Cell-promoted nanoparticle aggregation decreases nanoparticle-induced hyperthermia under an alternating magnetic field independently of nanoparticle coating, core size and subcellular localization

Raquel Mejías^{1,#}, Patricia Hernández Flores^{1,#}, Marina Talelli^{1,3}, José L. Tajada-Herráiz¹, María E.F. Brollo², Yadileiny Portilla¹, María P. Morales², Domingo F. Barber^{1,*}.

¹ Department of Immunology and Oncology, and NanoBiomedicine Initiative, Centro Nacional de Biotecnología (CNB/CSIC), Darwin 3, Campus de Cantoblanco, 28049 Madrid, Spain

² Department of Energy, Environment and Health, Instituto de Ciencia de Materiales de Madrid (ICMM/CSIC), Sor Juana Inés de la Cruz 3, Campus de Cantoblanco, 28049 Madrid, Spain

³ Current address: RAFARM S.A., 12 Korinthou Str., 15451, N. Psihico, Athens, Greece

These authors contributed equally to this work

* Address correspondence to dfbarber@cnb.csic.es

ABSTRACT

Magnetic hyperthermia has a significant potential to be a new breakthrough for cancer treatment. The simple concept of nanoparticle-induced heating by the application of an alternating magnetic field has attracted much attention, as it allows the local heating of cancer cells, which are considered more susceptible to hyperthermia than healthy cells, while avoiding the side effects of traditional hyperthermia. Despite the potential of this therapeutic approach, the idea that local heating effects due to the application of alternating magnetic fields on magnetic nanoparticle-loaded cancer cells can be used as a treatment is controversial. Several studies indicate that the heating capacity of magnetic nanoparticles is largely reduced in the cellular environment due to increased viscosity, aggregation and dipolar interactions. However, an increasing number of studies, both *in vitro* and *in vivo*, show evidence of successful magnetic hyperthermia treatment on several different types of cancer cells. This apparent contradiction might be due to the use of different experimental conditions. Here we analyze the effects of several parameters on the cytotoxic efficiency of magnetic nanoparticles as heat inductors under an alternating magnetic field. Our results indicate that cell-nanoparticle interaction reduces the cytotoxic effects of magnetic hyperthermia, independently of nanoparticle coating and core size, the cell line used and the subcellular localization of nanoparticles. However, there seems to occur a synergistic effect between the application of an external source of heat and the presence of magnetic nanoparticles, leading to higher toxicities than those induced by heat alone, or the accumulation of nanoparticles within cells.

KEYWORDS: Magnetic hyperthermia, Alternating magnetic field, SPION, nanoparticle aggregation, lysosomal permeabilization, oxidative damage, cell death.

INTRODUCTION

The development of new nanomaterials, especially magnetic nanoparticles (MNPs), is one of the most active research fields today. Because of their tunable physicochemical properties, MNPs have a wide variety of applications in biomedicine and life sciences, such as cell isolation, imaging, drug delivery and biosensing ¹; and provide the possibility to combine several of these applications at the same time. Among MNPs, superparamagnetic iron oxide nanoparticles (SPIONs) are usually preferred. SPIONs show no residual magnetization in the absence of an external magnetic field. This is of great importance in biomedical applications, since a permanent magnetic behavior would lead to nanoparticle aggregation and blood vessel embolization. Besides, SPIONs are well tolerated and have low toxicity profiles even in long-term studies ², and they undergo a process of biotransformation from SPIONs to other non-superparamagnetic iron forms, which allows their clearance ³⁻⁶. SPIONs have been in clinical use for years, and several types have been approved by the European Medicines Agency (EU) and by the Food and Drug Administration (USA) for use in human patients, especially as antianemic drugs and contrast agents for magnetic resonance imaging (MRI) ⁷. However, in the last years there has been a growing interest in other biomedical applications, such as thermal therapy, especially for cancer treatment. Cancer cells are considered to be more susceptible to hyperthermia than healthy cells due to their higher metabolic rates ⁸ and hyperthermic inhibition of DNA repair ⁹. In addition to direct effects of heat on tumor cells, it has been observed that hyperthermia improves tumor antigen presentation, activation of dendritic cells and NK cells, and leukocyte trafficking through endothelium ¹⁰, enhancing the anti-tumor immune response. Hyperthermic cancer therapy also improves the effectiveness of other therapies, such as chemotherapy and radiation ¹¹, which prompted the use of systemic hyperthermia as an adjuvant therapy for cancer. Despite these promising observations, clinical benefits of hyperthermia are limited by toxicity resulting from diffuse heating of healthy tissue ¹² and the resistance of some cancers to traditional hyperthermia ¹³. For these reasons, localized magnetic hyperthermia (MHT) has a special relevance, as we can increase the temperature only in those areas where SPIONs are present, without affecting other tissues. MHT is based on the accumulation of nanoparticles in the target site, and the application of an alternating magnetic field (AMF) of sufficient strength and frequency to induce nanoparticle heating. Heat from magnetic nanoparticles is generated due to the delay in the relaxation of the magnetic moment of the particles through either the rotation of the magnetic moment in the particle (Néel relaxation) or the rotation of the particle itself (Brownian relaxation), when the particles are exposed to AMF with alternation times shorter than the magnetic relaxation times of the particles ¹⁴. The heating capacity of magnetic nanoparticles and the effects of MFH depend on the physicochemical properties of magnetic nanoparticles (size, aggregation state, crystallinity) ¹⁴, their magnetic properties (saturation magnetization and anisotropy), the frequency and intensity of the AMF, and the cell type ¹⁵. Local MHT, which has a significant potential to be a new breakthrough for cancer treatment, has been successfully used in a number of studies reporting effective MHT in both preclinical ¹⁶⁻²⁰ and clinical studies ²¹⁻²⁵. Moreover, several groups have recently reported cell death after MHT treatment, without perceptible temperature rise ²⁶⁻³⁰. The mechanisms responsible for these observations have not been elucidated yet, but some data indicate that these effects are related to an increase in lysosomal permeability, which correlates with increased production of reactive oxygen species (ROS), enhanced activity of the lysosomal protease cathepsin D in the cytoplasm, and decreased tumor cell viability ³¹⁻³³. This lysosomal permeabilization might be caused by mechanical rotation or vibration of SPIONs affecting the lipid membrane stability, since it has been observed that dynamic magnetic fields induce a slow rotation of lysosome-targeted SPIONs, causing the tearing of the lysosomal membrane, and resulting in apoptosis activation ³⁴. Other possible cause for increased lysosomal permeability might be a very localized

intracellular heat release from SPIONs, which also enhances the generation of ROS by the iron oxide surface of the nanoparticles through the Fenton reaction^{32, 34-35}, which is known to be temperature dependent³⁶. Recently, it has been observed that the application of an AMF increases the generation of ROS by SPIONs both in suspensions³⁷ and within cells³⁸, suggesting that in response to AMF application, SPIONs convert the energy they absorb into heat, enhancing ROS generation at their surfaces³². However, in the last years a number of studies³⁹⁻⁴³, indicate that the heating capacity of SPIONs is largely reduced when internalized by cells, due to increased viscosity of the environment, nanoparticle aggregation and dipolar interactions between particles (as previously observed by our group⁴⁴), which would justify the reduction of mechanical and thermal effects of SPIONs when subjected to an AMF. These contradictory observations make it rather difficult to draw general conclusions, partly because these studies were conducted using different nanoparticle types, concentrations, AMF amplitudes, frequencies, and cancer cell lines. In order to assess the factors affecting the cytotoxic efficiency of nanoparticles within cells under an AMF, here we present a systematic study using different nanoparticle coatings, cell lines, subcellular localizations, and nanoparticle core sizes, in an attempt to isolate the source of the high variability of the results obtained from different studies on cellular magnetic hyperthermia.

RESULTS AND DISCUSSION

Influence of nanoparticle coating on MHT

Nanoparticle coatings play essential roles in biomedical applications. Surface coating affects nanoparticle uptake and biodistribution⁴⁵, and may vary the protein adsorption on the nanoparticle surface in physiological medium, modifying the composition of the protein corona⁴⁶, which in turn can influence nanoparticle stability, aggregation, and magnetic behavior⁴⁷. For MHT, we analyzed 14 nm core-sized MNPs with two different coatings (Figure 1a): A positively charged coating of (3-aminopropyl)triethoxysilane (APS), and a negatively charged coating of dimercaptosuccinic acid (DMSA). Physicochemical characteristics of both types of nanoparticles are summarized in Table 1.

Table 1. Main physicochemical characteristics of the 14 nm core-sized MNPs used.

MNP	Core size (nm)	Coating	% (wt.) coating	Hydrodynamic diameter (nm)	ζ-potential pH 7 (mV)
APS-MNPs	13.7 ± 2	3-aminopropylsilane	1	222 (0.28)	+ 20
DMSA-MNPs	13.7 ± 2	Dimercaptosuccinic acid	3	88 (0.16)	- 29

Note: Hydrodynamic diameter and polydispersity index are represented as Z-average number in intensity and standard deviation/mean size.

When an AMF (250 kHz, 25 kA/m) was applied to 10 mg/ml MNP suspensions in water, we observed an increase in temperature of 5.2 and 5.7 °C over room temperature RT (Figure 1b). In these conditions, specific absorption rate (SAR) values for APS-MNPs and DMSA-MNPs were 16.7 and 16.0 W/g Fe respectively. The heating capacity of both MNP types decreased when medium viscosity increased (0.1% w/v agarose; Figure 1b), as described for similar MNPs⁴⁸.

We analyzed MNPs toxicity on murine pancreatic adenocarcinoma Pan02 cells, using the PrestoBlue assay (Figure 1c). Cultured Pan02 cells were exposed to different concentrations (ranging from 10 to 500 µg Fe/ml) of APS-MNPs or DMSA-MNPs for 24 h. Viability was not significantly affected by APS- or DMSA-MNPs at all nanoparticle concentrations tested, although at the highest concentrations (over 250 µg Fe/ml) mean cell viability was reduced below 90%. These data indicate that these types of

MNPs exert little toxicity in this cell line, with no notable influence on cell proliferation.

Figure 1. Nanoparticle characterization, toxicity, cell uptake and subcellular distribution on Pan02 cells. (a) Representative TEM images of 14 nm core-sized APS-MNPs and DMSA-MNPs. Scale bar: 50 nm (b) Heating curves of 14 nm core-sized APS-MNPs (top) and DMSA-MNPs (bottom) suspensions (10 mg Fe/ml) in water or 0.1% agarose, under an AMF (250 kHz, 25 kA/m (Vpp)). The coil was maintained at RT (dotted lines). (c) PrestoBlue viability assay of Pan02 cells incubated with various APS- or DMSA-MNP concentrations for 24 h. Data were normalized to untreated cells, and are shown as mean $\pm$ SD (3 independent experiment in triplicates). One-way ANOVA showed no statistically significant differences. (d) Quantification of cell-associated MNPs by ICP-OES. The total amount of iron was divided by the number of cultured cells for each sample. Data are shown as mean $\pm$ SD (n = 3-4). One-way ANOVA followed by Bonferroni multiple comparisons, *p < 0.05, **p < 0.01, ***p < 0.001. (e) Representative TEM images of MNP-loaded Pan02 cells. Images on the right are magnifications of the boxed areas in the images on the left. Scale bar: 100 nm.

To analyze nanoparticle uptake, we incubated Pan02 cells with several concentrations

(ranging from 50 to 200 $\mu\text{g Fe/ml}$) of APS- or DMSA-MNPs for 24 h, and evaluated nanoparticle association with cells by ICP-OES (Figure 1d). APS-MNP levels reached 32 pg Fe/cell, while Pan02 cells accumulated only 5 pg Fe/cell after incubation with the highest concentration of DMSA-MNPs. This difference might be due to the nature of the coating, since it has been described that positively charged nanoparticles are internalized more easily by cells than neutral or negatively charged nanoparticles⁴⁹. Besides, positive coatings induce the formation of larger nanoparticle aggregates, which facilitates the adsorption to the cell surface and subsequent internalization⁵⁰⁻⁵¹. Since no significant differences were found between the two highest doses of APS-MNPs, we chose the 150 $\mu\text{g Fe/ml}$ concentration for future experiments.

We determined subcellular localization of MNPs by TEM. Both types of nanoparticles were found inside double-membrane vesicles, identified as lysosomes (Figure 1e), but we observed clear differences between them. TEM images confirmed higher levels of internalized APS-MNPs, which occupied practically all the lysosomal compartment and showed a greater degree of compaction compared to DMSA-MNPs, which formed small aggregates separated from each other.

In order to investigate whether APS- or DMSA-MNPs exposed to an AMF induce the thermal-independent cytotoxic effects described by other groups, we subjected MNP-loaded Pan02 cells to an AMF (250 kHz, 25 kA/m) for 1 h, with constant control of the temperature of the culture medium, which was maintained at 37°C. As controls we used untreated cells at standard culture conditions (SC), and cells incubated at 37°C without CO₂ or relative humidity control (EC), in similar conditions to the interior of the AMF generator (V3) device. After treatment, cells were tested for cell viability, lysosomal permeability, ROS generation and Hsp70 expression.

To analyze cell viability after treatment, cells were stained with annexin V and propidium iodide (PI), and analyzed by flow cytometry, which provides quantitative and qualitative information about the type of cell death. Since it has been described that the concentration of nanoparticles and their aggregation can affect the MHT process and its outcome^{39-40, 52}, important differences between APS- and DMSA-MNPs could be expected. However, AMF application did not induce significant changes in the percentage of dead cells compared to control groups (Figure 2a). We observed that the presence of APS-MNPs significantly increased the percentage of early and late apoptotic cells compared to control cells not exposed to MNPs, regardless of whether they were subjected to the AMF. This result suggests some toxicity of APS-MNPs on Pan02, which correlates with the results obtained from PrestoBlue assay. We observed a non-significant increase of DMSA-MNP-induced cell death compared to cells without MNPs, except for controls maintained at standard culture conditions, which implies a toxic effect of DMSA-MNPs only when there are additional stress factors acting on Pan02 cells.

To evaluate if APS- or DMSA-MNPs induced increased lysosomal permeability during AMF application, we used the acridine orange (AO) staining. In the nucleus and cytoplasm AO concentration is low, and the molecules can be found in monomeric form, which emits green fluorescence. However, when the molecule diffuses into the lysosomes, the acid pH leads to AO protonation, which prevents it from freely diffusing back into the cytosol. Consequently, the increase in its concentration leads to monomer stacking and red fluorescence emission⁵³. We proceeded as previously described³¹, where they analyzed the reduction of red fluorescence as an indicator of increased lysosomal permeability, but also took into account the changes in green fluorescence, which represent variations in plasmatic membrane permeability.

Figure 2. Effects of AMF application on MNP-loaded Pan02 cells. Pan02 cells were analyzed after incubation with APS- or DMSA-MNPs for 24 h (150 μ g Fe/ml) and exposed to AMF (AMF), or were maintained under standard conditions (SC), or incubated at 37°C without CO₂ and relative humidity control (EC) for 1 h. For each culture condition cell unexposed to MNPs were used as negative controls (-). (a) Cell death percentage was analyzed by flow cytometry after staining with Annexin V and PI. Data are shown as mean $\pm$ SD (n = 3). Two-way ANOVA followed by '-' vs. 'MNP' contrast, *p < 0.05, **p < 0.01, ***p < 0.001. (b) Lysosomal permeability was assessed by flow cytometry after staining with acridine orange. PEI-treated cells (100 μ g/ml) were used as a positive control. Data were normalized to -/SC cells, and are shown as mean $\pm$ SD (n = 3-5). One-way ANOVA followed by Bonferroni multiple comparisons, or two-way ANOVA followed by '-' vs. 'MNP' contrast, *p < 0.05, **p < 0.01, ***p < 0.001. (c) ROS generation analysis was determined by flow cytometry after incubation with the H₂DCFDA fluorescent probe. H₂O₂-treated cells were used as a positive control (1 mM, 30 min). Data were normalized to -/SC cells, and are shown as mean $\pm$ SD (n = 3-4). Two-way ANOVA followed by '-' vs. 'MNP' contrast, *p < 0.05, **p < 0.01, ***p < 0.001. (d) Hsp70 expression was analyzed by RT-qPCR. Cells incubated at 42°C were used as a positive control. Data were normalized using the 2^{- $\Delta\Delta$ Ct} method and are shown as mean $\pm$ SD (n = 3). Two-way ANOVA followed by '-' vs. 'MNP' and 'SC' vs. 'EC' vs. 'AMF' contrasts, *p < 0.05, **p < 0.01, ***p < 0.001.

Treated cells were stained with AO and analyzed by flow cytometry. As a positive control for permeability we used polyethylenimine (PEI)-treated cells, since PEI is a well-known membrane permeabilizer, frequently used in cell transfection⁵⁴. We

observed that APS-MNPs induced a decrease in red and green fluorescence compared to cells non-exposed to MNPs (Figure 2b), indicating increased permeability in both lysosomal and plasmatic membranes. The decrease in fluorescence intensity could be due to the fact that nanoparticles increase the scattering of light in all directions in a dose-dependent manner⁵⁵, but we also hypothesize that a real increase in membrane permeability might be induced by the cationic nature of APS⁵⁶. However, exposure to AMF had no additional effect in membrane permeability. DMSA-MNPs did not induce any changes in fluorescence intensity, regardless of AMF application, which supports the idea of the influence of the positive charges of the APS as inductors of increased membrane permeability and/or lower intracellular nanoparticle concentration. In PEI-treated cells red and green fluorescence also decreased significantly compared to untreated cells.

It has been described that iron oxide MNPs may generate ROS through Fenton reaction^{32, 35}, which could be enhanced by local hyperthermic effects when MNPs are exposed to an AMF³⁷⁻³⁸. However, this process may be deeply influenced by the nature of nanoparticle coating and concentration^{38, 57}, cell type⁵⁷ or duration of exposure to AMF⁵⁷⁻⁵⁸. Here, we analyzed ROS levels using 2',7'-dichlorodihydrofluorescein (H₂DCFDA), a non-fluorescent derivative of fluorescein that is known to convert to the highly fluorescent 2',7'-dichlorofluorescein upon cleavage of its acetate groups by esterases or oxidation. Cells exposed to APS-MNPs, regardless AMF application, showed a decrease in fluorescence intensity compared to cells non-exposed to MNPs (Figure 2c), which could be explained as in the previous section. DMSA-MNPs, however, had no effect on fluorescence intensity, indicating that MNPs do not generate ROS with or without exposure to AMF.

Cells respond to stress by adaptive changes that protect them from cell death. Heat shock proteins (HSPs) are a group of molecular chaperones that play a protective role against several stress factors, including hyperthermic exposure⁵⁹. HSPs, and particularly hsp70, are overexpressed in response to stimuli that induce protein denaturation, such as heat, oxidative stress, heavy metals and infections, among others⁶⁰. We analyzed Hsp70 expression in Pan02 cells by RT-qPCR (Figure 2d). Incubation with APS-MNPs led to higher Hsp70 expression levels compared to cells non-exposed to MNPs, but AMF application did not produce an additional increase. This suggests that APS-MNPs induced some AMF-independent cell stress. In contrast, DMSA-MNPs did not produce an increase in Hsp70 expression levels compared to untreated cells, although a significant increase was observed in EC samples, probably due to the high variability of data obtained from them. In any case, the increase in Hsp70 expression is again AMF-independent. These results indicate that there were no intracellular hyperthermic effects that could go unnoticed when measuring the temperature of the culture medium, or they were too weak to elicit an observable cellular response.

Influence of subcellular localization on MHT

Subcellular localization of MNPs might also influence the MHT process⁶¹. In fact, cell membrane hyperthermia has been proposed as a new mode of MHT⁶² in which MNPs are selectively located on the cell membrane. Due to its low thermal conductivity, heating of the plasmatic membrane might be easier to achieve than conventional intracellular hyperthermia, and lower nanoparticle concentrations would be needed. In this mode of hyperthermia the increase of temperature could compromise the integrity of the cell membrane, leading to cell death. In order to find out whether, under these conditions, MHT thermal effects were observed, we used the human T cell acute lymphoblastic leukemia Jurkat cell line. These cells are non-phagocytic, and previous unpublished data from our group indicate that APS-MNPs are not accumulated inside Jurkat cells, but they remain adsorbed to the external surface of the cell membrane (Figure 3a), in accordance with observations from other groups using different types of nanoparticles⁶³⁻⁶⁴. We discarded the use of DMSA-MNPs with this cell line, since this

type of nanoparticles showed very little interaction with Jurkat cells surface (unpublished data).

MNPs toxicity was analyzed on Jurkat cells using the PrestoBlue assay (Figure 3b). Cultured cells were exposed to different concentrations of APS-MNPs (ranging from 10 to 500 $\mu\text{g Fe/ml}$) for 24 h. At all tested concentrations viability was over 100% compared to untreated cells maintained at standard culture conditions, although no statistically significant differences were found. This result suggests a slight induction of T cell proliferation by MNPs, as described for other metal oxide nanoparticles⁶⁵.

Figure 3. Subcellular distribution, nanoparticle toxicity and cell uptake on Jurkat cells. (a) Representative confocal microscopy image of Jurkat cells incubated with APS-MNPs for 2 h (150 $\mu\text{g Fe/ml}$). Scale bar: 10 μm . (b) PrestoBlue viability assay of Jurkat cells incubated with various APS-MNP concentrations for 24 h. Data were normalized to untreated cells, and are shown as mean $\pm$ SD (3 independent experiment in triplicates). One-way ANOVA showed no statistically significant differences. (c) Quantification of cell-associated MNPs by ICP-OES after a 2-h incubation. The total amount of iron was divided by the number of cultures cells for each sample. Data are shown as mean $\pm$ SD (n = 3). One-way ANOVA showed no statistically significant differences.

We also characterized the uptake of APS-MNPs by Jurkat cells. Previous experiments in our group indicate that this interaction occurs in the first 2 h of incubation, so we exposed cultured Jurkat cells to different concentrations of APS-MNPs (ranging from 50 to 200 $\mu\text{g Fe/ml}$) during that period of time, and next we analyzed the samples by ICP-OES. The average amount of MNPs associated with Jurkat cells increased up to ~ 10 pg Fe/cell, as a function of the initial nanoparticle concentration. However, no statistical significance was found (Figure 3c). For subsequent experiments on Jurkat cells a concentration of 150 $\mu\text{g Fe/ml}$ was selected.

In order to investigate the efficiency of APS-MNPs as inductors of cell membrane magnetic hyperthermia under an AMF, we incubated Jurkat cells with MNPs for 2 h. MNP-loaded Jurkat cells were exposed to an AMF (250 kHz, 25 kA/m) for 1 h, with constant temperature of the culture medium (37°C). As controls, we used untreated cells incubated at 37°C without CO_2 or relative humidity control (EC). After treatment, cells were stained with annexin V and PI, followed by flow cytometry analysis (Figure 4a). A toxic effect of APS-MNPs alone was evidenced in Jurkat cells, since the percentage of early and late apoptotic cells was significantly higher than that observed for cells non-exposed to MNPs, regardless AMF application.

TEM images confirmed the presence of APS-MNPs predominantly on the plasmatic membrane (Figure 4b), as previously observed by confocal microscopy (see Figure 3a). We observed areas of membrane disruption after MFH treatment. However, some control cells presented similar alterations, probably due to rough handling of the samples, making it rather difficult to draw conclusions from this experiment.

Jurkat cells response to heat was evaluated by HSP70-1B expression analysis (Figure 4c). As a control we used cells incubated at 42°C in a water bath for 1 h. AMF application did not produce a significant effect on expression levels compared to other experimental conditions. Taken together, these results indicate that the combination of MNPs and AMF did not exert a hyperthermic effect on Jurkat cells. Therefore, the increased cell death showed by the annexin V/PI assay and the disruption of cell

membrane observed in TEM images may be due to thermal-independent factors. One of the possibilities is the generation of mechanical effects that compromise the integrity of the plasmatic membrane, leading to programmed cell death as observed in other studies^{34, 66}.

Figure 4. Effects of AMF application on MNP-loaded Jurkat cells. Jurkat cells were analyzed after incubation with APS-MNPs for 2 h (150 μ g Fe/ml) and exposed to AMF (AMF), or were maintained at 37°C without CO₂ and relative humidity control (EC) for 1 h. For each culture condition cell unexposed to MNPs were used as negative controls (-). The analyses were carried out on adhered and suspended cells. **(a)** Cell death percentage was analyzed by flow cytometry after staining with Annexin V and PI. Data are shown as mean $\pm$ SD (n = 3). Two-way ANOVA followed by 'L' vs. 'APS' contrast, *p < 0.05, **p < 0.01, ***p < 0.001. **(b)** Representative TEM images of MNP-loaded Pan02 cells. Images on the right are magnifications of the boxed areas in the images on the left. Membrane disruptions can be observed (arrowheads). Scale bars: 1 μ m (left), 100 nm (right). **(c)** Hsp70 expression was analyzed by RT-qPCR. Cells incubated at standard culture conditions (SC) or at 42°C were used as negative and positive controls respectively. Data were normalized using the $2^{-\Delta\Delta Ct}$ method and are shown as mean $\pm$ SD (n = 3). Two-way ANOVA showed no statistically significant differences.

Influence of nanoparticle core size on MHT

Although other groups have observed cell death induction in MNP-loaded cells exposed to an AMF without an observable temperature increase^{26-31, 38}, we did not obtain the same results under our experimental conditions. This might be due to the differences in the temperature reached in the sample vessel in each AMF generator, due to their particular designs of coils, cooling systems and electronics beneath temperature control, so in the following experiments we eliminated temperature limitation in the sample vessel. Besides, we included different core-sized APS-MNPs. Large nanoparticles are usually preferred for hyperthermia studies because they show higher SAR values than smaller nanoparticles⁶⁷. However, there is evidence that the use of smaller particles may be more efficient for MHT in biosystems⁶⁸. A possible explanation for these observations is based on the mechanisms involved in thermal induction mediated by MNPs. SPIONs may transform the energy of the applied AMF by means of two types of relaxation. Néel relaxation occurs when the reorientation of the

magnetic moment occurs while the particle is immobile, while in Brown relaxation the particle itself rotates at the same time that its magnetic moment is reoriented. For nanoparticles above a critical size, Brown relaxation predominates, but in smaller particles the main mechanism of relaxation is that of Néel¹⁴. Bearing in mind that Brown relaxation is affected by the viscosity of the medium, which is higher inside the cells, smaller particles might be more efficient for MHT. For this reason, we synthesized and tested APS-MNPs of three different core sizes: 6, 8 and 14 nm (Figure 5a). Physicochemical characteristics of all nanoparticle samples are summarized in Table 2.

Table 2. Main physicochemical characteristics of 6, 8, and 14 nm core-sized APS-MNPs.

MNP	Core size (nm)	% (wt.) coating	Hydrodynamic diameter (nm)	ζ-potential pH 7 (mV)
APS-6 nm-MNPs	6.3 ± 1	4.9	49 (0.20)	+30
APS-8 nm-MNPs	8.2 ± 1	5.5	54 (0.15)	+33
APS-14 nm-MNPs	13.7 ± 2	3.5	62 (0.19)	+32

Note: Hydrodynamic diameter and polydispersity index are represented as Z-average number in intensity and standard deviation/mean size.

It is important to note that the percentage of coating for APS-14 nm-MNPs is now higher than in previous experiments, providing increased stability and reduced interaction between magnetic cores, thus reducing the hydrodynamic diameter (see Table 1).

When an AMF (250 kHz, 25 kA/m) was applied to 10 mg/ml MNP suspensions in water, we observed an increase in temperature of 1.9, 2.8 and 4.1°C over the temperature of the sample vessel (31°C) in the AMF generator device (Figure 5b). Under these conditions, SAR values for 6, 8 and 14 nm core-sized APS-MNPs were 9.0 ± 3.9, 8.3 ± 4.1 and 12.7 ± 4.4 W/g Fe respectively (mean ± SD, n ≥ 6). According to previous studies⁴⁸, 6 and 8 nm core-sized APS-MNPs present Néel relaxation predominantly, while in APS-14 nm-MNPs both relaxation modalities contribute to heat dissipation.

The analysis of nanoparticle toxicity on Pan02 cells (MNP concentration ranging from 0 to 500 µg Fe/ml, incubated overnight) showed no significant reduction of cell viability compared to untreated cells for all APS-MNP samples (Figure 5c), although at the maximum concentration APS-6 nm-MNPs decreased cell viability to 83%. In this experiment, we did not observe the non-significant reduction of viability showed previously for 14 nm core-sized nanoparticles (Figure 1c), probably due to the improvement in the percentage of coating achieved in this sample. For further characterization of MNP effects on Pan02 cells, we chose a concentration of 250 µg Fe/ml, the maximum dose of particles that induced no effects on cell viability.

The average amount of MNPs internalized by Pan02 cells after overnight was 18.54, 32.50 and 49.70 pg Fe/cell for 6, 8 and 14 nm core-sized APS-MNPs respectively, as determined by ICP-OES (Figure 5d). An important aspect observed in the internalization tests was the high variability of the results depending on the day in which the test was performed. This fact has been described by other groups⁶⁹⁻⁷⁰, and it is hypothesized to be caused by changes in the endocytic capacity of cells as a function of the increase in passage number⁷¹.

Figure 5. Characterization, toxicity and cell uptake of different core-sized MNPs on Pan02 cells. (a) Representative TEM images of 6, 8 or 14 nm core-sized APS-MNPs. Scale bar: 50 nm. (b) Heating curves of 6, 8 or 14 nm core-sized APS-MNPs suspensions (10 mg Fe/ml) in water (orange line) under an AMF (250 kHz, 25 kA/m (Vpp)). The coil was maintained at 31°C (black line). Data are shown as mean $\pm$ SD ($n \geq 6$). (c) PrestoBlue viability assay of Pan02 cells incubated overnight with 6, 8 or 14 nm core-sized APS-MNPs at different concentrations. Data were normalized to -/SC cells, and are shown as mean $\pm$ SD ($n = 4-5$). One-way ANOVA followed by Bonferroni multiple comparisons, * $p < 0.05$. (d) Quantification of cell-associated MNPs by ICP-OES. The total amount of iron was divided by the number of cultures cells for each sample. Colors indicate different MNP batches, while symbols indicate independent experiments performed on different dates. Dose: 250 $\mu\text{g Fe/ml}$. Data are shown as mean $\pm$ SD ($n \geq 8$). One-way ANOVA followed by Bonferroni multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Once we characterized all APS-MNP samples and their cellular uptake, we proceeded with the MHT tests. Pan02 were incubated overnight with 6, 8 or 14 nm core-sized APS-MNPs, we subjected them to an AMF (250 kHz, 25 kA/m) for 1 h, and we used PrestoBlue to analyze cell viability at different time points after treatment (1, 2, 3, 4 or 24 h) in order to better characterize cell death kinetics. As controls, we incubated Pan02 cells, with and without APS-MNPs in water bath at 37°C, with no CO₂ supply or

relative humidity control, in similar conditions to the sample vessel in the V3 device. The combination of AMF with all APS-MNPs types significantly reduced cell viability compared to controls, but this effect was observable at different time points post-treatment (Figure 6): 24 h for APS-6 nm-MNPs and APS-8 nm-MNPs, and 3 h for APS-14 nm-MNPs. Although previous experiments did not show a significant decrease in cell viability at a dose of 250 $\mu\text{g Fe/ml}$ (Figure 5c), here we observed a toxic effect of the three types of APS-MNPs alone on Pan02 cells, probably due to the absence of standard culture conditions, which might affect cell viability in a synergistic manner with APS-MNPs. AMF application had also a negative effect on viability, regardless of the presence of APS-MNPs, contrary to what has been observed in other studies using fields of similar intensity and frequency^{27-28, 57}. Statistical significances between treatment groups are summarized in Table 3.

Figure 6. Cell viability after AMF application to MNP-loaded Pan02 cells. Pan02 cells were analyzed after incubation overnight with 6, 8 or 14 core-sized APS-MNPs (250 $\mu\text{g Fe/ml}$) and exposed to AMF (AMF), or were maintained at 37°C in a water bath without CO₂ and relative humidity control (W) for 1 h. For each culture condition cell unexposed to MNPs were used as negative controls (-). PrestoBlue viability assay was performed 1, 2, 3, 4 and 24 h after treatment. Data were normalized to -/SC cells, and are shown as mean $\pm$ SD ($n \geq 6$). Statistical significances are summarized in Table 3.

Table 3. Statistical analysis of Pan02 cell viability assay after AMF application (Figure 6).

APS-6 nm-MNP		Time post-treatment (h)				
		1	2	3	4	24
Factorial ANOVA	Exposed to MNPs	—	*	*	**	***
	Exposed to AMF	—	*	*	**	***
	Combined MNPs + AMF	—	—	—	—	*
Contrast	' - ' vs. 'MNP'	—	*	*	**	***
	'W' vs. 'AMF'	—	*	*	**	***

APS-8 nm-MNP		Time post-treatment (h)				
		1	2	3	4	24
Factorial ANOVA	Exposed to MNPs	*	***	***	*	***
	Exposed to AMF	*	***	***	***	***
	Combined MNPs + AMF	—	—	—	—	***
Contrast	' - ' vs. 'MNP'	*	***	***	*	***
	'W' vs. 'AMF'	*	***	***	***	***

APS-14 nm-MNP		Time post-treatment (h)				
		1	2	3	4	24
Factorial ANOVA	Exposed to MNPs	*	—	*	*	***
	Exposed to AMF	—	—	*	**	***
	Combined MNPs + AMF	—	—	*	*	***
Contrast	' - ' vs. 'MNP'	—	—	*	*	***
	'W' vs. 'AMF'	—	—	*	***	***

Note: First, a factorial ANOVA test was carried out with two factors: exposure to MNPs, and exposure to AMF. In case of finding significant differences in any of the main effects, the analysis continued with the following contrasts: contrast of differences on cell viability between samples exposed or not to MNPs (' - ' vs. 'MNP'), and contrast of differences on cell viability between samples exposed or not to AMF ('W' vs. 'AMF'). *p < 0.05, **p < 0.01, ***p < 0.001.

TEM images taken 1 h after treatment showed no alterations in cells unexposed to MNPs, regardless of AMF application (Figure 7a, b). In these cells, organelles were well conserved and cell and nuclear membranes were intact. Similar results were observed for cells exposed to 6, 8 or 14 nm core-sized APS-MNPs without AMF application (Figure 7c, e, g). MNPs were confined in closed endosomes/lysosomes distributed in the cytoplasm. Apart from that, we observed no structural changes in organelles or membranes. However, APS-MNP-loaded cells that were subjected to AMF showed characteristic features of the first phases of apoptosis (Figure 7d, f, h). Electron dense nuclei were observed due to the presence of condensed chromatin, numerous vacuoles distributed throughout the cytoplasm, and apoptotic bodies on the cell surface, as described by other researchers⁷⁰, although lysosomal membranes seemed to be unaltered, and MNPs remained highly compacted inside the lysosomal compartment. No cells with necrotic morphology were found, despite having been described in some previous similar studies on MHT⁶⁹. Results were the same for all different core-sized nanoparticles.

Figure 7. Representative TEM images of Pan02 cells 1 h after treatment. Pan02 cells were analyzed after incubation overnight without MNPs (a, b) with 6 nm core-sized APS-MNPs (c, d), 8 nm core-sized APS-MNPs (e, f) or 14 nm core-sized APS-MNPs (g, h). All MNP concentrations were 250 $\mu\text{g Fe/ml}$. After incubation cells were exposed to AMF (AMF, images b, d, f, h), or maintained at 37C in a water bath without CO_2 and relative humidity control for 1 h (W, images a, c, e, g). Arrowheads: orange - MNPs, green - vacuoles, blue - apoptotic bodies, red - condensed chromatin. Scale bars: 1 μm .

A caspase-3 activation assay confirmed apoptosis as the cell death modality observed after treatment. Cleaved caspase-3 was detected by Western blot at 1, 6 and 24 h after AMF application. As a positive control of caspase-3 activation we incubated Pan02 with 500 nM gemcitabine for 48 h ⁷², and as a hyperthermic caspase-3 activation control we used cells incubated in a water bath at 45°C for 30 min. No effect was observed at 1 h post-treatment, while at later time points only samples exposed to APS-MNPs and AMF showed caspase-3 activation (Figure 8a), correlating with TEM images. Cleaved caspase-3 levels seemed to be higher in treated cells than in the hyperthermic positive control, indicating a stronger apoptotic response after MHT. These results were observed in 20% of the samples at 6 h post-treatment, and 100% of the samples after 24 h (not shown). As we can see, although a decrease of cell viability on APS-MNP-loaded cells was detected in the PrestoBlue assay after treatment (Figure 6, Table 3), this effect is not mediated by apoptosis, which indicates that reduced cell viability might be not due to cell death, but a decrease in metabolic and/or proliferative rates.

Changes in lysosomal permeability were tested using AO. All treated cells and controls were stained with AO and analyzed by flow cytometry. Three different core-sized nanoparticles, regardless of AMF application, showed a significant decrease in red and green fluorescence intensity compared to cells unexposed to MNPs (Figure 8b), as previously observed for APS-coated nanoparticles (Figure 2b). When APS-MNP-loaded cells were exposed to AMF, the reduction in fluorescence intensity was even more pronounced, indicating higher lysosome and membrane permeabilities.

Figure 8. Effects of AMF application on Pan02 cells loaded with different core-sized APS-MNPs. Pan02 cells were analyzed after incubation overnight with 6, 8 or 14 core-sized APS-MNPs (250 μ g Fe/ml) and exposed to AMF (AMF), or were maintained at 37°C in a water bath without CO₂ and relative humidity control (W) for 1 h. For each culture condition cell unexposed to MNPs were used as negative controls (-). (a) Western blot analysis of cell lysates (1 h post-treatment). Gemcitabine-treated cells (G; 500 nM, 48 h) or cells incubated at 45°C (30 min) were used as positive controls. Cleaved caspase-3 was detected as an index of caspase-3 activation, and β -actin was used as a loading control. (b) Lysosomal permeability was assessed by flow cytometry after staining with acridine orange (0-2 h post-treatment). PEI-treated cells (100 μ g/ml) were used as a positive control. Data were normalized to -/W cells, and are shown as mean $\pm$ SD ($n \geq 6$). One-way ANOVA followed by Tamhane multiple comparisons, or two-way ANOVA followed by 'W' vs. 'MNP' contrast, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. U: undetermined. (c) ROS generation analysis was determined by flow cytometry after incubation with the H₂DCFDA fluorescent probe (0-2 h post-treatment). H₂O₂-treated cells were used as a positive control (1 mM, 30 min). Data were normalized to -/W cells, and are shown as mean $\pm$ SD ($n = 3$). Student's t test (-/W vs. H₂O₂), or two-way ANOVA, * $p < 0.05$. (d) Hsp70 expression was analyzed by RT-qPCR (1 h post-treatment). Cells incubated at 42°C were used as a positive control. Data were normalized using the $2^{-\Delta\Delta C_t}$ method and are shown as mean $\pm$ SD ($n = 3$). Two-way ANOVA followed by 'W' vs. 'MNP' contrast, * $p < 0.05$.

Changes in intracellular ROS levels were analyzed after using the fluorescent probe H₂DCFDA. As a positive control we use cells treated with 1 mM H₂O₂ for 2 h. No significant differences were found in cells incubated with 6, 8 or 14 nm core-sized APS-MNPs, independently of AMF application, compared to untreated controls, although the combination of APS-MNP and AMF showed a tendency to increase ROS levels (Figure 8c).

Finally, we analyzed Hsp70 expression in Pan02 cells by RT-qPCR (Figure 8d). Incubation with 6, 8 or 14 nm core-sized nanoparticles did not induce any effect on Hsp70 expression compared to untreated cells, while the application of AMF on Pan02 cells loaded with any of the three types of APS-MNPs led to higher Hsp70 expression levels compared to cells unexposed to AMF. However, AMF application to cells without MNPs produced a similar effect. This observation, together with the fact that the three

types of nanoparticles showed similar results in all the tests carried out, regardless of their SAR or their relaxation mechanism, led us to consider the presence of a so far unnoticed external heat source. To determine if this was the case, we applied the AMF for 1 h to a culture dish containing 2 ml of complete culture medium. In a first analysis the coil of the V3 device was refrigerated, and the temperature was constantly maintained at 37°C (as in the experiments described in the first two sections), while in the second case temperature control was removed (as in the experiments in this third section). The measurement of the medium temperature at the end of the AMF application was 37°C in the first case, and 43.5°C in the second, which suggests that coil heating was responsible for the increase in the medium temperature, as observed in other studies⁷⁰, and might also be responsible for many of the effects observed in this section, although not all of them can be explained by this external heat source. For instance, data from cells exposed to AMF and any of the APS-MNPs types compared to those obtained from cells exposed to AMF without MNPs showed clear differences in cell viability, morphology, caspase-3 activation, and lysosomal permeability, although all these groups were subjected to the same external heat source. Besides, we know that the interaction of AMF and APS-MNPs is not responsible of these results by itself, as we observed in the experiments performed with temperature control at 37°C in previous sections. Therefore, there seems to be a synergistic effect between the application of an AMF to cells containing magnetic nanoparticles, and the external heating of the samples.

Influence of cell-induced nanoparticle aggregation on MNP magnetic behavior

Our results did not show the hyperthermic effects described by other authors for similar particles and field conditions²⁶⁻³⁰, some of which even reported an increase in the effects mediated by MHT compared to external hyperthermia⁷³⁻⁷⁴. This suggests that the heating capacity of the MNPs used in this study was reduced when associated to cells compared to their performance in water. Although it has been described that the contact of MNPs with cells may affect their heating capacity, probably due to nanoparticle aggregation³⁹⁻⁴⁰, the mechanisms underlying this process have not been completely elucidated yet. Previous results of our group support the fact that the magnetic properties of MNPs are affected when in contact with cells due to increased viscosity of the environment, nanoparticle aggregation and dipolar interactions between particles⁴⁴ leading to a magnetic relaxation process that takes place over a wide range of frequencies. This result may explain the heating inefficiency of nanoparticles observed in our results regardless MNPs cell location.

In order to determine whether nanoparticles orientation during cellular aggregation could be the cause of this different magnetic behavior observed, MNP aggregates were isolated from lysosomes and orientation analyzed using bright and dark field transmission electron microscopy. This technique has been used to locate and identify crystalline nanoparticles in complex biological matrices⁷⁵. When APS-MNP aggregates from Pan02 lysosomes were analyzed in bright field mode, inorganic nanoparticles with high mass and crystallinity appear dark (Figure 9). In contrast, in dark field mode, the incident beam of electrons is tilted an angle that corresponds to the direction of the diffraction, and thus, inorganic nanoparticles with the same crystallographic orientation appear bright. It can be observed that bright spots with sizes similar to the particles are dispersed over the aggregate with random orientation. The formation of large oriented aggregates, chains or closed flux assemblies are not observed⁷⁶. Same results were obtained in the case of DMSA-MNP aggregates. These nanoparticles aggregation in a non-controlled way could be the cause of this different magnetic behavior before and after cell contact and could explain the inefficient response to de AMF.

The formation of large oriented aggregates, chains or closed flux assemblies are not observed, neither before⁷⁶ nor after applying the alternating magnetic field⁷⁷. Previous studies show that high-frequency hysteresis loops carried out on particles with sizes ranging from 6 to 350 nm present a susceptibility χ that increases for large particles

(>20 nm) but it is almost field independent for the smaller ones. This suggests that the applied field induces chain ordering in large particles but not in the smaller ones due to the competition between thermal and dipolar energy.

Figure 9. Electron diffraction patterns assigned to a spinel iron oxide structure (on the left), bright field images (central) and dark field images obtained by tilting the electron beam an angle that corresponds to the diffraction direction (on the right), marked with a yellow ring in the diffraction pattern.

CONCLUSIONS

As we have seen, when we analyzed our particles as mediators of cellular MHT, we did not observe the hyperthermic effects described by other authors²⁶⁻³⁰, some of which even reported an increase in the effects mediated by MHT compared to external hyperthermia⁷³⁻⁷⁴. Our results suggest that MNPs heating capacity was reduced when associated to cells compared to their performance in water, indicating that the contact of MNPs with cells may affect their heating capacity, probably due to nanoparticle aggregation. According to our results, MNP aggregation seems to be independent of the cell type, incubation time or nanoparticle coating, supporting the idea that contact with cells is sufficient to induce nanoparticle aggregation, as previously proposed³⁹⁻⁴⁴. MNP aggregation in a non-controlled way could be one of the causes of the different magnetic behavior observed in cell-induced aggregation, and could explain the inefficient response to de AMF and reduced heating capacity. Our results suggest that the implementation of biomedical applications based on magnetic hyperthermia will require first the development of new approaches for the coating of nanoparticles that avoid cell-induced aggregation, as for example the one recently proposed in Clauson et al.⁷⁸. In addition, in future studies where the possible biomedical application of intracellular hyperthermia is analyzed, the use of temperature-sensitive fluorescent nanoparticles with aggregation-induced emission⁷⁹ could be of great help.

MATERIALS AND METHODS

Iron oxide nanoparticles. Magnetite (Fe_3O_4) nanoparticles were synthesized by the coprecipitation method as previously described⁸⁰ with slight modifications in order to obtain different core sizes. To synthesize 6 nm-core sized nanoparticles 425 ml of an aqueous solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.09 mol) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.054 mol) were added to

75 ml of KOH (25%) at a rate of 40 ml/s. To produce 8 nm-core sized nanoparticles we followed the same procedure but in this case NH₄OH (25%) was used as alkaline medium. 14 nm-core sized nanoparticles were obtained as described for 8 nm-cored sized particles, but at a addition rate of 0.2 ml/s, followed by heating at 90°C for 3 hours. After core synthesis, a standard protocol was used to oxidize magnetite to maghemite (γ -Fe₂O₃), activating the particle surface for coating⁴⁸. In order to obtain positively charged nanoparticles we added 1.22 ml of (3-aminopropyl)triethoxysilane (APS; 0.005 mol) to a mixture of 10 ml of nanoparticles (28 g Fe₂O₃/L) and 10 ml of methanol at a very slow rate (10 μ l/s), followed by vigorous stirring for 12 h. Then, methanol was evaporated using a rotary evaporator, and the mixture was purified by dialysis to remove unreacted APS and remaining traces of methanol. For negatively charged nanoparticles 5 mg of dimercaptosuccinic acid (DMSA; 0.027 mol) were added to a suspension of 10 ml of nanoparticles (4.3 g Fe₂O₃/L) at pH 3 with gentle stirring. Next, the pH of the mixture was raised to 11 by adding KOH, and sonicated for 15 min. Finally, nanoparticle suspension was dialyzed, and the pH adjusted to 7 with diluted HNO₃.

Nanoparticle characterization. Particle size and shape were determined by transmission electron microscopy (TEM). A drop of nanoparticle suspension was placed on a carbon-coated copper grid, and the solvent allowed to evaporate at room temperature (RT). Images were captured using a 100 keV JEOL-JEM 1010 microscope equipped with a Gatan Orius 200 SC digital camera. Mean particle size and distribution were evaluated by measuring the largest internal dimension of at least 300 particles with the software Image J (NIH, USA), followed by data fitting to a log-normal distribution to obtain the mean size and standard deviation (σ). The amount and composition of the coating were determined by Fourier transform infrared spectroscopy (FTIR). Samples were prepared by diluting iron oxide powder (2 wt. %) in KBr and compressing the mixture into a pellet, and spectra recorded between 4000-400 cm⁻¹ in a Bruker IFS 66 V-S spectrometer. Simultaneous thermogravimetric analysis (TG) and differential thermal analysis (DTA) were performed on a Seiko TG/TDA 320U device. For these analyses samples were heated from RT to 900°C at 10°C/min with a continuous air supply of 100 ml/min. Colloidal characterization was performed by dynamic light scattering (DLS) using a ZetaSizer Nano ZS (Malvern). Hydrodynamic size values were the result of three different measurements at different dilutions. ζ -potential was determined at 25°C, as a function of pH, using 0.01 M KNO₃ as the electrolyte. Iron concentration was measured with a Optima 2100 DV inductively coupled plasma optical emission spectrometer (ICP-OES; PerkinElmer).

Alternating magnetic field generator. A custom-made device (V3) was used for MHT experiments. This equipment, which has been described in detail elsewhere⁸¹, generates an AMF of 250 kHz, and a peak-to-peak voltage (V_{pp}) of 25 kA/m. The V3 device has a water flow cooling system in order to avoid Joule heating effect. The temperature of the samples was measured using a fiber optic sensor (PRB-G40, OSENSA Innovations) or a non-contact infrared temperature probe (MLX90614, Melexis).

Thermal characterization of nanoparticles. To analyze the heating efficiency of the nanoparticles, 150-250 μ l of 10 mg Fe/ml dispersion of nanoparticles in distilled water or 0.1% w/v agarose (Conda). Samples were placed in the hyperthermia device and the coil temperature was fixed at RT. The AMF was applied for 15 min, and the sample temperature was monitored every 0.5 s. SAR values were calculated according to the equation:

$$SAR \left(\frac{W}{g \text{ Fe}} \right) = \frac{C_{water}}{C_{Fe}} \times \frac{\Delta T}{\Delta t}$$

Where C_{water} is the calorific capacity of water (J/gK), c_{Fe} is the concentration of the magnetic material in the dispersion (g Fe/L), and $\Delta T/\Delta t$ is the initial slope of the heating curve.

Cell culture. The murine pancreatic adenocarcinoma Pan02 cell line (C57BL/6 background) was cultured in DMEM (Biowest) supplemented with 10% (v/v) FBS (Gibco Life sciences), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (all from Biowest). The human T-cell acute lymphoblastic leukemia Jurkat cell line was cultured in RPMI 1640 (Biowest) supplemented as described for Pan02 cells. Both cell lines were maintained in standard culture conditions (37°C, 5% CO₂ and 90% relative humidity).

Nanoparticle uptake and distribution. To determine nanoparticle uptake, cellular iron content after incubation with magnetic nanoparticles was quantified. Cell samples were collected, washed three times with PBS, and the number of cells counted using a Neubauer chamber. Next cells were centrifuged (300 rcf, 3 min), supernatant discarded and cell pellets digested at 90°C in 900 μ l of 65% (v/v) HNO₃ in water for 1 h, and 1 ml of 30% (w/v) H₂O₂ in water. Next, 8 ml of distilled water were added, and iron quantification determined by ICP-OES. Untreated cells (without nanoparticles) were also analyzed to assess the amount of endogenous iron. The amount of iron obtained from each sample was divided by the number of cells per sample. Nanoparticle distribution was analyzed by TEM. After incubation with nanoparticles, cells were washed three times with PBS, and then fixed in 2% glutaraldehyde and 1% tannic acid in HEPES (2 h, RT). Cells were rinsed with HEPES, post-fixed with 1% osmium tetroxide (1 h) and 2% uranyl acetate (30 min; both at 4°C), dehydrated with a series of acetone solutions and gradually infiltrated with Epon resin. The resin was allowed to polymerize (60°C, 48 h), and ultrathin sections (60–70 nm) were obtained with a diamond knife mounted on a Leica EM UC6 ultramicrotome. Sections were supported on a formvar/carbon-coated nickel grid and observed using a JEOL-1011 electron microscope (acceleration voltage 100 kV). Images were obtained with an Erlangshen ES1000W camera.

Cell viability. Cell viability was determined by the PrestoBlue assay (Invitrogen). 5×10^3 Pan02 cells or 10^5 Jurkat cells were cultured in 96-well plates (100 μ l complete medium) for 24 h. Then, various concentrations of MNPs in complete medium were added (0–1 mg Fe/ml; 100 μ l) in triplicate, to achieve final concentrations between 0 and 500 μ g/ml in a final volume of 200 μ l. Cells were incubated for 20 h, and 100 μ l of medium were aspirated from each well and replaced with 100 μ l PrestoBlue reagent (1:5 in complete medium). After 4 h, plates were centrifuged (1467 rcf, 15 min), 100 μ l of supernatant from each well were transferred to a new plate, and fluorescence was evaluated at different times in a TECAN Infinite 200 Pro fluorometer (560 nm excitation wavelength, 590 nm emission wavelength). Cell viability was expressed as the percentage of fluorescence of treated cells compared to untreated cells.

AMF application. For each MHT experiment cells were seeded in 35 mm dishes and incubated for 24 h. Then, MNPs were added (0.25 mg/ml) and incubated overnight. Cells without MNPs were used as controls. After washing three times with PBS and replacement of complete medium, plates were placed in the V3 device, and AMF (25 kA/m, 250 kHz) was applied for 1 h. Coil temperature was controlled throughout the AMF application. Additional dishes were maintained at standard culture conditions, or in water baths at different temperatures and used as controls.

Acridine orange assay. Lysosomal stability after treatment was analyzed using the fluorescent probe acridine orange (AO), which emits green fluorescence at low concentration (e.g. cytoplasm), while when entrapped in lysosomes at high concentration and acidic environment emits red fluorescence. After treatment, cells were incubated with 0.2 μ g/ml AO in complete culture medium for 15 min at standard cultured conditions, washed three times with PBS, and collected for fluorescence measurement by flow cytometry in the FL4 channel.

Analysis of ROS generation. In order to determine ROS accumulation within cells, we used 2',7'-dichlorodihydrofluorescein (H₂DCFDA), a non-fluorescent derivative of fluorescein that is known to convert to the highly fluorescent 2',7'-dichlorofluorescein upon cleavage of its acetate groups by esterases or oxidation. After treatment, cells were incubated with 0.05 mM H₂DCFDA in PBS for 15 min at standard culture conditions. As controls, we used cells treated with 1 mM H₂O₂ for 2 h. Next, cells were washed three times with PBS, and collected for fluorescence analysis by flow cytometry in the FL1 channel.

Hsp70 gene expression. The induction of Hspa1b expression was investigated by quantitative RT-PCR. Immediately and 1 h after hyperthermia treatment, total RNA was extracted from cells using the PureLink RNA Mini Kit (Applied Biosystems) following manufacturer's instructions. RNA concentration was determined by absorbance measurements at 260 and 280 nm in a NanoDrop 1000 spectrophotometer (Thermo Scientific) and 40 ng RNA/sample were converted into cDNA using a MultiScribe reverse transcription-based reaction kit (Applied Biosystems) in the presence of a RNase inhibitor (N8080119, Applied Biosystems) in a MyCycler thermocycler (Bio-Rad; 25°C-10 min, 37°C-2h, 85°C-5 min, 4°C ∞). For the RT-qPCR we used specific murine or human Hsp70 and β -actin primers, all from Sigma-Aldrich, summarized in Table 4.

Table 4. RT-qPCR primer list

Gene Symbol	Official Full Name	Gene ID	Species	Sequence
Hspa 1b	Heat shock protein 1B	15511	Mouse	F: AAACAGACTCTTTGCACTTG R: TAACAGTCAACGCAATTACC
HSPA 1B	Heat shock protein family A (Hsp70) member 1B	3304	Human	F: CCTATGTCATTTCTGGTTCAG R: TTAAAGGGAACGAAACACC
Actb	Actin, beta	11461	Mouse	F: GATGTATGAAGGCTTTGGTC R: TGTGCACTTTTATTGGTCTC
ACTB	Actin beta	60	Human	F: GACGACATGGAGAAAATCTG R: ATGATCTGGGTCATCTTCTC

The reaction was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems), in an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems; 95°C-15 s, 60°C-60 s, 40 cycles). Melting curves were generated in order to verify the specificity of the amplification (15 s, from 60°C to 95°C). β -actin was used as an endogenous control for gene expression. Data were acquired using the SDS 2.4 software, and analyzed with the Expression Suite 1.1 software (both from Applied Biosystems), according to the $2^{-\Delta\Delta C_t}$ method⁸².

Cell death analysis. FITC-Annexin V and propidium iodide (PI) double stain was used to detect cell death. Immediately (Pan02) or 24 h (Jurkat) after treatment, cells were harvested, centrifuged (300 rcf, 3 min), and resuspended in binding buffer (SouthernBiotech). Next, 10 μ l of FITC-Annexin V (SouthernBiotech) were added, and cells were incubated for 15 min at 4°C in the dark. Subsequently, 380 μ l of binding buffer were added to each tube and immediately before introducing the samples in the flow cytometer (FC500 MCL; Beckman Coulter), we added 10 μ l of PI (SouthernBiotech). The data acquired from 3 x 10⁴ Pan02 cells or 10⁵ Jurkat cells per sample were analyzed with the KALUZA Analysis 1.5a software (Beckman Coulter).

TEM was also used to determine the apoptotic or necrotic morphology of treated cells. Samples were prepared and images acquired as described nanoparticle distribution analysis.

Confocal microscopy. Jurkat cells were incubated with 150 $\mu\text{g Fe/ml}$ APS-MNPs for 2 h. Cell membranes were stained with wheat germ agglutinin (WGA) Alexa Fluor 594 conjugate (Life Technologies), following manufacturer's instructions. Images were taken in a Leica TCS SP5 confocal microscope (Leica Microsystems) with a 561-nm excitation laser, using a 63x/1.4 NA oil immersion objective.

Caspase-3 activation assay. At different time points after treatment (1, 6 and 24 h), treated cells and control cells (500 nM gemcitabine for 48 h) were lysed in lysis buffer (1% Triton-X 100, 50 mM Tris, 150 mM NaCl, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 2 nM okadaic acid) (45 min, 4°C). The lysate was clarified by centrifugation and 12 μg of total protein from each sample, determined with the Micro BCA protein assay kit (Pierce), was mixed with Laemmli sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β -mercaptoethanol, 0.02% bromophenol blue) and loaded on 12% SDS-polyacrylamide gels for electrophoresis. Proteins were transferred to a 0.2 μm -pore nitrocellulose membrane (BioRad), which was blocked with 10% w/v non-fat milk in Tris-buffered saline (TBS). For immunoblotting, we used specific primary antibodies for cleaved caspase-3 (Cell Signalling), and β -actin (Sigma). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako), protein bands were visualized using ECL Western Reagent (GE Healthcare) and developed on A-Plus Medical film (Konica Minolta).

Magnetic isolation of Pan02 cell's lysosomes and TEM study of the internalized MNPs. Pan02 cells were seeded at a density of 7×10^6 in a Petri dish (P-100, Falcon) and then allowed to adhere for 24 h. After this, magnetic nanoparticles were added at concentration of 125 $\mu\text{g Fe/mL}$ and incubated during 24 h at a temperature of 37°C. At that point, the majority of the MNPs were within the lysosomes. The non-internalized MNPs were washed away with PBS. The cells were harvested after incubation with trypsin for 3 min and centrifugation at 1500 rpm during 3 min at 4°C. Then, to magnetically isolate the lysosomes, the protocol of Bertoli *et al.*⁸³ was followed. Finally, the MNPs internalized in the lysosomes were observed using TEM

Statistical analysis. Statistical analysis was performed using the SPSS Statistics 24 software (IBM). We applied a two-tailed Student's *t* test for dichotomous variables. When comparing three or more categories, we used a one-way ANOVA for one factor, or a two-way ANOVA for multiple factors. For non-normal data or heteroscedasticity a Box-Cox transformation was applied. If heteroscedasticity was not solved, the nonparametric Welch and Brown-Forsythe alternatives were used. For *post hoc* multiple comparisons we used the Bonferroni correction in homoscedastic conditions, and the Tamhane method in heteroscedastic conditions. Data were considered significantly different at a value of $p < 0.05$ (*), and highly significant at $p < 0.01$ (**) or $p < 0.001$ (***).

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REFERENCES

- Colombo, M.; Carregal-Romero, S.; Casula, M. F.; Gutierrez, L.; Morales, M. P.; Bohm, I. B.; Heverhagen, J. T.; Prosperi, D.; Parak, W. J. Biological Applications of Magnetic Nanoparticles. *Chem. Soc. Rev.* **2012**, *41*, 4306-4334.
- Patil, R. M.; Thorat, N. D.; Shete, P. B.; Bedge, P. A.; Gavde, S.; Joshi, M. G.; Tofail, S. A. M.; Bohara, R. A. Comprehensive Cytotoxicity Studies of Superparamagnetic Iron Oxide Nanoparticles. *Biochem. Biophys. Rep.* **2018**, *13*, 63-72.
- Levy, M.; Luciani, N.; Alloyeau, D.; Elgrabli, D.; Deveaux, V.; Pechoux, C.; Chat, S.; Wang, G.; Vats, N.; Gendron, F.; Factor, C.; Lotersztajn, S.; Luciani, A.; Wilhelm, C.; Gazeau, F. Long Term in Vivo Biotransformation of Iron Oxide Nanoparticles. *Biomaterials* **2011**, *32*, 3988-3999.
- Mejias, R.; Gutierrez, L.; Salas, G.; Perez-Yague, S.; Zotes, T. M.; Lazaro, F. J.; Morales, M. P.; Barber, D. F. Long Term Biotransformation and Toxicity of Dimercaptosuccinic Acid-Coated Magnetic Nanoparticles Support their Use in Biomedical Applications. *J. Control. Release* **2013**, *171*, 225-233.
- Rojas, J. M.; Gavilan, H.; Del Dedo, V.; Lorente-Sorolla, E.; Sanz-Ortega, L.; da Silva, G. B.; Costo, R.; Perez-Yague, S.; Talelli, M.; Marciello, M.; Morales, M. P.; Barber, D. F.; Gutierrez, L. Time-Course Assessment of the Aggregation and Metabolization of Magnetic Nanoparticles. *Acta Biomater.* **2017**, *58*, 181-195.
- Mazuel, F.; Espinosa, A.; Luciani, N.; Refay, M.; Le Borgne, R.; Motte, L.; Desboeufs, K.; Michel, A.; Pellegrino, T.; Lalatonne, Y.; Wilhelm, C. Massive Intracellular Biodegradation of Iron Oxide Nanoparticles Evidenced Magnetically at Single-Endosome and Tissue Levels. *ACS Nano* **2016**, *10*, 7627-7638.
- Anselmo, A. C.; Mitragotri, S. Nanoparticles in the Clinic. *Bioeng. Transl. Med.* **2016**, *1*, 10-29.
- Roti Roti, J. L. Cellular Responses to Hyperthermia (40-46 degrees C): Cell Killing and Molecular Events. *Int. J. Hyperthermia* **2008**, *24*, 3-15.
- Oei, A. L.; Vriend, L. E.; Crezee, J.; Franken, N. A.; Krawczyk, P. M. Effects of Hyperthermia on DNA Repair Pathways: One Treatment to Inhibit them All. *Radiat. Oncol.* **2015**, *10*, 165.
- Skitzki, J. J.; Repasky, E. A.; Evans, S. S. Hyperthermia as an Immunotherapy Strategy for Cancer. *Curr. Opin. Investig. Drugs* **2009**, *10*, 550-558.
- Rao, W.; Deng, Z. S.; Liu, J. A Review of Hyperthermia Combined with Radiotherapy/Chemotherapy on Malignant Tumors. *Crit. Rev. Biomed. Eng.* **2010**, *38*, 101-116.
- van der Zee, J. Heating the Patient: a Promising Approach? *Ann. Oncol.* **2002**, *13*, 1173-1184.
- Burke, A. R.; Singh, R. N.; Carroll, D. L.; Wood, J. C.; D'Agostino, R. B., Jr.; Ajayan, P. M.; Torti, F. M.; Torti, S. V. The Resistance of Breast Cancer Stem Cells to Conventional Hyperthermia and their Sensitivity to Nanoparticle-Mediated Photothermal therapy. *Biomaterials* **2012**, *33*, 2961-2970.
- Rosensweig, R. E. Heating Magnetic Fluid with Alternating Magnetic Field. *J. Magn. Magn. Mater.* **2002**, *252*, 370-374.
- Goya, G. F.; Asin, L.; Ibarra, M. R. Cell Death Induced by AC Magnetic Fields and Magnetic Nanoparticles: Current State and Perspectives. *Int. J. Hyperthermia* **2013**, *29*, 810-818.

16. Araya, T.; Kasahara, K.; Nishikawa, S.; Kimura, H.; Sone, T.; Nagae, H.; Ikehata, Y.; Nagano, I.; Fujimura, M. Antitumor Effects of Inductive Hyperthermia Using Magnetic Ferucarbotran Nanoparticles on Human Lung Cancer Xenografts in Nude Mice. *Onco. Targets Ther.* **2013**, *6*, 237-242.
17. Balivada, S.; Rachakatla, R. S.; Wang, H.; Samarakoon, T. N.; Dani, R. K.; Pyle, M.; Kroh, F. O.; Walker, B.; Leaym, X.; Koper, O. B.; Tamura, M.; Chikan, V.; Bossmann, S. H.; Troyer, D. L. A/C Magnetic Hyperthermia of Melanoma Mediated by Iron(0)/Iron Oxide Core/Shell Magnetic Nanoparticles: a Mouse Study. *BMC Cancer* **2010**, *10*, 119.
18. Du, L.; Zhou, J.; Wang, X.; Sheng, L.; Wang, G.; Xie, X.; Xu, G.; Zhao, L.; Liao, Y.; Tang, J. Effect of Local Hyperthermia Induced by Nanometer Magnetic Fluid on the Rabbit VX2 Liver Tumor Model. *Prog. Nat. Sci.* **2009**, *19*, 1705-1712.
19. Jordan, A.; Scholz, R.; Maier-Hauff, K.; van Landeghem, F. K.; Waldoefner, N.; Teichgraeber, U.; Pinkernelle, J.; Bruhn, H.; Neumann, F.; Thiesen, B.; von Deimling, A.; Felix, R. The Effect of Thermotherapy Using Magnetic Nanoparticles on Rat Malignant Glioma. *J. Neurooncol.* **2006**, *78*, 7-14.
20. Kossatz, S.; Ludwig, R.; Dahring, H.; Ettelt, V.; Rimkus, G.; Marciello, M.; Salas, G.; Patel, V.; Teran, F. J.; Hilger, I. High Therapeutic Efficiency of Magnetic Hyperthermia in Xenograft Models Achieved with Moderate Temperature Dosages in the Tumor Area. *Pharm. Res.* **2014**, *31*, 3274-3288.
21. Johannsen, M.; Gneveckow, U.; Taymoorian, K.; Thiesen, B.; Waldoefner, N.; Scholz, R.; Jung, K.; Jordan, A.; Wust, P.; Loening, S. A. Morbidity and Quality of Life During Thermotherapy Using Magnetic Nanoparticles in Locally Recurrent Prostate Cancer: Results of a Prospective Phase I Trial. *Int. J. Hyperthermia* **2007**, *23*, 315-323.
22. Johannsen, M.; Gneveckow, U.; Thiesen, B.; Taymoorian, K.; Cho, C. H.; Waldoefner, N.; Scholz, R.; Jordan, A.; Loening, S. A.; Wust, P. Thermotherapy of Prostate Cancer Using Magnetic Nanoparticles: Feasibility, Imaging, and Three-Dimensional Temperature Distribution. *Eur. Urol.* **2007**, *52*, 1653-1661.
23. Maier-Hauff, K.; Rothe, R.; Scholz, R.; Gneveckow, U.; Wust, P.; Thiesen, B.; Feussner, A.; von Deimling, A.; Waldoefner, N.; Felix, R.; Jordan, A. Intracranial Thermotherapy Using Magnetic Nanoparticles Combined with External Beam Radiotherapy: Results of a Feasibility Study on Patients with Glioblastoma Multiforme. *J. Neurooncol.* **2007**, *81*, 53-60.
24. Wust, P.; Gneveckow, U.; Johannsen, M.; Bohmer, D.; Henkel, T.; Kahmann, F.; Sehouli, J.; Felix, R.; Rieke, J.; Jordan, A. Magnetic Nanoparticles for Interstitial Thermotherapy--Feasibility, Tolerance and Achieved Temperatures. *Int. J. Hyperthermia* **2006**, *22*, 673-685.
25. Maier-Hauff, K.; Ulrich, F.; Nestler, D.; Niehoff, H.; Wust, P.; Thiesen, B.; Orawa, H.; Budach, V.; Jordan, A. Efficacy and Safety of Intratumoral Thermotherapy Using Magnetic Iron-Oxide Nanoparticles Combined with External Beam Radiotherapy on Patients with Recurrent Glioblastoma Multiforme. *J. Neurooncol.* **2011**, *103*, 317-324.
26. Asin, L.; Goya, G. F.; Tres, A.; Ibarra, M. R. Induced Cell Toxicity Originates Dendritic Cell Death Following Magnetic Hyperthermia Treatment. *Cell Death Dis.* **2013**, *4*, e596.
27. Creixell, M.; Bohorquez, A. C.; Torres-Lugo, M.; Rinaldi, C. EGFR-Targeted Magnetic Nanoparticle Heaters Kill Cancer Cells Without a Perceptible Temperature Rise. *ACS Nano* **2011**, *5*, 7124-7129.

28. Marcos-Campos, I.; Asin, L.; Torres, T. E.; Marquina, C.; Tres, A.; Ibarra, M. R.; Goya, G. F. Cell Death Induced by the Application of Alternating Magnetic Fields to Nanoparticle-Loaded Dendritic Cells. *Nanotechnology* **2011**, *22*, 205101.
29. Rodriguez-Luccioni, H. L.; Latorre-Esteves, M.; Mendez-Vega, J.; Soto, O.; Rodriguez, A. R.; Rinaldi, C.; Torres-Lugo, M. Enhanced Reduction in Cell Viability by Hyperthermia Induced by Magnetic Nanoparticles. *Int. J. Nanomedicine* **2011**, *6*, 373-380.
30. Villanueva, A.; de la Presa, P.; Alonso, J. M.; Rueda, T.; Martínez, A.; Crespo, P.; Morales, M. P.; González-Fernández, M. A.; Valdés, J.; Rivero, G. Hyperthermia HeLa Cell Treatment with Silica-Coated Manganese Oxide Nanoparticles. *J. Phys. Chem.* **2010**, *114*, 1976-1981.
31. Domenech, M.; Marrero-Berrios, I.; Torres-Lugo, M.; Rinaldi, C. Lysosomal Membrane Permeabilization by Targeted Magnetic Nanoparticles in Alternating Magnetic Fields. *ACS Nano* **2013**, *7*, 5091-5101.
32. Clerc, P.; Jeanjean, P.; Hallali, N.; Gougeon, M.; Pipy, B.; Carrey, J.; Fourmy, D.; Gigoux, V. Targeted Magnetic Intra-Lysosomal Hyperthermia Produces Lysosomal Reactive Oxygen Species and Causes Caspase-1 Dependent Cell Death. *J. Control. Release* **2018**, *270*, 120-134.
33. Connord, V.; Clerc, P.; Hallali, N.; El Hajj Diab, D.; Fourmy, D.; Gigoux, V.; Carrey, J. Real-Time Analysis of Magnetic Hyperthermia Experiments on Living Cells under a Confocal Microscope. *Small* **2015**, *11*, 2437-2445.
34. Master, A. M.; Williams, P. N.; Pothayee, N.; Pothayee, N.; Zhang, R.; Vishwasrao, H. M.; Golovin, Y. I.; Riffle, J. S.; Sokolsky, M.; Kabanov, A. V. Remote Actuation of Magnetic Nanoparticles for Cancer Cell Selective Treatment Through Cytoskeletal Disruption. *Sci. Rep.* **2016**, *6*, 33560.
35. Wang, B.; Yin, J. J.; Zhou, X.; Kurash, I.; Chai, Z.; Zhao, Y.; Feng, W. Physicochemical Origin for Free Radical Generation of Iron Oxide Nanoparticles in Biomicroenvironment: Catalytic Activities Mediated by Surface Chemical States. *J. Phys. Chem. C* **2013**, *117*, 383-392.
36. Pereira, M. C.; Oliveira, L. C. A.; Murad, E. Iron Oxide Catalysts: Fenton and Fenton-Like Reactions - a Review. *Clay Miner.* **2012**, *47*, 285-302.
37. Wydra, R. J.; Oliver, C. E.; Anderson, K. W.; Dziubla, T. D.; Hilt, J. Z. Accelerated Generation of Free Radicals by Iron Oxide Nanoparticles in the Presence of an Alternating Magnetic Field. *RSC Adv.* **2015**, *5*, 18888-18893.
38. Wydra, R. J.; Rychahou, P. G.; Evers, B. M.; Anderson, K. W.; Dziubla, T. D.; Hilt, J. Z., The role of ROS generation from magnetic nanoparticles in an alternating magnetic field on cytotoxicity. *Acta Biomater.* **2015**, *25*, 284-90.
39. Etheridge, M. L.; Hurley, K. R.; Zhang, J.; Jeon, S.; Ring, H. L.; Hogan, C.; Haynes, C. L.; Garwood, M.; Bischof, J. C. Accounting for Biological Aggregation in Heating and Imaging of Magnetic Nanoparticles. *Technology (Singap World Sci)* **2014**, *2*, 214-228.
40. Di Corato, R.; Espinosa, A.; Lartigue, L.; Tharaud, M.; Chat, S.; Pellegrino, T.; Menager, C.; Gazeau, F.; Wilhelm, C. Magnetic Hyperthermia Efficiency in the Cellular Environment for Different Nanoparticle Designs. *Biomaterials* **2014**, *35*, 6400-11.
41. Soukup, D.; Moise, S.; Cespedes, E.; Dobson, J.; Telling, N. D. In Situ Measurement of Magnetization Relaxation of Internalized Nanoparticles in Live Cells. *ACS Nano* **2015**, *9*, 231-240.

42. Moise, S.; Cespedes, E.; Soukup, D.; Byrne, J. M.; El Haj, A. J.; Telling, N. D. The Cellular Magnetic Response and Biocompatibility of Biogenic Zinc- and Cobalt-Doped Magnetite Nanoparticles. *Sci. Rep.* **2017**, *7*, 39922.
43. Cabrera, D.; Coene, A.; Leliaert, J.; Artes-Ibanez, E. J.; Dupre, L.; Telling, N. D.; Teran, F. J. Dynamical Magnetic Response of Iron Oxide Nanoparticles Inside Live Cells. *ACS Nano* **2018**, *12*, 2741-2752.
44. Fortes Brollo, M. E.; Hernandez Flores, P.; Gutierrez, L.; Johansson, C.; Barber, D. F.; Morales, M. D. P. Magnetic Properties of Nanoparticles as a Function of their Spatial Distribution on Liposomes and Cells. *Phys. Chem. Chem. Phys.* **2018**, *20*, 17829-17838.
45. Feng, Q.; Liu, Y.; Huang, J.; Chen, K.; Huang, J.; Xiao, K. Uptake, Distribution, Clearance, and Toxicity of Iron Oxide Nanoparticles with Different Sizes and Coatings. *Sci. Rep.* **2018**, *8*, 2082.
46. Sakulkhu, U.; Mahmoudi, M.; Maurizi, L.; Salaklang, J.; Hofmann, H. Protein Corona Composition of Superparamagnetic Iron Oxide Nanoparticles with Various Physico-Chemical Properties and Coatings. *Sci. Rep.* **2014**, *4*, 5020.
47. Yuan, Y.; Borca Tasciuc, D. A. Comparison Between Experimental and Predicted Specific Absorption Rate of Functionalized Iron Oxide Nanoparticle Suspensions. *J. Magn. Magn. Mater.* **2011**, *323*, 2463-3469.
48. de la Presa, P.; Luengo, Y.; Multigner, M.; Costo, R.; Morales, M. P.; Rivero, G.; Hernando, A. Study of Heating Efficiency as a Function of Concentration, Size, and Applied Field in γ -Fe₂O₃ Nanoparticles. *J. Phys. Chem.* **2012**, *116*, 25602-25610.
49. Frohlich, E. The Role of Surface Charge in Cellular Uptake and Cytotoxicity of Medical Nanoparticles. *Int. J. Nanomedicine* **2012**, *7*, 5577-5591.
50. Safi, M.; Courtois, J.; Seigneuret, M.; Conjeaud, H.; Berret, J. F. The Effects of Aggregation and Protein Corona on the Cellular Internalization of Iron Oxide Nanoparticles. *Biomaterials* **2011**, *32*, 9353-9363.
51. Calatayud, M. P.; Sanz, B.; Raffa, V.; Riggio, C.; Ibarra, M. R.; Goya, G. F. The Effect of Surface Charge of Functionalized Fe₃O₄ Nanoparticles on Protein Adsorption and Cell Uptake. *Biomaterials* **2014**, *35*, 6389-6399.
52. Cabrera, D.; Camarero, J.; Ortega, D.; Terán, F. J. Influence of the Aggregation, Concentration and Viscosity of the Nanomagnetism of Iron Oxide Nanoparticle Colloids for Magnetic Hyperthermia. *J. Nanopart. Res.* **2015**, *17*, 121.
53. Pierzynska-Mach, A.; Janowski, P. A.; Dobrucki, J. W. Evaluation of Acridine Orange, LysoTracker Red, and Quinacrine as Fluorescent Probes for Long-Term Tracking of Acidic Vesicles. *Cytometry A* **2014**, *85*, 729-737.
54. Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring Polyethylenimine-Mediated DNA Transfection and the Proton Sponge Hypothesis. *J. Gene Med.* **2005**, *7*, 657-663.
55. Zucker, R. M.; Massaro, E. J.; Sanders, K. M.; Degn, L. L.; Boyes, W. K. Detection of TiO₂ Nanoparticles in Cells by Flow Cytometry. *Cytometry A* **2010**, *77*, 677-685.
56. Leroueil, P. R.; Hong, S.; Mecke, A.; Baker, J. R., Jr.; Orr, B. G.; Banaszak Holl, M. M. Nanoparticle Interaction with Biological Membranes: Does Nanotechnology Present a Janus Face? *Acc. Chem. Res.* **2007**, *40*, 335-342.
57. Asin, L.; Ibarra, M. R.; Tres, A.; Goya, G. F. Controlled Cell Death by Magnetic Hyperthermia: Effects of Exposure Time, Field Amplitude, and Nanoparticle Concentration. *Pharm. Res.* **2012**, *29*, 1319-1327.

58. Sadhukha, T.; Niu, L.; Wiedmann, T. S.; Panyam, J. Effective Elimination of Cancer Stem Cells by Magnetic Hyperthermia. *Mol. Pharm.* **2013**, *10*, 1432-1441.
59. Mosser, D. D.; Caron, A. W.; Bourget, L.; Denis-Larose, C.; Massie, B. Role of the Human Heat Shock Protein hsp70 in Protection Against Stress-Induced Apoptosis. *Mol. Cell. Biol.* **1997**, *17*, 5317-5327.
60. Srivastava, P. Roles of Heat-Shock Proteins in Innate and Adaptive Immunity. *Nat. Rev. Immunol.* **2002**, *2*, 185-194.
61. Kobayashi, S.; Ohki, A.; Tanoue, M.; Inaoka, Y.; Murase, K. Comparative Study of Extracellular and Intracellular Magnetic Hyperthermia Treatments Using Magnetic Particle Imaging. *Open Journal of Applied Sciences* **2017**, *7*, 647-660.
62. Lunov, O.; Zablotskii, V.; Pastor, J. M.; Pérez-Landazábal, J. I.; Gómez-Polo, C.; Syrovets, T.; Simmet, T. Thermal Destruction on the Nanoscale: Cell Membrane Hyperthermia with Functionalized Magnetic Nanoparticles. *AIP Conf. Proc.* **2010**, *1311*, 288.
63. Lorenz, M. R.; Holzapfel, V.; Musyanovych, A.; Nothelfer, K.; Walther, P.; Frank, H.; Landfester, K.; Schrezenmeier, H.; Mailander, V. Uptake of Functionalized, Fluorescent-Labeled Polymeric Particles in Different Cell Lines and Stem Cells. *Biomaterials* **2006**, *27*, 2820-2828.
64. Sabuncu, A. C.; Grubbs, J.; Qian, S.; Abdel-Fattah, T. M.; Stacey, M. W.; Beskok, A. Probing Nanoparticle Interactions in Cell Culture Media. *Colloids Surf. B Biointerfaces* **2012**, *95*, 96-102.
65. Simón-Vázquez, R.; Lozano-Fernández, T.; Dávila-Grana, A.; González-Fernández, A. Metal Oxide Nanoparticles Interact with Immune Cells and Activate Different Cellular Responses. *Int. J. Nanomedicine* **2016**, *11*, 4657-4668.
66. Kim, D. H.; Rozhkova, E. A.; Ulasov, I. V.; Bader, S. D.; Rajh, T.; Lesniak, M. S.; Novosad, V. Biofunctionalized Magnetic-Vortex Microdisks for Targeted Cancer-Cell Destruction. *Nat. Mater.* **2010**, *9*, 165-171.
67. Tong, S.; Quinto, C. A.; Zhang, L.; Mohindra, P.; Bao, G. Size-Dependent Heating of Magnetic Iron Oxide Nanoparticles. *ACS Nano* **2017**, *11*, 6808-6816.
68. Wang, C.; Hsu, C. H.; Li, Z.; Hwang, L. P.; Lin, Y. C.; Chou, P. T.; Lin, Y. Y. Effective Heating of Magnetic Nanoparticle Aggregates for in Vivo Nano-Theranostic Hyperthermia. *Int. J. Nanomedicine* **2017**, *12*, 6273-6287.
69. Guardia, P.; Di Corato, R.; Lartigue, L.; Wilhelm, C.; Espinosa, A.; Garcia-Hernandez, M.; Gazeau, F.; Manna, L.; Pellegrino, T. Water-Soluble Iron Oxide Nanocubes with High Values of Specific Absorption Rate for Cancer Cell Hyperthermia Treatment. *ACS Nano* **2012**, *6*, 3080-3091.
70. Blanco-Andujar, C.; Ortega, D.; Southern, P.; Nesbitt, S. A.; Thanh, N. T.; Pankhurst, Q. A. Real-Time Tracking of Delayed-Onset Cellular Apoptosis Induced by Intracellular Magnetic Hyperthermia. *Nanomedicine (Lond)* **2016**, *11*, 121-136.
71. Yang, Y.; Wang, Q.; Song, L.; Liu, X.; Zhao, P.; Zhang, F.; Gu, N.; Sun, J. Uptake of Magnetic Nanoparticles for Adipose-Derived Stem Cells with Multiple Passage Numbers. *Sci. China Mater.* **2017**, *60*, 892-902.
72. Huang, P.; Plunkett, W. Induction of Apoptosis by Gemcitabine. *Semin. Oncol.* **1995**, *22* (4 Suppl 11), 19-25.
73. Sanz, B.; Calatayud, M. P.; Torres, T. E.; Fanarraga, M. L.; Ibarra, M. R.; Goya, G. F. Magnetic Hyperthermia Enhances Cell Toxicity with Respect to Exogenous Heating. *Biomaterials* **2017**, *114*, 62-70.

74. Ogden, J.; Tate, J.; Strawbridge, R.; Ivkov, R.; Hoopes, P. Comparison of Iron Oxide Nanoparticle and Waterbath Hyperthermia Cytotoxicity. *Proc. SPIE Int. Soc. Opt. Eng.* **2009**, *7181*, 71810K.
75. Klein, N. D.; Hurley, K. R.; Feng, Z. V.; Haynes, C. L. Dark Field Transmission Electron Microscopy as a Tool for Identifying Inorganic Nanoparticles in Biological Matrices. *Anal. Chem.* **2015**, *87*, 4356-4362.
76. Wei, A.; Kasama, T.; Dunin-Borkowski, R. E. Self-Assembly and Flux Closure Studies of Magnetic Nanoparticle Rings. *Journal of Materials Chemistry* **2011**, *21*, 16686-16693.
77. Morales, I.; Costo, R.; Mille, N.; Silva, G. B. D.; Carrey, J.; Hernando, A.; Presa, P. High Frequency Hysteresis Losses on gamma-Fe(2)O(3) and Fe(3)O(4): Susceptibility as a Magnetic Stamp for Chain Formation. *Nanomaterials (Basel)* **2018**, *8*, 970.
78. Clauson, R. M.; Chen, M.; Scheetz, L. M.; Berg, B.; Chertok, B. Size-Controlled Iron Oxide Nanoplatfoms with Lipidoid-Stabilized Shells for Efficient Magnetic Resonance Imaging-Trackable Lymph Node Targeting and High-Capacity Biomolecule Display. *ACS Appl. Mater. Interfaces* **2018**, *10*, 20281-20295.
79. Wang, Z.; Yong, T. Y.; Wan, J.; Li, Z. H.; Zhao, H.; Zhao, Y.; Gan, L.; Yang, X. L.; Xu, H. B.; Zhang, C. Temperature-Sensitive Fluorescent Organic Nanoparticles with Aggregation-Induced Emission for Long-Term Cellular Tracing. *ACS Appl. Mater. Interfaces* **2015**, *7*, 3420-3425.
80. Luengo, Y.; Nardecchia, S.; Morales, M. P.; Serrano, M. C. Different Cell Responses Induced by Exposure to Maghemite Nanoparticles. *Nanoscale* **2013**, *5*, 11428-11437.
81. Barber, D. F.; Mejías, R.; Tajada-Herráiz, J. L.; Pérez-Yagüe, S.; Talelli, M. Device for Generating Hyperthermia in Cells by Means of Magnetic Nanoparticles (MNPs), Method for Determining Temperature in Cells Using Said Device, and Use of the Device to Determine the Temperature in Cells. *Patent Pub. No. WO/2016/059283 April 21, 2016*.
82. Livak, K. J.; Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402-408.
83. Bertoli, F.; Davies, G. L.; Monopoli, M. P.; Moloney, M.; Gun'ko, Y. K.; Salvati, A.; Dawson, K. A. Magnetic Nanoparticles to Recover Cellular Organelles and Study the Time Resolved Nanoparticle-Cell Interactome Throughout Uptake. *Small* **2014**, *10*, 3307-3315.

Abstract Graphic