

A new method for the aqueous functionalization of superparamagnetic Fe₂O₃ nanoparticles

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A new methodology for the synthesis of hydrophilic iron oxide nanoparticles has been developed. This new method is based on the direct chemical modification of the nanoparticles' surfactant molecules. Using this methodology both USPIO (ultrasmall super paramagnetic iron oxide) (hydrodynamic size smaller than 50 nm) and SPIO (super paramagnetic iron oxide) (hydrodynamic size bigger than 50 nm) were obtained. In addition, we also show that it is possible to further functionalize the hydrophilic nanoparticles via covalent chemistry in water. The magnetic properties of these nanoparticles were also studied, showing their potential as MRI contrast agents. Copyright © 2008 John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: nanoparticles; contrast agents; colloids; magnetic properties; molecular imaging

1. INTRODUCTION

In the last few years we have witnessed major development in the synthesis and applications of nanoparticles (NP). This is specially true in the field of superparamagnetic iron oxide nanoparticles. Publications in this area have focused on the synthesis of the iron oxide core (1–3), the stabilization of nanoparticles in water and their functionalization for biomedical applications (4–10). It is generally accepted that the best synthetic routes for the preparation of iron oxide cores are based on high-temperature decomposition of organic precursors. This method renders nanoparticles with a narrow size distribution, with a well-defined shape and with good crystallinity (11). However, the drawback of this method is that the NPs are coated with an organic surfactant, usually oleic acid, requiring extra steps to obtain water stable colloids (9,10). To date, there has been no general methodology for the synthesis of iron oxide nanoparticles in water that features all of the following: (a) reproducible control of the size; (b) control of surface composition; (c) small polydispersity; (d) NP ready for further functionalization by covalent bonding; (e) compatibility with aqueous media.

At present there are two main methods to prepare water-based colloids of iron oxide. The micelle-like approach, where the oleic acid is masked by the use of amphiphilic molecules [Fig. 1(a)] and the ligand exchange approach, where the surfactant is removed, at least partially, by a second ligand bearing hydrophilic groups [Fig. 1(b)].

These two methods have allowed the synthesis of a large number of NP with interesting properties (12–16). However, these two approaches are still not ideal, having important drawbacks such as a lack of reproducibility in the control over surface composition, although with exceptions such as magnetolipo-

somes (17–19), and the difficulties of functionalization through covalent chemistry in water.

Herein we report a new methodology for the synthesis of iron oxide nanoparticles based on the decomposition of organic precursors followed by covalent functionalization of the surfactant molecules by oxidation (2,20,21). The resulting nanoparticles are stable in water and can be further functionalized via the formation of covalent bonds. In addition, we report preliminary magnetic measurements that demonstrate the potential of these nanoparticles as MRI contrast agents. Our new synthetic methodology has been shown to have all the desirable features for the reproducible synthesis of iron oxide nanoparticles compatible with aqueous media.

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Contract/grant sponsor: MEC; contract/grant numbers: NAN2004-08805-C04 -01, SAF2008-05412. Contract/grant sponsor: Regional government of Madrid CAM; contract/grant number: S0505-AGR-0187.

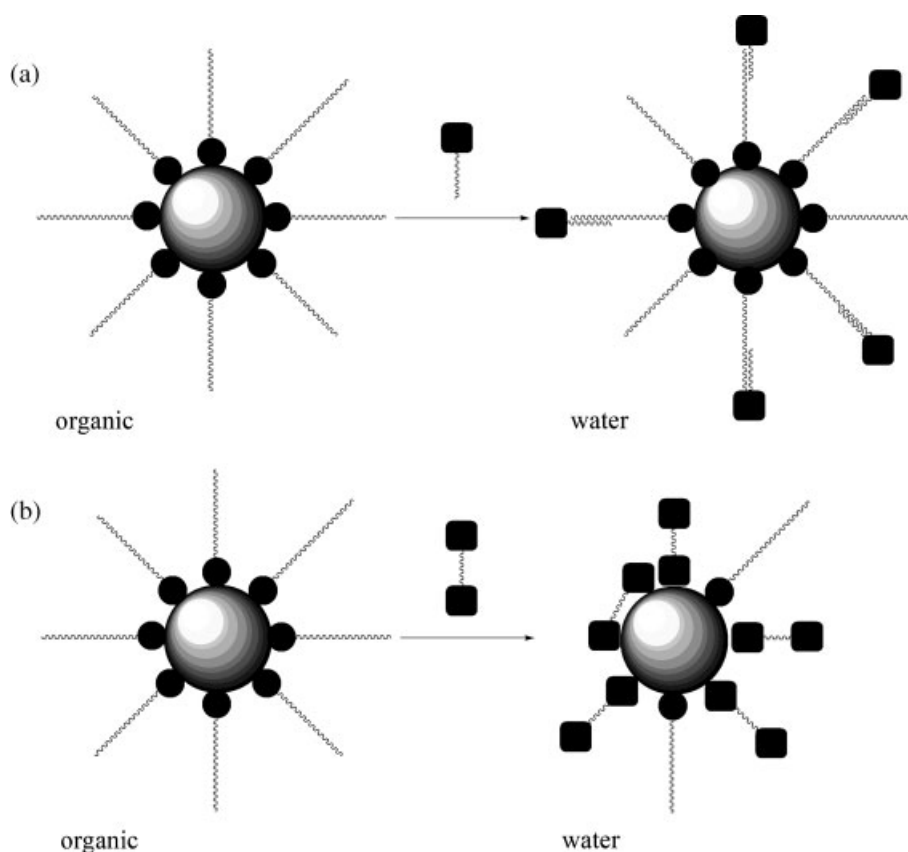


Figure 1. Transferring hydrophobic nanoparticles to water; (a) micelle-like approach, (b) ligand exchange approach.

2. RESULTS AND DISCUSSION

Iron oxide nanoparticles were synthesized by the decomposition of iron acetylacetonate. These nanoparticles showed a mean hydrodynamic size, measured by photon correlation spectroscopy (PCS), of 10 nm and a very narrow size distribution (11). A transmission electron microscopy (TEM) image of this sample is shown in Fig. 2.

To obtain a stable colloidal dispersion in water our approach is based on chemically modifying the oleic acid used to passivate the molecules, rather than the established approach of masking the oleic acid or exchanging it with other molecules. More specifically the double bond of the oleic acid was oxidized using potassium permanganate, yielding a second carboxylic acid functionality (NP-azelaic acid, Fig. 3). This transforms the oleic acid coating agent into azelaic acid, resulting in nanoparticles with good water stability and a functional group that can be potentially used for further functionalization of the NP.

In order to obtain a high enough concentration of the oxidant (i.e. KMnO_4) in the organic solvent, the phase transfer catalyst benzyltrimethyl-ammonium chloride (BTACl) was used. The final step in the oxidation method was the cleavage of the organo-manganese intermediate formed between the permanganate ion and the double bond, in acid or in basic media.

Fourier transform infrared (FT-IR) spectroscopy was used to characterize the composition of the NP after oxidation of the double bond. Figure 4 shows the spectra for NP-oleic acid (top spectrum), NP-azelaic acid (acid oxidation, 1, spectrum a) and NP-azelaic acid (basic oxidation, 2, spectrum b).

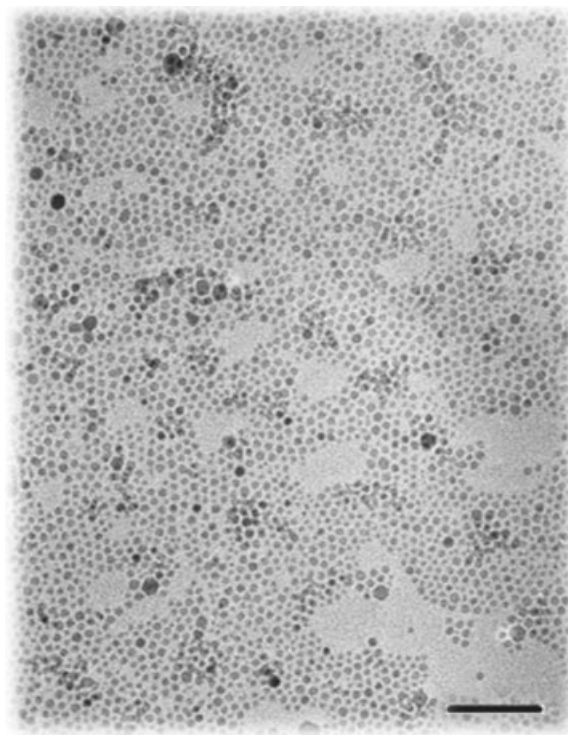


Figure 2. TEM image of NP-oleic acid prepared by the decomposition of organic precursors (scale bar is 50 nm).

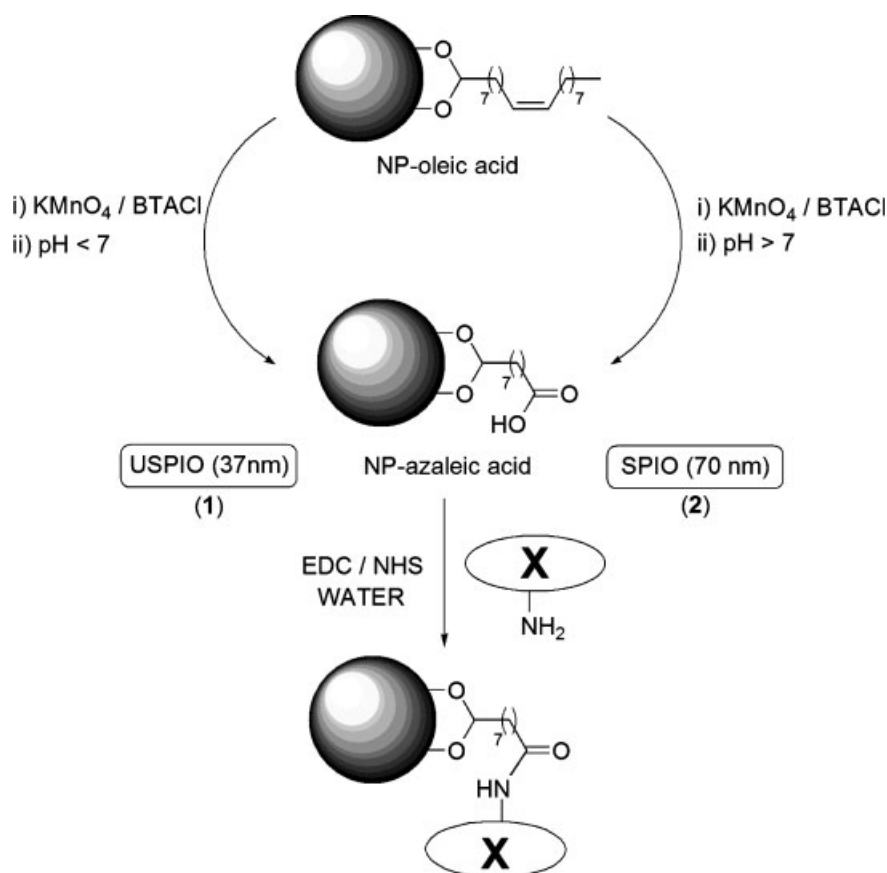


Figure 3. New approach for the synthesis of hydrophilic nanoparticles and their functionalization in water.

The spectrum for the hydrophobic NP shows signals corresponding to the oleic acid moiety (2918 , 2850 , 1411 , 1621 and 1567 cm^{-1}) and for the Fe–O (from magnetite) at 594 cm^{-1} . The formation of azelaic acid is supported by several bands. The broadened $\text{C}=\text{O}$ peak at 1625 cm^{-1} due to the presence of two carbonyl groups in the molecule and the disappearance of the signal at 1567 cm^{-1} is assigned to the double bond in oleic acid. Other bands assigned to azelaic acid are at 984 , 1036 , 1126 and 1192 cm^{-1} (22,23). Owing to the strong oxidation process used for the surface modification of the NP, a slight oxidation of the iron oxide NP from magnetite to maghemite takes place. This is shown by the appearance of two weak bands at 634 and 445 cm^{-1} in the FT-IR spectrum. To further prove the oxidation of the double bond, the organic layer of the reaction was analysed by ^1H NMR spectroscopy. The organic solvent (a mixture of chloroform and hexane) was evaporated under reduced pressure and the residue dissolved in deuterated chloroform. The signals corresponding to the nonanoic acid are clearly seen [0.90 (t, 3H), 1.27 (m, 10H), 1.66 (m, 2H) and 2.38 (t, 2H) ppm]. In addition there is no evidence of the presence of oleic acid in the organic phase (see Supporting Information).

The sizes of the resulting hydrophilic nanoparticles, determined by photon correlation spectroscopy (PCS), were $37 \pm 4\text{ nm}$ (PDI 0.18) for 1 (USPIO) and $70 \pm 7\text{ nm}$ (PDI 0.25) for 2 (SPIO). For the particles studied in our work, all the PCS measurements showed very good reproducibility (see Table 1). In addition, there was an excellent correlation between the different ways of

reporting the value. For example, the values for one of the samples obtained with the oxidation in acid media were: Z-average = 39 nm , intensity = 43 nm , volume = 27 nm and number = 21 nm . The differences in size between the particles resulting from the oxidation in acid and basic conditions could be attributed to the greatest oxidation capacity of permanganate in acid media, allowing the use of a lower concentration of the oxidant and the phase transfer catalyst. There was a small increase in the size of the NP from the hydrophobic state to the water colloid. This degree of broadening of the size distribution of the aggregates is unavoidable but can be drastically reduced by using the method described herein, as can be seen from the PDI values.

In order to gain some insight into the stability of the nanoparticles in water their zeta potentials (ζ) were determined (see Fig. 5). A similar profile was obtained for both nanoparticles 1 and 2; however the stability, at physiological pH, of the NP obtained via the acid oxidation (1) was higher (-46 mV) than that for those obtained under basic conditions (-20 mV). This can be attributed to the slightly higher agglomeration of 2, with the basic conditions, compared with 1, which leads to a smaller density of carboxylic acid groups on the NP's surface and, therefore, to a reduced stability. Suspensions of the iron oxide NP (both obtained in acidic or basic medium) remained stable in water for months without a significant variation of the hydrodynamic size.

The ζ potential values were in agreement with the presence of azelaic acid on the NP's surface. The isoelectric point for these

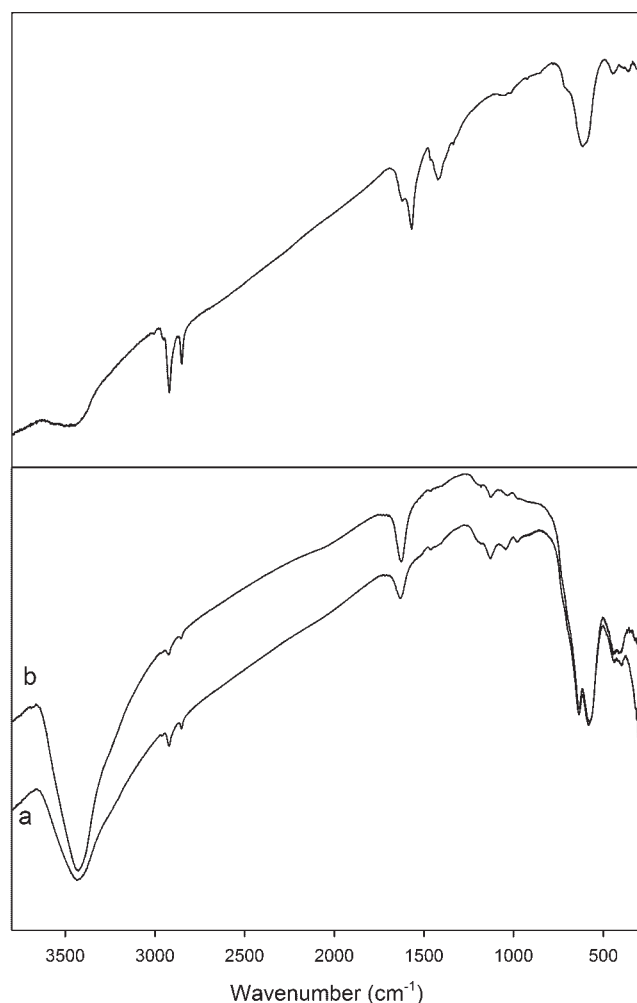


Figure 4. Infrared spectra for NP-oleic acid (top spectrum) and hydrophilic nanoparticles 1 and 2 (spectra a and b respectively).

particles was approximately 4.5 for NP 1 and 5.7 for NP 2, in accordance with the second pK_a value of azelaic acid, which is 5.3.

In order to investigate the reproducibility of the synthetic method, the reactions were repeated 10 times and similar results were obtained on all occasions (see Table 1 for a selection of some of the repeats). These results clearly highlight the

Table 1. Size, size distribution and surface composition for selected repetitions of the oxidation method, both in acidic and basic conditions

Sample	Size (Z-average) (nm)	PDI	Zeta (mV) (pH 7)
1 (repeat 1)	37 ± 4	0.20	-46 ± 4
1 (repeat 2)	38 ± 6	0.18	-44 ± 6
1 (repeat 3)	40 ± 7	0.21	-45 ± 3
2 (repeat 1)	70 ± 7	0.25	-20 ± 7
2 (repeat 2)	74 ± 9	0.30	-21 ± 6
2 (repeat 3)	68 ± 5	0.23	-20 ± 6

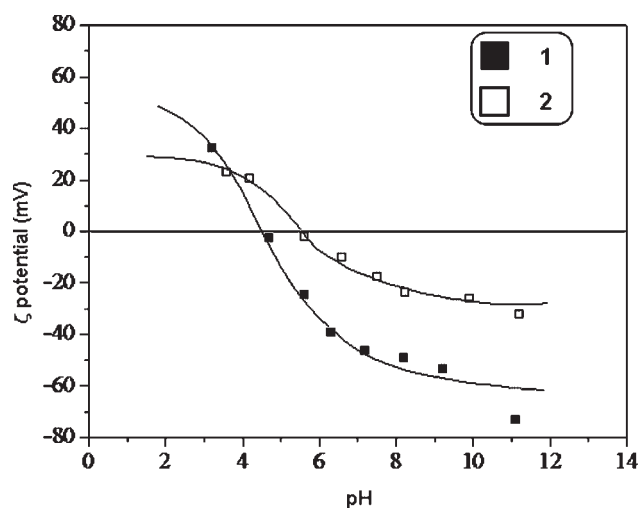


Figure 5. Profile of the ζ potential vs pH for the oxidized particles.

reproducibility of the method regarding the size, surface composition and size distribution of the NPs.

TEM images were obtained for the two types of oxidized nanoparticles. Figure 6 shows the low aggregation for NP 1, at two magnifications, when transferring the NP from the hydrophobic phase to water. A certain distance between particles is clearly observable, precluding the aggregation and formation of large lumps (for more TEM images see supporting information).

To study the magnetic properties of the new nanoparticles, magnetization curves were performed for the NP-oleic acid in hexane and both 1 and 2 in water (Fig. 7). The three samples show a superparamagnetic behaviour with saturation magnetization values of $87 \text{ emu g}^{-1} \text{ Fe}$ for NP-oleic acid, $77 \text{ emu g}^{-1} \text{ Fe}$ for 1 and $63 \text{ emu g}^{-1} \text{ Fe}$ for 2. The expected value for bulk magnetite at room temperature is $110 \text{ emu g}^{-1} \text{ Fe}$, which means that particles prepared in this work present a certain degree of surface oxidation (as confirmed by FT-IR spectra) increasing from the hydrophobic sample to hydrophilic ones.

The different susceptibility at low field can be related to the size of the aggregates in suspension (24,25); thus the greater susceptibility observed for sample 2 is consistent with a larger aggregate size as observed in the PCS measurements.

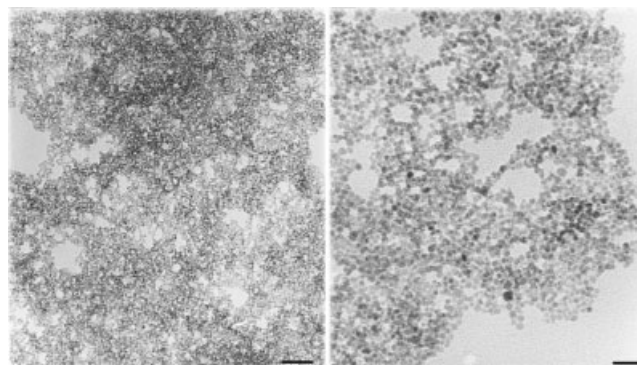


Figure 6. TEM images for NP-azelaic acid 1; scale bars are 50 nm (left) and 20 nm (right).

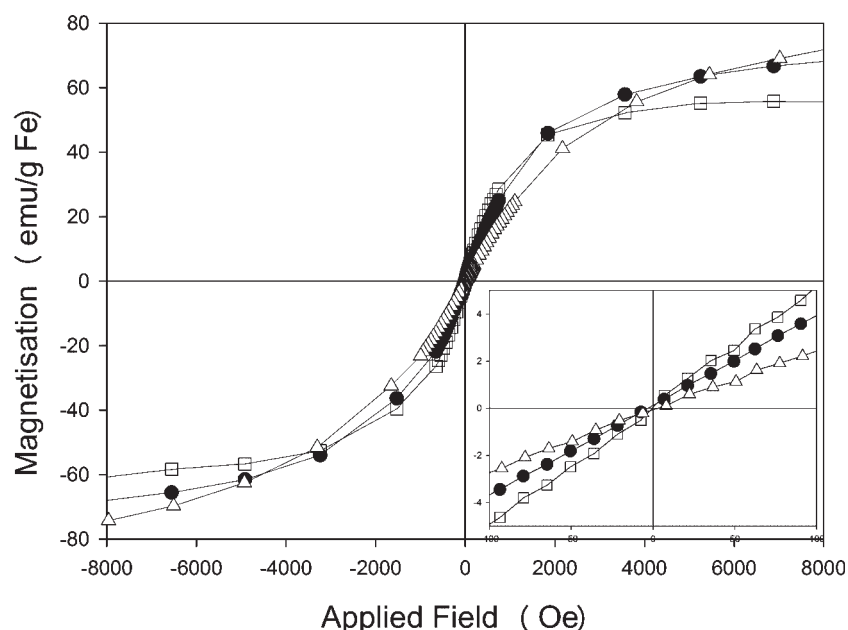


Figure 7. Main panel: magnetization curves at 298 K for hydrophobic NP (Δ), NP 1 ($\bullet$) and NP 2 ($\square$). Inset: enlargement of the magnetization curves between -100 Oe and 100 Oe.

Finally, to test the behaviour of this hydrophilic NP as contrast agents for MRI, we measured the longitudinal (T_1) and transversal (T_2) relaxation times (see Supporting Information). For 1 the values were $r_1 = 4 \text{ s}^{-1} \text{ mM}^{-1}$ and $r_2 = 115 \text{ s}^{-1} \text{ mM}^{-1}$, while for NP 2, $r_1 = 2.3 \text{ s}^{-1} \text{ mM}^{-1}$ and $r_2 = 110 \text{ s}^{-1} \text{ mM}^{-1}$, respectively. The measured relaxivity values were in agreement with the hydrodynamic sizes values, as the r_2/r_1 ratio was higher for those nanoparticles that showed a slightly higher degree of aggregation. This ratio was 47.8 for NP 2 and 28.7 for NP 1.

As described above, the acid functionality introduced via our new methodology increases considerably the stability of the NP in aqueous media. This, together with their relaxivity values and the possibility for further functionalization (by reacting the terminal carboxylic acid), suggests that these nanoparticles could yield useful MRI contrast agents.

To demonstrate the possibility of attaching a range of different groups on the surface of the hydrophilic nanoparticles, we carried out two different reactions with NP 1 (see Fig. 8). One of these reactions was the attachment of glucose to the nanoparticles via the formation of an amide with the terminal carboxylic acid of the surfactant [Fig. 8(a)]. The coating with saccharides has been previously shown to be interesting due to the increased stability of the resulting nanoparticles and also their specific binding to target cells (26). We carried out this modification using EDC/NHS as coupling agents. The resulting nanoparticles have a mean hydrodynamic size of 45 nm (PDI 0.28), and a good stability in water (-19.6 mV at pH 7). Finally, in the IR spectrum, the bands for the sugar group can be clearly observed (bands at 1030 and 1536 cm^{-1} ; Fig. 8(b)).

The second reaction studied was the attachment of a fluorophore (2-hydroxy-3-amino-naphthalene sulfonic acid) to the NP surface to yield a dual MRI/optical probe [Fig. 8(c)]. The hydrodynamic size of the resulting nanoparticles was 75 nm (PDI 0.17), while a strong fluorescence emission at 550 nm [see

Fig. 8(d)] was observed (when exciting the NP at 276 nm) with a very good stability in water (-30 mV at pH 7).

The number of azelaic acid and dye molecules per particle were quantified by means of ^1H NMR spectroscopy. First, we calculated the number of azelaic acid per particle; a sample of NP 1 was dissolved in concentrated HCl (HCl 37%, room temperature), the solvent was then eliminated under reduced pressure and the residue washed with CDCl_3 . A ^1H NMR spectrum (see Supporting Information, Fig. S6) was recorded, showing the signals corresponding to azelaic acid [0.30 (m, 6H), 0.95 (m, 4H), 1.3 (m, 4H)] with the corresponding integrations. It is important to mention that no resonances corresponding to oleic acid were detected in this sample. In order to quantify the amount of azelaic acid, 3-aminopyridine (2.7 mg) was added as internal standard (see Fig. S7). Two signals from acid (those at 0.30 and 0.95 ppm) and the two signals from the standard were used to quantify the former (the results were consistent regardless of the resonances chosen). The azelaic acid concentration in the sample was determined to be 0.25 mM , which corresponds to approximately 40 molecules of acid per nanoparticle (27–29). Using a similar approach, we determined the amount of fluorescent dye present in NP 4. In this case, the standard used for comparison was azelaic acid, giving a concentration of dye 0.02 mM , which corresponds to, approximately, 3.5 molecules of dye per particle.

In conclusion, a new method for the covalent functionalization of superparamagnetic nanoparticles in water has been developed. Minor changes in the synthetic experimental conditions yielded two different types of hydrophilic superparamagnetic nanoparticles. Compared with other common methodologies, the one presented here has several advantages, namely: the methodology shows a reproducible control over size, size distribution and surface composition; oleic acid is not removed from the nanoparticles' surface but modified to make them hydrophilic; the resulting nanoparticles are water stable and can yield either USPIO or SPIO

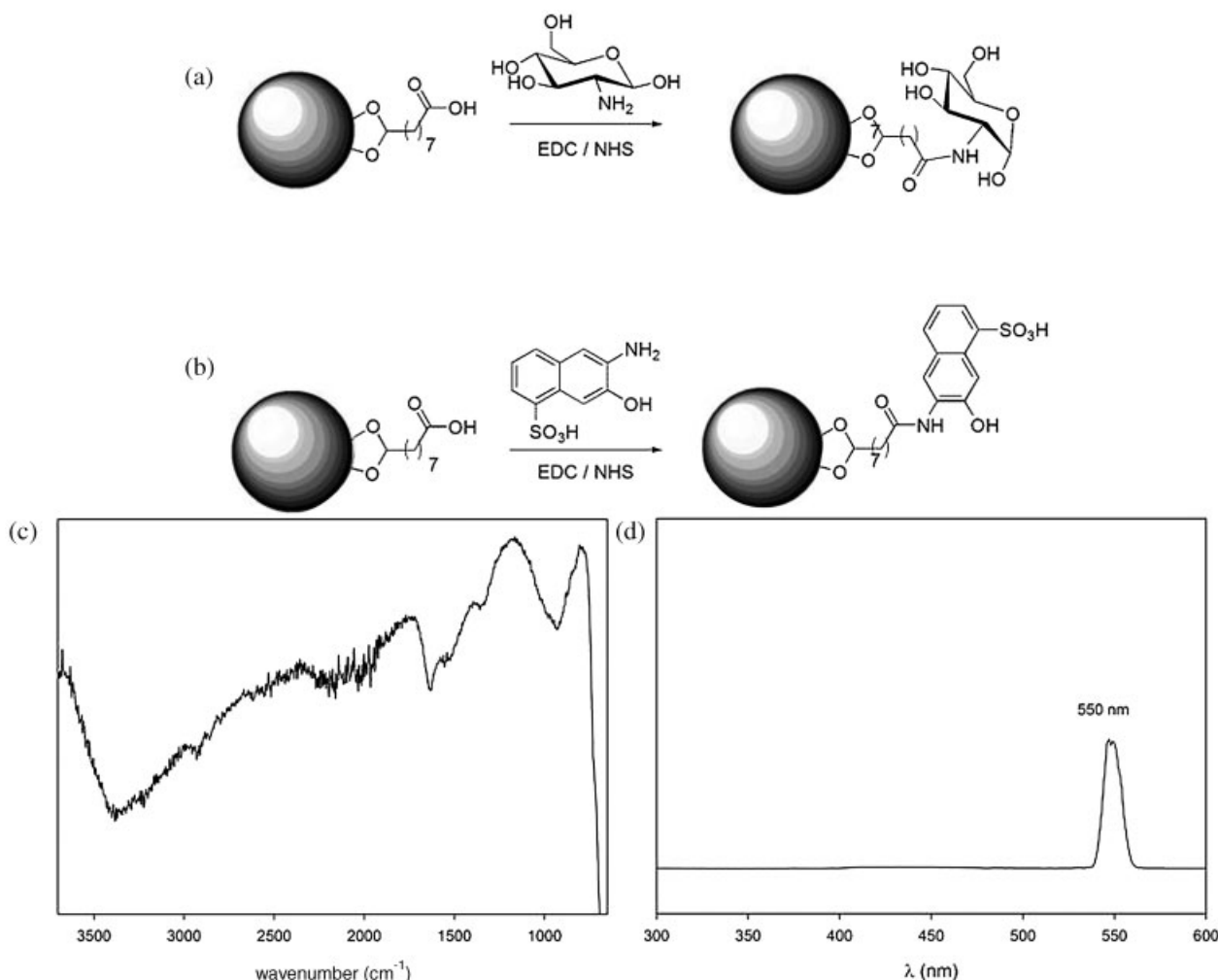


Figure 8. Covalent functionalization in water. (a) Reaction with amino glucose; (b) IR spectrum of sample 3; (c) reaction with 3-hydroxy-4-amino-naphthalene sulfonic acid and (d) fluorescence emission spectrum of sample 4 (excitation at 276 nm).

particles; these nanoparticles can be further functionalized in water by reacting the carboxylic acids with amines.

3. EXPERIMENTAL SECTION

3.1. Synthesis of magnetic NP-oleic acid

The nanoparticles were synthesized using iron acetylacetonate as precursor and phenyl ether as solvent. A mixture of Fe(acac)₃ (2 mmol, 0.71 g), 1,2-hexadecanediol (2.38 g, 10 mmol), oleic acid (1.69 g, 6 mmol), oleylamine (1.60 g, 6 mmol) and phenyl ether (20 ml) were added to a three-neck flask. The reaction mixture was heated under mechanical stirring and a flow of nitrogen gas until a temperature of 200°C was reached. This temperature was kept for 120 min and then the solution was heated to reflux (254°C) for 30 min in a nitrogen atmosphere. After this time, the solution was cooled down to room temperature.

To remove side products the reaction mixture was mixed with ethanol and centrifuged at 8500 rpm for 10 min. Finally, the resulting nanoparticles were mixed with 20 ml of hexane and

0.05 ml of oleic acid and centrifuged at 8500 rpm (5650 g) in order to remove aggregates and obtain a stable suspension.

3.2. Synthesis of NP-azelaic acid (1)

KMnO₄ (0.3 g, 1.9 mmol) and benzyltrimethyl-ammonium chloride (0.8 g, 4.3 mmol) were dissolved in chloroform (60 ml). This solution was added to a concentrated dispersion of NPs coated with oleic acid in a hexane–chloroform mixture (20:20 ml). The solution was mechanically stirred and heated at reflux for 4 h. After this time 50 ml of AcOH–AcO[−] buffer (pH 2.9) was added and the mechanical stirring and heating continued for 20 h. After cooling, NaHSO₃ (3 × 2 ml) was added to eliminate the excess of permanganate; finally the dispersion was washed several times with water and chloroform obtaining the colloidal dispersion.

3.3. Synthesis of NP-azelaic acid (2)

KMnO₄ (0.5 g, 3.2 mmol) was dissolved in chloroform (40 ml), together with benzyltrimethylammonium chloride (1 g, 5.4 mmol). The solution was added to a concentrate dispersion of

NP coated with oleic acid in a hexane–chloroform mixture (20:20 ml). The mixture was heated at reflux and mechanically stirred for 4 h; after this time 20 ml of NaOH (1%) solution was added, maintaining heating and stirring for another 32 h. After cooling, NaHSO_3 was added (3×2 ml) to eliminate the excess of permanganate; finally the dispersion was washed several times with water and chloroform obtaining the colloidal dispersion.

3.4. Synthesis of NP-glucose (3)

Amino-glucose hydrochloride (0.085 g, 0.40 mmol), *N*-hydroxysuccinimide (0.080 g, 0.70 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (0.077 g, 0.40 mmol) were dissolved in 5 ml of distilled water. A 5 ml aliquot of NP-azelaic acid (1) (4 mg ml^{-1} in Fe) suspended in water was added and the pH adjusted to 9 with a 1% solution of NaOH. The mixture was shaken in a vortex for 5 h and the resulting nanoparticles were separated with a magnet and washed with water. This last washing process was repeated four times to yield a stable water colloid of 3.

3.5. Synthesis of NP-naphthalene sulfonic acid (4)

3-Hydroxy-4-amino-naphthalenesulfonic acid (0.053 g, 0.22 mmol), *N*-hydroxysuccinimide (0.024 g, 0.21 mmol) and EDC (0.021 g, 0.11 mmol) were dissolved in 5 ml of distilled water. A 5 ml aliquot of NP-azelaic acid (1) (4 mg ml^{-1} in Fe) suspended in water were added and the pH adjusted to 9 with a 1% solution of NaOH. The mixture was shaken in a vortex for 5 h and the resulting nanoparticles were separated with a magnet and washed with water. This washing process was repeated until a colourless solution was obtained upon separation of the particles to yield a stable water colloid of 4.

3.6. Relaxometry

All images were acquired at 298 K in a Bruker Biospec spectrometer (Bruker Biospec 47/40, Bruker Biospin, Germany, 4.7 T) equipped with an 11.2 cm gradient probe capable of producing gradients of up to 200 mT m^{-1} . Images to obtain T_2 parametric maps were acquired using a normal spin echo sequence consisting of 90° pulse and a train of equally spaced 180 pulses with switching phases (Carr–Purcell–Meiboom–Gill–Hill pulse sequence). Images were acquired with 20 TEs (6–120 ms) and with a TR of 5 s (TE = time of echo; TR = time of repetition). Other imaging parameters included 100 kHz spectral width, 6 cm square field of vision, 2 mm slice thickness, and 64×64 acquisition matrix size.

T_1 parametric maps were extracted from saturation recovery imaging data using a single spin echo sequence with the shortest TE and sequence parameters (6 ms), and variable TR from 50 to 3000 ms, tailored to provide sampling times with adequate coverage of the T_1 recovery curve. Other imaging parameters included were identical to T_2 acquisitions. Both T_1 and T_2 values were obtained from nonlinear least squares fitting routines included in commercial software (Bruker Paravision 3.0, Bruker Biospin, Germany).

3.7. TEM microscopy

A transmission electron microscope (Jeol-2000 FXII) operating at 200 keV was used. A drop of the corresponding nanoparticles suspension was deposited onto a carbon mesh on a copper grid and left to evaporate at room temperature.

3.8. Magnetic measurements

Magnetic characterization of the samples was carried out in a vibrating sample magnetometer using 100 μl of solution in a special sample holder. Magnetization curves were recorded at room temperature by first saturating the sample in a field of 1 T. The magnetization values were normalized to the amount of iron to yield the specific magnetization ($\text{emu g}^{-1} \text{ Fe}$). The initial susceptibility (χ) for the suspensions was measured in the field range ± 100 Oe and the saturation magnetization values (M_s) were evaluated by extrapolating to infinite field the experimental results obtained in the high field range where the magnetization linearly increases with $1/H$.

3.9. Photon correlation spectroscopy and ζ potential

Hydrodynamic size and evolution of the ζ potential vs pH for the hydrophilic suspensions were evaluated in a Zetasizer NANO-ZS device (Malvern Instruments). Hydrodynamic size was measured at pH 7. Zeta potential was measured in a special ζ potential cuvette with KNO_3 at 0.01 M concentration at different pH, provided with a He–Ne laser of 633 nm wavelength.

Acknowledgements

This work was carried out under financial support from MEC (NAN2004-08805-C04-01 and SAF2008-05412) and the regional government of Madrid CAM (S0505-AGR-0187).

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