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Enhancing Therapeutic Efficacy through Designed Aggregation of Nanoparticles

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Abstract

Particle size is a key determinant of biological performance of sub-micron size delivery systems. Previous studies investigating the effect of particle size have primarily focused on well-dispersed nanoparticles. However, inorganic nanoparticles are prone to aggregation in biological environments. In our studies, we examined the consequence of aggregation on superparamagnetic iron oxide (SPIO) nanoparticle-induced magnetic hyperthermia. Here we show that the extent and mechanism of hyperthermia-induced cell kill is highly dependent on the aggregation state of SPIO nanoparticles. Well-dispersed nanoparticles induced apoptosis, similar to that observed with conventional hyperthermia. Sub-micron size aggregates, on the other hand, induced temperature-dependent autophagy through generation of oxidative stress. Micron size aggregates caused rapid membrane damage, resulting in acute cell kill. Overall, this work highlights the potential for developing highly effective anticancer therapeutics through designed aggregation of nano delivery systems.

Keywords

superparamagnetic iron oxide; magnetic hyperthermia; particle size; aggregation; necrosis; apoptosis; autophagy

1.0 Introduction

Nano drug delivery systems are of considerable interest in cancer therapy because of their ability to accumulate passively in tumors (1–3). A key area of research is the effect of size of the delivery system on therapeutic effectiveness (4–6). Several reports suggest that the optimal size of nanoparticles for tumor therapy is around 100 nm (3). However, the presence of proteins can lead to aggregation of nanoparticles in biological environment (7–10). For example, depending on the surface properties, nanoparticles can form sub-micron to micron

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size aggregates in the presence of serum proteins (11, 12). The effect of aggregation on the therapeutic performance of nanoparticles has not been previously investigated.

In our studies, we examined the consequence of aggregation on superparamagnetic iron oxide (SPIO) nanoparticle-induced magnetic hyperthermia (MH). Superparamagnetic materials generate heat when placed in alternating magnetic field (13, 14). Depending on the size of the material, heat is generated through either Néel or Brownian relaxation or a combination of the two (15, 16). Heat generated and the associated temperature change from such relaxation phenomena dissipate over short distances due to the high thermal conductivity of water and can therefore be used for highly focused heating (17, 18). This concept is utilized in MH, where an external magnetic field is applied to SPIO nanoparticles trapped in tumor tissue that results in local heating and tumor cell kill (18).

We hypothesized that the aggregation state of SPIO nanoparticles will affect their magnetic properties, which, in turn, will affect their tumor cell kill effectiveness. To test this hypothesis, we fabricated SPIO nanoparticle aggregates of various sizes and evaluated their ability to induce MH in cell culture models as well as in a mouse subcutaneous model of lung cancer.

2.0 Materials and Methods

2.1 Materials

Ferrous chloride tetrahydrate, ferric chloride hexahydrate, myristic acid, Pluronic F127, ascorbic acid, potassium hydroxide, 1,10-phenanthroline and sodium acetate were purchased from Sigma (St. Louis, MO). Penicillin/streptomycin, fetal bovine serum, Dulbecco's phosphate buffered saline, F-12K (Kaighn's modification) and trypsin-EDTA solution were obtained from Invitrogen Corporation (Carlsbad, CA). Cytotox 96® non-radioactive cytotoxicity assay and CellTiter 96® AQueous one solution cell proliferation assay kit were purchased from Promega (Madison, WI). The commercial SPIO nanoparticles (Nanomag®) were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). For Western blotting, antibody against HSP70 was from Enzo Life Sciences (Farmingdale, NY), anti-GAPDH-HRP and anti- β -actin-HRP were from Sigma, anti-PARP1, anti-cleaved PARP1, anti-LC3B and HRP-linked anti-rabbit and anti-mouse IgG were from Cell Signaling Technology (Danvers, MA).

2.2 Methods

2.2.1 Synthesis of SPIO nano and micro aggregates—SPIO nanoparticles were synthesized by condensation of 0.82 g ferric chloride hexahydrate and 0.33 g ferrous chloride tetrahydrate dissolved in 30 ml of degassed and nitrogen-purged water with 3 ml of 5 M ammonium hydroxide. The reaction was allowed to continue for 30 minutes under magnetic stirring (200 gauss magnetic stir bar, 1600 rpm) and nitrogen gas bubbling through the reaction mixture. Iron oxide formed was washed three times with 30 ml of nitrogen-purged water, followed by magnetic separation and heating on a hot (80°C) oil bath. About 100 mg of myristic acid was added to the heated mixture, which was stirred for another 30 min at 1600 rpm using a 200 gauss magnetic stir bar. Excess myristic acid was removed by

two washes with acetone, followed by two washes with water to remove excess acetone. Each wash was followed by magnetic separation of nanoparticles. The myristic acid-coated particles were then suspended in 30 ml water using a water-bath sonicator and 100 mg of Pluronic F127 was added and stirred overnight. The final dispersion was fractionated by centrifugation at 800 g for 6 minutes. The supernatant contained nano aggregates. The pellet was dispersed in 15 ml of water and 20 mg Pluronic F127 by sonication for 1 hour and was comprised of micro aggregates.

Coumarin-6 labeled SPIO nanoparticles were synthesized by incorporating coumarin-6 into the fatty acid layer of SPIO nanoparticles dispersed in water (19). One ml of 500 µg/ml coumarin-6 in ethanol was added to 100 mg magnetite equivalent of SPIO NP in 10 ml of water and stirred overnight. The coumarin-6 labeled SPIO nanoparticles were washed three times in water followed by magnetic separation of nanoparticles. The final product was dispersed in 10 ml of water and 5 mg Pluronic F127 and inspected under a fluorescent microscope to confirm the presence of dye-label on particles.

2.2.2 Characterization of SPIO nanoparticles

2.2.2.1 Particle size—The hydrodynamic diameter of SPIO nanoparticles was determined by dynamic light scattering. About 1 mg of SPIO nanoparticles was dispersed in 2 ml of deionized water by sonication, and the dispersion was subjected to particle size analysis using a Delsa™ Nano C Particle Analyzer (Beckman, Brea, CA). Measurements were performed at 25° C and at a 165° scattering angle. Mean hydrodynamic diameter was calculated based on size distribution by weight, assuming a lognormal distribution. For transmission electron microscopy (TEM), a drop of an aqueous dispersion of SPIO nanoparticles was placed on a Formvar®-coated copper grid (150 mesh, Ted Pella Inc. Redding, CA). Excess liquid was removed using a filter paper, and the grid was allowed to air-dry. Particles were imaged using a JEOL JEM-1210 transmission electron microscope (Peabody, MA). The diameters of at least 100 different particles were measured using ImageJ software and averaged to determine the mean Feret's diameter.

2.2.2.2 Composition—The form of iron oxide was assessed by Fourier-transformed infrared spectroscopy (FTIR). About 5 mg of SPIO nanoparticles was analyzed using Vertex 70 FT-IR spectrophotometer (Bruker Optics Inc., Billerica, MA). Scans were performed from 4000 cm⁻¹ to 400 cm⁻¹ and each spectrum was obtained by averaging 16 interferograms at a resolution of 2 cm⁻¹. The data was collected and analyzed using OPUS software (Bruker Optics Inc., Billerica, MA). Magnetic properties were determined with an accurately weighed sample of SPIO nanoparticles, which was sprinkled on a lightly greased silicon wafer and placed in a vibrating sample magnetometer (Micromod Model 3900, Princeton, NJ) operating at room temperature. Magnetization curves were recorded in magnetic fields ranging from -1 T to 1 T, at increments of 0.002 T. The curve was normalized to the mass of magnetite to obtain the saturation magnetization per gram of magnetite. The composition of SPIO nanoparticles was estimated using 1,10 phenanthroline-based iron assay (20). SPIO nanoparticles were dissolved in 12 N hydrochloric acid. The solution was then diluted with distilled water to obtain a final acid concentration of 0.2 N. To this acid solution, 10 mg/ml ascorbic acid, 1.2 mg/ml 1,10 phenanthroline, 22.4 mg/ml

potassium hydroxide and 123 mg/ml sodium acetate were added in a volume ratio of 1:1:1:1:5. Absorbance of the resultant solution was measured at 490 nm using a microplate reader (ELx800 Absorbance Microplate Reader, Biotek, Winooski, VT). Solutions of commercial iron oxide powder and ferric chloride (hexahydrate) in 0.2 N hydrochloric acid were used as standards.

2.2.2.3 Heating rate—The heating rates for different formulations were determined by placing 0.5 ml of the formulation (2 mg/ml or 1 mg/ml magnetite equivalent) in AMF. Temperature measurement was performed every 2 seconds using a fluoroptic probe inserted in the middle of the sample. Sample temperature was monitored for 30 seconds to ensure temperature stability, after which AMF was turned on. Specific loss power (SLP) was calculated from the initial heating rate (60 seconds) as the product of magnetite concentration, temperature change and the specific heat capacity of water. The contribution of the polypropylene sample tube (0.6 ml volume) to heat loss was ignored in SLP calculations.

2.2.3 Cell culture studies

A549 (human lung adenocarcinoma) and A549-Luc (stable luciferase expressing) cells were used in the study. Both cells were propagated using F-12K medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution and maintained at 37 °C and in 5% carbon dioxide.

2.2.3.1 Cell death after magnetic hyperthermia—About 1 million cells were suspended in 500 µl of F-12K (with 10% FBS) and mixed with 500 µl SPIO nano or micro aggregates in saline to make a final concentration of 1.75 mg/ml magnetite. The suspension was placed in AMF (6 kA/m, 386 kHz) for 5, 15 or 30 min, maintaining the temperature of the cell suspension between 43 – 46°C. Cells not exposed to AMF, with or without SPIO nanoparticles, served as control. Additionally, cells were kept in a water bath for 30 min at 46 °C, to serve as conventional hyperthermia controls. Magnetic hyperthermia induced using a commercial formulation of dextran-coated SPIO (Nanomag[®]-D-SPIO) was also used for comparison of *in vitro* efficacy. Cells were pelleted 2 hr following treatment, and the amount of LDH released by the cells in the supernatant was determined. LDH released by same number of freeze-thaw lysed cells was used to calculate 100% cell death. In addition, 50,000 treated cells were plated per well in a 24-well plate and assayed for cell survival after 24 hours by MTS assay.

2.2.3.2 Instantaneous PI uptake during AMF exposure—About 1 million cells were suspended in 500 µl of F-12K (with 10% FBS) and incubated with 500 µl nano or micro aggregates or Nanomag[®] in saline (final concentration of 1.75 mg/ml magnetite) at 4°C for 30 minutes. To each tube, 10 µM PI was added, and the treated cells were subjected to 30 minutes of AMF, while maintaining the cell suspension temperature below 5°C with an ice bath. Cells not exposed to AMF, with or without SPIO nanoparticles, were used as controls. Following AMF exposure, cells were immediately washed with buffer, resuspended in medium and kept on ice. PI present in cells was detected in the FL-3 channel of the flow

cytometer (BD, FACSCalibur™). Data from 10,000 cells in each group were analyzed using Cyflogic software.

2.2.3.3 Reactive oxygen species (ROS) generation—Immediately prior to the addition of SPIO formulations, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) (7.5 μM) and PI were added to the cell suspension. Cells were subjected to flow cytometric analysis after 2 hours. The deacetylated and oxidized product, 2',7'-dichlorofluorescein, formed due to ROS generation in the cells, was detected in the FL-1 channel while PI fluorescence was detected in the FL-3 channel.

2.2.3.4 Western blot analysis—Cells were lysed using RIPA buffer containing protease inhibitor cocktail (Sigma) on ice. Cell lysates were centrifuged at 13,000 rpm for 10 min to pellet the insoluble cellular debris, and the supernatant was analyzed. Protein concentration in the supernatant was determined by the bicinchoninic acid assay (Thermo Scientific). Protein samples (15 μg) were loaded onto a 4 – 15% Tris-hydrochloride gel (Bio-Rad Laboratories) and, after electrophoresis for 40 min at 100 V, transferred onto a nitrocellulose membrane (Whatman) using a Criterion blotter (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat dry milk in tris-buffered saline containing Tween 20 (TBST) for 1 hr. The membrane was then incubated overnight at 4° C with primary antibodies against PARP1, cleaved PARP1, HSP70, LC3B, GAPDH-HRP or β-actin-HRP, diluted in either 5% non-fat dry milk in TBST or 5% BSA in TBST. After three 8-min washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit (for PARP1, cleaved PARP1 and LC3B) or anti-mouse (for HSP70) IgG in 5% non-fat dry milk in TBST for 1 hr and then washed three times with TBST. Protein bands were then visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). For densitometric quantification, immunoblots were digitized on a flatbed scanner and the signal intensities of the visualized bands were quantified using ImageJ (NIH) and OriginPro 8 software (OriginLab Corporation, Northampton, MA).

2.2.3.5 Confocal microscopy—Following incubation with coumarin-6 labeled SPIO nanoparticles and AMF exposure, cells were stained with LysoTracker® red DND-99 (Invitrogen) for 30 minutes at 37°C or Texas red® -X conjugated wheat germ agglutinin (Invitrogen) for 10 minutes at 37°C and washed with phosphate buffered saline. Ten μl of the cell suspension was added to a glass slide, mounted by a cover glass and sealed with clear nail polish. The slides were imaged using a 40×/1.30 numerical aperture oil-immersion objective in an Olympus FluoView FV1000 BX2 upright confocal microscope (Olympus Corporation). The images were analyzed using FV1000 Viewer software (Olympus Corporation) and ImageJ (NIH).

2.2.3.6 Effect of aggregate size on cell kill by magnetic hyperthermia—To further investigate the effect of aggregate size on cell kill by magnetic hyperthermia, we performed additional fractionation of SPIO aggregates. The final dispersion containing myristic acid-coated and Pluronic-stabilized SPIO nanoparticles was fractionated by centrifugation at 400 g for 6 minutes. The residue from this step was termed Fraction A. The supernatant was further centrifuged at 600 g for 6 minutes, 800 g for 6 minutes and 1200 g

for 6 minutes, and the residue was separated from the supernatant following each fractionation. The residues from each of the centrifugation runs were labeled Fractions B–D, and the final supernatant was labeled Fraction E. The hydrodynamic size of the fractions A, B, C, D and E was 1000 nm (1003 ± 90 nm), 750 nm (748 ± 44 nm), 405 nm (406 ± 17 nm), 345 nm (344 ± 8 nm) and 220 nm (221 ± 2 nm), respectively.

About 1 million cells were suspended in 500 μ l of F-12K medium (with 10% FBS) and mixed with 500 μ l of SPIO Fraction A, B, C, D or E in saline, resulting in a final concentration of 1.75 mg/ml magnetite. The suspension was placed in AMF (6 kA/m, 386 kHz) for 15 min, maintaining the temperature of the cell suspension between 43 and 46°C. Cells not exposed to AMF, with or without SPIO nanoparticles, served as controls. Cells were pelleted 2 hrs following treatment, and the amount of LDH released by the cells in the supernatant was determined. LDH released by same number of freeze-thaw lysed cells was used to normalize for 100% cell death.

2.2.4 *In vivo* tumor growth studies

The study was carried out in compliance with protocol approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Female BALB/c-nude mice (C.Cg/AnNTac-*Foxn1*^{nu} NE9; Taconic Farms), four to six weeks of age, were used for the studies. One million A549-Luc cells were injected subcutaneously in the abdominal region of the mice, and the tumor dimensions were measured every other day using digital calipers. The tumor volume (V) was calculated using the formula $V = 0.5(L \times W^2)$, where L and W are the longest and shortest diameters, respectively. When the tumor volume reached 150 mm³, 40 μ l of saline or 225 μ g magnetite equivalent of nano or micro aggregate suspension was injected intratumorally and allowed to distribute inside the tumor for 24 hours (21). After 24 hours, animals in the MH groups were anesthetized, and their abdominal region (tumor region) was exposed to AMF (6 kA/m, 386 kHz) for 30 minutes. The tumor temperature was measured using an infrared camera (Infrared solutions), where snapshots of the AMF exposed tumor region of the mice were acquired every 2 minutes. The temperature elevation in the tumor during MH was added to the normal body temperature (assumed to be 37°C) to obtain the calculated tumor temperatures. Tumor volumes were measured every other day after treatment by calipers, and bioluminescence imaging (IVIS Spectrum, PerkinElmer) of the tumors was performed every week. Development of 1000 mm³ tumors or 30 days after cell injection (whichever came first) marked the end of the study for each animal. At the end of the study, the tumors were excised, formalin fixed, paraffin embedded, sectioned and stained with Prussian blue (for iron) or hematoxylin and eosin (for cellular structure). In a separate set of animals, the tumors were excised immediately after MH treatment and processed for Prussian blue and hematoxylin-eosin staining.

2.2.5 Statistical analysis

Statistical analyses were performed using one-way ANOVA, followed by Bonferroni-Holm testing for comparison between individual groups. A probability level of $P < 0.05$ was considered significant.

3.0 Results and Discussion

3.1 Effect of aggregate size on *in vitro* cytotoxicity

To study the effect of aggregation on therapeutic performance, we used a synthetic technique that facilitated the aggregation of SPIO nanoparticles. The final product was fractionated into micro and nano size aggregates. Nano aggregates had an average hydrodynamic diameter of 207 ± 3 nm. Micro aggregates had an average size of 1052 ± 65 nm. The aggregates were composed of 12 ± 3 nm iron oxide cores (Figure 1a–d), which were confirmed to be magnetite (Figure 1e). Both nano and micro aggregates had high saturation magnetization of 72.9 and 67.7 emu/g of magnetite, respectively, with negligible remanence (1.64 and 1.44 emu/g of magnetite, respectively) and coercivity (2.73 and 2.36 Oe), verifying their superparamagnetic nature (Figure 1f). The heating profiles for the two fractions were virtually identical (Figure 1g). The specific loss powers for the aggregates were also similar (89.6 ± 2.8 W/g and 93 ± 5.6 W/g for nano and micro aggregates, respectively). As a control, we used a commercial well-dispersed 100-nm SPIO nanoparticle formulation (Nanomag[®]; specific loss power of 68.9 ± 4.3 W/g) in our studies.

Nano aggregate-mediated hyperthermia resulted in a time-dependent decrease in cell survival. Lactate dehydrogenase (LDH) release was not significantly increased at two hours following MH treatment (Figure 2a); however, less than 10% of the treated cells survived at 24 hours (Figure 2b). Similar inefficient early LDH release and higher cell death at 24 hours was also observed in cells subjected to conventional hyperthermia or MH using a commercial well-dispersed 100-nm SPIO nanoparticle formulation (Nanomag[®]). This was not surprising, given that hyperthermia has been shown to induce apoptotic cell death, a slow process that takes several hours (22–24). Both Nanomag[®]-mediated MH and conventional hyperthermia resulted in PARP1 cleavage (Figure 2c), confirming the induction of apoptosis with these treatments. However, nano aggregate-induced MH resulted in a decrease in the levels of intact 116 kDa PARP1 protein (Figure 2c, Supporting Information Figure S1a), without a concomitant increase in levels of the cleaved PARP1 fragment (89 kDa) (Figure 2c, Supporting Information Figure S1b), a marker for caspase-dependent apoptosis (25). The lysosomal enzyme cathepsin, which is released after lysosomal rupture such as during autophagy (26), can cleave PARP1 into fragments of different sizes (44, 55, 62 and 74 kDa) (27), and thereby decrease PARP1 levels without a detectable increase in the 89 kDa fragment. Expression of LC3B-II (Figure 2c), an autophagy marker (28), further points to autophagy as the primary cell kill mechanism with nano aggregate-mediated MH.

Autophagy is a form of programmed cell death mediated by the lysosomal machinery and intracellular membrane rearrangement, which leads to massive degradation of cellular components (29). Autophagy involves sequestration of cytoplasmic components in a membrane vesicle called autophagosome, which then fuses with the lysosome to form an autolysosome. Proteases within the autolysosome degrade the sequestered cytoplasmic components. Oxidative stress has been shown to play an important role in inducing autophagy (30, 31). We determined the level of reactive oxygen species (ROS) produced by cells subjected to different treatments. The ROS levels remained unchanged after

conventional hyperthermia and Nanomag[®]-induced MH. However, nano aggregates resulted in elevated cellular ROS levels (Figure 2d). One of the effectors of oxidative stress is GAPDH (32), which causes an elevation of cellular glycolysis and drives autophagy (33). MH using SPIO nanoparticle formulations resulted in elevated GAPDH protein levels, and this increase was greatest in cells subjected to nano aggregate-mediated MH (Figure 1c, Supporting Information Figure S1c).

With micro aggregates, we observed significant LDH release from treated cells at 2 hours following MH treatment (~90% cell kill with 30 min of MH, Figure 2a). Acute LDH release with micro aggregates indicated that the cell membrane was compromised rather quickly, pointing to a necrotic cell death mechanism (34). To further determine the mechanism of necrosis and to confirm membrane damage, cells incubated with SPIO aggregates were subjected to alternating magnetic frequency (AMF) at a low temperature (<5°C). Acute membrane damage induced by this treatment was determined by monitoring the 'instantaneous' propidium iodide (PI) uptake by treated cells (35). Only the cells treated with micro aggregates demonstrated instantaneous PI uptake in the absence of temperature elevation (Figure 3a). Neither nano aggregates (Figure 3b) nor Nanomag[®] (Figure 3c) was effective in inducing instantaneous PI uptake. We further investigated the effect of size of aggregates on cytotoxicity. Interestingly, these studies suggest that the cytotoxic effect evolves into necrotic mechanism around 400 nm (Figure 3d). In addition, we evaluated the behavior of the different formulations under varying magnetic field frequencies. Our results showed that only micro aggregates induced significant instantaneous PI uptake in the presence or absence of bulk temperature elevation at all the frequencies tested (Figure 3e–g). Interestingly, the field frequency had a larger effect on micro aggregate-induced acute cell kill in the absence of bulk temperature elevation.

Additionally, while conventional hyperthermia and Nanomag[®] or nano aggregate-induced MH caused an elevation of heat shock protein 70 (Hsp70), micro aggregate-mediated MH showed no such increase (Supporting Information Figure S1d,e). This further verified the role of temperature elevation as the primary mechanism of cell death with sub-micron aggregates and a temperature-independent cell death mechanism with micro aggregates. Induction of Hsp70 expression also suggests the possibility of triggering Hsp70-mediated resistance to thermotherapy (36, 37).

3.2 Mechanism of micro aggregate-mediated membrane damage

The mechanism of membrane damage induced by micro aggregates was also assessed by an examination of the cellular distribution of the aggregates. Coumarin-6 (a lipophilic green fluorescent dye) was incorporated in SPIO nanoparticles to aid in the visualization of particles (38). Confocal microscopic images showed the presence of aggregates on cell surfaces and in the lysosomes (Figure 4a). In the absence of AMF, incubation with micro aggregates did not affect the cell membrane integrity (Figure 4b). Exposure to AMF caused blebbing of the membrane at points where the micro aggregates contacted the cell membrane (Figure 4b). The membrane blebs were large and characteristic of necrotic blebs (39). Additionally, the blebbed cells also stained positive for PI, indicating the loss of membrane integrity in these cells (Figure 4c). Thus, these observations support the hypothesis that the

cell membrane damage is a consequence of the direct contact of SPIO nanoparticles with the membrane. Others have suggested that SPIO nanoparticles induce membrane damage as a result of intense local heat generation (40, 41). However, additional studies are needed to investigate the specific mechanism of membrane damage caused by micro aggregates.

3.3 *In vivo* efficacy of micro aggregate-induced magnetic hyperthermia

To determine whether differences in aggregate size affects *in vivo* therapeutic efficacy as well, we evaluated the anticancer efficacy of micro and nano aggregates in mice bearing subcutaneous A549 tumor xenografts. Mice received either saline or 225 µg magnetite equivalent of nano or micro aggregate suspension by intra-tumoral injection. Animals receiving the aggregates without exposure to AMF served as control groups. Tumor temperatures were monitored using infrared thermometry, and the magnetic field conditions were optimized to ensure that the tumor temperature did not increase beyond 46°C. The average tumor temperature was 43.5°C in both the micro and nano aggregate groups, and highest temperature did not exceed 45.7°C (Figure 5a). Nano aggregate-mediated MH resulted in a moderate decrease in tumor growth rate ($p = 0.085$) relative to that in animals that received nano aggregates but were not exposed to AMF. Micro aggregate-mediated MH showed near complete inhibition of tumor growth (Figure 5b), which was statistically significant ($p < 0.001$). The results from the bioluminescence imaging reinforced this finding, with micro aggregate-mediated MH being highly effective in decreasing the tumor-associated luminescence (Figure 5c). Histological analysis of the tumor sections revealed that micro aggregate-mediated MH resulted in the formation of “hollow” tumors, with a large central acellular core and a small outer rim of cells (Figure 6a, Supporting Information Figure S2). Immediately after exposure to AMF, cells surrounding the SPIO aggregates (confirmed by Prussian blue staining) were characterized by altered morphology, decreased cell volume, and increased nuclear density (Figure 6c,e). Tumor tissue sections from the control group (injected with micro aggregates but not exposed to AMF) showed no such changes in the cellular morphology around the SPIO aggregates (Figure 6b,d). Because all the experimental parameters (dose of SPIO, duration of AMF exposure and the temperature elevation) were similar for both nano and micro aggregates, the enhanced effectiveness of micro aggregates is likely due to local membrane damage.

It is noteworthy that despite their inhomogeneous distribution within the tumor, a complete inhibition of tumor growth was observed for micro aggregate treatment. A number of studies show that treatments that induce acute necrosis in the tumors are able to stimulate anti-tumor immune response, which reinforces the anticancer effectiveness of the treatment (42, 43). Thus, the acute cell damage and necrosis induced by micro aggregates could stimulate a bystander anti-tumor immune response, which would affect the growth of the entire tumor. The animal model used here (SCID mice) precludes the generation of humoral and T-cell mediated immune responses. However, these mice have functional natural killer (NK) cells that can mount an effective anticancer immune response (44). In addition, an increase in local tumor temperature (hyperthermia) would likely reinforce the anticancer benefits of acute necrosis induced by micro aggregates. Induction of necrosis rather than apoptosis by micro aggregates may have additional benefits as well. Tumor cells can develop resistance to apoptotic pathways through upregulation of heat shock proteins (36, 37). Because

necrotic cell death is often rapid (few minutes), necrosis is less prone to these resistance mechanisms. While an extensive investigation into induction of necrosis and generation of anticancer immune response by micro aggregates was beyond the scope of this report, these *in vivo* results confirm that larger aggregates are more effective in killing tumor cells than smaller sub-micron size aggregates.

Intravenous delivery of micro aggregates is likely not practical due to concerns of embolism (45, 46) and because the particle size is too large to take advantage of the enhanced permeation and retention effect (3). However, the particle size range of micro aggregates is particularly suitable for inhalation delivery (47, 48) to treat lung tumors (48–50). Also, several types of solid tumors (liver, kidney, prostate, and lung) are treated clinically by local interventions using physical ablation techniques (microwave, radiofrequency) (51–53). Such cancers will benefit from treatment with the micro aggregates. Alternately, an *in situ* aggregation approach can also be envisioned, where sub-micron size particles injected intravenously aggregate into micron size aggregates in the tumor microenvironment.

4.0 Conclusions

Inorganic nanoparticles have the propensity to aggregate when introduced into a biological milieu. Our studies showed that the particle size of aggregates affects the therapeutic performance of SPIO nanoparticles. Well-dispersed SPIO nanoparticles induce apoptosis, similar to that observed with conventional hyperthermia. Nanometer size aggregates, on the other hand, induce temperature-dependent autophagy through generation of ROS. Micron size aggregates caused rapid membrane damage and acute cell kill *in vitro*, which translated into effective *in vivo* tumor growth inhibition. Overall this work highlights the potential for developing highly effective anticancer therapeutics through simple yet often overlooked modifications of delivery systems such as their state of aggregation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of SPIO aggregates

(a) Representative TEM image of SPIO nano aggregate showing a 100–200 nm aggregate composed of 12 ± 3 nm SPIO core (average Feret's diameter of 100 different SPIO cores measured using ImageJ software). Scale bar, 50 nm. (b) TEM image of micro aggregates showing a large electron dense particle (about 1.5 μm in size). Scale bar, 500 nm. (c) Higher magnification image of the boxed area of (B) demonstrating that the micro aggregates are also composed of the smaller SPIO cores tightly packed together to form the larger structure. Scale bar, 50 nm. (d) TEM image of uranyl acetate counterstained SPIO nano aggregates showing that the SPIO cores are enveloped by myristic acid/pluronic F127 coating to form stable aggregates. The size of micro aggregates interfered with the visualization of coating on them. (e) FT-IR spectrum of SPIO nanoparticles. Presence of characteristic magnetite bands at 570 cm^{-1} and 400 cm^{-1} (arrow) and absence of maghemite bands at 700 cm^{-1} and $630\text{--}660\text{ cm}^{-1}$ (box) indicate that the primary form of iron oxide is magnetite⁴. (f) Magnetization curves of nano aggregates (NA) and micro aggregates (MA) recorded on a vibrating sample magnetometer. The curve was normalized to the weight of magnetite added to obtain saturation magnetization per gram of magnetite. The sigmoidal curve is characteristic of superparamagnetic substances. The data shows that the saturation magnetization of nano and micro aggregates was similar ($\sim 70\text{ emu/g magnetite}$). Additionally, exposure of micro aggregates to AMF did not alter their saturation magnetization or superparamagnetic properties. (g) Heating rates of the different SPIO formulations (1.75 mg/ml magnetite equivalent) placed in an alternating magnetic field of 6 kA/m and operating at a frequency of 386 kHz. The initial temperature was equilibrated to 37°C , and temperature of the SPIO NP dispersion was sampled at 5 second intervals using a fluoroptic probe following the application of magnetic field. The magnetic field was stopped when the temperature reached about 45.5°C and restarted at 43.5°C . The graph shows the similarity in the heating profiles of nano and micro aggregates during the three AMF on/off

cycles. In spite of the slower heating rate of the well-dispersed particles, the temperature was still maintained between 43°C and 46°C in this group.

Figure 2. Effect of SPIO nanoparticle aggregate size on the mechanism of cell kill by MH
 Effect of nano aggregate (NA), micro aggregate (MA) and commercial nanoparticle (Nanomag®; NM)-mediated MH and conventional hyperthermia (WBH) on (a) cell death quantified by LDH release from the cells 2 hours after treatment and (b) cell survival 24 hours after treatment measured by MTS assay. A549 cell suspension containing different SPIO formulations (1.75 mg/ml magnetite equivalent) were subjected to 5 min, 15 min or 30 min of AMF after the suspension temperature reached 43°C. Untreated cells or cells incubated with SPIO formulations without AMF exposure (-MH) were used as controls. Conventional hyperthermia involved maintaining cells at 46°C for 30 min on a water bath. Data shown is mean $\pm$ S.D., n = 3. * P < 0.00001 vs. cells exposed to 30 min heating with different treatment; # P < 0.001 vs. corresponding unheated control. (c) Western blotting analysis of expression of PARP1 (116 kDa), cleaved PARP1 (89 kDa), LC3B-I (16 kDa), LC3B-II (14 kDa), GAPDH (37 kDa) and beta actin (44 kDa) 24 hours after MH treatment as in (A) and (B). (d) ROS generation after different treatments. CM-H2DCFDA was added to A549 cells immediately before exposure to alternating magnetic field and after 2 hours, the cells were analyzed by flow cytometry to determine ROS levels. The graph shows a representative ROS profile of cells after treatment. Neither conventional hyperthermia nor Nanomag® mediated MH resulted in the elevation of ROS production relative to that in untreated cells. However, both nano and micro aggregate mediated MH led to an elevation

of ROS levels in the cells. The concentration of different SPIO aggregates used was 1.75 mg/ml magnetite equivalent.

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Figure 3. Effect of SPIO nanoparticle aggregate size on acute cell kill

Flow cytometric analysis of instantaneous PI uptake by cells incubated with (a) micro aggregates, (b) nano aggregates and (c) Nanomag® and subjected to AMF under low temperature (<5°C). PI was added to the cell suspension immediately prior to AMF exposure (30 min), washed after treatment to eliminate the excess PI and analyzed by flow cytometry. Data shown is representative PI profile of the different treatments (n = 3). Micro aggregates showed an increase in PI positive population after AMF exposure. (d) The effect of SPIO nanoparticle aggregate size on cell kill by MH was studied. A549 cell suspension containing different SPIO formulations (1.75 mg/ml magnetite equivalent) was subjected to 15 min of AMF after the suspension temperature reached 43°C. Untreated cells or cells

incubated with SPIO formulations without AMF exposure (–MH) were used as controls. Cell death was quantified by LDH release from the cells 2 hours after treatment. Data shown is mean $\pm$ S.D., n = 3. *P < 0.05 Vs Untreated. To study the effect of magnetic field frequency on cell kill by SPIO nanoparticle aggregates, A549 cell suspension containing different SPIO formulations (1.75 mg/ml magnetite equivalent) was subjected to 30 min of AMF, **(e)** with temperature elevation (43 – 46°C) or **(f)** at low temperature (<5°C). Cell death was quantified by LDH release from the cells 2 hours after treatment **(e)** or by instantaneous PI uptake **(f)**. **(g)** Heating rates at different frequencies for **(e)**. The operating frequency of AMF was varied from 186 to 386 kHz. Data shown is mean $\pm$ S.D., n = 3. NA = nano aggregates, MA = micro aggregates, NM = Nanomag[®]. # P < 0.01 Vs all other treatments.

Figure 4. Micro aggregate mediated membrane damage through direct contact

(a) Confocal microscopic images of cells incubated with coumarin-6 labeled SPIO micro aggregates (green). Lysosomes were stained with Lysotracker red®. Representative images showing the location of lysosomes (left), micro aggregates (center) and their colocalization (right) are shown. The merged image shows aggregates on the surface of cells (white arrows) and in lysosomes (yellow arrows). Scale bar, 5µm. (b) Confocal microscopy of cells incubated with coumarin-6 labeled SPIO micro aggregates before (top) and after (bottom) AMF exposure at low temperature (<5°C). The cell membrane was labeled with Texas red®

labeled wheat germ agglutinin (left). The coumarin-6 labeled SPIO micro aggregates (center) were present both on the cell membrane and inside the cells. The cell membrane was intact in cells incubated with particles in the absence of AMF (top right), indicated by the smooth cell membrane staining. Exposure to AMF resulted in cell membrane blebbing (bottom right, yellow arrows) at the sites where the aggregates contacted the cell membrane (white arrows). (c) Phase contrast (left) and fluorescence microscopic image (middle) of cells incubated with SPIO micro aggregates and PI and exposed to low temperature AMF. The merged image (right) shows PI positive, extensively blebbed cells (black arrows) and the healthy cells devoid of blebbing or PI staining.

Figure 5. Effect of SPIO aggregate mediated MH on *in vivo* tumor growth

(a) Representative photograph of a mouse placed in the radiofrequency coil (left) during nano aggregate (NA) (top) or micro aggregate (MA) (bottom) mediated MH. The black arrow indicates the location of the subcutaneous tumor xenograft. The temperature elevation during MH was measured using infrared thermometry. Infrared thermometric images of the temperature difference before and at the end of MH treatment is shown (right). The tumor temperature (black oval) was calculated for both nano and micro aggregate mediated MH (table). Note that the elevated temperature at the edges of the coil is due to the heating of the coil during AMF exposure. (b) The tumor growth was monitored every alternate day after MH treatment. The graph shows tumor growth with different treatments (225 μ g magnetite equivalent for nano and micro aggregates) injected intratumorally as a function of time after treatment. Data shown is mean $\pm$ S.D., $n = 6$. * $P < 0.001$ and # $P < 0.1$ vs. control. Representative bioluminescence images of mice bearing luciferase transfected A549 subcutaneous xenografts after different treatments are shown. Mice were injected with 40 μ l (c) saline, (d, e) 225 μ g magnetite equivalent of SPIO nano aggregates and (f, g) 225 μ g magnetite equivalent of SPIO micro aggregates intratumorally when the tumor volumes reached 150 mm^3 . After 24 hours, the (e) nano aggregate and (g) micro aggregate treated animals were subjected to magnetic hyperthermia for 30 minutes. Tumor bioluminescence

was measured before MH treatment (Day 0) and on days 2, 7, 14, 21 and 28. The images are from an animal in each group monitored over 4 weeks after treatment. The bioluminescence intensity of all images was scaled as shown in the right.

Figure 6. Histology of tumors exposed to MH treatment

(a) Hematoxylin and eosin (H&E) staining (left) of a tumor after 30 days of micro aggregate mediated MH. The tumor is partially acellular due to the MH treatment. Prussian blue staining (right) performed on a subsequent section (5 μ m apart) shows the presence of SPIO particles at the necrotic margins of the tumor. (b) H&E staining of a tumor section (400 $\times$ magnification) from animals receiving no treatment. The tumor cells have a 'swollen' appearance with large lightly stained nucleus. (c) H&E staining (400 $\times$ magnification) of a micro aggregate injected tumor that was not exposed to an AMF. The micro aggregates can

be identified by their brown color. Cells adjacent to the particles have similar morphology as the untreated tumor (B). **(d)** H&E staining (400× magnification) of a tumor collected immediately after micro aggregate mediated MH. Cells around the particles have altered morphology characterized by cell shrinkage and denser nuclear stain. **(e)** Lower magnification (100×) of (D) showing a distinct region around the micro aggregates (black bordered area) where the cells have a different morphology compared to the rest of the tumor.