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The use of magnetic nanoparticles as contrast agents in magnetic resonance imaging (MRI) has been extensively explored in recent years due to their unique properties and potential applications in various medical fields. These nanoparticles are commonly divided into two types: T₁ or T₂ contrast agents, with the relaxation time being the primary criterion for classification (T₁ or T₂ contrast agent).

Magnetic nanoparticles have been investigated in the last decade for multiple purposes in nanomedicine such as drug delivery nanocarriers, hyperthermia and magnetic resonance imaging contrast agents. In the field of MRI contrast agents, many efforts have been undertaken to develop more powerful and sensitive materials that on the one hand could improve the current contrast agents in terms of relaxation times, both longitudinal or T₁, and transverse or T₂, and on the other hand, could increase the circulation time and reduce cytotoxicity. MRI contrast agents are sorted into two types: T₁ contrast agents, which are commonly called positive contrast agents due to the signal enhancement they produce on T₁-weighted images; and T₂ or negative contrast agents, which produce darkening on T₂-weighted images. The ability of given contrast agent to decrease the T₁ or T₂ relaxation times of the surrounding water protons is called relaxivity, r₁ or r₂ (expressed in mM⁻¹ s⁻¹), and the ratio r₂/r₁ indicates the capacity of the material to be used as T₁ or T₂ contrast agent. Lower values of r₂/r₁ (between 1-3) lead to T₁ type, higher values (> 10) to T₂ type, and the intermediate values, between 3-10, can be considered as dual T₁ and T₂ contrast agents.

Gadolinium (Gd) chelates are the most commonly used MRI contrast agents in the clinical practice. They produce T₁ shortening of the water protons through chemical exchange of the coordinated water with the bulk water, which translates into signal enhancement on T₁-weighted images (positive contrast). These contrast agents are rapidly excreted through renal filtration and are normally used to visualize areas with leaky vessels (inflammation, tumors, etc.), where they are retained due to the enhanced permeability and retention (EPR) effect or passive targeting. For molecular imaging, which is typically based on active targeting, longer circulation times and higher sensitivity are required. Gd nanoparticles arose as a promising alternative to Gd chelates because they combine both very high relaxivity, due to the concentration of Gd ions per nanoparticle, and long circulation times. However, it has been shown that Gd
ions, leached from either complexes or nanoparticles could become highly toxic in vivo, thus leading to important renal problems such as nephrogenic systemic fibrosis. On the contrary, superparamagnetic iron oxide nanoparticles (SPIONs) show very low toxicity. Indeed, SPIONs have been approved by the FDA and have been applied successfully in the clinical practice as bowel contrast agent (i.e., Lumirem® and Gastromark®) and also for liver/spleen imaging (i.e., Endorem® and Feridex IV®). Another advantage of SPIONs is that they can be easily synthesized with size control and low cost production via thermal decomposition methods. In terms of relaxivity, they show very high $r_2/r_1$ values, being postulated good $T_2$-type contrast agents for molecular imaging. However, one of the main concerns regarding $T_2$ contrast is that the $T_2$-weighted MRI sequences used to visualize the signal voids they produce are prone to susceptibility artifacts caused by air, hemorrhage or metal implants, which can lead to misinterpretations, and consequently positive contrast is usually preferred.

It was recently reported that decreasing the size of the iron oxide nanoparticles below 4 nm enhances their efficiency as $T_1$ contrast agents and hence it could be used as a strategy to obtain dual contrast agents. Unfortunately the increase in $r_1$ was accompanied by a significant decrease of $r_2$, leading to low $r_2/r_1$ values, and limiting the use of these nanoparticles as $T_1$ contrast agents. Manganese doped-iron oxide nanoparticles are good candidates to overcome this limitation. The incorporation of a paramagnetic ion in the spinel ferrite structure induces an increase in the mass magnetization of the material leading to an improvement of the magnetic properties of the materials. The use of manganese for biomedical application, however, brings another concern into the picture, the high toxicity of Mn$^{2+}$ ions. It becomes then crucial to avoid metal leaching from the nanoconjugates. Many different coating strategies have been developed to protect the inorganic core of the magnetic nanoparticles from the surrounding media, and to increase their stability in biological environments. The coating strategies can be sorted in two main categories, the micellar encapsulation and the ligand exchange. Micellar encapsulation is the most universal and extended method, based on the addition of a second layer of an amphiphilic polymer that interacts in a non-covalent manner with the outer hydrophobic layer of the nanoparticle. This interaction is not stable enough and further functionalizations are required to enforce the stability of the organic shell around the inorganic core. The other coating strategy, ligand exchange, requires the use of a ligand molecule much more reactive and with stronger binding than the surfactant attached to the nanoparticle during the nanoparticle synthesis. Thus, a standard ligand molecule would present an anchor group in one side that binds to the nanoparticle surface and a reactive group on the other side. The stability in aqueous media of the ligand-exchanged magnetic nanoparticles depends somehow on the binding interaction between the anchor group of the ligand and the surface of the magnetic nanoparticles, and also on the hydrophilic balance of the functionalized nanoparticles.

The polyethylene glycol (PEG) chain is an ideal hydrophilic spacer for the ligand exchange because it not only modulates the hydrophilic balance of the magnetic nanoparticles, but also supports resistance to protein adsorption and helps the nanoparticles to improve their circulation life time and to decrease the reticuloendothelial clearance rate.

We report herein a facile method to functionalize manganese ferrite nanoparticles (MNPs) by a simple ligand exchange. The ligand molecule presents an anchor group (gallol derived) that binds strongly to the MNP surface and a tunable and biocompatible hydrophilic PEG part that allows the stabilization of the MNPs in aqueous media. Also, the ligand exchange takes place by a gentle shaking in few seconds without perturbing the magnetic characteristics of the manganese ferrite nanoparticles. Furthermore, we demonstrate the possibility to tune the relaxivities $r_1$ and $r_2$ of PEGylated MNPs based on their size and their use as MRI contrast agents with long circulation times and potential tumour targeting.

Results and Discussion

Synthesis of water soluble Manganese Ferrite Nanoparticles

Monodisperse manganese ferrite nanoparticles (MNPs) with different diameter sizes were synthesized by following the seed-mediated growth protocol previously described by Sun and coworkers. The 6 nm MNP seeds were synthesized and by a successive growth, 7.5, 9, 12 and 14 nm MNPs, were synthesized. These MNPs were capped with oleic acid to stabilize them in organic solvents such as toluene or hexane, but a further approach is required to transfer them to aqueous media. The easy and novel proposed approach to render hydrophilic manganese ferrite nanoparticles is based on the ligand exchange method developed by us on super-paramagnetic iron oxide nanoparticles (SPIONs). The success of this approach, reported elsewhere, relied on several factors: i) high yield and stability of SPIONs in aqueous media and ii) low-cost and facile synthesis of the PEG-Gallol derived ligand with the possibility to modify further the end group of the PEG chain (for instance -OH or –NH$_2$). Therefore, we synthesized the Gallol-PEG-OH ligand following the reported procedure (see Experimental Section) with a modification in the length of the PEG chain to 3000 Da of molecular weight. We have chosen a 3000 Da PEG spacer because it is within the optimal molecular weight range (1500-5000 Da) to minimize the phagocytic response of macrophages thus extending the nanoparticles circulation time. The anchor of the ligand is the gallol group, which probably reacts through strong electrostatic interactions between the MNP surface and the hydroxyl groups of the gallol derived in the same way as iron oxide nanoparticles. The ligand exchange procedure (see details in Experimental Section) takes place by a gentle shaking in the presence of a nucleophilic base as triethylamine (Fig. 1A).

Indeed, the replacement occurs smoothly and instantaneously, thus leading to water soluble manganese ferrite nanoparticles.
One of the main problems during the transfer of the NPs to water is the aggregation with the consequent precipitation of the particles and the modification and/or loss of their magnetic properties. This aggregation could be possibly related to several physicochemical factors such as weak ligand-NP interactions, poor hydrophilic/hydrophobic balance, and surface charge density among others. Also, it is important to take into account the physiological factors that could affect the magnetic properties and stability of the MNPs in vivo (discussed in detail in the in vivo MRI studies section). In our procedure, the two first problems are solved with the use of a gallol derived and a 3000 Da PEG spacer; the third one, the surface charge density over the nanoparticle, is solved by minimizing the charges of the ligand, the more neutral it is, the more stable our nanoparticles will be. Therefore, the end group of the ligand in our case is a hydroxyl group. Moreover, that functionality will allow us to attach vectors such as biomolecules or drugs for targeting and treatment of different diseases in future investigations.

**Characterization of water soluble MNPs**

As mentioned above, the properties of the nanoparticles could potentially be affected with the functionalization. To demonstrate that our ligand exchange method on manganese ferrite nanoparticles does not affect the magnetic and physicochemical properties of the as-prepared nanoparticles, we performed an exhaustive characterization of the MNPs in water solution. Firstly, the mean diameter sizes of the MNPs were determined by TEM and dynamic light scattering (DLS). TEM characterization could not confirm the presence of the Gallol-PEG-OH on the MNPs, but it was observed that the Gallol-PEG-MNPs were well dispersed and separated on the TEM grid with respect to the as-prepared MNPs (Fig. 1B-D, and Fig. S1 and S2). On the contrary, by DLS we could observe a notable increase (between 14 and 19 nm) in the hydrodynamic diameter of the manganese nanoparticles, indicating a change in the coating of the MNPs (Fig. 2A). Moreover, negative and minimum (Z)-potential values were measured under physiological pH and osmolality conditions for the water soluble MNPs (Fig. 2A), in accordance with the premises commented above for long-time circulation nanoparticles in vivo. Then, the presence of the ligand Gallol-PEG-OH attached to the MNPs was confirmed by FTIR spectroscopy, the FTIR spectrum exhibited the corresponding signals to the PEG in comparison with the starting manganese ferrite nanoparticles, which exhibited the oleic acid (surfactant) signals (Fig. 2B, and Fig. S3).
To demonstrate that the signals corresponded to the ligand attached to the manganese ferrite nanoparticles and they were not due to the ligand in solution, two different purification methods were applied: i) dialysis through a membrane (MWCO: 25 kDa) during 3 days exchanging milli-Q water at 3 and 8 hours with fresh milli-Q water, and ii) further purification into a centrifugal PALL filter (MWCO: 100 kDa) at 450 rcf, being this step repeated until a clear solution was obtained (Fig. S4 and Fig. S5). It was observed that during the first purification by dialysis, the MNPs did not collapse, thus indicating the high stability of the functionalized MNPs in water. The percentage of the organic shell around the MNPs was evaluated by thermo-gravimetric analysis (TGA), giving a range of 42-53% of organic material (Fig. S6). Based on the TGA results and the TEM average diameters, the dispersant packing density revealed a proportional increase from 1.1 to 2.8 molecules per nm² for the 6 and 14 mM PEGylated MNPs respectively. This corresponds to higher dispersant packing density values for larger nanoparticles due to lower steric hindrance among the ligands (Table S1). The magnetic properties of the MNPs (6, 7.5, 9, 12 and 14 nm) were investigated by Vibrating Sample Magnetometer (VSM) at room temperature. The values of mass magnetization (Ms) of the water soluble MNPs were in good agreement with the findings obtained by Cheon and coworkers for manganese-doped iron oxide nanoparticles, in which the bigger the magnetic nanoparticles, the higher the Ms values, thus showing Ms values of 50, 60, 72, 92 and 106 emu/g (Mn+Fe) for 6, 7.5, 9, 12 and 14 nm size water soluble manganese ferrite nanoparticles, respectively (Fig. 2C). In addition, X-ray diffraction (XRD) analysis of the MNPs revealed that the mild conditions of ligand exchange did not affect the structure of the nanoparticles observing the characteristic peaks for the ferromagnetic spinel phase (Fig. 2D, and Fig. S7).

To evaluate the feasibility of the MNP-GA-PEG-OH as potential T₁ and T₂ MRI contrast agents, the relaxivities were measured on a 1.5 T relaxometer, and a 9.4 T pre-clinical MRI scanner. In the case of transverse relaxivity, r₂ a similar trend was observed in both magnetic resonance spectrometers (1.5 and 9.4 T), bigger size MNPs (such as 14 nm) exhibited higher relaxivity values (301 and 336 mM⁻¹s⁻¹ respectively) in agreement with the mass magnetization values trend (Fig. 3A). This means that the Ms value of the manganese ferrite nanoparticle directly correlates with NP size, as commented above, due to well-known spin canting effects that affect the smaller magnetic nanoparticles dramatically, and in the same degree their r₂. On the contrary, r₁ did not show the same trend, being significant at low magnetic field, but negligible at high field (Fig. 3B). This might be due to the fact that r₁ is dominated mainly by interactions between bulk water protons and electron spins of the nanoparticles, and the diffusion time of water close to the inner sphere of the MNP. Therefore, the r₁ values for the MNPs-GA-PEG-OH were found similar independently of their sizes, between 7 and 18 mM⁻¹s⁻¹ at 1.5 T, but with a resulting r₂/r₁ < 5 for the smaller MNPs (6 and 7.5 nm). This r₂/r₁ values fall within the intermediate range that characterizes dual T₁ and T₂ contrast agents. In contrast, at 9.4 T the r₂/r₁ ratios increased dramatically, above 78, for 6 and 7.5 nm MNPs because at very high magnetic fields r₁ decreases to values proximate to zero, whereas r₂ increases, which limits the use of these MNPs-GA-PEG-OH as T₂ contrast agents at the high fields typically used in preclinical studies (Fig. 3C). Therefore, for the in vivo studies in animal models we used T₁-weighted MRI sequences, as discussed in detail below. However, it is noteworthy to mention that at clinical magnetic fields they could be used as dual contrast agents, i.e., both T₁ and T₂, which is the ultimate goal of the PEGylated nanoparticles developed in the current work.
number. This is in close agreement with the flow cytometry data performed at the highest dose of nanoparticles. As it is shown in the scatter plot, PC-3 treated cells were no necrotic and the analysis of the subG1 cell cycle revealed no features of apoptosis, with no differences compared to untreated PC-3 cells (Fig. 4C). Thus, taken together MNP-GA-PEG-OH with a ranging size from 6 to 14 nm seem to be compatible with key parameters related to cell viability.

In vivo MRI Studies

For in vivo studies, two extreme manganese nanoparticles were chosen based on their size and relaxivity values, the smallest one, 6 nm MNP-GA-PEG-OH, which presents a hydrodynamic diameter in physiological medium of 20 nm and a $r_2$ of 64 mM$^{-1}$s$^{-1}$ at high field, and the biggest one, 14 nm MNP-GA-PEG-OH with a hydrodynamic diameter in physiological medium of 32 nm and $r_2$ of 336 mM$^{-1}$s$^{-1}$ at high field. A five times higher $r_2$ value in the 14 nm MNPs could lead to the use of less contrast agent material, therefore improving the efficiency and cost-effectiveness of the synthesis. Although at first sight the difference in size is not noticeable, at nanometer scale an increase of 60% in the hydrodynamic diameter could possibly affect the in vivo distribution and circulation times, which are two of the main concerns when considering the use of magnetic nanoparticles as contrast agents. Therefore, after intravenous injection of the MNPs, we followed the time-courses in vivo for 1 h in different tissues, and quantified $T_2$ changes at 0, 1, 24 and 48 h. For this purpose, 6 and 14 nm MNP-GA-PEG-OH (5 mg Fe-Mn per Kg of body weight) were intravenously injected in the tail vein of Balb/c mice, $T_2$-weighted MR images acquired every 2 minutes, and the time-courses calculated from regions of interest (ROIs) in kidneys, liver, spleen and muscle (Fig. 5). Both MNPs exhibited similar liver uptake rates (Fig. 5, black curves) with a maximum concentration of MNPs (maximal signal decay) at 6 min after injection, which remained stable for the rest of the time course. Significant kidney accumulation was also observed for both MNPs, as shown in Fig. 5 (red curves).

Interestingly, some renal clearance seemed to happen as well (Fig. 6), in spite of the hydrodynamic diameters (HD) of our MNPs (20 and 32 nm), which exceed the well established size exclusion limit (6-8 nm) for glomerular filtration. To demonstrate that our MNPs are partially excreted through the kidneys, we collected urine from 1 to 24 h after intravenous injection of our MNPs and analyzed it by transmission electron microscopy and Energy dispersive X-Ray (EDX) analysis (Fig. S8), which revealed the presence of the MNPs, although we could not verify their size due to interferences with the organic material present in the urine. A possible explanation for this phenomenon is that the MNPs shrink or lose part of the organic shell in the kidneys, likely related to the acidic environment, being thus able to cross the glomerular barrier. Muscles did not show any change in $T_2$ (Fig. 5, brown curve), indicating that, as expected, our MNPs did not reach the extravascular extracellular space.

![Image](image-url)
Long term clearance was evaluated by quantitative $T_2$ mapping at 0, 1, 24 and 48 h. Fig. 7 and Fig. 8 show representative high resolution $T_2$-weighted images and the $T_2$ values measured at the different experimental times. Both liver and kidneys showed significant decrease in $T_2$ at 1 h, with $\Delta T_2$ of -6 and -7 in the liver and -19 and -19.8 in the kidneys for the 6 and 14 nm MNPs, respectively, indicating that our MNPs are slowly taken up by the reticuloendothelial system (RES), whereas they continue circulating in the bloodstream, and hence passing through the kidneys where they are partially excreted to urine, as mentioned above. As nicely shown by Choi et al., retention of PEGylated NPs within the renal corpuscles of the kidney cortex is size dependent, with maximal retention times for NPs in the range of 76-96 nm of hydrodynamic diameter, and virtually no retention at all for smaller NPs, with HD around 25 nm, which is very close to the HD of the MNPs reported herein. These small NPs were only found in peritubular capillaries. Hence, the MRI contrast detected in the kidneys can be attributed to circulating MNPs that pass through the peritubular capillaries and return to the bloodstream until they are fully excreted through the liver. For the 6 nm MNPs the $T_2$ values recovered only partially in the liver after 48 h ($\Delta T_2$ = -1.6), but were fully restored to normal values in the kidneys after 24 h ($\Delta T_2$ = -0.1), suggesting that these MNPs are no longer circulating in the blood and that a small amount is still trapped in the kupffer cells after 48 h. However, for the 14 nm MNPs nor the liver neither the kidneys showed full recovery to normal $T_2$ values after 24 h, and only after 48 h the kidneys restored the normal values ($\Delta T_2$ = +0.1), which suggests longer circulation times for these MNPs as compared to the smaller one. These circulation times are also in good agreement with those reported by Choi et al., where they described blood circulation half-lives ranging from 7 to 38 h for NPs with HD between 25 and 164 nm, being half-lives and HD inversely correlated. According to this finding, the 6 nm (20 nm HD) MNP is expected to have longer circulation times as compared to the 14 nm (32 nm HD) MNP. However, our data show the opposite. This finding could be explained by the unexpected...
renal clearance we described above, which very likely acts more efficiently for the smaller one. The long circulating times together with the high relaxivity of our MNPs makes them an excellent candidate for MRI-based molecular imaging. In addition, the smaller MNPs, with sizes between 6 and 7.5 nm, show $r_2/r_1$ values within the intermediate range at 1.5 T, holding promise as dual contrast agents for clinical applications.

![Fig. 8](image)

**Table 2**

<table>
<thead>
<tr>
<th>14 nm MNPs</th>
<th>$T_2 (ms^{-1})$</th>
<th>$\Delta T_2 (ms^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidneys</td>
</tr>
<tr>
<td>0</td>
<td>20.2</td>
<td>52.7</td>
</tr>
<tr>
<td>1</td>
<td>13.2</td>
<td>32.9</td>
</tr>
<tr>
<td>24</td>
<td>17.9</td>
<td>44.4</td>
</tr>
<tr>
<td>48</td>
<td>19.4</td>
<td>52.8</td>
</tr>
</tbody>
</table>

Fig. 8. Representative $T_2$-weighted MR images at different experimental times after the intravenous injection of 14 nm MNP-GA-PEG-OH. A-D) $T_2$-weighted images of kidneys and muscle at 0, 1, 24 and 48 h respectively after injection. E-H) $T_2$-weighted images of liver and spleen at 0, 1, 24 and 48 h after injection. Table 2 shows average $T_2$ and $\Delta T_2$ values of the different tissues.

**Experimental Materials**

Reagents were obtained from commercial suppliers and used without further purification. Iron (III) acetyl acetone, Manganese (II) acetylacetonate, Oleic acid 99%, Oleylamine, Benzyl ether, 1,2-Hexanediol, Gallic acid, Poly ethyleneglycol 3kDa, Triethylamine, 4-Dimethylaminopyridine were supplied by Sigma Aldrich and Acros Organics. As solvents, Milli-Q water (18.2 MΩ, filtered with filter pore size 0.22 µM) from Millipore, toluene, isopropanol, ethanol, acetone, hexane, and anhydrous tetrahydrofuran were used (HPLC grade, Acros Organics). Purification of the water-soluble magnetic nanoparticles from free reagents was performed on a centrifuge filters PALL with a molecular cut-off of 100 kDa. Dynamic light measurements were performed on a Zetasizer Nano ZS90 (Malvern, USA). FTIR spectra were recorded with a FTIR-4100 Jasco. $^1$H-NMR spectrum was recorded on a NMR Bruker Ascend™ 400MHz spectrometer. Fe and Mn concentrations were determined on an Inductively Coupled Plasma High Resolution Mass Spectroscopy (ICP-HRMS, Element XR, Thermo Fisher). TEM images were obtained on a FEI Tecnai G2 Twin microscope operated at an accelerating voltage of 100 kV. TEM samples were prepared by dropping the solution on a carbon-coated copper grid and...
letting the solvent evaporate. Thermogravimetric analysis (TGA) was carried out by using a METTLER TOLEDO model TGA/DSC 1 in the temperature range 30–800 °C with a heating rate of a 10 °C/min under N₂ flow (50 mL/min). X-ray diffraction patterns were collected with a PANalytical X’Pert PRO MPD diffractometer equipped with CuKα1 radiation in the 20 range of 10–80°. Samples were prepared by drop casting of a high concentrated magnetic nanoparticle solution onto a glass. The magnetization-field M-H curves were performed in a Vibrating Sample Magnetometer (VSM) at room temperature.

Synthesis of 3 kDa pegylated galol anchor (Gallol-PEG)
The synthesis of ligand Gallol-PEG was performed similar to a previously established synthetic route, briefly 8.824 g of 3 kDa molecular weight polyethylene glycol (2.94 mmol), 0.5 g of gallic acid (2.94 mmol) and 71 mg of 4-Dimethylaminopyridine (DMAP, 0.294 mmol) were dissolved in 200 mL of anhydrous THF in a round bottom flask under inert atmosphere. A solution of Dicyclohexil carbodiimide (DCC) (3.03 g, 14.7 mmol, 50% purity) was added to the mixture of 2 mmol of iron acetate, 3.85 mmol of triethylamine and 1 mL of oleic acid capped manganese ferrite NPs (10 g/L in toluene) was shaking in a separation funnel. The mixture was diluted with 5 mL of toluene and 5 mL of Milli-Q water. After shaking, 10 mL of acetone was added to transfer completely the NPs into the aqueous phase. The aqueous phase was collected and placed in a rotovaporator to remove the residual organic solvents. After that, the PEG-MNPs were purified in centrifuge filters PALL (Molecular weight cut-off: 100 kDa) at 450 rcf. The purification step was repeated minimum 3 times, adding physiological medium, till the filtered solution was completely clear. The solution of PEG-MNPs was placed onto a permanent magnet (0.6 T) for 5 min to remove the bigger aggregates.

In vitro Relaxivities

Typically, a solution of 1.0 mL of Gallol-PEG (0.1 M in CHCl₃), 50 uL of triethylamine and 1.0 mL of oleic acid capped manganese ferrite NPs (10 g/L in toluene) was shaking in a separation funnel. The mixture was diluted with 5 mL of toluene and 5 mL of Milli-Q water. After shaking, 10 mL of acetone was added to transfer completely the NPs into the aqueous phase. The aqueous phase was collected and placed in a rotovaporator to remove the residual organic solvents. After that, the PEG-MNPs were purified in centrifuge filters PALL (Molecular weight cut-off: 100 kDa) at 450 rcf. The purification step was repeated minimum 3 times, adding physiological medium, till the filtered solution was completely clear. The solution of PEG-MNPs was placed onto a permanent magnet (0.6 T) for 5 min to remove the bigger aggregates.

Synthesis of manganese ferrite nanoparticles (NPs)

Monodisperse manganese ferrite nanoparticles were synthesized following the seed-mediated growth protocol reported by Sun et al for iron oxide nanoparticles. Synthesis of 6 nm MnFe₂O₄ nanoparticles seeds: 2 mmol of iron acetylacetonate, 1 mmol of manganese acetylacetonate, 10 mmol of hexadecanediol, 6 mmol of dodecylamine, 6 mmol of oleylamine and 20 mL of benzyl ether were mixed and magnetically stirred. The solution was heated to 200 °C for 2 h under a flow of nitrogen, followed by increasing the temperature to reflux (300 °C) for 1 h. The mixture was cooled down to room temperature by removing the heating mantle. Finally, the sample was washed several times using ethanol, acetone and isopropanol as precipitation agents and centrifugation, followed by re-dispersion in toluene. Synthesis of 7.5 nm MnFe₂O₄ nanoparticles via 6 nm MnFe₂O₄ seeds: 80 mg of 6 nm MnFe₂O₄ seeds dispersed in hexane (1 mL) were added to the mixture of 2 mmol of iron acetylacetonate, 1 mmol of manganese acetylacetonate, 10 mmol of hexadecanediol, 6 mmol of dodecylamine, 6 mmol of oleylamine and 20 mL of benzyl ether were mixed and magnetically stirred. The solution was heated to 60 °C for 1 h under vacuum to remove the hexane, and then the mixture was heated to 200°C for 2 h under a nitrogen flow. Soon after, the mixture was allowed to reflux (300 °C) for 1 h. The mixture was cooled down to room temperature by removing the heating mantle. Finally, the sample was washed several times using ethanol, acetone and isopropanol as precipitation agents and centrifugation, followed by re-dispersion in toluene.

Similarly, 80 mg of 7.5 nm MnFe₂O₄ seeds reacted with 2 mmol of iron acetylacetonate, 1 mmol of manganese acetylacetonate, 10 mmol of hexadecanediol, 6 mmol of dodecylamine, 6 mmol of oleylamine and 20 mL of benzyl ether led to 9 nm MnFe₂O₄ nanoparticles. Using the same seed-growth procedure, 12 and 14 nm MnFe₂O₄ nanoparticles were synthesized.

Ligand exchange on manganese ferrite nanoparticles (MNPs)

Typically, a solution of 1.0 mL of Gallol-PEG (0.1 M in CHCl₃), 50 uL of triethylamine and 1.0 mL of oleic acid capped manganese ferrite NPs (10 g/L in toluene) was shaking in a separation funnel. The mixture was diluted with 5 mL of toluene and 5 mL of Milli-Q water. After shaking, 10 mL of acetone was added to transfer completely the NPs into the aqueous phase. The aqueous phase was collected and placed in a rotovaporator to remove the residual organic solvents. After that, the PEG-MNPs were purified in centrifuge filters PALL (Molecular weight cut-off: 100 kDa) at 450 rcf. The purification step was repeated minimum 3 times, adding physiological medium, till the filtered solution was completely clear. The solution of PEG-MNPs was placed onto a permanent magnet (0.6 T) for 5 min to remove the bigger aggregates.

Cytotoxicity assays

The PC-3 human prostate cancer cell line was kindly provided by Dr. M. Japon (Department of Endocrine Tumorigenesis and Hormonal Regulation of Cancer, Biomedicine Institute of Seville, IBIS, CSIC-University of Seville, Spain). The cells were maintained in RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (BioWhittaker), 1% L-glutamin, 100 IU/mL penicillin and 100 IU/mL streptomycin (BioWhittaker) in a humidified atmosphere of 5% CO₂ at 37 °C. PC-3 cells were seeded overnight in 96-well plates at a density of 40,000 cells per well, in a final volume of 200 μL. For 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) determinations, the media was replaced the day after with fresh media containing MNP-GA-PEG-OH at concentrations ranging from 100 μg/mL to 100 ng/mL for 24 h. After treatment, the supernatant of each well was saved for LDH measurement and replaced by 100 μL of media containing MTT labelling reagent (Roche Diagnostics GmbH, Mannheim, Germany) at a final concentration of 0.5 mg/mL. After further incubation for 4
hours in a humidified atmosphere (37 °C, 5% CO₂), 100 μL of the solubilization solution was added to each well. The plates stood overnight in the incubator in a humidified atmosphere after complete solubilisation of the formazan crystals. Then, spectrophotometric absorbances were determined using a microplate (ELISA) reader at 570 nm with 660 nm as reference wavelength. LDH release after 24 h incubation with MNP-GA-PEG-OH was determined in the saved supernatant according to manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany). Each experimental condition was performed in triplicate. For detection of apoptotic cells by flow cytometry, PC-3 cells were seeded overnight in 12-well plates at a density of 120,000 cells per well, in a final volume of 1 mL, and treated as stated before. At the end of the experiment, the cells were fixed in an ice-cold solution of 70% ethanol for at least 24 h, incubated 10 minutes in DNA extraction buffer (Na₂HPO₄ 0.2M, pH 7.8), and then incubated with 0.1% (w/v) RNAse and 50 μg/mL propidium iodide at 37°C for 30 min before analysis for DNA content by flow cytometry using cell quest software.

**In vivo MRI**

Mice experiments were performed in accordance with the ethical guidelines of Andalusian government. Female Balb/c mice with ca. 30 g in weight, provided by Janvier Labs were used. All the experiments were performed on a 9.4 T Bruker Biocor system equipped with a 400 mT/m gradients and a 40 mm quadrature bird-cage resonator. High resolution T₂-weighted images were acquired using a turbo-RARE sequence with respiratory gating (TE = 16 ms, TR = 1000 ms, 4 averages, 156 μm in-plane resolution and 1 mm slice thickness). Quantitative T₂ measurements were also performed using a multi-echo spin echo sequence (TEs ranging from 7 ms to 448 ms, TR = 3500 ms, FOV = 4 cm, matrix size = 128x128, slice thickness = 1 mm). The time-courses were followed by using a turbo-RARE with the same parameters indicated above, but only 1 average to improve temporal resolution (1 image every 2 minutes). The acquisition scheme was as follows: T₂-weighted, quantitative T₂, intravenous injection of the MNPs, time-course for one hour, quantitative T₂ and T₂ weighted. The MNPs were administered intravenously via tail vein at a concentration of 5 mg Fe/Mn per kg.

**Conclusions**

In this work, a new facile and fast ligand exchange method to transfer in aqueous media different sizes manganese iron oxide nanoparticles has been developed. A complete characterization over the nanoparticles confirmed the innocuous ligand exchange approach thus keeping the magnetic properties of the as-prepared MNPs, such as X-ray diffraction patterns of spinel metal ferrite crystal and high saturation magnetization values, and also these water soluble MNPs presented a low degree of cytotoxicity. The 6 nm MNP-GA-PEG-OH resulted to be good dual T₁ and T₂ contrast agents at clinical magnetic field, whereas the 14 nm MNP-GA-PEG-OH behave as excellent T₂ contrast agents at both low and high fields. The possibility to tune easily the low-cost ligand synthesis will enable to further functionalize the MNPs for targeting strategies. The water soluble MNPs displayed a high dense PEG core shell that prevents the rapid uptake by the reticuloendothelial system and permits long blood circulation times up to 24 h. The total clearance of the MNPs from the body took 24 h and 48 h for the 6 nm and 14 nm MNPs, respectively. The combination of low toxicity, excellent T₂ relaxation times, as well as excellent r₂/r₁ values at low field, together with long circulation times, make these nanomaterials a very promising contrast agent for MRI-based molecular imaging at clinical magnetic fields. The specific targeting of these MNPs could lead to the development of new methods for early diagnosis of different diseases and the tailoring healthcare to patients’ individual needs through a personalized medicine approach based on MNPs.

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**Notes and references**

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† Electronic Supplementary Information (ESI) available: Additional experimental details included TEM images of MNPs, size distribution graphs, thermogravimetric analysis, FTIR spectra, and XRD patterns of MNPs. See DOI: 10.1039/b000000x/