Stability and Relaxation Mechanisms of Citric Acid Coated Magnetite Nanoparticles for Magnetic Hyperthermia

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ABSTRACT: Magnetite (Fe3O4) nanoparticles are proper materials for Magnetic Fluid Hyperthermia applications whenever these conjugate stability at physiological (neutral pH) medium and high specific dissipation power. Here, magnetite nanoparticles 9–12 nm in size, electrostatically stabilized by citric acid coating, with hydrodynamic sizes in the range 17–30 nm, and well dispersed in aqueous solution were prepared using a chemical route. The influence of media acidity during the adsorption of citric acid (CA) on the suspension’s long-term stability was systematically investigated. The highest content of nanoparticles in a stable suspension at neutral pH is obtained for coating performed at pH = 4.58, corresponding to the larger amount of CA molecules adsorbed by one carboxylate link. Specific absorption rates (SARs) of various magnetite colloids, determined calorimetrically at a radio frequency field of 265 kHz and field amplitude of 40.1 kA/m, are analyzed in terms of structural and magnetic colloid properties. Larger dipolar interactions lead to larger Néel relaxation times, in some cases larger than Brown relaxation times, which in the present case enhanced magnetic radio frequency heating. The improvement of suspension stability results in a decrease of SAR values, and this decrease is even large in comparison with uncoated magnetite nanoparticles. This fact is related to interactions between particles.

1. INTRODUCTION

Stable dispersion of magnetic nanoparticles (MNPs) has attracted much attention, in a first stage because of their rheological properties that allow dynamic control by applied fields and more recently due to their widespread biomedical diagnostic and therapeutic applications such as visualization agents in magnetic resonance imaging, therapeutic carriers in drug delivery, heat intermediaries in cancer treatment therapies, and labelers for in vitro and in vivo separation experiments. The usefulness of the magnetic colloids for biomedical application depends on their biocompatibility, the stability of the magnetic nanoparticles in solution at neutral pHs, and the capability of MNP surfaces to become chemically functionalized.

Among many studied materials, Fe and its oxides are the unique FDA (US Food and Drug Administration) accepted ones. The amphoteric surface of Fe oxides facilitates their functionalization. Subsequently, many protocols have been developed for its preparation by either high-temperature decomposition of an organic iron precursor or low-temperature coprecipitation. The MNPs produced by high-temperature decomposition methodology display better structural and magnetic properties but also hydrophobicity, and further work on MNP surfaces for their stabilization in neutral conditions is needed. Stability in suspension is a major requirement for any biomedical application that involves injection in a living human being and results from the interplay between attractive dipolar and van der Waals interactions and repulsive electrostatic and steric interactions.

Steric stabilization includes coating with nonionic surfactant molecules, polymers, and inorganic layers as silica or some noble metals. Among the routes employed for obtaining the water-soluble functionalized iron oxide MNPs, the addition of small biocompatible organic molecules, such as amino acids, peptides, citric acid, and cyclodextrin, presents the advantages of combining electrostatic and steric stabilization, assuring coating biocompatibility and providing functionality for biomolecule conjugation. Comparing polymeric stabilization with electrostatic one presents the advantage of reversibility from the aggregated state by dilution but usually involves the binding of a large macromolecule resulting in a coated MNP with a large overall size which strongly modifies its hydrodynamic behavior. So, a renewed interest has appeared in iron oxide nanoparticles synthesized by coprecipitation and

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stabilized by functionalization of their surface using small molecules with large deprotonation ability like organic polyprotic acids, leading to electrosteric stabilization. Citric acid (AH$_3$ with A = C$_6$H$_5$O$_7$) is a small molecule that has three carboxyl and one hydroxyl groups and is known to chemisorb to the iron oxide nanoparticle by forming a carbonate group with the Fe–OH molecules present on the nanoparticle surface, leaving one or two carboxyl groups negatively charged that can be used for other purposes. A positively charged drug$^{16}$ and/or a fluorescent molecule, like for example rhodamine$^{18}$, can be bonded to them for applications in drug delivery or for in vitro labeling studies, respectively. As the AH$_3$ molecule is small the nanoparticles’ hydrodynamic radius are not much enlarged, which is of importance in magnetic hyperthermia therapy applications. Differences evidenced in the rheological and microstructural properties of biocompatible citric acid coated magnetites prepared by different protocols based on the coprecipitation method have been recently discussed elsewhere.$^{15}$

Magnetic Fluid Hyperthermia is a therapy to treat cancer tumors. Briefly, MNPs exposed to a radio frequency (rf) field absorb energy from the applied field and dissipate this energy through magnetic relaxation effects locally raising the tumor temperature above 43 °C and selectively killing tumor cells.$^{7}$ The figure of merit of a given nanoparticle for this application is given by the specific absorption rate (SAR), which is defined as the heat power absorbed from the rf field per gram of magnetic material. Nowadays, active research is being executed on MNPs’ synthesis protocol to improve specific absorption rates to reduce the amount of material that has to be incorporated into a given tumor for its treatment and also to extend this kind of therapy to smaller tumors, which requires the attainment of larger SAR values. For single-domain MNPs, relaxation processes are of the Néel and Brown types. These processes correspond to the nanoparticle magnetic moment switching among its easy axis directions and the viscous friction due to the Brownian rotational diffusion of particles in the fluid, respectively. As both processes take place in parallel, the heating is driven by the one having the shorter characteristic time.$^{19}$ From the stabilization ways previously outlined emerges the possibility of either using MNPs with large hydrodynamic sizes (polymeric like coating) possibly resulting in a Néel driven process or using MNPs covered with small organic molecules, as the ones studied here, which may dissipate through a Brown type process. The question of which of these mechanisms optimizes the heating process is still an open question, whose answer requires more experimental research.

In this work, highly stable and biocompatible magnetic suspensions of citric acid coated Fe$_3$O$_4$ nanoparticles in water were produced as biomedical colloids suitable for energy dissipation under an external ac magnetic field in the rf range and appropriated for magnetic hyperthermia therapy. Moreover, the specific heating efficiency at a frequency of 265 kHz and field amplitudes of 20, 27.8, and 40.1 kA/m were determined and are discussed here in terms of the MNP structural and magnetic parameters. Optimum synthesis conditions for long-term suspension stability, as well as the performance of the obtained ferrofluids for rf heating, are determined for a wide range of experimental conditions. A detailed analysis of SAR measured values in terms of magnetic and structural colloid properties is addressed. Finally, the coated MNPs were efficiently internalized by human lung adenocarcinoma A549 cells, and its viability was analyzed by flow cytometry using Annexin V - Propidium Iodide (PI) markers.

### 2. EXPERIMENTAL DETAILS

Fe$_3$O$_4$ nanoparticles (core) were prepared by coprecipitation of ferric chloride and ferrous chloride in the presence of excess ammonia NH$_4$OH solution (AS) via a modified Massart method.$^{20,21}$ The so obtained magnetite cores were negatively charged by CA adsorption over its surfaces. The coating was performed without leaving the wet route, to avoid nanoparticle agglomeration and Brownian movement. The amino groups of AMPS and CS$_i$$(i = 1–6)$ labeled colloids synthesized by one-step coprecipitation at 60 °C, followed by citric acid adsorption at pH$_{ads}$ medium acidity and finally suspended at pH$_{susp}$ [X] stands for concentration as mass of magnetite per solution volume; typical error is ±0.2. Data derived from SQUID-DC magnetometry: $M_s$ for magnetic core diameter, $d_{TEM}$ for nanoparticle diameter retrieved from TEM images, $d_1$ for weight (averaged nanoparticle hydrodynamic diameter), $T_B$ for blocking temperatures determined from ZFC curve maximum, $T_B$ for blocking temperature for same size noninteracting nanoparticles, and SAR for Specific Absorption Rate at 40.1 kA/m and 265 kHz in Watts per gram of magnetite.

<table>
<thead>
<tr>
<th>$[X]$ (g/L)</th>
<th>pH$_{ads}$ ± 0.01</th>
<th>pH$_{susp}$ ± 0.01</th>
<th>$M_s$ (Am$^2$/kg)</th>
<th>$a_{ds}$ (nm)</th>
<th>$d_{TEM}$ (nm)</th>
<th>$T_B$ (K)</th>
<th>$T_B$ (K)</th>
<th>SAR (W/g)</th>
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</thead>
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<tr>
<td>CS$_1$</td>
<td>13.4</td>
<td>4.58</td>
<td>7.44</td>
<td>16.0</td>
<td>7.1</td>
<td>2.5</td>
<td>5.3</td>
<td>9.5 ± 1.6</td>
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<td>10.7</td>
<td>4.91</td>
<td>7.34</td>
<td>9.7</td>
<td>7.0</td>
<td>2.4</td>
<td>7.5</td>
<td>8.2 ± 2.4</td>
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<tr>
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<td>5.50</td>
<td>6.97</td>
<td>24.9</td>
<td>6.0</td>
<td>2.8</td>
<td>3.1</td>
<td>9.3 ± 1.7</td>
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<td>26.0</td>
<td>7.1</td>
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<td>3.4</td>
<td>11.9 ± 3.5</td>
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<tr>
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<td>6.88</td>
<td>7.10</td>
<td>17.7</td>
<td>5.8</td>
<td>2.5</td>
<td>4.0</td>
<td>9.4 ± 2.0</td>
</tr>
<tr>
<td>CS$_6$</td>
<td>7.4</td>
<td>7.08</td>
<td>7.22</td>
<td>11.5</td>
<td>6.7</td>
<td>2.4</td>
<td>6.4</td>
<td>10.9 ± 1.8</td>
</tr>
</tbody>
</table>

$^{a}$CS$_i$ $(i = 1–6)$ labeled colloids synthesized by one-step coprecipitation at 60 °C, followed by citric acid adsorption at pH$_{ads}$ medium acidity and finally suspended at pH$_{susp}$. $\pm$ denotes standard deviation, and $\delta$ for twice the magnetically frustrated layer thickness. $d_{TEM}$ stands for nanoparticle diameter retrieved from TEM images, $d_1$ for weight (averaged nanoparticle hydrodynamic diameter), $T_B$ for blocking temperatures determined from ZFC curve maximum, $T_B$ for blocking temperature for same size noninteracting nanoparticles, and SAR for Specific Absorption Rate at 40.1 kA/m and 265 kHz in Watts per gram of magnetite.

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by means of a permanent magnet, mixed with a CA aqueous solution (0.02 g/mL), and left to react at 60 °C during 90 min to obtain citric acid coated magnetite. To yield uncoated magnetite (M), the black precipitate was washed several times and resuspended in water at a pH close to neutral (7–7.4).

The pH at which CA was adsorbed to the MNP surface (pH_m) was varied from 4.58 to 7.08. AS (0.25% w/w) was used to adjust the suspension pH to pH_m close to 7. Then the suspension was again placed in a permanent magnet during 600 s. By this way six colloids (C_i with i = 1 to 6 for increasing pH_m) were prepared. Each one of them was then divided into two parts: the suspension of MNPs that were immobilized with a permanent magnet (CP_i) and the suspension of the MNPs which remained suspended under the moderate magnetic field created by the magnet (CS_i). Details about the preparation of the six CS_i colloids of Fe_3O_4 nanoparticles coated at different pH_m are given in Table 1. pH measurements were carried out with a pH-meter with ±0.01 pH accuracy.

Common and standardized chemical volumetric analysis was used for colloid concentration [x] determination, expressed as magnetite mass per solvent volume with an accuracy of 2%. K_2Cr_2O_7 was used as titrant.

Phase identification and core particle characterization were performed by X-ray diffraction (XRD) and transmission electron microscopy (TEM) on dried samples. Dried coating easily hydrates, hindering sample preparation for XRD analysis, especially in the more efficiently coated cases. The patterns were determined with an X'Pert Diffractometer within a 2θ range from 20 to 80°. TEM images were obtained with a TEM JEOL JEM 2100 microscope.

Zeta-potential measurements and thermogravimetry (TG) combined with differential thermal analysis (DTA) were used to confirm coating achievement. Measurements were carried out on dried powder samples with a Shimatsu TG-50 and DTA-50 system. During measurements the samples were kept inside platinum crucibles and heated at a constant rate of 10 K/min under a flux of 50 mL/min of N_2. The laser doppler electrophoretic method was used to measure zeta potentials (ζ [mV]) of 1 mL of colloid with a Malvern Zetasizer nano ZS90 device. The zeta potential was obtained by the application of the Henry equation using the Hu device. The zeta potential was obtained by the application of [mV] of 1 mL of colloid with a Malvern Zetasizer nano ZS90 device.

Hydrodynamic sizes were obtained from the inverse Laplace transformation of the measured time correlation function assuming the Stokes–Einstein relation between relaxation times and hydrodynamic diameters. Each sample was measured 10 times. Mean values (d_H) of the weight-average hydrodynamic sizes are reported in Table 1.

Time-dependent calorimetric experiments were conducted exposing 1 mL of the colloid, held in a clear glass Dewar, to rf fields of 20, 27.8, and 40.1 kA/m amplitude and 265 kHz frequency. The temperature was sensed during treatment with a fiber optic sensor placed at the center of the sample. The sensor was connected to a calibrated signal conditioner (Neoptix) with an accuracy of ±0.1 C. Colloid temperature was kept below 65 °C to minimize evaporation and prevent colloid destabilization.

The SAR parameter was obtained from the initial slope of the heating curve with the expression

$$ SAR = \frac{C \cdot \partial T}{[X] \cdot \partial t} $$

where C is the volumetric heat capacity of the solvent ($C = 4.18 J/K cm^3$) and [X] is the MNP concentration given as mass of Fe_3O_4 per solvent volume unit.

Viability and discrimination of apoptotic and necrotic cells were determined by double-labeling for the Annexin V and PI assay and analyzed by flow cytometry using a PARTEC PAS III cytometer. It is known that the plasmatic membrane loses phospholipid asymmetry in the apoptosis, resulting in the exposure of phosphatidylserine (PS) residues at the outer plasma membrane leaflet. Annexin V interacts strongly and specifically with PS, and it is used to detect apoptosis by targeting for this asymmetry. Annexin V does not bind to normal live cells since the molecule is not able to penetrate the phospholipid bilayer. In necrotic cells the integrity of the plasma membrane is lost, and then Annexin V is able to bind to the cell. To discriminate between necrotic and apoptotic cells PI (a membrane-impermeable DNA stain) is simultaneously used.

As49 cells were grown as monolayer in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10 vol % fetal bovine serum and 0.5 mg mL^-1 of streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. For the MNP uptake, the cultures at 60% confluence (24 h incubation time) were washed with phosphate buffered saline (PBS) and were incubated, in 18 mL of DMEM doped with three distinct concentrations of 34, 67, and 135 µg Fe_3O_4/mL of culture medium, in a 75 cm² flask during 12 h. Then, cells were washed four times with PBS to remove the nonincorporated MNPs and trypsinized from each flask. From each As49 internalized culture, 10⁶ cells were resuspended in Annexin-binding buffer and stained with 5 µL of Annexin and 5 µL of propidium iodide. Cells incubated without nanoparticles at the same condition of the internalized ones (36 h incubation time) were used as control.

3. RESULTS AND DISCUSSIONS

3.1. Stability Analysis. The crucial step in the preparation of a stable aqueous suspension of MNPs resides on their surfaces engineering, in this case on the CA adsorption efficiency. Representative DTA-TG curves of citric acid and the CS_i colloid are shown in Figure 1a and b, respectively. The DTA curve in Figure 1(a) displays a sharp endothermic peak at 157 °C without weight loss at the corresponding TG, assigned to fusion ($ΔH_{fus} = −0.04 kJ/g$), and a broad endothermic feature (centered at 217 °C), assigned to citric acid decomposition ($ΔH = −0.55 kJ/g$), happening in many steps. TG data show an abrupt single-step weight loss (onset at 209 °C) of 87.5% due to CA decomposition. On the other hand, no fusion peak appears at the DTA curve (Figure 1b) of coated nanoparticles, indicating the absence of free CA precipitation, and exothermic peaks related to magnetite oxidation commencing around 200 °C are superimposed to attached CA decomposition. The TG curve displays a two-step
weight loss (onsets at 175 and 222 °C) of 7.3 and 2.5% which is attributed to desorption of citric acid molecules from the nanoparticle surface. Two-step weight loss is an indication of a bilayer coating formation: at the first layer the CA molecules are chemically attached to the MNP surface and the second is bound to the first through hydrogen bonds. The latter desorbs at lower temperature. Two-step weight loss has been observed at the MNP coated by bilayers of n-alkanoic acids. From the comparison of these thermogravimetric curves it is inferred that the binding of the CA molecule to the MNP surface was accomplished.

Both types of aqueous suspensions (CP, and CS) are very stable, and the MNPs remain suspended for periods of several months, CS colloids being extremely stable. For CS colloids the separation of the MNPs from the dispersion media was not even possible by three times centrifugation at 13 000 rpm during 600 s. The mass fraction ($f$) of suspended nanoparticles, defined as the quotient of concentrations, measured at pH$_{\text{sus}}$, $f = [CS]/[C]$, was used as a parameter to quantify the CA adsorption efficiency and as a long-term stability criterion. In Figure 2, it can be seen that $f$ decreases monotonically with pH$_{\text{ads}}$. The aqueous citric acid solution left to react with the oxide particles during the synthesis consists of a pH-dependent mixture of AH$_3$, AH$_2^-$, AH$^-$, and A$^3^-$. The molar fractions $x_i$ ($y = AH_3$, AH$_2^-$, AH$^-$, and A$^3^-$) calculated using the known acid dissociation constants $pK_{a1} = 3.13$, $pK_{a2} = 4.76$, and $pK_{a3} = 6.40$ are included in Figure 2. The larger $f$ value is achieved for CA adsorption at pH$_{\text{ads}} = 4.58$, when 60% of CA molecules were negatively charged as AH$_2^-$ and 40% as AH$^-$, The decrease of AH$_2^-$ and AH$^-$ occurring at pH larger than 5.5 disfavors stability. At pH values lower than 4.58, there is a large dissolution rate, and the MNP dissolves during preparation.

Zeta-potential ($\zeta$) measurements of CS$_2$, CS$_6$, CS$_{10}$, and u colloids are shown in Figure 3, and the mean $\zeta$ values against pH are displayed in Figure 4. The isoelectric point (IEP) of coated nanoparticles is close to 2, and for pH > 4 the largest mean value of $\zeta$ is close to $-36$ mV. Results suggest that at larger pH$_{\text{sus}}$ stability occurs when a larger amount of AH$_2^-$ is present at binding conditions, i.e., a larger amount of CA binds to the nanoparticle surfaces by one carboxylate leaving two free dangling ends. Further deprotonation of an already bonded molecule occurs at pH value larger than $pK_{a3} = 6.40$, providing electrostatic and steric stabilization. Mean $\zeta$ values as large as $-36$ mV confirm that the particles have become highly negatively charged and that the coating was accomplished. Our results differ from a previous work where the largest amount of magnetic nanoparticles in stable suspension at pH$_{\text{sus}} = 10.1$ was obtained with CA adsorption at 80 °C and pH$_{\text{ads}} = 5.2$ where 70% the molecules are twice deprotonated as AH$^2^-$. 

Figure 1. TG and DTA plots of neat citric acid at the upper part (a) and CS$_2$ colloid at the lower part (b).

Figure 2. Dots stand for the fraction ($f$) of MNP that remains stable at neutral pH after exposure to 0.1 T during 600 s, used as a long-term stability parameter, triangles for acid dissociation constants ($pK_a$), and pH$_{\text{ads}}$ for adsorption pH. Lines stand for theoretical calculations of molar fraction of the species composing the aqueous citric acid solution vs adsorption pH$_{\text{ads}}$ using equilibrium dissociation constants for deprotonation reactions.

Figure 3. Zeta potential ($\zeta$) distribution of coated MNPs measured at neutral suspension pH.

Figure 4. Mean zeta potential ($\zeta$) against suspension pH$_{\text{sus}}$. Lines stand to guide the eye and IEP for the isoelectric point.
and 25% once deprotonated; however, the largest $|\zeta|$ determined in both works coincide because both were measured at pH values larger than $pK_a$ where the three carboxyl groups are dissociated. A largest $\zeta$ value of $-25.6$ at pH 6 was obtained in other recent work \textsuperscript{16} where coated magnetite was optimized for drug delivery. A lower $\zeta$ value means less charge and may be related to a shorter adsorption time (30 min less).

3.2. Structural and Magnetic Analysis. X-ray diffraction patterns for $u$ and CS$i$ samples present typical X-ray diffraction patterns of magnetite (see Figure S1 in Supporting Information). The diffraction patterns display well-defined peaks belonging to the cubic spinel structure, indicating that the samples are single phase. The whole patterns were refined, including peak broadening due to crystallite size, using a cubic spinel structure (space group Fd3m) with lattice parameter $a = 8.378$ Å and the O atoms arranged in a face-centered-cubic lattice. Figure 5a displays a typical TEM image of $u$-MNPs ($d_{TEM} = 9.9 \pm 2.4$ nm). These particles are not regularly faceted; instead they show spheroid-like forms and are quite aggregated. A representative TEM image of a coated colloid (CS$i$) is shown Figure 5b and a single CS$i$ particle in Figure 5c. The selected area electron diffraction pattern indicates good crystallinity (see inset of Figure 5c). The MNPs are spherical, more uniform, and further apart from each other than uncoated nanoparticles. Chain-like arrangements due to dipolar interactions appear. Mean size values estimated from TEM data are listed in Table 1. CS$i$ and CP$i$ colloids display similar mean size; for example, $d_{TEM}$ for CP$i$ is $10.8 \pm 2.7$ nm.

Magnetic analysis was performed on dried colloid samples and on frozen uncoated and both CS$i$ and CP$i$ coated colloids. Each sample is a collection of single domain particles of magnetic volume $V$ and giant magnetic moment $\mu = M_s \rho V$ ($\rho$ is the mass density). Particle magnetocrystalline anisotropy axes are randomly distributed. The energy of this ensemble placed in a magnetic field $H$ arises from three main contributions, the magnetic anisotropy energy, the particle magnetic moment Zeeman interaction with the applied field, and the dipolar interaction among particles.

The first and third energy terms dominate at low field, and then determine the magnetization behavior as a function of temperature in FC and ZFC curves displayed in Figure 6 for $u$ and CS$i$ samples. The absence of a maximum in the ZFC curve of the $u$ sample indicates a blocking temperature close to room temperature due to aggregation and strong dipolar interaction between particles. The constancy of $M$ almost in the whole FC curve is also a signature of a highly interacting system. The ZFC curve of CS$i$ exhibits a maximum at the blocking temperature $T_B = 92$ K, and ZFC bifurcates from the FC curve at the irreversibility temperature $T_i = 140$ K. For temperatures higher than $T_B$ magnetization temperature dependence strongly departs from the Curie-like behavior ($M \sim H/T$) expected for a noninteracting system. As temperature decreases from $T_i$ the FC magnetization increases. Comparing ZFC–FC curves of uncoated and coated MNPs it is clear that citric acid coating prevents strong aggregation and lowers dipolar interaction, but still the system behaves as an interacting superparamagnet.
The mean particle magnetic moment $\langle \mu \rangle$ in all the studied samples is around $1.5 \times 10^5 \mu_B$; the anisotropy energy barrier $K_{\text{eff}}V$ is much smaller than the magnetic moment field interaction energy $\mu_BH$ and the magnetization of the particles at a given temperature and field can be modeled as

$$M(T, H) = N \int_0^\infty \mu L \left( \frac{\mu H}{k_B T} \right) f(\mu) d\mu + \chi_s H$$

where the Langevin function $L(x) = \coth(x) - 1/x$, being $x = \mu_B H/k_B T$, is convoluted with a log-normal distribution of magnetic moments $f(\mu) = \exp[-(\ln(\mu/\mu_m)/2\sigma^2)/\mu_m\sigma(2\pi)^{1/2}]$, and $\chi_s$ is the high field susceptibility related to surface magnetic disorder induced by the lack of symmetry. Not fully coordinated atoms at the surface of the particle lead to a magnetically frustrated layer which does not saturate even at 10 K and 2000 kA/m (see inset in Figure 7).

The second energy term dominates at high fields and is the main contribution to the hysteresis loops shown in Figure 7.

![Figure 7. Specific magnetization loops measured at 290 K for various colloids. Inset: CS$_2$ measurement at 10 K. Solid lines stand for best Langevin fits using eq 2.](image)

The colloids CS$_2$ and CP$_2$ under study here are single domain and behave as interacting superparamagnets. For CS$_{10}$, CS$_{20}$, and CS$_{50}$ samples the size derived from magnetic cycles is larger than the mean sizes derived by TEM, while for CS$_3$, CS$_4$, and CS$_5$, the agreement is good. This discrepancy can be ascribed to the fact that TEM, although being a more direct determination, involves a smaller number of particles than the macroscopic magnetic measurement.

A charged particle moving in a solvent drags with it a layer of fluid molecules and counterions, resulting in an entity with a quite large hydrodynamic diameter $d_{\text{hl}}$. In effect, in this study it was found that $d_{\text{hl}}$ values are 1 order of magnitude larger than the overall nanoparticle size including CA coating (see Table 1).

### 3.3. Specific Absorption Rates (SARs)

The time required to reach a given temperature, at fixed frequency, decreases with increasing field amplitude. SAR represents the power released per gram of magnetite upon ac-field application and is mainly given by the magnetization loop area. SAR values listed in Tables 1 and 2 were obtained from the initial slope of the heating curves using eq 1. These values range from 5.2 to 41.5 W/g for CS$_2$ colloids, from 29 to 104 W/g for CP$_2$, and 203 W/g for the $\mu$-MNP colloid. The inspection of Figure 9 shows that for our samples there is no clear relation...
from the linear relation expected for SAR plotted against dependence, plotted in Figure 11, displays an increasing is the out-of-phase magnetic susceptibility. SAR for

Table 2. Magnetic Characterization and Radiofrequency Dissipation of Magnetite Stable Aqueous Colloids and Uncoated Colloid

<table>
<thead>
<tr>
<th>colloid</th>
<th>$\mu$</th>
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<th>CP6</th>
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<td>$[X]$</td>
<td>8.28</td>
<td>6.1</td>
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</table>

$\sigma_{d_m}$ is the size distribution of MNPs having diameters $d_m$. Data derived from SQUID-DC magnetometry: $M_s$ stands for specific magnetization, $d_m$ for magnetic core diameter, and $\delta$ for twice the magnetically frustrated layer thickness.

The processes take place in parallel ($\tau^{-1} = \tau_0^{-1} + \tau_N^{-1}$) and prevails the one having the shorter relaxation time.

$\tau_0$ values calculated at 300 K using data listed in Table 1 are plotted vs SAR in Figure 12.

![Figure 11](image1.png) Specific absorption rate (SAR) field amplitude dependence. Dotted lines stand for second-order polynomial fits.

![Figure 12](image2.png) Brown relaxation times $\tau_N = (3\eta V_i)/(k_B T)$ calculated with $k_B = 1.38 \times 10^{-23}$ J K$^{-1}$, viscosity $\eta = 8.90 \times 10^{-4}$ Pa s, and $T = 300$ K. Neel relaxation times $\tau_N = \tau_0 \exp(k_B V_i/k_B T)$ of isolated nanoparticles calculated using size-dependent anisotropy energy density $k_B V_i$ and $\tau_0$ interpolated from data in refs 31 and 32. Relaxation times $\tau_{bulk}$ for the Neel mechanism taking into account size dependence and interaction.
interaction of the nanoparticles. Gilmore et al. 31 in magnetite particles grown inside three protein cages of distinct size, where the cage served to constrain particle size and shape and to mute interparticle interaction. Using this relation with $K(d) = 2 \tan(h d/4)$, as in ref 31, we have retrieved $K_{\text{eff}}$ for each particle size listed in Table 1. The corresponding $r_0$ values were interpolated from the $r_0(d)$ relation built from data in refs 31 and 32. Evaluation of $r_0$ disregarding interactions results in values in the range $0.1$ – $0.6 \times 10^{-9}$ s.

To take into account that the magnetic moments of interacting particles fluctuate with different relaxation times than those of isolated ones, a total energy barrier given by $E_{\text{int}} = K_{\text{eff}} V + E_{\text{int}}$ was assumed. 33 The interaction energy $E_{\text{int}}$ was deduced from the shift of the ZFC maximum (Figure 6), disregarding $r_0$ dependence with interactions, with respect to a noninteracting ensemble as $E_{\text{int}} = k_B(T_{\text{int}} - T_0)\ln(t_0/r_0)$. Here $T_0$ is the blocking temperature expected for noninteracting particles of sizes listed in Table 1, as derived from the $K_{\text{eff}}$ and $r_0$ values mentioned above; $T_{\text{int}}$ is the blocking temperature resulting from ZFC measurements (see Table 1); and $t_0 \sim 100$ s is the SQUID measuring time. Then, Néel relaxation time for interacting particles $\tau_{\text{int}}$ was obtained with eq 4 with $\sigma = (K_{\text{eff}} V + E_{\text{int}})$. The results are shown in Figure 12 where the relaxation times are plotted vs SAR values determined with $H_0 = 40.1$ kA/m, i.e., maximum used $H_0$ value, larger than the anisotropy field. Interaction results in slower magnetic relaxation. A crossover between $\tau_{\text{int}}$ and $\tau_0$ behavior appears at 12.8 W/g. The larger SAR values come out with the more interacting colloids, i.e., for the particles displaying larger $\tau_{\text{int}}$ (large $E_{\text{int}}$ values). In those cases the process seems to be driven by a Brownian mechanism, while for the other cases the Néel mechanism prevails.

The uncoated magnetite results in a very unstable suspension but produces the largest SAR of 200 W/g. Although particle size is similar to those of coated particles, the hydrodynamic size is 1.5 μm (polydispersity index $\sigma_{\text{PI}} = 0.5$) indicating the presence of large clusters of particles moving in the fluid. Blocking temperature is around 300 K, due to strong interactions. The analysis outlined above for coated colloids, when applied to the uncoated colloids, results in relaxation times various orders of magnitude larger. Still $\tau_0 < \tau_{\text{int}}$ supporting the idea that interactions play an important role in magnetic fluid hyperthermia increasing nanoparticle energy dissipation. SAR values against $M_i$ are plotted in Figure 13 for all CPs, CSs, and $u$ colloids. An increasing behavior is observed, as expected from the $X_0$ dependence with $M_i$ irrespective of nanoparticle size and dispersion. Lowering of $M_i$ is assigned to a larger magnetically frustrated layer. Those CSs colloids of MNP coated at lower $p\text{H}_{\text{ads}}$ and displaying larger electrostatic stability present lower $M_i$ values. The corresponding CP colloids display larger $M_i$ and SAR values consistently with the idea of MNPs having less coating material on their surfaces and being more effectively attracted to the magnet. These observations suggest that citric acid may have an influence on the surface of the MNP, producing larger frustrated layers which on one side improve nanoparticle suspension stability but on the other one lower SAR values. The optimum synthesis condition to accomplish both properties, extremely high stability in suspension at neutral pH and large SAR values, is obtained by citric acid coating at $p\text{H}_{\text{ads}} = 6.25$.

3.4. In Vitro Biocompatibility of CA-Coated MNPs Internalized in A549 Cells. The materials involved in the synthesis procedure (water, CA, and magnetite) are known to display good biocompatibility. However, since interfacial processes may take place when MNPs are dispersed in biological media changing their surface reactivity and consequently their toxicity, 34 we have tested the viability of our nanoparticles in A549 cells. Figure 14 shows the cytomteric analysis of double-labeling for Annexin V and PI assay for the A549 cell without MNP exposure and internalized with three distinct CS4 colloid concentrations. Viable cells are negative for both labelers (DL quadrant); apoptotic cells are PI negative and annexinV positive (DR quadrant); while necrotic cells are both labelers (DL quadrant); apoptotic cells are PI negative and annexinV positive (DR quadrant); while necrotic cells are PI negative and Annexin V positive (DR quadrant); while necrotic cells are PI negative and annexinV positive (DR quadrant); while necrotic cells are PI negative and Annexin V positive (DR quadrant). After internalization with CS4 colloids at concentrations of 34, 67, and 135 μg Fe/cm$^3$/mL, the percentages of viable cells are 94.7%, 95.1%, and 81.3%, respectively. Only for the highest concentration, viability decrease compared with 84.7% of control cells is observed. The fact that the control culture shows a lower viability than the lower concentrated cultures may be due to an overgrown control culture. It is concluded that 12 h exposure to CA-coated Fe$_3$O$_4$ nanoparticles at low concentration is not altering A549 cell viability. Then, citric acid coated magnetite aqueous suspensions at neutral pH were internalized without causing cell toxicity.

**CONCLUSIONS**

We have presented a detailed and extensive study of synthesis conditions, magnetic properties, and radiofrequency dissipation efficiency of various biocompatible citric acid coated magnetite aqueous suspensions at neutral pH. The preparation method enables us to control the long-term stability of the suspension varying the citric acid adsorption pH. The largest stability at neutral medium is achieved for the nanoparticles coated at pH 4.58, where citric acid mainly binds to the particle surface by one carboxylate, leaving two free and charged dangling ends. Citric acid influences the surface of the MNP, producing thick magnetically frustrated layers which on one side improve nanoparticle suspension stability but on the other one reduce SAR values. Finally, our results indicate that dipolar interactions between the nanoparticles play a key role in the Néel relaxation mechanism and dissipation efficiency. Magnetic relaxation becomes slower for interacting colloids, and heating efficiency increases. The largest SAR values arise from the more interacting nanoparticles, a case where Néel relaxation times become larger than Brown relaxation times, leading the Brown dissipation mechanism to prevail. It is shown that SAR data cannot be easily reproduced using the magnetic data and the
most currently used theoretical expressions, due to the presence of dipolar interactions between the nanoparticles. Interaction energy was included in the theoretical expressions through the quantity \( \sigma = (K_{\text{eff}}V + E_{\text{int}})/k_B T \) and its value inferred from the blocking temperature shift.

**ASSOCIATED CONTENT**

- Supporting Information
  - Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

- AS, ammonia solution; CA, citric acid; CP, ith pellet colloid; CS, ith supernatant colloid; DTA, differential thermal analysis; DLS, dynamic light scattering; \( d_H \), hydrodynamic diameter; DMEM, Dulbecco’s modified eagle medium; FC, field cooling; IEP, isoelectric point; ISP, interacting superparamagnet; MNP, magnetic nanoparticles; PI, propidium iodide; \( \nu K_{\text{a}} \), acid dissociation constant; \( \nu \)-MNP, uncoated magnetic nanoparticles; rf, radiofrequency; SAR, specific absorption rate; TEM, transmission electron microscopy; TG, thermogravimetry; XRD, X-ray diffraction; ZFC, zero field cooling; \( \sigma_{\text{PI}} \), polydispersity index

**REFERENCES**

(3) Gupta, A. K.; Gupta, M. Biomaterials 2005, 26, 3995–4021.
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(15) Raikher, Y. L.; Shliomis, M. I. The effective field method in the orientational kinetics of magnetic fluids and liquid crystals, Advances in Chemical Physics 87; Wiley: New York, 1994; Chapter 8, p 595.


(25) Ralikh, Y. L.; Shliomis, M. I. The effective field method in the orientational kinetics of magnetic fluids and liquid crystals, Advances in Chemical Physics 87; Wiley: New York, 1994; Chapter 8, p 595.


Figure 1: X-ray diffraction pattern of dried powder retrieved from $u$ and $CS_6$ colloids with cubic spinel structure (space group Fd3m) indexation.

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