

# Free-Radical Formation by the Peroxidase-Like Catalytic Activity of $\text{MFe}_2\text{O}_4$ ( $\text{M} = \text{Fe}$ , $\text{Ni}$ , and $\text{Mn}$ ) Nanoparticles

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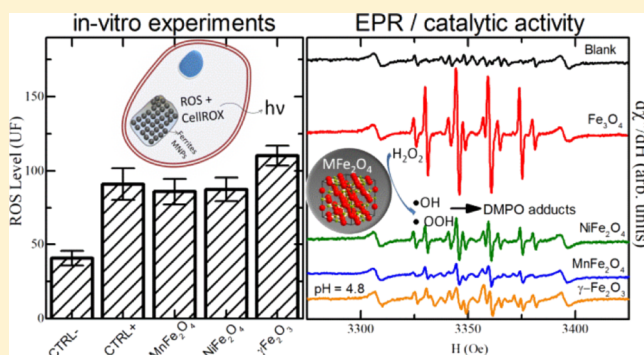
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## Supporting Information

**ABSTRACT:** Ferrite magnetic nanoparticles (MNPs) have peroxidase-like activity and thus catalyze the decomposition of  $\text{H}_2\text{O}_2$ -producing reactive oxygen species (ROS). Increasingly important applications of these ferrite MNPs in biology and medicine require that their morphological, physicochemical, and magnetic properties need to be strictly controlled. Usually, the tuning of their magnetic properties is achieved by the replacement of Fe by other 3d metals, such as Mn or Ni. Here, we studied the catalytic activity of ferrite MNPs ( $\text{MFe}_2\text{O}_4$ ,  $\text{M} = \text{Fe}^{2+}/\text{Fe}^{3+}$ , Ni, and Mn) with the mean diameter ranging from 10 to 12 nm. Peroxidase-like activity was studied by electron paramagnetic resonance (EPR) using the spin-trap 5,5-dimethyl-1-pyrroline N-oxide at different pHs (4.8 and 7.4) and temperatures (25 and 40 °C). We identified an enhanced amount of hydroxyl ( $\cdot\text{OH}$ ) and perhydroxyl ( $\cdot\text{OOH}$ ) radicals for all samples, compared to a blank solution. Quantitative studies show that  $[\cdot\text{OH}]$  is the dominant radical formed for  $\text{Fe}_3\text{O}_4$ , which is strongly reduced with the concomitant oxidation of  $\text{Fe}^{2+}$  or its substitution (Ni or Mn). A comparative analysis of the EPR data against in vitro production of ROS in microglial BV2 cell culture provided additional insights regarding the catalytic activity of ferrite MNPs, which should be considered if biomedical uses are intended. Our results contribute to a better understanding of the role played by different divalent ions in the catalytic activity of ferrite nanoparticles, which is very important because of their use in biomedical applications.



## INTRODUCTION

Ferrite magnetic nanoparticles (MNPs) are currently being used in many different clinical protocols approved by regulatory agencies such as the FDA.<sup>1</sup> Mixed ferrites with the general formula  $\text{M}_x\text{Fe}_{3-x}\text{O}_4$  (i.e., incorporating other 3d metallic ions within the spinel structure) have attracted interest in biomedicine because their magnetic properties can be adjusted for specific purposes, for example, to serve as magnetic resonance imaging (MRI) contrast agents<sup>2</sup> or to tune the heating response in magnetic hyperthermia.<sup>3</sup>

The peroxidase-like activity of the bulk iron oxides catalyzed by Fenton-based reactions involves the decomposition of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) into reactive oxygen species (ROS) such as the radicals  $\cdot\text{OH}$  and  $\cdot\text{OOH}$ .<sup>4</sup> Hence, for simplification, here we consider the peroxidase-like activity of

MNPs as the catalytic activity that involves the decomposition of  $\text{H}_2\text{O}_2$  into  $\cdot\text{OH}$  and  $\cdot\text{OOH}$  radicals. This peroxidase-like activity of iron oxides has many well-documented differences with the enzymatic activity of actual peroxidases regarding their dependence on pH, temperature, and  $\text{H}_2\text{O}_2$  concentration.<sup>5,6</sup> Nevertheless, the enzymatic-like activity of natural iron oxides and hydroxides containing  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  is known to play a significant role in several geological-dependent biological processes of plants and microorganisms.<sup>7</sup>

In 2007, Gao et al.<sup>8</sup> measured the peroxidase-like catalytic activity of magnetite ( $\text{Fe}_3\text{O}_4$ ) nanoparticles under different

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experimental conditions, reporting a strong increase in the catalytic activity with (a) decreasing nanoparticle size, suggesting a surface-related effect, and (b) increasing  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ratios, indicating an active role of ferrous iron. These results were immediately recognized as extremely relevant because of the ubiquity of iron oxide MNPs in medicine and biology protocols.<sup>9–11</sup> On the other hand, Gumpelmayer et al.<sup>12</sup> have recently reported that removing free iron ions from the surface of  $\text{Fe}_3\text{O}_4$  nanoparticles with a Chelex buffer eliminates their intrinsic peroxidase-like activity. It is important to note that the two works mentioned above were done using commercial  $\text{Fe}_3\text{O}_4$  MNPs, which provide very few details about their composition and surface properties. Moreover, in both cases, the data on the peroxidase-like activity were relative to the oxidation of a chromogenic substrate. Although recent works have provided a better characterization of the ferrite MNPs and linked the MNP coatings to their peroxidase-like activity,<sup>13</sup> quantitative data of ROS production from well-characterized ferrite nanoparticles under different physiological conditions are still lacking. In particular, further experimental work is required to assess whether  $\text{Fe}_3\text{O}_4$  nanoparticles are capable of triggering peroxidase-like catalytic mechanisms *in vitro* and *in vivo*.

It is known that for bulk materials or nanoparticles, the incorporation of 3d transition metals (especially  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ni}^{2+}$ ) into the tetrahedral and octahedral sites of the spinel structure strongly affects the reactivity toward  $\text{H}_2\text{O}_2$ .<sup>14</sup> The incorporation of Co or Mn yields a measurable increase in the formation of  $\bullet\text{OH}$  species, while  $\text{Ni}^{2+}$  ions inhibit this reaction.<sup>14–17</sup> Using a luminol-based chemiluminescent reaction, Shi et al.<sup>18</sup> have found that  $\text{CoFe}_2\text{O}_4$  ferrite nanoparticles were able to catalyze  $\text{H}_2\text{O}_2$  decomposition. However, the limitation of this nonspecific technique is that it cannot be used to differentiate among the radical species produced in the reaction, thus hindering their quantification.

The relevance of the catalytic activity of ferrite nanoparticles has been clearly illustrated by Clerc et al.,<sup>19</sup> who proposed a dual effect in oncological treatments by combining the peroxidase-like activity of ferrite nanoparticles in the acidic pH of lysosomes with their heating ability for magnetic fluid hyperthermia, a synergistic approach that could improve apoptosis of target cells.

Electron paramagnetic resonance (EPR) is perhaps the most sensitive measurement technique used to identify and quantify individual free-radical species.<sup>20–26</sup> Because of the high reactivity and short half-life of the free radicals, this technique requires the use of a diamagnetic “spin-trap” compound, such as the 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), that generates a more stable paramagnetic radical-adducted species, which can be readily detected. The EPR technique has already been used to study the peroxidase-like activity of  $\text{Fe}_3\text{O}_4$  nanoparticles in the decomposition rate of  $\text{H}_2\text{O}_2$  at some physiological pHs.<sup>27</sup> The authors observed a substantial increment of  $\bullet\text{OH}$  formed by the nanoparticles at pH 4.8 (similar to lysosomal conditions) and almost null catalytic activity of the nanoparticles at pH of 7.4 (similar to the cytoplasm of healthy cells). More recently, EPR spectroscopy using DMPO was applied to elucidate the main mechanisms of the Fenton-like and Haber–Weiss-like catalytic reactions. These studies were able to associate the formation of the free radical  $\bullet\text{OH}$  with the activity of  $\text{Fe}^{2+}$  and the formation of  $\bullet\text{OOH}$  with the activity of  $\text{Fe}^{3+}$ .<sup>4</sup> Although DMPO is a good tool to study the formation of the free radical  $\bullet\text{OH}$  (with a

lifetime in the order of several minutes), it can also be used to identify the free radical  $\bullet\text{OOH}$ . Nevertheless, the analyses of the later one need to be carefully adjusted because the amounts of  $\bullet\text{OOH}$  (as determined by the adduct DMPO– $\text{OOH}$ ) are underestimated because it has a relative slow kinetic of formation and a short half-life (50 s).<sup>28–30</sup>

In this work, we have focused on the role of  $\text{Fe}^{2+}$  in the peroxidase-like catalytic activity of different ferrite nanoparticles with the general formula  $\text{M}_x\text{Fe}_{3-x}\text{O}_4$  ( $\text{M} = \text{Fe}$ ,  $\text{Ni}$ , and  $\text{Mn}$ ) using EPR and the spin-trap DMPO. We identified the formation of the free radicals  $\bullet\text{OH}$  and  $\bullet\text{OOH}$  from  $\text{H}_2\text{O}_2$  generated under different pHs (4.8 and 7.4) and temperatures (25 and 40 °C). Absolute quantification was performed for the radical  $\bullet\text{OH}$ , which is the dominant species in the catalytic activity of  $\text{Fe}^{2+}$  ion. A comparative study among the nanoparticles of the radical  $\bullet\text{OOH}$  formation was also performed. Because the passivation of the ferrite particle surface can play a major role in the catalytic activity,<sup>31</sup> we have also analyzed the influence of two chelating agents with distinct interaction properties in respect to the metallic ions.

To compare the peroxidase-like activity among the samples, we focused on the concentration of the free radical  $\bullet\text{OH}$  ( $[\bullet\text{OH}]$ ). The morphology and composition of the nanoparticle were characterized by transmission electron microscopy (TEM) and X-ray photoelectron spectroscopy (XPS), respectively. The amount of the free radical  $\bullet\text{OH}$  ( $[\bullet\text{OH}]$ ) produced is related to the composition, specifically to the  $\text{Fe}^{2+}/\text{Fe}$  ratio in ferrite. Finally, the results obtained from the EPR experiments contrasted with those obtained from the *in vitro* experiments in BV2 cells through fluorescence to assess the peroxidase-like activity of the dextran-coated MNPs.

## ■ EXPERIMENTAL METHODS

**Materials.** The following chemical reagents were used in the synthesis and functionalization of the nanoparticles. The organometallic precursors  $\text{Fe}^{3+}$  acetylacetonate ( $\text{Fe}(\text{acac})_3$ , 97%),  $\text{Ni}^{2+}$  acetylacetonate ( $\text{Ni}(\text{acac})_2$ , 95%),  $\text{Mn}^{2+}$  acetylacetonate ( $\text{Mn}(\text{acac})_2$ , technical grade), 1,2-octanediol (98%), oleic acid (analytical standard), oleylamine (70%), benzyl ether (98%), methanol (reag. Ph. Eur.), ethanol (96%), acetone (reag. Ph. Eur.), chloroform (>99%), toluene (99.8%), dextran (analytical standard,  $M_w$  12 000), and ammonium hydroxide (27%) were purchased from Sigma-Aldrich (USA). Hydrochloric acid (HCl, 37%), iron chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 97%), potassium phosphate ( $\text{K}_3\text{PO}_4$ , 98%), and potassium thiocyanate (KSCN, >99.9%) used in the determination of nanoparticle composition by UV analysis; DMPO (>97%) and dimethyl sulfoxide (DMSO, >99%) used in the EPR experiments; as well as *tert*-butyl hydroperoxide (TBHP) solution (Luperox TBH70X, 70% in water) used for *in vitro* experiments were also purchased at Sigma-Aldrich. Quartz tubes (2 mm thickness) for Q-band used in the EPR experiments were purchased from Wilmar (USA). A MgO crystal doped with  $\text{Mn}^{2+}$  ions was selected among other crystals to have nearly zero  $\text{Cr}^{3+}$  impurities, thus providing clean central spectra while providing a calibrating intensity and line position reference. Phosphate-buffered saline (PBS), fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), and CellROX Green Reagent were purchased from Thermo Fisher Scientific (USA).

**Synthesis of the Nanoparticles.** The MNPs used in this work were synthesized through the high-temperature decomposition of  $\text{Fe}(\text{acac})_3$ ,  $\text{Ni}(\text{acac})_2$ , and  $\text{Mn}(\text{acac})_2$  precursors,

following the synthesis route described elsewhere.<sup>32,33</sup> The nanoparticles obtained with this procedure are coated mainly by oleic acid and partially by oleylamine. The organic surface coating was removed in order to disperse the nanoparticles in water. To perform this step, the nanoparticles were dispersed in methanol for 8 h at 40 °C and then in acetone for 48 h at 40 °C. After this treatment, the absence of oleic acid and oleylamine coating was verified by Fourier transform infrared spectroscopy (FTIR), as shown in Figure S1 presented in the [Supporting Information](#). Afterward, the chemically etched nanoparticles were coated with dextran for in vitro experiments. For this purpose, the nanoparticles were dispersed with 10 times the mass of dextran in an ammonium hydroxide solution (0.1 N, pH = 11) for 48 h under stirring, followed by magnetic separation and washing several times with ultrapure water. An oxidized Fe<sub>3</sub>O<sub>4</sub> MNP sample was prepared by following the previous methodology without using dextran. The MNPs made of Fe<sub>3</sub>O<sub>4</sub> as well as its oxidized counterpart ( $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>), Ni<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub>, and Mn<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> were labeled Fe-MNP sample, oxFe-MNP sample, Ni-MNP sample, and Mn-MNP sample, respectively.

**Characterization of the Nanoparticles.** The morphology and size dispersion of the nanoparticles were analyzed from the TEM images taken in an F20 TECNAI microscope operating at 200 kV. The analysis of the nanoparticle surface coating was done by FTIR performed in a Spectra Two spectrometer (PerkinElmer), using a uATR optical configuration.

To obtain the oxidation state of the metallic ions in the nanoparticles, X-ray photoelectron spectroscopy (XPS) measurements were performed with a SPECS spectrometer, using a 150 mm PHOIBOS hemispherical analyzer in the energy range of Al K $\alpha$  (1486.6 eV) and a chamber pressure of 10<sup>-10</sup> mBar. In order to determine the Fe<sup>2+</sup>/Fe ratio in each sample, their XPS spectra in the Fe 2p energy range were analyzed. The fitting procedure used for the Fe 2p<sub>3/2</sub> peak for each sample was similar to that reported by Grosvenor et al.<sup>34</sup> In this way, the binding energy for each sample was calibrated using the C 1s peak position with a reference value of 284.8 eV; a Shirley-type background was used in the fitting in order to remove most of the extrinsic loss structure; and the Fe 2p<sub>3/2</sub> envelope was fitted using multiplets (three peaks corresponding to Fe<sup>2+</sup> and four for the Fe<sup>3+</sup> in the ferrite structure), with correlations among the binding energy and area of the peaks. High-energy peaks corresponding to the surface structures and shake-up-related satellites were also added. The Ni/Fe and Mn/Fe ratios were obtained by analyzing the XPS spectra of the samples in the Fe–Ni–Mn 3p energy range. In this way, the area of each peak referent to the respective element was obtained by numerical integration,<sup>35–37</sup> and the Ni/Fe and Mn/Fe ratios were calculated taking into account the corresponding photoionization cross section in this energy range.<sup>38</sup>

The Fe<sup>3+</sup> and total Fe content in the nanoparticles was obtained by UV–visible (UV–vis) spectrophotometry following the procedure described below. The as-synthesized particles were washed several times with toluene to remove the excess oleic acid followed by a wash in HCl (6 N) for 10 min at 60 °C and then placed into two tubes. To obtain the total iron concentration, one of the tubes was incubated with 5  $\mu$ L of 3% H<sub>2</sub>O<sub>2</sub> with the aim to oxidize all Fe<sup>2+</sup> to Fe<sup>3+</sup>; the second tube was incubated with 5  $\mu$ L of ultrapure water to determine the initial Fe<sup>3+</sup> amount. Iron standard solutions were prepared

by dissolving FeCl<sub>3</sub>·6H<sub>2</sub>O in ultrapure water (0–150 ppm) and processed for total iron determination. Finally, Fe<sup>3+</sup> concentration was determined by adding 400  $\mu$ L of 0.5 M KSCN and measuring the absorbance of the red complex at 480 nm. Because the Fenton reaction can catalyze KSCN oxidation in acidic media,<sup>39</sup> the H<sub>2</sub>O<sub>2</sub> concentration was carefully selected. In our experiments, after increasing the H<sub>2</sub>O<sub>2</sub> concentration by a factor of 10, a decrease in sample absorbance was observed, possibly because of KSCN oxidation. In contrast, the absorbance of standard solutions was not altered when increasing H<sub>2</sub>O<sub>2</sub>, probably because the Fenton reaction depends mainly on the Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub> ratio and we used Fe<sup>3+</sup> to create the calibration curve. To assess the performance of the method, pure magnetite ([Fe<sup>3+</sup>] = 66.6%) was introduced as a standard to validate our results. The absorbance spectra of Fe<sup>2+</sup>, Ni<sup>2+</sup>, and Mn<sup>2+</sup>, before and after oxidation, were also recorded.

**Catalytic Activity Measured by EPR.** EPR measurements were performed in an ELEXSYS II-E500 spectrometer (Bruker) with an X-band resonant cavity (9.4 GHz) at specific temperatures of 25 and 40 °C. The spectra were acquired with an attenuation of 10 dB (20 mW microwave power) and 1 Oe of amplitude of the modulation field. The solutions for the EPR experiments were prepared by dispersing 120  $\mu$ g of nanoparticles in 200  $\mu$ L of an acetate buffer solution (pH = 4.8), or in phosphate buffer solution (pH = 7.4). In each solution, 50  $\mu$ L of DMPO/DMSO solution with a DMSO concentration of 0.33 g/mL (0.56 M) and 10  $\mu$ L of H<sub>2</sub>O<sub>2</sub>·30% were added (0.49 M). The starting point of the reaction was assumed to be the moment H<sub>2</sub>O<sub>2</sub> was added. In order to quantify the amounts of the free radicals, the EPR spectrum of each solution in a quartz tube was recorded simultaneously with the MgO pattern crystal, doped with a known concentration of Mn<sup>2+</sup> attached to the tube. The dependences of the obtained EPR spectra with the microwave power for 15, 20, 25, and 30 dB were recorded in order to analyze the effect of the signal saturation and deviations from the expected linear dependence at lower attenuations and were considered for the quantification. The collected spectra were fitted with the software SPIN from Bruker using the same procedure for all measurements in the following order: first, the ferromagnetic resonance of the ferrite nanoparticles was subtracted and then the resonance lines were adjusted with the hyperfine parameters of five different species. In order to identify each species, we used the fitted values of hyperfine constant in the Spin Trap database of the National Institute of Environmental Health Sciences—NIEHS, USA (<https://tools.niehs.nih.gov/stdb/index.cfm>). The •OH, •OOH, •CH<sub>3</sub>, and •N free-radical concentrations were obtained by comparing the EPR-fitted spectrum intensities of every species with the intensity of the MgO/Mn<sup>2+</sup> pattern, as reported elsewhere.<sup>40,41</sup>

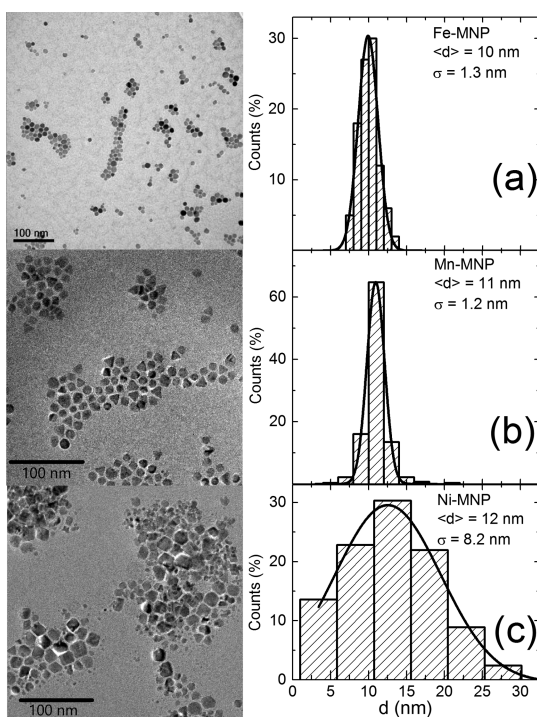
**In Vitro Tests of ROS Production.** The in vitro production of ROS by the MNPs was tested in BV2 cells (mouse, C57BL/6, microglia) from ATCC (Manassas, VA, USA). Cells were routinely cultured in DMEM supplemented with 10% FBS at 37 °C in a 5% CO<sub>2</sub> atmosphere. Control groups consisted of untreated cells (negative control) and cells treated with 10 or 20  $\mu$ M of TBHP (positive control). Experimental groups were made of cells treated with Fe<sub>3</sub>O<sub>4</sub>, NiFe<sub>2</sub>O<sub>4</sub>, or Mn<sub>0.66</sub>Fe<sub>2.34</sub>O<sub>4</sub> nanoparticles at a concentration of 50  $\mu$ g/mL. Both hydroperoxide and MNP concentrations were tested before and proved to be noncytotoxic. The quantification of ROS produced in vitro was performed with cells seeded

in 96-well plates at a density of  $4 \times 10^3$  cells/well, allowing cells to attach overnight before incubating with the different treatments for 24 h. For the evaluation of ROS production at 40 °C, the cells were incubated till the last hour at this temperature. After this time, the CellROX reagent was added at a final concentration of 50  $\mu$ M and incubated for another 30 min at 37 °C. Then, the cells were washed three times with PBS and fluorescence was measured with a Synergy HT plate reader (Biotek, Winooski, VT, USA) using a 485/20 nm excitation and 530/25 nm emission filter set, using the comparative relative fluorescence units (RFU).

For the statistical analysis of the ROS determination in vitro results, InfoStat software (Universidad Nacional de Córdoba, Argentina) was used. Statistics were performed using one-way ANOVA and Tukey multiple comparisons test when applicable. Differences were considered significant if  $p < 0.05$ . Population statistics are presented as mean  $\pm$  standard error of the mean.

## RESULTS

**Morphology and Composition.** The peroxidase-like catalytic activity of MNPs is known to be highly dependent on the surface area and the particle composition. Therefore, a proper nanoparticle characterization is essential in order to understand the dynamics of free-radical formation. Figure 1



**Figure 1.** Representative TEM images for samples (a) Fe-MNP, (b) Mn-MNP, and (c) Ni-MNP. Right panels: the respective diameter histogram fitted with a Gaussian distribution and the obtained values for the mean diameter  $\langle d \rangle$  and dispersion  $\sigma$ .

shows the representative TEM images of Fe-MNP, Mn-MNP, and Ni-MNP, where different shapes can be observed (rounded for Fe-MNP and a mixture of rounded and faceted for Ni-MNP and Mn-MNP). Size histograms were obtained from the TEM images (right panels, Figure 1) and then fitted with a Gaussian distribution to obtain the mean diameters ( $\langle d \rangle$ ) and standard deviations ( $\sigma$ ) that are given in Table 1.

When considering the Fenton-based reactions, the  $\text{Fe}^{2+}/\text{Fe}$  molar ratio in the nanoparticles is critical in order to understand the catalytic activity of the samples.<sup>1–5</sup> For this reason, we used X-ray photoelectron spectroscopy (XPS) to analyze the changes in the oxidative state of iron in the nanoparticle after the incorporation of Ni and Mn in the spinel structure. Table 1 gives the  $\text{Fe}^{2+}/\text{Fe}$  ratio, as well as the Mn/Fe and Ni/Fe ratios, obtained from the XPS spectra. Figures S2 and S3 in the Supporting Information show the spectra with the corresponding fitting curves, together with a table with the fitting parameters for the spectra Fe 2p<sub>3/2</sub> and 3p (Figure S4 in the Supporting Information), respectively.

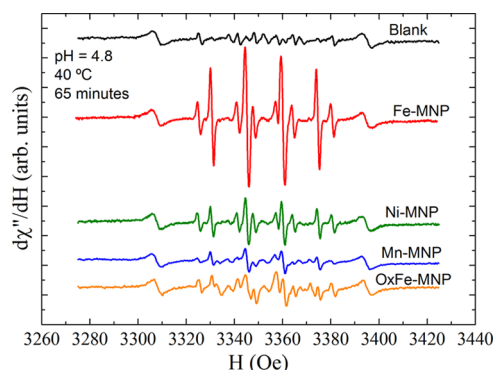
From different studies in these systems detailed in the literature, it is known that system prepared in similar way that our samples present a ferrite structure. Thus, we consider a general formula of  $\text{MFe}_2\text{O}_4$  for samples Fe-MNP, Mn-MNP and Ni-MNP, and  $\text{Fe}_{2.67}\text{O}_4$  for the sample oxFe-MNP, despite a small stoichiometry deviation with oxygen vacancies taking place. Thus, according to the XPS analysis, the complete formula of the samples can be written as Fe-MNP =  $\text{Fe}_{0.81}^{2+}\text{Fe}_{2.19}^{3+}\text{O}_4$ ; Mn-MNP =  $\text{Mn}_{0.33}\text{Fe}_{0.61}^{2+}\text{Fe}_{2.06}^{3+}\text{O}_4$ , and Ni-MNP =  $\text{Ni}_{0.84}\text{Fe}_{0.30}^{2+}\text{Fe}_{1.86}^{3+}\text{O}_4$ . To provide further support for the obtained data, the  $\text{Fe}^{2+}/\text{Fe}$  molar ratios were also obtained by UV–visible spectrophotometric quantification of  $\text{Fe}^{3+}$  ions by the  $\text{Fe}^{3+}$ –thiocyanate complex formation ( $\lambda_{\text{absorption}} \approx 465$  nm), after particle dissolution in HCl 6 N (see Table 1). The UV–visible spectra of the different transition-metal ions are given in Figure S5 of the Supporting Information. For total Fe quantification in each sample, a strong oxidation process was induced by adding  $\text{H}_2\text{O}_2$  to the solution prior  $\text{Fe}^{3+}$  quantification. The  $\text{Fe}^{2+}/\text{Fe}$  ratios obtained from Fe–thiocyanate UV–visible spectrophotometry are in good agreement with the values obtained from XPS. Both measurements show a decrease in  $\text{Fe}^{2+}$  content after Mn and Ni substitution, indicating a preferential substitution of  $\text{Fe}^{2+}$  by Mn and Ni. In addition, oxidation of Fe-MNP samples (exposed 48 h to an alkaline aqueous solution of pH = 12, 40 °C) resulted in almost complete oxidation of  $\text{Fe}^{2+}$ , as determined by the  $\text{Fe}^{3+}$ –thiocyanate method, showing a  $\text{Fe}^{2+}/\text{Fe}$  ratio of about 0.07.

**Catalytic Activity by EPR.** The catalytic activity of the nanoparticles in buffer solutions (pH = 4.8 and pH = 7.4) after addition of  $\text{H}_2\text{O}_2$  was recorded by EPR spectroscopy at 40 °C with the addition of the spin-trap DMPO in DMSO to detect and identify the free radicals formed in the peroxidase reaction. Figure 2 shows the EPR spectra recorded at 40 °C in acetate buffer solution (pH = 4.8) for the Fe-MNP, Ni-MNP, and Mn-MNP samples and the oxidized form of Fe-MNP (oxFe-MNP). Here, nanoparticle activities were referred to the blank sample spectra, which correspond to the same solutions but without the nanoparticles. It should be noticed that the intensities in the EPR spectra of the nanoparticle containing solutions are larger than blank samples in every case (Figure 2). Moreover, a remarkable enhancement of the EPR intensity was observed for the dispersion containing the  $\text{Fe}_3\text{O}_4$  MNPs (Fe-MNP). After a detailed analysis, it was observed that each EPR spectrum was composed of the resonance signals of five different paramagnetic species (Figure 3). The contributions detected are (i) the outer lines corresponding to the central resonance lines of the  $\text{Mn}^{2+}$  ions in the MgO crystal;<sup>42</sup> (ii) the DMPO/ $\cdot\text{OH}$  free-radical spectrum composed of four resonance lines with a 1:2:2:1 intensity ratio that results from the splitting of the resonance line because of the hyperfine

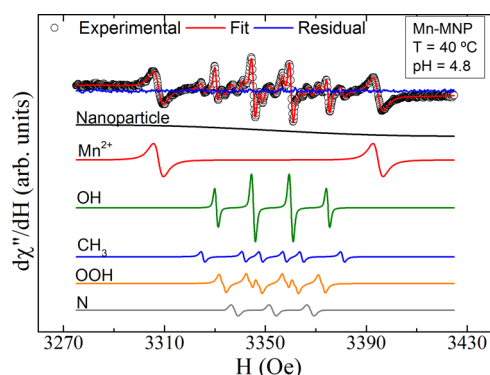
Table 1. Morphological and Compositional Parameters of the Samples<sup>a</sup>

technique	parameter	sample			
		Fe-MNP	Mn-MNP	Ni-MNP	oxFe-MNP
TEM <sup>b</sup>	$\langle d \rangle$ (nm)	10	11	12	10
	$\sigma$ (nm)	1.3	1.2	8.2	1.3
XPS	Mn/Fe		0.12 (2)		
	Ni/Fe			0.40 (1)	
	Fe <sup>2+</sup> /Fe	0.27 (2)	0.23 (2)	0.14 (2)	
Fe <sup>3+</sup> –thiocyanate method	Fe <sup>2+</sup> /Fe	0.27 (3)	0.23 (3)	0.09 (4)	0.07 (4)

<sup>a</sup>The standard errors in the compositional analyses are given in parentheses which are in reference to the last digit. <sup>b</sup> $\langle d \rangle$  is the main diameter and  $\sigma$  is the dispersion obtained from the respective histogram fit with a Gaussian distribution.



**Figure 2.** EPR spectra of DMPO spin adducts obtained with Fe-MNP, Ni-MNP, Mn-MNP, and oxFe-MNP nanoparticles in DMPO/DMSO containing solutions after H<sub>2</sub>O<sub>2</sub> addition. Top spectrum corresponds to the blank solution (without nanoparticles). Data were measured 65 min after H<sub>2</sub>O<sub>2</sub> addition at  $T = 40$  °C in pH = 4.8.



**Figure 3.** EPR spectrum and the corresponding fitting with the residual obtained for the Mn-MNP sample in DMPO/DMSO containing solution measured 60 min after addition of H<sub>2</sub>O<sub>2</sub> at  $T = 40$  °C in pH = 4.8 (top curve). Each DMPO-adduct radical component of the fitted spectra is shown separately in the spectra below (continuous line).

interaction of the electronic spin with the nuclear spin of the nitrogen and the hydrogen ions; (iii) the DMPO/•OOH radical spectrum with six lines resulting from the convolution of 12 resonances, corresponding to the hyperfine interaction of the electron spin with a nitrogen and two hydrogen ions; (iv) the DMPO/•CH<sub>3</sub> radical contribution, probably resulting from a secondary reaction of the DMSO with the H<sub>2</sub>O<sub>2</sub> or the free radical •OH; and (v) three broader lines that are related to the interaction of the electron spin of oxidized DMPO with a nitrogen ion (DMPO/•N). Table 2 summarizes the hyperfine parameters obtained for each radical in all spectra. It is worth

noting that the different paramagnetic species can be well characterized with the  $g$ ,  $H_{\text{SF}}$ , and  $w$  parameters that remain unchanged in the different experimental conditions, for all samples. Only changes in areas were observed. Finally, only the cavity and Quartz tube signal were measured and found to have only a very small and very broad contribution, which does not interfere with the free-radical contribution.

To simplify the comprehension of spectral analysis, Figure 3 shows the breakdown of the Mn-MNP EPR spectrum obtained at pH 4.8 at 40 °C after 60 min of reaction, with the corresponding fitting and the residual curve. As observed, there is an excellent agreement between the data and the fitted values. In the bottom panel, each component of the spectra is presented separately, corroborating that this technique allows for both the identification of all the free radicals formed and their quantification (by comparing the intensity signals of each radical contributions to the Mn<sup>2+</sup> pattern sample). Because DMPO/•CH<sub>3</sub> radicals are formed as a consequence of the reaction between the •OH radical with DMSO, the total [•OH] was calculated as the contribution of the intensities of the •OH signal plus the •CH<sub>3</sub> signal. Moreover, as the •N radical contribution was present in all spectra with similar area, including the blank sample, it was not considered in the discussion.

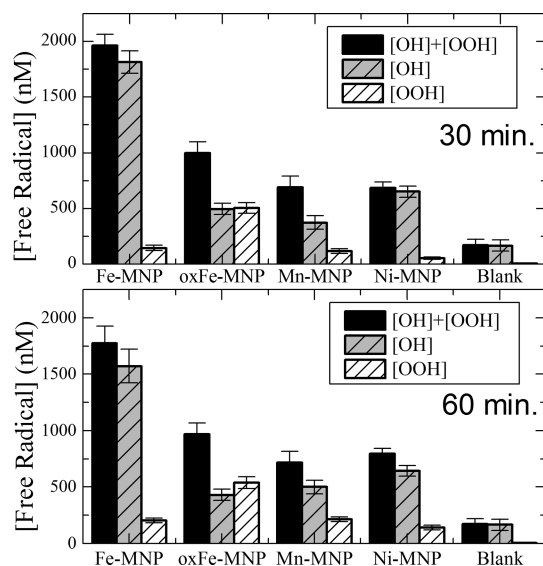
The [•OH] and [•OOH] measured for each ferrite ( $T = 40$  °C, pH = 4.8) are shown in Figure 4. This figure corresponds to the qualitative features observed in Figure 2; that is, the catalytic activity of Fe-MNP considering [•OH] is remarkably higher than that observed for the other nanoparticle samples. The Fe-MNPs enhance the production of the [•OH] free radical by a factor of  $\sim 10$  when compared to the blank solution. As can be observed from Figure 2, the oxidized form of magnetite (oxFe-MNP) presents a reduction of EPR intensities, which was further reflected as a strong decrease of [•OH] as calculated in Figure 4, related to the smaller Fe<sup>2+</sup> content in this sample.

The solutions containing Mn and Ni nanoparticles also displayed higher values of [•OH] compared to the blank solution; yet these values were lower than the values obtained for the solution with the Fe-MNP. Nevertheless, [•OH] was strongly reduced in Mn-MNP relative to the Fe-MNP samples, while the levels of [•OOH] were comparable between them. On the other hand, in the solution containing Ni-MNPs, the amount of both radicals was reduced. These results confirm the crucial role played by Fe<sup>2+</sup> in the formation of •OH radicals because Fe<sup>2+</sup> was oxidized (oxFe-MNP) or substituted by Mn and Ni ions in the Mn-MNP and the Ni-MNP samples, respectively. Figure S6 of the Supporting Information shows the catalytic activity of each ferrite sample in terms of the free radical •OH defined as the [•OH] produced in each sample

Table 2. EPR Parameters Obtained from the Fitting of the EPR Spectra for the Different Samples<sup>a</sup>

	Mn <sup>2+</sup> /MgO	•OH	•CH <sub>3</sub>	•OOH	•N
<i>g</i>	2.0005(3)	2.0023(3)	2.0024(3)	2.0023(3)	2.0023(3)
<i>w</i> [Oe]	4.3(5)	1.4 (2)	1.4 (2)	1.5 (2)	2.1 (4)
LS	0.2 (2)	0.98 (2)	0.98 (2)	0.98 (2)	0.98 (2)
<i>H</i> <sub>sf</sub> [Oe]	87.0 (1)				
<i>H</i> <sub>sf</sub> <sup>N</sup> [Oe]		14.7 (2)	16.2 (2)	14.4 (1)	14.8 (1)
<i>H</i> <sub>sf</sub> <sup>H</sup> [Oe]		14.9 (2)	23.0 (2)	10.4 (2)/1.4 (3)	

<sup>a</sup> *g* = gyromagnetic ratio; *w* = line width; LS = line shape coefficient (1 for a Lorentzian distribution and 0 for a Gaussian one); *H*<sub>sf</sub> is the hyperfine of Mn<sup>2+</sup>/MgO for *I* = 5/2; and *H*<sub>sf</sub><sup>N</sup> and *H*<sub>sf</sub><sup>H</sup> are the hyperfine coupling fields between the electronic spin and the nuclear spins of the N and H ions, respectively. The standard errors are given in parentheses which are in reference to the last digit.

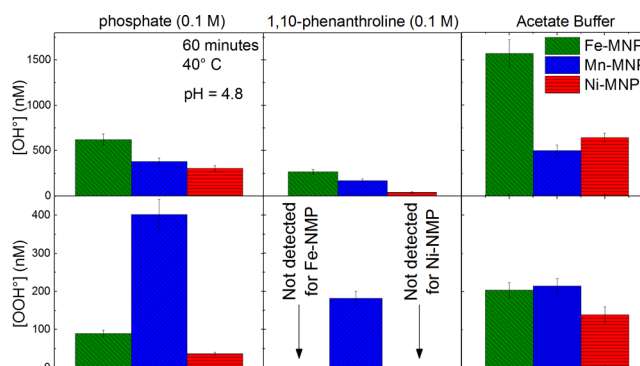


**Figure 4.** Total concentration of free radicals generated at 30 and 60 min after addition of H<sub>2</sub>O<sub>2</sub> to a DMPO/DMSO solution in acetate buffer solution (black bar) calculated as the sum of [•OH] and [•OOH] for each ferrite sample and the blank, at 40 °C in pH = 4.8. The error bars refer to the standard errors.

divided by the [•OH] obtained in the blank solution. Regarding the temporal evolution, no changes in radical production were observed in this catalytic activity of each ferrite in the first 120 min of reaction. When experiments were performed in pH = 7.4, as shown in Figure S7 of the Supporting Information, there were no significant differences in the catalytic activity of the samples and the blank, indicating the absence of peroxidase-like activity at this pH.

The quantitative analysis of the total [•OOH] produced by the samples is hard because of the limitations imposed by the DMPO/•OOH adduct, even in the presence of DMSO as a stabilizer. Nevertheless, a comparative study among the samples is valid, which indicates that the oxidation of the Fe-MNP (oxFe-MNP) and the incorporation of Mn lead to an increase in the [•OOH]/[•OH] ratio when compared to the other two samples. It also shows that part of the DMPO/•OH adduct detected arises from the decomposition of the DMPO/•OOH adduct.<sup>28–30</sup> As expected, [•OOH] (related to the Fe<sup>3+</sup> content) for oxFe-MNP, even underestimated, increases compared to the Fe-MNP sample. The [•OOH]/[•OH] ratio also increases for the Mn-MNP in comparison to the ratio of the Fe-MNP sample; yet not in the same magnitude as observed for the oxFe-MNP sample, denoting the possibility of some activity of the Mn ion in the production of this free-radical species.

To elucidate the effect of the different surface metallic ions in the nanoparticle's catalytic activity, we studied the free-radical formation by EPR in the presence of anionic ligands that form different complexes with the metallic ions, similar to that used to study the catalytic activity of other nanomaterials.<sup>43</sup> For this purpose, we have used the K<sub>3</sub>PO<sub>4</sub> and 1,10-phenanthroline as anionic ligands. It is known that phosphate ions have strong affinity for Fe<sup>3+</sup> ions,<sup>44</sup> and hence, they are able to coordinate with the Fe<sup>3+</sup> ions in the nanoparticle surface. An effective interaction with both Fe ions is expected, yet it would be weaker with Fe<sup>2+</sup> ions than with Fe<sup>3+</sup> ions.<sup>31</sup> Phosphates also have some coordination ability with manganese and nickel ions,<sup>45</sup> although lower than the one observed with Fe<sup>3+</sup> ions. In the case of 1,10-phenanthroline compound, this has stronger affinity for Ni<sup>2+</sup> and Fe<sup>2+</sup> ions<sup>46</sup> and lower affinity for Mn<sup>2+</sup> ions.<sup>47,48</sup> Figure 5



**Figure 5.** [•OH] and [•OOH] obtained from the EPR fitting procedure for every ferrite sample measured at 40 °C and in acetate buffer (pH 4.8) containing 0.1 M of phosphate, 0.1 M of 1,10-phenanthroline, and in only acetate buffer as a control. The reaction time is 60 min. The error bars refer to the standard errors.

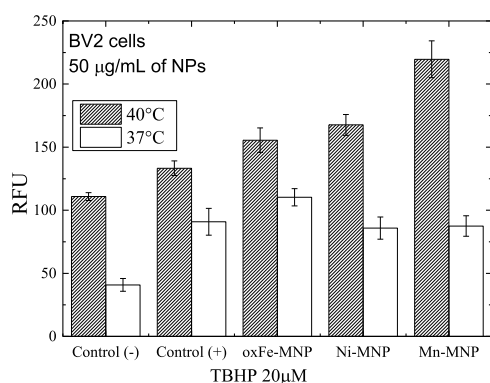
shows [•OH] and [•OOH], after 60 min of reaction, obtained from the EPR fitting procedure using both ligands for all ferrite samples. As explained before, the solutions were prepared by dispersing the nanoparticles in acetate buffer at pH = 4.8 containing phosphate (0.1 M) or 1,10-phenanthroline (0.1 M). Figure S9 of the Supporting Information shows the temporal dependence of total concentration of radicals for the samples in the presence of both ligands. In the case of Fe-MNP with the phosphate ligands, a strong reduction of [•OH], from ~2000 to ~600 nM, was observed. For the other two samples, Mn-MNP and Ni-MNP, a reduction was also observed but less pronounced: from ~450 to 300 nM for the Mn-MNP sample and from ~500 to ~250 nM for the Ni-MNP sample. When the 1,10-phenanthroline ligand was added to the solution, a

strong reduction in [ $\bullet\text{OH}$ ] was obtained for all samples. It is known that the functionalization of the nanoparticle's surface with anionic ligands with strong affinity for metallic cations changes the peroxidase-like activity in the ferrite nanoparticles.<sup>45</sup> Specifically, our results indicate that the use of anionic ligands with strong affinity for iron oxides can inhibit the peroxidase-like activity of ferrites where one of Fe ions dominates.

Because phosphate and 1,10-phenanthroline are scavengers of  $\bullet\text{OH}$ , it is expected that they would stabilize the DMPO/ $\bullet\text{OOH}$  adduct. Considering this, the [ $\bullet\text{OOH}$ ] also decreases in the presence of phosphate in both Fe-MNP and Ni-MNP samples when compared to the acetate buffer solution, whereas it is doubled for the Mn-MNP sample. For the 1,10-phenanthroline ligand, no contribution of the  $\bullet\text{OOH}$  species was observed for the Fe-MNP and Ni-MNP samples. For the Mn-MNP sample, a similar amount of [ $\bullet\text{OOH}$ ] was observed in 1,10-phenanthroline and in acetate buffer solution. These results suggest that the Mn ions in our ferrite nanoparticles present some catalytic activity increasing the [ $\bullet\text{OOH}$ ] radical in physiological conditions, which is not completely unexpected because the catalytic activity of Mn ions in oxides has been reported.<sup>49</sup>

Concerning the temperature dependence of the catalytic activity of the nanoparticles, Figure S8 in the [Supporting Information](#) compares the [ $\bullet\text{OH}$ ] and [ $\bullet\text{OOH}$ ] for the Fe-MNP sample measured at 25 and 40 °C at pH = 4.8 for different times, showing that the increment with the temperature is only about 10%, which is not as significant as the changes in the particle composition or the pH.

**In Vitro Experiments.** Figure 6 gives the relative ROS production in BV2 cells exposed to 50  $\mu\text{g/mL}$  of oxFe-MNP,



**Figure 6.** Intracellular ROS levels (in RFUs) of BV2 cells exposed to 50  $\mu\text{g/mL}$  of oxFe-MNP, Ni-MNP, and Mn-MNP at 37 °C (white bars) and 40 °C (dashed bars). The bars from negative control (–) and positive control with 20  $\mu\text{M}$  TBHP (+) are also included ( $p > 0.05$ ).

Ni-MNP, or Mn-MNP and incubated at 37 and 40 °C for 60 min. Additionally, the negative (without nanoparticles or TBHP) and positive (20  $\mu\text{M}$  TBHP) controls at the two temperatures are shown. The procedure used to coat Fe-MNP with dextran is analogous to the protocol to produce the oxidized oxFe-MNPs. Also, Mn-MNP and Ni-MNP samples were coated with dextran for in vitro experiments; hence, possible oxidation of  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$  cannot be ruled out.

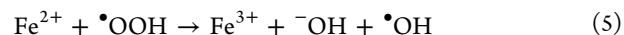
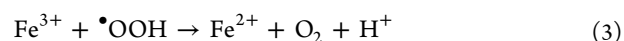
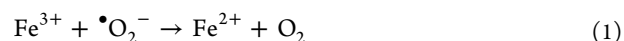
For the experiments performed at 37 °C, the ROS levels measured for the nanoparticles cannot be considered statistically different from the positive control. Yet the oxFe-MNP

sample presents an ROS level slightly higher than the samples of Mn-MNP and Ni-MNP. This tendency is similar to that observed for the catalytic activity determined for each sample in the EPR measurements. Comparing the ROS level for the in vitro experiments at 37 °C, the relation oxFe-MNP/Mn-MNP/Ni-MNP = 1:0.79:0.78 is obtained. The relation 1:0.77:0.86 is obtained for the total amount of free radicals in the EPR experiments at 60 min of reaction.

Incubation of cells at 40 °C for 1 h increased ROS production in both the control and the experimental groups, mostly because of the enhancement of cell stress at such a high temperature. A stable increment in the measured levels of ROS is observed for the negative control in comparison to the value at 37 °C, which is close to the value obtained for the positive control. In fact, comparing both controls, the obtained value cannot be taken as statistically different despite a tendency of having a higher value in the positive control as indicated. However, the ROS levels measured for oxFe-MNP and Ni-MNP are similar and statistically higher than that of the negative control. Interestingly, the Mn-MNP sample showed considerably higher ROS activity than the other samples, as well as a higher activity than the positive control at the same temperature.

## DISCUSSION

$\bullet\text{OH}$  formation is the critical point concerning the toxicity associated with the peroxidase-like activity of ferrite nanoparticles, which is iron-catalyzed by the Haber–Weiss reaction, which makes use of Fenton chemistry<sup>50,51</sup>



The Haber–Weiss reaction chain has different starting points, one related to the direct reaction of the  $\text{Fe}^{2+}$  with  $\text{H}_2\text{O}_2$  and other related to a previous reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . This is an interesting point, as an oxidation of the nanoparticle surface Fe is expected when the nanoparticles are in aqueous solution or during the functionalization process with dextran; however, free radicals can be also produced starting from the reaction described in eqs 1 and 3 but considering that the chain reaction starting from eq 1 has a faster kinetics.

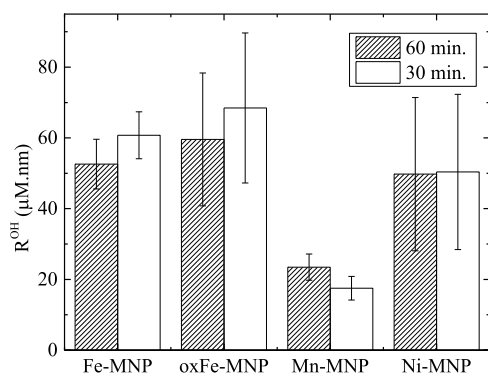
It is expected that the peroxidase-like activity of the ferrite nanoparticles depends on their surface-to-volume ratio. Indeed, Gao et al.<sup>8</sup> have reported that the catalytic reaction of ferrite diminishes with increasing particle size. In addition to the surface area, other factors related to the surface of the iron oxide are important to determine the peroxidase-like activity of the iron oxides. The ability to binding and the dissociation of oxygen molecules to the surface of the particles are critical factors with a complexity; for stance, in molecules with an active Fe ion in the core, the dioxygen binding and dissociation dynamics of the resulting iron peroxide species are key points for the catalytic activity of the molecule.<sup>52</sup> Besides the surface, the structure and superficial plane are also important in the

peroxidase-like activity of oxides with spinel structure, including maghemite and the magnetite,<sup>53</sup> among other 3d transition-metal oxides with similar structure.<sup>54</sup> Further, the surface reduction and oxidation kinetics in the oxide system also play fundamental roles in the catalytic activity of the metal oxide systems, as studied by Righi and Magri.<sup>55</sup> These authors showed theoretically that the vacancies are a determining factor for the oxidation/reduction reactions in the (100) plane of the maghemite, which can be related with the Haber–Weiss chain reactions through the reaction described in eq 1. In addition, Wang et al.<sup>56</sup> showed recently that the population of  $e_g$  orbitals plays a fundamental role in the peroxidase-like catalytic activity of perovskite oxide-based nanosystems.

As discussed above, prediction of the peroxidase-like activity of the ferrite nanoparticles are complex and involve several physical–chemical factors. Nevertheless, a deeper analysis of our results allows to investigate the role of the surface chemical catalytic activity among the different ferrites studied, with respect to the production of the  $\bullet\text{OH}$  radical as determined by EPR ( $[\bullet\text{OH}]_{\text{EPR}}$ ). For this, we first consider the influence of the particle size by normalizing  $[\bullet\text{OH}]_{\text{EPR}}$  for each sample with the surface-to-volume ratio weighted by the size distribution as obtained from TEM analyses (i.e.,  $[\pi d_{\text{TEM}}^2]/[(\pi/6)d_{\text{TEM}}^3] \times f(d)$ ). Here, we assume that the atoms acting in the catalytic reaction are localized in the last atomic layer: the superficial plane. We also normalize  $[\bullet\text{OH}]_{\text{EPR}}$  with the  $[\text{Fe}^{2+}]/[\text{Fe}]$  ratio obtained from the XPS results. This normalized amount was defined by the parameter  $R^{\text{OH}}$  as follows

$$R^{\text{OH}} = [\bullet\text{OH}]_{\text{EPR}} \times ((\pi d_{\text{TEM}}^2)/((\pi/6)d_{\text{TEM}}^3) \times f(d)) / ([\text{Fe}^{2+}]/[\text{Fe}]) \quad (7)$$

Figure 7 presents the  $R^{\text{OH}}$  values calculated from the EPR results at 30 and 60 min of reaction for samples Fe-MNP, oxFe-MNP, Mn-MNP, and Ni-MNP.



**Figure 7.** Quantity  $R^{\text{OH}}$  calculated with eq 4 from the value of  $[\bullet\text{OH}]$  determined by EPR at 30 and 60 min of reaction, pH = 4.8 and  $T = 40^\circ\text{C}$ , using the respective  $[\text{Fe}^{2+}]/[\text{Fe}]$  ratio estimated from the XPS results and the average surface calculated from the diameter distribution obtained from the TEM analyzes. The error bars refer to the calculated dispersion.

oxFe-MNP, Ni-MNP, and Mn-MNP. Despite the error bars, mainly because of the size dispersion, the resulting values for the formation of  $\bullet\text{OH}$  indicate the key role played by  $\text{Fe}^{2+}$  by the almost constant value of  $R^{\text{OH}}$  after normalization, excepting for sample Mn-MNP that presents a smaller  $R^{\text{OH}}$  value.

The substitution of  $\text{Fe}^{2+}$  by Mn and Ni ions in the spinel structure reduces the catalytic activity that produces the free radical  $\bullet\text{OH}$ . In fact, the Mn-MNP sample showed a lower

amount of  $[\bullet\text{OH}]$  than expected. Probably, this is related to a competitive catalytic reaction involving the Mn ions and the free radical  $\bullet\text{OOH}$ . This Mn activity is indicated by the EPR measurements in the presence of phosphate and 1,10-phenanthroline. As mentioned before, the phosphate anion and  $\text{Fe}^{2+}$  form a complex in the surface of the nanoparticles, leading to a strong reduction in the amount of radical  $[\bullet\text{OH}]$  in the solution of Fe-MNPs. As expected, the reduction of  $[\bullet\text{OH}]$  was not that significant in the Ni-MNP and Mn-MNP samples because of the lower concentration of  $\text{Fe}^{2+}$  in these nanoparticles. This result may indicate that the Mn ions catalyze the production of the  $[\bullet\text{OOH}]$  radical. The catalytic activity of  $\text{Mn}^{3+}$  ions with  $e_g$  hybridization is reported in the literature.<sup>49,56</sup> Despite the fact that our results clearly indicate the role in the peroxidase-like catalytic activity of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  in the octahedral site of the ferrites, deeper studies on the structure and cationic distribution in the spinel structure must be performed in order to fully elucidate the activity of all cations in this chemical reaction.

Concerning the in vitro cell culture results, we emphasize that the catalytic activity as inferred from EPR data and fluorescence in vitro should be compared with caution. Not only do EPR and photoluminescent protocols have different specificities but they were also measured in different conditions. It is important to mention other remarkable differences between the ROS level determined in the in vitro experiments and EPR quantification of the free radicals produced. First, the oxidative stress of the cell and the influence on its metabolism should be taken into account, as shown by the ROS level measured for the negative and positive controls in the in vitro experiments at  $40^\circ\text{C}$ . Second, the acetate buffer used in the EPR experiments was different from the carbonate buffer used in the culture media or the phosphate-rich media in intracellular localization. In the proximity of these chelates of transition metals, the process could lead to the formation of metallic complexes on the surface of the nanoparticles, directly affecting the catalytic activity of the nanoparticles.<sup>45</sup> Third, the concentration of nanoparticles used in the EPR measurements was  $\sim 600 \mu\text{g/mL}$ , while in cell culture experiments, this concentration would have induced toxicity issues. At the same time, the kinetics of the reactions should also be taken into consideration as EPR, photoluminescent protocols involve very different time scales (seconds and hours, respectively), and hence, direct comparison of data from both techniques would require additional experiments to evaluate the time evolution of the reactive species production in each media with the same particle concentration. Fourth, our dextran-coated nanoparticles tend to be localized in the lysosome<sup>57</sup> (pH = 4.8), but different coatings may result in other areas of different pH conditions (e.g., pH 7.4), where the ferrites displayed no catalytic activity in the EPR experiments. Therefore, it is important to know the intracellular localization of the nanoparticles in the cell for a better comparison with the EPR studies. Finally, we remark the procedure used to obtain the coating is also important, as it may result in the oxidation (or in other cases the reduction) of the metallic ions in the surface of the particles. Our procedure to obtain the dextran-coated nanoparticles results in the oxidation of  $\text{Fe}^{2+}$  ions, affecting directly the catalytic activity, and probably the Ni and Mn ions are also affected in this process. Thus, deeper studies are necessary for a better compression of this point. Despite this, we found a relatively good correspondence between in

vitro and EPR experiments, with both indicating a catalytic activity of the nanoparticles that increases the amount of ROS. These results are relevant for the applications of ferrite nanoparticles in biomedical protocols, and they indicate that the composition and the oxidation state of the nanoparticles are critical. EPR is a useful technique to evaluate the activity of the nanoparticles, and it can complement the in vitro evaluations.

For the Mn-MNP, the higher ROS levels detected for in vitro experiments are probably related to the amount of radical  $\bullet\text{OOH}$  produced, including the catalytic activity of  $\text{Mn}^{3+}$  ions present in the sample that is not correctly detected by EPR using DMPO as a spin trap. Concerning the Mn ferrite, more detailed studies on the Mn ionic valence, its distribution in the crystalline sites of the ferrite, and the correct measurement of the  $[\bullet\text{OOH}]$  produced using another spin-trap specific for this radical are needed.

## CONCLUSIONS

From the EPR measurements, we were able to quantify the formation of  $[\bullet\text{OH}]$  and  $[\bullet\text{OOH}]$  resulting from the catalytic activity (pH = 4.8 and 40 °C) of the Mn-MNP, Ni-MNP, and Fe-MNP and its oxidized form (oxFe-MNP). Our results indicate that the production of the  $[\bullet\text{OH}]$  radical was triggered by the presence of  $\text{Fe}^{2+}$ . Because of this, the total amount of free radical produced by the catalytic activity of the nanoparticles was reduced when Mn and Ni ions were used to substitute  $\text{Fe}^{2+}$  in the ferrite crystal lattice. Accordingly, lower concentration of this radical was also observed in the oxidized magnetite. No radical formation was evidenced for any sample at pH = 7.4. In vitro measurements of ROS formation in BV2 cells exposed to the dextran-coated nanoparticles at 37 and 40 °C showed measurable catalytic activity of the nanoparticles. For 37 °C, in vitro results are in conformity with the EPR experiments.

An additional outcome stemming from the above results is the significance of keeping the experimental conditions as invariable as possible if a direct comparison between different techniques is made. Nanoparticles with high peroxidase-like catalytic activity could be of interest for oncological applications, whereas for MRI, ophthalmology, or drug delivery, the design of MNPs with minimum peroxidase-like activity is desired. Therefore, the control of surface oxidation or functionalization is a key issue for a safe and efficient design of these materials. We believe that our work provides a basic outline to better engineer and produce ferrite nanoparticles with predictable catalytic impact on desired applications, regardless of whether these applications are in the field of biomedicine, industry, or environmental sciences.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcc.9b05371.

Fitted  $\text{Fe } 2p_{3/2}$  and  $3p$  XPS spectra of samples Fe-MNP, Mn-MNP, and Ni-MNP, including the respective tables with main parameters obtained from the XPS analyses; UV–visible spectrophotometry results; concentration of the free radicals of the different samples as a function of the reaction time measured by EPR; concentration of free radicals as a function of time reaction measured by EPR for sample Fe-MNP at different temperatures (25

and 40 °C) and pHs (4.8 and 7.0); and time dependence of  $[\bullet\text{OH}]$  and  $[\bullet\text{OOH}]$  for samples Fe-MNP, Mn-MNP, and Ni-MNP in acetate buffer (pH 4.8) containing 0.1 M of phosphate and 1,10-phenanthroline (PDF)

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A.C.M.M. and E.L.W. contributed equally. This manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

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