

**Ph.D. thesis**

**Synthesis and stabilization of magnetic fluids under physiological  
conditions with the view of biomedical applications**

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## 1. Introduction

Preparations of stable water based magnetic fluids (MFs) are nowadays of renewed interest, due, in particular, to biomedical applications. Magnetic fluids, or ferrofluids as they are often called consist mainly of nanosized magnetic particles, typically iron oxides ( $\text{Fe}_3\text{O}_4$  or  $\gamma\text{-Fe}_2\text{O}_3$ ) suspended in a liquid carrier. Water-based magnetic fluids containing magnetic nanoparticles (e.g., magnetite or maghemite), coated with a stabilizing layer can be manipulated by an external magnetic field gradient in such way that the entire liquid can move according to the inhomogeneous magnetic field.

Hyperthermia, targeted drug delivery, cell separation and magnetic resonance imaging (MRI) are just a few examples of the large number of possible applications in medical diagnosis and therapy. Most of these applications require the magnetic nanoparticles to be non-toxic, chemically stable, uniform in size, and well dispersed under physiological conditions.

In biocompatible MFs various coatings have been developed using biocompatible molecules such as dextran, polyethylene glycol (PEG), polyvinyl alcohol (PVA) and phospholipids. Recently coatings with dendrimers and silica have also been designed. Many important applications of MFs are known in the field of biotechnology and biomedicine, such as cell labeling and separation, magnetic resonance imaging (MRI) contrast agent (replace the most commonly used T1 MRI contrast agent, i.e., gadolinium (Gd) chelates), enzyme and protein separation, targeted drug delivery, magnetic hyperthermia, etc. The biocompatible magnetic iron oxide nanoparticles are not toxic at all, and they can be accumulated in different tissues depending on the particle size, which can increase the contrast between the different organs in living systems.

Hyperthermia is a promising therapeutic method in the cancer healing. The superparamagnetic particles can heat the surrounding area if an alternating (AC) magnetic field is used. If the used frequency is properly chosen, the nanoparticles can be induced only without affecting the iron content of the hemoglobin in the blood. One of the key points of the therapeutic use of hyperthermia is to selectively transport enough magnetic particles to the intended target tissue to generate enough heat locally by exposure to a tolerable level of magnetic fluid that does not in itself cause any undesirable side effects.

Biological media has a well defined pH and salt content (i.e., the blood pH is about 7.2-7.4 and the NaCl concentration is 0.15 mol/l). The colloidal stability of water based magnetic fluids is critical in general, it depends on the electrostatic repulsion influenced by

the electrolyte concentration and pH of the solution, as well as the specific ion-adsorption, besides the steric repulsion forces and the magnetic attractive interactions.

The main aim of my thesis was to prepare well stabilized magnetite-containing magnetic fluids using different stabilizers (citric acid (CA), polyacrylic acid (PAA), and sodium-oleate (NaOA)) to protect the particles from the aggregation under physiological conditions.

One of the aims of my work was to investigate the effect of molecular oxygen present in the synthesis of the nanoparticles, concerning changes in their magnetic behavior, morphology, and crystal structure.

I planned to stabilize the magnetite with 3 different carboxylated compounds. One of them is citric acid (CA), a well known complexing agent, the other one is a macromolecule, poly(acrylic acid) (PAA), and the third one is sodium oleate (NaOA), an amphiphilic surfactant. In this way, I could study the differences between stabilizing efficiency of compounds having similar chemical interactions between the active sites on the magnetite surface and the adsorbed carboxylic groups, which stabilize nanoparticles through the electrostatic, steric and the combined electrosteric interactions. I intended to characterize the adsorbed amount of CA, PAA and NaOA on the magnetite surface, and follow the changes in the pH dependent particle size and surface charge by dynamic light scattering (DLS) and electrophoretic mobility measurements.

My aim was to prepare stable magnetic fluids and characterize their enhanced salt tolerance with correct coagulation kinetics measurements to predict their behaviour under physiological conditions. I also planed to measure the cytotoxic and antiproliferative effects of the magnetic fluids on HeLa adenocarcinoma cell lines.

My further aim was to show differences among the contrasting performance (i.e., proton relaxation effect) of the tested magnetic fluids stabilized by different hydrophilic layers (i.e., CA, PAA, NaOA) at different field strengths using  $H^1$ -NMR (0.47 and 9.4 T) and MRI devices to compare them with the approved contrast agents Resovist and Gd-complexes. Considering the recent trend of MRI development, it is important to predict the behavior of the nanoparticles under different magnetic fields. Finally, I also planed to measure the magnetic hyperthermic heating effect of the above samples with a home made instrument.

## **2. Experimental methods**

The co-precipitation method was used to synthesize magnetite nanoparticles. Samples were identified using X-ray diffraction (XRD) and particle morphology was characterized by

transmission electron microscopy (TEM); the magnetic properties or the transformation to maghemite was measured by Mössbauer spectroscopy and vibrating sample magnetometer (VSM). The attenuated total reflection Fourier transform infrared (ATR-FTIR) measurements and the dynamic light scattering (DLS) instrument were also used for the characterization of the samples after the stabilization procedure.

The adsorption isotherms of the CA, PAA and NaOA on the magnetite nanoparticles were determined by the batch method. The magnetite suspensions (dialyzed against 0.001 mol/l HCl) were equilibrated with the series of stabilizer solutions in closed test tubes for 24 h at room temperature. The NaCl concentration was kept constant at 0.01 mol/l and the pH was adjusted at ~6 with 0.1 or 1 mol/l HCl and NaOH. The equilibrium concentration was determined by measuring the absorbance of supernatants at appropriate wavelength after perfect separation of the solid particles. The equilibrium concentration of CA was determined by enzymatic Boehringer Mannheim citric acid assay. The amount of dissolved Fe in the equilibrium supernatants after CA adsorption was measured by inductively coupled plasma (ICP) emission spectroscopy.

The pH dependent particle size and electrophoretic mobility of the naked magnetite nanoparticles and of those in the presence of stabilizers of increasing concentration were determined in diluted samples using NanoZS apparatus of Malvern. The enhanced salt tolerance of naked and stabilized magnetite nanoparticles was characterized by coagulation kinetic measurements to study the colloidal stability under physiological conditions (pH~7; 0.15 mol/l NaCl). The biocompatibility, cytotoxic and antiproliferative effects were measured in vitro on the HeLa (cervix adenocarcinoma) human cell line by using the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) cell viability assay.

The contrast effect of the differently stabilized magnetic nanoparticles were measured with a GE Excite HD (1.5 T) clinical MRI instrument (Euromedic Diagnostics Ltd.), whereas Bruker MiniSpec Mq 20 (0.47 T) and Bruker DRX 400 (9.4 T) instruments were used for the <sup>1</sup>H-NMR measurements. The T1 (spin-lattice) and T2 (spin-spin) relaxation were measured and the corresponding r1 and r2 relaxivities were calculated.

I also measured the magnetic hyperthermic heating effect of the samples with a home-made instrument. The alternating magnetic field was induced in a coil. I made the measurements in a model system, in which the circumstances in the living system might be simulated. The temperature increase was induced by applying AC field, then a specific absorption rate (SAR; measured in W/g Fe) was calculated. The parts of the instrument were

an oscilloscope (CS-4128), a generator (TR-0458), a power amplifier (Philips, PM5175), a coil and a digital thermometer (GTH 175/MOP).

All experiments were performed at room temperature ( $25 \pm 1^\circ\text{C}$ ). All reagents were of analytical grade product of Fluka or Sigma-Aldrich, and Milli-Q water was used.

### **3. Summary of new scientific results**

#### **T1. Mössbauer spectroscopy of synthetic magnetite nanoparticles**

The Mössbauer spectroscopy results showed that the freeze-dried synthetic magnetite nanoparticles, prepared and kept under nitrogen atmosphere were magnetite with 4-12 nm particle size. I also got evidence that their transformation in the presence of air to maghemite starts right after their synthesis.

#### **T2. Adsorption of the carboxylated compounds on magnetite nanoparticles**

By measuring CA adsorption on magnetite surface I showed that the adsorption isotherm is of high affinity (H) type; citrate ions adsorbed on the surface by coordinating  $\equiv\text{FeOH}$  sites via one or two of their carboxylate functionalities through a water bridge with an outer-sphere complexation. The surface coverage reached the high affinity limit at  $\sim 0.13$  mmol CA on 1 g magnetite. The increasing CA adsorption measured with increasing CA equilibrium concentration may be caused by the formation of new binding sites due to the increasing dissolution of surface Fe ions, and also by the surface oligomerization of citrate. The dissolution of Fe(III)-ions from magnetite crystals with increasing CA loading became evident from the rising yellowish color and huge iron concentration of supernatants ( $\sim 0.45$  mmol/L), and supported by the decreasing saturation magnetization value observed with increasing citrate concentration. Evidence for the CA oligomerization was the peaks observed at  $1734\text{ cm}^{-1}$  and  $837\text{ cm}^{-1}$  in the ATR-FTIR spectra.

Monolayer coverage of magnetite was reached in the presence of  $\sim 0.35$  mmol PAA monomer (COOH) on 1 g magnetite. Polyacrylate adsorbed on the surface by coordinating in a monodenate form (via one carboxylate group to the  $\equiv\text{FeOH}$  site) with chemisorption, through the exchange of the surface  $-\text{OH}$  groups from the coordination zone of the surface  $\equiv\text{Fe}$  ions (inner-sphere complexation). The evidence for this type of bonding is that the Fe-OH peak ( $3695\text{ cm}^{-1}$ ) in the ATR-FTIR spectra disappears after PAA adsorption. After its adsorption the polymer binds to the surface by some of its carboxylate groups, whilst the rest

of the molecule makes hydrophilic loops and tails in the coating to form a thick steric protecting layer around the nanoparticles. In the presence of poly(acrylic acid) the saturation magnetization curve of magnetite is decreased, because of the iron dissolution from the crystal during the adsorption, in a same way as in the case of citrated magnetic fluids.

The adsorption isotherm of the amphiphilic Na-oleate from its aqueous solution was found to exhibit a two-step shape. In the first step oleate is chemisorbed on the surface due to complex formation ( $\sim 1$  mmol NaOA /g magnetite), while the second layer of oleate anions can be adsorbed at the hydrophobic shell of the oriented surfactant molecules of the first layer via hydrophobic interaction ( $\sim 2$  mmol NaOA/ g magnetite). In the ATR-FTIR spectra the same peak disappeared at  $3965\text{ cm}^{-1}$  as in the case of PAA stabilized magnetite, giving evidence for the chemisorption in the first layer.

### **T3. Modification of electrophoretic mobility and aggregation of magnetite nanoparticles**

The electrophoretic measurements gave evidence that the citrate, poly(acrylate) and oleate adsorption on the magnetite surface changes the surface charge at the physiological pH range (pH=6-9), and the particles become overcharged. The carboxylated nanoparticles have large negative electrophoretic mobility values (CA:  $-2 \cdot 10^{-8}\text{ m}^2/\text{Vs}$ , PAA:  $-3.3 \cdot 10^{-8}\text{ m}^2/\text{Vs}$ , NaOA:  $-4.5 \cdot 10^{-8}\text{ m}^2/\text{Vs}$ ). The iron-oxide particles charged positively at pH $\sim 6$  are neutralized by a certain amount of the added stabilizers (CA $\sim 0.15$  mmol/g; PAA $\sim 0.3$  mmol/g; NaOA $\sim 0.75$  mmol/g), then their further adsorption leads to first charge reversal then overcharging. These results are in a good agreement with the adsorption measurements; namely with the values corresponding to the high affinity part of the citrate adsorption isotherm ( $\sim 0.13$  mmol CA/g), to the saturation part of the PAA adsorption isotherm ( $\sim 0.35$  mmol PAA/g) and to the first layer formation ( $\sim 1$  mmol NaOA /g magnetite) of the Na-olate adsorption.

By measuring the change of the hydrodynamic diameter I found that the use of the stabilizers leads to a pH independent particle size at pH 5-10. However, the particle size of magnetite was not changed in the presence of citrate and oleate, whereas after the adsorption of poly(acrylate) the particle size increased by a factor of two. This thick adsorption layer is typical in the presence of polyelectrolytes.

#### **T4. *The salt tolerance of stabilized magnetite suspensions***

The enhanced salt tolerance of magnetite nanoparticles was investigated by coagulation kinetic measurements in order to characterize the colloidal stability under physiological conditions (pH~7; 0.15 mol/l NaCl). The critical coagulation concentrations of the diluted samples gave evidence about the following behavior of the studied magnetic fluids:

- the small citrate molecules in the adsorbed layer could not protect the magnetic nanoparticles from the adhesion, because the stabilization is only electrostatic, and thus the particles aggregate already in the presence of small amount of NaCl (0.07-0.09 mol/l).;
- aggregation could not be detected if the magnetite was covered with oleate double layer up to 0.2-0.25 mol/l NaCl concentration;
- PAA can effectively prevent the aggregation of magnetic particles due to the steric and electrostatic repulsive barrier of the ionized layer of polyacrylate coating on magnetite. In the presence of PAA the critical coagulation concentration was found to be 0.3 mol/l, which is much higher than that of either citrate or oleate stabilization.

By means of this colloidal stability measurement, which has never been used before in the field of magnetic fluids; I determined the critical coagulation concentration of the diluted samples, giving evidence about the behavior of magnetic fluids in the presence of electrolyte, and predicting their *in vitro* aggregation.

#### **T5. *In vitro experiments with HeLa cells***

The cytotoxic effect of the magnetic fluids was studied using HeLa adenocarcinoma cells. None of the samples demonstrated a pronounced cytotoxic effect against the cell line, or prevented the cell growing, thus, the samples were not found to have any antiproliferative effect. In other words, all the stabilized magnetic fluids are biocompatible.

In the case of the citrated magnetic fluids a terrifyingly large amount of nanoparticles were able to pass through the cell membrane and being internalized in the cell around the nucleus, which was easily visualized by means of a light microscope. This *in vitro* aggregation behavior was the same as predicted from the simple coagulation kinetics test, since the CA stabilized nanoparticles aggregated under physiological conditions. However, the magnetic fluids stabilized by polyacrylate or an oleate double layer did not show micron sized aggregates after their interaction with the cells.

#### **T6. Testing the decreasing proton relaxation in the presence of different hydrophilic surface covered magnetite nanoparticles in order to MRI contrast enhancement**

The T1 and the T2 relaxations of naked magnetite measured with a clinical MRI instrument (1.5 T) resulted in an extremely high relaxivity values ( $r_1 = 8.9 \text{ mM}^{-1}\text{s}^{-1}$  and  $r_2 = 429.5 \text{ mM}^{-1}\text{s}^{-1}$ ). The citrated, poly(acrylated) and oleated magnetic fluids have shown smaller  $r_1$  parameters (CA:  $3.5 \text{ mM}^{-1}\text{s}^{-1}$ ; PAA:  $3.9 \text{ mM}^{-1}\text{s}^{-1}$  and NaOA:  $1.1 \text{ mM}^{-1}\text{s}^{-1}$ ) as compared with naked magnetite. Similar behavior was also observed concerning the  $r_2$  relaxivity values (CA:  $155.7 \text{ mM}^{-1}\text{s}^{-1}$ ; PAA:  $232.4 \text{ mM}^{-1}\text{s}^{-1}$ ; NaOA:  $240.2 \text{ mM}^{-1}\text{s}^{-1}$ ). The contrasting effect of magnetic nanoparticles with different hydrophilic coverages, measured at 1.5 T has to be compared to that of Gd-chelates ( $r_1 = 4.7 \text{ mM}^{-1}\text{s}^{-1}$ ;  $r_2 = 5.3 \text{ mM}^{-1}\text{s}^{-1}$ ). The  $r_1$  relaxivity is in the same range, but the Gd-complex has an extremely low  $r_2$  relaxivity comparing with the iron-oxide nanoparticles.

I found that increasing field strength (0.47; 1.5 and 9.4 T) induces a decline in the  $r_1$  relaxivity value ( $27.8$ ;  $8.9$  and  $0.5 \text{ mM}^{-1}\text{s}^{-1}$ , respectively) of naked magnetite as well as in that of the stabilized magnetic fluids (CA:  $15.7$ ;  $3.5$  and  $0.8 \text{ mM}^{-1}\text{s}^{-1}$ ; PAA:  $17.7$ ;  $3.9$  and  $0.5 \text{ mM}^{-1}\text{s}^{-1}$ ; NaOA:  $4.5$ ;  $1.1$  and  $0.2 \text{ mM}^{-1}\text{s}^{-1}$ , respectively). Determining the  $r_2$  values under the same circumstances the results showed a maximum behavior with increasing field strength for both the naked ( $297.9$ ;  $429.5$  and  $301.8 \text{ mM}^{-1}\text{s}^{-1}$ , respectively) and the stabilized samples (CA:  $143.9$ ;  $155.7$  and  $95.8 \text{ mM}^{-1}\text{s}^{-1}$ ; PAA:  $186.5$ ;  $232.4$  and  $154 \text{ mM}^{-1}\text{s}^{-1}$ ; NaOA:  $176.4$ ;  $240.2$  and  $138.9 \text{ mM}^{-1}\text{s}^{-1}$ , respectively).

#### **T7. Magnetic hyperthermic measurements**

The measurements with the home-made hyperthermic instrument showed that the heating effect is increased with increasing magnetite concentration (in the content 0.1-1 g magnetite/10 ml, the SAR value was found to be 2.1-10 W/g Fe). To compare the SAR parameters of naked (2.1-3.3 W/g Fe) with that of the stabilized (2.5-3.5 W/g Fe) magnetite suspensions at the same concentration (0.5 g magnetite/ 10 ml), it can be stated that the presence of stabilizing layers did not influence significantly the heat release. The amount of generated heat was smaller in each case (2.1-10 W/g Fe) than the results in the literature (~10-200 W/g Fe).



## **Possible practical application**

The synthesized magnetite nanoparticles were stabilized with different carboxylated compounds in order to prepare magnetic fluids. These were stable under physiological conditions and their biocompatibility was confirmed in biological tests. These magnetic fluids have a noteworthy properties such as contrasting or heating effects, so they may be susceptible in the clinical MRI diagnosis or in the therapeutic application of hyperthermia; thereby hopeful theranostic agents.

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