms. Relaxation can arise from dipolar mechanisms or from diffusion of water molecules near the gadolinium complex.

In tissue, two general factors control the efficiency of which a complex influences relaxation rates; the chemical environment *in vivo* and compartmentalization of the complexes in the tissue. In addition, macromolecular binding of the agent to molecules in the tissue have great effect on relaxation, which can potentially cause relaxitity enhancement. However, tissue water compartmentalized into interstitial and intracellular space can have slow exchange rates and this can potentially decrease the relaxivity of an agent since only a part of the tissue water is encountering the paramagnetic centers of the agent [30].

In addition, factors affecting relaxation are also pH, viscosity and temperature of the environment. According to an experiment conducted in water within this work, pH is increasing with increasing

Gd concentration. In samples with same Gd concentration (1.2 mM), relaxation times caused by the Gd_2O_3 nanoparticles are short for pH around 6, but when pH is increased, so are the relaxation times, which is shown in figure 6.

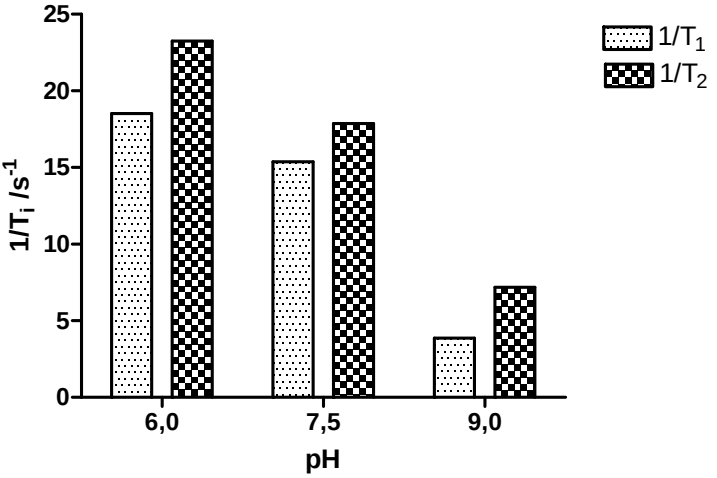

Figure 6. *Graph of relaxation rates for Gd_2O_3-DEG nanoparticles (conc. 1.2 mM) in different pH.*

Regarding viscosity, relaxation is often fast in inert surroundings due to the slower molecular motion, which is shown in section 3.3.3. Temperature affects relaxivity due to influences on motional energy [15]. Higher temperatures mean faster molecular motion and T_1 times increase with increasing temperatures.

3.1.3 Different contrast agents

MRI with contrast agents is now a well established technique for clinical diagnosis and the use of such agents to obtain improved

images has played a great role for the increased utility of MRI. Today there are mainly two contrast agents used in MRI; superparamagnetic iron oxide particles (SPIOs) and paramagnetic chelates of particularly gadolinium, but manganese are also used. Manganese and gadolinium agents are used to obtain positive contrast in MR images whereas iron oxide particles are mainly used to obtain negative contrast. A drawback with negative contrast agents is that they create signal voids in the images and therefore the contrast could be difficult to detect. Loss of signal in tissue pathologies is not unusual and the presence of such voids makes it complicated to discriminate between the contrast, tissue and image artifacts. Different chelates of Gd^{3+} are the most commonly used clinical agents. The Gd^{3+} ion is strongly paramagnetic due to its seven unpaired f-electrons. However, Gd^{3+} chelates have low relaxivity compared to what is theoretically possible and they are not very selective [9]. There is a demand for new, more efficient and tissue specific agents that could be used as molecular markers and magnetic tracers and possibly could generate great contrast with low doses.

3.2 Lanthanides and gadolinium

In 1794, the Finnish chemist and Professor J. Gadolin, examined a recently discovered mineral, gadolinite, and from this mineral he isolated a new oxide, yttria (named after the small village Ytterby in the Swedish archipelago outside Stockholm where gadolinite once was found). Yttria was shown to content yttrium (Y), terbium (Tb), erbium (Er), ytterbium (Yb), scandium (Sc), holmium (Ho), thulium (Tm), gadolinium (Gd), dysprosium (Dy) and lutetium (Lu). These elements

are some elements of the lanthanides, or rare earth metals [31, 32]. Gadolinia, the oxide of gadolinium, was discovered from yttria in 1886 by the French chemist Paul Émile Lecoq de Boisbaudran. In nature gadolinium is present only in chemical compounds and in mixtures with other rare earth metals. Gadolinium can be separated chemically and the metal is silver white, shining and soft. It has been found to be ferromagnetic below 19°C and thereafter it is paramagnetic. Because of its magnetic properties it has come to medical use in MRI [33].

3.3 Gadolinium agents

Gadolinium based contrast agents are normally used to shorten T_1 and to give positive contrast (brightness) in MR images. Available agents today are different kinds of gadolinium ion based chelates that are relatively stable molecules. They are, however, nonspecific and have short rotational correlation time that limits the proton relaxivity [34, 35]. This has led to immense attempts to develop more efficient contrast agents and examples of such agents are for instance Gd^{3+} based agents with higher molecular weight like Gd-DTPA functionalized polymers, Gd-DTPA terminated dendrimers, Gd complex loaded liposomes as well as high density lipoprotein nanoparticles or micelles among many others [36]. Another route of development is the recent investigations of different constellations of crystalline nanoparticles of gadolinium oxide, which have high number of gadolinium atoms even in very small size particles. These particles exhibit higher relaxivities than the gadolinium chelates do.

3.3.1 Gd-chelates and safety

There are a variety of Gd-chelates used today and they are considered safe. However, in patients with renal dysfunction, the administration of Gd-based contrast agents is linked to the disorder NSF (nephrogenic systemic fibrosis) [37, 38, 39]. NSF is a scleroma-like fibrotic skin disorder seen in patients with renal impairment where deposits of collagen in tissues, skin thickening and hyperpigmentation are hallmarks. The disorder can have systemic involvement and can in severe cases be fatal [40]. It is shown that Gd-chelates with a nonionic linear structure have been more associated with development of NSF. However, since these facts have been reported, changes in MRI clinical practice have eliminated new NSF cases. For example, it is important that renal status of the patient is evaluated prior to administration and a proper agent and dose is chosen. In addition, patients should be closely monitored after administration and risk patients could undergo dialysis after examination [38].

3.3.2 Gd_2O_3 nanoparticles

One advantage of using nanoparticle based systems as contrast agents compared to chelate complexes is the possibility for nanoparticles to yield higher relaxivity due to higher density of gadolinium in a compact core since the number of gadolinium atoms is very high even for small size particles [41, 42]. In addition, benefits of supplementary features such as luminescence or different targeting as well as functionalizing through embedding the particles in organic or inorganic polymers could be obtained. Another benefit with a particulate agent embedded in a polymer could implicate less leakage

of Gd ions, which would be a desirable feature when dealing with safety issues [43].

3.3.3 Relaxivity of Gd_2O_3 nanoparticles in different media

In this work, two different preparations of Gd_2O_3 nanoparticles have been studied in various environments. Particles received directly after synthesis, capped with DEG (diethylene glycol), were studied in water, 1 M hydroxylamine buffer, cell culture medium (RPMI 1640) as well as in agarose gels. In addition, Gd_2O_3-DEG nanoparticles were received after synthesis and dialysis and studied in H_2O, cell culture medium and inside cells. Dialysis was performed to exclude ions and large aggregates in the original solution. The effects of dialysis were investigated in Ahrén et al. [43] and it was observed that relaxivity is decreasing with dialysis time. During this work it was discovered that relaxivity is decreasing by approximately two-thirds and then remain constant between 1-3 days of dialysis time, which is why we choose to work with particles dialyzed for 24 h. The lower relaxivity of dialyzed particles can be explained by loss of ions that contributed to the higher relaxivity of undialyzed samples, as well as loss of DEG coating, resulting in smaller particles with higher rotational motion.

Relaxivity was notably changed in the different environments. In the cell culture medium samples, macromolecular binding, which slow down rotational motion, is a very likely explanation to the increased relaxivities compared to H_2O or buffer. In addition, the viscosity of the surroundings has effect on rotational motion of the particles, which could alter the relaxivity. In agarose gel and cell culture medium, we obtain higher relaxivity compared to H_2O and buffer, with similar r_1

for agarose and cell culture medium. We do not observe an increase in r_2 compared to r_1 in the agarose gel as in the cell culture medium. Instead, r_1 and r_2 are similar (table 1). In these samples, however, $1/T_2$ (relaxation rate) is increased.

Inside cells there appears to be no difference in r_1 compared to cell culture medium, but we observe an increase in r_2 (table 2). This might be explained by the compartmentalization of the internalized particles, where they might act like larger particles. This would have greater effect on the transversal relaxivity. High macromolecular content inside the cells can also explain the higher relaxivity.

Table 1. *Relaxivity (r_1 and r_2) in $s^{-1}mM^{-1}$ for undialyzed Gd_2O_3-DEG in water, hydroxylamine buffer, cell culture medium and agarose gel measured at 1.5 T, 21-23°C.*

	H_2O	Buffer	RPMI 1640	1% Agarose
r_1 Gd_2O_3	9.2	9.8	13.9	14.3
r_1 Gd-DTPA	4.7	5.6	5.1	5.7
r_2 Gd_2O_3	11.3	11.9	22.3	14.3
r_2 Gd-DTPA	5.3	6.2	6.4	7.5
r_2/r_1 Gd_2O_3	1.2	1.2	1.6	1.0
r_2/r_1 Gd-DTPA	1.1	1.1	1.3	1.3

Table 2. *Relaxivity (r_1 and r_2) in $s^{-1}mM^{-1}$ of dialyzed Gd_2O_3-DEG in water, cell culture medium and inside cells, measured at 1.5 T, 21-23°C.*

	H_2O	H_2O*	RPMI 1640	THP-1 cells
r_1 Gd_2O_3	2.8	2.7	3.6	4.1
r_2 Gd_2O_3	3.0	3.7	12.9	17.4
r_2/r_1 Gd_2O_3	1.1	1.4	3.5	4.2

***** *Particles were dialyzed for 72 h instead of 24 h.*

Chapter 4

Cell labeling

4.1 Cell biology

There are a lot of different cell types in nature and they are classified into two major groups; prokaryotic cells and eukaryotic cells. Prokaryotic cells are single-celled organisms like bacteria and eukaryotic cells are cells from plants and animals. All cells are surrounded by a phospholipid membrane, but unlike prokaryotes, eukaryotic cells also comprise of extensive internal membranes that enclose specific compartments (organelles) and separate them from the rest of the internal parts outside the nucleus (cytoplasm). The largest organelle in an eukaryotic cell is the nucleus, which contain most of the DNA. In addition, other important organelles are mitochondria, where the cell's energy metabolism is carried out; endoplasmatic reticula, where glycoproteins and lipids are synthesized; Golgi, which distribute membrane structures to appropriate places in the cell; peroxisomes, where fatty acids and

amino acids are degraded; ribosomes, which produce proteins; lysosomes (in animal cells), which degrade worn-out cell constituents and foreign materials taken up by the cell. Some of these organelles are visible in figure 7.

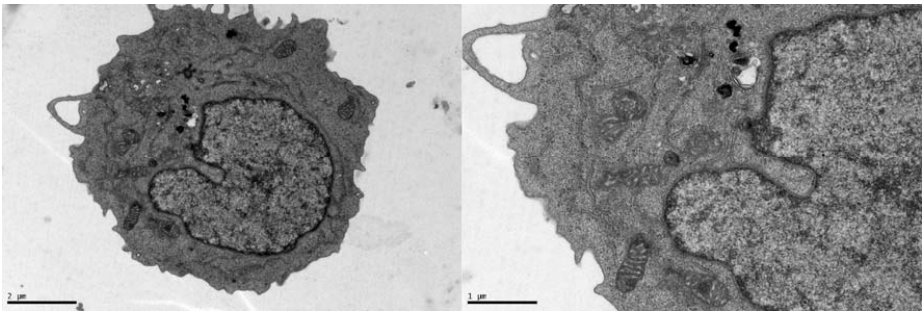

Figure 7. *Electron microscopy images of Ba/F3 cell. The large structure in the middle of the cell is the nucleus. Another visible organelle is for example mitochondria, which shows in the magnified image at the right.*

In addition, the cytoplasm of eukaryotic cells contains an array of fibrous proteins that usually is called cytoskeleton. These different cytoskeletal filaments give stability to the cell and help the cell to maintain shape. They also allow for cellular movement and arrange the transportation of structures and molecules within the cell [44].

Cells can internalize objects from its surrounding by processes like phagocytosis or endocytosis. Phagocytosis is a process where filaments of the cytoskeleton work to move large parts of the cell so the cell engulfs bacteria or other large particles. Endocytosis is a process in which a smaller region of the membrane invaginates to form a new intracellular membrane coated vesicle about 0.05-0.1 µm in diameter. Relatively few cell types carry out phagocytosis whereas most eukaryotic cells continually engage in endocytosis where the cell nonspecifically take up small parts of extracellular fluid and any material that is located within this fluid. Endocytosis can also be a

specific uptake process that is receptor-mediated. In this case a specific receptor located in the cell membrane bind to an extracellular macromolecule (ligand) that the receptor recognizes. Following this binding the cell membrane with the receptor-ligand complex undergoes endocytosis and becomes a transport vesicle inside the cell. Phagocytosis and endocytosis are mechanisms which are used in this thesis to internalize Gd_2O_3 nanoparticles into cells [44].

4.1.1 Hematopoietic cells

Hematopoietic cells are cells of the blood system and all cellular elements of blood derive from the same precursor cells; the pluripotent hematopoietic stem cells in the bone marrow. The different types of blood cells are summarized in figure 8.

In this thesis two types of hematopoietic cells were used; THP-1 cells and Ba/F3 cells. THP-1 cells are monocytes (white blood cells) and they can be phagocytic. They give rise to mature macrophages which are the 'cleaners' of the immune system in the body. Ba/F3 cells are lymphoid progenitors, which give rise to the lymphocytic B-cells. B-cells can, when activated, differentiate to plasma cells that secrete antibodies [45].

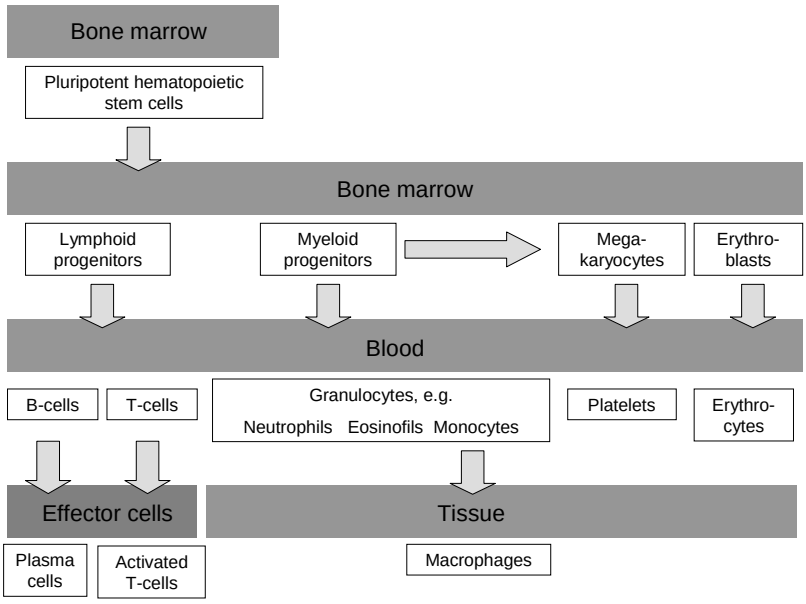

Figure 8. *Schematic illustration of cellular elements of the blood that arise from hematopoietic stem cells in the bone marrow. This is a generalized figure from Janeway et al. [45].*

4.1.2 THP-1 cells

THP-1 is a monocytic cell line derived from the peripheral blood from a one year old male with acute monocytic leukemia. THP-1 monocytes have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins. They produce lysozymes and are known to be phagocytic [46, 47]. If treated with phorbol esters, THP-1 monocytes can differentiate into macrophage like cells, which mimic native monocyte-derived macrophages. Differentiated THP-1 cells behave more like these native monocyte-derived macrophages than other well established cell lines, such as U937, HL-60 and HELA [48]. THP-1 cells grow in suspension (RPMI 1640 medium) with 10% FCS, 2 mM

L-glutamine and 1% penicillin-streptomycin in culture flasks at 37°C in 5% CO_2 atmosphere. They are very tolerable and a suitable cell type to be studied in this work.

4.1.3 Ba/F3 cells

Ba/F3 is a murine bone marrow-derived progenitor B-cell line established from peripheral blood of mice [49]. It is dependent on Interleukin-3 (IL-3) for viability and proliferation and the cells are classified as early cells of the lymphoblastiod lineage [50]. The cells grow in suspension (RPMI 1640) with 10% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin, 25 mM Hepes, 50 µM 2-merkaptoetanol and 5% IL-3. They are cultured in flasks at 37°C in 5% CO_2 atmosphere.

4.2 Cell labeling with nanoparticles

There are several methods to label different cell types with magnetic nanoparticles [51]. SPIOs are most frequently used for cell labeling experiments. However, they usually generate negative contrast, even though there are known methods and MRI sequences that can generate positive contrast from SPIOs [3, 52, 53]. Since SPIOs are superparamagnetic they have great influence on the transverse magnetization and relaxation, T_2 and T_2*. This is due to susceptibility effects since superparamagnetic materials create large distortions in the magnetic field. If using SPIOs as a marker for stem cell migration, it could potentially become hard to visualize the particles since stem

cells are believed to migrate near bone tissue, which signal is also extinguished in MR images. Therefore the migration could be difficult to follow. A particulate agent that generates positive contrast could be an important complement to SPIOs. For this, Gd_2O_3 nanoparticles possess desirable properties [54].

Small size iron oxide particles (<100 nm) facilitates transport across cell membranes and labeling is usually performed by incubating the cells of interest with the contrast agent *in vitro*. The uptake of the contrast agent then occurs through phagocytosis/endocytosis, but transfection agents or electroporation are common labeling techniques as well [55]. This has been used to image atherosclerosis and other inflammatory processes [56], but stem cell labeling has also been performed despite the difficulties with signal voids. Clinically available SPIO agents are normally liver-specific and used to detect liver tumours [57]. In this work, SPIOs are used in paper 3 to compare uptake with Gd_2O_3-DEG nanoparticles.

4.2.1 Labeling with Gd_2O_3 nanoparticles

There is reason to believe that if it is possible to label cells with SPIOs the same would be applicable for Gd_2O_3 nanoparticles. However, it is important to design the particles to be suitable as a cell labeling agent [58]. Particles size and functionalization of the particles have been shown to be important for cell uptake. Vance et al. [58] suggests that particles should be between 25-50 nanometers to be internalized most efficiently into cells. The Gd_2O_3 nanoparticles used in this work is mainly covered with DEG (diethylene glycol) with particle size between 2-10 nanometers. The small particle size allows for

functionalization of the particles of approximately 15-40 nanometers to the final optimal radius for cells to ingest. During the work with this thesis functionalization has been performed with PEG (polyethylene glycol). PEG has shown to be non-immunogenic, non-antigenic and protein resistant [59]. Within this work cell labeling studies with Gd_2O_3-PEG have been conducted, however results from these MRI measurements show no signal. Thus, we conclude that the PEG-nanoparticles are not taken up by the cells used in this work, and therefore not suitable for cell labeling without further modification. On the other hand, if one were to design a blood pool contrast agent, Gd_2O_3-PEG could be suitable since the kidney ultra filtration could be slowed down due to the capping and particles would thus have a prolonged circulation time. In addition, shielding with PEG reduces degradation of the particles and, as mentioned above, minimizes recognition of the immune system [60].

We have shown in paper 2 that DEG-covered Gd_2O_3 nanoparticles are ingested in cells with good results [61]. However, both gadolinium and DEG are known to be toxic, which imply that this capping is not suitable for cell labeling. Viability studies in this work show that for a short period of time (2-48 h) the cells withstand the treatment of the particles, which is also confirmed by Faucher et al. [62]. Therefore we conclude that for these types of studies, the DEG-capped particles are not hazardous to the cells and our results would therefore be reliable. However, when designing the particles for clinical applications, other capping that stabilizes the particles further is necessary.

4.2.2 Transfection agents

Transfection agents are macromolecules possessing electrostatic charges used for nonviral transfections of DNA into nuclei of cells [63]. This property is also applied when transfecting cells with magnetic tracers for MRI. There is a variety of different transfection agents available such as polycationic amines, dendrimers and lipid-based agents. In this thesis work the transfection agent protamine sulfate was used. Protamine sulfate is a low-molecular weight polycationic peptide that is well tolerated by cells. It is FDA approved as an antidote to heparin anticoagulant and commonly it is used to facilitate gene transfection of cells and thus, combined with nanoparticles to label cells for MRI [64]. It works by altering the charge between extracellular macromolecules and the cell surface and thereby facilitates contact and thus, makes it easier for the cells to ingest the macromolecules into the cells.

4.2.3 Cell tracking

As a part of the project to develop methods for tracing stem cells with MRI, labeled cells have also been studied embedded in gelatin phantoms. We wanted to investigate how few cells that could be detected with the available MR-equipment. THP-1 cells were used for this purpose. The monocytic cells were treated with 2.0 mM Gd_2O_3-DEG nanoparticles for 2 h with 1 million cells in each sample. After incubation the cells were washed twice in cell culture medium. Each sample were then diluted to get appropriate amounts of cells for adding 2 µl from each sample to spots on to a gelatin layer in a custom made Plexiglas box. The samples were left to air dry and a top coat of

gelatin were added and left to stiffen prior to MRI. The gelatin phantom was scanned at room temperature with a SenseFlex-L coil in a Philips Achieva 1.5 T clinical scanner. A T_1-weighted inversion recovery sequence was used with TR=1400 ms and TE=5 ms. Slice thickness was 3 mm. It is clear from figure 9 that samples with both 10000 and 5000 cells are visible and for a trained eye it is possible to discern the sample with 1000 cells (encircled sample). To reach the goal to be able to visualize very few cells, the method needs to be more sensitive. It would be desirable to have higher loading frequency of nanoparticles into the cells as well as another coil that allows for higher resolution. In addition, more efficient particles contributing to higher relaxivity would be beneficial.

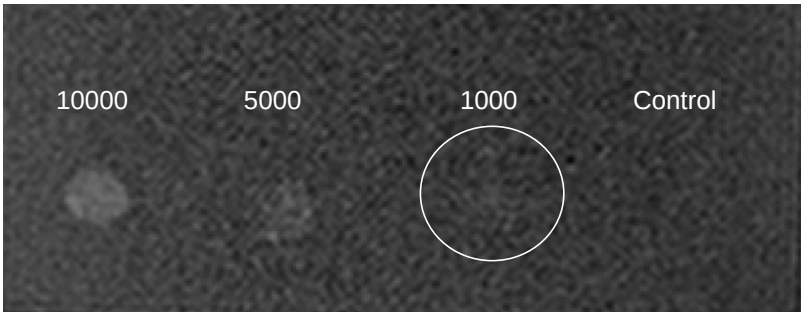

Figure 9. *Image of Gd_2O_3 incubated THP-1 cell samples embedded in gelatin. Samples containing 10000 and 5000 cells are clearly visible and the sample containing 1000 cells is vaguely visible.*

Chapter 5

Fluorescence

5.1 Fluorescence physics

In fluorescence microscopy, the specimens of interest are usually treated with special reagents to accomplish the fluorescent signals. The molecules of these reagents are able to absorb light for an extremely short time and then emit the light. The emitted light is shifted to a longer wavelength than the excitation (absorption) wavelength, for example, blue light is absorbed and green light is then emitted immediately afterwards. Green is changed to yellow, yellow to red and so on. This shift is termed Stokes shift after its discoverer. In fluorescence, the wavelength of the emitted light is about 20 to 50 nanometers longer than absorbed exciting light. Fluorescence molecules can only absorb light of a certain wavelength. Each of the various fluorochromes exhibits its own, specific excitation spectrum depending on the internal structure of the fluorescence molecules and their surroundings. Furthermore, not every photon is absorbed, but only a fraction of incoming photons. The total energy of absorbed

fraction of photons is not entirely converted to emitted light. Good fluorescence probes feature a high quantum yield, which is describing the ratio of the emitted to the absorbed photons. This is very useful for microscopy. Nowadays, fluorescence methods have made it possible to specifically couple the fluorescence molecules with biological substances, for instance antibodies and thereby there are probes that can target specific structures and tissues of interest [65, 66].

5.2 Applications

Fluorescence microscopy, as well as other optical imaging techniques, are well established and highly developed for *in vitro* and *ex vivo* applications in molecular and cellular biology. An extension of this toward non-invasive *in vivo* imaging represents an interesting future for extracting biological information from living subjects [67]. One approach to *in vivo* molecular MRI could be the design of a multimodal contrast agent with optical and magnetic properties for dual imaging. Such agent could be useful in preoperative diagnosis and in intra-operative surgical resection of brain tumors or other lesions [68, 69]. For this purpose, lanthanides (rare-earths) possess suitable properties. Rare-earth doped nanoparticles are promising materials for fluorescent labeling, as they usually have narrow emission spectra, long lifetimes, and minimized photobleaching. Pure gadolinium oxide is fluorescent with an emission spectrum showing a broad band centered at 530 nm. Doped with 10% europium, the emission spectrum shows a narrow peak at 600-620 nm [70]. Other lanthanide ions, such as Tb^{3+} and Yb^{3+}, can also be of consideration when doping for example Gd_2O_3 nanoparticles.

5.3 Fluorescence studies in this thesis

The original idea was to use the fluorescent properties of gadolinium and investigate the possibilities to detect particle uptake. Images of Gd_2O_3-DEG particles lighting up cell organelles (probably lysosomes or endosomes) inside the cells were obtained (figure 10) [71]. This experiment lead to an extended collaboration with the department of Physics, Chemistry and Biology at Linköping University to visualize

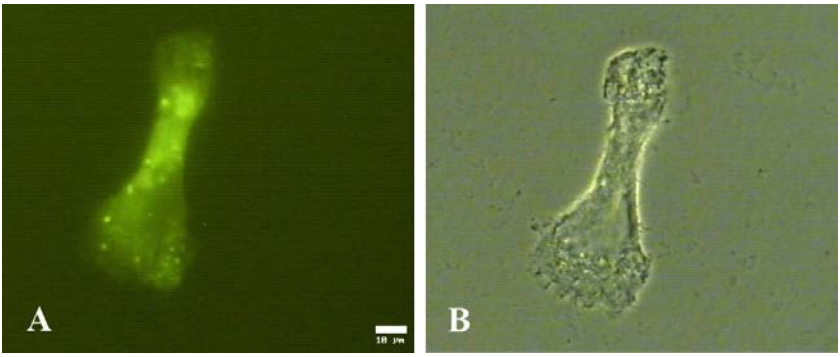

Figure 10. *A) shows fluorescence microscopy image of THP-1 cell incubated with Gd_2O_3 nanoparticles. B) shows confocal microscopy image of the same cell.*

the weakly fluorescent gadolinium particles doped with another, more strongly fluorescent lanthanide (terbium), and monitor them inside cells. We also investigated the relaxation properties of these particles and these studies are described in Paper 4 of this thesis. The idea was to examine the possibilities to develop a multi-modal contrast agent that could be detected not only by MRI, but also by fluorescence microscopy.

Chapter 6

Papers

Paper 1: **High Proton Relaxivity for Gadolinium Oxide Nanoparticles.** Engström M et al. *MAGMA,* 2006; 19: 180-186.

Paper 2: **Positive MRI Enhancement in THP-1 Cells with Gd_2O_3 Nanoparticles.** *Contrast Media and Mol. Imaging,* 2008; 3: 106-111.

Paper 3: **Detection of Gd_2O_3 in Hematopoietic Progenitor Cells for MRI Contrast Enhancement.** *Submitted to Contrast Media and Mol. Imaging on Mars 3, 2011.*

Paper 4: **Synthesis and Characterization of Tb^{3+} Doped Gd_2O_3 Nanocrystals: A Bifunctional Material with Combined Fluorescent Labeling and MRI Contrast Agent.** *J. Phys. Chem. C,* 2009; 113: 6913-6920.

6.1 Summary of papers

6.1.1 Paper 1

High Proton Relaxivity for Gadolinium Oxide Nanoparticles.

Nanosized materials of Gd_2O_3 can provide high contrast enhancement in MRI and nanoparticles are promising candidates for molecular imaging because they convey the possibility of high relaxivity per molecular binding site. In this study, we investigated proton relaxation enhancement by ultrasmall (5-10 nm) Gd_2O_3 nanocrystals. Nanocrystals coated with diethylene glycol (DEG) were syntesized, characterized by X-ray photoelectron spectroscopy (XPS), and investigated by MRI relaxometry. Relaxivity of Gd_2O_3 was compared with gadolinium chelates in clinical use today.

Particle synthesis

Gadolinium oxide nanocrystals were synthesized by the polyol method in two different ways. A mixture of NaOH, DEG and either $Gd(NO_3)_3$ or $GdCl_3$ was heated to 140˚C and when reactants had dissolved, the temperature was raised to 180˚C and held constant for 4 h, yielding a dark yellow colloid.

XPS

The chemical composition of the nanocrystals was investigated by X-ray photoelectron spectroscopy.

Relaxation time measurements

Samples of Gd_2O_3-DEG and Gd-DTPA (Magnevist®) were prepared in 10 mm NMR test tubes with H_2O, 1 M hydroxylamine buffer ($NH_2(OH)/NH_3(OH)^+$) and in RPMI 1640 cell culture medium (Gibco, Invitrogen) in concentrations of 0.1 to 1.6 mM. During measurement the tubes were immersed in a bowl with saline and pH of the samples were measured by a Metrohm 744 pH meter and also checked by pH-indicator strips (Merck). T_1 and T_2 relaxation times were measured with a 1.5 T Philips Achieva clinical scanner using the head coil. A 2D mixed multiecho SE interleaved with a multiecho IR sequence was used for the measurements. Imaging parameters were varied to minimize the standard deviations in relaxation time calculations. Gd contents in nanoparticle stem solutions were determined by inductively coupled plasma sector field mass spectrometry (ICP-SFMS) at Analytica AB (Luleå, Sweden).

Results and discussion

Gd_2O_3-DEG nanoparticles induced higher proton relaxivities compared to Gd-DTPA (relaxivity constants are given in table 3). The relaxivities due to the nanoparicles were twice that of the chelate. This is, to our knowledge, the first time relaxation behaviour for such Gd_2O_3 nanoparticles have been reported. Another interesting observation was the marked T_1-reducing effect and simulated signal increase at low concentrations (~<0.7 mM). The concentration range below 0.6 mM in plasma is most relevant for clinical use. At the recommended dose of Magnevist, 0.1 mmol/kg, the detected plasma concentration of Gd is 0.6 mM at 3 min after injection.

Table 3. *Relaxivity constants (r_1, r_2) in s^{-1} mM^{-1}, standard deviation (SD), and p values for Gd-DTPA and Gd_2O_3-DEG in H_2O, buffer and cell culture medium (RPMI 1640) measured at 1.5T, 21-23°C.*

	r_1	SD	p	r_2	SD	p	pH
H_2O							
Gd-DTPA	4.7	±0.1	<0.0001	5.3	±0.2	<0.0001	5.4
Gd_2O_3-DEG(1)	9.2	±0.3	<0.0001	11.3	±0.4	<0.0001	6.3-7.2
Buffer							
Gd-DTPA	5.6	±0.3	<0.0001	6.2	±0.3	<0.0001	7.2
Gd_2O_3-DEG(2)	9.8	±0.5	<0.0001	11.9	±0.7	<0.0001	7.4
RPMI							
Gd-DTPA	5.1	±0.1	<0.0001	6.4	±0.1	<0.0001	7.3
Gd_2O_3-DEG(1)	13.2	±0.7	<0.0001	24.6	±2.3	0.0003	7.3
Gd_2O_3-DEG(2)	13.9	±0.8	<0.0001	22.3	±1.9	0.0017	7.3

6.1.2 Paper 2

Positive MRI Contrast Enhancement in THP-1 Cells with Gd_2O_3 Nanoparticles.

During the past decades MRI has become a well established technique for clinical diagnosis and the use of contrast agents to obtain improved images has played a great role for the increased utility of MRI. In this study we examined nanoparticles of Gd_2O_3 as a cell labeling contrast agent for MRI.

Particle synthesis

Nanoparticles of Gd_2O_3 were synthesized by the polyol method. $GdCl_3$ was dissolved in 10 ml DEG (diethylene glycol) by heating the mixture to 140˚C. Solid NaOH was dissolved in 5 ml DEG and subsequently added to the Gd solution. The temperature was then raised to 180˚C and held constant for 4 h under reflux and magnetic stirring, yielding a colloid. This particle solution was filtered and dialyzed to exclude large particles and ions.

Characterization

The particles were characterized by high-resolution electron microscopy (TEM) and photoelectron spectroscopy (XPS).

Cell labeling

THP-1 monocytes were incubated in different concentrations of Gd_2O_3 for 2 h at 37˚C in 5% CO_2 atmosphere. After incubation cells were washed and prepared for MRI measurements. In addition, cell viability after incubation with Gd_2O_3 for different period of times was studied with typan blue coloring/bürker chamber counting.

Relaxation time measurements

Cell samples were measured with a Philips Achieva 1.5 T MR-scanner using the head coil. T_1 was measured with inversion recovery pulse sequence with TE=29 ms, TR=3000 ms (for samples with Gd incubation conc. 1.0-2.5 mM) and IR=50-2900 ms (9 measure points). The 0.5 mM (incubation conc.) sample was measured with TR=10000 ms and IR=50-5000 ms (10 measure points). T_2 was measured with a multi echo sequence, TE=20 ms, TR=1000 ms, number of echoes were 16 and the flip angle was 70°. FOV for the measurements was 200 mm and slice thickness was 5 mm.

Results and discussion

Relaxivity results show that intracellular Gd_2O_3 shorten relaxation times with increasing concentration. For r_1, the relaxivity of particles in monocytes was not significantly different compared to particles in cell culture medium (p=0.36). For r_2, the relaxivity between samples in monocytes and samples in cell culture medium was significantly different (p=0.02). For monocyte samples r_1 was 4.1 s^{-1} mM^{-1} and r_2 was 17.4 s^{-1} mM^{-1}. Corresponding r_1 and r_2 for particles in medium were 3.6 s^{-1} mM^{-1} and 12.9 s^{-1} mM^{-1}, respectively.

Table 2. *Relaxivity constants (r_1, r_2) in s^{-1} mM^{-1}, standard deviation (SD), p-values and r_2/r_1 for Gd_2O_3 in cell culture medium and THP-1 cells.*

	r_1	SD	p	r_2	SD	p	r_2/r_1
RPMI 1640	3.6	±0.27	0.0009	12.9	±1.02	0.0062	3.5
THP-1 cells	4.1	±0.42	0.0023	17.4	±0.94	0.0003	4.2

This study indicates a potential for Gd_2O_3 nanoparticles to be used as a cell labeling contrast agent and signal intensity image shows high contrast enhancement for cell samples incubated with 1.5 mM Gd_2O_3. Cell viability after incubation observed in this study did not decrease to any significant degree.

6.1.3 Paper 3

Detection of Gd_2O_3 in Hematopoietic Progenitor Cells for MRI Contrast Enhancement

The importance of having specific and efficient contrast agents increases as the possible applications of MRI broadens. There has been a huge development in fields of molecular imaging, which benefits of having access to targeting agents. Previous studies have shown that Gd_2O_3 nanoparticles can generate high relaxivity and positive signal and in this study the aim was to improve methods for monitoring hematopoietic stem cell migration by MRI. By using Gd_2O_3 nanoparticles as cell labeling agent the uptake was compared in two different hematopoietic progenitor cell types and it was investigated whether the transfection agent protamine sulfate increased the particle uptake.

Particle Synthesis and Characterization

The particles used in this study were obtained from the same procedures as described in Paper 2.

Cell labeling

THP-1 and Ba/F3 progenitor cells were incubated in two different concentrations of Gd_2O_3 and two different concentrations of SPIOs, with or without previous exposure to transfection agent protamine sulfate. After incubation cells were washed and SPIO-incubated samples were analyzed with Prussian blue staining and Gd_2O_3 nanoparticle samples with Electron Microscopy to assess whether

particles were intracellular. In addition, samples were prepared for MRI measurements. Cell viability of Ba/F3 after incubation with Gd_2O_3 for different period of times was studied with typan blue coloring/bürker chamber counting. Viability of THP-1 cells after incubation was previously studied in Paper 2.

Relaxation time measurements

T_1 relaxation times were measured with inversion recovery (IR) sequences; TE=29 ms, TR=10 s and IR delay times were 125, 300, 500, 1000, 2000 and 5000 ms. FOV was 200 mm and slice thickness=5 mm. T_2 was measured with a multi-echo Spin Echo sequence with 16 echoes at multiples of 20 ms, TR=1000 ms. FOV=200 mm and slice thickness=5 mm. Sample tubes were immersed in a holder in a bowl of water, temperature was 21±1°C. Measurements were performed in a Philips Achieva 1.5 T clinical scanner using a head coil.

Results and Discussion

Both Prussian blue staining and Electron Microscopy reveal intracellular particles. Protamine sulfate treated samples appeared to have ingested more particles than samples not exposed to protamine sulfate. Uptake percentage of Gd_2O_3 nanoparticles are quite low; 0.2-4% in Ba/F3 and 1-5% in THP-1, however not unexpected. Corresponding uptake for SPIOs were 2-11% in Ba/F3 and 2-20% in THP-1. This might be explained by the larger particle size of SPIOs, which might be optimal for recognition and uptake by the cells. Relaxivity measures of all samples treated with Gd_2O_3 was; r_1 5.1±0.3 and r_2 14.9±0.7 (s^{-1} mM^{-1}). For all samples treated with SPIOs r_1 was 2.1±0.5 and r_2 was 57.2±16.7 (s^{-1} mM^{-1}). Brighter signal from samples incubated with the higher concentrations of Gd_2O_3 nanoparticles supports the fact that high signal intensity could be obtained

intracellularly. The viability of cells in this study remained unaffected by the incubation of the gadolinium nanoparticles and cells were intact at the time for MRI. We conclude that Gd_2O_3 nanoparticles are continuously promising as a positive intracellular MRI contrast agent that has potential to be developed as a stem cell targeting agent.

6.1.4 Paper 4

Synthesis and Characterization of Tb^{3+} Doped Gd_2O_3 Nano-crystals: A Bifunctional Material with Combined Fluorescent Labeling and MRI Contrast Agent Properties.

Molecular imaging with aid of targeted contrast agents like fluorescent probes has been improving in the past decade regarding, for instance, detection limits, imaging modalities and engineered functionality. Recently, the development of engineered nanoparticles with multifunctional features has emerged. Rare-earth based nanoparticles are promising types of luminescent materials and ion-doped lanthanide oxide nanoparticles are highly photostable, exhibit long luminescence lifetimes and narrow emission bands. In this study, rare-earth oxide Gd_2O_3 nanoparticles doped with Tb_3^+ ions were synthesizised and capped with different organic acids. For some samples, polyethylene glycol (PEG)-containing molecules were coupled to the acids. Studies on the possibility of using these particles as a fluorescent cellular labeling agent and MRI contrast agent is presented.

Particle synthesis

Terbium-doped gadolinium oxide nanoparticles were synthesized by the polyol method. For the 5% Tb-doped nanocrystals, 5.7 mmol $GdCl_3$ and 0.3 mmol $TbCl_3$ were dissolved in 30 ml DEG, constantly stirred and heated in silicon oil bath at 140-160˚C for 1 h. Then, 7.5 mmol NaOH dissolved in 30 ml DEG was added. After complete dissolution of the reactants, the solution was refluxed at 180˚C for 4 h under stirring. For the 20% Tb-doped Gd_2O_3, the above procedure was followed, except for adding 1.1 mmol of $TbCl_3$ and no addition of NaOH. The synthesized suspensions were then filtered to remove large particles and agglomerations. Then the particles were functionalized with organic acids and PEG (see Paper 4 for details).

Characterization

High-resolution transmission electron microscopy (HRTEM), x-ray photoelectron spectroscopy (XPS), energy disperse x-ray (EDX) and infrared spectra (IR) were carried out to characterize the synthesized material. Optical properties were characterized by photoluminescence (PL) measurements.

Cell labeling

Cell labeling was carried out with Terbium doped Citric Acid (CA) capped Gd nanoparticles. The Terbium content was for the cell studies in this work 5%. A monocytic cell line, THP-1 cells, was differentiated to macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA). Cells were harvested and treated with 1.0 mM Tb:Gd_2O_3:CA nanoparticles for 2 h, 1 million cells/sample. After incubation the cells were washed twice in RPMI 1640 medium and twice in phosphate-buffered saline (PBS, 1X solution), centrifugation

at 1050 rpm for 8 minutes in each washing. Finally the THP-1 cells were mounted on adhesion slides and fixated (4% paraformaldehyde, PFA). The cells were then treated with anti-fading medium prior to examination by fluorescence microscopy. In addition, dermal fibroblast cells form *Xenopus laevis* were labeled with the Tb:Gd_2O_3:CA nanoparticles, but were not, however, used by the author of this thesis and are therefore not addressed in this summary.

Relaxation time measurements

Proton relaxation times were measured with a 1.5 T Philips Achieva whole body scanner using the head coil. A 2D mixed multiecho SE interleaved with a multiecho IR sequence was used (see Paper 1 for details regarding imaging time parameters and other MRI settings).

Results and discussion

Images of THP-1 cells incubated with the 5Tb:Gd_2O_3:CA nanoparticles, show bright yellow-green grains in the cytoplasm (figure 11). Because the cells are highly phagocytic, these bright grains most probably correspond to particle containing endosomes or lysosomes.

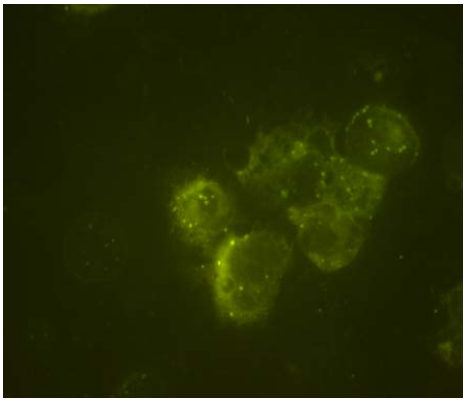

Figure 11. *Fluorescence microscopy image of THP-1 cells that have taken up 5% Tb doped Gd particles into endosomes or lysosomes.*

MRI relaxivity studies were performed with PEGylated Tb-containing Gd particles in water and compared with undoped Gd-PEG particles and a commercial agent, Gd-DTPA. The T_1 relaxivity of the different PEGylated particles was 2-3 times higher than Gd-DTPA. This

indicates a possibility to use lanthanide doped Gd_2O_3 particles as a multimodal MRI contrast agent.

6.2 Author's contributions

Throughout my thesis work, I collaborated in an interdisciplinary research network covering medicine, biology, chemistry and physics, which was both inspiring and productive. This resulted in me getting the chance to learn and understand more about other disciplines other than the biomedical field, which is my educational background. Within my project I learned about MRI physics, which I was not originally trained for and this was sometimes a challenge. I was also trained in cell culturing and I worked independently with two different cell lines and was responsible for maintenance and methods necessary regarding this.

6.2.1 Paper 1

In this study, I contributed to the study design and implementation of the MRI experimental setup and I performed all the laboratory work regarding this as well as the MRI measurements. I also analyzed and summarized the MRI results and constructed figures for the manuscript and in addition, obtained the statistical MRI data. This was an intense learning phase and I was especially acquiring MRI knowledge. The synthesis and particle characterization was performed by our collaborators at the department for Physics, Chemistry and Biology at Linköping University and with this work I was first introduced to interdisciplinary work within this research field.

6.2.2 Paper 2

I this study, I was responsible for the study design and implementation of the cell and MRI experiments. I independently performed all the laboratory work and I also performed the MRI measurements and data analysis. In this work, our collaborators in chemistry and physics (at the department of Physics, Chemistry and Biology) performed synthesis, dialysis and characterization of the particles. I was responsible for summarizing these results, writing the major part of the manuscript as well as corresponding with the journal.

6.2.3 Paper 3

After discussing possible applications with my colleges, I came up with the idea of using a progenitor stem cell line to investigate the possibility to use the Gd_2O_3 nanoparticles as a potential stem cell marker. I acquired the Ba/F3 cell line and transfection agent protamine sulfate from our collaborator Jan-Invar Jönsson at the department of Clinical and Experimental Medicine, Experimental Hematology Unit at Linköping University. I performed all the laboratory part of the work from cell culturing, cell labeling to MRI studies. I also analyzed and summarized the results and was responsible for writing the manuscript and corresponding with the journal. The synthesis and particle characterization was performed, as in all our previous studies, by our collaborators at the department for Physics, Chemistry and Biology at Linköping University.

6.2.4 Paper 4

I came up with the original idea of using undoped Gd particles for observing internalized particles using fluorescence microscopy combined with the MRI studies. With this information in hand I wanted to evaluate if the auto fluorescence of Gadolinium would be sufficient for observing intracellular particles. My first pilot study was presented at ESMRMB in Basel in 2005 and the idea was to investigate if this could be a standard method to determine whether the particles were ingested in the cells or not. However, the auto fluorescence of Gadolinium particles was not strong enough for our purposes. Our collaborators at the department of Physics, Chemistry and Biology initiated a project on doped Gadolinium particles to obtain a multi-modal contrast agent where the lanthanides Europium and Terbium were used to intensify the particle fluorescence. The following work in this project resulted in Paper 2. I was responsible for planning and performing the cell studies regarding THP-1 cells and I was also responsible for the MRI measurements and involved in the analysis of these results. This included for instance cell culturing, cell labeling, preparing samples for MRI and fluorescence microscopy and operating the MR-scanner. Regarding the manuscript I contributed with information to the sections about THP-1 cells and MRI. The efforts behind this paper indeed intensified our interdisciplinary work and enhanced my understanding of the importance of joint knowledge in different scientific fields.

6.2.5 Pilot projects

Besides the work that resulted in the included papers, I have conducted a series of pilot projects aiming to develop new methods for tissue characterization by means of nanoparticle contrast enhancement in MRI;

To study **enhancement in biological environment** PEGylated Gd_2O_3 nanoparticles have recently been studied in pieces of salmon. This is a project in which I had an operative key role. Visualization was carried out in both MRI and CT to investigate the possibility to achieve a multi-modal contrast agent suitable for examinations in these two modalities.

In a project aiming for **cell tracking**, I embedded labeled cells in gelatin phantoms to investigate how few cells could be detected with the available MR-equipment. This is a part of the stem cell project towards the aim to develop a method sensitive enough to monitor stem cell migration by MRI and results are described in chapter 4.

Another project aimed to study the tissue composition of **atherosclerotic plaques**. I labeled macrophages, which then were incubated with surgically removed atherosclerotic plaques. Cells appeared to attach to the plaque surface, which could be detected with MRI. This project resulted in a larger study which is to be carried out in the near future. This study is to be conducted with MRI, CT, ultrasound followed by final histology of the plaques and thus, investigates the possibility to label and visualize atherosclerosis.

***In vivo* imaging** was conducted during a practice visit at AstraZeneca in Mölndal (at the end of my PhD education as a part of the AgoraLink project). I had the opportunity to bring our PEGylated nanoparticles and perform studies in higher magnetic fields (4.7 and 9.4 T). Studies were performed with particles in water at both field strengths as well as *in vivo* in rats at 4.7 T.

Chapter 7

Discussion

The papers included in this thesis consider studies of different preparations of Gd_2O_3 nanoparticles for contrast enhancement in MRI. Excellent relaxivity properties are reported and positive contrast is obtained for the Gd_2O_3 nanoparticles in different environments, such as water, buffers and cell culture medium as well as inside cells.

Gd nanoparticles in solution show increased relaxivity compared to commercial agent Gd-DTPA and have a T_1-reducing effect and strong signal intensity at low concentrations. It is considered that the concentration range below 0.6 mM in plasma is most relevant for clinical use [72] and in Paper 1, the Gd_2O_3 nanoparticles show high signal intensity below this concentration.

According to these results, the Gd_2O_3 particles in the size range of about 5-10 nm, show paramagnetic behavior. They show high r_2 values and consequently higher r_2/r_1 quote in cells as well as in cell culture medium than in H_2O. This indicates that the T_2 effect is

higher when the surroundings are not as homogeneous as in aqueous solution. In this case, the water exchange may be altered as the particles are captured in small enclosed compartments like the endosomes or lysosomes of cells. Also, in these compartments, as well as bound to for instance different proteins in the medium, the nanoparticles may act like larger particles, which would have greater effect on the transversal relaxivity than the longitudinal. However, we still observe a clear T_1 enhancement in T_1-weighted images. Particles in for example RPMI cell culture medium increase both r_1 and r_2 compared to particles in water. Macromolecular binding increases rotational correlation time, which increases the relaxivity [30] and therefore, any proteins bound to particles in the RPMI-medium lead to higher relaxivity. However, since we observe this T_1 enhancement, although the very high r_2, Gd_2O_3 nanoparticles are considered suitable for T_1 contrast enhancement. Comparing relaxation of Gd_2O_3 nanoparticles in the two different cell types, THP-1 and Ba/F3, there is no noticeable difference. The relaxivity curves in Paper 3 with overall relaxivity in all cells regardless of cell type, fit on the same line.

The cell labeling protocols used in these studies have consequently been improved and elaborated on. The differences are whether the THP-1 cells were matured to become macrophages (treated with PMA) or not or whether transfection agent protamine sulfate was used. PMA-stimulating the cells to differentiate them to macrophages were found to be unnecessary for our purposes since the THP-1 cells ingested comparable amounts of particles without the stimulation. Therefore, in Paper 2 and 3, the THP-1 cells were used as monocytes. In Paper 3, we investigated the possibility to label a progenitor stem cell (Ba/F3) and increase particle uptake in THP-1 cells by treating

the cells with transfection agent protamine sulfate. Ba/F3 cells are not phagocytic and would therefore have slower mechanisms for internalizing the particles and thus, a transfection agent was believed to provide an enhanced uptake. Using a transfection agent did increase the uptake noticeably compared to untreated cells. However, from the studies performed leading to the results in Paper 2, we observed that it is possible to obtain high uptake in phagocytic cells without a transfection agent. This might depend on the status on the cells in the culture when exposing them to the particles and on the inherent natural and biological differences of cells. In electron microscopy images of Gd_2O_3 nanoparticles in Ba/F3 cells we observe some particles close to the cell membrane and located between aggregated cells. This might indicate that all measured Gd is not intracellular. For future work, investigations regarding cell aggregation must be performed to ensure that particles do not get stuck between cells.

Regarding the toxicity of gadolinium, it is clear that especially Gd^{3+} ions are toxic and some effects are blocking of calcium channels, which disturbs many actions achieved by Ca^{2+}, inhibition of phagocytosis in liver macrophages (Kupffer cells) and affecting the cytochrome P450 system [73]. Recently, it has also been noticed that gadolinium-based contrast agents have caused nephrogenic systemic fibrosis (NSF) in patients with renal impairment [37-40, 74]. It is therefore very important that the Gd_2O_3 nanoparticles are designed in such way that free Gd could not be released or that particles could stay in the circulation for such a long time risking getting caught in the tissue. In this thesis, the toxicity of Gd_2O_3 particles has been studied as viability in two different cell types to confirm the methodology of the experiments and these results were surprisingly good with regards to

the alledged effects of gadolinium. During 8 days of exposure to Gd_2O_3, the viability of the THP-1 cells did not decrease below acceptable levels for cell experiments. However, THP-1 is a tumor-transformed cell line and might perhaps have developed strategies for survival that normal cells do not have. In Ba/F3 cells, the viability was monitored during 72 h of exposure to the Gd_2O_3-DEG nanoparticles and during this time, viability was not significantly reduced.

The major part of this thesis concerns pure Gd_2O_3 nanoparticles capped with DEG. Our interdisciplinary team involving research groups in medicine, biology, chemistry and physics, have performed and are currently performing several studies regarding capping and functionalization of these particles to make them non-toxic and clearable, although able to stay in circulation long enough to be detectable with MRI-techniques [75]. Work is also ongoing regarding whether to design a specific contrast agent to target certain tissue, for instance atherosclerotic plaques or stem cells. It has to be considered whether the particles should be coated to stay outside cells or if they should be designed to be an intracellular agent. When targeting stem cells to monitor stem cell migration, the agent is preferred to be able to get inside cells. It is beneficial if the particles could also be coated with organic fluorophores to get a bifunctional nanoparticle with both magnetic and luminescent properties [36]. For this reason, it is interesting to further investigate the Tb_3^+-doped particles since the results of this thesis work suggest that doped Gd_2O_3 nanoparticles have contrast enhancement as well as fluorescent properties. This could open new diagnostic possibilities for designing a positive contrasted molecular imaging, multimodal agent. Such an agent can then be considered as an MRI enhancer using common 1.5 T clinical systems, combined with different fluorescent techniques.

Future work in this project will include investigations regarding exploring other methods to internalize the particles inside cells. This could for instance involve using other transfection agents or electroporation of the cells. The electroporation method might not be good when working with proper stem cells since they are sensitive and might not tolerate that treatment. However, Walczack et al. [55] reports successful attempts to label stem cell with proper calibrated magnetoelectroporation. In addition, further studies include capping and functionalization to obtain more effective internalization as well as proper design for designated purposes. In this area the possibilities are many and there are numerous of interesting alternatives.

Chapter 8

Conclusions

- ☆ Gd_2O_3 nanoparticles posses appropriate properties for contrast enhancement in MRI.

- ☆ It is possible to increase the positive contrast with Gd_2O_3 nanoparticles compared to commercial Gd-chelates.

- ☆ With proper capping and functionalization the particles could be a suitable cell labeling agent when positive contrast is required.

- ☆ Doped with other lanthanides, the Gd_2O_3 nanoparticles can be a multi-modal contrast agent for imaging in both MRI and fluorescence imaging modalities.

- ☆ Thus, we see a possibility to design a contrast agent that could visualize stem cell migration with positive contrast enhance-ment.

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