Abstract

The necessity for synthesis of nanoparticles with well controlled size and morphology emerged with the development in recent years of novel advanced applications especially in biomedical related fields. These applications require nanoparticles with more complex architecture such as multifunctional nanoparticles (i.e. core–shell structures) that can carry several components with different embedded functionalities. In this thesis, we developed core–shell nanoparticles (CSNPs) with finely tuned silica shell on iron oxide core as model system for advanced applications in nanomedicine such as MRI, drug delivery and hyperthermia.

The synthesis of monodispersed, and well separated, single iron oxide core–silica (SiO₂) shell nanoparticles for biomedical applications is still a challenge. Substantial amount of aggregated and multicore CSNPs are generally the undesired outcome. In this thesis, synthesis of monodispersed, free of necking, single core iron oxide-SiO₂ shell nanoparticles with different distinct overall size and tuneable shell thickness was performed using an inverse microemulsion method. The influence of the reaction time, hydrodynamic conditions and precursor concentration on the synthesis process and thickness of the silica layer was investigated and the process was optimised. The residual reactions during the post synthesis processing were inhibited using a combination of pH adjustment and alternating shock freezing with ultracentrifuging.

The second part of the thesis is concerning thorough characterisation of the CSNPs with different shell thickness. The non-aggregated tuneable shell CSNPs maintained the superparamagnetic character of the cores with high magnetisation, showing great potential for their applications in nanomedicine. Magnetic measurements and relaxivity tests were performed and the comparison of the CSNPs with commercial products revealed the fact that relaxation time ratios (r₂/r₁) of the CSNPs obtained are higher than those of the commercially available MRI contrast agents which indicates a better T2 contrast.

In the last part of the thesis the in-vitro toxicity investigation results are reported. For the investigation of cytotoxicity (3- 4, 5-dimethyldiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide (MTT) assay was performed and the secretion of pro-inflammatory cytokines TNF-α and IL-6 was determined using enzyme-linked immunosorbert assay (ELISA). The cells were exposed to a wide range of concentrations of nanoparticles (between 0.5 µg/ml to 100 µg/ml). The cell toxicity results indicated no severe toxic effects on human monocyte-derived macrophages (HMDM) as model system. The internalisation of the nanoparticles by HMDM was monitored using transmission electron microscopy (TEM).

The CSNPs have the capacity of forming stable colloidal dispersions at physiological pH, with desired magnetic properties, low toxicity, and the potential for further functionalisation via surface modification of the silica shell or by adding new components (i.e. quantum dots, therapeutics). These characteristics make them highly promising for drug delivery, medical imaging, hyperthermia, magnetic cell marking and cell separation as well as many other biomedical applications.
LIST OF PAPERS

This thesis is based on following publications:


Carmen Vogt, Muhammet S. Toprak, Sophie Laurent, Jean-Luc Bridot, Robert N. Müller, Mamoun Muhammed, High quality and tuneable silica shell - magnetic core nanoparticles, accepted for publication in Journal of Nanoparticle Research, DOI: 10.1007/s11051-009-9661-7

Andrea Kunzmann, Carmen Vogt, Neus Feliu, Fei Ye, Tina Thurnherr, Sophie Laurent, Jean-Luc Bridot, Robert Müller, Muhammet S. Toprak, Harald F. Krug, Bengt Fadeel, Mamoun Muhammed, Biocompatibility of tuneable silica shell-magnetic core nanoparticles evaluated in vitro using primary human monocyte-derived macrophages, manuscript

Other work not included in the thesis:
Conference presentations

Marian, Carmen M.; Fornara, Andrea; Qin, Jian; Muhammed, Mamoun "Optimized Magnetic Nanoparticle for High Resolution Contrast in MRI (poster)" 8th International Conference on Nanostructured Materials, Aug 20-25, 2006, Bangalore/India


Carmen M. Vogt, Muhammet Toprak, Jingwen Shi, Neus Feliu Torres, Bengt Fadeel, Sophie Laurent, Jean-Luc Bridot, Robert N. Müller, Mamoun Muhammed, Optimised synthetic route for tuneable shell SiO₂@Fe₃O₄ core-shell nanoparticles, MRS Fall Meeting, December 1-5, 2008, Boston, USA


Carmen Vogt, Andrea Kunzmann, Britta Andersson, Fei Ye, Neus Feliu Torres, Tina Thurnherr, Sophie Laurent, Jean-Luc Bridot, Robert Muller, Muhammet Toprak, Harald F. Krug, Annika Scheynius Bengt Fadeel, Mamoun Muhammed "Tunable superparamagnetic Fe₃O₄-SiO₂ core-shell nanoparticles: synthesis, characterization, and in vitro compatibility with immune-competent cells”, 34th International Conference and Exposition on Advanced Ceramics and Composites, 24-29 January 2010, Daytona Beach, USA

Contributions of the author

Paper 1. Planning and performing the experiments, characterizing the samples, evaluation of the results and writing the manuscript.

Paper 2. Planning and performing experiments, performing part of the characterization of the samples, evaluation of the results and writing the manuscript.

Paper 3. Planning and performing synthesis experiments, performing part of the characterization of the samples, evaluation of parts of results and writing parts of the manuscript.
### ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Fe(CO)$_5$</td>
<td>Iron pentacarbonyl</td>
</tr>
<tr>
<td>Fe(acac)$_3$</td>
<td>Iron Acetylacetonate</td>
</tr>
<tr>
<td>Fe(cup)$_3$</td>
<td>Iron (N-nitrosophenylhydroxylamine)</td>
</tr>
<tr>
<td>R$_{1,2}$</td>
<td>T1 or T2 relaxivity [s$^{-1}$]</td>
</tr>
<tr>
<td>r$_{1,2}$</td>
<td>T1 or T2 relaxation [s$^{-1}$mM$^{-1}$]</td>
</tr>
<tr>
<td>$\mu$</td>
<td>magnetic permeability [H/m]</td>
</tr>
<tr>
<td>$\mu_0$</td>
<td>magnetic permeability of vacuum $4\pi \times 10^{-7}$ [H/m]</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>B</td>
<td>magnetic induction [T] or [G]</td>
</tr>
<tr>
<td>CSNP</td>
<td>core – shell nanoparticle</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>D$_c$</td>
<td>single domain critical size [m]</td>
</tr>
<tr>
<td>DDS</td>
<td>drug delivery system</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>anisotropy energy [eV]</td>
</tr>
<tr>
<td>ED</td>
<td>Electron Diffraction</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E$_T$</td>
<td>thermal energy J or eV</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FEG</td>
<td>Field Emission Gun</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>H</td>
<td>magnetic applied field [A/m] or [Oe]</td>
</tr>
<tr>
<td>h</td>
<td>Planck constant $6.626 \times 10^{-34}$ [Js]</td>
</tr>
<tr>
<td>HMDM</td>
<td>human monocyte-derived macrophages</td>
</tr>
<tr>
<td>HRTEM</td>
<td>High-Resolution Transmission Electron Microscopy</td>
</tr>
<tr>
<td>ICDD</td>
<td>International Centre for Diffraction Data</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann constant $1.381 \times 10^{-23}$ [JK$^{-1}$]</td>
</tr>
<tr>
<td>$k_{\text{eff}}$</td>
<td>anisotropy constant [J/cm$^3$]</td>
</tr>
<tr>
<td>LDH</td>
<td>lactic acid dehydrogenase</td>
</tr>
<tr>
<td>M</td>
<td>magnetization [A/m] or [emu/cm$^3$]</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>$M_s$</td>
<td>saturation magnetisation [Am$^2$/kg] or [emu/g]</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylidiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>n</td>
<td>refractive index of the medium [dimensionless]</td>
</tr>
</tbody>
</table>
OI  optical imaging
PBMC  peripheral blood mononuclear cells
PBS  phosphate buffered saline
PEG  poly(ethylene glycol)
PEO  poly(ethylene oxide)
PET  Positron Emission Tomography
PLA  Polylactic acid/ polylactide
PLGA  poly(lactic-co-glycolic acid)
PLL A  poly(L,L-lactide)
PMMA  Poly(methyl methacrylate)
PVA  poly(vinyl alcohol)
RES  reticulo-endothelial system
RPMI  Roswell Park Memorial Institute medium
SD  standard deviation
SEM  Scanning Electron Microscopy
SPECT  Single Photon Emission CT
SPION  superparamagnetic iron oxide nanoparticle
T  absolute temperature  [K]
T1  Longitudinal/spin-lattice relaxation  [s]
T2  Transverse/spin-spin relaxation  [s]
Tb  blocking temperature  [K]
TC  Curie temperature  [K]
TEM  Transmission Electron Microscopy
TEOS  Tetraethyl orthosilicate
texp  experimental time  [s]
TN  Néel temperature  [K]
TNF  tumour necrosis factor
US  ultrasound
V  volume  [m³]
Vb  blocking volume  [m³]
VSM  vibrating sample magnetometer
WHO  World Health Organisation
XRD  X-ray diffraction
Λ  radiation wavelength  [m]
α  semiangle of the lens  [rad]
χ  magnetic susceptibility  [dimensionless]
δ  resolution  [m]
τ0  characteristic relaxation time  [s]
τb  Brownian relaxation time of magnetic particle  [s]
τN  Néel relaxation time of magnetic particle  [s]
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1 Introduction

Nanomedicine is defined as the application of nanometer range technologies in medical areas (from biomedical imaging to drug delivery and therapeutics). This is a new, fast expanding and growing medical field. The overall goal of nanomedicine is the same as it is for traditional medicine: early and accurate diagnosis, effective treatments that are free of side effects, and non-invasive evaluation of the efficacy of the treatment.

Nanotechnology that nanomedicine is using brings not just improvement of the existing techniques but also provides completely new tools and capabilities\(^1, 2\). Nanotechnology is a very broad interdisciplinary research field that involves various areas of science as chemistry, physics, engineering, biology, medicine and erodes the traditional boundaries between them\(^3\). In a more technical definition, nanotechnology is the understanding and control of matter at dimensions between 1 and \(~100\) nanometers, where unique phenomena enable novel applications\(^4\). Indeed, the physical and chemical properties of materials can radically change as their size is scaled down to small clusters of atoms. The high ratio surface area to volume, large percentage of surface atoms compared with bulk materials are the results of the small size\(^5\). Due to their size, the nanodevices (such as nanoparticles) possess biomimetic features, being in the same range of dimension as antibodies, membrane receptors, nucleic acids and proteins, among other biomolecules\(^6\).

Nanoparticles are powerful tools for imaging, diagnosis and therapy due to their intrinsic properties as detailed above. In recent years, a new generation of nanoparticles arises, with improved performances for the same type of applications and with a complex structure compared with the simple designed nanoparticles. These complex nanoparticles are called multifunctional nanoparticles due to the fact that the components in their structure can carry different functionalities which allow achieving multiple tasks simultaneously.
1.1 Objectives

For *in-vivo* and *in-vitro* applications in nanomedicine, nanoparticles have to meet some specifications: small overall size, high surface area for surface functionalisation; high colloidal stability; and the ability to pass the biological barriers\(^7\). However, especially for the *in-vivo* applications, the particles should meet more strict requirements: non-toxicity, non-immunogenicity, long-term retention within blood circulation, and the ability to reach and pass through the endothelial capillary membranes without embolism of the bigger vessels\(^8\). These additional requirements directed the research towards the development of new nanoparticles with well-defined size and shape, no aggregation and that at the same time can exert different functions (multifunctional nanoparticles).

This work is part of a project focusing on understanding the influence of engineered nanoparticles (composition, size, surface modification, etc.) on the immunity system.

The objective of this thesis is to develop an optimized route and synthesize biologically relevant multifunctional CSNPs with various distinct overall sizes. Specifically iron oxide core – silica shell nanoparticles are fabricated with a great control of the silica shell thickness and no residual aggregation.

The influence of various synthesis parameters on the silica shell thickness is studied and the post synthesis treatment is adjusted to have a high control on the thickness of the shell and on the aggregation of the resulted particles.

The applicability of the particles with different thickness of the silica (thus different overall size) as Magnetic Resonance Imaging (MRI) contrast agents was studied by magnetic measurements and by measuring the relaxivity properties of the particles.

The investigation of the cytotoxicity and uptake of the CSNPs using as cell model human monocyte-derived macrophages (HMDM) was performed, as a step further in order to assess the applicability of these particles for *in-vivo* applications.
1.2 Outline

This thesis describes the development of finely tuned silica shell on magnetic iron oxide core nanoparticles for different applications in medicine, such as MRI contrast agents and magnetically driven drug delivery systems.

Developing nanoparticles for applications in medical related area implies adopting identical strategies as all the drugs or devices to be used in humans have to follow. The main idea behind the work is similar to the concept of developing a new drug/ imaging contrast agent. The development process needs to follow some strictly regulated iterative stages which are distributed in two big parts: preclinical and clinical study. The preclinical study is the path of the drug from the discovery, development till clinical application. The role is to assess if the drug may be tested safely in humans and also has the potential to be effective in certain human disease states (diagnostic and/treatment). It starts with the idea of the new drug and identification of all specific requirements that has to be complied. During the development process careful physical and chemical characterization has to be performed. The product will be further characterized extensively in-vitro and in-vivo to evaluate its safety (short term and long term toxicity), the molecular paths of drug actions, and its pharmacokinetic properties (absorption, distribution, metabolism and excretion). The long process ends with the clinical phase, the final stage before the drug is introduced into the market.

In this thesis, we present the beginning of the process of developing a new multifunctional system based on CSNPs, precisely the synthesis, characterisation and in-vitro cytotoxicity assessment of the CSNPs.

Chapter 1 gives an overview on different multifunctional nanoparticles for applications in medical area with specifically detailing the system that we focus in this thesis: iron oxide core – silica shell nanoparticles. In this respect, we give an overview of the synthesis methods and the magnetic properties of the iron oxide nanoparticles. Also the two commonly used methods of producing the silica shell of the CSNPs are presented. The toxicity and cytotoxicity of the nanoparticles used in medicine is briefed.

Chapter 2 describes the experimental procedures followed for the synthesis of the SPION core, the preparation of the CSNPs with the influencing parameters, and characterization of nanoparticles. The experimental work performed for the synthesis of the magnetic iron oxide nanoparticles core with defined size and of the silica shell – magnetic core nanoparticles is detailed. The study of the parameters that influence the synthesis process like: stirring rate and reaction time has been performed to finely tune the silica shell thickness
and the overall size of the final nanoparticles. All the materials were characterized by different techniques to evaluate the physical and chemical properties. Specific experiments have been performed to evaluate the cytotoxicity and the inflammatory response of the cells to the presence of nanoparticles with different silica shell thickness as well as to interrogate the possible cellular uptake of the CSNPs.

**Chapter 3** discusses the results of the synthesis and characterisation of the iron oxide core – silica shell nanoparticles. The morphology and structure studies of the magnetic nanoparticles used as core in the synthesis of the CSNPs are summarised. The optimisation process with the influence of different parameters on the silica shell thickness is presented. Furthermore, the control of the final CSNPs agglomeration degree by the modification of post synthesis process is included. At last the magnetic evaluation of the core and CSNPs and also the *in-vitro* cytotoxicity and uptake studies results are reported.

In **chapter 4** the work presented in this thesis is summarised while the planned continuation studies are described in **Further work**.
1.3 Multifunctional nanoparticles

The new generation of nanoparticles in nanomedicine are nanoparticles that have combined components with different functionalities in one single entity and thus can achieve different tasks simultaneously (active targeting a certain type of cells or compartment, imaging them and delivering an active compound – drug); a schematic representation is given in fig. 1.1.

![Multifunctional nanoparticles with various ligands performing different functions](image)

**Figure 1.1.** Multifunctional nanoparticles with various ligands performing different functions

A large quantity of therapeutics can be incorporated or attached on the surface of the multifunctional nanoparticles while other components can be used for targeting and/or imaging.

The multifunctional nanoparticles can be classified under three major categories:

**Liposomes and micelles**

The development of liposomes as drug delivery vesicles (fig 1.2. a) is one of the earliest forms of nanomedicine. With sizes varying from 100 nm to few micrometers they can deliver inside the phospholipid bilayered membrane vesicles from small molecules to proteins, peptides, DNA, magnetic nanoparticles etc. By PEGylation the circulation time is increased and by combining them with a therapeutic agent the multifunctionality of the vesicles is increased⁹.

Micelles (unilamellar lipid vesicles of 20 to 100 nm) present similar features (long circulating behaviour, high biocompatibility and possibility of increasing the functionality number) as liposomes being one of the favourite ways of delivering hydrophobic drugs. Specifically, the hydrophobic parts form the core to minimize their exposure to aqueous
surroundings, whereas the hydrophilic blocks form the corona-like shell to stabilize the core through direct contact with water (fig 1.2. b). The hydrophobic core is capable of carrying pharmaceuticals (poorly water soluble drugs), with high loading capacity (5–25% weight). Its hydrophilic shell provides not only a steric protection for the micelle (with increased stability in blood) but also functional groups suitable for further micelle modification\textsuperscript{10}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{liposome_micelles}
\caption{Schematic representation of (a) liposome and (b) micelles structure}
\end{figure}

**Polymeric carriers**

The *polymeric nanoparticles* are one of the most favourite systems in designing multifunctional nanoparticles. The size can vary from 20 nm to 200 nm with a large engineering flexibility and sustained release characteristics (fig 1.3. a). Using polymers that are biodegradable (dextran\textsuperscript{11,12}, starch\textsuperscript{13}, poly(ethylene glycol) (PEG)\textsuperscript{14,15}, poly(lactic-co-glycolic acid) (PLGA)\textsuperscript{16,17} etc.) the biocompatibility is one leading feature and the drug release profile is highly adjustable.

*Dendrimers* are a relatively novel class of polymers with highly ordered structure. The structure of these polymers is repeated branching around the central core that results in a nearly-perfect three dimensional geometrical pattern (fig 1.3.b). The dendritic branching results in a semiglobular to globular architecture with a high density of cavities interbranches that present a high density of functional groups on the surface. The functionalising possibility, symmetry perfection, usually diameter in the range of 10 to 100 nm, and internal cavities provide many potential applications in biochemistry, gene therapy, and nanomedicine. Nanoparticles coated with dendrimers can alter the charge, functionality, and reactivity and enhance their stability and dispersibility\textsuperscript{18,19}. 
Hydrogels, three dimensional, hydrophilic polymeric networks capable of imbibing large volume of water or biological fluids are used as controlled drug release systems, microfluidic devices, biosensors, tissue implants, and contact lenses. Responsive hydrogels are a group of hydrogels sensitive to specific changes in their environment: pH, ionic strength and temperature. Unique properties can be achieved by incorporation of various nano materials such as metal/metal oxides nanoparticles or biological molecules into the hydrogel matrix. These nanocomposites of responsive hydrogels can exhibit multiple properties with capability of distance triggering to be exploited in drug delivery systems combined with MRI contrast agents and hyperthermia treatment20, 21.

Figure 1.3. (a) Polymeric nanoparticles, (b) dendrimeric nanoparticles (the light grey dots represent hydrophilic drugs and the dark grey dots the hydrophobic drug)

Core – shell structures
The core – shell architecture is another attractive alternative approach for developing multifunctional nanoparticles for MRI and drug delivery as well as for hyperthermia. The visualisation of the nanoparticles is done via the functionality of the core while the shell, apart from the function of protecting the core is also the scaffold for adding further functions: drug delivery (drug incorporation and controlled released either via diffusion or active delivery via pH, temperature modulation), active targeting by surface modification with specific ligands or even a tool for an alternative way of visualisation by incorporating a different imaging agent.

A large variety of combinations of materials that are assembled in core-shell architecture can be found in the literature. As core, quantum dots23-25, fluorophores26-28 or magnetic nanoparticles (iron oxides: maghemite29, 30 and magnetite31-33, ferrites34, 35, FePt29, 36, 37, FeCo38, 39) are the most researched moieties. The most commonly used coating layers are
either organic layers (poly(methyl methacrylate) (PMMA)\textsuperscript{30}, polylactic acid (PLA)\textsuperscript{40, 41} poly(lactic-co-glycolic acid) (PLGA)\textsuperscript{16, 42}, polyvinyl alcohol (PVA)\textsuperscript{25, 43}) or inorganic materials as silica (amorphous\textsuperscript{32, 44, 45}, mesoporous\textsuperscript{46, 47}), apatite\textsuperscript{48}, metallic layer as gold\textsuperscript{33, 49, 50} and silver\textsuperscript{51, 52}.

1.4 Iron oxide core – silica shell multifunctional nanoparticles

The CSNPs are excellent candidates for increasing the intrinsic functionalities and building up progressively more complicated structures.

From the different CSNPs presented in the section 1.3 we have chosen silica shell-iron oxide core structure as the model system to study due to the high biocompatibility of both components and great potential for building up multifunctional nanoparticles with complex structure.

The magnetic core can be used for specific targeting by an external field. In addition, it allows localization through MRI and visualization of the effects of the active substance incorporated in/adsorbed on the surface of the shell. Additionally, the magnetic core can potentially be used as an alternative treatment modality via hyperthermia therapy.

Silica offers an inert coating material on the magnetic core that inhibits their aggregation in liquid media, enhances their chemical stability and offers a versatile platform for surface modification. Furthermore, dye incorporation or quantum dots inclusion into the shell adds an extra function of detection and localisation under different radiation wavelength.

In the following sections the synthesis techniques of magnetic iron oxide nanoparticles and their magnetic properties and the silica coating methods are briefly described.

1.4.1 Iron oxide nanoparticles: synthesis and magnetic properties.

Iron oxide synthesis methods

Two major magnetic iron oxide phases are used in medical applications: magnetite (Fe\textsubscript{3}O\textsubscript{4}) and maghemite (\(\gamma\)-Fe\textsubscript{2}O\textsubscript{3}). The preparations methods are critical in determining their properties: particle size and size distribution, shape, surface properties, magnetic properties and they are consequently influencing their applications\textsuperscript{53}.

Numerous methods can be applied to synthesise magnetic iron oxide nanoparticles. The most commonly used methods are: coprecipitation, sol-gel synthesis, microemulsions, hydrothermal reactions, polyol method, thermal decomposition of precursors apart from less
common techniques as spray and laser pyrolysis\textsuperscript{54-56}, electrochemical methods\textsuperscript{57, 58} and flow injection synthesis\textsuperscript{59}.

The coprecipitation method is probably the simplest one allowing the preparation of large quantities of nanoparticles in one batch. The iron oxides are prepared by mixing ferrous and ferric salts in aqueous media (non oxidizing environment) at high pH\textsuperscript{60, 61}. The precipitated iron oxides’ properties (size, shape, composition) depend on the type of salts used as precursors (nitrates, sulfates, chlorides), molar ratio of the starting precursors, reaction temperature, pH and ionic strength of the media\textsuperscript{62}. Significant control on the size of the resulting particles can be achieved if during the formation of iron oxide nanoparticles chelating organic anions (carboxylate ions: citric, gluconic, oleic acid), or polymers as: PVA, dextran, starch are added\textsuperscript{63}.

Another method that is used for the synthesis of iron oxide nanoparticles is sol-gel technique. Sol-gel methods generally refer to the hydrolysis and condensation of metal alkoxides precursors that result in a colloid/“sol” of oxide nanoparticles. The “sol”, by further condensation and polymerisation leads to a three dimensional network called “gel” containing the liquid phase\textsuperscript{64}. In order that the nanoparticles acquire the final crystalline state, the gel has to be subjected to elevated temperatures. The properties of the gel are influenced by the solvent used, temperature, type of precursors utilised, pH and hydrodynamic conditions\textsuperscript{53}. This method is also suitable for production of large quantities of iron oxide nanoparticles.

Water-in-oil microemulsions, called also inverse microemulsions, are alternatively used to obtain iron oxide nanoparticles with narrow size distribution and controlled morphology. The nanosized droplets of water dispersed in the oil phase and stabilised by surfactant molecules (at the oil/water interface) are confined environments limiting the growth in the formation process of the nanoparticles\textsuperscript{65}. Varying the size of the aqueous pool phase (or the ratio between the organic and water phase) is the main factor regulating the water droplet size, hence the iron oxide phase size, even if other factors as reaction temperature, concentration of the reactants, flexibility of the surfactant film may also influence\textsuperscript{66-68}.

Another route used for synthesising iron oxide nanoparticles is the hydrothermal method. In this method known as a simple, versatile and environmentally friendly route (no organic solvent involved, no post reaction treatment as calcination necessary)\textsuperscript{69}, the reactions are carried out in aqueous media at high pressures (>2000 psi) and elevated temperatures (200-300°C). The reactions conditions: solvent content (higher water content conducted to larger nanoparticles), temperature and time (the longer the reaction time the bigger size iron oxide
nanoparticles)\(^{70}\) have an important influence on the resulting nanoparticles all the mentioned parameters influencing the nucleation and growth processes.

The polyol method is a facile method of synthesising nanoparticles with well defined size and shape, narrow size distribution compared with the aqueous route\(^{71}\). The solvents (ex. ethylene glycol, diethylene glycol, polyethylene glycol) dissolve the inorganic precursors, but due to their high boiling point they also allow a wide range of temperatures for performing the reaction. Moreover they act as reducing agents but also as stabilising agents preventing the aggregation of newly formed particles\(^{72}\). The obtained nanoparticles are easily dispersible in polar solvents being coated with a hydrophilic ligand.

A high degree of monodispersity and a greater control on the size of the nanoparticles can be achieved using synthesis methods as high temperature decomposition of organic iron precursors. Iron oxide particles with well control on the size ranging from 4 to 20 nm\(^{73-75}\) are resulting from the thermal decomposition of Fe(Cup)\(_3\), Fe(CO)\(_5\), Fe(acac)\(_3\) in the presence of surfactants. Using iron chloride salts or other iron oxides as precursors is a much more environmental friendly alternative and is becoming an increasingly used method for obtaining easily tuneable size iron oxide nanoparticles\(^{76-79}\). The nanoparticles obtained are dispersible in organic solvent, various treatments being necessary to make these particles dispersible in water.

**Magnetic properties**

The magnetic nanoparticles and the structures, in which the magnetic nanoparticles are constituents, have to undergo complex magnetic characterisations in order to assess their magnetic properties and the potential applicability in medical areas where these features are essential (i.e. MRI).

When the magnetic properties of a material are assessed, the value of magnetic moment for the sample is a sum of the magnetic moments of the atoms from the sample. This value is influenced by several parameters, some sample dependants: the size of the sample and the magnitude of the magnetic ordering, other measurement conditions as temperature and applied magnetic field.

\(M\), is the magnetisation of a sample and is defined as the magnetic moment \(m\) per unit volume \(V\):

\[
M = \frac{m}{V} \tag{1.1}
\]

Two notions are used to quantify the response of a material to magnetic field.
$\chi$ is the susceptibility or the variation of the magnetisation $M$ in applied magnetic field $H$.

\[ \chi = \frac{M}{H} \quad (1.2) \]

The permeability $\mu$ is the magnetic induction variation $B$ with the applied magnetic field $H$.

\[ B = \mu_0 (M + H) \quad (1.3) \]
\[ \mu = \frac{B}{H} \quad (1.4) \]

and $\mu_0$ is the permeability of vacuum:

\[ \mu_0 = 4\pi \times 10^{-7} \text{ } H / m \quad (1.5) \]

The iron atom, due to its four unpaired electrons in 3d orbitals, has a high magnetic moment. When iron atoms form crystals, different magnetic behaviors can occur (figure 1.4).

![Diagram of magnetic behaviors](image)

Figure 1.4. Alignment of individual atomic magnetic moments in different types of materials.\textsuperscript{64}

In the materials with a paramagnetic behaviour, the magnetic moments of the atoms are randomly aligned, the crystal having a zero net magnetic moment. If this crystal is under an external magnetic field, the crystal will have a small net magnetic moment due to the alignments of some of the moments.

A ferromagnetic crystal has all the moments aligned even in the absence of a magnetic field. In a ferrimagnetic crystal the net magnetisation is the sum of individual atomic magnetic moments that are arranged antiparallel and that have different strength. If the antiparallel moments are equal in magnitude, the crystal is antiferromagnetic and the net magnetic moment is zero.
The ordered arrangement of the magnetic moments decreases with the increase in the temperature (due to thermal fluctuations of the magnetic moments of the atoms). For ferro-, ferri- and antiferromagnetic materials is defined a critical temperature below which a spontaneous magnetisation is present: Currie temperature \((T_c)\) for ferro- and ferrimagnetic materials and Néel temperature \((T_N)\) for antiferromagnetic materials.

Large particles and bulk materials have a multidomain structure, with regions of uniform magnetisation being separated by domain walls (fig 1.5).

![Figure 1.5. The multidomain structure of a bulk magnetic material.](image)

When the particle size of the material decreases the number of the domains decreases until it reaches one single domain (below a critical size \(D_C\), material dependent).

\[
D_C \approx 18 \sqrt{\frac{A K_{\text{eff}}}{\mu_0 M^2}}
\]  

(1.6)

where \(A\), exchange constant, \(K_{\text{eff}}\) the anisotropy constant, \(\mu_0\) vacuum permeability and \(M\) is the saturation magnetisation\(^{63}\).

A single domain particle uniformly magnetised has all the spins aligned in the same direction that results in very high coercivity\(^{63}\).

When a ferromagnetic material of magnetisation \(M\) is subjected to a magnetic field \(H\), a magnetisation curve with a hysteresis is obtained (Figure 1.6(a)). The graph shows the fact that \(M\) increases under an increasing \(H\) till it reaches a maximum value called saturation magnetisation \(M_s\). When \(H\) decreases, the domains do not return to their original orientation, the material having a residual magnetisation called remnant magnetisation \(M_R\) which can be annulated by applying a magnetic field in the opposite direction of the initial field, \(H_C\).
Another important parameter that characterises the monodomain particles is the anisotropy energy. It represents the preference of the magnetisation to orient along preferential directions in the nanoparticles (relative to the crystallographic directions). The axes along which the magnetic energy is minim are called easy axes or anisotropy directions (fig 1.7).

The anisotropy energy is then a function of the tilt angle:

$$ E = K_{\text{eff}} V \sin^2 \theta $$

(1.7)

where $K_{\text{eff}}$ is the effective uniaxial magnetocrystalline anisotropy constant, $V$ is the volume of the particles and $\theta$ is the angle between the magnetisation direction and the easy axis. The anisotropy energy is dependent on several factors as: shape of the crystal, bulk magnetocrystalline anisotropy and the agglomeration degree that give rise to dipole interaction between neighbouring particles.

Figure 1.7. Variation of the magnetic energy with the angle relative to the easy axis$^{53}$
For single nanodomain particles the anisotropy energy is comparable with the thermal energy

\[ E_T = k_B T \]  \hspace{1cm} (1.8)

where \( k_B \) is Boltzmann constant and \( T \) is the absolute temperature.

When \( k_B T \ll K_{\text{eff}} V \), the magnetic moment is fixed along the easy axis of the magnetisation, thermal energy being too small to overcome the anisotropy barrier. The nanoparticles are called then thermally blocked particles with the magnetic moment “blocked” along a specific easy axis (crystallographic direction).

When \( k_B T \gg K_{\text{eff}} V \) the system behaves paramagnetic with the magnetic moments randomly arranged, the thermal energy being high enough to deviate the magnetic moment from the easy axis. The atoms are ferromagnetically coupled resulting a “super” moment inside of each particle, phenomenon called superparamagnetism\(^8\).

In a magnetic field the superparamagnetic nanoparticles have no remanent magnetisation, no coercivity while maintaining a very high saturation magnetisation\(^5\) (figure 1.6(b)).

Characteristic of superparamagnetic nanoparticles is Néel relaxation time, \( \tau_N \), the time necessary for magnetisation to return to equilibrium after perturbation:

\[ \tau_N = \tau_0 e^{K_F / k_B T} \]  \hspace{1cm} (1.9)

where \( \tau_0 \) is the characteristic relaxation time (anisotropy energy dependent), \( k_B \) Boltzmann constant, \( T \) the absolute temperature.

The equation 1.9 shows that the Néel relaxation time is dependent on the nanoparticle volume and the temperature.

As a conclusion, the magnetic properties of single domain iron oxide nanoparticles are the saturation magnetisation, the Néel relaxation time, both being functions of nanoparticles’ size (inclusive aggregation degree) and the anisotropy constant.

### 1.4.2 Silica coating methods

Silica is one of the most researched materials for coating magnetic and metallic nanoparticles. It increases stability of the nanoparticles in suspensions (due to the coulomb repulsion – silica is negatively charged at physiological pH - and also isolating the magnetic dipoles through the silica shell) but also improves the chemical stability and biocompatibility of the core particles.

Two main approaches are followed for the formation of CSNPs with silica as the shell; both of them are originally used for producing silica nanoparticles. The first is the well known
Stöber method\textsuperscript{81} in which silica is formed on the surface of the iron oxide cores by hydrolysis and condensation of a precursor as tetraethyl orthosilicate (TEOS). In basic media TEOS undergoes hydrolysis and polycondensation reactions that results in formation of amorphous silica. The method is very versatile, the adjustment of the silica shell being possible by varying several parameters: the solvent type (higher molecular weights alcohols form larger core- shell nanoparticles), the concentration of iron oxide nanoparticles (lower concentrations of iron oxide conduct to bigger composites nanoparticles), the concentration of the silica precursor, the concentration of ammonia catalyst and the water\textsuperscript{45, 82-84}.

The synthesis of uniform silica shells, with single core and controlled thickness is still a challenge, although the method is relatively simple and a lot of research was and is still conducted on core-shell nanoparticles with silica shell,. The microemulsion method is an alternative to Stöber technique\textsuperscript{85, 86}. Water-in-oil microemulsion (w/o) or inverse microemulsion was extensively studied and used for the production of silica nanoparticles with a narrow size distribution\textsuperscript{47, 87-91}. This route is becoming favoured for the synthesis of CSNPs, the inverse micelles being used as a confined and controlling factor for the growth of the silica shell\textsuperscript{92-96}.

Similar factors as for Stöber methods are used to control the thickness of the silica shell (iron oxide nanoparticles concentration, silica precursor concentration, ammonia concentration). To them are added the factors that are used in controlling the size of the droplets of the water phase as: ratio of oil phase to water phase, the surfactant to co-surfactant ratio and the type of co-surfactant\textsuperscript{89}. Still, the particles produced are strongly necked and aggregated due to the condensation reactions that continue during the post synthesis processing, even though the method has the advantage of greatly controlling the thickness of the silica shell. The aggregation and necking result in an increase of the overall size of the particles, decrease in the surface area and reduced stability of the particles in colloids.

In order to synthesis silica nanoparticles with larger size a seed particles growth using Stöber method is used\textsuperscript{97-100}. This method can also be used for synthesising CSNPs with large average size, small CSNPs being used as seeds in order to grow thicker silica shells. The concentration of the seeds and the amount of the silica precursor are the main features that are controlling the growth of a monodispersed population of CSNPs with no new (empty) silica formed\textsuperscript{97, 101}.

This thesis is specifically addressing solving the aggregation issue of the particles obtained by inverse microemulsion method and, in addition, synthesising a large variety of sizes from $\sim 22$ to $\sim 120$ nm using either the microemulsion system either the seeded particle
growth via Stöber method. Well-dispersed, perfectly separated (without the presence of interparticle bridging) and single core-shell nanoparticles with an excellent control of the shell thickness are obtained.

1.5 Cytotoxicity of nanoparticles

The biomedical area sees in the recent years an “invasion” of the nanoparticles that find applications in most of the fields, with medical imaging and drug delivery being the most representative of them. As the number of applications increases, it is extremely important to understand and quantify the potential toxicity effects of the new form of materials\textsuperscript{102}.

The \textit{in-vitro} toxicological studies provide a critical support for the possible applications of the nanoparticles in biological fields but also help in assessing the safety and potential effects that might appear \textit{in-vivo}\textsuperscript{103}. These studies should include, short and long term toxicity, uptake studies and possible mechanisms of uptake, translocation pathways and interactions with cells, receptors and signaling pathways involved\textsuperscript{104}.

The result of the synthesis and characterization of the nanoparticles is a comprehensive evaluation of their physical – chemical characteristics. Eventual variability in the raw materials suppliers or methodologies to manufacture nanoparticles should also be noted\textsuperscript{105}. All these characteristics might have an effect on the response of the exposed cells and tissues to the presence of the nanoparticles.

Dose in toxicology is defined as the quantity of the tested material that the biological system is exposed to. Expressing dose as gram of sample per unit of relevant medium that they are exposed or as number of particles it can result in very different results concerning toxicity\textsuperscript{106}. A size dependency of the toxic effect rather than concentration dependency is increasingly reported with the smaller size nanoparticles often showing an increased toxicity compared with the larger ones\textsuperscript{107}. This effect is related to the stability of the particles in biological media as the smaller particles can easily enter the cells while bigger ones (i.e. aggregated ones) find difficult to cross the cellular membrane\textsuperscript{108, 109}. Structure and time of exposure have an important impact also on the toxic response\textsuperscript{104}.

Other factors depending on the technique used in cytotoxicity evaluation may also influence the interpretation of the \textit{in-vitro} testing. Possible interactions between the tested nanoparticles and different chemicals involved in various steps of the procedure (e.g. the dye used for colorimetric measurements) should be considered\textsuperscript{102}. Making use of more than one assay, and adding another dimensionality to the tests (i.e. complimentary long term toxicity
assessment\textsuperscript{110} to the short term toxicity testing) in determining the nanoparticles toxicity could be a safe way of producing reliable data.

When assessing the biocompatibility of a material \textit{in vitro}, it should be kept in mind that in order to mimic the conditions from the tissues and organs were several cell types coexist and interact to each other and with the extracellular matrix, model systems based on primary cells are offering a higher level of complexity\textsuperscript{111} than immortalized cell lines. In addition, another important aspect that has to be addressed is the effect of nanoparticles on the immune system\textsuperscript{105, 112, 113}. Macrophages, being phagocytic cells, are the first line of defence against invading microorganism, particles and cellular debris and thus playing an essential role in the innate immunity\textsuperscript{114}. The macrophages function must be, in consequence, well assessed when utilising nanoparticles which intended applications are biorelated areas\textsuperscript{115}. In addition, the biodistribution of the nanoparticles in animal models and humans may be highly determined by their uptake by the reticulo-endothelial system (RES), macrophages being part of this system along with monocytes and other phagocytic cells\textsuperscript{116-118}. Furthermore, macrophages can be obtained as primary cells and thus being closer to the \textit{in vivo} situation. Thus, primary human macrophages are an excellent system for \textit{in vitro} testing of nanoparticles that are intended for biomedical applications.

\textit{In vitro} cytotoxicity tests are an essential part of assessing the toxicity of the cells but additional tests \textit{in-vivo} should be done in order to confirm/disconfirm the results of the \textit{in-vitro} testing. Moreover, it is important to assess administration route toxicity dependence, biodistribution of the nanoparticles, the specific accumulation sites, eventual inflammatory phenomenon that can appear on the accumulation sites, the uptake by the RES organs and also the clearance pathways of the nanoparticles from the organism.

### 1.6 Biomedical applications of multifunctional nanoparticles

The major aim of medicine is an early and accurate diagnosis of clinical conditions, and to provide an efficient treatment with minimum or no side effects. The development of nanotechnology brought the realisation of this goal closer than ever. In this respect, new materials and devices that can work in nanoscale emerged (i.e. nanoparticles) and they are new powerful tools for imaging, diagnosis and therapy\textsuperscript{6}.

The smallest capillaries in the human body are 4-6 \textmu m diameter. When considering possible medical applications (\textit{in-vivo} or \textit{in-vitro}), the nanoparticles have to possess some
basic properties, specifically: a size of less than 20 nm (especially for intravenous (i.v.) administration); high surface area with a larger platform for surface functionalisation; high colloidal stability (with minimum agglomeration and aggregation); and the ability to pass the biological barriers. It is believed that nanoparticles smaller than 50 nm can enter most of the cell types and those smaller than 20 nm can cross endothelial barriers of the blood vessels and enter the surrounding tissues. However, in particular for the in-vivo applications, the particles should adhere to more strict requirements: non-toxicity, non-immunogenicity, long-term retention within blood circulation, and the ability to reach and pass through the endothelial capillary membranes without embolism of the bigger vessels. Additionally, the demand for nanotools that can perform multiple tasks concurrently give rise to the concept of multifunctionality and in particular to multifunctional nanoparticles as simultaneous imaging agent for diagnosis, drug delivery carrier for therapy and post treatment follow up.

In the following section we detail the multifunctionality applicability in magnetic resonance imaging as our CSNPs are intended to be used as contrast agent for MRI.

1.6.1 Magnetic Resonance Imaging (MRI)

MRI, among other non-invasive imaging techniques such as computed tomography (CT), positron emission tomography (PET), single photon emission CT (SPECT), ultrasound (US) and optical imaging (OI), is one of the mostly used methods in research as well as in clinics due to its capability of imaging the soft tissue structures. Primarily used for producing anatomical images, MRI gives also information on the physiochemistry of the tissue, flow, diffusion and motion.

The fundamental principle of MRI is that unpaired nuclear spins (mainly from hydrogen atoms in water - 70% to 90% of most tissues - and organic compounds) align themselves when exposed to a magnetic field. A temporary radiofrequency pulse change the alignment of the spins, and their return to baseline (relaxation) is recorded as a modification in electromagnetic flux. Protons from different tissues react differently giving a picture of anatomical structures. Two independent processes, longitudinal (or spin-lattice) relaxation (T1-recovery) and transverse (or spin-spin) relaxation (T2-decay), can be monitored to generate an MR image.
MR imaging is considered to be highly sensitive (to show pathological changes in a tissue) but not so specific (i.e. different pathologies have similar appearances). The development of chemically synthesized contrast agents has helped MRI become the pre-eminent imaging technique in clinical diagnosis and a powerful tool for biomedical research. Contrast agents help to improve the specificity by inducing a change in the behaviour of the surrounding tissue (affecting the behaviour of protons in their proximity), better tissue characterisation and a reduction in image artefacts. They enhance the image contrast between normal and diseased tissue and indicate the status of organ function or blood flow after administration by increasing the relaxation rates of water protons in tissue in which the agent accumulates.

Apart from general requirements as tolerance, safety, toxicity, stability, osmolality, biodistribution, elimination and metabolism, contrast agents have to possess specific prerequisites as adequate relaxivity and susceptibility.

Currently available MRI contrast agents can be categorized into two broad groups: $T_1$-agents and $T_2$-agents. $T_1$-agents generally increase the longitudinal (or spin-lattice) relaxation rates ($1/T_1$) of water protons in tissue more than the transverse (or spin-spin) relaxation rates ($1/T_2$). Soluble paramagnetic agents as gadolinium and manganese chelates are having dominantly $T_1$-lowering effect with an increase in signal intensity on $T_1$ weighted images (positive contrast agents). The superparamagnetic iron oxide nanoparticles (SPION) are $T_2$-agents increasing the $1/T_2$ of tissue selectively leading to decreases in signal intensity on $T_2$ weighted images (negative contrast agents). The ability of the agent to reduce the $T_1$- and $T_2$-
relaxation times are respectively described by the $R_1(1/T_1)$ and $R_2(1/T_2)$ relaxivity values of the agent and is defined by:

$$R_{1,2} = R^o_{1,2} + r_{1,2}C$$

(1.12)

where $R_{1,2}$ (unit s$^{-1}$) is the respective $T_1$ or $T_2$ proton relaxation rate in the presence of the contrast agent, $R^o_{1,2}$ are the relaxation rates in the absence of contrast agent and $C$ is the contrast agent concentration (unit mM). The constant of proportionality $r_{1,2}$ (unit s$^{-1}$ mM$^{-1}$) is called relaxivity and is a measure of the increase in the relaxation rate of protons per unit concentration of contrast medium$^{128}$.

The ability of SPIONs to perform as MR imaging contrast agents is actively investigated in the last 20 years$^9$. Magnetic nanoparticles with controlled size, morphology and magnetic properties are used but in order to increase their stability and nontoxicity in physiological conditions, and to allow functionalization and targeting, these materials are surface modified or coated with organic or inorganic layers$^{129}$. Most clinical approved MRI contrast agents SPION based (Ferridex®, Combidex®, Resovist®, and AMI-228/Ferumoxytrol) are dextran or other carbohydrate coated or citrate-stabilized particles (VSOP-C184)$^{129, 130}$ but SPION with other coating materials (polymers, metals (gold, silver), silica) for MRI are either in the development phase or in different phases of the preclinical study$^{53}$. The coating layer is used as platform for further functionalisation via conjugation with multiple targeting entities (peptides, antibodies). The functionality of these particles further increases with the addition of other imaging moieties (as dyes or quantum dots) and/or therapeutic agents (multimodal imaging)$^{129}$.

When multifunctional nanoparticles used as MRI contrast agents are coupled with molecular probes that recognise certain tissue signatures, then the visualisation has an enhanced specificity to certain modification in the targeted tissue$^9$. Multifunctional nanoparticles find application in diagnosing some of the most prevalent diseases in humanity.

The multimodal specific imaging of cardiovascular diseases is one of the important impact applications. Multifunctional magnetic nanoparticles are proposed as MRI contrast agents for several clinical cardiovascular applications as myocardial injuries, and atherosclerosis. The increased uptake of SPION by the macrophages (hallmark cells in atherogenesis) and further functionalisation of SPION with adhesion molecule (V-CAM) – target for endothelial and macrophage cells – will improve the early diagnosis and decrease the morbidity and mortality associated with atherosclerosis$^9, 129$. 
Cancer imaging is another extremely important application of multifunctional magnetic nanoparticles. The early detection of the disease associated with early treatment increase the curability chances with dramatic social and economic impact. The current resolution limit of 2-3 mm (for liver tumours) and lymph metastases of 5-10 mm can be decreased significantly by active targeting (antigen – antibody reaction) of a specific marker expressed on the tumour cells or SPION labelling with a precursor lesions specific antigen\textsuperscript{129}. The drug loading of the targeted nanoparticles adds to the sensitive MRI agent particles the function of controlled drug carrier. Covalently bound methotrexate on the magnetic nanoparticles and pH induced release of the antitumoral agent into the specific tumoral cells allowed MRI localising the nanoparticles before and after the drug release\textsuperscript{131}.

If a cell can be sufficiently loaded with magnetic nanoparticles, cell tracking by MRI is another possible application with the resolution near to the size of the cell. Localising preloaded cells with functionalised magnetic nanoparticles after transplantation or transfusion, apoptosis detection or monitoring enzymatic activities are just few of the new highly specialised multimodal MRI applications in medicine\textsuperscript{9, 53}. 

2 Experimental

2.1 Magnetic core - silica shell nanoparticles

2.1.1 Synthesis of superparamagnetic iron oxide nanoparticles (SPION)
SPION were prepared by high temperature decomposition of the iron complex precursor (iron oleate) in a high boiling temperature solvent (1-octadecene) through a two-step method described in detail elsewhere. In a typical experimental procedure iron oleate was produced by reacting FeCl$_3$·6H$_2$O with sodium oleate in a solvent mixture composed of ethanol, distilled water and hexane in ratios. In order to obtain the SPION, the iron oleate was decomposed in 1–octadecene using oleic acid as capping agent. The resulting particles were separated by precipitation, centrifugation and resuspension, successively, in ethanol and hexane. Finally, the particles were resuspended in cyclohexane and kept at 4°C until further use.

The synthesis of SPION was also done by one-pot modified method which includes the dissolution of FeO(OH) in oleic acid, formation of iron oleate complex and thermal decomposition at elevated temperatures taking place successively by heating up the mixture under stirring and N$_2$ purging. A typical synthesis starts from 2 mmol of iron oxide hydroxide and 8 mmol of oleic acid heated up to 320°C and kept at that temperature for 45 minutes. The obtained oleic acid capped particles are separated by several cycles of precipitation with ethanol and redispersion in cyclohexane. The final dispersion media is cyclohexane, the suspension being kept at 4°C until further use.

2.1.2 SPION - silica CSNPs preparation
The SPION obtained by both methods were subsequently coated with silica shell by the inverse microemulsion method in a Triton X100/hexanol/water/cyclohexane system. The microemulsion system was formed by mechanically mixing the SPION suspension in cyclohexane with the water phase and the surfactant (Triton X100) and co-surfactant (hexanol). The addition of the silica precursor (TEOS) was done drop wise after the formation of the microemulsion and the hydrolysis and condensation reactions were allowed to take place under continuous stirring for a determined time. The synthesised core – shell particles were collected by destabilization of the microemulsion via (i) pH adjustment to approximately 2 and (ii) successive cycles of liquid N$_2$ cooling, centrifuging at high rpm and redispersing the
collected particles in ethanol and finally in water. The particles were suspended in water and kept at 4°C until further use.

A seeded particle growth based on Stöber method was used in order to obtain particles with large average diameter of ~120 nm. In a typical experiment H2O and ethanol (v:v ratio of 2:3) were mixed with a certain volume of core-shell nanoparticles aqueous suspension (~30 nm). NH4OH was used to adjust the pH of the reaction to 8-9. As the condensation time was maintained fixed (2h), the final diameter of the core-shell nanoparticles is adjusted by varying the quantity of TEOS added. The particles were washed by several cycles of ultracentrifuging at high rpm and low temperature (2 °C) and redispersion in water.

2.2 Characterization

The fabricated materials (cores and CSNPs) were analyzed at various stages of the synthetic process in order to understand and control the mechanism of formation of the silica shell and the overall size of the nanoparticles. Several techniques including atomic absorption spectroscopy and inductively coupled plasma, transmission electron microscopy, scanning electron microscopy, Fourier transform infrared spectroscopy, X-ray powder diffraction and vibrating sample magnetometer have been used for their analysis and each of these techniques are described in detail below.

Atomic absorption flame spectroscopy (AAS). The amount of iron oxide nanoparticles in cyclohexane suspension and of iron oxide core – silica shell composites in the final water suspension was measured by AAS.

The sample preparation for this technique consist of digesting a certain volume of particles with concentrated hydrochloric acid (HCl) after removing the organic solvent and the capping agent by heating the suspension to 600°C for 2h. The resulting solution was diluted to a certain volume and the iron concentration was measured with Varian SpectrAA-220 and triplicate measurements were performed for concentration determination of the analyte. For measuring the iron oxide core nanoparticles concentration from the core – shell samples, a definite volume of suspension is treated first with HF 5% to dissolve the silica shell and then with concentrated HCl to digest the iron oxide cores. The following processing and measurements of the concentration is identical with the iron concentration in core suspension samples as described above.
Transmission electron microscope (TEM). The morphology, size and size distribution study of the iron oxide cores and iron oxide core – silica shell nanoparticles were investigated with transmission electron microscope (TEM).

The analyses were performed with a JEOL 2100 high resolution FEG-TEM unit using an acceleration voltage of 200 kV.

In any type of microscopy, the image is formed as a result of the interaction of the incident particles with the samples. In electron microscopy, when the particles (electrons) hit the sample, different interactions can occur. These interactions and the generated signals are the basis of the various imaging and spectroscopy methods that can be utilised in electron microscopy. From the incident electron beam only a small part is passing through the sample with the energy unchanged. Most of the incident electrons will have their initial energy reduced as a result of inelastic interactions (X-rays, Auger electrons, secondary electrons, plasmons, phonons, UV quanta or cathodoluminescence) (figure 2.1.). A small part of the electrons will have their energy preserved but their trajectory will be modified as a result of elastic interaction with the sample.

**Figure 2.1.** Scheme of electron-matter interactions arising from the impact of an electron beam onto a specimen.

In TEM the transmitted electrons through the thin sample are used to form a map of local densities and diffraction information (ED) when crystalline samples are analysed.

Samples for TEM imaging were prepared by dipping a carbon coated 200 mesh copper TEM grid in sonicated particle suspension and allowing drying at room temperature over
night. Gatan DigitalMicrograph 3.11.2 for GMS 1.6.2 with Orius SC1000 2 camera was used as image processing software. The particle size distribution for each sample was determined by measuring the maximum dimension of at least 300 particles in random fields of view from several TEM micrographs.

High resolution TEM (HRTEM) and electron diffraction analysis (ED) was performed to study the crystal structure and lattice orientation of the iron oxide core nanocrystals.

**Scanning electron microscopy (SEM).** SEM was performed in order to confirm the morphology and the size distribution of the core shell nanoparticles.

The possibilities of analysing just very thin specimens on a very small area are important limitations of the TEM. These constraints were the incentives for developing electron microscopes that can analyse bulk (thick) samples on a much larger area - SEM. In SEM, as in TEM, the dual character of electrons (charged particles and wave) is exploited to image the sample. The incoming (primary) electrons can transfer energy to the atomic electrons that are present in the sample, which can then be released as secondary electrons. Another possibility for the primary electrons is that they are backscattered (“reflected”) at the surface of the specimen.

The beam of primary electrons is scanned across the specimen simultaneously in two perpendicular directions covering a square/rectangular area of the sample (raster). Collecting the secondary electrons from each point of the sample an image can be formed.

The analysis was performed with a Zeiss Ultra 55 scanning electron microscope (SEM). The samples for SEM were prepared by dropping the particles suspension on a carbon tape and let it dry in ambient conditions overnight.

**Fourier Transform Infrared Spectroscopy (FTIR).** The study of the surface of the particles is performed using FTIR analysis.

The investigation was performed using Nicolet Avatar IR 360 E.S.P. spectrophotometer with an Attenuated Total Reflectance (ATR) crystal and scans were performed in the range of 400 to 4000 cm\(^{-1}\).

FTIR is based on the fact that most molecules absorb light in the infra-red region of the electromagnetic spectrum (0.8 µm to 1 mm wavelength), this absorption corresponding specifically to the bonds existing in the molecule. The sample is irradiated with a broad spectrum of IR light and the absorbance at particular frequencies is plotted after the data is treated with a Fourier transformation. The resulting spectrum is a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms in the molecule. As each material is a unique combination of atoms, the infrared
spectrum is compound or bond specific. Therefore, infrared spectroscopy is used for qualitative analysis but also can be utilised for quantitative investigations as the intensity of the peaks in the spectrum is a direct indication of the amount of sample present.

The analysis of the samples is done either in solid (dry) or colloidal form. Due to the ATR crystal in the structure of the FTIR no sample preparation is required (apart from evaporating the solvent to obtain the dry powder when the study is done in dry form).

**X-ray powder diffraction (XRD).** The X-ray powder diffraction (XRD) patterns of the core nanoparticles as well of the CSNPs with different shell thickness were recorded and used for identification of the crystalline phases and to calculate the crystallites sizes.

When radiation hits a sample, it is either scattered (in an elastic manner or inelastic) or absorbed. When the scattering is entirely elastic, the energy of the primary radiation remains unchanged, no energy being lost. The regular arrangement of the atoms in a crystalline material interacts elastically with the radiation that has the wavelength comparable with the interatomic distance.

The principle of XRD powder diffraction is based on the elastic interaction of the X-ray radiation with samples. X-rays are easily produced with wavelengths matching the unit cell dimensions of crystals ($10^{-10}$ m). The result is a diffraction spectrum in which the intensity of the scattered radiation is plotted as a function of the scattering angle. The diffraction angles and the diffraction intensities are a function of the crystal structure.

The diffraction pattern obtained by XRD powder diffraction can help in identifying the phase composition of a sample, in calculating the unit cell lattice parameters and the crystallites sizes but can also give information about residual strains (macro or micro-strains).

The spectra were recorded on a Philips X’pert pro super Diffractometer with Cu Kα radiation ($\lambda=1.5418$ Å). The samples (core and CSNPs) for recording the pattern were prepared by allowing them to form, by solvent evaporation, a thick layer on a clean glass slide.

**Vibrating sample magnetometer (VSM).** The magnetizations were measured using VSM.

VSM uses an induction principle, and is widely utilised for characterizing the magnetic properties of materials. If a sample of any material vibrates with sinusoidal motions in a uniform magnetic field a sinusoidal electrical signal (a current or voltage) will be induced in a sensing coil. The electrical signal has the same frequency as the vibration frequency of the sample and its amplitude is proportional to the magnetic moment.
In a VSM the sample is mounted on the end of a rigid rod attached to a mechanical resonator which oscillates the sample (usually in a vertical direction) at a fixed frequency. Surrounding the sample is a set of sensing coils. As the sample moves, its magnetization, $M$, alters the magnetic flux through the coils. This produces an AC voltage directly proportional to $M$, which can be detected and amplified by an amplifier. The external magnetizing field is usually provided by a horizontal electromagnet. VSM measures permanent moments and hysteresis curves of materials. The fitting of the profiles gives several parameters as the crystal radius ($r$) and the specific magnetization ($M_S$).

The measurements were performed on a VSM-NUOVO MOLSPIN/ Newcastle Upon Tyne, UK using a known volume and concentration of colloidal particle suspension. The magnetic field was varied in the range of -1T to 1T.

### 2.3 Cytotoxicity assessment

The cytotoxicity studies presented in this section were generated in the Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet.

Three different Fe$_3$O$_4$-SiO$_2$ CSNPs with average diameters of ~30 nm, ~50 nm and ~120 nm were studied.

The cell model chosen is human monocyte-derived macrophages (HMDM). The cells were differentiated from peripheral blood mononuclear cells (PBMC) obtained from healthy donors (Karolinska university Hospital, Sweden) by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). The PBMC were collected and washed three times with phosphate-buffered saline (PBS) before $10^7$ cells were resuspended in 80 µl MACS buffer (0.5% BSA, 2 mM EDTA in PBS). PBMC were incubated with anti CD14 beads for monocyte isolation according to manufacturer’s instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14 positive cells were resuspended in RPMI (Roswell Park Memorial Institute medium) containing 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin M-CSF. Macrophages were generated by adding 50 ng/ml of the recombinant cytokine M-CSF and the cells were cultured for three days. The macrophage phenotype was confirmed by analyzing the F4/80 expression using flow cytometry (Becton Dickinson).

Cell viability of HMDM was assessed based on the mitochondrial conversion of 3-(4, 5-dimethylidiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide (MTT)\textsuperscript{133}. Approximately 100 000 PBMC were seeded into a 96-well plate and differentiated as described above. Cells were
exposed to particles in doses ranging from 0.5 µg to 100 µg/ml for 24 h and 48 h in RPMI containing 10% FBS. After exposure, the supernatant was removed and cells were washed once with PBS (pH = 7.4). 100 µL of MTT solution (0.5 mg/ml) was added and incubated with cells for 3 h at 37 °C. Finally, 50 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. MTT conversion was quantified by measuring the absorbance at 570 nm using a spectrophotometer (Infinite F200, Tecan, Männedorf, Switzerland). The percentage of cell viability was calculated relative to untreated cells.

For the detection of the release of the cytokines the supernatants of exposed cells (with doses of 0.5 – 100 µg/ml) were collected at 24 h and 48 h and kept at -80 ºC until analyzed. IL-6 and TNF-α was determined by ELISA kits (Mabtech, Nacka, Sweden) according to manufacturer’s instruction. The absorbance was measured at 405 nm using a spectrophotometer ((Infinite F200, Tecan, Männedorf, Switzerland).

### 2.4 Uptake studies

The cells incubation with the nanoparticles was done in the Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet and the TEM sample preparation and section analysis were recorded at Materials-Biology Interactions Laboratory, Swiss Federal Laboratories for Materials Testing and Research (EMPA).

The same set of three different Fe₃O₄-SiO₂ CSNPs with average diameters of ~30 nm, ~50 nm and ~120 nm were used to study the cellular uptake.

For TEM examination, the cells were exposed to nanoparticles in concentration of 50 µg/mL. After 2 h or 24 h, cells were washed three times with PBS, pelleted and sucked up in a cellulose capillary tube. Fixation of the cells was performed with a 0.2 M sodium cacodylate buffer at pH 7.4 containing 3% glutaraldehyde. The cells are following a post-fixation process in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.4 at 4 °C for 30 min and dehydration by rinsing with a graded ethanol series followed by acetone. The embedding in Epon resin, cutting ultrathin section (~50 nm) and staining with 2% uranyl acetate and lead citrate are the last steps of the procedure before analysing in TEM the cells sections that potentially have uptake of the nanoparticles. The analysis was done in a Zeiss 900 TEM at the accelerating voltage of 80 kV.
3 Results and discussion

3.1 Morphology and structure studies of magnetic nanoparticles

The SPION nanoparticles with average size of 9.5 (±1.2) nm were obtained by the procedures described in 2.1.1 section. The nanoparticles, possessing a spherical shape, have a narrow size distribution and they are highly crystalline as observed from HRTEM image (fig. 3.1.). From the HRTEM images the $d$ spacing are corresponding to the planes $\{220\}$, $\{331\}$ and $\{422\}$ of magnetite (or maghemite) crystals. The two phases have a similar crystalline structure (ccp lattice of oxygen atoms with $1/3$ Fe$^{II}$ ions in octahedral sites and $2/3$ Fe$^{III}$ ions distributed between octahedral and tetrahedral sites for magnetite and just Fe$^{III}$ ions randomly distributed between octahedral and tetrahedral sites for maghemite) and physical properties. That is the reason why, according to ICDD standards, the peaks displayed on the diffractogram are characteristic to magnetite phase (Fe$_3$O$_4$) (ICDD#: 00-002-1035) and maghemite ($\gamma$-Fe$_2$O$_3$) (ICDD#: 00-013-0458). From XRD measurements, the average crystallite size was also calculated using Scherrer’s equation and a value of 10.5 nm was obtained.

Figure 3.1. HRTEM image of the core iron oxide nanoparticles synthesised by thermal decomposition. The inset represents FFT performed on a selected area of SPION
3.2 Core-shell nanoparticles: optimization of the synthetic route

The SPION were used as core for the fabrication of CSNPs in an inverse (w/o) microemulsion Triton-X100/hexanol/water/cyclohexane system. The continuous phase (oil phase) consisted of cyclohexane with Triton-X100/hexanol as the surfactant/co-surfactant couple. The hydrophobic oleic acid capping on the surface of SPION make them easily dispersible in the oil phase (cyclohexane).

The hydrolysis and condensation reactions of the silica precursors (TEOS) take place in the confined space of the water droplets dispersed in the oil (cyclohexane) phase.

First the hydrolysis proceeds in basic media following a nucleophilic substitution, in which the hydroxyl anion attacks the silicon.

\[
\text{Si(OR)}_4 + x\text{H}_2\text{O} \leftrightarrow \text{Si(OR)}_{4-x}\text{(OH)}_x + x\text{ROH} \quad (3.1)
\]

where R=C\text{\textsubscript{2}}H\text{\textsubscript{5}}

\[
\Xi -\text{Si-OH}^- + \text{OH-Si} \leftrightarrow \Xi -\text{Si-O-Si} + \text{H}_2\text{O} \quad (3.3)
\]

\[
\Xi -\text{Si-OH}^- + \text{RO-Si} \leftrightarrow \Xi -\text{Si-O-Si} + \text{ROH} \quad (3.4)
\]

Interaction between the hydrolyzed species results in formation of silicon-oxygen-silicon bonds (condensation reaction). This takes place by the attack of the deprotonated silanol on a protonated silanol or ethoxysilane group. The byproducts of the reactions are either water or ethanol\textsuperscript{135}.

The mechanism of silica coating on SPION is presented in detail in Paper 2. Shortly, the ligand exchange of the capping oleic acid species with hydrolysed TEOS is followed by subsequent phase transfer of SPION from the oil phase to the water phase. In the water droplets the reaction continues resulting in the formation of coated SPION with various thickness of silica.
The formation of single core coated with silica layer with no silica nanoparticles or multicores beads produced is ensured by a careful adjustment of the amount of magnetic particles in microemulsion.

In order to obtain a stability region of the microemulsion so that we attain a high experimental reproducibility and in consequence good results reliability, a large number of experiments have been performed. The concentration survey generated a series of values for stable microemulsions with the molar ratio of components: H₂O/Triton-X100: 6.3; Triton-X100/hexanol: 0.5; and TEOS/ H₂O: 0.008.

The representative TEM image of CSNps shows the homogeneous coating of the magnetic core (fig. 3.2.a). The XRD analysis of the cores and the iron oxide core - silica shell nanoparticles (fig. 3.2. b) confirm the high crystallinity of the magnetic core. For the CSNPs the XRD graph displays an amorphous peak from the silica shell, the rest of the peaks being identical with the bare SPION, only with reduced intensity.

For optimising and controlling the synthetic process of the formation of the silica layer we studied the influence of various experimental parameters: the effect of the stirring rate, the reaction time and the post synthesis washing steps.

The hydrodynamic conditions were studied by varying the stirring rate from 400 rpm to 1200 rpm. Regardless the stirring rate, the formation of single core SPION-silica shell nanoparticles took place with no empty silica particles observed (fig. 3.3). Experimental observations showed that the stirring rate plays an important role in keeping the microemulsion system stable. At low stirring rate (400 rpm up to 600 rpm) a high percentage
of the core-shell particles formed in the system attached to the glass walls of the reactor. At higher stirring rates this fact was not observed probably because the stirring speed was high enough to overcome the physical interactions between the newly formed particles and the reactor’s walls.

**Figure 3.3.** TEM images of the SPION core – silica shell nanoparticles at stirring rates of: (a) 400 rpm, (b) 800 rpm, (c) 1200 rpm

The silica shell growth following two hours of stirring at different rates are presented in fig. 3.4.: at all stirring rates the overall size of SPION-silica nanoparticles remains constant with a slight increase in the size and polydispersity at higher stirring rates.

**Figure 3.4.** Size dependence of the CSNPs on the stirring rate for reaction duration of 2 h (the line is inserted as a guide for the trend)

In general, for all the reaction times the stirring rate had only a minimum influence on the size dispersion and homogeneity of CSNPs.

**Thickness control of silica shell.** For applications in biomedical field, it is crucial to accurately control the silica shell thickness. Therefore, we studied the silica shell thickness variation as a function of time, the amount of TEOS and the SPION core size being
maintained constant. The series of TEM images of the particles obtained are presented in figure 3.5.

![TEM images of the SPION core – silica shell nanoparticles with various silica shell thickness: (a) 5.2 nm; (b) 6.5 nm (c) 10.4 nm, (d) 13.2 nm, (e) 17.4 nm and (f) 51.5 nm](image)

**Figure 3.5. TEM images of the SPION core – silica shell nanoparticles with various silica shell thickness: (a) 5.2 nm; (b) 6.5 nm (c) 10.4 nm, (d) 13.2 nm, (e) 17.4 nm and (f) 51.5 nm**

The SEM images (fig. 3.6) complement the TEM images confirming the high degree of monodispersity of the CSNPs and shape.

![SEM images of silica – SPION nanoparticles](image)

**Figure 3.6. SEM images of silica – SPION nanoparticles**

The investigation of the TEM images reveals the fact that even after a reaction time of 2h the silica shell has a considerable thickness of 5.2 ±1.7 nm. For the identical set of initial conditions, the thickness of the silica shell increases linearly with time, reaching a maximum value and remaining almost constant if the reaction time is further increased. This is attributed to the slow reaction kinetics caused by the low amount of the remaining TEOS. The
measurements of the thickness of the silica shell as a function of time are summarized in fig. 3.7.

**Figure 3.7.** (a) Silica layer thickness of SPION core-silica shell nanoparticles as a function of time. The inset represents the silica layer thickness as a function of unreacted TEOS in the microemulsion (the continuous line represents the data trend). The point * indicates the addition of extra TEOS after the maximum size is reached for the initially adopted conditions

The addition of fresh TEOS after the stabilisation of the size, increase the silica shell thickness further without destabilising the microemulsion. This demonstrates that the CSNPs in microemulsion can still be active nucleation sites for the formation of new silica layer.

**Growth of larger average size CSNPs.** The size of the water phase droplets is the limiting factor of the growth of the particles in the inverse microemulsion system. The increase of the particles size over a certain value will have as consequence a decreased stability of the microemulsion. This fact will have as effect a broadening of the size distribution of the resulting particles. Consequently, the seeded particle growth using Stöber method was utilised for obtaining core-shell nanoparticles with larger size (~120 nm). As seeds CSNPs with average diameter of ~30 nm were used which were obtained through silica coating in inverse microemulsion. The hydrolysed species of the precursor (TEOS) polymerise on the surface of the seed particles via a surface growth dominated process (fact confirmed by the non existence of the empty silica particles) which results in the increase of the silica layer thickness of the CSNPs. The thickness of the silica layer is controlled by the
amount of the silica precursor added, the other variables (the seeds concentration, water to alcohol ratio, quantity of ammonia, reaction time) being maintained constant\textsuperscript{97-101}.

**Density of the silica shell.** In order to assess the density of coating silica layer etching experiments were performed. CSNPs with different shell thickness were treated with a concentrated solution of HCl for 5 minutes. The TEM images of the resulting structures (fig. 3.8) revealed that the silica layer is porous enough to allow access of the acid to the SPION core and enable it to be dissolved completely. Earlier Mornet et al\textsuperscript{136} reported similar hollow structures. The porous silica shell can be used as a matrix on/in which therapeutics can be adsorbed and released via local heat generated in the core (due to SPION) using AC magnetic field or pH change in the environment. Hollow, porous and intact silica spheres obtained by HCl treatment are structures which could be used for confined zone synthesis within silica capsules or for controlled drug release induced by the thickness of the silica shell\textsuperscript{136}. When diluted HCl was used, partially dissolved cores with different shapes were also obtained \textsuperscript{93}. Extended exposure to acid had no effect on the hollow silica structures.

![Figure 3.8. TEM images of the CSNPs with silica shell thickness of ~ 10.5 nm after treated with conc. HCl.](image)

**Control of aggregation and agglomeration of the CSNPs.** The microemulsion synthesis process has its limitations. One important shortcoming is the relatively low yield of the synthesis process. Another drawback is the fact that the resulting core-shell nanocomposites are strongly necked due to residual reactions leading to formation of silica layer connecting particles together, as previous reports on CSNPs \textsuperscript{17, 85, 93, 94, 96, 119} showed. While the problem of low yield of the synthetic procedure is difficult to solve, due to low concentration of the reactants, eliminating the interparticle aggregation and/agglomeration is
feasible. We investigated various adjustments of the post-synthesis processing that would lead to solving the necking and irreversible agglomeration problems of nanoparticles in the separation procedure. Methods already described such as alternating cycles of ethanol or acetone addition for destabilising the microemulsion and centrifuging or diluting the system with an excess of solvent (cyclohexane) did not result in separated nanoparticles. A combination of two new steps in the separation process produced dramatically improved quality particles with no aggregation or necking retained. First the pH adjustment of the aqueous phase in the pH range of 1-2 minimized the condensation rate of TEOS. Second, the kinetic destabilization of the microemulsion by shock cooling causes phase separation, slowing down the reaction and making the separation of nanoparticles from the remaining unreacted precursor considerably easier. Using this procedure we successfully fabricated highly separated, non aggregated magnetic core - silica shell nanoparticles.

3.3 Magnetic characterization of the SPION and CSNPs

VSM analyses performed on the CSNPs with different sizes are presented in figure 3.9. The calculated magnetic core sizes of 9.5 nm and 12.3 from VSM measurements are in good agreement with the ones obtained from TEM demonstrating that iron oxide nanoparticles are single crystalline. Furthermore, core-shell nanocomposites retained the superparamagnetic character of the magnetic core particles, with the normalized Ms values (expressed in emu per unit weight of core-shell sample) of 12.5 (20.7), 6.6 (9), and 3.0 (0.5) emu/g for the samples coated with different silica layer thickness (figure 3.9 a and b) which correspond to various weight ratios of diamagnetic silica layer / magnetic core of ~3.2 (0.8), ~7 (4.2) and ~18 (79). Magnetization per unit weight of sample decreases as the thickness of the silica shell increase for the same magnetic core size of SPION. Furthermore, there is an exponential decrease of magnetisation with the increase of the weight of the silica in the composites (figure 3.9 c). These nanocomposite structures are sufficiently magnetically responsive for medical imaging or targeted drug delivery applications.
Figure 3.9. (a) Magnetic measurements on the CSNPs performed by VSM with a silica thickness of ~5.6 nm (■), ~9 nm (●) and ~13.6 nm (▲). (b) Magnetic measurements on the core-shell nanocomposite spheres performed by VSM with a silica thickness of ~6.5 nm (■), ~17.4 nm (●) and ~51.5 nm (▲), (c) Saturation magnetisation versus SiO$_2$/Fe$_3$O$_4$ weight ratio (the line is inserted as a guide for the trend)

Relaxivity studies were performed on the CSNPs having as core SPION obtain by thermal decomposition of FeO(OH). The results are summarized in table 3.1. With the increase of the silica layer thickness, the interactions between the surrounding water molecules with the magnetic iron oxides core decrease$^{137-139}$. This is reflected in the observed trend of decrease in the $r_1$ and $r_2$ values, in both 20 MHz and 60 MHz fields, with the increase of the thickness of silica layer. A comparison was done with two commercial T2 contrast agents: Resovist® (Schering AG, Germany) and Feridex® (Advanced Magnetics, Cambridge, Massachusetts). The $r_2/r_1$ ratios of the core shell nanoparticles obtained are generally higher than those of Resovist® and Feridex® at 0.47T (20 MHz) and 1.41 T (60 MHz) which indicates a better T2 contrast in case our core-shell particles are used.
**Table 3.1.** The relaxivity values of iron oxide core–silica shell nanoparticles with different thickness of the silica layer versus other commercial iron oxide based MRI contrast agents, Resovist® and Feridex®. The values shown are measured at 20 MHz (0.47 T) and 60 MHz (1.41 T) in water (37°C).

<table>
<thead>
<tr>
<th>Name</th>
<th>Surface coating</th>
<th>$r_1$(s⁻¹mM⁻¹) 20 MHz</th>
<th>$r_1$(s⁻¹mM⁻¹) 60 MHz</th>
<th>$r_2$(s⁻¹mM⁻¹) 20 MHz</th>
<th>$r_2$(s⁻¹mM⁻¹) 60 MHz</th>
<th>$r_2/r_1$ 20 MHz</th>
<th>$r_2/r_1$ 60 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂-SPION (1)</td>
<td>SiO₂ 5.6 nm</td>
<td>4.1</td>
<td>2.1</td>
<td>97.9</td>
<td>101.7</td>
<td>23.73±0.9</td>
<td>48.15±1.1</td>
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<td>SiO₂-SPION (2)</td>
<td>SiO₂ 9 nm</td>
<td>2.2</td>
<td>0.8</td>
<td>40.5</td>
<td>48.6</td>
<td>18.55±0.5</td>
<td>61.42±1.6</td>
</tr>
<tr>
<td>SiO₂-SPION (3)</td>
<td>SiO₂ 13.6 nm</td>
<td>1.5</td>
<td>0.7</td>
<td>34.6</td>
<td>41.2</td>
<td>23.15±0.8</td>
<td>60.35±1.8</td>
</tr>
<tr>
<td>SiO₂-SPION (4)</td>
<td>SiO₂ 6.5 nm</td>
<td>7.4±0.3</td>
<td>1.7±0.1</td>
<td>106.6±3.2</td>
<td>123.2±0.7</td>
<td>14.4</td>
<td>72.4</td>
</tr>
<tr>
<td>SiO₂-SPION (5)</td>
<td>SiO₂ 17.4 nm</td>
<td>1.4±0.1</td>
<td>0.7±0.03</td>
<td>85.9±2.6</td>
<td>100.3±0.9</td>
<td>61.3</td>
<td>143.2</td>
</tr>
<tr>
<td>SiO₂-SPION (6)</td>
<td>SiO₂ 51.5 nm</td>
<td>0.9±0.04</td>
<td>0.5±0.02</td>
<td>38.9±0.7</td>
<td>46.9±0.9</td>
<td>43.2</td>
<td>93.8</td>
</tr>
<tr>
<td>Resovist®140,141</td>
<td>carboxydextran</td>
<td>24.9</td>
<td>10.9</td>
<td>177</td>
<td>190</td>
<td>7.10</td>
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</tr>
<tr>
<td>Feridex®137</td>
<td>dextran</td>
<td>40</td>
<td>-</td>
<td>160</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

### 3.4 Cytotoxicity assessment

The cytotoxicity effects of three different sizes of SiO₂-Fe₃O₄ core-shell nanoparticles (~30 nm, ~50 nm and ~120 nm) in HMDM cells was investigated.

The cytotoxicity effect of the nanoparticles on the HMDM cells was investigated by MTT assay (which gives information on the cellular mitochondrial function) and by the quantification of the cytokines release (proteins produced by the cells in response to an immune stimulus).

The mitochondrial functionality of HMDM, after exposure to different core-shell nanoparticles sizes for 24 h and 48 h, was not significantly affected, regardless of the nanoparticles concentrations used. However, when the dose of 100 µg/ml of CSNPs was used for incubation with the cells for 24 h and 48 h, a trend of decrease in mitochondria functionality was observed (figure 3.10).
Figure 3.10. Mitochondrial functionality in HMDM. The results are presented for 24 h and 48 h incubation with CSNPs, as percent cell viability mean ± SD from three independent experiments using cells from different healthy blood donors.

TNF-α and IL-6 as representative for pro-inflammatory cytokines were chosen for investigation on HMDM cells. As positive control, bacterial lipopolysaccharide (LPS) which is a stimulator of both TNF-α and IL-6 cytokines release in macrophages was chosen. Comparatively, the cytokine release (figure 3.11) was not triggered by exposure to the CSNPs, which further confirm the cellular biocompatibility of the CSNPs with HMDM cells.

Figure 3.11. TNF-α secretion (a) and IL-6 secretion (b) in HMDM. Results are presented for 24 h and 48 h incubation with CSNPs as cytokine release (pg/ml) ± SD from three independent experiments using cells from different healthy blood donors.
3.5 Uptake studies

The cellular uptake studies were done in HMDM cells on the same set of CSNPs, having overall size of \( \sim 30 \text{ nm} \), \( \sim 50 \text{ nm} \) and \( \sim 120 \text{ nm} \).

The dose (50 \( \mu \text{g/ml} \)) was chosen as the dose with low toxicity but high enough to allow a high probability of visualization of the nanoparticles internalization process. The 2 h incubation time is low enough to permit the uptake of the nanoparticles but too short for the toxicity to manifest.

Figure 3.12 presents the TEM images of the cellular uptake by HMDM of the three sizes of the CSNPs. All particles, independent on the size, are successfully uptake by the cells, being visible in the cytosol, but not in the nucleus. The active uptake mechanism (endocytosis) is indicated by the presence of the particles in membrane – enclosed vesicles (endosomes). Further studies using inductively coupled plasma-mass spectrometry (ICP-MS) method are needed for quantitatively assessing the uptake efficiency of the particles in HMDM cells.
Figure 3.12. TEM micrographs at various magnifications of the uptake in HMDM cells exposed to the CSNPs of ~30 nm (A-A’’), ~50 nm (B-B’’) and ~120 nm (C-C’’). Scale bars: A-C: 2 µm; A’-C’: 500 nm; A’’-C’’: 200nm.

The core – shell structure and the initial size is well preserved for all sizes of the nanoparticles, a good proof that the particles are stable at the low pH present in the endosomes.
4 Conclusions

The objective of this work is to obtain biologically relevant CSNPs with iron oxide as core and silica coating, with high biocompatibility that can be utilised as MRI contrast agents.

To the best of our knowledge, until now, the synthesis of single core iron oxide silica shell nanoparticles, well-dispersed with no aggregation and with a wide range of distinct sizes is not reported in the literature. Furthermore, the in vitro investigation of their cytotoxicity and the uptake studies is also not reported.

We successfully synthesised finely adjustable silica shell on iron oxide core nanoparticles via an optimised inverse microemulsion method in combination with Stöber method for the synthesis of bigger particles. CSNPs with a narrow size distribution having crystalline magnetic core and a tuneable thickness amorphous, porous silica shell were obtained by adjusting the amount of silica precursor and the reaction time. A thorough investigation of the parameters that can influence the thickness of the silica shell was performed. The monodisperse single central core-silica shell adjustable thickness CSNPs were free of residual aggregation due to a modified post synthesis processing procedures (pH adjustment to the region of minimum condensation of TEOS and shock cooling the solution that results in phase separation).

The CSNPs with various thickness of the silica shell preserved the superparamagnetic character of the core particles, with an observed tendency of decrease in the magnetization saturation with the increase of the silica shell thickness. The relaxivity studies revealed that the ratio $r_2/r_1$ for all the thicknesses of the silica shell studied is higher than the values for the commercially available T2 contrast agents investigated (Resovist® and Feridex®).

As the intended applications for these nanocomposites are in biomedical area, their cytotoxicity assessment is compulsory. Using HMDM possess the advantage of modelling the possible immune response from the macrophages. MTT assay on HMDM cells, even though showed apparent minor toxic effects, revealed a dose dependent ($\mu$g of sample/ml of medium) increase toxicity. The cytokine release by HMDM was not affected which indicates the absence of an inflammatory response in the presence of the CSNPs. The TEM studies of the cellular uptake confirmed the presence of the particles inside of the cytosol of the HMDM after 2h of incubation, regardless of the size of the CSNPs. Additionally; the particles preserved their initial core – shell morphology. Further ICP-MS studies, ongoing, are necessary to quantify the efficiency of the nanoparticles uptake.
The CSNPs synthesized in this work, due to the physical characteristics (monodispersed, non aggregated, with a tuneable silica shell), magnetic properties (superparamagnetic, high $r_2/r_1$ ratio) and their minor toxicity appear to be excellent candidates for biomedical applications such as MRI. Nevertheless, animal studies need to be performed in order to confirm their biocompatibility, their biodistribution and suitability as imaging agents.
Future work
The work presented in this thesis on the design and fabrication of highly monodisperse, nonaggregated CSNPs with a finely adjustable silica shell, with confirmed low toxicity, are a solid platform for the incoming planned work.

Future planned work has different directions.

First we will concentrate on increasing the functionality and complexity of the system. Quantum dots inclusion and/ drug loading in the silica shell is one of the directions. This type of multifunctional nanoparticles will aid the in-vivo imaging/tracking using confocal microscopy. Surface modification of the silica shell with molecules that might enhance the cellular uptake will be another direction of research (active targeting function).

More extended magnetic studies will be followed by a detailed investigation of the influence of the silica thickness and the addition of new components to the system on the relaxivity behaviour of the CSNPs.

The experiments concerning the toxicity, uptake study of the CSNPs will continue with extension of the study on different cell lines (dendritic cells). Studies on the protein coating of the particles when placed in plasma and the influence of the protein corona on the cellular uptake are another direction of research that will be pursued.

The same strategy of cell toxicity battery of tests will be applied also on the coming new complex architecture nanoparticles (the SPION core – silica shell with quantum dots embedded in the silica shell and/surfaced modified).

These collaborative studies will allow us to further develop and fully characterise the multifunctional CSNPs before introducing them in the in–vivo testing stage.
Acknowledgements

And now, to the part of the thesis that attracts the most readers…..

My foremost gratefulness goes to Prof Mamoun Muhammed, my supervisor, for embarking me in this scientific journey through the opportunity he gave me in joining Functional Materials Division. His sharp criticism makes me become aware of my weaknesses but his humane attitude shows me that science is just one part of life.

I have to thank Dr. Muhammet Toprak, my second supervisor, for his understanding and kindness in tutoring me. His systematic and logical thinking combined with a sympathetic character make from him an excellent scientist with a great heart.

My thoughts of special thanks are going to Professor Bengt Fadeel and Professor Annika Scheynius, Neus Feliu Torres, Jingwen Shi, Andrea Kunzmann and Britta Andersson from Karolinska Institutet for the kind treatment you give to my babies (the CSNPs) in the thorough cytotoxicity studies but also for the rich scientific discussions we have. I have to also thank Professor Harald Krug, Tina Thurnherr and Liliane Diener at EMPA for the excellent TEM work they do on visualising the cell uptake of the core shell nanoparticles.

I also want to thank Dr. Sophie Laurent, Dr. Jean-Luc Bridot and Professor Robert Müller from University of Mons-Haineaut, Belgium for the great help in performing the magnetic measurements.

Life without friends would be empty. I am blessed to be part of a group where friendship, fruitful discussions and sharing scientific ideas are part of everyday live. Thank you Jian (Sylvan) for you brilliant advices and support whenever I ask for, Andrea and Sverker for the agreeable coffee breaks, Shanghua (Peter), Ying and Xiaodi (Yang) for the pleasant lunch companies, Abhi, Fei, and Robina for enjoyable long times spend together in the lab and to Salam, Mazhar and Terrance for your friendship. Hans and Wubeshet, thank you for you patience and kindness!

To my dear friend Anna, you showed me the meaning of true friendship. Thank you!

If I am what I am now is because of my parents’ continuous support, the power of never give up and the will for incessant improvement they implanted in us (me and my sister). I miss you enormously! I know that the only valid way of thanking you is by never forgetting where I come from. My sister Oana, no matter how much space is in between us, you will always be my dear one that I want to argue with!

And at last but most important, I have to thank first to God from the bottom of my hearth for giving me the most wonderful and supportive husband, Uli, and the most cherished baby in the world, Johannes.
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