Particle and Particle Systems Characterization

HoF₃ and DyF₃ Nanoparticles as Contrast Agents for High-Field Magnetic Resonance Imaging.

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<p>Please note, if you are submitting a revision of your manuscript, there is an opportunity for you to provide your responses to the reviewers later; please do not add them to the cover letter.</p>	<p>and DyF3 Nanoparticles as Contrast Agents for High-Field Magnetic Resonance Imaging” by Daniel González-Mancebo, Ana Isabel Becerro, Cristina Rojas, Maria L García-Martín, Jesús M de la Fuente and Manuel Ocaña (Full Paper, No. ppsc.201700116).</p> <p>We are very grateful to the reviewer 1 for his/her revision which we have accurately followed and has clearly benefited the manuscript.</p> <p>We have made the corresponding changes in the text (highlighted in yellow), as commented and detailed on the following pages. In order to facilitate the revision, we have reproduced the reviewer’s questions in blue and answered below each one in black. A clean copy of the revised manuscript has been uploaded as well.</p> <p>Looking very much to hearing from you soon,</p> <p>Dr. Ana Isabel Becerro Materials Science Institute of Seville CSIC- University of Seville</p>
<p>Do you or any of your co-authors have a conflict of interest to declare?</p>	<p>No. The authors declare no conflict of interest.</p>

**HoF₃ and DyF₃ Nanoparticles as Contrast Agents
for High-Field Magnetic Resonance Imaging.**

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Abstract

Clinical contrast agents (CAs) currently used in magnetic resonance imaging (MRI) at low fields are less effective at high magnetic fields. The development of new CAs is mandatory to improve diagnostic capabilities of the new generation of high field MRI scanners. The purpose of this study is to synthesize uniform, water dispersible LnF_3 ($\text{Ln} = \text{Ho}, \text{Dy}$) nanoparticles and to evaluate their relaxivity at high magnetic field (9.4 T) as a function of size and composition. Two different types of HoF_3 NPs were obtained by homogeneous precipitation in ethylene glycol at 120 °C. The use of holmium acetate as holmium precursor led to rhombus-like nanoparticles while smaller, ellipsoid-like nanoparticles were obtained when nitrate was used as the holmium salt. To explain this behaviour, the mechanism of formation of both kinds of particles was analysed in detail. Likewise, rhombus-like DyF_3 nanoparticles were prepared following the same method as for the rhombus-like HoF_3 nanoparticles. The transversal and longitudinal magnetic relaxivity values of the nanoparticles at high magnetic field were evaluated as a function of particle size and composition finding, to the best of our knowledge, the highest transverse relaxivity values at 9.4 T described in the literature for this kind of CAs. Finally, it was demonstrated that the three types of NPs showed negligible cytotoxicity for C6 rat glioma cells for concentrations up to 0.1 mg mL^{-1} .

1. Introduction

Magnetic resonance imaging (MRI) is one of the most powerful diagnostic tools in modern clinical medicine. It has extremely high tissue penetration and does not use ionizing radiations or radioactive agents, which are clear advantages compared to other diagnostic techniques such as X-ray computed tomography (CT), single photon emission computed tomography (SPECT), and positron emission tomography (PET).^[1] MRI signal indicates differences in the proton density, water motion or in the longitudinal (T_1) and transverse (T_2) relaxation times of the water protons, between tissues. Intrinsic contrast arising from differences in one or more of these parameters is sometimes very subtle between normal and abnormal regions, and therefore the use of contrast agents (CAs) is necessary to induce additional contrast.^[2] Clinically used CAs are currently based on gadolinium chelates,^[3] which shorten T_1 (positive CAs) and superparamagnetic iron oxide (SPIO),^[4] which shorten T_2 (negative CAs). Although not yet commercialized, Ln-based inorganic nanoparticles (NPs) appear as interesting alternative MRI CAs due to their excellent contrast enhancement compared to Gd chelates and their long circulation times, which allow for longer scans.^[5,6,7] In addition, these NPs can be easily functionalized thus allowing target specificity, which leads to reduced dose and minimized damage to normal tissues.^[8,9]

Classical MRI scanners have been operated at low magnetic fields (≤ 3 T) but the trend is to use high field magnets, which increase sensitivity and shorten the acquisition times. Current clinical CAs are less effective at ultra-high magnetic fields than at lower fields. For example, the optimum magnetic field strength for Gd^{3+} chelates is < 1 T and SPIO suffers magnetization saturation at 1.5 T.^[10] Therefore, new CAs need to be developed to improve diagnostic capabilities of the new generation of high field MRI scanners. Dy^{3+} and Ho^{3+} are two ions that are suitable for high field T_2 CAs due to their large magnetic moment (~ 10.6 and $10.0 \mu_B$, respectively) without saturation of the magnetization at very high magnetic fields.^[11] Holmium-containing materials are particularly interesting because it has been reported that Ho-based particles can be used for the radionuclide therapy of cancer cells and the paramagnetic properties could then be used to monitor the therapy by means of MRI.^[12] A few studies have been recently published on Dy- and Ho-based NPs that have shown to be good candidates for T_2 contrast agents at high magnetic field (7 T – 11.7 T). However, they have been limited to $NaDyF_4$ NPs^[13,14,15,16] and $NaHoF_4$ NPs.^[17,18,19]

1 In this study, we address the synthesis of uniform and well disperse HoF_3 and DyF_3 NPs
2 with different size and shape to assess their suitability as high field MRI CAs. While the
3 synthesis of HoF_3 NPs has not been reported up to now in the literature, the synthesis of
4 DyF_3 NPs has been recently published although the NPs are clearly irregular in shape
5 and show a high degree of aggregation,²⁰ which make them useless for bio-applications.
6 This is, therefore, the first report in the literature about the synthesis of uniform, well-
7 dispersed HoF_3 and DyF_3 NPs. The synthesis method consists in a simple and quick
8 polyol-based process at 120 °C which rendered water dispersible NPs. The as prepared
9 HoF_3 and DyF_3 NPs were thoroughly characterized in terms of their morphology and
10 crystalline structure and the formation mechanism was analysed in detail using XRD,
11 DLS, HRTEM, and HAAF-STEM. Finally, the transversal and longitudinal magnetic
12 relaxivity values of the NPs at high field (9.4 T) were evaluated as a function of particle
13 size, shape and composition finding, to best of our knowledge, the highest transverse
14 relaxivity values at 9.4 T described in the literature for this kind of CAs.
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29 **2. Results and discussion**

30 *2.1. Morphology and crystal structure of the HoF_3 nanoparticles*

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32 The formation of uniform particles in solution requires a homogeneous precipitation
33 process, which can be achieved through a slow and controlled release of either the
34 precipitating anions or cations, or both, in the reaction medium.^[21] We have used two
35 different approaches consisting in *i*) the controlled release of just the fluoride anions,
36 while the Ho^{3+} cations were freely release to the reaction medium from holmium nitrate,
37 and *ii*) the controlled release of both the fluoride anions and the Ho^{3+} cations from
38 holmium acetate ($\text{Ho}(\text{CH}_3\text{CO}_2)_3$). In both cases, the fluoride anions were thermally
39 released from the ionic liquid 1-butyl,2-methylimidazolium tetrafluoroborate
40 ($[\text{BMIM}]\text{BF}_4$) as a consequence of its hydrolysis with the hydration water molecules of
41 the metal precursors.^[22] Ionic liquids are non-volatile, non-flammable organic salts with
42 low melting point, which can be used also as solvents or additives in inorganic
43 synthesis.^[23] They show a superior capability for solvation and stabilization of the metal
44 cations, thus acting as capping agents or surfactants in the synthesis of inorganic
45 NPs.^[22,24,25] In all cases, ethylene glycol was used as solvent due to its well-documented
46 properties as cation-complexing and surface protection.^[26] In fact this solvent has been
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reported to be very adequate for the synthesis of uniform inorganic NPs, including lanthanide fluorides^[27,28,29]

TEM micrographs as well as histograms showing the dimensions of the holmium fluoride NPs obtained following the two different approaches defined above and described in the *Experimental section* are shown in Figure 1. The HoF₃ NPs synthesized from Ho(NO₃)₃ (hereafter called HoF-el), showed an ellipsoid-like morphology (Figure 1a), with a long axis of ~70 nm and a short one of ~30 nm (Figure 1b). Some particles lay with their flat face perpendicular to the TEM grid plane (dark contrast particles), from which a particle thickness of 10-15 nm can be estimated. A different morphology was obtained for the holmium fluoride synthesized from holmium acetate. This second morphology consisted of rather uniform, well-dispersed, rhombus-like NPs (Figure 1c), with average long and short diagonals of around 110 nm and 50 nm, respectively (Figure 1d). Many particles with more open ends could also be observed in the micrograph, which exhibited very similar dimensions to the rhombus-like NPs. To know the origin of the two different shapes (rhombuses and open-end NPs) observed in Figure 1c, HAADF-STEM images were taken at two different orientations of the TEM sample holder (0° and 70°) (Figure 2). It can be observed that while particle 1 shows a clear rhombus shape laying horizontally in the image recorded at 0° (Figure 2, top), it shows clear open ends when rotated 70° along the long axis of the rhombus (Figure 2, bottom). On the other hand, particle 3 (open ends) shows the opposite behavior, so that it transforms into a rhombus-like particle when rotated 70°, although in this case the rotation is slightly out of the horizontal axis of the particle. This result indicates that the two different shapes observed in Figure 1c are only two different views of the same NP. A thickness of ~40 nm of the rhombus was estimated from these images. The fact that the short axis dimension (~50 nm) of the rhombus face and the thickness (~40 nm) of the particle are quite similar allows the particles to lay either with their rhombus face or with their lateral face parallel to the TEM grid, which explains the observation of both shapes in the TEM image of Figure 1c.

DLS measurements conducted in water suspensions of the HoF-el and HoF-rh NPs at pH= 6.8 are shown in Figure 3, top. The average sizes obtained were 74(20) nm for HoF-el and 94(30) nm for HoF-rh. These values are close to the large dimension of the corresponding particles obtained from the TEM micrographs, indicating that all three NPs were well dispersed in water without the need of any further surface modification.

1 The Zeta potential of the HoF-el and HoF-rh were 38(5) mV and 42(5) mV,
2 respectively, which suggest that the colloidal stability of the suspensions was mainly
3 due to the electrostatic repulsion among positively charged particles.
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6 Figure 3, bottom shows the XRD patterns of the HoF-el and HoF-rh NPs. Both patterns
7 contain the same set of reflections, which matched well with PDF 00-023-0284 (ICDD,
8 2014) corresponding to orthorhombic HoF₃. Comparison of the intensities of the 020,
9 111 and 210 reflections with those of the PDF of orthorhombic HoF₃ indicates a
10 preferred orientation of the 020 and 210 families of planes in the two kinds of particles.
11 It is noteworthy as well that the XRD pattern of the HoF-el sample showed significantly
12 broader reflections than those of HoF-rh, which is a clear indication of the smaller
13 crystallite size of the HoF-el NPs. In addition, a clear anisotropic line broadening can be
14 observed in the XRD pattern of the HoF-el sample (compare, for example, the width of
15 the 020 and 111 reflections). This anisotropic behavior is characteristic of particles with
16 a high aspect ratio like platelets or ribbons, and suggests that the HoF-el particles are
17 flat. We have obtained the size of the coherent diffraction domains of both samples after
18 applying the Scherrer formula to the three most intense reflections (020, 111 and 210).
19 Crystallite size values between 5 nm and 15 nm for the HoF-el sample and between 30
20 nm and 50 nm for the HoF-rh sample were obtained. The values obtained for the HoF-el
21 sample are smaller than the particle dimensions and reflects the polycrystalline nature of
22 the HoF-el NPs. The case of the rhombus-like particles is clearly different, as the
23 biggest crystallite size obtained from the XRD pattern is close to two dimensions of the
24 NP (the short axis of the rhombus and its thickness), thus suggesting that these particles
25 could be formed by single crystals.
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43 Further information on the single- or polycrystalline nature of the rhombus-like NPs
44 was obtained from HRTEM. The DDP obtained from the square area located in the
45 middle of the rhombus (Figure 4a) shows well-defined spots, characteristic of a single
46 crystal. However, the DDP calculated from the edge of the NP shows elongated spots
47 compatible with the presence of crystallites that are slightly misoriented. These data
48 allow concluding that the rhombus-like NPs are formed by an ordered aggregation of
49 smaller subunits. The crystallites must be perfectly oriented in the middle of the NP,
50 thus producing a DDP characteristic of a single crystal. The orientation of the
51 crystallographic axes of the HoF₃ crystal structure in the building units could be
52 determined from the HRTEM and the DDP spots obtained for single domains (Figure
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4b). Two sets of planes with an interplanar distance of 3.4 Å and 2.9 Å are clearly observed in the HRTEM image and in the DDP of the blue square, which correspond to the 020 and 210 planes, respectively, of the orthorhombic HoF₃ phase, in good agreement with the XRD data. The whole set of spots in the DDP correspond to the [001] zone axis of such structure, indicating that the crystallite grains attach to each other through the crystalline faces parallel to the *c* crystallographic axis and that such axis is therefore perpendicular to the rhombus face of the particle.

Although the XRD pattern of the HoF-el samples clearly demonstrated that the NPs were flat and that they were formed by smaller constituent subunits, we have also carried out a HRTEM study on this type of particles to analyze if the crystallites are oriented to each other as well as to know, in such case, the orientation of the *c* crystallographic axis of the HoF₃ structure respect to the particle surface. The HRTEM image of a single HoF-el NP (Figure 5a) is in good agreement with the XRD data as it shows that it is formed by small crystalline grains. The grains are somehow oriented to each other as inferred from the slightly elongated spots of the DDP calculated from the whole particle (Figure 5b). The higher magnification HRTEM image of Figure 5c shows crystallites of around 5 – 15 nm size, in agreement with the XRD data. The DDP calculated from one of those crystallites (white quadrangle in Figure 5c) shows the 020 and 210 planes of the orthorhombic HoF₃ phase, in good agreement with the preferred orientation observed in the XRD pattern of this sample. The set of spots observed in the DDP corresponds to the [001] zone axis of orthorhombic HoF₃, which suggests that the crystallites are attached to each other through the crystalline faces parallel to the *c* crystallographic axis. These data allow concluding that also in this case the NPs are formed by an ordered aggregation of smaller subunits.

To corroborate the formation mechanism suggested by the XRD and electron diffraction data, time-dependent experiments were carried out in the synthesis conditions of the HoF-rh and HoF-el NPs. Each reaction was quenched by immersing the reactor in an ice bath after aging. Figures 6a and 6b show TEM micrographs and XRD patterns, respectively, of the products obtained after aging for 15 min, 1.5 h, 2.5 h, and 4 h the corresponding solutions leading to the HoF-rh NPs. Before 10 minutes no precipitation was observed, while elongated particles, with approximate dimensions 250(30) nm length and 40(5) nm width, were formed after 10 minutes of aging. The corresponding XRD pattern showed broad reflections compatible with orthorhombic HoF₃ symmetry.

A significantly lower peak width is observed for the 020 than for the rest of reflections in the pattern, suggesting flat NPs. Applying the Scherrer formula to several single reflections, we obtained crystallite sizes in the range 5 – 10 nm, while the width of the 020 reflection indicated a crystallite size of 25 nm. In addition, a clear preferred orientation of the 020 planes is observed in the pattern. A detailed observation of the TEM micrograph of these particles (Figure 7a) clearly shows that they are formed by smaller subunits. The DDP obtained from the whole particle (insert in Figure 7a) shows elongated spots that suggest that the constituent subunits are already slightly oriented from the very early stages of the reaction. The HRTEM micrograph (Figure 7b) showed spherical crystalline grains of 5 – 10 nm with lattice fringes at 3.4 Å corresponding to the 020 planes, in good agreement with the XRD results. Increasing reaction time to 1.5 hours led to a decrease in the length of the particles (~200(30) nm) and to an increase in their width (100(10) nm) (Figure 6a). A clear anisotropic peak broadening was still observed in the corresponding XRD pattern (Figure 6b), suggesting that the particles are still quite flat at this stage. The 1.5 hrs TEM image of Figure 6a showed that some of the particles (with a darker contrast) lay with their flat face perpendicular to the TEM grid plane, from which a particle thickness of 20-30 nm can be estimated. Likewise, a dark contrast can be observed in the central part of each particle. The contrast increased with prolonging reaction time, as clearly observed in the particles obtained after 2.5 hours of aging, which showed smaller dimensions: 120(10) x 90(10) nm). A detail TEM study has been carried on the latter particles to shed some light on the origin of the dark contrast. The BF and HAADF-STEM line profiles obtained along the long axis of the particle shown in Figure 8a have been plotted in Figure 8b. These profiles indicate that the contrast is due to both, a diffraction contrast and a higher thickness in the center of the particle compared with the ends. In fact, the DDPs obtained from the HRTEM image of Figure 8c, calculated from the center and from one end of the particle, showed very well defined spots in the former and more elongated in the latter, suggesting a higher degree of ordering of the crystallites in the center of the NP. The narrower reflections observed in the corresponding XRD pattern, in comparison with the 1.5 hours pattern, clearly indicate a higher degree of crystallinity of the sample, in agreement with the TEM observations.

Finally, after 4 hours aging, the particles appeared completely formed (Figure 6a) and their XRD pattern (Figure 6b) clearly showed all the reflections expected for orthorhombic HoF₃, as described in the former subsection.

In contrast with the slow kinetics of the HoF-rh NPs, the ellipsoid-like NPs formed very quickly after only 10 minutes reaction (Figure 9a). At earlier stages (5 minutes) the reaction product consisted of an amorphous gel-like precipitate, as shown in the corresponding TEM micrograph, while longer reaction times (up to 4 hours) did not alter either their size, morphology or crystallinity, as shown in the corresponding XRD patterns of Figure 9b.

With the information obtained from the XRD and TEM data described above, the following formation mechanism can be envisaged for the rhombus- and ellipsoid-like NPs (Figure 10). The fluoride anions required for the precipitation of the HoF₃ phase are provided by the BF₄⁻ anions of the ionic liquid.^[22] The BF₄⁻ anions undergo fast hydrolysis on heating with the hydration water molecules of the corresponding holmium salt (Ho(CH₃CO₂)₃·xH₂O and Ho(NO₃)₃·5H₂O), according to the reaction:

On the other hand, the Ho³⁺ cations are released from the nitrate and acetate precursors. The free Ho³⁺ cations can then react with the free fluoride anions to form HoF₃ nuclei. Such nuclei grow and constitute the primary particles, which join together to form the particles observed after 10 minutes reaction. A certain orientation of the crystallites already exists at this reaction stage, possibly caused by the known capping effect of the ionic liquid, as commented above.^[30] The role of the holmium precursor is crucial from this very early stage of the reaction. While there is a controlled release of Ho³⁺ cations from the acetate salt due to the well-known complexing capacity of the acetate anion,^[26] the Ho cations are free in the solution when nitrate is used as the holmium precursor, thus accelerating the reaction rate and giving rise to different particle morphologies. Elongated ribbon-like particles are formed from acetate while smaller and the less anisometric ellipsoid-like nanoparticles are obtained from Ho³⁺ nitrate. The latter morphology seems to be much more stable than the former as demonstrated by the absence of any morphological or crystallographic change with increasing reaction time in the case of the ellipsoid-like NPs. However, the ribbon-like particles are clearly unstable in these conditions as inferred from the significant reorganization of the

primary units into wider, shorter particles when the aging time is increased to 1.5 and 2.5 hours. This process progresses until the system reaches an equilibrium state with rhombus geometry. During the reorganization, the primary units attach to each other through the crystalline faces parallel to the c axis of the HoF_3 crystal structure, as indicated by HRTEM.

2.2. Morphology and crystal structure of the DyF_3 nanoparticles

In order to evaluate the influence of the lanthanide nature on the magnetic relaxivity of the lanthanide fluoride NPs, we have synthesized DyF_3 NPs following the same method used to obtain the HoF -rh NPs. The method rendered well-dispersed DyF_3 NPs, hereafter called DyF -rhNPs, with the same morphology as that of the HoF -rh NPs (Figure 11a). Figure 11b shows the histograms obtained after measuring the long and short dimensions of the NPs in a hundred NPs from TEM images, which gave an average of 110 nm x 50 nm, as obtained for the HoF -rh NPs. The particles are well dispersed in water as inferred from the DLS curve of Figure 11c, which indicates an average hydrodynamic diameter of 100(35) nm. The Zeta potential value was 43(6) mV, very close to the one obtained for the HoF -rh NPs, which suggests that also in this case, the colloidal stability of the suspension was mainly due to the electrostatic repulsion among positively charged particles. Finally, the XRD pattern shown in Figure 11d is compatible with orthorhombic DyF_3 .

2.3. Magnetic resonance relaxivity

Magnetic relaxivities of the HoF -el, HoF -rh and DyF -rh NPs were evaluated at high magnetic field (9.4 T). With this purpose, the longitudinal (T_1) and transversal (T_2) proton relaxation times were measured in aqueous suspensions of the NPs containing different concentrations of Ho^{3+} and Dy^{3+} ions at 298 K. Figure 12 shows the $1/T_1$ and $1/T_2$ values obtained at 9.4 T as a function of the Ln^{3+} concentration for all three NPs. The slopes of the curves gave the relaxivity values r_1 and r_2 shown in Table 1. The following comments can be made in view of the relaxivity values obtained:

(1) Relaxivity measurements performed at 9.4 T showed r_2 values much higher than the r_1 ones for all three nanoparticle types, which indicate that the synthesized LnF_3 NPs are adequate as negative contrast agents.^[31] This behavior is due to the anisotropic $4f$ electron ground state of both Dy^{3+} and Ho^{3+} ions, which give rise to a short electronic

relaxation time (T_{1e}) on the order of 10-13 sec, leading to a minor contribution to T_1 relaxation, according to the SBM theory.^[32] However, both Dy^{3+} and Ho^{3+} ions own large magnetic moments (10.63 μ_B and 10.60 μ_B , respectively) which make them very efficient as T_2 MRI CAs.

(2) It can be observed in Table 1 that the r_2 value obtained for the HoF-rh NPs (608.39 $mM^{-1}s^{-1}$) is significantly higher than that obtained for the HoF-el NPs (349.98 $mM^{-1}s^{-1}$). This difference can be ascribed to the different size of both particles and is in good agreement with the behavior of $NaHoF_4$,^[17] $NaDyF_4$,^[18,14] and Dy_2O_3 ^[33] NPs, where increasing transversal relaxivity was also observed with increasing particle size. This trend has been explained using two different approaches. On one hand, the increase in relaxivity with increasing particle size has been attributed to the higher magnetization of big NPs compared with the smaller ones as a result of the *spin-canting* effect. Such effect appears in the nanoscale regime and consists in the tilting of the surface spins, which significantly influences the magnetic moments of the NPs. The spin-canting effect is size-dependent so that as the nanoparticle size increases the surface effect becomes less pronounced and results in a higher magnetization value for the big NPs compared with the smaller ones.^[34] On the other hand, the increase in transversal relaxivity with increasing nanoparticle size has been explained in terms of the diffusion correlation time τ_D of the NPs ($\tau_D = r_c^2/D$, where r_c is the radius of the nanoparticle and $D = 2.5 \times 10^{-9} m^2 s^{-1}$ is the diffusion coefficient of water at 25 °C). For small nanoparticle sizes ($\tau_D < 1.5 \times 10^{-6} s$), which is the case for our three particle types (HoF-el, HoF-rh and DyF-rh), the relaxivity is described in terms of the outer-sphere theory,^[35,32] which indicates that r_2 is proportional to τ_D and, therefore, to the particle size.

(3) The r_2 value of the HoF-rh NPs (608.39 $s^{-1} mM^{-1}$) is higher than that of the DyF-rh NPs of the same size (380.37 $s^{-1} mM^{-1}$), in spite of the slightly larger effective magnetic moment of Dy^{3+} compared to that of Ho^{3+} . A similar trend was described for spherical $NaHoF_4$ (17 nm) and rod-like $NaDyF_4$ (19 x 25 nm) NPs, which showed r_2 values of 130.6 and 91.4 $mM^{-1}s^{-1}$, respectively at 9.4 T.^[18] It is in turn remarkable that the relaxivity values obtained for any of our LnF_3 NPs are higher than those reported for $NaHoF_4$ and $NaDyF_4$.

(4) It is well known that the higher the r_2/r_1 ratio, the higher the contrast efficiency for a T_2 MRI CA. [36] The r_2/r_1 ratios of the three LnF_3 NPs synthesized in this work are significantly higher than that of Resovist (215 at 9.4 T), a clinically approved SPIO negative MRI contrast agent. [37]

These results strongly suggest the potential applicability of the synthesized HoF_3 and DyF_3 NPs as high field MRI contrast agents.

2.4. Cytotoxicity assay

The three types of NPs (HoF-el , HoF-rh and DyF-rh) were submitted to biocompatibility studies by evaluating the viability of C6 rat glioma cells with the MTT assay. The results showed that cell viability remained around 90% in all three cases (Figure 13) even at the high concentration of 100 $\mu\text{g/mL}$, concluding that there was an absence of mitochondrial damage after exposure to HoF-el , HoF-rh or DyF-rh NPs.

3. Conclusions

We have successfully synthesized for the first time uniform, orthorhombic HoF_3 NPs with a rhombus-like morphology (110 nm x 50 nm) using a polyol-based precipitation method at 120 °C with lanthanide acetate and $[\text{BMIM}]\text{BF}_4$ as the holmium and fluoride sources, respectively. Smaller ellipsoid-like HoF_3 NPs (70 nm x 30 nm) were obtained following the same methodology but using $\text{Ho}(\text{NO}_3)_3$ as the holmium precursor. This result was explained on the basis of the complexing capacity of the acetate anion. While the ellipsoid NPs are simply formed by the attachment of smaller constituent subunits, the formation mechanism of the rhombuses is more complex, involving a considerable reorganization of the constituent subunits from elongated NPs to rhombus-like NPs. In order to analyze the influence of the lanthanide nature on the magnetic relaxivity of the lanthanide fluoride NPs, uniform, rhombus-like DyF_3 NPs were also obtained following the same method as used for the rhombus-like HoF_3 NPs. Relaxivity measurements performed at 9.4 T showed r_2 values much higher than the r_1 ones for all three nanoparticle types, which indicate that the LnF_3 NPs are adequate as negative contrast agents. Likewise, the r_2 values were found to be dependent on the NP size, so that the bigger the NP, the higher the r_2 value. The rhombus-like HoF_3 NPs rendered higher r_2 values higher than the DyF_3 NPs with the same morphology. It was found that the r_2/r_1

values were much higher than that reported for Resovist, a clinically approved SPIO negative MRI contrast agent. Finally, cytotoxicity assays showed absence of mitochondrial damage after exposure of C6 cells to any of the three types of NPs. These results indicate that the synthesized NPs are potentially useful as high field MRI negative CAs.

4. Experimental

4.1. Materials

Holmium acetate hydrate ($\text{Ho}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), holmium nitrate hydrate ($\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, 99.9%), dysprosium acetate hydrate ($(\text{CH}_3\text{CO}_2)_3\text{Dy} \cdot x\text{H}_2\text{O}$ (99.9%), 1-butyl-2-methylimidazolium tetrafluoroborate ($[\text{BMIM}]\text{BF}_4$, $\text{C}_8\text{H}_{15}\text{BF}_4\text{N}_2$, $\geq 97\%$), and ethylene glycol (EG, $\text{C}_2\text{H}_6\text{O}_2$, 99.5%), all of them purchased from Sigma Aldrich.

4.2. Synthesis of the NPs

Rhombus-like HoF_3 NPs were obtained according to the following method: Holmium acetate ($\text{Ho}(\text{CH}_3\text{CO}_2)_3$) was dissolved in EG with magnetic stirring at $70\text{ }^\circ\text{C}$ to obtain a 0.04 M solution (total volume 2.5 mL). The solution was then cooled down to room temperature, admixed with 2.5 mL of $[\text{BMIM}]\text{BF}_4$ and magnetically stirred for 5 minutes at room temperature to favor homogenization. The final solution was aged for 4 hours in tightly closed test tubes using an oven preheated at $120\text{ }^\circ\text{C}$. The resulting dispersion was cooled down to room temperature, centrifuged to remove the supernatants and washed, twice with ethanol and once with double distilled water. For some analyses, the powders were dried at room temperature. Several experiments were also conducted during shorter reaction times to find out the reaction mechanism for the formation of the NPs. An ice bath was used to quench the reactions after aging. Rhombus-like DyF_3 NPs were obtained from Dy acetate ($\text{Dy}(\text{CH}_3\text{CO}_2)_3$) following the same method as for the rhombus-like HoF_3 NPs. Ellipsoid-like HoF_3 NPs were synthesized following the same method described above for the rhombus-like particles but using $\text{Ho}(\text{NO}_3)_3$ as the Ho source instead of $\text{Ho}(\text{CH}_3\text{CO}_2)_3$.

4.3. Characterization techniques

1 The shape and size of the nanoparticles was examined by *transmission electron*
2 *microscopy (TEM, Philips 200CM)*. Particle size distributions were obtained from the
3 micrographs by counting several hundreds of particles, using the free software *ImageJ*.
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6 To gain additional information on the morphological and structural features of the
7 synthesized nanoparticles, they were also characterized by *High Angle Annular Dark*
8 *Field Scanning TEM (HAADF-STEM)* and *High Resolution TEM* by using a FEI
9 Tecnai field emission gun STEM microscope (G2F30) working at 300 kV (0.2 nm point
10 resolution) and equipped with a HAADF detector from Fischione (0.16 nm point
11 resolution in STEM-HAADF mode). A Fischione ultra-narrow gap tomography holder
12 was used to acquire HAADF-STEM images from 0° to 70°. The *Digital Diffraction*
13 *Patterns (DDP)* were obtained from the HRTEM images using the *Tecni Imaging and*
14 *Analysis (TIA)* software.
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18 Additional information on the size and colloidal stability of the particles in aqueous
19 suspension (0.5 mg·mL⁻¹ of solid) was obtained from *Dynamic Light Scattering (DLS)*
20 measurements. The experiments were carried out using a Malvern Zetasizer Nano-ZS90
21 equipment, which was used as well to measure the Zeta potential of the suspensions.
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24 The crystalline structure of the particles was assessed by *X-ray diffraction (XRD)* using
25 a Panalytical, X' Pert Pro diffractometer (CuK α) with an X-Celerator detector over an
26 angular range of 10° < 2 θ < 120°, 2 θ step width of 0.02°, and 10 s counting time. The
27 crystallite size was estimated using the Scherrer formula from the full width at half
28 maximum of a single reflection. A shape factor of k = 0.9 and an instrumental
29 broadening factor of 0.112 were used for the calculation.
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32 ¹H NMR relaxation times, T₁ and T₂, were measured at 9.4 T using a Bruker Biospec at
33 different concentrations of Ho³⁺ and Dy³⁺ (0.2, 0.4, 0.6, 0.8, and 1.0 mmol dm⁻³) in
34 water at 298 K. T₁ was determined either using inversion-recovery or saturation
35 recovery sequences and T₂ was determined using the Carl-Purcell-Meiboom-Gill
36 (CPMG) sequence. Relaxivities (r₁, r₂) were obtained from the slopes of the curves 1/T₁
37 or 1/T₂ vs. the concentration of Ho³⁺ and Dy³⁺ expressed in mM.
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Cytotoxicity assay: C6 rat glioma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37° C in an incubator with a humidified

atmosphere containing 5% CO₂. To perform the cytotoxicity assays, C6 cells were plated at a density of 1 x 10⁴ cells/well in a 96-well plate at 37°C in 5% CO₂ atmosphere (200 µL per well, number of replicates = 5). After 24 h of culture, the medium in the wells was replaced with fresh medium containing the magnetic NPs in varying concentrations from 0.5 µg/mL to 500 µg/mL. After 24 h, the medium was removed and 200 µL of fresh medium with MTT (0.5 mg/mL) was added to each well. After 2 h of incubation at 37°C and 5% CO₂, the medium was removed and the formazan crystals were solubilized with 200 µL of DMSO. Two controls were performed to adequately evaluate cytotoxicity: cells unexposed to the NPs were used as negative control, and cells exposed to Triton X-100 (1% v/v) for 15 min prior to the MTT procedure, were used as positive control. The absorbance of each well was read on a microplate reader (Dynatech MR7000 instruments) at 550 nm. The relative cell viability (%) and its error with respect to the control wells containing cell culture medium without nanoparticles was calculated as follows:

$$Relative\ Cell\ Viability\ (RCV)\ (\%) = \frac{[Abs]_{test}}{[Abs]_{control}} \times 100$$

$$Error\ (\%) = RCV_{test} \times \sqrt{\left(\frac{[\sigma]_{test}}{[Abs]_{test}}\right)^2 + \left(\frac{[\sigma]_{control}}{[Abs]_{control}}\right)^2}$$

where σ is the standard deviation.

Acknowledgements

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Table 1: Proton relaxivity values (r_1 and r_2) obtained for water suspensions of the different HoF_3 and DyF_3 NPs at 9.4 T and 298 K.

LnF ₃ NPs (dimensions)	B= 9.4 T		
	r ₁ (s ⁻¹ mM ⁻¹)	r ₂ (s ⁻¹ mM ⁻¹)	r ₂ /r ₁
HoF-el (70x30 nm)	0.95	349.98	368.40
HoF-rh (110x50 nm)	0.59	608.39	1031.17
DyF-rh (110x50 nm)	0.68	380.37	559.37

Figure captions

Figure 1: TEM micrographs and size distribution plots obtained from TEM images of HoF-el (a,b) and HoF-rh (c,d) NPs obtained in the conditions described in the experimental section.

Figure 2: HAADF-STEM images of the HoF-rh sample, recorder at 0° (top) and 70° (bottom) tilt angle.

Figure 3: *Top:* DLS plots showing the particle size distribution of the HoF-el (a) and HoF-rh (b). *Bottom:* XRD patterns of the HoF-el (a) and HoF-rh (b) NPs. The lines at the bottom of the figure are the reflections corresponding to orthorhombic HoF₃ (ICDD PDF 00-023-0284).

Figure 4: (a) HRTEM image of a HoF-rh NP. The inset shows two DDPs obtained from center (red) and edge (blue) areas. (b) Higher magnification image corresponding to the white rectangle area shown in (a), showing single domains (dashed circles). The insert is an indexed DDP calculated from the blue square.

Figure 5: (a) TEM image of a HoF-el NP, (b) DDP pattern obtained from the whole nanoparticle, (c) HRTEM micrograph of the HoF-el NP shown in (a).

Figure 6: TEM micrographs (a) and XRD patterns (b) of the NPs observed after different aging times in the experimental conditions leading to HoF-rh NPs.

Figure 7: (a) Detail of one NP shown in Figure 6a after 10 minutes reaction. (b) High magnification of the white square in figure 7a. The inset is the DDP obtained from the marked area.

Figure 8: (a) HAADF-STEM image of a NP shown in Figure 6a after 2.5 h reaction and BF-STEM and HAADF-STEM line profiles along the long axis of the NP. (b) HRTEM image of the same NP. The insets are DDPs obtained from the center (red) and edge (green) areas.

Figure 9: TEM micrographs (a) and XRD patterns (b) of the NPs observed after different aging times in the experimental conditions leading to HoF-el NPs.

Figure 10: Schematic illustration of the formation mechanism of the ellipsoid-like and rhombus-like NPs showing the aggregation of their small constituents as well as their tridimensional morphology.

Figure 11: TEM micrograph (a), size distribution plots obtained from several TEM images (b), DLS plots showing the particle size distribution (c), and XRD patterns (d) of the rhombus-like DyF₃NPs. The lines at the bottom of plot (d) are the reflections corresponding to orthorhombic DyF₃ (ICDD PDF 00-032-0352).

Figure 12: T_1 and T_2 proton relaxivity plots obtained at room temperature using a 9.4 T magnet in aqueous suspensions of HoF-el (a), HoF-rh (b), and DyF-rh (c) NPs *versus* the lanthanide concentration.

Figure 13: Cytotoxicity assay of C6 cells incubated with different concentrations of HoF-el, HoF-rh and DyF-rh NPs for 24 h.

HoF₃ and DyF₃ Nanoparticles as Contrast Agents for High-Field Magnetic Resonance Imaging.

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Abstract

Clinical contrast agents (CAs) currently used in magnetic resonance imaging (MRI) at low fields are less effective at high magnetic fields. The development of new CAs is mandatory to improve diagnostic capabilities of the new generation of high field MRI scanners. The purpose of this study is to synthesize uniform, water dispersible LnF_3 ($\text{Ln} = \text{Ho}, \text{Dy}$) nanoparticles and to evaluate their relaxivity at high magnetic field (9.4 T) as a function of size and composition. Two different types of HoF_3 NPs were obtained by homogeneous precipitation in ethylene glycol at 120 °C. The use of holmium acetate as holmium precursor led to rhombus-like nanoparticles while smaller, ellipsoid-like nanoparticles were obtained when nitrate was used as the holmium salt. To explain this behaviour, the mechanism of formation of both kinds of particles was analysed in detail. Likewise, rhombus-like DyF_3 nanoparticles were prepared following the same method as for the rhombus-like HoF_3 nanoparticles. The transversal and longitudinal magnetic relaxivity values of the nanoparticles at high magnetic field were evaluated as a function of particle size and composition finding, to the best of our knowledge, the highest transverse relaxivity values at 9.4 T described in the literature for this kind of CAs. Finally, it was demonstrated that the three types of NPs showed negligible cytotoxicity for C6 rat glioma cells for concentrations up to 0.1 mg mL^{-1} .

1. Introduction

Magnetic resonance imaging (MRI) is one of the most powerful diagnostic tools in modern clinical medicine. It has extremely high tissue penetration and does not use ionizing radiations or radioactive agents, which are clear advantages compared to other diagnostic techniques such as X-ray computed tomography (CT), single photon emission computed tomography (SPECT), and positron emission tomography (PET).^[1] MRI signal indicates differences in the proton density, water motion or in the longitudinal (T_1) and transverse (T_2) relaxation times of the water protons, between tissues. Intrinsic contrast arising from differences in one or more of these parameters is sometimes very subtle between normal and abnormal regions, and therefore the use of contrast agents (CAs) is necessary to induce additional contrast.^[2] Clinically used CAs are currently based on gadolinium chelates,^[3] which shorten T_1 (positive CAs) and superparamagnetic iron oxide (SPIO),^[4] which shorten T_2 (negative CAs). Although not yet commercialized, Ln-based inorganic nanoparticles (NPs) appear as interesting alternative MRI CAs due to their excellent contrast enhancement compared to Gd chelates and their long circulation times, which allow for longer scans.^[5,6,7] In addition, these NPs can be easily functionalized thus allowing target specificity, which leads to reduced dose and minimized damage to normal tissues.^[8,9]

Classical MRI scanners have been operated at low magnetic fields (≤ 3 T) but the trend is to use high field magnets, which increase sensitivity and shorten the acquisition times. Current clinical CAs are less effective at ultra-high magnetic fields than at lower fields. For example, the optimum magnetic field strength for Gd^{3+} chelates is < 1 T and SPIO suffers magnetization saturation at 1.5 T.^[10] Therefore, new CAs need to be developed to improve diagnostic capabilities of the new generation of high field MRI scanners. Dy^{3+} and Ho^{3+} are two ions that are suitable for high field T_2 CAs due to their large magnetic moment (~ 10.6 and $10.0 \mu_B$, respectively) without saturation of the magnetization at very high magnetic fields.^[11] Holmium-containing materials are particularly interesting because it has been reported that Ho-based particles can be used for the radionuclide therapy of cancer cells and the paramagnetic properties could then be used to monitor the therapy by means of MRI.^[12] A few studies have been recently published on Dy- and Ho-based NPs that have shown to be good candidates for T_2 contrast agents at high magnetic field (7 T – 11.7 T). However, they have been limited to $NaDyF_4$ NPs^[13,14,15,16] and $NaHoF_4$ NPs.^[17,18,19]

1 In this study, we address the synthesis of uniform and well disperse HoF₃ and DyF₃ NPs
2 with different size and shape to assess their suitability as high field MRI CAs. While the
3 synthesis of HoF₃ NPs has not been reported up to now in the literature, the synthesis of
4 DyF₃ NPs has been recently published although the NPs are clearly irregular in shape
5 and show a high degree of aggregation,²⁰ which make them useless for bio-applications.
6 This is, therefore, the first report in the literature about the synthesis of uniform, well-
7 dispersed HoF₃ and DyF₃ NPs. The synthesis method consists in a simple and quick
8 polyol-based process at 120 °C which rendered water dispersible NPs. The as prepared
9 HoF₃ and DyF₃ NPs were thoroughly characterized in terms of their morphology and
10 crystalline structure and the formation mechanism was analysed in detail using XRD,
11 DLS, HRTEM, and HAAF-STEM. Finally, the transversal and longitudinal magnetic
12 relaxivity values of the NPs at high field (9.4 T) were evaluated as a function of particle
13 size, shape and composition finding, to best of our knowledge, the highest transverse
14 relaxivity values at 9.4 T described in the literature for this kind of CAs.
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29 **2. Results and discussion**

30 *2.1. Morphology and crystal structure of the HoF₃ nanoparticles*

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32 The formation of uniform particles in solution requires a homogeneous precipitation
33 process, which can be achieved through a slow and controlled release of either the
34 precipitating anions or cations, or both, in the reaction medium.^[21] We have used two
35 different approaches consisting in *i*) the controlled release of just the fluoride anions,
36 while the Ho³⁺ cations were freely release to the reaction medium from holmium nitrate,
37 and *ii*) the controlled release of both the fluoride anions and the Ho³⁺ cations from
38 holmium acetate (Ho(CH₃CO₂)₃). In both cases, the fluoride anions were thermally
39 released from the ionic liquid 1-butyl,2-methylimidazolium tetrafluoroborate
40 ([BMIM]BF₄) as a consequence of its hydrolysis with the hydration water molecules of
41 the metal precursors.^[22] Ionic liquids are non-volatile, non-flammable organic salts with
42 low melting point, which can be used also as solvents or additives in inorganic
43 synthesis.^[23] They show a superior capability for solvation and stabilization of the metal
44 cations, thus acting as capping agents or surfactants in the synthesis of inorganic
45 NPs.^[22,24,25] In all cases, ethylene glycol was used as solvent due to its well-documented
46 properties as cation-complexing and surface protection.^[26] In fact this solvent has been
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reported to be very adequate for the synthesis of uniform inorganic NPs, including lanthanide fluorides^[27,28,29]

TEM micrographs as well as histograms showing the dimensions of the holmium fluoride NPs obtained following the two different approaches defined above and described in the *Experimental section* are shown in Figure 1. The HoF₃ NPs synthesized from Ho(NO₃)₃ (hereafter called HoF-el), showed an ellipsoid-like morphology (Figure 1a), with a long axis of ~70 nm and a short one of ~30 nm (Figure 1b). Some particles lay with their flat face perpendicular to the TEM grid plane (dark contrast particles), from which a particle thickness of 10-15 nm can be estimated. A different morphology was obtained for the holmium fluoride synthesized from holmium acetate. This second morphology consisted of rather uniform, well-dispersed, rhombus-like NPs (Figure 1c), with average long and short diagonals of around 110 nm and 50 nm, respectively (Figure 1d). Many particles with more open ends could also be observed in the micrograph, which exhibited very similar dimensions to the rhombus-like NPs. To know the origin of the two different shapes (rhombuses and open-end NPs) observed in Figure 1c, HAADF-STEM images were taken at two different orientations of the TEM sample holder (0° and 70°) (Figure 2). It can be observed that while particle 1 shows a clear rhombus shape laying horizontally in the image recorded at 0° (Figure 2, top), it shows clear open ends when rotated 70° along the long axis of the rhombus (Figure 2, bottom). On the other hand, particle 3 (open ends) shows the opposite behavior, so that it transforms into a rhombus-like particle when rotated 70°, although in this case the rotation is slightly out of the horizontal axis of the particle. This result indicates that the two different shapes observed in Figure 1c are only two different views of the same NP. A thickness of ~40 nm of the rhombus was estimated from these images. The fact that the short axis dimension (~50 nm) of the rhombus face and the thickness (~40 nm) of the particle are quite similar allows the particles to lay either with their rhombus face or with their lateral face parallel to the TEM grid, which explains the observation of both shapes in the TEM image of Figure 1c.

DLS measurements conducted in water suspensions of the HoF-el and HoF-rh NPs at pH= 6.8 are shown in Figure 3, top. The average sizes obtained were 74(20) nm for HoF-el and 94(30) nm for HoF-rh. These values are close to the large dimension of the corresponding particles obtained from the TEM micrographs, indicating that all three NPs were well dispersed in water without the need of any further surface modification.

1 The Zeta potential of the HoF-el and HoF-rh were 38(5) mV and 42(5) mV,
2 respectively, which suggest that the colloidal stability of the suspensions was mainly
3 due to the electrostatic repulsion among positively charged particles.
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6 Figure 3, bottom shows the XRD patterns of the HoF-el and HoF-rh NPs. Both patterns
7 contain the same set of reflections, which matched well with PDF 00-023-0284 (ICDD,
8 2014) corresponding to orthorhombic HoF₃. Comparison of the intensities of the 020,
9 111 and 210 reflections with those of the PDF of orthorhombic HoF₃ indicates a
10 preferred orientation of the 020 and 210 families of planes in the two kinds of particles.
11 It is noteworthy as well that the XRD pattern of the HoF-el sample showed significantly
12 broader reflections than those of HoF-rh, which is a clear indication of the smaller
13 crystallite size of the HoF-el NPs. In addition, a clear anisotropic line broadening can be
14 observed in the XRD pattern of the HoF-el sample (compare, for example, the width of
15 the 020 and 111 reflections). This anisotropic behavior is characteristic of particles with
16 a high aspect ratio like platelets or ribbons, and suggests that the HoF-el particles are
17 flat. We have obtained the size of the coherent diffraction domains of both samples after
18 applying the Scherrer formula to the three most intense reflections (020, 111 and 210).
19 Crystallite size values between 5 nm and 15 nm for the HoF-el sample and between 30
20 nm and 50 nm for the HoF-rh sample were obtained. The values obtained for the HoF-el
21 sample are smaller than the particle dimensions and reflects the polycrystalline nature of
22 the HoF-el NPs. The case of the rhombus-like particles is clearly different, as the
23 biggest crystallite size obtained from the XRD pattern is close to two dimensions of the
24 NP (the short axis of the rhombus and its thickness), thus suggesting that these particles
25 could be formed by single crystals.
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28 Further information on the single- or polycrystalline nature of the rhombus-like NPs
29 was obtained from HRTEM. The DDP obtained from the square area located in the
30 middle of the rhombus (Figure 4a) shows well-defined spots, characteristic of a single
31 crystal. However, the DDP calculated from the edge of the NP shows elongated spots
32 compatible with the presence of crystallites that are slightly misoriented. These data
33 allow concluding that the rhombus-like NPs are formed by an ordered aggregation of
34 smaller subunits. The crystallites must be perfectly oriented in the middle of the NP,
35 thus producing a DDP characteristic of a single crystal. The orientation of the
36 crystallographic axes of the HoF₃ crystal structure in the building units could be
37 determined from the HRTEM and the DDP spots obtained for single domains (Figure
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4b). Two sets of planes with an interplanar distance of 3.4 Å and 2.9 Å are clearly observed in the HRTEM image and in the DDP of the blue square, which correspond to the 020 and 210 planes, respectively, of the orthorhombic HoF₃ phase, in good agreement with the XRD data. The whole set of spots in the DDP correspond to the [001] zone axis of such structure, indicating that the crystallite grains attach to each other through the crystalline faces parallel to the *c* crystallographic axis and that such axis is therefore perpendicular to the rhombus face of the particle.

Although the XRD pattern of the HoF-el samples clearly demonstrated that the NPs were flat and that they were formed by smaller constituent subunits, we have also carried out a HRTEM study on this type of particles to analyze if the crystallites are oriented to each other as well as to know, in such case, the orientation of the *c* crystallographic axis of the HoF₃ structure respect to the particle surface. The HRTEM image of a single HoF-el NP (Figure 5a) is in good agreement with the XRD data as it shows that it is formed by small crystalline grains. The grains are somehow oriented to each other as inferred from the slightly elongated spots of the DDP calculated from the whole particle (Figure 5b). The higher magnification HRTEM image of Figure 5c shows crystallites of around 5 – 15 nm size, in agreement with the XRD data. The DDP calculated from one of those crystallites (white quadrangle in Figure 5c) shows the 020 and 210 planes of the orthorhombic HoF₃ phase, in good agreement with the preferred orientation observed in the XRD pattern of this sample. The set of spots observed in the DDP corresponds to the [001] zone axis of orthorhombic HoF₃, which suggests that the crystallites are attached to each other through the crystalline faces parallel to the *c* crystallographic axis. These data allow concluding that also in this case the NPs are formed by an ordered aggregation of smaller subunits.

To corroborate the formation mechanism suggested by the XRD and electron diffraction data, time-dependent experiments were carried out in the synthesis conditions of the HoF-rh and HoF-el NPs. Each reaction was quenched by immersing the reactor in an ice bath after aging. Figures 6a and 6b show TEM micrographs and XRD patterns, respectively, of the products obtained after aging for 15 min, 1.5 h, 2.5 h, and 4 h the corresponding solutions leading to the HoF-rh NPs. Before 10 minutes no precipitation was observed, while elongated particles, with approximate dimensions 250(30) nm length and 40(5) nm width, were formed after 10 minutes of aging. The corresponding XRD pattern showed broad reflections compatible with orthorhombic HoF₃ symmetry.

A significantly lower peak width is observed for the 020 than for the rest of reflections in the pattern, suggesting flat NPs. Applying the Scherrer formula to several single reflections, we obtained crystallite sizes in the range 5 – 10 nm, while the width of the 020 reflection indicated a crystallite size of 25 nm. In addition, a clear preferred orientation of the 020 planes is observed in the pattern. A detailed observation of the TEM micrograph of these particles (Figure 7a) clearly shows that they are formed by smaller subunits. The DDP obtained from the whole particle (insert in Figure 7a) shows elongated spots that suggest that the constituent subunits are already slightly oriented from the very early stages of the reaction. The HRTEM micrograph (Figure 7b) showed spherical crystalline grains of 5 – 10 nm with lattice fringes at 3.4 Å corresponding to the 020 planes, in good agreement with the XRD results. Increasing reaction time to 1.5 hours led to a decrease in the length of the particles (~200(30) nm) and to an increase in their width (100(10) nm) (Figure 6a). A clear anisotropic peak broadening was still observed in the corresponding XRD pattern (Figure 6b), suggesting that the particles are still quite flat at this stage. The 1.5 hrs TEM image of Figure 6a showed that some of the particles (with a darker contrast) lay with their flat face perpendicular to the TEM grid plane, from which a particle thickness of 20-30 nm can be estimated. Likewise, a dark contrast can be observed in the central part of each particle. The contrast increased with prolonging reaction time, as clearly observed in the particles obtained after 2.5 hours of aging, which showed smaller dimensions: 120(10) x 90(10) nm). A detail TEM study has been carried on the latter particles to shed some light on the origin of the dark contrast. The BF and HAADF-STEM line profiles obtained along the long axis of the particle shown in Figure 8a have been plotted in Figure 8b. These profiles indicate that the contrast is due to both, a diffraction contrast and a higher thickness in the center of the particle compared with the ends. In fact, the DDPs obtained from the HRTEM image of Figure 8c, calculated from the center and from one end of the particle, showed very well defined spots in the former and more elongated in the latter, suggesting a higher degree of ordering of the crystallites in the center of the NP. The narrower reflections observed in the corresponding XRD pattern, in comparison with the 1.5 hours pattern, clearly indicate a higher degree of crystallinity of the sample, in agreement with the TEM observations.

Finally, after 4 hours aging, the particles appeared completely formed (Figure 6a) and their XRD pattern (Figure 6b) clearly showed all the reflections expected for orthorhombic HoF₃, as described in the former subsection.

In contrast with the slow kinetics of the HoF-rh NPs, the ellipsoid-like NPs formed very quickly after only 10 minutes reaction (Figure 9a). At earlier stages (5 minutes) the reaction product consisted of an amorphous gel-like precipitate, as shown in the corresponding TEM micrograph, while longer reaction times (up to 4 hours) did not alter either their size, morphology or crystallinity, as shown in the corresponding XRD patterns of Figure 9b.

With the information obtained from the XRD and TEM data described above, the following formation mechanism can be envisaged for the rhombus- and ellipsoid-like NPs (Figure 10). The fluoride anions required for the precipitation of the HoF₃ phase are provided by the BF₄⁻ anions of the ionic liquid.^[22] The BF₄⁻ anions undergo fast hydrolysis on heating with the hydration water molecules of the corresponding holmium salt (Ho(CH₃CO₂)₃·xH₂O and Ho(NO₃)₃·5H₂O), according to the reaction:

On the other hand, the Ho³⁺ cations are released from the nitrate and acetate precursors. The free Ho³⁺ cations can then react with the free fluoride anions to form HoF₃ nuclei. Such nuclei grow and constitute the primary particles, which join together to form the particles observed after 10 minutes reaction. A certain orientation of the crystallites already exists at this reaction stage, possibly caused by the known capping effect of the ionic liquid, as commented above.^[30] The role of the holmium precursor is crucial from this very early stage of the reaction. While there is a controlled release of Ho³⁺ cations from the acetate salt due to the well-known complexing capacity of the acetate anion,^[26] the Ho cations are free in the solution when nitrate is used as the holmium precursor, thus accelerating the reaction rate and giving rise to different particle morphologies. Elongated ribbon-like particles are formed from acetate while smaller and the less anisometric ellipsoid-like nanoparticles are obtained from Ho³⁺ nitrate. The latter morphology seems to be much more stable than the former as demonstrated by the absence of any morphological or crystallographic change with increasing reaction time in the case of the ellipsoid-like NPs. However, the ribbon-like particles are clearly unstable in these conditions as inferred from the significant reorganization of the

primary units into wider, shorter particles when the aging time is increased to 1.5 and 2.5 hours. This process progresses until the system reaches an equilibrium state with rhombus geometry. During the reorganization, the primary units attach to each other through the crystalline faces parallel to the c axis of the HoF_3 crystal structure, as indicated by HRTEM.

2.2. Morphology and crystal structure of the DyF_3 nanoparticles

In order to evaluate the influence of the lanthanide nature on the magnetic relaxivity of the lanthanide fluoride NPs, we have synthesized DyF_3 NPs following the same method used to obtain the HoF -rh NPs. The method rendered well-dispersed DyF_3 NPs, hereafter called DyF -rhNPs, with the same morphology as that of the HoF -rh NPs (Figure 11a). Figure 11b shows the histograms obtained after measuring the long and short dimensions of the NPs in a hundred NPs from TEM images, which gave an average of 110 nm x 50 nm, as obtained for the HoF -rh NPs. The particles are well dispersed in water as inferred from the DLS curve of Figure 11c, which indicates an average hydrodynamic diameter of 100(35) nm. The Zeta potential value was 43(6) mV, very close to the one obtained for the HoF -rh NPs, which suggests that also in this case, the colloidal stability of the suspension was mainly due to the electrostatic repulsion among positively charged particles. Finally, the XRD pattern shown in Figure 11d is compatible with orthorhombic DyF_3 .

2.3. Magnetic resonance relaxivity

Magnetic relaxivities of the HoF -el, HoF -rh and DyF -rh NPs were evaluated at high magnetic field (9.4 T). With this purpose, the longitudinal (T_1) and transversal (T_2) proton relaxation times were measured in aqueous suspensions of the NPs containing different concentrations of Ho^{3+} and Dy^{3+} ions at 298 K. Figure 12 shows the $1/T_1$ and $1/T_2$ values obtained at 9.4 T as a function of the Ln^{3+} concentration for all three NPs. The slopes of the curves gave the relaxivity values r_1 and r_2 shown in Table 1. The following comments can be made in view of the relaxivity values obtained:

(1) Relaxivity measurements performed at 9.4 T showed r_2 values much higher than the r_1 ones for all three nanoparticle types, which indicate that the synthesized LnF_3 NPs are adequate as negative contrast agents.^[31] This behavior is due to the anisotropic $4f$ electron ground state of both Dy^{3+} and Ho^{3+} ions, which give rise to a short electronic

relaxation time (T_{1e}) on the order of 10-13 sec, leading to a minor contribution to T_1 relaxation, according to the SBM theory.^[32] However, both Dy^{3+} and Ho^{3+} ions own large magnetic moments (10.63 μ_B and 10.60 μ_B , respectively) which make them very efficient as T_2 MRI CAs.

(2) It can be observed in Table 1 that the r_2 value obtained for the HoF-rh NPs (608.39 $mM^{-1}s^{-1}$) is significantly higher than that obtained for the HoF-el NPs (349.98 $mM^{-1}s^{-1}$). This difference can be ascribed to the different size of both particles and is in good agreement with the behavior of $NaHoF_4$,^[17] $NaDyF_4$,^[18,14] and Dy_2O_3 ^[33] NPs, where increasing transversal relaxivity was also observed with increasing particle size. This trend has been explained using two different approaches. On one hand, the increase in relaxivity with increasing particle size has been attributed to the higher magnetization of big NPs compared with the smaller ones as a result of the *spin-canting* effect. Such effect appears in the nanoscale regime and consists in the tilting of the surface spins, which significantly influences the magnetic moments of the NPs. The spin-canting effect is size-dependent so that as the nanoparticle size increases the surface effect becomes less pronounced and results in a higher magnetization value for the big NPs compared with the smaller ones.^[34] On the other hand, the increase in transversal relaxivity with increasing nanoparticle size has been explained in terms of the diffusion correlation time τ_D of the NPs ($\tau_D = r_c^2/D$, where r_c is the radius of the nanoparticle and $D = 2.5 \times 10^{-9} m^2 s^{-1}$ is the diffusion coefficient of water at 25 °C). For small nanoparticle sizes ($\tau_D < 1.5 \times 10^{-6} s$), which is the case for our three particle types (HoF-el, HoF-rh and DyF-rh), the relaxivity is described in terms of the outer-sphere theory,^[35,32] which indicates that r_2 is proportional to τ_D and, therefore, to the particle size.

(3) The r_2 value of the HoF-rh NPs (608.39 $s^{-1} mM^{-1}$) is higher than that of the DyF-rh NPs of the same size (380.37 $s^{-1} mM^{-1}$), in spite of the slightly larger effective magnetic moment of Dy^{3+} compared to that of Ho^{3+} . A similar trend was described for spherical $NaHoF_4$ (17 nm) and rod-like $NaDyF_4$ (19 x 25 nm) NPs, which showed r_2 values of 130.6 and 91.4 $mM^{-1}s^{-1}$, respectively at 9.4 T.^[18] It is in turn remarkable that the relaxivity values obtained for any of our LnF_3 NPs are higher than those reported for $NaHoF_4$ and $NaDyF_4$.

(4) It is well known that the higher the r_2/r_1 ratio, the higher the contrast efficiency for a T_2 MRI CA. [36] The r_2/r_1 ratios of the three LnF_3 NPs synthesized in this work are significantly higher than that of Resovist (215 at 9.4 T), a clinically approved SPIO negative MRI contrast agent. [37]

These results strongly suggest the potential applicability of the synthesized HoF_3 and DyF_3 NPs as high field MRI contrast agents.

2.4. Cytotoxicity assay

The three types of NPs (HoF-el , HoF-rh and DyF-rh) were submitted to biocompatibility studies by evaluating the viability of C6 rat glioma cells with the MTT assay. The results showed that cell viability remained around 90% in all three cases (Figure 13) even at the high concentration of 100 $\mu\text{g/mL}$, concluding that there was an absence of mitochondrial damage after exposure to HoF-el , HoF-rh or DyF-rh NPs.

3. Conclusions

We have successfully synthesized for the first time uniform, orthorhombic HoF_3 NPs with a rhombus-like morphology (110 nm x 50 nm) using a polyol-based precipitation method at 120 °C with lanthanide acetate and $[\text{BMIM}]\text{BF}_4$ as the holmium and fluoride sources, respectively. Smaller ellipsoid-like HoF_3 NPs (70 nm x 30 nm) were obtained following the same methodology but using $\text{Ho}(\text{NO}_3)_3$ as the holmium precursor. This result was explained on the basis of the complexing capacity of the acetate anion. While the ellipsoid NPs are simply formed by the attachment of smaller constituent subunits, the formation mechanism of the rhombuses is more complex, involving a considerable reorganization of the constituent subunits from elongated NPs to rhombus-like NPs. In order to analyze the influence of the lanthanide nature on the magnetic relaxivity of the lanthanide fluoride NPs, uniform, rhombus-like DyF_3 NPs were also obtained following the same method as used for the rhombus-like HoF_3 NPs. Relaxivity measurements performed at 9.4 T showed r_2 values much higher than the r_1 ones for all three nanoparticle types, which indicate that the LnF_3 NPs are adequate as negative contrast agents. Likewise, the r_2 values were found to be dependent on the NP size, so that the bigger the NP, the higher the r_2 value. The rhombus-like HoF_3 NPs rendered higher r_2 values higher than the DyF_3 NPs with the same morphology. It was found that the r_2/r_1

values were much higher than that reported for Resovist, a clinically approved SPIO negative MRI contrast agent. Finally, cytotoxicity assays showed absence of mitochondrial damage after exposure of C6 cells to any of the three types of NPs. These results indicate that the synthesized NPs are potentially useful as high field MRI negative CAs.

4. Experimental

4.1. Materials

Holmium acetate hydrate ($\text{Ho}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), holmium nitrate hydrate ($\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, 99.9%), dysprosium acetate hydrate ($(\text{CH}_3\text{CO}_2)_3\text{Dy} \cdot x\text{H}_2\text{O}$ (99.9%), 1-butyl-2-methylimidazolium tetrafluoroborate ($[\text{BMIM}]\text{BF}_4$, $\text{C}_8\text{H}_{15}\text{BF}_4\text{N}_2$, $\geq 97\%$), and ethylene glycol (EG, $\text{C}_2\text{H}_6\text{O}_2$, 99.5%), all of them purchased from Sigma Aldrich.

4.2. Synthesis of the NPs

Rhombus-like HoF_3 NPs were obtained according to the following method: Holmium acetate ($\text{Ho}(\text{CH}_3\text{CO}_2)_3$) was dissolved in EG with magnetic stirring at 70°C to obtain a 0.04 M solution (total volume 2.5 mL). The solution was then cooled down to room temperature, admixed with 2.5 mL of $[\text{BMIM}]\text{BF}_4$ and magnetically stirred for 5 minutes at room temperature to favor homogenization. The final solution was aged for 4 hours in tightly closed test tubes using an oven preheated at 120°C . The resulting dispersion was cooled down to room temperature, centrifuged to remove the supernatants and washed, twice with ethanol and once with double distilled water. For some analyses, the powders were dried at room temperature. Several experiments were also conducted during shorter reaction times to find out the reaction mechanism for the formation of the NPs. An ice bath was used to quench the reactions after aging. Rhombus-like DyF_3 NPs were obtained from Dy acetate ($\text{Dy}(\text{CH}_3\text{CO}_2)_3$) following the same method as for the rhombus-like HoF_3 NPs. Ellipsoid-like HoF_3 NPs were synthesized following the same method described above for the rhombus-like particles but using $\text{Ho}(\text{NO}_3)_3$ as the Ho source instead of $\text{Ho}(\text{CH}_3\text{CO}_2)_3$.

4.3. Characterization techniques

1 The shape and size of the nanoparticles was examined by *transmission electron*
2 *microscopy (TEM, Philips 200CM)*. Particle size distributions were obtained from the
3 micrographs by counting several hundreds of particles, using the free software *ImageJ*.
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6 To gain additional information on the morphological and structural features of the
7 synthesized nanoparticles, they were also characterized by *High Angle Annular Dark*
8 *Field Scanning TEM (HAADF-STEM)* and *High Resolution TEM* by using a FEI
9 Tecnai field emission gun STEM microscope (G2F30) working at 300 kV (0.2 nm point
10 resolution) and equipped with a HAADF detector from Fischione (0.16 nm point
11 resolution in STEM-HAADF mode). A Fischione ultra-narrow gap tomography holder
12 was used to acquire HAADF-STEM images from 0° to 70°. The *Digital Diffraction*
13 *Patterns (DDP)* were obtained from the HRTEM images using the *Tecni Imaging and*
14 *Analysis (TIA)* software.
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18 Additional information on the size and colloidal stability of the particles in aqueous
19 suspension (0.5 mg·mL⁻¹ of solid) was obtained from *Dynamic Light Scattering (DLS)*
20 measurements. The experiments were carried out using a Malvern Zetasizer Nano-ZS90
21 equipment, which was used as well to measure the Zeta potential of the suspensions.
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24 The crystalline structure of the particles was assessed by *X-ray diffraction (XRD)* using
25 a Panalytical, X' Pert Pro diffractometer (CuKα) with an X-Celerator detector over an
26 angular range of 10° < 2θ < 120°, 2θ step width of 0.02°, and 10 s counting time. The
27 crystallite size was estimated using the Scherrer formula from the full width at half
28 maximum of a single reflection. A shape factor of k = 0.9 and an instrumental
29 broadening factor of 0.112 were used for the calculation.
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32 ¹H NMR relaxation times, T₁ and T₂, were measured at 9.4 T using a Bruker Biospec at
33 different concentrations of Ho³⁺ and Dy³⁺ (0.2, 0.4, 0.6, 0.8, and 1.0 mmol dm⁻³) in
34 water at 298 K. T₁ was determined either using inversion-recovery or saturation
35 recovery sequences and T₂ was determined using the Carl-Purcell-Meiboom-Gill
36 (CPMG) sequence. Relaxivities (r₁, r₂) were obtained from the slopes of the curves 1/T₁
37 or 1/T₂ vs. the concentration of Ho³⁺ and Dy³⁺ expressed in mM.
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41 *Cytotoxicity assay:* C6 rat glioma cells were cultured in Dulbecco's Modified Eagle
42 Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum
43 (FBS) and 1% penicillin/streptomycin at 37° C in an incubator with a humidified
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atmosphere containing 5% CO₂. To perform the cytotoxicity assays, C6 cells were plated at a density of 1 x 10⁴ cells/well in a 96-well plate at 37°C in 5% CO₂ atmosphere (200 µL per well, number of replicates = 5). After 24 h of culture, the medium in the wells was replaced with fresh medium containing the magnetic NPs in varying concentrations from 0.5 µg/mL to 500 µg/mL. After 24 h, the medium was removed and 200 µL of fresh medium with MTT (0.5 mg/mL) was added to each well. After 2 h of incubation at 37°C and 5% CO₂, the medium was removed and the formazan crystals were solubilized with 200 µL of DMSO. Two controls were performed to adequately evaluate cytotoxicity: cells unexposed to the NPs were used as negative control, and cells exposed to Triton X-100 (1% v/v) for 15 min prior to the MTT procedure, were used as positive control. The absorbance of each well was read on a microplate reader (Dynatech MR7000 instruments) at 550 nm. The relative cell viability (%) and its error with respect to the control wells containing cell culture medium without nanoparticles was calculated as follows:

$$Relative\ Cell\ Viability\ (RCV)\ (\%) = \frac{[Abs]_{test}}{[Abs]_{control}} \times 100$$

$$Error\ (\%) = RCV_{test} \times \sqrt{\left(\frac{[\sigma]_{test}}{[Abs]_{test}}\right)^2 + \left(\frac{[\sigma]_{control}}{[Abs]_{control}}\right)^2}$$

where σ is the standard deviation.

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Table 1: Proton relaxivity values (r_1 and r_2) obtained for water suspensions of the different HoF₃ and DyF₃ NPs at 9.4 T and 298 K.

LnF ₃ NPs (dimensions)	B= 9.4 T		
	r_1 (s ⁻¹ mM ⁻¹)	r_2 (s ⁻¹ mM ⁻¹)	r_2/r_1
HoF-el (70x30 nm)	0.95	349.98	368.40
HoF-rh (110x50 nm)	0.59	608.39	1031.17
DyF-rh (110x50 nm)	0.68	380.37	559.37

Figure captions

Figure 1: TEM micrographs and size distribution plots obtained from TEM images of HoF-el (a,b) and HoF-rh (c,d) NPs obtained in the conditions described in the experimental section.

Figure 2: HAADF-STEM images of the HoF-rh sample, recorder at 0° (top) and 70° (bottom) tilt angle.

Figure 3: *Top:* DLS plots showing the particle size distribution of the HoF-el (a) and HoF-rh (b). *Bottom:* XRD patterns of the HoF-el (a) and HoF-rh (b) NPs. The lines at the bottom of the figure are the reflections corresponding to orthorhombic HoF₃ (ICDD PDF 00-023-0284).

Figure 4: (a) HRTEM image of a HoF-rh NP. The inset shows two DDPs obtained from center (red) and edge (blue) areas. (b) Higher magnification image corresponding to the white rectangle area shown in (a), showing single domains (dashed circles). The insert is an indexed DDP calculated from the blue square.

Figure 5: (a) TEM image of a HoF-el NP, (b) DDP pattern obtained from the whole nanoparticle, (c) HRTEM micrograph of the HoF-el NP shown in (a).

Figure 6: TEM micrographs (a) and XRD patterns (b) of the NPs observed after different aging times in the experimental conditions leading to HoF-rh NPs.

Figure 7: (a) Detail of one NP shown in Figure 6a after 10 minutes reaction. (b) High magnification of the white square in figure 7a. The inset is the DDP obtained from the marked area.

Figure 8: (a) HAADF-STEM image of a NP shown in Figure 6a after 2.5 h reaction and BF-STEM and HAADF-STEM line profiles along the long axis of the NP. (b) HRTEM image of the same NP. The insets are DDPs obtained from the center (red) and edge (green) areas.

Figure 9: TEM micrographs (a) and XRD patterns (b) of the NPs observed after different aging times in the experimental conditions leading to HoF-el NPs.

Figure 10: Schematic illustration of the formation mechanism of the ellipsoid-like and rhombus-like NPs showing the aggregation of their small constituents as well as their tridimensional morphology.

Figure 11: TEM micrograph (a), size distribution plots obtained from several TEM images (b), DLS plots showing the particle size distribution (c), and XRD patterns (d) of the rhombus-like DyF₃NPs. The lines at the bottom of plot (d) are the reflections corresponding to orthorhombic DyF₃ (ICDD PDF 00-032-0352).

Figure 12: T_1 and T_2 proton relaxivity plots obtained at room temperature using a 9.4 T magnet in aqueous suspensions of HoF-el (a), HoF-rh (b), and DyF-rh (c) NPs *versus* the lanthanide concentration.

Figure 13: Cytotoxicity assay of C6 cells incubated with different concentrations of HoF-el, HoF-rh and DyF-rh NPs for 24 h.

Figure 1

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 5

Figure 7

Figure 9

Figure 9

Figure 10

Figure 11

Figure 13