Published in final edited form as:

ACS Appl Mater Interfaces. 2017 November 15; 9(45): 39283–39302. doi:10.1021/acsami.7b15116.

Hydroxyapatite as a Vehicle for the Selective Effect of Superparamagnetic Iron Oxide Nanoparticles against Human Glioblastoma Cells

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Abstract

Despite the early promises of magnetic hyperthermia (MH) as a method for treating cancer, it has been stagnating in the past decade. Some of the reasons for the low effectiveness of superparamagnetic nanoparticles (SPIONs) in MH treatments include (a) low uptake in cancer cells; (b) generation of reactive oxygen species that cause harm to the healthy cells; (c) undeveloped targeting potential; and (d) lack of temperature sensitivity between cancer cells and healthy cells. Here we show that healthy cells, including human mesenchymal stem cells (MSCs) and primary mouse kidney and lung fibroblasts, display an unfavorably increased uptake of SPIONs compared to human brain cancer cells (E297 and U87) and mouse osteosarcomas cells (K7M2). Hydroxyapatite (HAP), the mineral component of our bones, may offer a solution to this unfavorably selective SPION delivery. HAP nanoparticles are commended not only for their exceptional biocompatibility but also for the convenience of their use as an intracellular delivery agent. Here we demonstrate that dispersing SPIONs in HAP using a wet synthesis method could increase the uptake in cancer cells and minimize the risk to healthy cells. Specifically, HAP/SPION nanocomposites retain the superparamagnetic nature of SPIONs, increase the uptake ratio between U87 human brain cancer cells and human MSCs versus their SPION counterparts, reduce migration in a primary brain cancer spheroid model compared to the control, reduce brain cancer cell viability compared to the treatment with SPIONs alone, and retain the viability of healthy human MSCs. A functional synergy between the two components of the nanocomposites was established; as a result, the cancer versus healthy cell (U87/MSC) selectivity in terms of both the

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b15116.

Scanning electron micrographs of SPION/HAP nanohybrids and of its individual components; uptake of different types of HAP nanoparticles by hMSCs and by E297 and U87 glioblastoma cells; actin cytoskeleton anisotropy in U87 cells following the uptake of SPION/HAP nanohybrids, its individual components and MBs; and programming code used to quantify intracellular nanoparticle localization (PDF)

uptake and the toxicity was higher for the composite than for SPIONs or HAP alone, allowing it to be damaging to cancer cells and harmless to the healthy ones. The analysis of actin cytoskeleton order at the microscale revealed that healthy MSCs and primary cancer cells after the uptake of SPIONs display reduced and increased anisotropy in their cytoskeletal arrangement, respectively. In contrast, the uptake of SPION/HAP nanocomposites increased the cytoskeletal anisotropy of both the healthy MSCs and the primary cancer cells. In spite of the moderate specific magnetization of HAP/SPION nanohybrids, reaching 15 emu/g for the 28.6 wt % SPION-containing composite, the cancer cell treatment in an alternating magnetic field resulted in an intense hyperthermia effect that increased the temperature by ca. 1 °C per minute of exposure and reduced the cell population treated for 30 min by more than 50%, while leaving the control populations unharmed. These findings on nanocomposites of HAP and SPIONs may open a new avenue for cancer therapies that utilize MH.

Graphical Abstract

Keywords

brain tumor; cancer; cytoskeleton; glioblastoma; iron oxide; magnetite; hydroxyapatite; uptake

1. INTRODUCTION

Cancer is the second leading cause of death in the United States,¹ and no treatment offers a panacea for the various types of cancer that exist. Superparamagnetic iron oxide nanoparticles (SPIONs), in turn, have been approved by the FDA as contrast agents in magnetic resonance imaging, but their potential applications are numerous. One such application is magnetic hyperthermia (MH) where magnetic particles subjected to an alternating magnetic field generate heat² through multiple mechanisms including (i) hysteresis losses, (ii) Néel relaxation, (iii) Brownian relaxation, and (iv) frictional losses, causing the deterioration of cancer cells without the use of strong chemotherapeutics.^{2–4} MH was once considered the most prospective potential fourth leg of the traditional cancer therapy triad including surgical resection, chemotherapy, and radiation therapy⁵ but has been in steady decline over the past two decades.⁶ This has been in spite of the moderately promising clinical trials; for example, patients with glioblastoma multiforme subjected to six sessions of MH treatment following injection of SPIONs into the tumor area exhibited an

increased median survival time: 13.4 vs 6.2 months.⁷ One of the reasons for this decline is the never verified assumption that cancer cells are more susceptible to heat than healthy cells, on which MH as a ubiquitous cancer therapy was based. In reality, this heat sensitivity varies between different types of cells and tissues.⁸ Thus, to revitalize MH, it is necessary to focus on another effect where this selectivity can be established. In this study, this effect is tied to the fact that SPION effectiveness in MH is hindered also by the low uptake in cancer cells and the generation of reactive oxygen species that cause harm to the healthy cells in the body.⁹ To that end, we examine the variability in the uptake of magnetic nanoparticles delivered with the help of a carrier, hydroxyapatite (HAP), as well as in the cytoskeletal actin microfilament organization following the uptake.

The choice of HAP, the synthetic version of the mineral component of our bones,¹⁰ is justified not only by its bioresorbability, facile synthesizability, and cytophilicity^{11,12} but also by the convenience of its use as a nonviral and nonimmunogenic transfection agent.^{13,14} Technically, both HAP and SPIONs begin to dissolve in the moderately acidic environment of the late endosome, allowing for the endosomal escape of the cargo before its lysosomal degradation. Because of these general properties of HAP, including the ability to transfect an array of different cell lines,¹⁵ its applicability need not be limited to bone, and here we extend it to a brain cancer model. The favorability and naturalness of combining SPIONs and HAP have been previously verified. For example, even though under normal conditions magnetite nanoparticles convert to maghemite at 300 °C and then to hematite at 350 °C,¹⁶ interspersing of SPIONs through HAP leads to stabilization of the maghemite phase up to 600 °C and retention of strong magnetism even past that point,¹⁷ indicating an intimate interaction between HAP and SPIONs, which is the basis for the proposed use of HAP as a chaperone-like carrier of SPIONs. The toxicity of SPIONs was also ameliorated following their embedment into a HAP matrix.¹⁸ Taking advantage of the ability of HAP to complex and be doped with an array of catalytic ions and of the ability of SPIONs to be magnetically separated, the combination of these two materials has been considered a route to efficient “green” heterogeneous catalysts in organic chemistry.¹⁹ A similar manipulability was achieved by combining SPIONs with microcrystalline calcite, another major biomineral.²⁰ On top of this, deposits in the world’s largest underground iron ore mine, in Kiruna, are of an iron oxide–apatite form,²¹ and their geological presence, like that of nelsonites, another iron oxide–apatite ore, is abundant all across the planet, prompting us to repursue the geo-inspired design of advanced materials elaborated previously²² in search of a solution for specific medical problems.

The hypothesis laying the foundation for this study is that dispersing SPIONs within HAP nanoparticles could increase the uptake ratio between cancer cells and healthy cells and thus enable a selective therapeutic activity, toxic for the cancer cells and harmless for the healthy ones. Correspondingly, the goal of this work has been the construction and characterization of a HAP/SPION nanocomposite as an MH device capable of confining the destructive effects onto cancer cells only while minimizing the risks imposed onto healthy ones.

2. MATERIALS AND METHODS

2.1. Synthesis of SPION, HAP, and HAP/SPION Nanocomposite

SPIONs were precipitated by adding a 100 mL aqueous solution containing 0.1 vol % Triton X-100 (Arcos Organics, New Jersey, NJ), 2 vol % ammonia (Sigma-Aldrich, St. Louis, MO), and 1 M NaOH (Fisher Scientific, Hampton, NH) at 1 drop per second to 100 mL of an aqueous solution containing 10 mM FeCl₃ (FeCl₃·6H₂O, Alfa Aesar, Haverhill, MA) and 5 mM FeCl₂ (FeCl₂·4H₂O, Alfa Aesar, Haverhill, MA) at 80 °C and under a stirring rate of 1800 rpm. The resulting dispersion of SPIONs was mixed vigorously at 1800 rpm for 1 h, thus creating the 100 mg/mL SPION stock solution. For individual assays, this stock solution was diluted with deionized water to 5 mg/mL standards. Final concentrations of nanoparticle compositions are given in Table 1. As an industry standard, commercially available magnetic iron oxide beads (MB) were diluted with deionized water to 5 mg/mL. MBs were used to determine the effectiveness and efficacy of a commercially available SPION against that of the SPIONs synthesized here.

Two forms of HAP were synthesized: (i) HAP prepared with no base added to precursor solutions, henceforth known as nHAP, and (ii) HAP prepared under alkaline conditions and known as sHAP. The two types of HAP were analyzed in parallel to eliminate the effects of surface composition or structural variance and increase the confidence that the experimentally observed effects are due to HAP itself. nHAP was synthesized using a modified procedure reported previously.²³ A 100 mL solution of 0.1 M calcium nitrate tetrahydrate (Fisher Scientific, Hampton, NH) and 100 mL of 0.06 M ammonium phosphate monobasic (Fisher Scientific, Hampton, NH) were mixed together dropwise by adding the calcium solution into the phosphate solution at 13.33 mL per minute for 1 h. sHAP was synthesized using the procedure described earlier.²⁴ The procedure was identical to that used to precipitate nHAP except the calcium and phosphate solutions contained 12 and 6 mL of ammonium hydroxide (Sigma-Aldrich, St. Louis MO), respectively. The final yield was 5 mg/mL (1 mg per 200 mL synthesis volume). Both HAP materials underwent the same washing procedure after synthesis. The milky suspensions were poured into 50 mL Falcon tubes and centrifuged at 3500 rpm for 5 min. The supernatant was discarded, and 35 mL of deionized water was poured into each tube, vortexed, then centrifuged again at 3500 rpm for 5 min. This procedure was repeated once more with 100% ethanol instead of deionized water. Following centrifugation, samples were dried for 48 h at 37 °C in an Incu-Shaker (Benchmark Scientific, South Plainfield, NJ).

Four HAP/SPION nanocomposites with two different SPION concentrations in both nHAP and sHAP were made by adding SPION suspensions into the calcium solution prior to precipitation of HAP. Final concentrations of each material are listed in Table 1. In short, adding 1 or 10 mL of 40 mg/mL SPION stock suspension to the 100 mL 0.1 M calcium solution as a precursor for HAP before mixing the calcium and the phosphate solutions yielded either 1Cs/nHAP or 10Cs/nHAP, respectively. As shown in Figure 1, these composites contained 3.8 and 28.6 wt % of the SPION component, respectively. Washing and drying of the precipitated nanocomposite was the same as that used for the preparation of HAP.

2.2. Physicochemical Characterization

Transmission electron microscopy (TEM) images were taken on a JEOL JEM 1220 Life Science TEM. Scanning electron microscopy (SEM) images were taken on a JEOL JSM 6320 FE-SEM. The aspect ratio of needle-like HAP particles was measured using MATLAB's (Natick, MA) Image Viewer app. Particle size and zeta potential were measured using dynamic light scattering (DLS) on a Zetasizer Nanoseries (Malvern, UK). Zeta potential measurements were conducted in the 2–10 pH range and scanned 30 times for 10 s at each data point. SQUID magnetometry was run on 1CsHAP and 10CsHAP compositions using a MPMS-5 Quantum Design SQUID magnetometer operated at room temperature and under a maximum applied magnetic field of $H = 5$ kOe.

X-ray powder diffraction (XRD) analysis was conducted on a Bruker D2 Phaser diffractometer. Scans were performed in the 10–90° range, with a 0.0012 step size and 1 s of scan time per step for a total scan time of 2 h. The crystallinity of the HAP phase was calculated using a previously reported procedure.²⁵ Specifically, per eq 1, the degree of crystallinity, X_c , is related to the intensity of the (300) reflection, I_{300} , and the intensity of the hollow between (112) and (300) reflections, $V_{112-300}$, which disappears in noncrystalline samples.

$$X_c \approx 1 - \left(\frac{V_{112-300}}{I_{300}} \right) \quad (1)$$

Iron oxide phase composition was quantified using another procedure reported previously.²⁶ Specifically, per eq 2, the wt % of maghemite (γ -Fe₂O₃) is directly related to the intensity fraction of the maghemite-derived (511) reflection, $I_{(511)\text{maghemite}}$, and the magnetite-derived (511) reflection, $I_{(511)\text{magnetite}}$ in the 56–58.5 2θ range. The wt % of magnetite (Fe₃O₄) was 100% – wt % of maghemite. The diffraction patterns were smoothed using MATLAB before quantification.

$$\left[\frac{I_{(511)\text{maghemite}}}{I_{(511)\text{magnetite}} + I_{(511)\text{maghemite}}} \right] = 1.0136w_{\text{maghemite}} - 0.2371 \quad (2)$$

The average crystallite size was calculated by employing the Scherrer equation on a Bruker's EVA software, correlating integral breadths of the most prominent reflections for HAP and SPIONs to the crystallite dimensions, specifically (002), (211), and (310) for HAP and (311) and (400) for SPIONs.

2.3. Cell Culture

Primary lung and kidney fibroblasts were isolated from lungs and kidneys of two 8-week old, female C57BL/6J mice. In brief, organs were placed on Petri dishes with Hanks' Balanced Salt Solution (Life Technologies, Carlsbad, CA) and continuously minced with a sterile razor blade until a homogeneous mixture was achieved. The mixture was transferred

into a 50 mL Falcon tube containing 5 mg of collagenase type IV plus 20 mL of DMEM with 10% FBS and 5% antibiotic–antimycotic (Life Technologies, Carlsbad, CA) and incubated at 37 °C. For every kidney and liver, 5 and 10 mL of DMEM were used, respectively. After 30 min, the solution containing the tissue was shaken by hand for 2 min before being incubated for another 30 min. This procedure was repeated twice. After the final incubation, the solid cell debris was allowed to settle by gravity and cell suspensions were transferred to 10 cm culture plates with 10 mL of the suspension per dish.

K7M2-pCI Neo mouse osteosarcoma cell line was purchased from the American Type Culture Collections (Manassas, VA). U87-MG and E297 human glioblastoma cells were a gift from Herbert H. Engelhard (UIC, Department of Neurosurgery). U87 is an established cell line dating back to 1968.²⁷ E297 is a patient cell line extracted in 1991²⁸ that does not require immunosuppressants to grow tumors on rats.²⁹ Human mesenchymal stem cells (hMSCs) were a gift from Anne George (UIC, Department of Oral Biology). All cell lines except for hMSCs were maintained in DMEM with 10% FBS, 1% antibiotic–antimycotic (Life Technologies, Carlsbad CA). Human MSCs were maintained in MesenPRO RS culture medium composed of 10 mL of MesenPRO RS growth supplement and 5 mL of GlutaMAX-I in 500 mL of MesenPRO RS basal medium. Cell lines were grown to confluency before being plated on 12 mm circular glass coverslips or in 48-well culture plates.

2.3.1. Cell Viability Assay—A 12 mM 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) stock solution was prepared by adding 1 mL of sterile phosphate-buffered saline (PBS) to a 5 mg vial of MTT and vortex-mixing to ensure complete dissolution. Nanoparticles were diluted into 5 mg/mL standards in water and sonicated for 1 min. Cell lines were grown in 48-well plates until confluency. Upon confluency, 250 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ of nanoparticles was added to each well. Each plate contained four wells of negative control (growth medium alone), four wells of sample solution control (5% v/v water in media), four wells of sample controls (either 250 or 50 $\mu\text{g/mL}$ of nanoparticles in media without the cells), four wells of 250 $\mu\text{g/mL}$ nanoparticle samples, and four wells of 50 $\mu\text{g/mL}$ nanoparticle sample. To account for the effect of nanoparticles per se on the absorbance in the lysate, the sample control absorbance was subtracted from that of the nanoparticle samples. After 24 h of incubation, cells were washed with PBS, and 275 μL of 1:10 MTT/media v/v was added into each well. After 4 h of incubation at 37 °C, 211 μL of the solution was carefully removed and 125 μL of DMSO was added to each well. Plates were placed in a 37 °C incubator shaker at 120 rpm for 30 min before measuring the absorbance at 570 nm using the BMG LABTECH FLUOstar Omega microplate reader. Viability was expressed in percentages and normalized to the absorbance of both the negative and sample control.

2.3.2. Nanoparticle Uptake Analysis—Cell cultures on coverslips were treated with 250 $\mu\text{g/mL}$ of each nanoparticle system for 24 h before being washed, fixed, permeabilized, and stained. For comparison between SPIONs, nHAP, sHAP, and HAP/SPION nanocomposites, cells were fixed with 4% paraformaldehyde and then washed with the wash buffer (0.1 wt % Triton X and 0.1 wt % bovine serum albumin in PBS) and stained with 0.2 mL per well of the staining solution containing 1:4000 v/v AlexaFluor 568 Phalloidin as an

actin-staining reagent and 1:100 v/v Lonza's OsteoImage as HAP-staining reagent, along with one drop of NucBlue ReadyProbes as a cell nucleus-staining reagent. Fixed cells were incubated at room temperature for 2 h before being mounted and cured with ProLong Diamond antifade mounting agent. Samples that did not contain HAP were stained using the same protocol and reagents, excluding OsteoImage. To visualize SPIONs and MBs in an epifluorescence setting, actin was stained with both AlexaFluor 568 Phalloidin fluorescing in red and an overdose of ActinGreen 488 ReadyProbes Reagent fluorescing in green. This overdose effectively saturated the image background with green color and allowed for the visualization of SPIONs and MBs as a dark contrast to it. This contrast was exploited to determine the cell uptake of SPIONs and MBs and compare it between the cancerous, U87, and E297 cell lines and hMSCs.

For comparison of SPION uptake in three different types of cancer cells and three different types of normal cells, Prussian Blue was utilized as iron-staining reagent and Nuclear Fast Red as cell nucleus-staining reagent. Fixed cells were stained with 0.5 mL of 5 w/v % potassium hexacyanoferrate (II) trihydrate and incubated at room temperature for 5 min to allow Prussian blue pigment to form and then washed with distilled water before 0.5 mL of Nuclear Fast Red solution was added and incubated for 5 min at room temperature. Stained cells were washed with PBS before being mounted and cured with ProLong Diamond antifade mounting agent. The presence of blue color (Prussian blue) on slides seeded with cells confirmed the presence of ferric ions and SPIONs. The uptake was determined using the MATLAB code, Localization.m (Supporting Information).

Fluorescently stained cells were imaged using a Nikon Eclipse Ti fluorescent optical microscope with a DS-Fi2 color camera with a DS-U3 controller and a DS-Qi2 CCD camera with X-Cite 120 LED, respectively, using a Plan Apo λ 60x oil objective. Individual cells were cropped from composite images and processed using MATLAB to calculate (a) the amount of SPIONs or MBs uptaken by E297 using LocalizationBlackfromRB.m (Supporting Information); (b) the amount of SPION or MB uptake in U87 and MSCs using LocalizationBlackfromGreen.m (Supporting Information); and (c) the amount of uptaken HAP-containing materials using LocalizationGreen.m (Supporting Information). In short, images were split into their respective color channels, and the blue channel was subtracted from the green to reduce noise before being converted into black-and-white based on a specific color with a threshold. The number of pixels that exceeded the threshold was calculated and then multiplied by a factor of $0.05 \mu\text{m}/\text{pixel}$, determined by measuring the number of pixels within the scale bar of an image. The result equaled the number of micrometers in an image and was divided by the size of the nanoparticles in a sample determined by DLS to calculate its uptake, then normalized to the number of cells in the sample. Thus, derived uptake expressed in terms of the number of particles per cell was compared by measuring the surface area occupied per cell by the uptaken material using ImageJ software (NIH, Bethesda, MD).

2.3.4. Spheroid Migration Assay—Ten thousand E297 human glioblastoma cells were plated on Corning 96 well ultralow adhesion microplates for 96 h for multicellular spheroid formation. After 96 h, spheroids were individually plated onto 0.1% gelatin-coated 48 well plates and treated with $250 \mu\text{g}/\text{mL}$ of nanoparticle samples for 72 h. The plates were imaged

using a Nikon Eclipse Ti fluorescent optical microscope equipped with a DS-Fi2 color camera with DS-U3 controller using a Plan Fluor 10× lens. Images were stitched together using Adobe Photoshop (San Jose, CA, USA) and the length of each cell migration was measured with the Image Viewer app of MATLAB. Cell lengths were calculated by multiplying the pixel distance from the Image Viewer app by a factor of 1.36 $\mu\text{m}/\text{pixel}$, determined by measuring the number of pixels within the scale bar of an image.

2.3.5. Cytoskeletal Anisotropy Analysis—Individually cropped fluorescent cell images had their actin cytoskeletal patterns quantified using FibrilTool,³⁰ an ImageJ³¹ plug-in. In short, an entire cell was outlined to allow FibrilTool to provide the anisotropy of the actin cytoskeleton in a given region of interest. Cytoskeletal anisotropy was defined as a value between 0 for no order and 1 for perfectly ordered.

2.3.6. Magnetic Hyperthermia analysis—To assess the antitumoral activity of the MH effect resulting from the heat dissipation from HAP/SPION nanocomposites in an alternate magnetic radiofrequency field, E297 cells were seeded in rectangular, μ -Slide 8-well plates (*Ibidi*) and grown to confluency. The heating experiments were performed under ambient conditions and the wells containing the cells were held inside an insulating box to prevent the heat from dissipating. 10CsHAP particles were then added to the cells at the concentration of 5 mg/cm² in 100 μL of the DMEM cell culture medium and incubated for 30 min before plates were placed inside a multiturn helical induction coil of an induction heating system (Ultraflex SH2/350) and kept in the alternate magnetic field (1.16 μT , 350 kHz, 1 kW) for another 30 min, after which the particles were gently washed off from the cells and the cells were allowed to recover for 24 h. After the given period of time, an MTT assay was run to test for viability, as described in section 2.3.1. Temperature readings were made by periodically pulling the wells out of the coils and immersing the tip of a pocket-size thermometer with water-resistant case (Fisher Scientific Traceable Total-Range Thermometer) into the medium.

2.4. Statistical Evaluation

Outliers were removed using Tukey Fences³² where upper and lower bounds are defined as the 1.5 times the interquartile range added to the third quartile and 1.5 times the interquartile range subtracted from the first quartile, respectively. Any value beyond the bounds was considered an outlier. The data are expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined using a student's *t* test. A value of $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Structural Characterization of HAP/SPION Nanocomposites

The two components of nanocomposites, SPION and HAP, differed in morphology: while the narrowly dispersed SPIONs were round-shaped, having 25 nm in size on average, both sHAP and nHAP nanoparticles were rod-shaped, having average widths of 20 and 9 nm, respectively, and average lengths of 174 and 15.3 nm, respectively (Figure 2). Both components of the SPION/HAP composites were, thus, confirmed as nanosized, and the

morphological properties of the composites were dominated by their major phase, HAP. HAP nanoparticles precipitated under more alkaline conditions, sHAP, exhibited a higher degree of elongation, as measured by their aspect ratio of 8.7, as opposed to only 1.7 for nHAP. This difference in elongation depending on pH is explained by the hydroxyl ions filling the channels that pass straight through the center of the calcium hexagons of $P6_3/m$ symmetry and defining the c -axis of the hexagonal HAP unit cell.³³ Namely, lower concentrations of free hydroxyls at the lower pH of precipitation obstruct the uniaxial growth along the c -axis, as the result of which the aspect ratio of sHAP is significantly higher than that of nHAP. That HAP crystals observed here indeed elongate parallel to the c -axis, increasing the surface prominence of prismatic, $(hk0)$ planes, is confirmed by the pronounced difference in crystallite sizes measured along different crystallographic directions. Namely, the crystallite size estimated from the (002) reflection sandwiched between the two basal planes of HAP hexagons is in the range of 30 nm for all HAP and HAP/SPION nanocomposite samples, whereas that estimated from the (211) plane lying more parallel to the c -axis than (002) is in the sub-10 nm range (Table 2). In contrast, as expected from the approximate shape isotropy of SPIONs, the crystallite sizes estimated in [311] and [400] directions were almost identical, 19.6 and 22.6 nm, respectively, reflecting the particle aspect ratio estimated from TEM images: 1.1. This crystallite size was just slightly below the average particle size of 25 nm observed under TEM, suggesting the high degree of magnetic dipole ordering within the particle, as reflected in comparatively high magnetization values. Despite the round particle morphologies, the planar facets were prominent on the surface of SPIONs, facilitating the greater crystalline and magnetic dipole ordering compared to that seen on the convex surfaces curved at the atomic scale,³⁴ where glassy magnetic disorder caused by a high degree of spin canting is pronounced. The aspect ratio, along with the particle widths exhibited a drop as the amount of SPIONs in the solution precipitating HAP increased from 0 to 0.2 mg/mL to 2 mg/mL, specifically from 8.7 to 4.8 to 3.9, and this is visible from the corresponding TEM images (Figure 3). The particle width also dropped from 20 to 11 to 8 nm in the same range. The reason is that the increase in the concentration of SPIONs provided an additional hydrophilic nucleation surface in the solution, which increased the nuclei density and correspondingly dropped the particle size and decreased the crystallinity. Concordantly, the total crystallinity was indistinguishable between sHAP and nHAP, being around 32% for both, as estimated from eq 1, but decreased down to 20 and 23.5%, respectively, following the addition of SPIONs (Table 3).

Both SEM (Figure S1) and DLS confirmed moderate agglomeration of all particles. This is evidenced by the consistently larger hydrodynamic diameters of particles in suspension (Table 3) than those visualized under TEM (Figure 2). One of the central advantages of superparamagnetic particles is their lack of remanence, minimizing the magnetic dipole attraction between the adjacent particles in suspension and increasing their colloidal stability. However, in reality, even the minor amount of remanence is sufficient to cause a detectable level of aggregation in such colloidal systems. In contrast, the drive for aggregation in HAP systems comes predominantly from low particle surface charge and insufficient electrostatic repulsion between the particles, allowing the attractive forces of the equilibrium described by the DLVO theory to prevail. Such low surface charge gives HAP an

advantage over the systems that rely on high electrostatic charge, especially if positive, to permeate the plasma membrane and enter the cytoplasm, the reason being the proneness of the latter types of systems to react with the first oppositely charged barriers that they encounter en route to the target tissue while traveling through the vasculature.³⁵ Although moderate aggregation of nanoparticles in suspension is not ideal, the 300–500 nm hydrodynamic diameter range of HAP/SPION hybrids (Table 3) is adequate for parenteral application,³⁶ given that this submicron size range is still considerably lower than the 5–10 μm diameter of the smallest capillary or the dimensions of the discoid erythrocytes: $7 \times 1.7 \mu\text{m}$ on average. Intravenously administered formulations containing HAP particles with 20–60 μm in size were used earlier without any adverse effects.³⁷

X-ray diffractograms of SPIONs and different HAP samples and nanocomposites are shown in Figure 4a. Despite the broad diffraction peaks, which were suggestive of the low crystallinity entailing the nanosized character of both composite components, the characteristic peaks of both the $P6_3/m$ hexagonal crystal structure of HAP and Fd3m face-centered cubic, inverse spinel crystal structure of magnetite were detected in the corresponding XRD patterns. The following were the most intense reflections for HAP in the order of their intensity: (211) at 31.86° , (002) at 25.90° , (213) at 49.51° , (222) at 46.69° , (321) at 53.27° , (310) at 39.86° , (202) at 34.22° , and (502) at 63.07° . The following were the most intense reflections for SPIONs in the order of their intensity: (311) at 35.58° , (440) at 62.82° , (333) at 57.20° , (400) at 43.25° , and (220) at 30.21° . Magnetite reflections, specifically (311) and (400), were distinguishable only in the composites with a higher weight content of SPIONs. In addition to aforementioned HAP and SPIONs crystallinities and crystallite sizes in different crystallographic directions, these patterns indicated the mixed magnetite/maghemite composition of SPIONs (Table 3). Although the specific magnetization of maghemite is lower than that of magnetite,³⁸ this phase is being more favored for MH over magnetite because the extent of DNA damage is dependent on the Fe oxidation state³⁹ and lesser for Fe^{2+} than for Fe^{3+} .

3.2. Magnetic Properties of HAP/SPION Nanocomposites

Magnetic hysteresis curves for 1CsHAP and 10CsHAP nanocomposites are shown in Figure 4b. In spite of the relatively low content of SPIONs in nanocomposites, specific saturation magnetization (M_s) values were relatively high: 1.08 emu/g for 1CsHAP and 15.03 emu/g for 10CsHAP. Normalized to the weight percentage of the SPION component in 10CsHAP, M_s of pure SPIONs was in the excess of 50 emu/g. Given that M_s of bulk magnetite and maghemite are around 100 and 80 emu/g, respectively,⁴⁰ this value indicates a pronounced magnetic character of SPIONs incorporated into the nanocomposites. 1CsHAP has 7.5 times less of the SPION component per weight than 10CsHAP, yet the M_s value of 10CsHAP is 15 times higher than that of 1CsHAP, indicating the diamagnetic effect of the HAP component insulating SPIONs in the composite and interfering with the exchange interaction proportionally to its weight. With the SPION size of 20 nm, which is less than the predicted single domain size of ≈ 50 nm for cubic magnetite particles and ≈ 76 nm for the transition to two-domain particles,⁴¹ SPIONs analyzed here were single-domain. Correspondingly, as seen in Figure 4b, both 1CsHAP and 10CsHAP displayed superparamagnetic qualities. As seen in the inset of Figure 4b, 1CsHAP and 10CsHAP do have some remanence ($M_r = 0.048$

and 0.928 emu/g, respectively) and coercivity ($H_c = 31.5$ and 24.9 Oe, respectively), suggesting that a small portion of the magnetic domains remains blocked in the zero field. However, these values are significantly lower than those typifying bulk magnetite: ~15 times less of the M_s value for M_r , i.e., 6–7 emu/g, and $H_c = 250$ Oe. The SPION size of 20 nm is in the range near the maxima for Neel and Brownian relaxation thermal losses as a function of the particle size but below the range of dominance of hysteresis losses and has been considered ideal for MH applications.^{42,43}

3.3. Zeta Potential of HAP/SPION Nanocomposites

Comparative zeta potential vs pH curves for the two types of iron oxide nanoparticles and the two types of HAP nanoparticles studied, as well as for their composites, are shown in Figure 5. The zeta potential vs pH curves for both SPIONs and MBs were highly similar (Figure 5a). Dual points of zero charge (PZC) seen in both SPIONs and MBs are explained by the biphasic composition of the both. The composition of both SPIONs and MBs was biphasic, containing magnetite and maghemite (Table 3), endowing the zeta potential vs pH curves with the typical double hump character.⁴⁴ In maghemite, the concentration of oxygens is greater than in magnetite.⁴⁵ Since the oxygen groups on the iron oxide particle surface partially transform to hydroxyls, their negative charge will be higher and the point of zero charge lower than that of the less oxygenated particles. As a result, the higher PZC, detected at pH 7.2 for pure SPIONs, is magnetite-derived, as opposed to the lower one, detected at pH 3.5, which is maghemite-derived.

Unlike the zeta potential of SPIONs and MBs reaching the absolute value of 30 mV, the tentative threshold of colloidal stability, at $1.5 > \text{pH} > 8.2$, zeta potential of neither of the two types of HAP reached more than 10 mV in the acidic range or less than –25 mV in the alkaline range (Figure 5b). Low zeta potential decreases the stability of any colloid, including HAP.⁴⁶ At the physiological pH, the absolute zeta potential of all types of particles was lesser than 15 mV, i.e., within the window between –15 and 15 mV that is likely to cause the particles to agglomerate.⁴⁷ In addition, zeta potential curves of both 10C nanocomposites were dominated by HAP at $\text{pH} > 6$. At $\text{pH} < 6$, SPIONs dominated the zeta potential curves of both 10C nanocomposites (Figure 5c–d). This is explained by the dissolution of HAP at low pH values and the corresponding increase in the content of SPIONs in the composites.

3.4. Viability Assaying of HAP/SPION Nanocomposites

SPION/HAP nanohybrids reduced the viability of two glioblastoma cell lines, while mostly increasing the viability of hMSCs. As shown in Figure 6a–c, while the healthy cell viability for all six HAP-containing samples in the 50–250 $\mu\text{g/mL}$ dose range was above 80%, the cancer cell viability for both cell lines, U87 and E297, and for all the HAP-containing samples except 50 $\mu\text{g/mL}$ 10CsHAP in E297 was below it. SPION/HAP nanocomposites also did not reduce the viability of hMSCs when compared to any of the two experimental controls; in fact, they significantly increased their viability for both 50 and 250 $\mu\text{g/mL}$ doses of 10CnHAP, for 1 mg/mL dose of 1CsHAP and for 250 $\mu\text{g/mL}$ and 1 mg/mL doses of 10CsHAP (Figure 6a, d). At the same time, however, the nanocomposites reduced the viability of both human glioblastoma cell lines (Figure 6b,c). This selectivity is reiterated by

another observation: whereas an increase of the nanoparticle dose from 50 to 250 $\mu\text{g/mL}$ decreased the viability of the two cancer cell types, it increased the viability of hMSCs (Figure 6a–c). Both the synthesized and the commercial iron oxide nanoparticles, i.e., SPIONs and MBs, respectively, reduced the viability of hMSCs compared to the two experimental controls (Figure 6a). At the same time, SPIONs produced a less negative effect on the cancer cell lines than the nanocomposites did, reducing the viability of U87 cells, but not of E297 ones too (Figure 6b,c). The combination of SPIONs with HAP, therefore, not only produces the desired selective effects against healthy and cancer cell lines, but it also compensates for the negative effects that SPIONs alone cause to the healthy cells.

As seen in Figure 6, the selective effects are due to the selectivity of HAP carrier per se. While neither of the two HAP samples significantly lowered the viability of hMSCs and sHAP at the 250 $\mu\text{g/mL}$ dose even increased it significantly compared to the sample control, both types of HAP at both dosages decreased the viability of both glioblastoma cell lines down to 60% of the negative control and 67% of the sample control on average. Furthermore, as seen in Figure 6d, at the dose of 1 mg/mL and in a sparser cell population, HAP was the only sample that lowered the viability of a cancer cell line, specifically E297. As the content of HAP decreased and the content of SPIONs decreased in the sample, so did the viability of E297 cells recover. Meanwhile, the viability of U87 cells and MSCs remained unaffected. This positive cancer versus healthy cell specificity exhibited by HAP makes it theoretically capable of reinforcing the therapeutic effects of anticancer medications. Reports of similarly selective anticancer activities of HAP could be found in the literature. HAP, for example, imparted a cytotoxic effect on hepatoma cells, but not on healthy hepatocytes too.⁴⁸ Gastric cancer, liver cancer, and osteosarcomas cell lines also experienced a greater degree of proliferation inhibition than did the primary hepatocytes, lung fibroblasts, and keratinocytes.⁴⁹ Effects on lung cells challenged with HAP were similarly selective: toxic to alveolar adenocarcinoma A549 cells and harmless to normal bronchial epithelial 16HBE cells.⁵⁰ However, no cytotoxic effects of pure HAP on any glioblastoma cells have been reported previously.

Agglomeration is one of the factors of a potentially significant disparity between the nanoparticle/cell interactions in vitro and in vivo; namely, while aggregation in vivo would likely prevent the particles from reaching their target cells, this very same aggregation in cell culture may segregate the solids to the bottom of the well and bring in direct contact with the cells, thus fostering the uptake to a greater degree than would have occurred in very stable colloids. That gravity can be a more significant determinant of the uptake than particle size, shape, density, or concentration was demonstrated by comparing the uptake in inverted and in upright cell culture settings.⁵¹ This effect may explain how come the cytotoxicity of HAP and HAP/SPION particles in U87 or E297 cells was less than that caused by the less agglomerating SPIONs (Table 3) at the same concentration. Depending on the nanoparticle, however, agglomeration can also reduce the efficacy of vehicle delivery to the cells.⁵² Although there are uptake pathways that are more open when particles reach the cell membrane as agglomerates,^{53,54} agglomeration often reduces the Brownian motion and causes particle sedimentation in form of chunks too large to be uptaken by cells. However, it is difficult to untangle the pros from cons of agglomeration in the context of HAP/SPIONs: for, while agglomeration is a key to the capture of SPIONs by HAP, aggregation occurring

before HAP/SPION particles can reach the cells may unfavorably affect the cytotoxicity imparted onto cells.⁴⁸ With this in mind, the agglomeration rate of HAP/SPION particles needs to be determined as a function of the particle concentration before the conditions for their optimal uptake can be derived.

3.5. Tumor Spheroid Migration Assays

Measuring the cell migration out of E297 spheroids demonstrated that all of the nanoparticles acted as barriers for cancer cell migration (Figure 7). The control spheroids had the average migration of 478.5 μm , while the majority of the tested materials did not allow for more than 100 μm migration. Since it is generally established that thriving, robust cells migrate further and faster than the harmed cells,⁵⁵ this limited migration distance indicates a successful treatment. As seen in Figure 7, the control spheroid has long arms extending from it, but SPION and MB particle treatments block this extension. The resulting modified shape resembles that of a toroid, with a lesser cell density in the middle of the multicellular spheroid. Cells in the center of a tumor are more prone to necrosis because the obstructed diffusion of the material deep into a tumor without angiogenesis,⁵⁶ but since this effect does not directly correlate to the loss of viability, it can also be due to altered intercellular communication as the result of migration.⁵⁷ HAP and HAP/SPION nanocomposites also acted as barriers for migration, though without producing changes in contrast in the spheroid center. 1CnHAP elicited a lesser cell migration rate than its nHAP component, whereas the migrations over 1CsHAP and sHAP nanoparticles were comparable in magnitude. Due to the agglomeration of HAP and HAP/SPION particles, large crystals appear near the spheroids. Even if there was an open channel for migration, more prone to be present when irregularly shaped and large crystals are present, it was difficult for the tumor cells to migrate due to the cytotoxicity imparted by the material.

3.6. Comparative Cell Uptake Analysis

To effectively cause thermal inactivation and necrosis in cancer cells with MH, SPIONs need to be either taken up by the targeted tumor cells or lined against their outer membrane. However, all three types of healthy cells tested in this study, including human mesenchymal stem cells (MSCs) and primary mouse kidney and lung fibroblasts, displayed a greater uptake of SPIONs than all three of the cancer cell lines tested: K7M2, U87, and E297 (Figure 8a). Previous studies demonstrated a similar trend of the uptake of SPIONs being higher in healthy cells than in cancerous ones.⁵⁸ This disparity can lower the prospects of successful MH treatment of cancer and methods are needed to ensure the reversal of this trend. Specifically, carriers are required that would promote an increased uptake or membrane binding of SPIONs by the cancer cells compared to the healthy ones. In such a way, the chances of destroying the malignant cells would be increased and the risks of harming the healthy cells minimized.

The greater uptake of SPIONs by the healthy cells than by the cancer cells disproves the common assumption that cancer cells should uptake more nanoparticles than the healthy cells owing to their very nature—"greedy" and programmed for uncontrolled growth. The increased SPION uptake in lung fibroblasts versus kidney fibroblasts is disconcerting but expected. Lung tissue is regularly a site of accumulation for nanoparticles such as gold,⁵⁹

carbon nanotubes,⁶⁰ and SPIONs,⁶¹ only surpassed by the accumulation in the liver and the spleen. Lung tissue was shown to have greater iron levels than kidney tissue within the first 24 h following intravenous injection of SPIONs.⁶¹ Liver and spleen are expected to accumulate larger SPION concentrations as both organ systems are involved in the reticuloendothelial system (RES)^{62,63} and in recycling and metabolizing iron.⁶⁴

The fact that SPIONs are significantly more uptaken by the healthy cells (MSCs) than by the cancer cells (U87 and E297) does not make them an ideal candidate for MH, as their imparting a greater toxicity and necrosis to healthy tissues rather than to cancerous ones would be expected based on this difference in the uptake. To attempt to reverse the trend of 8–9 times greater uptake of SPIONs by hMSCs than by U87 cells (Figure 8a), SPIONs were combined with HAP and delivered as such to the cells. As seen from Figure 8b, the ratio between the amount of particles uptaken by U87 cells and by hMSCs increases for HAP/SPION nanocomposites compared to SPIONs alone. It also increases with the concentration of SPIONs in the nanocomposites, being higher for 10CsHAP than for 1CsHAP and for 10CnHAP than for 1CnHAP. Since the uptake efficiency in U87 cancer cells increases together with the SPION content in the nanocomposites, the logical question is who is the carrier of whom—HAP of SPIONs or SPIONs of HAP? Whichever the answer, the cancer versus healthy cell selectivity (U87 vs MSC) in terms of both the uptake and the toxicity was higher for the composite than for SPIONs or HAP alone. Unfortunately, the trend with E297 cells was such that there was no statistical difference between any of the same groups. This is in spite of the likeliness that E297 cells would exhibit a more malignant tumorigenicity than U87 cells, as explained in section 2.3. Interestingly, MBs are much less effectively uptaken into cells than SPIONs, even though they displayed no difference in the uptake between healthy and cancer cells. In terms of absolute amounts, averaged across all the cell lines, neither nHAP nor sHAP alone were uptaken more than SPIONs. Particle size averaging around 50 nm is often cited as the most optimal for the cell uptake,⁶⁵ markedly more efficient than sub-10 nm size range, possibly explaining for the greater uptake of larger sHAP particles compared to the smaller nHAP ones (Figure S3). Indeed, averaged along three dimensions, the size of sHAP nanoparticles was 70.32 nm, whereas the size of nHAP nanoparticles was 11.12 nm (Table 3). Even though sphericity appears to be directly proportional to uptake efficiency,⁶⁶ the more optimal size of sHAP nanoparticles in this case outweighs the more favorable shape of nHAP. Although HAP in all forms is readily uptaken by cells,^{11,67} particle size has a large effect on the uptake of the HAP,⁶⁸ possibly determining whether the particles are predominantly uptaken through macropinocytosis⁶⁹ or caveolae/clathrin-mediated endocytosis.⁷⁰ When it comes to formation of agglomerates in the solution, displayed by all six HAP-containing materials tested here (Table 3), dispersing them using citrate reduced both the particle uptake and the imposed cytotoxicity onto human macrophages in another study,⁷¹ reiterating the potential benefits of moderate agglomeration mentioned earlier in the discussion. Although smaller particles have the advantage of bypassing the non-specific capture by the RES and having longer pharmacokinetic half-lives^{72,73} early on it was recognized that targeted delivery of magnetic nanoparticles for MH would benefit from larger entities, in part because of their higher magnetic moment and the better navigability in the external field and in part because of the greater resistance to the blood flow.⁷⁴

Fluorescent images displaying the uptake of each nanoparticle system in hMSCs, U87 and E297 cells are shown in Figures 9, 10, and 11, respectively. As seen in Figures 10H,I and 11B,C,F,G, the nanoparticles exhibit a perinuclear localization, meaning that they are taken up by an endocytic pathway.⁷⁵ HAP particles appear to localize around the endoplasmic reticulum where they interfere with protein synthesis; as the cancer cell endocytoses more material, the cytotoxic effects become more pronounced.⁴⁹ Because SPIONs and MBs are invisible in the epifluorescence setting (Figures 9B,C, 10B,C, and 11B,C), the particles were visualized as a dark contrast to the background stained in green (Figure 12). MBs litter the cell and the surrounding area (Figure 12D,H), but the uptake is poor, and without a sufficient uptake MBs might not be an effective MH device. Unlike them, SPIONs are taken up well, though predominantly by the healthy hMSCs (Figure 12B,F), as observed previously (Figure 9). The clear appearance of the nuclei (Figures 10D, 11G, 12H, and 14), with magnetic nanoparticles surrounding them, suggests the predominant internalization of the particles rather than their mere adherence onto the cell membrane. Incubation times shorter than or equal to 5 min are used to achieve the sole external decoration of the cell membrane with SPIONs;⁷⁶ incubation in this study lasted 24 h to ensure thorough internalization of the nanocomposite particles. The significant difference in the uptake observed for SPIONs and MBs as well as for nHAP and sHAP suggests that chemical composition is a minor factor when it comes to determining the biological response to nanoparticles. Fine structural properties matter more in the design of biomedical nanoparticles than sheer chemistry. Moreover, the lack of correlation between the uptake amount and the reduction in viability suggests that what is being taken up matters more than how much it is being taken up when it comes to determining the effects of the uptake of a material on cells.

These findings suggest that combining SPIONs and HAP into nanocomposites with the purpose of delivering SPIONs using HAP nanoparticles as a carrier may be a promising method to increase the uptake in cancerous cells and minimize the risk imposed on healthy cells. However, it is apparent that further optimization of HAP nanoparticle properties for selective uptake is needed, with parameters such as surface charge and protein corona as possible considerations. Most critically, the more effective uptake of SPIONs than of nanocomposites is expected to be due to the greater colloidal stability of the former, as can be confirmed by the higher level of dispersibility of intracellularly located SPIONs (Figure 12B,F) than that of HAP nanoparticles and HAP-containing composites (Figures 9–11). Moderate agglomeration of HAP is necessary to capture SPIONs and ensure a higher magnetic moment of the system than that of Fe-doped HAP, which cannot exceed 5 emu/g,⁷⁷ but at the same time this agglomeration presents an obstacle to the effective nanoparticle uptake. Even though HAP nanoparticles in the culture medium form agglomerates, the two exist in an equilibrium and it would be erroneous to claim that “cells seldom encounter single HAP nanoparticles in the environment of cell culture or body fluid”.⁷⁸ This explains why a competing multitude of particle uptake pathways is at work at the HAP/cell interface, dependent on the cell line,⁷⁹ but also on various physicochemical particle properties,⁷⁰ in spite of the fact that HAP agglomerates exceeding 100 nm in size are expected to be mainly taken up by macropinosomes and HAP nanoparticles by dynamin-dependent caveolae and clathrin-coated vesicles. In addition, different biodistribution profiles are expected to result

depending on the extent of particle agglomeration.⁸⁰ Further studies will focus on optimization of the level of aggregation of the nanocomposites with the use of appropriate, biocompatible dispersion agents, e.g., citric acid or dextran.

3.7. Cytoskeleton Anisotropy Analysis

Anisotropy is the property of being directionally dependent; in the context of cell structure, it can define if cytoskeletal fibrils are highly ordered or disorganized. The cytoskeleton governs the shape of the cell, provides a scaffold for organelles, the uptake of materials and cell signaling.^{81,82} It is also involved in cancer cell metastasis and invasion.^{83,84} Alzheimer's disease demonstrates that alterations in the cytoskeleton affect the characteristics of the cell, in particular with the disintegration of microtubules leading to neurodegeneration.⁸⁵ Cytoskeletal disorganization also correlated with a progression from nontumorigenic to aggressive, malignant phenotype in mouse ovarian epithelial cells.⁸⁶ Elucidating the organization and structure of the cytoskeleton after the nanoparticle uptake can further our understanding of how a cellular system responds to foreign material and to what extent a nanoparticle system needs to be modified to increase the uptake.^{87–89} Following the uptake of SPIONs, microfilaments remodel, inducing increased endothelial cell permeability, while reactive oxygen species control the extent of the remodeling.⁹⁰ However, although actin cytoskeleton arrangement can determine the extent of the uptake, as it is inferable from the fact that macropinocytosis is an actin-dependent endocytic pathway,^{91,92} it is equally possible that changes in the cytoskeletal order are a downstream effect of the nanoparticle uptake.⁹³ To this date, no studies have been conducted with the goal of analyzing changes in the actin cytoskeleton after the uptake of SPIONs.

Figure 13 shows changes in the cytoskeleton anisotropy in hMSCs, U87, and E297 cells following the uptake of different nanoparticles. hMSCs exhibit a significant change in their cytoskeleton anisotropy during the uptake of SPIONs, 1CnHAP and 10CsHAP (Figure 13a). While SPION treatment decreased the anisotropy, creating a more disorganized cytoskeleton by 15.5% ($P = 0.02$), 1CnHAP and 10CsHAP treatments increased the anisotropy, forming a more organized cytoskeleton by 23.4% ($P = 0.0001$) and 16.75% ($P = 0.006$), respectively. Overall, all the nanoparticle samples containing HAP increased the cytoskeleton order in hMSCs, whereas all the nanoparticle samples containing iron oxide decreased it, indicating the markedly greater cytocompatibility of HAP compared to SPIONs. Furthermore, healthy MSCs exhibited a reduced anisotropy and less order in their cytoskeleton after the treatment with SPIONs (Figure 13a), causing an increase in the cell permeability to nanoparticles; in contrast, primary cancer cells (Figure 13b and Figure S4) exhibited an increased anisotropy and more order in the cytoskeleton arrangement after the treatment with SPIONs, correlating with a decrease in the cell permeability to nanoparticles. U87 cells displayed no significant change in the actin cytoskeleton anisotropy (Figure S4) and had the least amount of average change versus the control in all treatment groups, barely surpassing 5%. The primary, E297 glioblastoma cells exhibited the largest average change versus the control, greater than 10% for all the nanoparticle samples and nearly surpassing 30% following the treatment with sHAP. All the nanoparticle treatments caused a significant increase in actin filament anisotropy, indicative of increased intracellular ordering (Figure 13b). Such an increased organization in the actin cytoskeleton directly correlates with diminished spheroid migration.

If it is correct that the loss of cytoskeleton order entailed by a decreased anisotropy marks the transition of the cell toward malignancy, then it is possible that the nanoparticle treatments bring the E297 cells closer to their premalignancy state. Finally, the fact that hMSCs exhibit a reduced anisotropy and a less organized cytoskeleton when treated with SPIONs and E297 cells exhibit the opposite effect provides an indirect evidence in favor of the increased uptake of SPIONs in healthy cells compared to the cancerous ones.

3.8. Magnetic Hyperthermia Analysis

To prove the feasibility of HAP/SPION composites as a magnetic material for MH treatments, an aqueous medium containing the HAP/SPION sediment was first subjected to an alternating magnetic field (300 kHz, $1.16 \mu\text{T}$), and its temperature was measured as a function of time. As shown in Figure 14a, compared to the control, particle-free medium whose temperature was un-affected by the magnetic field, the temperature of the medium containing the HAP/SPION nanohybrid linearly rose from the fifth to the 30th minute of the experiment. The temperature of the medium increased by only 0.6°C during the first 5 min of the treatment in the magnetic field, but then subsequently increased at the rate of $1^\circ\text{C}/\text{min}$ throughout the rest of the experiment. This suggests that about 10 min of exposure to the external field—even if of a small intensity compared to the clinical setups, as is the case here—should be sufficient to raise the temperature of the tissue to 42°C , at which stage the necrotic effects of the heat should be significant.⁹⁴ Considering that the Curie points of maghemite and magnetite are at ~ 640 and 580°C , respectively, there was no sign of temperature plateauing upon prolonged exposure, showing that the diamagnetic insulation provided by HAP is not sufficient to yield a material with self-regulating thermal properties.

In the second step, E297 glioblastoma cells were incubated with $5 \text{ mg}/\text{cm}^2$ 10CsHAP for 4 h before being subjected to the aforementioned alternating magnetic field for 30 min and then allowed to recover for 24 h. As shown in Figure 14b, this treatment led to a significant reduction in the cancer cell population, specifically down to 43.5% compared to the untreated control. At the same time, the population treated with HAP/SPION under the same conditions but in the absence of the magnetic field showed no change in viability compared to the control population untreated with the nanocomposite. This demonstrates that the viability reduction effect is due to the MH effect and not the sole nanoparticle treatment. Although MH theoretically⁹⁵ and practically⁹⁶ benefits from superparamagnetic nanoparticles with high M_s , composites comprising maghemite and a diamagnetic component, having M_s as low as $3 \text{ emu}/\text{g}$, have achieved satisfying power and loss and heating profiles.⁹⁷ The heating effect was also achieved even with Fe-doped brushite with M_s of only $0.5 \text{ emu}/\text{g}$ ⁹⁸. Therefore, the successful achievement of the MH effect, both in terms of the temperature increase and the cell viability reduction, using 10CsHAP, a HAP/SPION nanohybrid with M_s of $15 \text{ emu}/\text{g}$ —which is 5–6 times lower than the bulk maghemite value—should not be surprising.

4. CONCLUSION

The goal of this work was the design, construction, and characterization of a hydroxyapatite–superparamagnetic iron oxide nanocomposite (HAP/SPION) usable as an

MH medical device capable providing a selectively toxic effect on cancer vs healthy cells. HAP/SPION nanohybrids synthesized hereby retained the superparamagnetic nature of their SPION component. They also increased the uptake ratio between U87 human glioblastoma cells and healthy MSCs compared to that exhibited by SPIONs alone, alongside reducing the brain cancer cell viability, thus allowing for the treatment of tumors even without the use of MH and slowing down cancer cell migration rate in the 3D tumor spheroid model, all the while maintaining the viability of healthy hMSCs. A functional synergy between the two components of the nanocomposites was established; as a result, the cancer versus healthy cell selectivity in terms of both the uptake and the toxicity was higher for the composite than for SPIONs or HAP alone, enabling it to be damaging to cancer cells and harmless to the healthy ones (Figure 15). A preliminary evidence on actin cytoskeletal rearrangement after nanoparticle uptake is also presented and it is shown that increased actin microfilament anisotropy directly correlates with the hindered cell migration and with a decrease in the cell permeability to nanoparticles.

The testing of HAP/SPION nanocomposites in vitro for MH effect resulted in a sufficient temperature increase to decimate the cancer cell population treated with the composite. This is in agreement with previous studies that demonstrated a finite heating efficiency of other types of HAP/SPION composites, albeit in the absence of cells.^{23,98} HAP/SPIONs have also been introduced into tumors in mice and shown to reduce their volumes after the MH treatment.⁹⁹ Moreover, there have been clinical trials with patients presenting glioblastoma multiforme, but only one group in Europe has been able to achieve positive results using MH and SPIONs.⁷ In the current treatment setup, MH may have to be applied concurrently with other cancer therapies treatments until the methods for effective delivery and magnetic field absorption through biological barriers become set in place. The next step will be to test the penetrability of the blood–brain barrier in vitro and following injection into the carotid artery in vivo, an effect that will be virtually impossible to proceed through the paracellular route because of the nanoparticle agglomeration, but conceivable through specific uptake, the efficiency of which was indirectly studied here.

The risk of thromboembolism due to parenteral application of these nanohybrids is minimal in the 0.05–1 mg/mL dose range tested in this study, which is comparable to both liposomal formulation doses (e.g., Doxil, 0.35 mg/mL) and typical IV administration doses of SPIONs for hyperthermia, ~1 mg/mL,¹⁰⁰ but lower than the IV-delivered HAP nanoparticles: 20 mg/mL.¹⁰¹ However, the blood flow presents one of the most critical hindrances to the effective localization and heat dissipation in the tumor area, the reason for which the most prospective MH applications rely on the injection of magnetic nanoparticles directly into the tumor area. The effects of the blood flow must be determined in more complex study settings, including in vivo. In vivo experiments will be also needed to assess if there are pharmacokinetic benefits of the HAP component compared to the fast clearance and low blood circulation half-life of pure SPIONs: 10 and 90 min for $t_{\alpha 1/2}$ and $t_{\beta 1/2}$, respectively.¹⁰² Although HAP microparticles did not exhibit any significantly longer pharmacokinetic half-life than SPIONs, their blood residence time was a bit longer,³⁶ which may be beneficial since the prolonged presence in the bloodstream increases the chances of reaching the tumorous tissue. HAP particles injected intravenously also inhibit platelet aggregation and the coagulation cascade,¹⁰³ which is an effect that can indirectly favor tumor targeting via

the enhanced permeability and retention (EPR) effect. Still, their contribution to arterial calcification¹⁰⁴ should not be neglected, especially in case of daily administrations. Since HAP, like SPIONs, predominantly accumulates in liver and spleen,^{105,106} it is likely that surface modifications shall be needed to improve the targeting potential of these nanohybrids. SPIONs are primarily recognized by the macrophages, which ingest them and degrade in the lysosome, thus largely preventing their arrival at the target destination.^{107,108} In contrast, biocompatible particles, especially if hydrophilic and minimally charged, all of which are properties possessed by HAP, avoid the recognition and removal from the circulation by the cells of the RES.¹⁰⁹ However, the idea that HAP, as a material native to the body, may take on the role of a Trojan horse, a chaperone of a kind, and allow SPIONs to circumvent the rapid recognition by the cells of the immune system may be lucid but unjustified. Still, countering the inflammatory response¹¹⁰ and the oxidative damage¹¹¹ frequently triggered by SPIONs, it is conceivable that the HAP component would have a positive effect on the overall safety profile in view of its excellent biocompatibility. Its intrinsic anticancer properties evidenced here present another reason in favor of its use. Whether all these benefits of the use of HAP in combination with SPIONs will outweigh its weaknesses, which include low colloidal stability, aggregation propensity and low chemical conjugation capacity, or this trend will swing in favor of other materials that transcend these weaknesses and that have been combined with SPIONs previously, such as silica,¹¹² polymers,¹¹³ or lipids,¹¹⁴ will be hopefully answered by future studies and metastudies. Until then, our approach will be to use these weaknesses as strengths, as exemplified in this study, where the aggregation of HAP was used to capture SPIONs into stable hybrid units and deliver them intracellularly. This approach agrees with the fundamental nature of HAP, a material that is mediocre in terms of all its properties, yet a material that won the evolutionary battle for a place within the skeletal base of our bodies.¹¹⁵

All in all, nanocomposites of HAP and SPIONs embody properties that may prove to be a new avenue for cancer therapies that utilize MH. HAP/SPIONs are biocompatible materials that can be utilized for localization to specific regions of the cell, decreasing systemic toxicity thereby, while preferentially harming cancer cells and sustaining healthy cells alive. Cumulatively, HAP/SPION nanocomposite or their analogues may provide the basis for a new generation of MH devices for cancer therapies, such that they minimize the use of harsh chemotherapeutics and risks imposed on healthy cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

NIH Grant No. R00-DE021416 and intramural University of Illinois at Chicago (UIC) fundings are acknowledged for support. We thank Maheshwar Iyer and Shreya Ghosh for TEM and SEM imaging at the Electron Microscopy Facility in the Research Resources Center at UIC; Chen Chen and Jeremiah Abiade for the SQUID measurements at the Northwestern University core research facility; and Neda Sadeghiani and Sean Tang for assistance with MH measurements at the Chapman University School of Pharmacy core facility.

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Figure 1.

Visual appearance and the weight percentage of SPIONs in different HAP/SPION nanocomposites.

Figure 2.

Transmission electron micrographs of (A) SPIONs, (B) nHAP, (C) sHAP, (D) 1CnHAP, (E) 1CsHAP, and (F) 10CsHAP.

Figure 3.
Transmission electron micrographs of (a) sHAP, (b) 1CsHAP, (c) 10CsHAP.

Figure 4. Normalized X-ray diffractograms of different nanoparticulate materials (a) and magnetic hysteresis curves for 1CsHAP and 10CsHAP (b).

Figure 5.

Zeta potential vs pH curves for nanoparticle systems: (A) SPIONs and MBs; (B) nHAP and sHAP; (C) SPIONs, nHAP, 1CnHAP, and 10CnHAP; (D) SPIONs, sHAP, 1CsHAP, and 10CsHAP. Data points are shown as averages with error bars representing the standard deviation.

Figure 6.

Viability of human mesenchymal stem cells (a, d), U87 (b, d) and E297 (c, d) glioblastoma cells following treatment with 50 µg/mL (ac), 250 µg/mL (a-c), or 1 mg/mL (d) of IONs, MBs, HAP (nHAP and sHAP), and HAP/SPION nanocomposites (1CnHAP, 1CuHAP, 10CnHAP, 10CuHAP) and normalized to the NC. NC refers to the negative control, and SC refers to the sample solution control. Data points are shown as averages with error bars representing the standard deviation of the mean. * refers to $P < 0.05$ vs SC. N denotes the number of experimental replicas. Dashed line drawn at 80% viability is a guide to the eye, showing that the healthy cell viability for all the HAP-containing samples is above it (a), while the cancer cell viability for all the HAP-containing samples except for 50 µg/mL 10CsHAP in E297 is below it (b, c).

Figure 7.

Average E297 spheroid migrations in different treatments: (A) control; (B) SPIONs; (C) SPIONs (same as B) with increased light through the sample; (D) MBs. Scale bars: (A) 500 μm ; (B–D) 100 μm ; (E) nHAP; (F) sHAP; (G) 1CnHAP; (H) 1CsHAP. Scale bars for E–H: 100 μm . Data points are shown as averages with error bars representing the standard deviation of the mean. Statistical difference of all the data points is significant compared to the control ($P < 0.05$).

Figure 8.

(a) Average number of SPIONs uptaken by different primary (blue bars) and cancer (red bars) cells, including hMSCs, lung fibroblasts, kidney fibroblasts, K7M2 osteosarcoma cells, and U87 and E297 glioblastoma cells after 24 h treatments. Uptake in cancer cell lines was statistically compared to that in primary cells. Data points are shown as averages with error bars representing the standard deviation of the mean. * refers to $P < 0.01$, and ** refers to $P < 0.0005$. (b) Uptake of nanoparticle systems by U87 cells normalized to the uptake by hMSCs. Data points are shown as averages with error bars representing the standard deviation of the mean. * refers to $P < 0.05$ of sample vs SPION uptake. Table inset defines the sample size for individual sample groups.

Figure 9.

Fluorescence images of hMSCs challenged with various nanoparticle systems: (A) control; (B) SPION; (C) MB; (D) nHAP; (E) sHAP; (F) 1CnHAP; (G) 1CsHAP; (H) 10CnHAP; (I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm .

Figure 10.

Fluorescence images of U87 glioblastoma cells challenged with various nanoparticle systems: (A) control; (B) SPION; (C) MB; (D) nHAP; (E) sHAP; (F) 1CnHAP; (G) 1CsHAP; (H) 10CnHAP; (I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm . White arrow in D denotes the perinuclear localization of the particles.

Figure 11.

Fluorescence images of E297 glioblastoma cells challenged with various nanoparticle systems: (A) control; (B) SPION; (C) MB; (D) nHAP; (E) sHAP; (F) 1CnHAP; (G) 1CsHAP; (H) 10CnHAP; (I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm . White arrow in F denotes the perinuclear localization of the particles.

Figure 12.

Fluorescence images of hMSCs (A, B, C, D) and U87 glioblastoma cells (E, F, G, H) challenged with SPIONs (A, B, E, F) and MBs (C, D, G, H) discernible in black. A, C, E, and G contain red and blue channels; B, D, F, and H contain red, blue and green channels. White arrow in H denotes a cell with no overlap between the particles (black) and the nucleus (blue), indicating predominantly internal, cytoplasmic localization of the particles.

Figure 13.

Change in the actin cytoskeleton anisotropy in hMSCs (a) and E297 cells (b) following the uptake of different nanoparticle systems. Data points are shown as averages with error bars representing the standard deviation of the mean. * refers to $P < 0.05$ versus the control. Table inset defines the number of measurements with columns as sample groups.

Figure 14.

(a) Temperature of the 100 μL aqueous medium either containing no particles (control) or containing 5 mg of 10CsHAP HAP/SPION nanohybrid as a function of time spent in an alternate magnetic field (300 kHz, 1.16 μT). (b) Viability of E297 cells normalized to the control, untreated cell population (E297) after being treated only with the magnetic field, H (300 kHz, 1.16 μT) (E297 + H), only with the 5 mg/cm² HAP/SPION nanohybrid (E297 + HAP/SPION), and with both the magnetic field and 5 mg/cm² HAP/SPION nanohybrid (E297 + HAP/SPION + H), allowing for the MH effect to take place. 10CsHAP composite was used as HAP/SPION. Data points are shown as averages with error bars representing the standard deviation of the mean ($n = 4$). * refers to $P < 0.01$ versus the control (E297) and ** refers to $P < 0.001$ versus the control (E297).

Figure 15.

Summation of the cancer versus healthy cell selectivity achieved by HAP/SPION nanocomposites. While SPIONs in the nanocomposite increase the uptake in cancer cells, HAP in it imposes a selectively toxic effect on cancer cells while leaving the healthy cells intact. Correspondingly, as immunofluorescent cell images show, in contrast to the viable appearance of cancer cells challenged with SPIONs, the cancer cells become morphologically deformed following the uptake of the nanocomposite, especially in the cytoplasmic regions in which they are located.

Table 1

Final Concentrations of Precursors for Each Nanoparticle System

nanoparticle system	final concentration of precursors					NH ₄ OH added
	[Fe ³⁺] (mM)	[Fe ²⁺] (mM)	[Ca ²⁺] (mM)	[PO ⁴⁻] (mM)		
SPION	5	2.5	0	0	0	yes
MB	n.a.	n.a.	0	0	0	n.a.
nHAP	0	0	50	50	30	no
sHAP	0	0	50	50	30	yes
ICnHAP	0.025	0.0125	50	50	30	no
ICsHAP	0.025	0.0125	50	50	30	yes
10CnHAP	0.25	0.125	50	50	30	no
10CsHAP	0.25	0.125	50	50	30	yes

Table 2

Crystallite Size of HAP and Magnetite in Nanocomposites

nanoparticle system	HAP crystallite size (nm)			magnetite crystallite size (nm)		
	(002)	(211)	(310)	(311)	(400)	
SPION	n.a.	n.a.	n.a.	19,60	22,60	
MB	n.a.	n.a.	n.a.	n.a.	n.a.	
nHAP	35,04	7,50	15,36	n.a.	n.a.	
sHAP	30,95	7,71	20,84	n.a.	n.a.	
1CnHAP	45,21	6,96	19,97	<i>a</i>	<i>a</i>	
1CsHAP	31,76	7,26	22,46	<i>a</i>	<i>a</i>	
10CnHAP	31,49	6,45	19,51	30,82	<i>a</i>	
10CsHAP	31,02	7,05	21,35	29,44	<i>a</i>	

^aIndeterminate as the composite contained the phase, but it was below the detection limit of the device.

Table 3

Hydrodynamic Diameters, Crystallinity, and the Phase Composition of Nanocomposite Phases

nanoparticle system	DLS diameter ^a (mean $\pm$ SD, nm)	TEM diameter ^b (mean $\pm$ SD, nm)	HAP crystallinity (%)	Fe ₃ O ₄ / γ -Fe ₂ O ₃ (%/%)
SPION	74.8 $\pm$ 9.4	25.22 $\pm$ 4.91	n.a. ^c	57.5/42.3
MB	33.2 $\pm$ 18.1	37.45 $\pm$ 4.97	n.a.	n.a.
nHAP	296.9 $\pm$ 30.8	11.12 $\pm$ 3.37	32.9	n.a.
sHAP	314.0 $\pm$ 39.7	70.32 $\pm$ 22.50	32.0	n.a.
1CnHAP	523.5 $\pm$ 60.1	14.41 $\pm$ 4.57	23.5	39.9/60.1
1CsHAP	480.6 $\pm$ 59.5	28.33 $\pm$ 17.00	20.0	69.4/30.6
10CnHAP	343.8 $\pm$ 35.7	n.a.	36.4	59.4/40.6
10CsHAP	343.8 $\pm$ 35.3	16.61 $\pm$ 4.63	20.8	44.4/55.6

^aAlso known as hydrodynamic diameter.^bTEM particle size expressed in diameters of hypothetic spheres obtained by averaging across three dimensions.^cn.a. = not applicable as the substance does not contain the phase and/or was not analyzed.