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## Magneto-mechanical destruction of cancer-associated fibroblasts using ultra-small iron oxide nanoparticles and low frequency rotating magnetic fields.

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#: equal participation



## ABSTRACT

The destruction of cells using the mechanical activation of magnetic nanoparticles by low-frequency magnetic fields constitutes a recent and interesting approach in cancer therapy. Here, we showed that superparamagnetic iron oxide nanoparticles as small as 6 nm were able to induce the death of pancreatic cancer-associated fibroblasts, chosen as a model. An exhaustive screening in the amplitude, frequency, and type (alternating vs rotating) of magnetic field demonstrated that the best efficacy was obtained for a rotating low-amplitude low-frequency magnetic field (1 Hz, 40 mT), reaching a 34% ratio in cell death induction; interestingly, cell death was not maximized for largest amplitudes of magnetic field. State-of-the-art kinetic Monte-Carlo simulations able to calculate the torque undergone by assemblies of magnetic nanoparticles explained these features and were in agreement with cell death experiments. Simulations showed that the force generated by the nanoparticles once internalized inside the lysosome was around 3 pN, which is in principle not large enough to induce direct membrane disruption. Other biological mechanisms were explored to explain cell death: the mechanical activation of magnetic nanoparticles induced the lysosome membrane permeabilization, the release of lysosome content and that cell death was mediated through a lysosomal pathway depending on Cathepsin-B activity. Finally, we showed that repeated rotating magnetic field exposure halted drastically the cell proliferation. This study established the proof-of-concept that ultra-small nanoparticles can disrupt tumor microenvironment through mechanical forces generated by mechanical activation of magnetic nanoparticles upon low-frequency rotating magnetic field exposure, opening new opportunities for cancer therapy.

**Keywords:** cancer, magnetic nanoparticles, targeted nanotherapy, rotating magnetic field, mechanical forces, lysosome



## Introduction

Iron oxide magnetic nanoparticles (IONPs), which display low toxicity and good biocompatibility in humans, are the subject of renewed interest in the perspective of cancer nanotherapy.<sup>1</sup> Over the past decades, a wide range of IONPs of controlled size, composition and functionality has been developed, providing an excellent resource for nanomedicine. Indeed, they have been already approved for clinical use as a contrast agent for magnetic resonance imaging and as iron supplement for the treatment of iron deficiency anemia.<sup>2-3</sup> Exposed to a high-frequency alternating magnetic field, IONPs release heat which can be used to develop biological approaches such as the eradication of cancer cells (magnetic hyperthermia),<sup>4-8</sup> the on-demand drug release,<sup>9</sup> the stimulation of neurons,<sup>10</sup> the induction of gene expression<sup>11</sup> or the disruption of extracellular matrix of the tumor microenvironment.<sup>12-13</sup> In particular, magnetic hyperthermia has been the subject of an active research activity in the past decades and tremendous progresses have been made in basic research on this promising cancer treatment.<sup>14</sup> Magnetic hyperthermia using IONPs under a high-frequency alternating magnetic field has distinctive benefits for tumor ablation in deep tissue because of the convenience of distant control and manipulation by the magnetic field. However, this approach, requiring magnetic fields with frequencies ranging from tens of kHz to MHz, generates eddy currents inside healthy tissues that may cause patient discomfort and adverse effects. But above all, applying high-frequency magnetic fields on a large scale (humans) remains a technical challenge, because of the strong heating of coils and magnetic cores under such magnetic fields.<sup>15</sup> Consequently, only few clinical trials have been performed applying a moderate frequency and amplitude of magnetic field (100 kHz, 20 mT) which caused several adverse effects due to heat diffusion to surrounding tissues and presented limiting factors such as the irregular intra-tumoral heat distribution.<sup>16-17</sup>

Recent reports highlighted new potentialities of IONPs for anti-cancer therapy.<sup>18</sup> Exposed to low-frequency magnetic fields, IONPs generate mechanical forces and torques, which are transmitted to the



materials they are in contact with. Such torques can be used to control the calcium entry within cells, improve gene transfection or tissue engineering, manipulate molecules, stimulate drug release, induce protein degradation, activate enzymes or destroy cancer cells.<sup>19-24</sup> The proof-of-concept of *in vitro* and *in vivo* targeting by IONPs of tumors overexpressing surface receptors could, moreover, allow it without any invasive intra-tumoral injections.<sup>25, 26</sup> This new approach could solve the problems related to magnetic hyperthermia mentioned above and would be highly beneficial to IONP-based anti-cancer therapy by offering a new opportunity to treat cancer. Several studies have already demonstrated that micrometric discs, spheres, or wires induce the death of cancer cells upon a low-frequency alternating (AMF) or rotating (RMF) magnetic fields.<sup>27-30</sup> So far, some studies have reported the death of cancer cells using IONPs exposed to AMF or RMF.<sup>31, 32-41</sup> But they did not explore stringently, theoretically as well as experimentally, for the optimal magnetic field parameters – e.g., type, amplitude, and frequency - able to induce torque leading to cell damages.

Several mechanisms protect a tumor against potential treatments. One of them is the tumoral microenvironment which creates a supportive scaffold for tumor cell proliferation and a barrier limiting the penetration and the dispersion of chemotherapeutic treatments into the tumor. Cancer-associated fibroblasts (CAFs) are key components of the tumor microenvironment, since they actively deposit extracellular matrix (ECM), by producing and secreting abundantly ECM proteins including types I and III collagen, that prevent the chemotherapeutic drugs from reaching their target.<sup>42</sup> Moreover, CAFs secrete factors in the extracellular medium (hormones, growth factors, chemokines...), promoting tumor growth and participating in the resistance acquisition of cancer cells to anti-cancer treatments such as in pancreatic cancer.<sup>43-44</sup> Among the precision nanotherapies aiming to deliver nanoparticles inside the tumor as a way of cancer control, the therapeutic targeting of CAFs using nano-agents constitute thus a new interesting strategy compared to main studies taking care of cancer cells.

In the present work, we develop a strategy targeting and killing CAFs by remote magneto-mechanical destruction. Interestingly, we show that it is possible to do it using nanoparticles as small as



6 nm, a puzzling result since the forces generated by such ultra-small MNPs is expected to be too small to disrupt cell membranes. We explain the origin of the observed effect and its magnetic field dependence using state-of-the-art magnetic simulations and shed light on cell death mechanisms. These data are very promising and show that ultra-small nanoparticles targeting lysosomes hold the potential for remote magneto-mechanical destruction of cells by disrupting their lysosomes, thus offering a new promising therapeutic strategy against tumors.

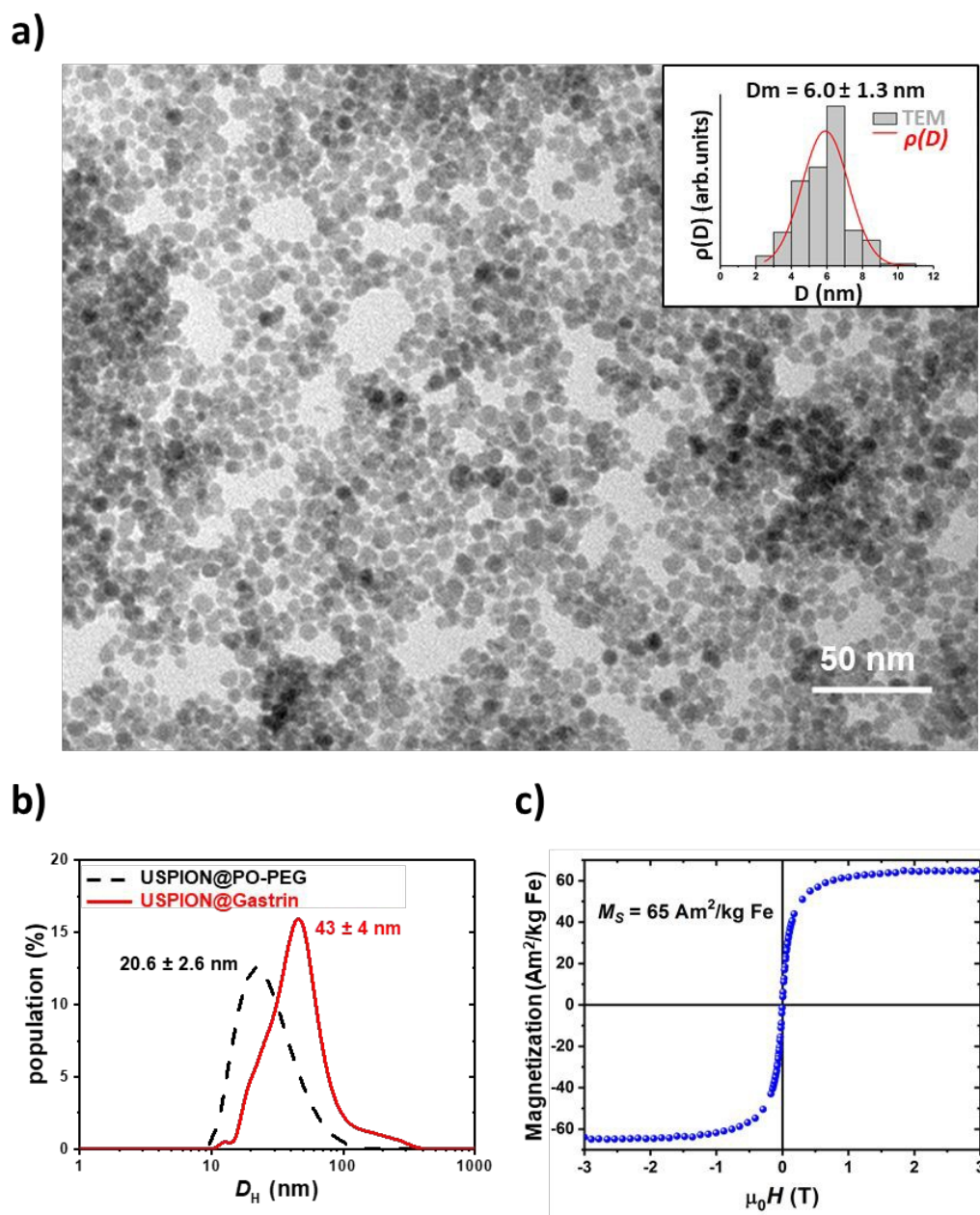
## Results

### USPION@Gastrin and specific CAF targeting

We first developed and characterized ultra-small superparamagnetic iron oxide nanoparticles (USPION) targeting pancreatic CAFs, chosen as a model. Then, we analyzed their uptake by pancreatic CAFs as well as their subcellular localization. 6 nm USPION were synthesized using microwave non aqueous sol-gel method.<sup>45</sup> As shown in previous studies, the USPIONs surface has to be functionalized in order to obtain bio-stability, biocompatibility and to allow specific targeting. Herein, the USPIONs surface was functionalized with phosphonate pegylated ligands bearing carboxylate functions as terminal group (PO-PEG-COOH). The phosphonate group shows high affinity for metal oxide surface whereas carboxylate groups could be used in a second step for covalent coupling of targeting molecules.<sup>26</sup> The magnetic core size, measured by TEM, equaled  $6.0 \pm 1.3$  nm (Figure 1a). The average number of PO-PEG-COOH per nanoparticle, evaluated using Fourier Transform Infrared Spectroscopy, was  $176 \pm 16$  (supplementary Figure S1). The hydrodynamic diameter in culture medium containing 0.5% FBS was found equal to  $20.6 \pm 2.6$  nm, indicating low aggregation (Figure 1b). As expected, 6 nm USPIONs exhibited a saturation magnetization of  $65.6 \pm 1.8$  Am<sup>2</sup>/kg Fe (Figure 1c).<sup>46</sup> 6 nm USPIONs were then decorated with a fluorophore (DY647), allowing their fluorescence emission detection, and with a synthetic peptide analog of gastrin, to selectively target pancreatic CAFs expressing the type 2 cholecystokinin receptor



(CCK2R). The analysis by DLS, in a biological medium enriched by 0.5% FBS, showed a distribution of the functionalized 6 nm USPIOs, called USPIO@Gastrin, of around  $43 \pm 4$  nm (Figure 1b). OPA assay indicates an average number of 26 peptides per nanoparticle using gastrin peptide calibration curve (Figure S2).



**Figure 1:** (a) TEM image of 6 nm USPIO@PO-PEG; insert) Particle size distribution deduced from TEM observations (bars), fit of the size distribution (line); (b) Hydrodynamic particle size distribution before (dashed black) and after functionalization with gastrin (full red) measured in volume by dynamic light scattering; (c) Hysteresis loop of 6 nm USPIO@PO-PEG measured at 300 K.





The cancer progression and aggressiveness are not only dictated by the tumor cells, but also involve the tumor microenvironment. In some types of cancer, the microenvironment may account for the largest portion of the tumor, such as in pancreatic adenocarcinoma whose stroma accounts for up to 80% of the tumor mass. Moreover, tumoral microenvironment constitutes a physical barrier limiting drug penetration and participates in the drug resistance acquisition by the tumor. Among the cells constituting the tumor microenvironment, CAFs deposit extracellular matrix and promote tumor growth and resistance acquisition of tumors. Here, we developed a nanotherapeutic strategy targeting the pro-tumoral CAFs of pancreatic adenocarcinoma, chosen as a model because pancreatic adenocarcinoma is a typical fibrotic tumor composed for up to 80% of its volume by a stiff stroma, and for which CAFs play a critical role in invasion and chemoresistance.<sup>44</sup> The type 2 cholecystokinin receptor, CCK2R, was showed to be expressed on cancer-associated fibroblasts (CAFs) in pancreatic adenocarcinoma. Moreover, its targeting, using an antagonist, inhibits the secretion of collagen, reduces fibrosis in the tumor microenvironment and halts the cancer progression, making the CCK2R a good candidate for pancreatic cancer pro-tumoral CAFs targeting.<sup>47-49</sup> The tumor microenvironment targeting strategy elaborated in this study requires a specific recognition of pancreatic CAFs expressing the CCK2R by USPION@Gastrin. We analyzed the CCK2R expression in two immortalized human CAF cell lines, called CAF1 and CAF2, isolated from the resected pancreas tissue of two patients undergoing operation for pancreatic cancer. Unfortunately, these cell lines do not express detectable level of CCK2R (Figure S3a). We therefore elaborated pancreatic CAFs expressing the CCK2R by permanently transfecting the CAF1 and CAF2 cell lines with an expression vector coding for the human CCK2R; two CAF cell lines expressing the CCK2R were respectively selected and termed CAF1-CCK2 and

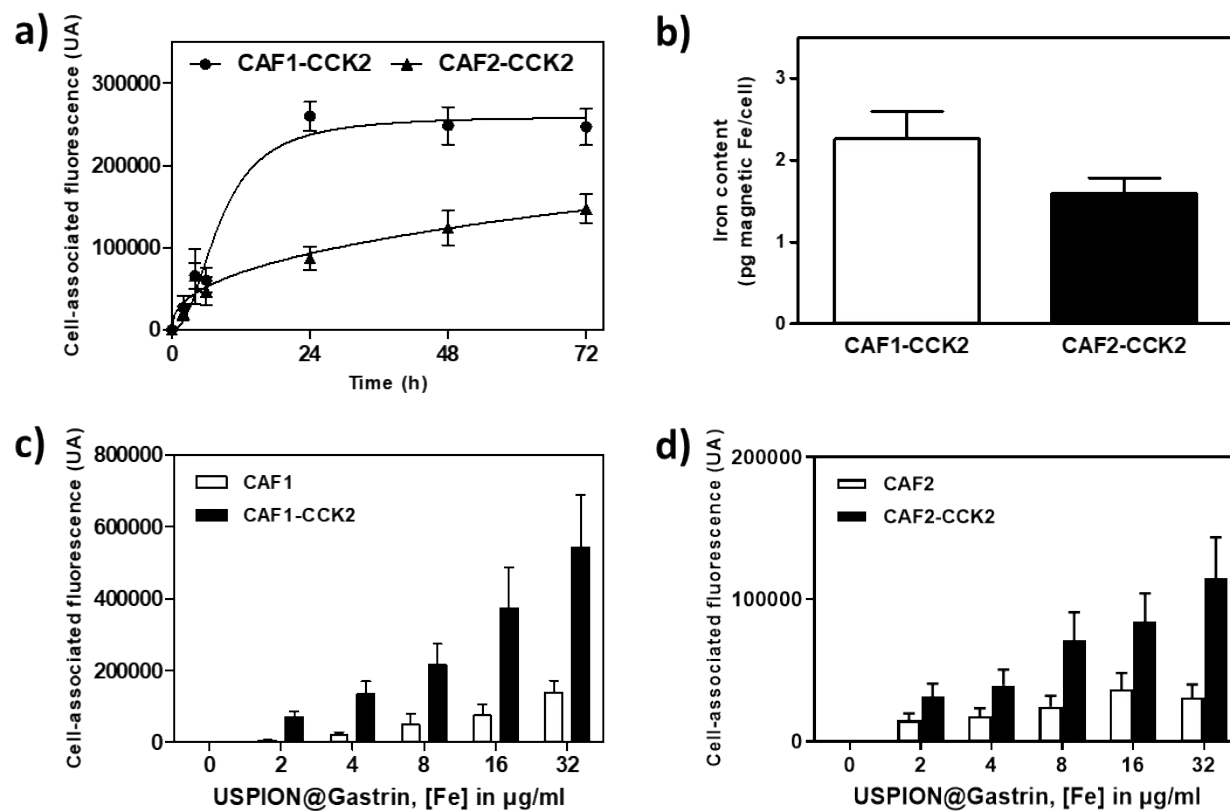


CAF2-CCK2 (Figure S3a). Of note, the CAF2-CCK2 cell line expresses the CCK2R with a 3.7-fold lower level than the CAF1-CCK2 cell line.

We first characterized the binding properties and the uptake of USPIO@Gastrin (Figures 2a-d). The kinetics of USPIO@Gastrin binding and internalization determined by flow cytometry indicate that the amount of USPIO@Gastrin taken up by CAF1-CCK2 and CAF2-CCK2 increased with the time of incubation and stabilized after 24h incubation for CAF1-CCK2 and still increased continuously for CAF2-CCK2 (Figure 2a). Furthermore, the amount of bound and internalized USPIO@Gastrin was higher and faster in CAF1-CCK2 cells than in CAF2-CCK2 cells, indicating that the USPIO@Gastrin uptake depends on the level of CCK2R expression at the cell surface. The global magnetic moment of Fe was evaluated by vibrating sample magnetometry as a quantitative method to measure magnetic nanoparticles quantity in cells (Figure 2b). After 24 h of incubation, magnetic measurements showed an amount of  $2.26 \pm 0.33$  and  $1.60 \pm 0.19$  pg of magnetic Fe per CAF1-CCK2 or CAF2-CCK2 cell, confirming that the USPIO@Gastrin uptake depends on the level of CCK2R expression at the cell surface. The specificity of USPIO@Gastrin binding and internalization were then determined by flow cytometry after 24 h of incubation (Figure 2c-d). The quantification of flow cytometry results indicated that the amount of USPIO@Gastrin taken up by CAF cells increased with the nanoparticle concentration and showed a high specific targeting of CAFs expressing the CCK2R since USPIO@Gastrin were taken up in higher amounts by CAF1-CCK2 and CAF2-CCK2 cells comparatively to CAF1 and CAF2 cells lacking the CCK2R (Figures 2c-d). Indeed, the uptake of USPIO@Gastrin by CAF1-CCK2 was  $13.0 \pm 0.1$ ,  $6.9 \pm 1.2$ ,  $4.2 \pm 1.4$ ,  $5.0 \pm 1.0$  or  $3.9 \pm 1.1$  higher comparatively to CAF1 cells, at the low extracellular concentrations of USPIO@Gastrin corresponding to 2, 4, 8, 16 or 32  $\mu\text{g Fe/ml}$  (Figure 2c). Similarly, the uptake by CAF2-CCK2 cells were  $2.1 \pm 0.6$ ,  $2.2 \pm 0.7$ ,  $2.9 \pm 0.8$ ,  $2.3 \pm 0.7$  or  $3.7 \pm 0.4$  higher than in CAF2 cells at the same USPIO@Gastrin concentrations (Figure 2d). Moreover, competition



studies showed that an excess of gastrin inhibited USPION@Gastrin uptake by CAF1-CCK2 and CAF2-CCK2 cells (Figures S3b, S3c). These results indicate that CAFs overexpressing specific surface receptors can be effectively targeted with magnetic nanoparticles, but also validate the use of gastrin to target pancreatic CAFs expressing the CCK2R.

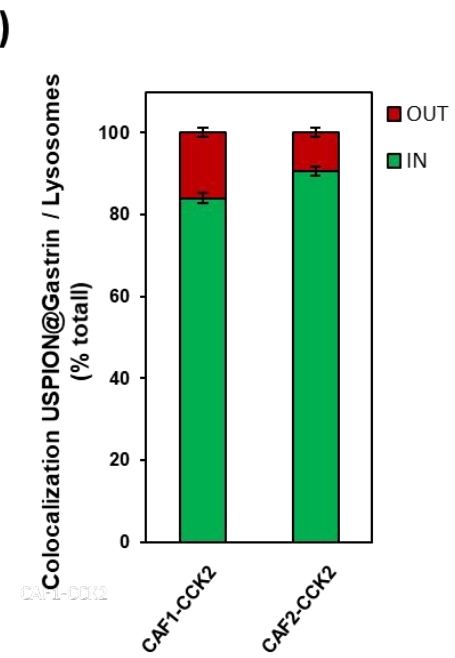
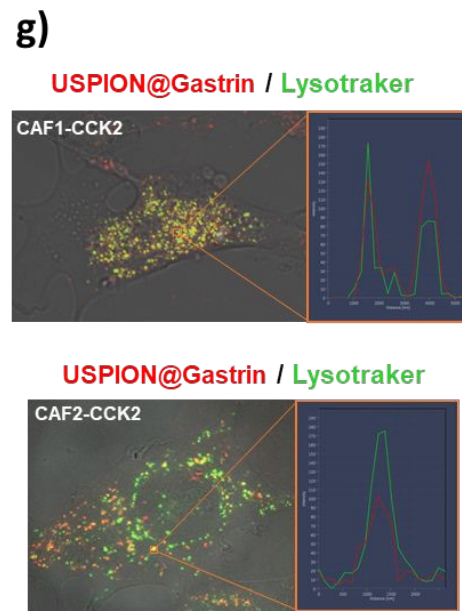
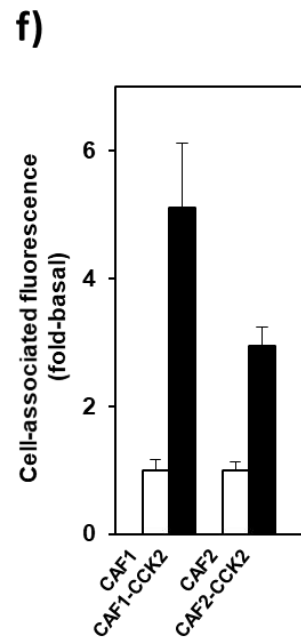
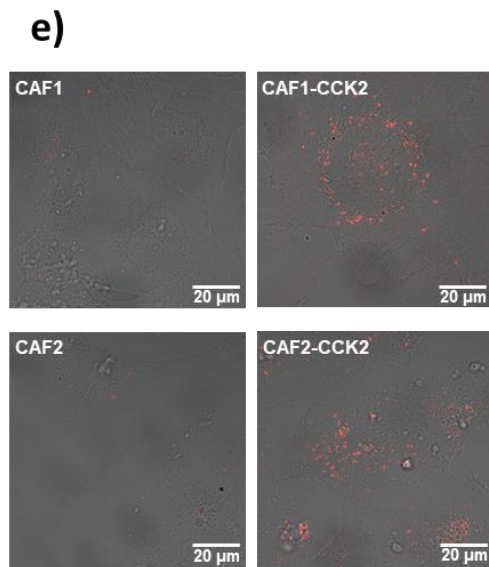
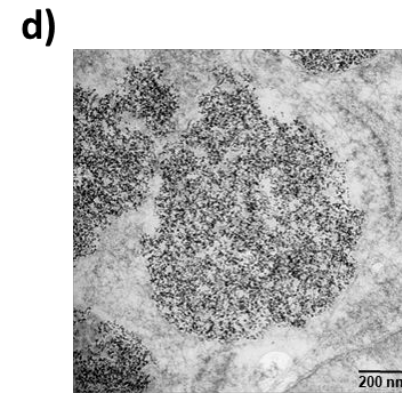
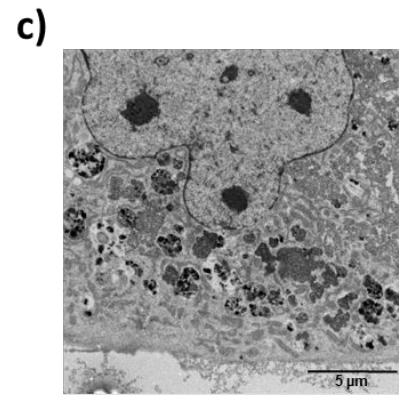
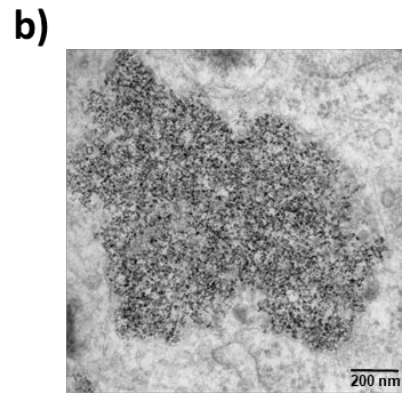
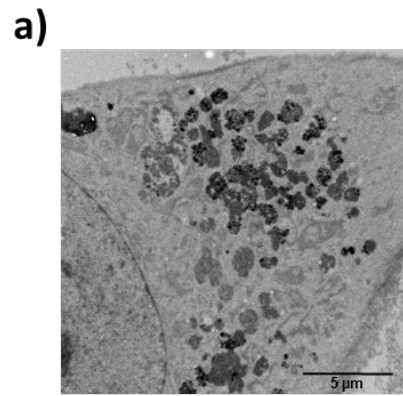


**Figure 2:** USPION@Gastrin specifically bind to CAF cells expressing the CCK2R, subsequently internalized and accumulated into lysosomes. (a) Kinetics of USPION@Gastrin uptake by CAF1-CCK2 and CAF2-CCK2. Cells were incubated with 16  $\mu\text{g/ml}$  of USPION@Gastrin for 2, 4, 6, 24 h, 48 h and 72 h. Fluorescence was measured by flow cytometry, and results are expressed as fluorescence associated with the cells and are the mean  $\pm$  SEM of at least three separate experiments. (b) CAF1-CCK2 and CAF2-CCK2 cells were incubated with 16  $\mu\text{g/ml}$  of USPION@Gastrin for 24 h. Fe contained in superparamagnetic MNPs was measured by vibrating sample magnetometer. (c) and (d) USPION@Gastrin uptake by CAF1 and CAF1-CCK2 (c) or CAF2 and CAF2-CCK2 (d) cells expressing or not the CCK2R. Cells were incubated with increased concentrations of USPION@Gastrin for 24 h. Cell-associated fluorescence was measured by flow cytometry, and results are expressed as fluorescence associated with the cells and are the mean  $\pm$  SEM of at least three separate experiments.



We next characterized the cellular fate of USPIO@Gastrin in the CAF cells (Figure 3a-h). The transmission electron microscopy (TEM) images of CAF1-CCK2 and CAF2-CCK2 cells indicated that USPIO@Gastrin were mainly localized in lysosomes (Figures 3a-d). Based on the TEM images, the concentration of NPs inside a lysosome was evaluated in the range of 500 to 5,000 nanoparticles per lysosome while the volume concentration of USPIO@Gastrin in lysosomes was estimated to be around 15%. Confocal microscopy study confirmed that USPIO@Gastrin accumulated in the lysosomal compartment of CAFs cells expressing the CCK2R after 24 h of incubation, whereas they are poorly present in the lysosomes of CAF1 and CAF2 cells devoid of CCK2R (Figure 3e-h). Figure 3e and 3f showed the USPIO@Gastrin internalization was respectively  $5.1 \pm 1.0$  and  $3.0 \pm 0.3$  more important in CAF1-CCK2 and CAF2-CCK2 cells than in CAF1 and CAF2 cells, in agreement with previous results obtained by flow cytometry analysis (Figures 2c-d). Analysis of USPIO@Gastrin / LysoTracker colocalization demonstrated that  $84.1 \pm 1.1$  and  $90.6 \pm 1.1$  % of USPIO@Gastrin uptaken by cells were localized inside the lysosomes of CAF1-CCK2 and CAF2-CCK2 cells, respectively (Figures 3g-h).





**Figure 3:** Subcellular colocalization of USPIO@Gastrin. (a-d) Electron microscopy analysis of USPIO@Gastrin accumulation in CAF1-CCK2 and CAF2-CCK2 cells. CAF1-CCK2 and CAF2-CCK2 cells were incubated with 16  $\mu\text{g/ml}$  of USPIO@Gastrin for 24 h. Image representative of USPIO@Gastrin accumulated in cells, (a) and (c), and in lysosomes, (b) and (d). (e-f) Analysis of USPIO@Gastrin localization by confocal microscopy. CAF1, CAF1-CCK2, CAF2 and CAF2-CCK2 cells were incubated with 16  $\mu\text{g/ml}$  of USPIO@Gastrin for 24 h, then observed by confocal microscopy (e). Quantification of USPIO@Gastrin uptake by analysis of confocal microscopy images (f). CAF1-CCK2 and CAF2-CCK2 cells were incubated with 16  $\mu\text{g/ml}$  of USPIO@Gastrin for 24 h, then with 10 nM LysoTracker Green for 15 minutes and observed by confocal microscopy (g). Quantification of USPIO@Gastrin accumulation in lysosomes by analysis of confocal microscopy images (h).

### Torque undergone by an assembly of USPIOs

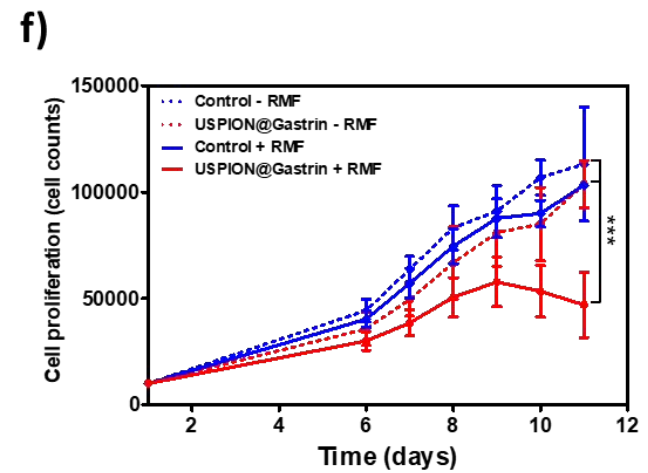
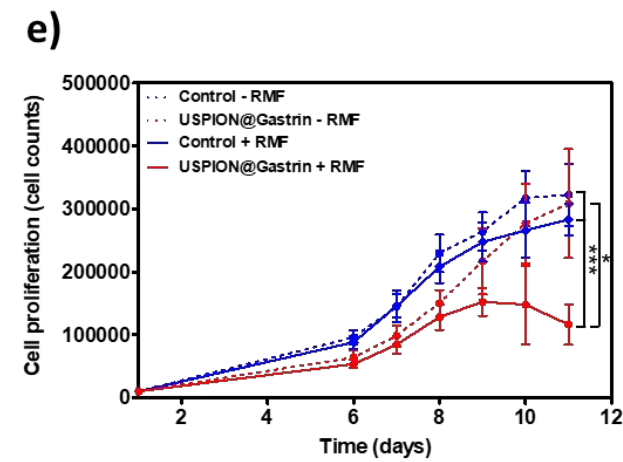
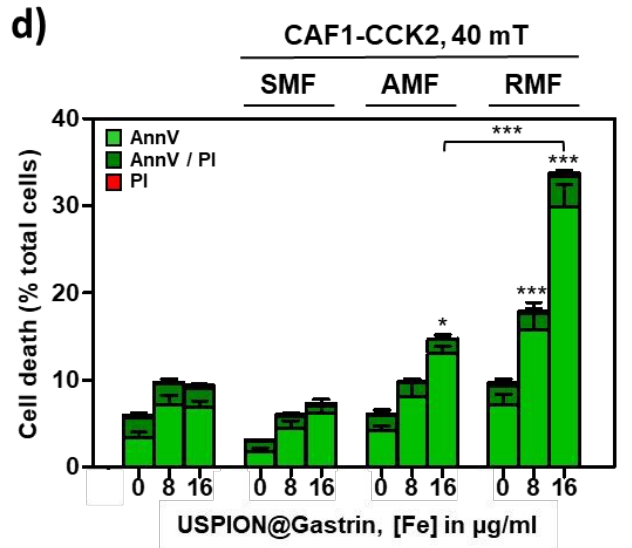
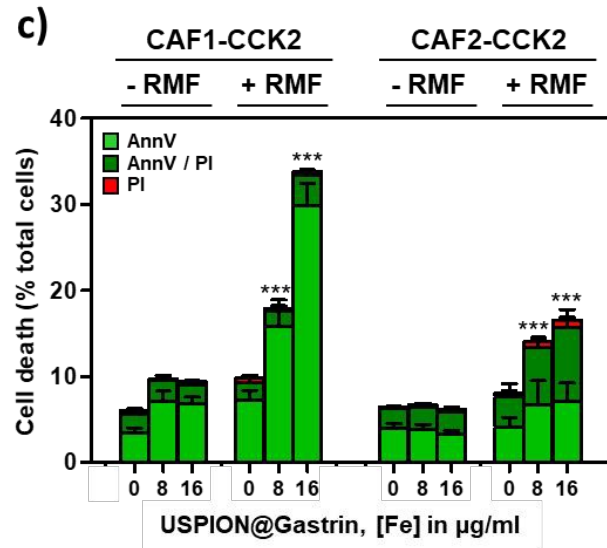
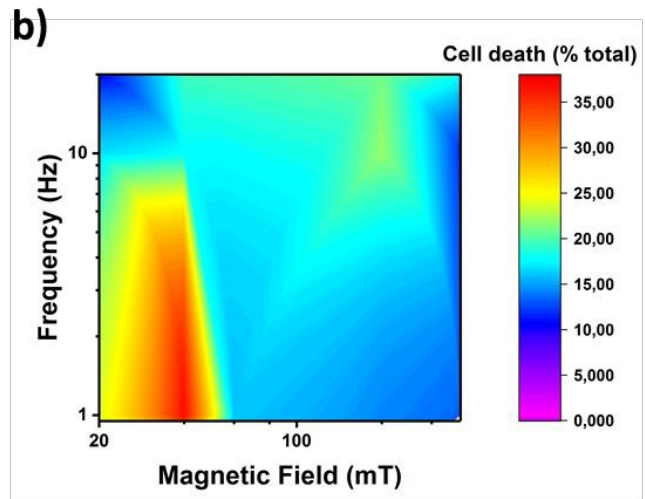
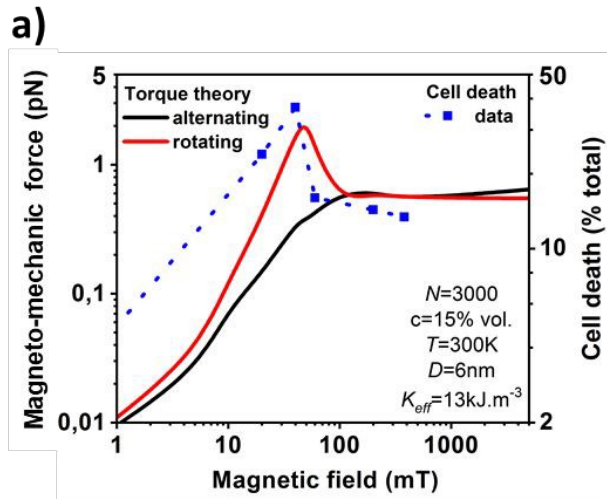
First, let us say a few words on the magnetic properties of the nanoparticles. In this type of nano-objects, the anisotropy of the individual particles comes from shape, surface and magnetocrystalline anisotropy. At a diameter of 6 nm, they are expected to single-crystal, single-domain and superparamagnetic at room temperature. When they are under the form of assemblies, an anisotropy originating from collective effects and dipolar interactions between the single objects emerges. This leads to a ferromagnetic-like behaviour of the assemblies, which generates torques well above the one expected for non-interacting nanoparticles (see Fig. 14 and discussion in Ref. [50]). In Ref. [50], one can read that “*Everything happens as if large anisotropy NPs in the superparamagnetic regime in a given range of concentrations behave exactly as low anisotropy noninteracting ferromagnetic NPs.* » Quantitatively, the total torque  $\tau$  of an assembly is expected to scale as  $\tau = \tau_{\text{ind}}\sqrt{N}$ , where  $\tau_{\text{ind}}$  is the torque generated by a single particle and  $N$  the number of particles in the assembly. However, it is shown in Ref. [50] that, under certain conditions, when the assembly presents a ferromagnetic-like behaviour, the torque can be larger than that.

Figure 4a illustrates the theoretical aspects related to the force generated by an assembly of USPIOs submitted to a rotating (RMF) or an alternating (AMF) magnetic field. The calculations have been performed for a nanoparticle diameter of 6 nm and using the magnetic parameters of magnetite (see



Methods; saturation magnetization  $M_s = 500 \text{ kA}\cdot\text{m}^{-1}$ , anisotropy  $K_{\text{eff}} = 13 \text{ kJ}\cdot\text{m}^{-3}$ ). The experimental magnetization value  $65 \text{ Am}^2\text{kg}^{-1}$  corresponds to  $336 \text{ kAm}^{-1}$ . Since the torque directly scales with the magnetization, the theoretical forces which will be presented below are reduced by approximately one third if the experimental value is used. The geometrical characteristics of the 6 nm USPIO assembly were evaluated from TEM images to be as close as possible of the studied system: the number of particles  $N$  in the assembly, corresponding to the average number of particles in the lysosomes, was  $N = 3000$  nanoparticles, with a volume concentration  $c = 15\%$ . First, the simulations showed that the force depends on the amplitude of the AMF: at lower amplitudes, the force increases with the field until it reaches a plateau of 0.7 pN for amplitudes larger than 100 mT. In contrast, the application of a RMF generates a strong torque peak, which occurs in a small range of field amplitude (20-60 mT), a behaviour that is due to a breaking of time reversal symmetry when the magnetic field rotates, as described in a previous study.<sup>50</sup> Only in a small range of RMF amplitude (30-60 mT), the generated forces are able to be above 1 pN, which is the lower limit to start breaking chemical and/or biological bond.<sup>18</sup> The highest theoretical force occurs for a RMF of 50 mT and reaches 3 pN. With amplitudes of RMF greater than 100 mT, the force reaches a plateau of 0.7 pN.







**Figure 4:** USPION@Gastrin generate a torque inducing the death of CAF-CCK2 cells upon low frequency rotating magnetic field. (a) Theoretical force generated by USPION@Gastrin under AMF or RMF, compared to the evolution of CAF1-CCK2 cell death after RMF exposure experimentally obtained, for different amplitudes of magnetic field. The force is calculated using monodisperse USPIONs of diameter  $d = 6$  nm, with a uniaxial anisotropy  $K_{\text{eff}} = 13 \text{ kJ.m}^{-3}$  and is the one generated by the 3000 nanoparticles assembly at a volume concentration of 15% corresponding to the average number of particles in the lysosomes and their volume concentration. (b) Quantification of CAF1-CCK2 cell death labeled with Annexin V/propidium iodide (AnnV/PI) according to RMF amplitude (mT) and frequency (Hz). CAF1-CCK2 cells were incubated for 24 h with USPION@Gastrin at  $[\text{Fe}] = 16 \mu\text{g/ml}$ , washed and exposed to RMF for 2 h. Dead cells were labeled with iFluor488-tagged AnnV/PI, 4h after magnetic field exposure, and counted by flow cytometry. (c) CAF1-CCK2 and CAF2-CCK2 cells were incubated with USPION@Gastrin for 24 h, washed, and exposed or not to RMF (40 mT, 1Hz) for 2 h. Dead cells were labeled AnnV/PI and counted 4 h after RMF exposure by flow cytometry. (d) CAF1-CCK2 cells were incubated with USPION@Gastrin for 24 h, washed, and exposed or not to static (SMF: 40 mT), alternating (AMF: 40 mT, 1 Hz) or rotating (RMF: 40 mT, 1Hz) for 2 h. Dead cells were labeled with AnnV/PI and counted 4 h after magnetic field exposure by flow cytometry. (e) and (f) CAF1-CCK2 and CAF2-CCK2 cells were incubated with USPION@Gastrin for 72 h, washed, and exposed or not to RMF (40 mT, 1 Hz) for 2 h, once/days during 6 days. Cell proliferation was evaluated by counting cells 24h after RMF exposure, during 6 days. Results are expressed as mean  $\pm$  SEM of at least three separate experiments. Statistical analysis was performed using one- or two-way ANOVA test.

## Induction of CAF death through low frequency rotating magnetic field actuation of USPION@Gastrin

Based on the previously described simulations, we first screened the efficiency of different amplitudes (20 to 380 mT) and frequencies (0.2 to 20 Hz) of rotating magnetic field (RMF) to induce the death of CAFs having accumulated USPION@Gastrin inside their lysosomes. For this purpose, CAF1-CCK2 cells expressing high level of CCK2R were incubated with USPION@Gastrin at 4, 8 or 16  $\mu\text{g}$  Fe/ml for 24 h, washed to eliminate unbound and non-internalized USPION@Gastrin and exposed to RMF for 2 h while temperature was maintained at 37°C. Controls were composed of cells lacking USPION@Gastrin and cells having internalized USPION@Gastrin but that were not exposed to RMF. After RMF exposure, the cells were cultured under standard conditions for recovery. The impact of treatment was determined by counting dead cells labeled with iFluor488-tagged annexin V (AnnV)



and/or propidium iodide (PI) by flow cytometry, 4 h after RMF exposure (Figure 4b, supplementary Figures S4-S8). As shown in Figure 4b, RMF application caused the cells having accumulated USPIO@Gastrin to die at most amplitudes and frequencies. Interestingly, the maximum effect was observed with the low frequency 1 Hz and the small amplitude of 40 mT leading to the death of  $17.9 \pm 3.0\%$  and  $33.8 \pm 2.8\%$  of CAF1-CCK2 cells incubated with 8 or 16  $\mu\text{g Fe / ml}$  of USPIO@Gastrin, respectively. This corresponds to a  $3.0 \pm 0.9$ - and  $5.6 \pm 1.7$ -fold increase in cell mortality ( $p < 0.05$  and  $P < 0.001$ , Figure 4c, supplementary Figure S4). Similarly, the 1 Hz / 40 mT RMF increased the cell mortality of CAF2-CCK2 cells incubated with 8 or 16  $\mu\text{g Fe / ml}$  of USPIO@Gastrin by  $2.2 \pm 0.4$ - or  $2.5 \pm 0.2$ -fold respectively (dead cells:  $14.0 \pm 1.7$  or  $16.5 \pm 0.4\%$  of CAF2-CCK2 cells incubated with 8 or 16  $\mu\text{g Fe / ml}$  of USPIO@Gastrin vs  $6.5 \pm 0.6\%$  CAF2-CCK2 cells lacking nanoparticles,  $p < 0.001$ , Figure 4c). No significant effect was observed on CAF1-CCK2 and CAF2-CCK2 cells lacking USPIO@Gastrin and exposed to RMF whatever the amplitude and frequency used, as well as on CAF1-CCK2 and CAF2-CCK2 cells incubated with USPIO@Gastrin but not exposed to RMF (Figure 4c, supplementary Figures S4-S8). Superimposed to the simulations, the cell death rate obtained experimentally at different amplitudes of RMF showed that the shape of the curve followed closely the force generated by USPIOs assembly, indicating that experimental data of RMF impact on cell death are in good agreement with the simulations of the magnetic torque (Figure 4a).

To compare the impact of different types of magnetic field, we then focused on CAF1-CCK2 cell line, which presented a better response to RMF exposure. CAF1-CCK2 cells having accumulated USPIO@Gastrin within their lysosomes were exposed to a 40 mT static (SMF), a 40 mT / 1 Hz rotating (RMF) or a 40 mT / 1 Hz alternating (AMF) magnetic field. After magnetic field exposure, the cells were cultured under standard conditions for recovery. The impact of treatment was determined by counting dead cells labeled with iFluor488-tagged Annexin V and/or PI by flow cytometry, 4 h after magnetic field exposure. As shown in Figures 4d, a significant increase of cell death was observed when USPIO@Gastrin were submitted to a 40 mT / 1 Hz RMF or AMF, whereas no significant effect was



observed upon a 40 mT SMF. Moreover, we demonstrate that RMF is the most efficient way to cause the death of cells having accumulated USPIO@Gastrin. Indeed, RMF caused the death of  $17.9 \pm 3.0\%$  and  $33.8 \pm 2.8\%$  of CAF1-CCK2 cells incubated respectively with 8 or 16  $\mu\text{g Fe / ml}$  of USPIO@Gastrin, whereas AMF killed only  $14.7 \pm 0.3\%$  CAF1-CCK2 cells ( $p < 0.001$ ) incubated with 16  $\mu\text{g Fe / ml}$  of USPIO@Gastrin. Of note, SMF, AMF or RMF did not affect the viability of cells devoid of USPIO@Gastrin.

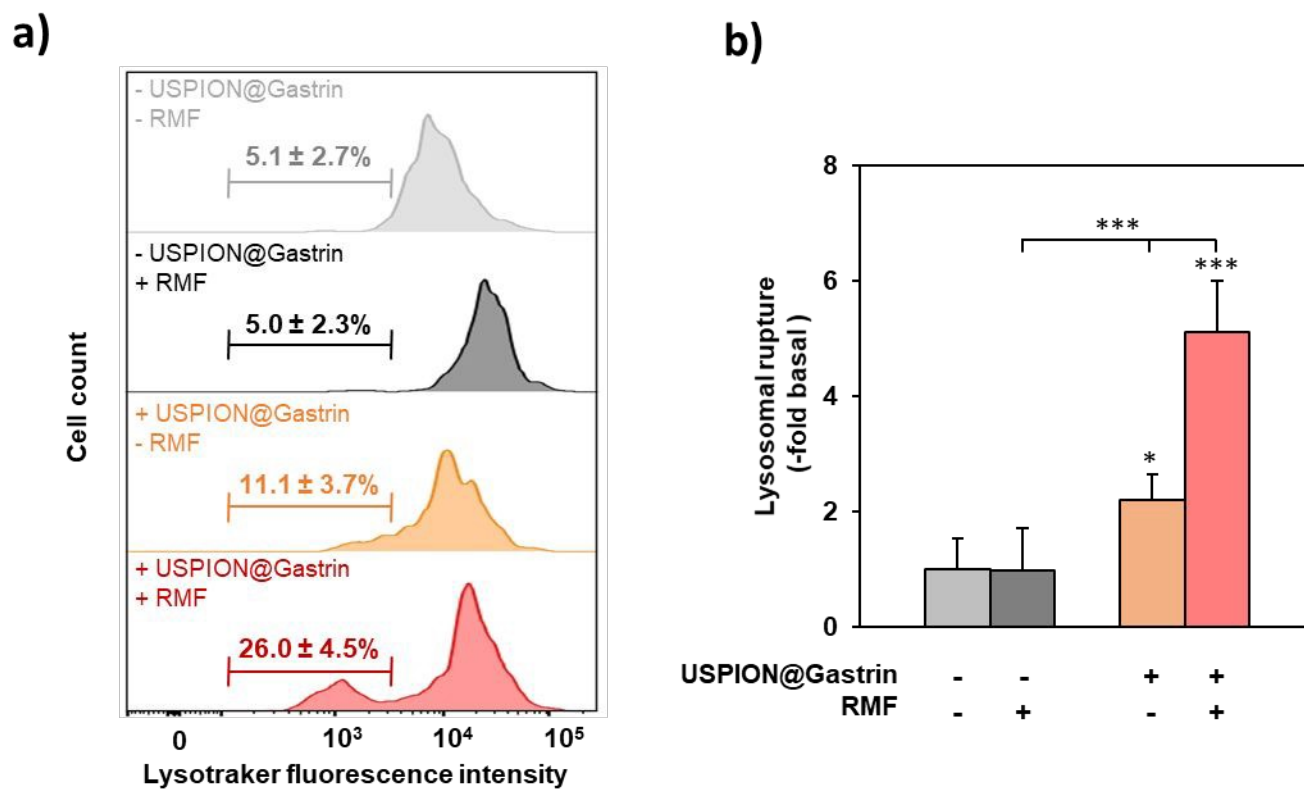
Finally, we analyzed the effect of repeated RMF treatment on cell proliferation of CAF1-CCK2 and CAF2-CCK2 cells (Figures 4e-f). CAF1-CCK2 and CAF2-CCK2 were incubated with USPIO@Gastrin at 16  $\mu\text{g Fe/ml}$  for 72 h, washed to eliminate unbound and non-internalized USPIO@Gastrin and exposed to RMF for 2 h once a day (from day 5 to 10) while temperature was maintained at  $37^\circ\text{C}$ . While the RMF treatment (40 mT, 1 Hz) had no significant effect on the number of nanoparticles-unloaded cells during the 6 days treatment period when compared to control cells, the proliferation of USPIO@Gastrin-loaded CAF1-CCK1 and CAF2-CCK2 cells after RMF treatment was significantly ( $p < 0.01$ ) inhibited (Figures 4e-f). Altogether, these results suggest that the attack on lysosomes *via* RMF activation of lysosome-accumulated USPIO@Gastrin prompts cell death and affects the growth of the cell population.

### **RMF induces the rupture of lysosomes containing USPIO@Gastrin**

Since conjugated magnetic nanoparticles with antibodies targeting the lysosomal protein LAMP1 were reported to induce lysosome membrane permeabilization (LMP) upon RMF,<sup>31</sup> we determined whether rotating USPIO@Gastrin nanoparticles, that were accumulated inside CAF-CCK2 lysosomes, caused lysosome damage. CAF1-CCK2 cells were incubated with USPIO@Gastrin for 24 h, washed to eliminate non-internalized nanoparticles and exposed to RMF for 2 h. Staining with LysoTracker, an acidotropic dye that fluorescently labels lysosomes and accumulates in unaltered lysosomes, was used to assess the effects of rotating USPIO@Gastrin on CAF1-CCK2 lysosomal integrity. When lysosome



ruptures, the dye does not accumulate in damaged lysosomes and is released into the cytosol, resulting in a population of cells with reduced fluorescence (pale cells). The decreased LysoTracker fluorescence was measured by flow cytometry. As shown in Figures 5a and b, cells treated with USPION@Gastrin and exposed to RMF show a significant 5.1 ± 0.9-fold increase of the number of pale cells (26.0 ± 4.5%,  $p < 0.001$ ), as compared to control cells lacking nanoparticles (5.1 ± 2.7%). No significant effect was observed on CAF1-CCK2 cells lacking USPION@Gastrin and exposed to RMF (5.0 ± 2.3% of pale cells), as well as on CAF1-CCK2 cells incubated with USPION@Gastrin but not exposed to RMF (11.1 ± 3.7% of pale cells). These results indicated that RMF exposure caused lysosome rupture of CAF1-CCK2 cells containing USPION@Gastrin.



**Figure 5:** RMF exposure caused the lysosomal rupture. CAF1-CCK2 cells were incubated with USPION@Gastrin at [Fe] = 16 µg/ml for 24 h, washed, exposed or not to RMF (40 mT, 1Hz) for 2 h, then incubated with 10 nM LysoTracker Green. Lysosome integrity was determined by analyzing LysoTracker fluorescence intensity by flow cytometry. (a) Representative peak diagrams of LysoTracker fluorescence intensity determined by flow cytometry. Bars indicate the percentage rate of pale cells with lysosomal rupture revealed by a decreased LysoTracker

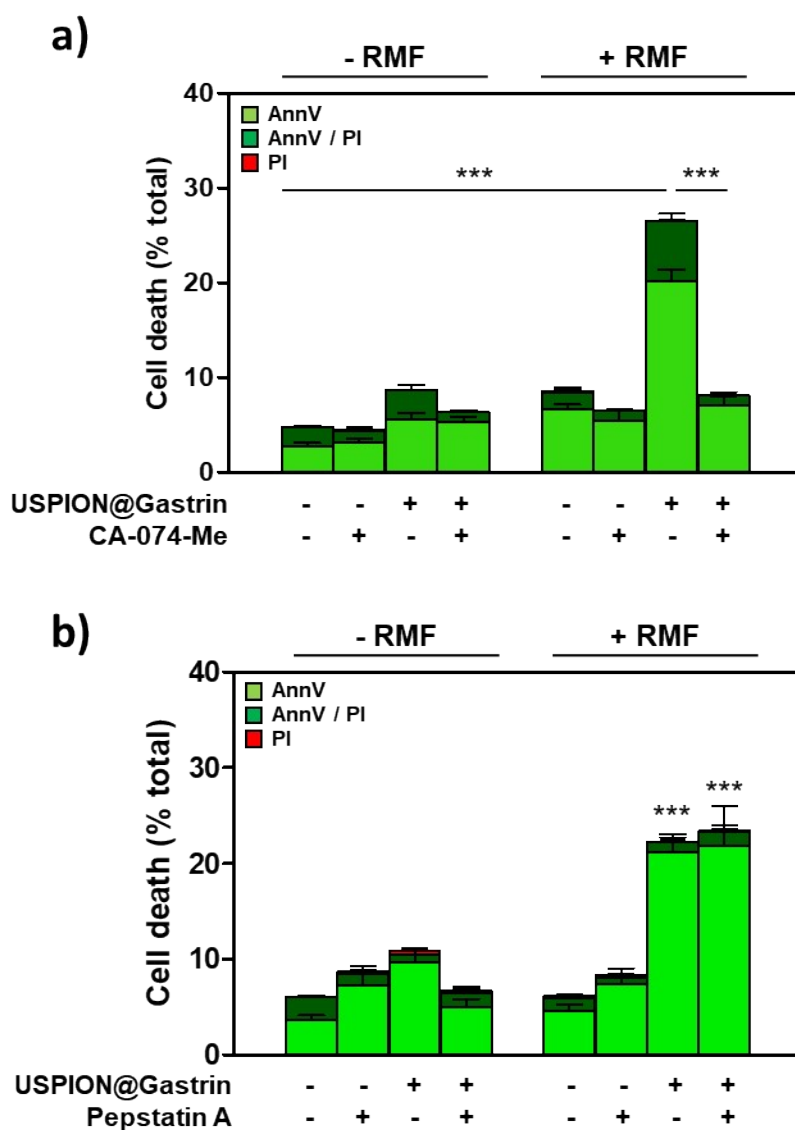


fluorescence intensity. (b) Quantification of lysosomal rupture was performed by counting the percentage of pale with a decreased LysoTracker fluorescence intensity by flow cytometry images and expressed as fold change of fluorescence intensity over control cells (in absence of USPION@Gastrin and RMF). Significant difference compared to the control condition corresponding to cells devoid of USPION@Gastrin in absence of RMF exposure was indicated above histogram bar. Statistical significances between other conditions are also indicated by lines/brackets. Results are the mean  $\pm$  SEM of three separate experiments. Statistical analysis was performed using one-way ANOVA test.

### CAFs death induced by USPION@Gastrin torque depends on lysosomal Cathepsin-B activity

Lysosome rupture is known to result in the leakage of lysosomal enzymes, including cathepsins, into the cytosol leading eventually to cell death. Such mechanism has been previously identified in response to intra-lysosomal magnetic hyperthermia, due to the release of local thermal energy from nanoparticles accumulated in lysosomes that required Cathepsin-B to kill cancer cells.<sup>5</sup> To investigate whether cathepsins could be involved in cell death in response to USPION@Gastrin-induced torque, RMF experiments were performed in presence or in absence of CA-074-Me, a selective inhibitor of Cathepsin-B, or Pepstatin A, an inhibitor of aspartyl peptidases including Cathepsin-D. After RMF exposure, the cells were cultured under standard conditions for recovery. The impact of treatment was determined by counting dead cells labeled with iFluor488-tagged AnnV and/or PI by flow cytometry, 4 h after RMF exposure (Figure 6a-b). As shown in Figure 6a, the effect of USPION@Gastrin torque on cell death was totally prevented by CA-074-Me (dead cells:  $7.6 \pm 0.7$  % vs  $27.5 \pm 1.0$  % in USPION@Gastrin-loaded CAF1-CCK2 cells treated in presence or in absence of CA-074 Me). In contrast, Pepstatin A did not prevent cell death induced by USPION@Gastrin torque under RMF (dead cells:  $24.4 \pm 3.7$  % vs  $22.3 \pm 2.0$  % in USPION@Gastrin-loaded CAF1-CCK2 cells treated in presence or in absence of Pepstatin A, respectively) (Figure 6b). These results indicate that CAFs death induced by USPION@Gastrin torque depends on Cathepsin-B activity.





**Figure 6:** CAFs death induced by USPION@Gastrin torque depends on lysosomal Cathepsin-B activity. CAF1-CCK2 cells were incubated with USPION@Gastrin at  $[\text{Fe}] = 16\mu\text{g}/\text{ml}$  for 24 h, washed, exposed or not to RMF (40 mT, 1Hz) for 2 h in absence or presence of CA-074-Me (a) or Pepstatin A (b) inhibitors. Dead cells were labeled with AnnV/PI, 4 h after magnetic field exposure, and counted by flow cytometry. Results are expressed as mean  $\pm$  SEM of at least three separate experiments. Statistical analysis was performed using one-way ANOVA test.

## Discussion

In the wider context of targeted nanotherapy by magnetic hyperthermia, we explored herein a new strategy consisting in the local release of mechanical energy based on the exposure of targeted 6 nm USPIOs to low-frequency magnetic fields. We first showed that functionalized USPIO (6 nm iron



oxide core) could target the lysosomes of pancreatic cancer-associated fibroblast (CAF), a key player in tumor microenvironment that promotes tumor growth and resistance. Pancreatic CAF expressing the CCK2 receptor were chosen as a model to do the proof-of-concept. Interestingly, such approach can be developed by targeting other receptors or protein surface able to internalize. We thus showed that the binding and internalization of USPIO@Gastrin depends on the presence and the expression level of the CCK2 receptor at the CAF surface, indicating that CAFs overexpressing specific surface receptors can be effectively targeted with functionalized magnetic nanoparticles. Then, we explored the opportunity offered by targeted USPIOs accumulated into CAF lysosomes to act as a therapeutic agent inducing their death upon low-frequency magnetic fields. Here, we aim at discussing the possible mechanisms of cell death, based on the results that we have obtained from magnetic simulations and experiments, and comparing our results with the literature.

An accurate screening in the characteristics of the magnetic field (amplitude, frequency, and type) demonstrated that, using 6 nm in diameter USPIOs, cell death is maximized when the field rotates, its amplitude is around 40 mT and the frequency is around 1 Hz. The former two characteristics (rotation and amplitude) are well predicted and understood by our magnetic simulations. Indeed, we had already theoretically shown that, when the magnetic field rotates instead of being alternating, a much more intense torque is generated in a specific range of magnetic field.<sup>50</sup> This theoretical behaviour is again illustrated in Fig. 4(a) with the specific parameters of our experiments.

For a relevant comparison of our experimental results with literature, it should first be recalled that our experiments were performed with single-domain objects, and that our simulations are only valid within this framework. The fact that a rotating field is more efficient than an alternating one has been previously found in cases where cell death was induced by rather large magnetic objects.<sup>40 37, 41</sup> With respect to single-domain objects, Li *et al.* found that increasing the field amplitude in the range [4-20 mT] increased cell death rate.<sup>32</sup> On the contrary, Master *et al.* concluded that amplitude had no influence on cell viability but with only two AMF amplitudes tested [62 and 125 mT].<sup>35</sup> Thus our result showing



that a large magnetic field can be less efficient than a smaller one has, to our knowledge, never been reported. It could appear at first sight counter-intuitive, but it is the natural result of our theoretical mentioned in the previous paragraph. The third characteristic permitting to optimize cell death – a frequency of 1 Hz – was not predicted by our simulations. In the latter, particles do not move, and it is not surprising that almost no frequency dependence of the torque amplitude is observed: generally speaking, to see significant changes in the magnetic response of immobilized superparamagnetic nanoparticles, excitation frequency should be changed by orders of magnitude. Li *et al.* and Chiriac *et al.* studies showed that increasing the frequency in the ranges [0-20 Hz] and [20-50 Hz], respectively, reduced the cell viability, whereas higher frequencies do not induce greater effects [70-100 Hz].<sup>32, 41</sup> In our study as well as in others, the optimal frequency able to induce cell death is not the largest one, but rather smaller frequencies ranging from 1 Hz to 50 Hz, which might have a bio-mechanical origin, such as the frequency-dependence of the cell response to a mechanical stimuli, or such as a resonance effect in the frequency-dependence of the mechanical response itself.<sup>32, 37, 41</sup>

Our magnetic simulations estimated that an assembly of 3,000 nanoparticles with a 6 nm crystalline core generate magnetic forces around 3 pN, a value which is well below the tens to hundreds of pN required to disrupt a bilayer membrane,<sup>18, 51</sup> suggesting that lysosome permeabilization or rupture induced by USPION@Gastrin torque may involve an alternative mechanism. The mechanical motion or vibration of small magnetic nanoparticles under low frequency magnetic fields have been described to manipulate macromolecules such as DNA (deoxyribonucleic acid) duplexes, enzymes or ion channels.<sup>10, 52-53</sup> Interestingly, plasma membrane disturbances induced by magnetic nanoparticles motion were shown to be associated with the activation of ion channels leading to increased levels of intracellular ions (such as calcium, sodium or potassium) and eventually to apoptosis.<sup>27-28, 33, 39, 54</sup> Several ion channels, transient receptor potential ion channels TRPML and two-pores channels TPC, have been identified in endo-lysosomal compartment whose overexpression in cancers increases cell proliferation, migration,





invasion and/or metastasis.<sup>55-57</sup> These channels are osmo-mechanical sensitive as they can be activated by both physical and chemical signals (oxidants, pH, and osmo-mechanical force). Noticeably, the magnetic forces of USPION@Gastrin assembly calculated by our simulations - few pN -, are large enough to activate such ion channels.<sup>18</sup> This suggests that dysregulation of lysosomal ion channels by USPION@Gastrin torque may be associated to impairment of lysosomal ion homeostasis, resulting in lysosomes dysfunction and permeabilization and thus cell death.<sup>58-59</sup> While few pN forces generated by the 6 nm nanoparticles assembly conducted to the death of 34% of cells upon 40 mT / 1 hz rotating field application, one can imagine that designing nanoparticles with better anisotropic and magnetic properties to respond to optimized magnetic fields parameters may promote the efficiency of magneto-mechanical cell destruction. In addition, nanoparticles surface functionalization (surface charge, size, shape, multiple active targeting...) increasing the nanoparticles uptake by cells may also enhance the efficiency of such strategy.

Last, this study highlights first data on the mechanisms at the origin of cell death that involves the lysosome damage and the lysosomal enzyme Cathepsin-B. Indeed, we showed that RMF exposure diminished the staining of lysosomes with the pH-independent LysoTracker and that CA-074-Me inhibitor prevents the effect of RMF on cell death. According to literature, these results indicate that lysosome permeabilization followed by the leakage of Cathepsin-B is likely at the origin of CAF death. Once released into the cytosol, Cathepsin-B was shown to activate apoptosis or non-apoptotic cell death pathways.<sup>60</sup> Thus, USPION@Gastrin torque upon RMF behaved as a lysosomotropic agent in CAF cells and induced cell death through a lysosomal cell death pathway. Among literature data investigating micro- or nanoparticles to induce cell death or tumor regression by mechanical forces, some studies suggested hypotheses about the mechanism of cell death depending on micro- or nanoparticles localization. Hence, plasma membrane disruption, lysosome damage or cytoskeleton disorganization have been mentioned to provoke cell death.<sup>30, 35, 38</sup> Of note, Shen *et al.* showed that the death of glioblastoma cancer cells induced by 62 nm-sized nanocubes upon RMF (40 mT, 15 Hz) was associated



with lysosome membrane permeabilization.<sup>38</sup> Further studies will allow determining the physic and cellular events leading to cell death. Interestingly, we showed here that such approach is also efficient on CAFs with ultra-small superparamagnetic nanoparticles (6 nm core), allowing intravenous injection and making this strategy an interesting therapeutic option to target different cellular components of a tumor by designing nanoparticles to develop multiple targeting strategies. Moreover, our study and others showed that intracellular cell membrane destruction is also efficient to induce cell death comparatively to the disruption from the cell surface.<sup>27-28, 38</sup>

## Conclusion

This study establishes the proof-of-concept that targeted 6 nm USPIOs can disrupt tumor microenvironment through mechanical forces upon rotating magnetic field exposure and opens new opportunities for cancer therapy. We demonstrated that USPIO surface functionalized with gastrin peptide internalized and accumulated into pancreatic CAFs lysosomes expressing the CCK2R, chosen as a model, awaiting the identification of a receptor or a set of receptors allowing the targeting the pro-tumoral CAFs subpopulations and/or cancer cells. The MNPs multi-functionalization to target different receptors expressed on tumor microenvironment and/or cancer cells will be certainly necessary in the future preclinical and clinical studies to obtain efficient tumor targeting and therapeutic response. We showed that the CCK2-targeting nanoparticles produce a torque inside lysosomes under rotating magnetic field, resulting in local mechanical damage of lysosome membranes and effective cell death. After consistent interaction with the lysosome membrane *via* mechanical force or by activating mechanosensitive ion channels on lysosome membrane during RMF, they efficiently damaged lysosomes, inducing the death of CAFs. Compared to the micro-scale magnetic materials, nanomaterials are easily internalized by cells. Moreover, the intracellular cell membrane destruction is also efficient to induce cell death comparatively to plasma membrane disruption. This remote magneto-mechanical



actuation approach that can be used to target cancer cells as well as cells from the tumor microenvironment is attractive with the advantage that low-frequency rotating magnetic fields are safe, penetrate human body without side effects and could considerably reduce the costs for instrumentation. In the context that the tumor microenvironment has an important role in tumor development, progression and metastasis, as well as in limiting drug penetration and efficacy or resistance, magneto-mechanical depletion of CAF population in tumors offers an interesting adjuvant anti-cancer approach that may increase the efficiency of conventional therapy such as chemotherapy and radiotherapy. Therefore, the present study offers a promising strategy for magnetic nanomedicines to non-invasively treat cancers, in particular cancer presenting a dense tumor microenvironment.

## Materials and methods

### Chemical materials

Iron (III) acetyl acetonate (>99.9%) and benzyl alcohol (99.8%) were purchased from Sigma Aldrich. Phosphonate – poly(ethylene glycol) PEG – carboxylic acid MW 2500 g/mol (PO-PEG-COOH) and citrate ions were respectively purchased from Specific Polymers (ref. SP-1P-10–002) and Sigma Aldrich (citrate ions). CA-074-Me and Pepstatin A inhibitors were from Sigma-Aldrich. Annexin V Binding Apoptosis Assay Kit was from AAT Bioquest, Inc. (Sunnyvale, CA, USA). LysoTracker® Green DND-26 was from Invitrogen.

### Synthesis and functionalization of USPION

6 nm USPIONS (ultra-small superparamagnetic iron oxide nanoparticles) were synthesized in benzyl alcohol under microwave heating as reported previously.<sup>45</sup> Briefly, iron (III) acetylacetonate (1.2 mmol) was added to 10 ml of benzyl alcohol and reacted at 210°C during 30 min under microwave irradiation on a Monowave 300 from Anton Paar. The resulting suspension was extracted from benzyl



alcohol to water by adding 10 ml of dichloromethane and 10 ml of citrate ions (3.3 mmol) dispersed in water. Immediately after, a phase separation occurs with the transfer of IONP within the upper water phase. Excess of citrate was removed using ultrafiltration process (Amicon 30 kDa, Merck Millipore). The coated citrate was removed with NaOH (1 mol/l) and IONPs were separated using a neodymium magnet. The solid ( $\text{Fe}_3\text{O}_4 = 40 \text{ mg}$ ) was redispersed in water at pH 2 using an HCl solution at  $10^{-1} \text{ mol/l}$  and surface functionalized by mixing the solution with a PO-PEG-COOH water solution (pH 2, 200 mg). Then, the excess of PO-PEG-COOH coating molecules was removed using ultrafiltration process (Amicon 30 kDa, Merck Millipore) and USPION@PO-PEG-COOH were dispersed in water at pH 7.4 using HCl ( $10^{-1} \text{ mol/l}$ ) and NaOH ( $10^{-1} \text{ mol/l}$ ) solutions.

The USPION@PO-PEG-COOH were then decorated with  $\sim 30$  molecules of a synthetic replicate of gastrin (Cys-Lys-Ser-Ser-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH<sub>2</sub>, Covalab) and  $\sim 20$  molecules of the fluorescent label NHS-DY647-PEG1 (Dyomics GmbH, Jena, Germany), and termed as USPION@Gastrin. An aliquot of USPION@PO-PEG-COOH was sonicated for 10 min on melting ice (Bioblock Scientific 88154). Firstly, the carboxylic acid functions at the outer surface of the IONP@PO-PEG-COOH were activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 2n COOH) and incubated with ethylenediamine (10n COOH) in MES buffer pH 4.5 at room temperature for 1 h. After 5 washes with 10 volumes of H<sub>2</sub>O, the second step was the linkage of the fluorophore NHS-DY647-PEG1 (20 molecules per IONP) in phosphate buffer pH 8.3, at room temperature for 1 h. Then, 3-Maleimidopropionic acid N-hydroxysuccinimide ester (BMPS, 2n COOH) in solution in DMF was added and incubated at room temperature for 1h. After 5 washes with 10 volumes of H<sub>2</sub>O, gastrin ( $\sim 100$  peptides per nanoparticles) in solution in 30% DMF, 70% H<sub>2</sub>O was added in phosphate buffer pH 7.2 and let to react for 2h. Finally, free maleimide functions were saturated by addition of an excess of 26cysteine (1000n COOH) in 50  $\mu\text{L}$  in phosphate buffer pH7.2. After 8 washes with 10 volumes of H<sub>2</sub>O, USPION@Gastrin were stored at 4°C.



## Physico-chemical characterization of USPION

Transmission Electron Microscopy (TEM) samples were prepared by depositing a drop of USPION suspension on carbon coated copper grids placed on a filter paper. TEM images were obtained using a FEI CM10 Microscope (Philips). The median diameter was deduced from TEM data measurements, simulating the diameter distribution with a lognormal function.

The average number of molecules of PO-PEG-COOH per nanocrystal was measured by Fourier Transform Infrared Spectroscopy. Infrared spectra in KBr pellets of various proportions of PO-PEG-COOH mixed with a constant amount of USPIONS were recorded. The normalized  $1110\text{ cm}^{-1}$  band was used for the establishment of the calibration curve and the average number of PO-PEG-COOH per nanoparticle was deduced from this curve.

Suspension of USPION@Gastrin at  $0.1\text{ mg/ml}$  was prepared in DMEM-F12 containing  $0.5\%$  FBS. The suspension was sonicated for 10 min on ice. After temperature equilibrium to  $20^\circ\text{C}$ , particle size was measured by dynamic light scattering using Nanotracs 250 (Microtrac, York, PA).

The magnetization curve of USPION@PO-PEG was measured on a VSM magnetometer (Quantum Design, Versalab) at room temperature. VSM measured magnetization cycles by applying a field from  $-3000$  to  $3000\text{ mT}$  with a sampling rate of approximately  $10\text{ mT/s}$ .

The average number of gastrin derivatives was measured by the OPA method after calibration curve establishment (supplementary Figure S2).  $50\ \mu\text{l}$  of the sample was diluted in  $50\ \mu\text{l}$  of NaOH  $2\text{N}$  and left overnight at  $60^\circ\text{C}$ .  $1\text{ ml}$  of OPA reagent was added to the mixture and fluorescence measurement was recorded. The average number of gastrin peptide per nanoparticle was deduced from a calibration. Before use, USPION@Gastrin were sonicated on ice for 10 min.

## Torque simulations

The magnetic properties of the USPIONS assembly have been calculated using a kinetic Monte Carlo program.<sup>50</sup> The torque is calculated using monodisperse USPIONS of diameter  $d = 6\text{ nm}$ , with a



uniaxial anisotropy  $K_{eff} = 13\text{kJ.m}^{-3}$ , displaying a magnetization per unit volume  $M_S = 500\text{ kA.m}^{-1}$  in a rotating or alternating magnetic field. In order to get the closest of the behavior of IONPs inside a lysosome, the magnetic interactions have been taken into account by studying a sphere containing a disordered assembly of 3000 particles at a volume concentration of 15%.

## Cell lines

Two immortalized human cancer-associated fibroblasts (CAF) cell lines were used. The immortalized human CAF1 line was established by retrovirus-mediated gene transfer of simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT) into human pancreatic fibroblasts isolated from the resected pancreas tissue of a patient undergoing operation for pancreatic adenocarcinoma cancer (PDAC), and obtained from Dr A. Masamune (Division of Gastroenterology, Tohoku University Graduate School of Medicine, Japan) <sup>61</sup>. The immortalized human CAF2 line was obtained from Dr C. Bousquet's laboratory (INSERM U1037-CRCT, France). CAF2 cells were isolated them from patient tumor tissues of pancreas surgically resected from PDAC patients, using the outgrowth method as already described, <sup>62</sup> and then immortalized through lentivirus-mediated gene transfer of human telomerase reverse transcriptase (hTERT). Briefly, small tissue blocks were cut (0.5–1 mm<sup>3</sup>) using a razor blade and were seeded in 10 cm<sup>2</sup> uncoated culture wells in the presence of DMEM-F12 containing 10% foetal calf de-complemented serum (FBS). Tissue blocks were cultured at 37°C in a humidified atmosphere at 95% air and 5% CO<sub>2</sub>. 18 h after seeding, culture medium was changed. CAFs grew out from the tissue blocks 1 to 3 days later. Fresh patient PDAC samples were obtained after informed consent in accordance with the declaration of Helsinki and stored at the « Cancer Research Center of Toulouse» collection (CRCT, INSERM U1037, N° CPP DC-2016-2654), according to the French law (declaration to the ministry of higher education and research). This study was approved by the ethic committee of the Institution.



Both cell lines were permanently transfected with the human CCK2 receptor (CCK2R) using the cDNA encoding CCK2R subcloned in pcDNA3 vector (BD Biosciences Clontech) and Lipofectamine 2000 (Invitrogen), and clones were selected with 400  $\mu\text{g/ml}$  geneticin (Sigma-Aldrich) and termed respectively CAF1-CCK2 and CAF2-CCK2. CAFs cells were cultured in DMEM/F-12 medium (DMEM/F-12 GlutaMAX™, ThermoFisher Scientific) containing 10% FBS, 100 IU/ml penicillin/streptomycin (Penicillin-Streptomycin P4333; Sigma-Aldrich) in a humidified atmosphere at 95% air and 5% CO<sub>2</sub> at 37°C. CAF1-CCK2 and CAF2-CCK2 cells were grown with 400  $\mu\text{g/ml}$  geneticin (Sigma-Aldrich).

### **Analysis of CCK2R expression in CAF cell lines**

50 × 10<sup>3</sup> cells/well were seeded into 24-well plates (Thermo Scientific™) and grown overnight. Cells were incubated with 10 nM Dy647-CCK9 agonist in presence or in absence of 10  $\mu\text{M}$  GV 150013X, a specific CCK2 receptor antagonist, for 1 h at 37°C, in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH 7.4 containing 0.5% FBS and 100 IU/ml penicillin-streptomycin. Then, cells were rinsed twice with ice-cold PBS containing 0.5% BSA and once with PBS alone. Cells were recovered and transferred to FACS tubes. Cell-associated fluorescence was determined using a BD FACSCalibur™ flow cytometer.

### **Analysis of cell viability**

10<sup>4</sup> cells/well of CAF1-CCK2 and CAF2-CCK2 were plated into 96-well plates (Thermo Scientific™). After overnight growth, cells were incubated with increased concentrations of USPIO@Gastrin in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH7.4 containing 0.5% FBS and 100 IU/ml penicillin-streptomycin for 24 h. Cell viability was determined using a MTT viability assay.



## Quantification of USPION@Gastrin binding and uptake by flow cytometry

$10^4$  cells/well were seeded into 24-well plates (Thermo Scientific™) and grown overnight. Cells were incubated with USPION@Gastrin (2 to 32  $\mu\text{g}$  magnetic Fe/ml) for 1 to 24 h at 37 C in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH 7.4 containing 0.5% FBS and 100 IU/ml penicillin-streptomycin. For competition experiments, USPION@Gastrin (16 magnetic Fe/ml) were incubated in presence or in absence of 10  $\mu\text{M}$  Gastrin-17, a specific CCK2 receptor agonist, for 24 h at 37°C. Then, cells were rinsed twice with ice-cold PBS containing 0.5% BSA and once with PBS alone. Cells were recovered and transferred to FACS tubes. Cell-associated fluorescence was determined using a BD FACSCalibur™ flow cytometer.

## USPION@Gastrin localization by confocal microscopy

$25 \times 10^3$  cells/compartiment were respectively seeded onto 4-compartment Cellview culture dishes (Greiner Bio-One). After overnight growth, cells were incubated with USPION@Gastrin (16  $\mu\text{g}$  magnetic Fe/mL) in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH7.4 containing 0.5% FBS and 100 IU/ml penicillin/streptomycin for 24 h. For lysosome staining, cells were incubated for 15-min in the presence of 10 nM LysoTracker Green DND-26 (excitation: 488 nm, Invitrogen). USPION@Gastrin (excitation: 633nm) and LysoTracker co-localization was analyzed using LSM780 confocal microscope (Zeiss).

## USPION@Gastrin localization by transmission electron microscopy

$10^4$  CAF1-CCK2 cells were grown in 6-dishes plates (Thermo Scientific™) and incubated with USPION@Gastrin (16  $\mu\text{g}$  magnetic Fe/mL) for 24 h. After removing medium, cells were fixed with 4% glutaraldehyde in Sorensen buffer for 4 h at 4°C. Fixed cells were washed with cold Sorensen buffer for 12 h and post-fixed in 1% osmium tetra oxide (osmium 2%, saccharose 0.25 M, Sorensen 0.05 M) for 1 h at 20°C, followed by washings with distilled water and uranyl acetate 2% for 12 h at 4°C. The cells





were dehydrated by sequential washings of 10 min in 30, 50, 70 and 95% ethanol followed by 3 washes of 15 min in absolute ethanol. Cells were then embedded in EMBED 812 resin for 12 h. Resin polymerization was obtained at 60°C for 48 h. Ultrathin sections of 70 nm were prepared, stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Hitachi HU12A, Japan) operating at 75 kV.

### Quantification of USPION@Gastrin uptake by vibrating sample magnetometer

Magnetic measurements using a vibrating sample magnetometer (PPMS, Quantum Design, USA) were used as a complementary approach to determine iron content. ~105 CAF1-CCK1 or CAF2-CCK2 cells were respectively seeded onto 6-well plates at a density and grown for 24 h. Cells were incubated with USPION@Gastrin (16 µg magnetic Fe/ml) in DMEM-F12 medium buffered with 10 mM HEPES buffer pH7.4 containing 0.5% FBS and 100 IU/ml penicillin-streptomycin for 24 h, at 37°C in a 5% CO<sub>2</sub> atmosphere, washed twice with ice-cold PBS. The suspension containing a known number of cells was put into a magnetic measurement capsule, the excess of liquid removed, and the remaining liquid allowed to evaporate. Hysteresis loops were measured at 300 K up to a magnetic field of 5 T. The linear slope corresponding to the diamagnetic signal was removed from raw measurements. The obtained magnetization was divided by the saturation magnetization of the nanoparticles (65 Am<sup>2</sup>/kgFe) and the number of cells to get the content of iron per cell.

### Cell treatment by low magnetic field application and setups

Cells exposure to magnetic fields was performed as follows. CAF1-CCK2 or CAF2-CCK2 cells were seeded into 4-compartment Cellview dishes (Greiner Bio-One) for cell proliferation or cell death analysis respectively, grown overnight and incubated with USPION@Gastrin (4, 8 or 16 µg magnetic Fe/ml) at 37°C in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH 7.4 containing 0.5% FBS and 100 IU/ml penicillin/streptomycin. After removing the medium, cells were rinsed twice with



incubation medium, incubated in incubation medium and then exposed to magnetic field. For experiments with a rotating magnetic field (RMF), the setup used is a homemade bench based on a magnet rotated by a motor. The magnet itself is a 2.5 cm thick cylindrical Halbach array magnet with an inner diameter of 6 cm and an outside diameter of 15 cm. At each magnetic field amplitude is associated a different magnet. Different amplitudes (20, 40, 60, 200, 380 mT) and frequencies (0.2, 1, 5, 10, 20 Hz) were assayed. The magnetic field amplitude varies weakly with the position at the scale of the Cellview dish, as shown by space-dependent magnetic field measurements: for the magnet rated as “380 mT” and “40 mT” the magnetic field equals  $380 \pm 50$  mT and  $41 \pm 1$  mT, respectively. The rotating magnet is surrounded by a Plexiglas chamber, the temperature inside which is regulated using a fan able to blow heated air. For experiments with a static magnetic field (SMF), this first setup is used without magnet rotation. For the comparison between RMF and AMF, a second home-made setup is used. In this second setup, the magnetic field is generated by an ensemble of 6 laminated silicon iron magnetic poles surrounded by copper wires in a configuration resembling the one of stators. The sample space has a diameter of 4.5 cm. Depending on the phase of the current sent into the coils surrounding the poles, either a rotating magnetic field or an alternating one is generated. The temperature inside the sample space and the coil is regulated using a similar device than with the previous magnetic field generator. In all these experiments, the temperature of the Cellview dish was maintained at  $37.0 \pm 0.2^\circ\text{C}$  during magnetic field exposure. At the end of the experiments, cells were placed in a humidified atmosphere at 5%  $\text{CO}_2$  and  $37^\circ\text{C}$ .

### Determination of cell death

$25 \times 10^3$  cells/compartiment of CAF1-CCK2 or CAF2-CCK2 cells were seeded into 4-compartment Cellview dishes (Greiner Bio-One) for cell proliferation or cell death analysis respectively, grown overnight and incubated with USPIO@Gastrin (4, 8 or 16  $\mu\text{g}$  magnetic Fe/ml) for 24 h at  $37^\circ\text{C}$  in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH 7.4 containing 0.5% FBS and 100



IU/ml penicillin/streptomycin. After removing medium, cells were rinsed twice with incubation medium, incubated in incubation medium with or without 10  $\mu$ M CA-074 Me or 10  $\mu$ M Pepstatin A for 1 h, and then exposed to magnetic field for 2 h. The effects of magnetic field treatments were investigated by labeling dead cells with Annexin V-iFluor488 (AnnV) and Propidium Iodide (PI) (excitation: 488 and 540 nm respectively, AAT Bioquest), 4 h after magnetic field exposure and counting them using a BD FACSCalibur™ flow cytometer.

### Cell proliferation analysis

10  $\times$  10<sup>3</sup> cells/compartment of CAF1-CCK2 or CAF2-CCK2 cells were seeded into 4-compartment Cellview dishes (Greiner Bio-One) for cell proliferation or cell death analysis respectively, grown overnight and incubated with USPIO@Gastrin (4, 8 or 16  $\mu$ g magnetic Fe/ml) for 72 h at 37°C in DMEM/F-12 medium buffered with 10 mM HEPES buffer pH 7.4 containing 0.5% FBS and 100 IU/ml penicillin/streptomycin. After removing medium, cells were rinsed twice with incubation medium, then exposed to magnetic field for 2 h once/day. The effects of magnetic field treatments were investigated on cell proliferation by counting the cell number by a cell counter (Beckman cell counter z2) after 24h of RMF treatment. Cells were counted once per day for 6 days.

### Lysosomal membrane permeabilization

CAF1-CCK2 and CAF2-CCK2 cells having internalized USPIO@Gastrin were exposed to low frequency RMF for 2 h, incubated with DMEM-F12 medium 0.5% FBS containing 10 nM LysoTracker Green DND-26 (Invitrogen, excitation wavelength: 488 nm) for 15 minutes, rinsed with incubation medium. Lysosome integrity was determined by analyzing LysoTracker fluorescence intensity by flow cytometry using a BD FACSCalibur™ flow cytometer.

### Statistical analysis



Results are expressed as the mean  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed using one- or two-way ANOVA test. Differences were considered significant when  $p < 0.05$ .

### Conflicts of Interest

The authors declare no conflict of interest.

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### Author contribution

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