Magnetic and relaxometric properties of polymer-based nanostructured bio-ferrofluids as novel MRI contrast agents

Authors and affiliations:

(Spain)

R. Bustamante$^{a,b}$, A. Millán$^a$, N.J.O. Silva$^c$, R. Piñol$^a$, L. Gavilondo$^a$, F. Palacio$^a$.

$^a$ Instituto de Ciencia de Materiales de Aragón, CSIC-Universidad de Zaragoza, 50009, Zaragoza, Spain.
$^b$ Centro de Estudios Avanzados de Cuba, Habana, Cuba.
$^c$ Departamento de Física and CICECO, Universidade de Aveiro, 3810-193 Aveiro, Portugal

(Italy)

H. Amiri$^{d,e}$, P. Arosio$^d$, M. Corti$^{e,f}$, A. Lascialfari$^{d,e,f}$

$^d$ Dipartimento di Scienze Molecolari Applicate ai Biosistemi, Università degli studi di Milano, I-20134 Milano, Italy.
$^e$ Dipartimento di Fisica “A. Volta”, Università degli studi di Pavia, I-27100 Pavia, Italy.
$^f$ Centro S3, CNR-Istituto di Nanoscienze, I-41125 Modena, Italy
Abstract

Here we show a study of the efficiency as Contras Agents (CA) for Magnetic Resonance Imaging (MRI) of a series of maghemite/polymer composite ferrofluids with different particle size. The ferrofluids are made of biocompatible components, contain anchoring groups for biofunctionalization, can incorporate fluorescent dyes, and have shown low cellular toxicity in previous studies. The maghemite particle sizes rank between 7.4 and 15 nm while the range of hydrodynamic sizes is 59 – 93 nm. The magnetic properties of the samples have been determined by means of magnetization and ac susceptibility measurements as a function of temperature and frequency. To cover most of the working frequencies in clinical NMR Imagers we have performed $^1$H nuclear magnetic resonance (NMR) experiments in the frequency range $10\text{KHz} \leq \nu \leq 200\text{MHz}$ for $T_1$ and $4\text{MHz} \leq \nu \leq 80\text{MHz}$ for $T_2$. It is found that both $r_1$ and $r_2$ (in mM$\text{Fe}^{-1}$ s$^{-1}$) strongly increases with the particle size. This behaviour is mostly due to an increase of the magnetic moment $\mu_{SP}$ of the nanoparticles with size, being well described by the model of proton relaxation induced by superparamagnetic nanoparticles proposed by A. Roch and R.N. Muller, where the low frequency $r_1$ and $r_2$ values are essentially proportional to $\mu_{SP}^2 \cdot N_{SP} \cdot R$ (where $N_{SP}$ the nanoparticles density and $R$ is the nanoparticles radius). A change in the relative contribution of magnetic anisotropy and water diffusion on the longitudinal relaxation was found for particle sizes over 8 nm. The NMR-dispersion (NMRD) profile shows that $r_2$ values for samples with sizes > 10 nm are comparable with or better than the ones of commercial samples, the best results obtained in particles with bigger magnetic cores (isn’t it the ratio $r_2/r_1$ what matters?). These results are corroborated by MRI experiments at $\nu=8.5\text{MHz}$, thus suggesting our samples as novel negative MRI contrast agents.

**Keywords:** MRI contrast agent, relaxometry, functionalized superparamagnetic nanoparticles
Introduction

The use of magnetic nanoparticles (MNPs) may lead to exciting new developments in biomedicine [1-3]. Attaching MNPs to a biological entity (e.g., cell, protein, enzyme, antibody, drug, DNA, etc.) permits performing a variety of operations (i.e., moving, fixing, counting, heating, locating, etc.) with minimal interaction thus leading to a large number of applications [4]. A good example is magnetic resonance imaging (MRI) which is the most promising non-invasive diagnostic technique in medicine [5].

Concerning biomedical uses, physical performance is always subordinated to biocompatibility. Materials that have shown excellent magnetic properties can find severe validation difficulties for clinical applications. Another desirable feature is the functionalization of the nanoparticles, that is to provide them with anchoring sites for biological vectors, luminescent labels, drugs, etc. The MNPs in the ferrofluids used here have been designed ad hoc for biomedical applications taking into account these requisites [6-8]: a) the magnetic core is maghemite, which is about the most biocompatible magnetic compound, the coating is made of biocompatible polymers, polyvinylpyridine (PVP) and polyethylenglycol (PEG), and the dispersing media is a phosphate buffer saline solution (PBS) with pH=7.4; b) it incorporates functional groups (-COOH) for the anchoring of biological vectors and it has also been functionalized with luminescent dyes for live cell studies. This type of bioferrofluids has shown low toxicity in cells cultures, human blood and in vivo experiments, and they are stable for years [8].

Despite the relatively high number of degrees of freedom for obtaining good images of the soft tissues of living beings, in some cases it is not possible to have enough image contrast to show the anatomy or pathology of interest. In such cases, one has to use contrast agents (CA), generally based on paramagnetic or superparamagnetic substances. The CAs used in MRI are selected to induce a shortening of the spin-lattice $T_1$ and/or spin-spin $T_2$ relaxation times of the hydrogen nuclei within the tissues/regions where they are delivered allowing a much better image contrast. Most commonly a paramagnetic CA, usually a gadolinium-based compound is used [9,10]. Gadolinium-enhanced tissues and fluids appear extremely bright on $T_1$-weighted images, and for this reason paramagnetic CA are called positive. More recently superparamagnetic (SP) CA, based on iron oxide nanoparticles [11,12], have become commercially available. The regions where such agents are delivered appear very dark and therefore they are called negative CA. The big advantage of this type of CA is their sensitivity that is expected to reach single cell level [13].

In order to exploit all the potential of MNPs in MRI it is necessary to determine the structure/performance relations that would lead to the optimal product. In fact, despite the great interest in synthesizing novel more efficient CAs, the influence of microscopic parameters like the kind of magnetic ion, the kind and thickness of the coating, the dimensions of the magnetic core and of the nanoparticle on the MRI efficiency, has been scarcely studied [14]. Studies of such kind on ferrites-based CA with different
magnetic core dimensions and an amphiphilic polymer or micelles coatings have been reported [15-18]. In particular, an optimum magnetic core diameter, \( d \approx 8-12\text{nm} \), has been suggested for these particles, all having the same coating but with a thickness slightly dependent on the magnetic core size. Other authors have shown that the kind and thickness of coating have a marked influence on the relaxivity, as deduced from preliminary studies on Mn-ferrites-based compounds [19].

Here we examine the relation between particle size, magnetic properties and CA efficiency in a polymer-based MNP system that is fully biocompatible, suitable for in vivo applications, with low cellular toxicity, and a high capacity for multifunctionalization. We have used the \(^1\text{H} \) NMR-dispersion-profile technique (\(^1\text{H} \) NMRD) to measure the longitudinal \( r_1 \) and transverse \( r_2 \) relaxivities, these parameters measuring the increase of the nuclear relaxation rates per unit of magnetic center. In some of the samples here investigated the transverse relaxivity, which is the most significant parameter for the efficiency of negative CAs, resulted comparable with or higher than commercial contrast agents. In order to confirm the MRI efficiency of our samples, we also performed some MRI in vitro experiments at \( \nu=8.5 \text{ MHz} \) using a low-field Imager.

This work has been carried out in parallel with similar studies about magnetic hyperthermia performances and toxicology. Thus, this is a first part of a general study aiming to develop a MNP system optimised for simultaneous diagnosis and therapy applications, the so-called theranostics.
Experimental

The synthesis of the ferrofluids was performed in two steps: 1) synthesis of maghemite/PVP nanocomposites, and 2) synthesis of ferrofluids (using the nanocomposites) in a PBS medium.

Maghemite/PVP nanocomposites were prepared by \textit{in situ} precipitation from iron–PVP coordination compounds, following the procedure described in [7]. A film of iron-polymer precursor was obtained by evaporation of a 50\% water:acetone solution containing 0.2 g of PVP (Aldrich, 60 kD), and variable amounts of FeBr$_2$ (Aldrich) and FeBr$_3$ (Aldrich). The precursor film was treated with 20 mL of 1 M NaOH solution for 1 h, washed with water and dried in open air to obtain a maghemite nanocomposite. The size of the maghemite nanoparticles in the composites was tuned by using different Fe(II)/Fe(III) and Fe/N ratios. Composites for samples A-C were prepared using a Fe(II)/Fe(III) ratio of 0.5 and Fe/N ratios of 0.5, 0.625 and 1 respectively. Composite for sample D was prepared using a ratio of 1 for both Fe(II)/Fe(III) and Fe/N.

The ferrofluids were prepared according to [6]. The maghemite/PVP nanocomposites were dispersed in an acidic solution at pH $\approx 3$. The resulting acidic ferrofluid was mixed with 0.18 mL of PEG (MW=200D) acrylate (PEG(200)-A) (Monomer&Polymer), and 0.02 g of PEG (MW=1000D) acrylate (PEG(1000)-A-COOH) (Monomer&Polymer), and was heated to 70°C during 24h. Then, Na$_2$HPO$_4$ was added for a 0.01 M final concentration, the pH was adjusted to 7.40 by addition of a 0.2 M NaOH solution, and the ionic strength was adjusted to 0.15 by addition of NaCl and KCl. Finally, the dispersion was filtered through a 0.22 $\mu$m membrane filter to obtain a bioferrofluid.

The total iron content in the samples was determined by atomic absorption in a plasma 40 ICP Perkin–Elmer spectrometer. The size of the maghemite nanoparticles was determined by transmission electron microscopy (TEM) images in a Philips CM30 microscope. The grids were covered by dip coating. The hydrodynamic size distribution of the dispersed nanoparticles in the ferrofluids was determined by Dynamic Light Scattering (DLS) using the Zetasizer Nano ZS of Malvern.

The magnetic properties of these ferrofluids were studied by means of dc magnetization as a function of field at room temperature and ac magnetic susceptibility measurements as a function of temperature and frequency in a MPMS-XL SQUID magnetometer from Quantum Design.

The nuclear magnetic resonance (NMR) contrast efficiency was assessed studying the increase of the nuclear relaxation rates per unit of magnetic center. The $^1$H NMR technique was employed to measure the longitudinal and transverse relaxivities in a wide range of frequencies covering most of the clinical imagers ($\nu \approx 8.5, 21$ and 63 MHz corresponding to 0.2, 0.5 and 1.5T respectively). For $10$kHz $\leq \nu \leq 10$MHz, the NMR data was collected with a Smartracer Stelar relaxometer using the Fast-Field-Cycling technique while for $\nu > 10$MHz a Stelar Spinmaster and an Apollo-Tecmag...
spectrometers have been used. Standard radiofrequency excitation sequences CPMG-like and saturation-recovery were applied to determine $T_2$ and $T_1$ values.

MRI experiments were performed at 8.5 MHz using an Artoscan Imager by Esaote SpA. The pulse sequences selected were: a) High resolution Gradient Echo with TR/TE/NEX = 1000ms/16ms/4, matrix = 256*192, FOV = 180*180, flip angle = $\pi$ and b) High resolution Spin Echo sequence with TR/TE/NEX = 1000ms/26ms/4, matrix = 192*192, FOV = 180*180.
Results and discussion

The characteristics of the ferrofluids are shown in table 1. TEM images (Fig. 1) show a uniform distribution of iron oxide nanoparticles encapsulated in a continuous polymer film. Most of the particles are rounded with an average size that increases regularly from 7.4 nm (sample A) to 15 nm (sample D) in relation to the Fe$_2$O$_3$/PVP and Fe(II)/Fe(III) ratios selected in the synthesis (Table 1). ED patterns on these particles are consistent with a maghemite crystal structure (central image in Fig. 1). A small number of particles are elongated.

Table 1. Characteristics of the ferrofluid samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe$_2$O$_3$/PVP$^a$</th>
<th>Fe(II)/Fe(III)</th>
<th>$D_h$(nm)$^b$</th>
<th>PDI$^c$</th>
<th>$D_p$(nm)$^d$</th>
<th>SD(nm)</th>
<th>$T_B$(K)$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5</td>
<td>0.5</td>
<td>59</td>
<td>0.16</td>
<td>7.4</td>
<td>1.2</td>
<td>40</td>
</tr>
<tr>
<td>B</td>
<td>0.625</td>
<td>0.5</td>
<td>62</td>
<td>0.18</td>
<td>8.6</td>
<td>2.0</td>
<td>45</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>0.5</td>
<td>92</td>
<td>0.15</td>
<td>10.8</td>
<td>2.9</td>
<td>160</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.9</td>
<td>93</td>
<td>0.14</td>
<td>15.0</td>
<td>3.7</td>
<td>200</td>
</tr>
</tbody>
</table>

$^a$Molar ratio; $^b$Hydrodynamic diameter; $^c$Polydispersity index as obtained from DLS; $^d$Maghemite particle diameter from TEM images; $^e$Blocking temperature from AC magnetic susceptibility measurements at 30 Hz.

Histograms of the hydrodynamic sizes for the series of samples are shown in Figure 2. In all cases a monomodal distribution of particle diameters was found. The average hydrodynamic diameter $D_h$ increases with the iron oxide/polymer ratio (Table 1) from 59 nm (sample A) to 92 nm (sample C), and it is hardly changing in samples with similar Fe$_2$O$_3$/PVP ratio but different particle size (samples C and D).

The average sizes of MNPs found by TEM represent about 10-15% of the average hydrodynamic sizes. Considering that at the pH of the medium (7.40) pyridine groups are hydrophobic and PEG residues are hydrophilic, the structure of the MNP@PVP@PEG beads in suspension could be as follows: the inside would be formed
by a single folded PVP chain holding the MNPs in the interior by N-Fe coordination bonds, and the outside would be formed by solvated PEG chains in a radial disposition.

The AC magnetic susceptibility as a function of temperature of samples A to D shows the characteristic behaviour of superparamagnetic systems, with the in- and out-of-phase components ($\chi'$ and $\chi''$, respectively) depending on frequency and with $\chi''$ having a maximum at blocking temperature ($T_B$), which depends on the frequency of the applied field following an Arrhenius like function [20,21]. This shows that at low temperatures the magnetic moment of the magnetic nanoparticles cannot reverse, i.e. it is not able to cross the anisotropy energy $E=KV_P$ ($K$ is the anisotropy constant characteristic of the material and $V_P$ is the volume of the magnetic nanoparticles) and follow the field. As temperature increases, the moments of the cores with lowest anisotropy energy start to reversal, being this process thermally activated and termed Néel relaxation. As expected from this model, at a given frequency, $T_B$ increases with the increase of $D_P$ (Fig. 3).

Below 273 K these water based ferrofluids are frozen and thermal activation is the only mechanism available for reversal. At room temperature, the nanoparticles whose $E$ is too high for the thermal activation to be effective (say $E\approx k_BT$) have the possibility of reversal by mechanical rotation in the fluid (Brownian relaxation mechanism). The onset of this mechanism leads to the sudden increase of $\chi''$ in samples with a relevant fraction of “larger” nanoparticles (i.e. nanoparticles with $E\leq k_BT$).
Figure 3. $\chi''$ vs. temperature for samples A to D. The $T_b$ increases with increasing average sizes. Color online.

From the plot of magnetization against the applied field (Fig. 4) it is evident that in the samples the magnetization increases with increasing particle size as previously found in maghemite/PVP composites [22]. Fitting these curves to a Langevin function [23] modified by adding a linear term it is possible to conclude that the average magnetic moment of the particles increase with the MNPs size. This model assumes that the core of the MNPs behave as a single spin (with the same value for all MNP), and that the magnetic moments at the MNPs surface have a linear behaviour with the field in the studied field range [22]. The linear term also accounts for the diamagnetic contribution of the polymer coating and fluid.

Figure 4. Magnetization per gram of iron oxide for the series of samples. Lines correspond to fitting to a Langevin modified function. Color online.
To evaluate the MRI contrast efficiency of the samples the nuclear longitudinal and transverse relaxivities \((r_1\) and \(r_2\)) were obtained from the spin-lattice \(T_1\) and the spin-spin \(T_2\) relaxation times measured at room temperature for each frequency, as [24]:

\[
r_i = \frac{[(1/T_i)_s - (1/T_i)_d]/c(Fe)}{
\]

Where \(i = 1, 2\), \(c(Fe)\) is the iron concentration in the sample (in mM), \((1/T_i)\) are the nuclear relaxation rates and the suffixes \(s\) and \(d\) stands for sample and dispersant (in our samples PBS at physiological pH) respectively.

The NMR-dispersion profiles for samples A to D and the commercial compound Endorem are shown in Figure 5. The longitudinal relaxivity curves are constant for low frequencies. In the range from 1 to ~10MHz \(r_1(\nu)\) shows a maximum for sample A and Endorem, with similar \(D_p\) (see Table 1), which is not present for the rest of the samples; apparently in our system there is a threshold in the particle size around 8 nm over which this maximum is not longer present. \(r_1(\nu)\) rapidly decreases for higher frequencies. The transverse relaxivity has a linear behaviour in the frequency range studied with a slope very close to zero.

![Figure 5. a) longitudinal \(r_1\) and b) transverse \(r_2\) relaxivities vs. frequency for samples A to D and the commercial CA, Endorem. Dashed lines indicate the operating frequencies of most clinical imagers (\(\nu \approx 8.5, 21\) and 63 MHz). Color online.](image)

Regarding the mechanisms that induce nuclear longitudinal relaxation in SP particles, it is worth to remind that the main mechanisms are [14,25]: (a) for \(\nu < 5\) MHz, the Neel relaxation of the molecular magnetization of the particles, giving a correlation time related to the magnetic anisotropy barrier, and an associated reversal time, \(\tau_N\), that follows the Arrhenius law; (b) for \(\nu > 5\) MHz, the Curie relaxation, which takes into account the sample magnetization through the Langevin function weighted by the spectral density function \(J^D(\omega_D)\), where \(\omega_D = 1/\tau_D\), \(\tau_D\) being the correlation time related to the diffusion of the water. While mechanism (a) gives a flattening of \(r_1(\nu)\) at frequencies \(\nu < 5\) MHz, mechanism (b) is responsible of the maximum in \(r_1(\nu)\) at higher frequencies \(\nu > 5\) MHz, see Endorem and B in Fig. 5a. In addition, for very small particles, with hydrodynamic diameter \(< 15\) nm, a “dispersion” at intermediate frequencies occurs [14]. As said above, no high-frequency maximum is observed in most of our samples. This fact can be tentatively attributed to the dominant role of the
magnetic anisotropy in our samples that overcomes the high frequency feature arising from Curie relaxation, possibly depressed by a scarce contribution of the diffusion process to $r_1(\nu)$.

A detailed discussion on the frequency dependence of longitudinal relaxivity in our system would require further experimental and theoretical investigations that are currently undertaken. Here, we will restrict to an analysis of the variation of $(1/T_1) = R_1(\nu)$ and $(1/T_2) = R_2(\nu)$ at low frequencies for different particle sizes. Roch and Muller proposed a theoretical model that relates $R_1$ and $R_2$ to the energy levels of a magnetic particle of spin $S$ obtained from a simplified Hamiltonian accounting for (magnetic) anisotropy energies [25]. This model is time-consuming and inapplicable to large particles with a high total spin, $S$. To overcome these limitations, the authors suggested an alternative heuristic model where $R_1$ and $R_2$ are expressed (Eqs. 31 and 32 in [25]) as the sum of two contributions corresponding to the limits of zero and high anisotropy in the complete theory, respectively. The expressions of $R_1$ and $R_2$ can be simplified (the Langevin term in particular) for low frequencies and still reproducing the increase of the absolute values of $r_1$ and $r_2$ with particle size in this frequency range, as follows:

\[ R_1 = \frac{1}{3} C_1 \times \{ 7 F \right[ \Omega(\omega_0, \omega_H, \tau_D, \tau_N) \right] + \left[ 7 Q + 3 \right] \times F(\omega_0, \tau_D, \tau_N) \} \]

\[ R_2 = \frac{1}{3} C_2 \times \{ 13 F \right[ \Omega(\omega_0, \omega_H, \tau_D, \tau_N) \right] + Q \left[ 7 F(\omega_0, \tau_D, \tau_N) \right] + 6 F(0, \tau_D, \tau_N) \}

+ 3 \left[ 3 F(0, \tau_D, \tau_N) \right] + 4 F(0, \tau_D, \tau_N) \}

\[ C_1 = \left( \frac{32 \pi}{1350000} \right) \frac{\mu^3_{SP}}{R D} \left( \frac{N_{SP}}{R D} \right) = \frac{32}{16} C_2 \]

where $\mu_{SP}$ is the magnetic moment of the nanoparticles, $\gamma_H$ the gyromagnetic ratio of protons, $N_{SP}$ the number of particles per litre, $R$ the particle radius, $D$ the diffusion coefficient, $\tau_D$ is the translational correlation time for the particles and the water molecules, $\tau_N$ is the Néel relaxation time, $\omega_0$ and $\omega_H$ are the electron and proton Larmor angular frequencies, and $F$ is a spectral density function accounting for the proton diffusion in the non uniform magnetic field created by $\mu_{SP}$, and its fluctuation around its mean value, $\omega_0$ is an adjustable parameter that considers the anisotropy field in the electron Larmor angular frequency ($\omega_0 < \omega_S$), and $P$ and $Q (P+Q \leq 1)$ are weighing factors for zero and high anisotropy energies, respectively. Figure 6 shows that this approximation is valid up to 1 MHz.
As $C_1$ and $C_2$ in Eq. 1 and 2 hardly change with the magnetic core diameter $D_P$, in the low frequency range, the most important contribution to the size dependence of $R_1$ and $R_2$ comes from the term $\mu_{SP}^2N_{SP}/R$. It is important to note that the iron concentration, commonly used to normalize the NMRD curves of different samples, is implicit in the particle density or number of particles per litre ($N_{SP}$). So that, for a fixed iron concentration, the particle density differs among samples with different particle size.

Figure 7 shows that $r_1$ and $r_2$ absolute values at low frequency increase quite linearly with $\mu_{SP}^2N_{SP}/R$. Therefore, the increase of $r_1$ and $r_2$ along the series of samples is caused by an increment of $\mu_{SP}$.
Samples with the highest $r_2$ values at 8.5MHz (C and D, Fig. 5b) were selected for MRI experiments. Prior to imaging, the iron concentration of all samples was carefully fixed at 0.02 g/L.

In Figure 8 images of samples C, D and the commercial CA are presented for two different pulse sequences a) High resolution Gradient Echo and b) High resolution Spin Echo. It is apparent for both sequences that sample D signal is darker than Endorem and therefore shows a better performance as contrast agent at the imager operating frequency (8.5MHz).
Conclusions

We report on the efficiency as CA for MRI of a series of maghemite/polymer composite ferrofluids, made of fully biocompatible ingredients, with several particle sizes. These ferrofluids have proven to be very interesting model systems for both fundamental studies of proton relaxation induced by superparamagnetic nanoparticles and practical MRI applications. It is found from the behavior of $r_1(\nu)$ that above a threshold particle size value of around 8 nm the relaxation process is dominated by the magnetic anisotropy contribution. Both longitudinal and transverse relaxivities show a strong increase with the particle size in relation to the increase of magnetic moment. This is in accordance with predictions from A. Roch and R.N. Muller theoretical model[25]. The sample with the highest particle size, D=15 nm, has demonstrated a capacity as MRI contrast agent superior to a well-known commercial product both in transverse relaxation measurements and in vitro MRI experiments.

Acknowledgments

Financial support from the Spanish Ministry of Science and Innovation research grants BFU2009-12763/BFI, MAT2007-61621, and Project Consolider-Ingenio in Molecular Nanoscience CSD2007-00010 are gratefully acknowledged. Thanks to EU-NoE MAGMANet for partly funding the project, and to E. Micotti, F. Orsini, and M. Pasin for their collaboration. R. Bustamante wants to thank ICMA-CSIC for the JAE-predoc grant.
References

[23]. P. Langevin, Annales de Chimie et de Physique, 5, 70 (1905).