Accounting for biological aggregation in heating and imaging of magnetic nanoparticles

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Aggregation is a known consequence of nanoparticle use in biology and medicine; however, nanoparticle characterization is typically performed under the pretext of well-dispersed, aqueous conditions. Here, we systematically characterize the effects of aggregation on the alternating magnetic field induced heating and magnetic resonance (MR) imaging performance of iron oxide nanoparticles (IONPs) in non-ideal biological systems. Specifically, the behavior of IONP aggregates composed of ~10 nm primary particles, but with aggregate hydrodynamic sizes ranging from 50 nm to 700 nm, was characterized in phosphate buffered saline and fetal bovine serum suspensions, as well as in gels and cells. We demonstrate up to a 50% reduction in heating, linked to the extent of aggregation. To quantify aggregate morphology, we used a combination of hydrodynamic radii distribution, intrinsic viscosity, and electron microscopy measurements to describe the aggregates as quasifractal entities with fractal dimensions in the 1.8–2.0 range. Importantly, we are able to correlate the observed decrease in magnetic field induced heating with a corresponding decrease in longitudinal relaxation rate (R_1) in MR imaging, irrespective of the extent of aggregation. Finally, we show *in vivo* proof-of-principle use of this powerful new imaging method, providing a critical tool for predicting heating in clinical cancer hyperthermia.

INNOVATION

We present two technical innovations which advance biomedical nanoparticle use beyond the assumption of well-dispersed, idealized systems. First, we develop a quantitative method to characterize biologically-relevant aggregation in terms of both degree and structure of the aggregates. Using IONPs as a model system, we then demonstrate the significant impact of aggregation on heating and imaging performance. Importantly, these new methods can now inform nanoparticle engineering design to either inhibit or encourage aggregation for specific applications for a variety of nanoparticles in the future. To illustrate this, our second technical innovation demonstrates the measurement and prediction of aggregation in a critical breakthrough for IONP-based cancer hyperthermia. Specifically we show that sweep imaging with Fourier transformation (SWIFT) MR imaging not only maintains signal integrity for IONP concentrations an order of magnitude higher than traditional MR approaches, but provides a platform to account for *in vivo* aggregation and directly predict local heating to better control biomedical outcomes.

INTRODUCTION

Nanoparticles are currently used for a wide variety of imaging and therapeutic applications in biomedicine^{1–5}. However, the presence of ions and proteins in the complex biological milieu of the body inevitably

leads to nanoparticle aggregation^{6,7} and it remains unclear how this affects their properties and functional performance. Using magnetic iron oxide nanoparticles (IONPs) as a model system, we show both the impact of biological (ionic and protein based) aggregation on alternating magnetic field induced heating and a new method to measure it with sweep imaging with Fourier transformation (SWIFT) magnetic resonance imaging (MRI)^{8–11}. While the application of IONPs in heating and imaging is known to be affected by nanoparticle aggregation, this influence is not well understood, with a range of studies suggesting contradictory effects on heating (Supplementary Information S1.1) and MRI contrast^{12–23}. Clearly then, there is a need to measure and account for aggregation effects on the heating and imaging of IONPs.

Described in detail subsequently, aggregation studies were conducted with commercially available Ferrotec EMG-308 composed of 10 ± 2.5 nm diameter superparamagnetic magnetite (Fe_3O_4) nanoparticles coated with an anionic surfactant in aqueous suspension. This system has been previously shown to heat reproducibly in dispersed solutions²⁴, hence it is a convenient model for systematic examination. Sub-micron IONP aggregates were characterized in suspensions, gels, and prostate cancer cells by comparing measured properties (hydrodynamic radii, intrinsic viscosities, and projected areas observed in electron microscopy images) to those expected for point-contacting quasifractal aggregates²⁵. Next, the heating and imaging behavior of these systems was characterized

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under varying aggregation conditions, where the degree of aggregation was controlled by suspension solute concentration. The heating of the IONPs in an alternating magnetic field was found to decrease by up to 50% with increasing extent of aggregation. The MR contrast of the IONPs in SWIFT MRI also exhibited a similar reduction in longitudinal relaxation rate (R_1) due to aggregation. Interestingly, SWIFT MRI demonstrates a strong correlation between longitudinal relaxation rate (R_1), IONP concentration ($[\text{Fe}]$), and extent of aggregation, thus allowing for direct prediction of IONP heating, regardless of the degree of aggregation in biological systems. Using cancer treatment by IONP hyperthermia as an example application, we then discuss the relevance for clinical use. These results suggest that biological aggregation is a critical factor for nanomaterial use in biological systems and that SWIFT MRI can be used to both measure and account for it in IONP applications.

RESULTS AND DISCUSSION

Characterizing nanoparticle aggregates

Nanoparticle aggregation in biological media is typically caused by electrostatic screening and protein bridging (Fig. 1a,b)^{6,7}. Phosphate buffered saline (PBS) and fetal bovine serum (FBS) were used to mimic these biological aggregation processes under controlled conditions. Populations of aggregates with repeatable mean hydrodynamic diameters (D_H , measured via dynamic light scattering) were produced by mixing the stock IONP solution with variable concentrations of PBS and FBS (Fig. 1c). A mean D_H of 52 nm was measured for the nanoparticles in pure water, suggesting some clustering for even the “dispersed,” aqueous case.

D_H provides a measure of the degree of aggregation, but does little to describe the geometry of the aggregates, which may also play a role in determining their effective magnetic behavior^{22,23}. While static light scattering can be used to examine aggregate morphologies in colloids²⁶, in this study, many aggregates had hydrodynamic sizes in the sub 100 nm range, which are difficult to detect at the single aggregate level with visible wavelength light scattering. Therefore, further methods needed to be developed which can be used for aggregate analysis in complex biological media (i.e. in the presence of extracellular and intracellular matrices). Here then, the aggregates were assumed to be quasifractal in nature, such that the number of IONPs per aggregate (N) is assumed equal to $k_f(R_g/a_{NP})^{D_f}$ (k_f : pre-exponential factor, R_g : radius of gyration, a_{NP} : average IONP radius, and D_f : fractal dimension)²⁵. In the quasifractal model, the fractal dimension is a key indicator of aggregate morphology; $D_f = 1.0$ corresponds to a straight chain aggregate, while $D_f = 3.0$ corresponds to a dense cluster. D_f was inferred in two ways— from direct examination of cryo-EM images (image method)²⁷ and from analysis of the suspensions’ intrinsic viscosities combined with D_H measurements (ensemble method) (Fig. 1d,e). The former enables inference of the most probable fractal descriptors (D_f , k_f , and N) for each aggregate observed in an electron microscopy image, while the latter simply allows for inference of the volume-weighted D_f and k_f in an entire suspension. Generally, in colloids, two limiting cases of aggregation are observed: (1) diffusion limited aggregation (DLA), in which aggregates take random walks in the absence of any long range attractive or repulsive interactions (though short range van der Waals forces may be present), and bind to one another upon collision; (2) reaction limited aggregation (RLA), in which particles may weakly repel one another, binding upon collision with a probability less than unity, but still aggregate via Brownian motion²⁸. DLA leads to aggregates with characteristic D_f values near 2.5 if aggregation primarily occurs through monomer (i.e. single particle) addition to aggregates, and D_f near 1.8 under instances where monomers are depleted and aggregate-aggregate collisions are abundant. Conversely, RLA leads to D_f near 3.0 with monomer-aggregate collisions, and D_f near 2.1 if monomers are completely depleted (i.e. with aggregate-aggregate collisions). As evidenced in Fig. 1, both the image and ensemble methods of aggregate analysis reveal that aggregates have fractal dimensions in the 1.8–3.0 range

(the expected range encompassing both limiting circumstances) and in most instances the volume averaged fractal dimensions are in the 1.8–2.1 range. In total, both aggregate analyses suggest that most suspensions are devoid of monomers. While the employed methods do not allow us to determine whether aggregation is more “DLA-like” or “RLA-like,” we can conclude that aggregate growth through aggregate-aggregate collisions is predominant in the test suspensions. Moreover, through the image method, we find that all suspensions had standard deviations of 0.2–0.3 and ~ 0.1 for D_f and k_f , respectively. Hence most observed aggregates in a given suspension had qualitatively similar morphologies. The image method also reveals that the average number of primary particles per aggregate increases with increasing solute concentrations, in line with what is suggested (but not proven) solely by D_H measurements.

Heating

Once we understood the makeup of the IONP aggregate populations, we investigated the resulting impact on performance. IONP-based cancer treatment relies on the fact that superparamagnetic and ferromagnetic nanoparticles produce heat in response to an alternating magnetic field^{24,29–32}. IONP heating is described by the specific absorption rate (SAR) which is a function of the excitation field (frequency and magnetic field strength), IONP properties, and suspending medium^{24,31}. SAR was characterized for each of the aggregated suspensions as shown in (Fig. 2a). Then heating versus IONP concentration was characterized for the suspended aqueous and 2X PBS (“highly aggregated”) populations (Fig. 2b). SAR_V is expected to vary linearly with IONP concentration in the absence of significant interparticle (or inter-aggregate) interactions³¹. This trend has been shown for dispersed suspensions²⁴ and appears to hold for the aggregated (2X PBS) case as well. Aggregates formed in PBS and FBS had comparable fractal descriptions and so heating in both suspensions followed a similar trend with D_H (Fig. 2c,d). Specifically, SAR decreased up to 30% with increasing aggregate size and plateaued for aggregate populations above about 500 nm in mean D_H for solutions. Finally, to better simulate conditions in a tissue environment, the PBS-induced aggregates were embedded in a 1% agarose mixture (common tissue phantom) and heated. The agarose environment caused an additional 20% decrease in SAR for all cases.

IONP heating in an alternating magnetic field is attributed to two loss mechanisms — Brownian rotation (frictional) and relaxation of the particles’ magnetization (Néelian or hysteresis)^{29–31}. Néelian relaxation was the dominant mechanism in these IONP heating experiments (Supplementary Information S1.3) and aggregation can interfere with this process in two ways. First, it is likely that the IONPs within the aggregates experience interactions through their locally induced magnetic fields (Fig. 2e). Such interparticle interactions are typically modeled in terms of dipole systems, where an increase in interactions is predicted to decrease the particles’ magnetization and heating^{13,22}. Interparticle interactions in these cases are largely determined by spacing, although aggregate size and shape will have some impact. Second, theoretical modeling assumes the particles’ anisotropic axis is free to align with the applied field³¹, but here the individual superparamagnetic IONPs were randomly confined in the aggregates and were likely unable to achieve optimal alignment when the excitation field was applied. Thus, each aggregate will have an effective anisotropic axis, which may be able to align in suspension, but may be further bound by the gel matrix (Fig. 2e). This restriction may explain the additional decrease in heating observed in the 1% agarose samples.

SWIFT MR imaging

Beyond heating, SWIFT and gradient echo (GRE) MRI techniques were used to image IONP populations in 1% agarose gels (Fig. 3a). The positive contrast generated by SWIFT shows clear advantages over GRE, where signal voids occur and the intensity is reduced to the level of noise for IONP concentrations above ~ 0.1 mg Fe/ml (Fig. 3b)¹¹. This agrees with previous studies showing that current imaging techniques are unable to

Figure 1 Controlled formation and characterization of IONP aggregates. **(a)** IONPs are incubated in various concentrations of PBS and FBS to simulate biological aggregation. Aggregation occurs due to charge shielding effects (PBS) or physical adsorption to proteins and neighboring particles (FBS). **(b)** The media lowers the energy barrier required for aggregation, allowing nanoparticles to escape a secondary energy minimum. **(c)** Mixing at controlled concentrations of PBS or FBS resulted in discrete populations of IONP aggregates, as measured by their hydrodynamic diameters (error bars: $\pm$ s.d., $n = 4$). **(d,e)** Cryo-EM image processing (image method) and NanosightTM/intrinsic viscosity (ensemble method) measurements are used to infer the average pre-exponential factor (k_f) and fractal dimension (D_f) of multiple and single aggregates, respectively, in suspensions, where $N = k_f (R_g/a_{NP})^{D_f}$ describes aggregate morphology. Reported values from image processing are the average of 20 or more aggregates for each suspension. Simulated aggregates with the average number of IONPs and the average reported D_f and k_f are displayed (image method: green, ensemble method: yellow). $N = 49, 79$, and 91 for the 1X, 1.75X, and 2X PBS suspensions, respectively, and $N = 54, 109$, and 98 for the 25%, 50%, and 100% FBS suspensions, respectively.

accurately measure IONP concentrations within the range used for clinical hyperthermia (~ 1 to 10 mg Fe/ml)^{5,33}. Conversely, the SWIFT technique preserves the short-lived signal of the water protons interacting with the IONPs, thereby maintaining signal integrity up to at least 3 mg Fe/ml^{10,11}. Thus, SWIFT can be used to measure the longitudinal relaxation rate ($R_1 = 1/T_1$) versus IONP concentration in dispersed and aggregated suspensions (Fig. 3c). Here, the dispersed IONP population maintains a linear relationship between concentration and R_1 throughout the measured range. This correlation remains, but weakens above 2 mg Fe/ml for the aggregated samples. Even more significant, the observed reductions in relaxivities (slope $\sim r_1 = R_1/[\text{Fe}]$) for the moderately (1.5X PBS) and highly (2X PBS) aggregated cases appear to correlate with the observed drops in heating, suggesting R_1 can be used as a measure of volumetric heating capacity, accounting for both localized IONP concentration and aggregation state (Fig. 3d). This has significant implications for clinical imaging and pre-treatment planning, where a map of R_1 can be used to better guide delivery and heating of IONPs for improved outcomes in cancer hyperthermia.

In vitro characterization

To further probe the relationship between IONP aggregation, heating, and MRI contrast, human prostate cancer cells (LNCaP-Pro5)³⁴ were incubated with IONPs for 24 hours and washed extensively to remove any loosely associated IONPs. TEM images of the IONP-exposed cells reveal large intracellular and tightly associated extracellular aggregates. The majority of internalized IONPs are clustered in intracellular compartments, forming much larger aggregates (Fig. 4a), but with similar fractal dimensions to those observed in PBS and FBS. Heating was measured for the IONP-loaded cells in suspension and agarose gel (Fig. 4b). While the cellularly associated IONPs demonstrated a higher degree of aggregation, there was no additional decrease in SAR as compared to 2X PBS in agarose. This suggests that there may be a practical limit to the effects of interparticle interactions and confinement on heating. The IONP-loaded cells in agarose were also imaged with SWIFT MRI and displayed markedly lower r_1 values than the dispersed and aggregated gels (Fig. 4c). We hypothesize two contributions to this decrease in intracellular relaxivity. First, TEM indicates that most intracellular aggregates are localized in cellular compartments, which limits the number of water protons able to access the IONPs and thus decreases the T_1 -weighted signal intensity. Second, as

Figure 2 Effects of aggregation on IONP heating. Two variations of SAR are used here: SAR_V (W/ml, heating in an IONP-laden volume) and SAR_{Fe} (W/g Fe, heating normalized based on IONP density, SAR_V = SAR_{Fe} × [Fe]). (a) SAR values were measured by placing the samples in an insulated induction coil, applying an alternating magnetic field at 190 kHz and 20 kA/m, and estimating heating from the temperature-time response. (b) Despite the observed changes in SAR_{Fe} with aggregation, SAR_V maintains a linear relationship with IONP concentration in the aggregated state. (c,d) SAR values decreased with increasing aggregate size and displayed significantly lower values in the 1% agarose gel. (d) Aggregate shape and packing density will determine the amount of interparticle magnetic interactions due to their dipolar behavior, but alignment of the randomly constrained IONPs within the aggregates may also play a role in the observed effects on heating. (All error bars: ± s.d., n = 4.)

intracellular aggregates are considerably larger than those in suspension the accessible surface area of each IONP is reduced, again limiting access of water and decreasing T_1 -weighted signal intensity. These results and conclusions agree with previously published work^{15,35,36}.

Despite the observed shift, the linear relationship between R_1 and [Fe] is maintained, so it is still possible to develop a correlation with

heating for the case of cellularly associated IONPs (Fig. 4d). In addition, the decrease in longitudinal relaxation is accompanied by a decrease in transverse relaxation (r_2 , the signal that determines the upper limit for SWIFT MRI IONP quantitation). In the present study, we were able to measure bulk cellular IONP concentrations greater than 4 mg Fe/ml, and by capitalizing on this change it will likely be possible to push these limits further.

These results point to two key limitations of the current approach. First, while linear trends were demonstrated between R_1 and [Fe] for the agarose gel and cellularly-associated samples, the relaxation rate was much lower in the cellular case. We believe this is driven by the high packing density and compartmentalization of the aggregates observed in the intracellular case, both of which reduce access to water protons. While this warrants further study, we believe the large shift in relaxation rate can be mitigated through an appropriate IONP coating which better controls aggregation, water access, and cellular uptake. Second, while the IONP concentrations measured by SWIFT MRI were an order of magnitude higher than possible with standard MR approaches¹¹, quantification was still limited to about 3 and 4 mg Fe/ml for the agarose gel and cellular cases, respectively. While this may be adequate in many cases, localized concentrations greater than 10 mg Fe/ml are possible in IONP-based hyperthermia treatments, so we are continuing to investigate the impact of the SWIFT MRI measurement parameters on these limits (i.e. increased acquisition bandwidth or multi-band-SWIFT³⁷). In addition, this limit is largely determined by the r_2 relaxation rate, so the impact of IONP coating on water access might again be an important control factor in designing a complimentary approach.

Preliminary *in vivo* investigation

Finally, to better demonstrate proof-of-concept of this technology, preliminary MR imaging was performed in a mouse hindlimb tumor model. Aqueous IONPs were directly injected into the LNCaP tumor at a dosage of 0.25 mg Fe/ml tumor and imaged 3 hours after injection (Fig. 5a,b). While we suspect the surfactant-coated IONPs experienced significant aggregation immediately upon administration and that this resulted in minimal diffusion from

the injection site, the longitudinal relaxation rate map still indicated a clear distribution of IONPs (Fig. 5c). Based on the correlation data in LNCaP cells, the maximum local IONP concentration and SAR_V would be about 0.55 mg Fe/ml and 0.07 W/ml, respectively. Continuing work aims to directly correlate IONP concentration, heating, and imaging contrast *in vivo*.

Figure 3 SWIFT MRI measures relaxivity and correlates it to the heating ability of concentrated IONPs in dispersed and aggregated systems. (a) Positive contrast is demonstrated with SWIFT MRI versus a decreasing signal-to-noise ratio with GRE. The increased intensity can be seen visually for phantoms with IONP concentrations ranging from 0 to 3.0 mg Fe/ml, where the signal voids are indistinguishable from one another in the negative contrast GRE images. (b) In the SWIFT images, the signal-to-noise ratio remains high even at increased IONP concentrations (15° flip angle). (c) R_1 values measured using SWIFT MRI show a strong correlation to IONP concentration and aggregation state, (d) offering the potential to use SWIFT MRI as a direct predictor for IONP heating, taking into account both localized concentration and aggregation state (R_1 was correlated with the experimental heating measurements described in Fig. 2).

In summary, our results demonstrate that aggregation can dramatically influence the ability of nanoparticles to image and treat disease in biological applications. Using IONPs as a model system, we have characterized their aggregation on the basis of number, size, and shape in solutions, gels, and cells. Using these well defined aggregates we then demonstrate a dramatic reduction in both heating potential and MRI contrast. Due to the correlation in these findings we also demonstrate that SWIFT MRI directly predicts the reduction in heating experienced during aggregation. Further, we show that SWIFT MRI can provide an imaging based prediction of *in vivo* IONP concentration. This ability can now be refined in future work to predict heating while accounting for aggregation in clinical cancer hyperthermia treatments.

More fundamentally, the experimental characterization of IONP aggregate size, geometry, and behavior can be combined with previous descriptions of dynamic, magnetic dipole interactions^{12,13,18,22} and aggregated relaxation^{15,16} to improve our understanding of the physical processes driving these changes in IONP heating and imaging. We speculate that enhanced performance of IONPs can be obtained through coatings that prevent or control interparticle interactions and subcellular localization. However, these methods and conclusions extend beyond the use of IONPs for heating and imaging. Biological aggregation is a critical consideration in the design and application of all nanomaterials intended

for use in biological systems. Technologies to study aggregate formation and its impact on performance, such as those presented here, are going to be necessary to advance the use of nanoparticles in medicine.

METHODS SUMMARY

Further experimental details can be found in **Supplementary Information S2**.

Preparation of IONP aggregates

Stock EMG-308 (Ferrotec USA Corp., Bedford, NH) IONP suspension was diluted in varying concentrations of PBS (0X, 1X, 1.25X, 1.5X, 1.75X, and 2X) and FBS (0%, 10%, 25%, 50%, and 100%). The aggregation reaction was allowed to proceed for exactly 4 hours, at which point measurements were made or the aggregates were fixed.

Dynamic light scattering (DLS) measurement of aggregate D_H

DLS was performed on a BIC 90Plus particle analyzer (Brookhaven Instruments Corp., Holtsville, NY). The incident 35 mW red diode laser generated 660 nm light and the solution viscosities were manually input based on intrinsic viscosity measurements for each solution.

Figure 4 Heating and imaging of IONPs *in vitro*. LNCaP cells were incubated with IONPs for 24 hours before subsequent analysis. (a) TEM shows large IONP aggregates both localized on the cell membrane and internalized in cellular compartments (average observed N of 374 and 248 for intracellular and extracellular aggregates, respectively). The cell image was created from a collection of TEM images taken at the same magnification. The aggregate shapes differ somewhat from those measured in PBS or FBS suspension (average $D_f \approx 1.9$ for both intracellular and extracellular aggregates), displaying more tightly packed structures of lower fractal dimension. (b) The reductions in SAR for the cellularly associated IONPs were similar to those for 2X PBS in agarose, (c) but a marked decrease in the longitudinal relaxivity was observed over the aggregated cases. (d) However, SWIFT MRI still appears to demonstrate a correlation with heating, even shifted to this lower relaxivity. (All error bars: $\pm$ s.d., $n = 4$.)

TEM preparation and imaging

Room temperature TEM images of IONPs were acquired with a FEI Tecnai T12 microscope (FEI, Inc., Hillsboro, OR) operating at 120 kV. A 200 mesh copper grid with formvar and carbon supports was dipped into a ~ 1 mg Fe/ml IONP suspension, then removed and allowed to dry before imaging. The LNCaP cells underwent a standard process of fixation, staining, dehydration, infiltration of polymer resin, and curing. The final polymer block was cut into ~ 60 nm slices with a microtome, deposited on a 200 mesh copper grid with formvar and carbon supports (Ted Pella Inc., Redding, CA), and imaged at 60 kV. Multiple images were taken at high magnification and later combined in a collage to obtain the high resolution image of the whole cell presented in Fig. 4. Cryogenic TEM

imaging was performed on a FEI Tecnai Spirit Bio-Twin microscope (FEI, Inc. Hillsboro, OR) at 120 kV and -179°C .

Characterization of IONP aggregate fractal geometry

Aggregates were assumed to be quasifractal in nature, such that each aggregate approximately obeys the relationship $N = k_f (R_g/a_{NP})^{D_f}$, where N is the number of IONPs comprising the aggregates, R_g is the aggregate radius of gyration, a_{NP} is the average core radius, and k_f and D_f are the pre-exponential factor and fractal dimension, respectively²⁵. D_f was inferred in two ways. In the first (image method), individual aggregates were imaged via Cryo-EM, and the two-dimensional radius of gyration, the perimeter, area, and longest end-to-end distance in the aggregate were

Figure 5 Preliminary SWIFT MR imaging in a mouse hindlimb tumor model. (a) A 0.25 mg Fe/ml tumor dose of aqueous IONPs was directly injected into an LNCaP, hindlimb tumor. (b) An MR image taken 3 hours after injection showed minimal diffusion from the injection site (likely due to rapid aggregation), (c) while the longitudinal relaxation rate map suggested a maximum local IONP concentration of about 0.55 mg Fe/ml based on the correlation data in LNCaP Cells.

measured and compared to values for computer generated aggregates of prescribed N_p , a_p , k_f , and D_f . Specifically, in this method individual aggregates were identified in images via setting a grayscale threshold specific for each image; darker values than the threshold denoted spaces where aggregates were present. Upon setting this threshold, a corresponding two-dimensional array was made where a value of “1” was assigned when a portion of an aggregate was detected, while a value of “0” was assigned where aggregates were not observed (lighter than the grayscale threshold). Individual aggregates were hence identified as contiguous spaces within two-dimensional arrays in which values of “1” had at least one neighboring “1” value. Similar two-dimensional arrays were constructed for computationally generated and deposited aggregates, facilitating one-to-one comparison in measured two-dimensional properties for imaged aggregates and computationally generated aggregates. In the second (ensemble method), the IONP’s hydrodynamic radii distribution functions were measured using a NanosightTM and the suspensions’ intrinsic viscosities were characterized. This information, along with the IONP mean radius (measured by TEM), were compared to the expected hydrodynamic radii distributions and intrinsic viscosities of computer generated aggregates of prescribed k_f and D_f with the most probable k_f and D_f then selected for each suspension.

Heating (SAR) measurements

These heating methods, experimental setup, and applied fields have been previously described in detail²⁴. Briefly, a 1 kW Hotshot inductive heating system with a 2.75-turn, water-cooled copper coil (Ameritherm Inc., Scottsville, NY) was used to generate the alternating magnetic field, adjustable up to 24 kA/m (volume-averaged peak-amplitude across the

sample) with an applied frequency fixed at $190 \pm 10\%$ kHz. Each 1 ml sample in a plastic microcentrifuge tube was insulated and centered in the inductive coil and heated for 3 minutes and the temperature was continuously recorded with a Luxtron 3100 fluoroptic thermometry system (Luxtron Inc., Santa Clara, CA). The SAR was estimated from the maximum linear fit within the initial minute of heating (typically the first 15–20 seconds), based on the rate of temperature rise method²⁹ and a custom fitting routine²⁴.

SWIFT MR imaging

The longitudinal relaxation time (T_1) was measured by the Look-Locker method with an ultra-short T_2 sensitive MRI sequence (SWIFT) for read out¹¹. Measurements were performed using a 9.4T magnet equipped with a DirectDrive spectrometer with a volume transmit/receive coil (Agilent Technologies, Santa Clara, CA). The pulse duration of the adiabatic saturation pulse was 500 μ s. Repetition time = 1.13 ms, flip angle = 1° , spectral width = 125 kHz, and field of view = $50 \times 50 \times 150$ mm³. The total scan time was ~ 10 minutes. The $T_{1\text{eff}}$ map was generated by performing three-parameter non-linear least squares fitting to the saturation-recovery equation on a pixel-by-pixel basis. The real T_1 map was acquired by doing Look-Locker correction on the $T_{1\text{eff}}$ map³⁸.

Preparation of *in vitro* samples

A prostate cancer cell line (lymph node cancer of the prostate, LNCaP-Pro5)³⁴ was incubated with 0.5 mg Fe/ml IONPs for 24 hours in monolayers in 75 cm² T-flasks at 37 °C and 5% CO₂. The cells were then washed with phenol-red free Hank’s balanced salt solution (HBSS) five times, to remove any non-cellularly associated IONPs, detached, pelleted, and then resuspended in cell media or a 1% agarose/HBSS mixture.

In vivo implantation and imaging

All animal procedures and care were approved by the University of Minnesota Institutional Animal Care and Use Committee. The male athymic nude mice (NU/J, The Jackson Laboratory, Bar Harbor, Maine, USA) weighing 20 to 25 g were housed according to these procedures. One mouse was used for proof-of-principle imaging experiments and received subcutaneous, hindlimb injections of 1 million LNCaP cells, prepared as previously described^{34,39}. Experiments were conducted after about 6 weeks and the tumors had reached an average diameter of about 7 mm. The animal was initially anesthetized in an induction chamber with 3–5% isoflurane and 50% nitrous oxide and then maintained during imaging via a nose cone at 1–2% isoflurane and 50% nitrous oxide. The aqueous IONP solution was directly injected into the tumor at a dosage of 0.25 mg Fe per milliliter tumor, immediately after baseline imaging. Three hours after IONP injection, the animal was again anesthetized for MR imaging. The SWIFT MR imaging protocols followed those described above. The animal was euthanized at the completion of the second imaging session.

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AUTHOR CONTRIBUTIONS

M.E., J.B., K.H., C.L.H., M.G., and C.J.H. conceived the experiments; M.E., K.H., J.Z., S.J., and H.R. performed and analyzed the experiments;

J.B., C.L.H., M.G., and C.J.H. oversaw the project; and M.E. wrote the manuscript, with all authors contributing significantly.

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SUPPLEMENTARY INFORMATION

S1. Additional results and discussion

Additional details on the characterization of the IONPs and aggregates are included in **Supplementary Figs. 1 to 3** and **Supplementary Tables 1 and 2**.

S1.1 Brief literature review — Impact of interparticle interactions on heating

While there are some examples in literature which touch on the effects of interparticle interactions on the ability of magnetic nanoparticles to

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Supplementary Figure 1 TEM characterization of aqueous EMG-308 IONPs. Commercially available EMG-308 was purchased from Ferrotec and used within 3 months. **(a,b)** TEM images reveal that the IONPs are polydisperse in size and shape. **(c)** The longest radius and the radius normal to it were measured using Image J (NIH), resulting in the values shown in the table. Scale bars represent 50 nm.

Supplementary Figure 2 X-ray diffraction pattern of EMG-308 IONPs. EMG-308 displays peaks characteristic of Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ phases. The small peak at $52^\circ 2\theta$ arises from the aluminum sample holder. A cobalt source was used for data collection, shifting the entire data set to higher degrees 2θ as compared to literature indices.

generate heat in response to an applied alternating magnetic field (summarized in **Supplementary Table 3**), these previous studies fail to provide a clear inference for clinical hyperthermia applications due to three main limitations. First, many of the studies do not reflect physical cases

relevant to biological aggregation (such as dried powders or idealized dipole systems). Second, very few of the studies directly investigate heating performance. While the qualitative impact on heating can be inferred from other physical behaviors (such as magnetic susceptibility or hysteresis), a quantitative correlation to heating behavior is not provided. Finally, aggregation is not typically measured directly or characterized appropriately. There is therefore a clear need for systematic study of biologically induced aggregation and its impact on magnetic nanoparticle heating behavior.

S1.2 Heating in redispersed suspensions

The reduction in heating attributed to aggregation in suspension (here the maximal case at 2X PBS) appeared to be at least partially reversible if the aggregates were redispersed. In this experiment, the IONPs were allowed to aggregate following the standard protocol, however, following the 4-hour aggregation period, the aggregated IONP solution was diluted with distilled water down to a 1X PBS concentration, vigorously pipetted, and then sonicated for 15 minutes. Afterwards, the IONP heating returned to 90% of the SAR value for well-dispersed conditions (**Supplementary Fig. 4**), suggesting that the aggregation process and accompanying reduction in heating are reversible.

S1.3 Heating in glycerol suspensions

IONP heating in an alternating magnetic field is generally attributed to two loss mechanisms, Brownian relaxation and relaxation of the particles' magnetization (Néelian or hysteresis)^{29–31}. Brownian heating relates to the frictional losses as the particles rotate with the dynamic field, while heating is also derived from the reversal of the IONP's internal magnetization. To isolate the two effects, heating experiments were performed in glycerol, which is an order of magnitude more viscous than water and, according to theory, should effectively eliminate the Brownian mode of heating. There was no significant difference in heating between glycerol and water for any of the fields studied (**Supplementary Fig. 5**), suggesting that Brownian contributions are absent even in the dispersed, aqueous suspensions. Therefore, heating is dominated by Néelian relaxation for these superparamagnetic IONPs^{30,31}.

S1.4 Heating at different field strengths

The heating response at a variety of field strengths was also compared for several of the aggregation end points. A complete summary of the SAR results is included in **Supplementary Fig. 6**. While the general trends observed at 20 kA/m remain consistent across the field strengths studied, there are some differences in the relative drops observed between aggregation states at the different field strengths. This can likely be attributed to changes in the complex magnetic susceptibility, which relates directly to heating^{24,31}, and is likely affected by the IONP aggregation state, in addition to the applied field.

Supplementary Figure 3 Digital photographs of IONP aggregation over time. While the well-dispersed solutions remain transparent, the PBS-incubated suspensions become increasingly cloudy over time as the aggregate sizes increase. The aggregates will settle out of suspension when they reach a large enough size, but can be temporarily resuspended for measurements by gently shaking by hand. These photos were taken at an IONP concentration of 0.25 mg Fe/ml for visual clarity.

Supplementary Table 1 Hydrodynamic diameters of IONP aggregates in PBS. Dynamic light scattering measurements were collected after 4 hours of incubation in various concentrations of phosphate buffered saline ($\pm$ s.d., $n = 4$).

Concentration PBS	Average hydrodynamic diameter (nm)
0	52 ± 1
1X	70 ± 1
1.25X	109 ± 3
1.5X	243 ± 23
1.75X	495 ± 58
2X	665 ± 150

Supplementary Table 2 Hydrodynamic diameters of IONP aggregates in FBS. Dynamic light scattering measurements were collected after 4 hours of incubation in various concentrations of fetal bovine serum ($\pm$ s.d., $n = 4$).

Concentration FBS	Average hydrodynamic diameter (nm)
10%	72 ± 2
25%	103 ± 2
50%	157 ± 7
100%	332 ± 33

S2. Detailed methods and materials

S2.1 Iron quantitation

A bulk colorimetric assay was used to quantify the IONP concentration in terms of mass iron per volume^{40–42}. This assay relies on the detection of a complex formed when Fe^{2+} ions react with ferrozine dye, so complete digestion and release of the iron was required. First, 300 μl of each sample (in triplicate) was added to a glass tube and concentrated hydrochloric acid was added at a 1:1 ratio. The tubes were sealed and held at 60 $^{\circ}\text{C}$ for 2 hours, vortexing every 30–45 minutes. The samples were then centrifuged at 400 RCF and 4 $^{\circ}\text{C}$ for 10 minutes. Next, 200 μl of the resulting supernatant was mixed at a 1:4 ratio with 2X PBS buffer. A serial 1:1 dilution with distilled water was used to prepare

seven 500 μl samples of decreasing concentration. Then, 50 μl of ferrozine reagent (6.5 mM ferrozine (Acros Organics, Inc., New Jersey, NJ), 13.1 mM neocuproine (Sigma-Aldrich Co., St. Louis, MO), 2 M ascorbic acid (Alfa Aesar, Ward Hill, MA), and 5 M ammonium acetate (Sigma-Aldrich Co., St. Louis, MO)) was added to each sample and set at room temperature for 30 minutes. Iron standards at 0, 1.25, 2.5, 5, 8, and 10 μg per ml were similarly prepared from a diluted stock iron standard (Sigma-Aldrich Co., St. Louis, MO). Duplicate 200 μl aliquots from each sample were then plated in a 96-well plate and the absorbance at 562 nm was measured in a Synergy HT spectroscopic plate reader (BioTek Instruments, Inc., Winooski, VT). The final iron concentrations were calculated from comparison with the absorbance of the iron standards.

S2.2 X-ray diffraction

X-ray diffraction was performed on a PANalytical X'pert MPD diffractometer (PANalytical, Inc., Westborough, MA) utilizing a cobalt source and an X'Celerator detector. Measurements were taken from $2\theta = 15$ to 85° with a step size of 0.02° and a dwell time of 100 seconds. Samples were prepared by drop coating from a concentrated aqueous IONP suspension onto a single quartz crystal sample holder.

S2.3 Preparation of IONP aggregates

The IONPs used in this study are designed to retain colloidal stability in aqueous suspension via an anionic surfactant; however, interference with the double-layer can lead to significant aggregation of the dispersed particles^{43–47}. Here we demonstrate that incubation with phosphate buffered saline (PBS, ionic) and fetal bovine serum (FBS, protein) solutions led to varying degrees of aggregation, depending strongly on the concentration of the aggregating solute. The stock IONP solution was diluted in varying concentrations of PBS (0X, 1X, 1.25X, 1.5X, 1.75X, and 2X) and FBS (0%, 10%, 25%, 50%, and 100%). While the aggregation protocol varied slightly depending on the sample preparation required for each measurement assay, the following consistencies allowed for repeatable aggregation between the protocols.

1. The aggregation reaction was allowed to proceed for exactly 4 hours, at which point measurements were made or the aggregates were fixed. The aggregation kinetics had slowed sufficiently by this point (data not shown) such that repeatable aggregate populations were formed.
2. Pipetting appeared to partially break up some of the larger aggregates. Once the aggregates were formed, mixing was accomplished by light vortexing or gentle shaking. If a pipette transfer was absolutely required, a large bore pipette was used.
3. All dilutions performed maintained the concentration of the aggregating solute (i.e. aggregates formed in 1X PBS were diluted only with 1X PBS).

Preparation of PBS solutions: Stock cellgro 10X PBS (Mediatech, Inc., Manassas, VA) was diluted with distilled water to the required concentrations. Dilutions involving the aqueous IONP solution accounted for this included volume, such that the reported PBS concentrations are that of the final solution.

Preparation of FBS solutions: Stock FBS (SAFC, St. Louis, MO) was diluted with distilled water to the required concentrations. The reported FBS concentrations are that of the initial FBS solution used for dilutions, without accounting for the added IONP suspension volume.

Preparation of agarose samples: 2% (by weight) agarose powder (Bio-Research Products Inc., North Liberty, IA) was dissolved at high temperature in PBS solution. This was then added at a 1:1 ratio to the aggregated solution (after the 4-hour time point), vortexed to mix thoroughly, and then immediately placed in an ice bath for 15 minutes to set.

Supplementary Table 3 Summary of literature related to magnetic nanoparticle interactions and heating. Previous studies include both experimental and theoretical work which provides some conclusions related to how interparticle interactions within aggregates may affect their ability to generate heat^{12–14,17–19,21–23,53–58}. However, these studies suffer from a number of limitations in describing the impact to magnetic nanoparticle based cancer hyperthermia.

Reference	Experimental	Theory/ Simulation	NP phase	Behavior measured or predicted	Inferred consequence for heating
Berkov ¹²		×	Fixed system	Hysteresis	Decrease
Burrows <i>et al.</i> ¹⁸		×	Fixed system	Hysteresis losses	It depends...
Castro <i>et al.</i> ¹⁴		×	Suspension	Aggregate formation	
Chantrell <i>et al.</i> ¹³		×	Solid matrix	Hysteresis, susceptibility	Decrease
Dennis <i>et al.</i> ¹⁷	×	×	Suspension	Neutron scattering, SAR	Increase
Dutz & Hergt ²¹	×		Dried powder	Hysteresis losses	Decrease
Dutz <i>et al.</i> ⁵³	×		Dried powder	Hysteresis losses	Slight increase
Eberbeck & Trahms ⁵⁴	×		Suspension	Magnetization	Decrease
Eggeman <i>et al.</i> ⁵⁵	×		Suspension	dT/dt	Increase
Haase & Nowak ²²		×	Fixed system	Hysteresis losses	It depends...
Linh <i>et al.</i> ⁵⁶	×		Suspension	SAR	Decrease
Martinez-Boubeta <i>et al.</i> ²³	×	×	Suspension	Hysteresis, SAR	It depends...
Serantes <i>et al.</i> ¹⁹	×	×	Suspension	SAR, hysteresis losses, susceptibility	Decrease
Urtizberea <i>et al.</i> ⁵⁷	×		Suspension	SAR, susceptibility	Decrease
Yuan & Borca-Tasciuc ⁵⁸	×	×	Suspension	SAR, susceptibility	Increase

Supplementary Figure 4 Heating in redispersed suspensions. While the significant amount of aggregation induced in 2X PBS results in a 30% decrease in SAR, this appears to be at least partially reversible when redispersing at lower PBS concentrations, resulting in a significant recovery in heating capability (error bars: $\pm$ s.d., $n = 4$).

Dynamic light scattering (DLS): Stock IONP suspension was diluted to 6 mg Fe/ml in the aggregating solution for a volume of 500 μ l. After 4 hours, this was diluted to a final volume of 2 ml and immediately measured.

Heating measurements in suspension: Stock IONP suspension was diluted to 6 mg Fe/ml in the aggregating solution for a volume of 500 μ l. After 4 hours, this was diluted to a final volume of 1 ml and immediately measured.

Heating measurements in agarose: Stock IONP suspension was diluted to 6 mg Fe/ml in the aggregating solution for a volume of 500 μ l. After 4 hours, this was diluted to a final volume of 1 ml with the 2% agarose/PBS solution and measured after setting.

SWIFT MRI measurements: Stock IONP suspension was diluted to 6 mg Fe/ml in the aggregating solution, for volumes of 17, 83, 167, 333, 417, and 500 μ l. After 4 hours, all solutions were diluted to a volume of 500 μ l. These solutions were then diluted to a final volume of 1 ml with the 2% agarose/PBS solution and vortexed at a low speed to mix thoroughly. Lastly, 400 μ l of this solution was quickly pipetted into an NMR tube and placed in an ice bath to set.

Cryo-EM imaging: Stock IONP suspension was diluted to 6 mg Fe/ml in the aggregating solution for a volume of 250 μ l. Aggregates at the 4 hour time point were diluted 1:10 and a small drop (2–4 μ l) was slowly pipetted to a 200 mesh lacey carbon grid at 100% humidity. The sample was blotted once for 2.5 s with 0 offset (no wait or drain times), then plunged into a small cup of liquid ethane surrounded by liquid nitrogen. Samples were vitrified with a FEI Vitrobot (FEI, Inc., Hillsboro, OR). All subsequent sample processing and transport were performed under liquid nitrogen (-179°C).

Viscosity and Nanosight measurements: Stock IONP solution was diluted to 3.75 mg Fe/ml in the aggregating solution, for a volume of 20 ml. After 4 hours, 5 ml was aliquoted out for Nanosight measurements and 15 ml was used for viscosity measurements.

S2.4 Preparation of LNCaP-IONP samples

A prostate cancer cell line (lymph node cancer of the prostate, LNCaP-Pro5)³⁴ was used for all cell studies. The cells were cultured in media composed of Dulbecco's modified Eagle medium (DMEM F12) supplemented with 10% FBS, 1% penicillin-streptomycin, and 10^{-9} M dihydrotestosterone. The cells were grown in monolayers in 75 cm^2 T-flasks at 37°C and 5% CO_2 . When they were about 60–80% confluent, they were seeded with cell media containing 0.5 mg Fe/ml IONPs and incubated for 24 hours. This concentration and time point

Supplementary Figure 5 Heating in glycerol suspensions. SAR was measured for a range of field strengths (5, 10, 15, and 20 kA/m) in aqueous and glycerol suspensions (error bars: $\pm$ s.d., $n = 4$). Glycerol's extremely high viscosity is expected to completely dampen any Brownian contributions to heating. The lack of significant difference between heating in aqueous and glycerol suspensions suggests that Brownian contributions are absent even in the dispersed, aqueous suspensions. (Note: the SAR calculations take into account the specific heat and density differences between water and glycerol²⁴.)

Supplementary Figure 6 Heating at different applied field strengths. The IONP heating measurements in distilled water, 1% agarose, 2X PBS, 2X PBS in 1% agarose, LNCaP cell suspensions, and LNCaP cells in 1% agarose were repeated at a variety of field strengths (5, 10, 15, and 20 kA/m) and $190 \pm 10\%$ kHz (error bars: $\pm$ s.d., $n = 4$).

have previously been shown to provide maximal cellular loading⁴². The cells were then washed with phenol-red free Hank's balanced salt solution (HBSS) five times, to remove any non-cellularly associated IONPs and then trypsinized (2.0 mL of 0.05% trypsin with 0.53 mM EDTA for 6–9 minutes) to detach the monolayer. The cells did not always fully detach from the trypsinization and so a cell scraper was employed in these cases. The detached cells were then resuspended in fresh media and counted. The average cell count was 7.5 ± 2 million cells per flask. While the specific treatment for cells depended on the measurements to be performed (below), generally they were then centrifuged at 400 RCF for 10 minutes to form a pellet. The resulting supernatant was removed and the pellet was resuspended in 2 ml or less of media. The bulk IONP concentration was measured by ferrozine assay after the other characterization was complete. The average intracellular IONP concentration was 47 ± 29 picograms iron per cell.

TEM imaging: One flask was prepared and centrifuged as described above. This cell pellet was resuspended in 2 ml of media and pipetted repeatedly to ensure cell clumps were separated and the cells were fully suspended. One ml of this cell suspension underwent a standard process of fixation, staining, dehydration, infiltration of polymer resin, and curing. The final polymer block was cut into ~ 60 nm slices with a microtome and deposited on a 200 mesh copper grid with formvar and carbon supports (Ted Pella Inc., Redding, CA). In detail, cells were washed with 0.1 M cacodylate buffer ("buffer") twice to remove excess cell growth media and then fixed by exposure to 2.5 M glutaraldehyde in buffer. The pellet was washed another two times and then exposed to 1% osmium tetroxide in buffer for 1 hour. After another two buffer washes, the pellet went through a series of dehydration steps wherein ethanol at 50%, 70%, 80%, 95%, and 100% was applied in sequence (multiple exposures at each concentration).

Finally, the pellet was exposed to three rinses of propylene oxide to fully remove any ethanol. Following this dehydration, the pellet was infiltrated with a 2:1 mixture of propylene oxide: EPON epoxy mix for 2 hours (uncovered), then two more exposures of a 1:1 mixture for 1 hour each (covered). The final wash was replaced with pure epoxy mix and allowed to infiltrate overnight. The following day, the resin was replaced. The mixture was placed in a curing oven at 40 °C for 24 hours, then 60 °C for 48 hours. The hardened block was removed from its casing (a 1 ml centrifuge tube) and allowed to cure for another 8 hours at 60 °C to ensure complete polymerization. The embedded block was then sectioned with a Leica EM UC6 Ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Heating measurements in suspension: Four flasks were prepared and centrifuged into a single pellet, as described above. This cell pellet was resuspended in 1 ml of media and pipetted repeatedly to ensure cell clumps were separated and the cells were fully suspended. The cells were pipetted for resuspension before each heating measurement.

Heating measurements in agarose: Four flasks were prepared and centrifuged into a single pellet, as described above. This cell pellet was resuspended in 0.5 ml of media and pipetted repeatedly to ensure cell clumps were separated and the cells were fully suspended. This cell suspension was then mixed with 0.5 ml of 2% agarose in HBSS and vortexed at a low speed. The gel was then placed in an ice bath to set.

SWIFT MRI measurements: Five flasks were prepared and centrifuged into a single pellet, as described above. This cell pellet was resuspended in 0.7 ml of media and pipetted repeatedly to ensure cell clumps were separated and the cells were fully suspended. Aliquots of 200, 150, 100, 50, 25, and 0 μ l of cell suspension were placed into NMR tubes and each was diluted with cell media to a final volume of 200 μ l. Next, 200 μ l of 2% agarose in HBSS was added and the samples were vortexed at a low speed. The gels were then placed in an ice bath to set.

S2.5 Dynamic light scattering of IONP aggregates

Dynamic light scattering was performed on a Brookhaven Instruments 90Plus particle analyzer (Holtville, NY). The incident 35 mW red diode laser generated 660 nm light. Hydrodynamic diameters were measured for four independent samples, each with five one-minute data collections (the average and standard deviation values were calculated from all the cumulative measurements). Solution viscosities were manually input based on the results of the kinematic viscosity measurements performed on the base solutions. Suspensions which yielded aggregate populations larger than 1000 nm were discounted as this size extends beyond the reliable operating range of the instrument.

S2.6 Transmission electron microscopy (TEM)

Room temperature TEM images of IONPs were acquired with a FEI Tecnai T12 microscope (FEI, Inc., Hillsboro, OR) operating at 120 kV. A 200 mesh copper grid with formvar and carbon supports was dipped into a ~1 mg Fe/ml IONP suspension, then removed and allowed to dry before imaging. LNCaP cells, prepared as described above, were imaged on the same microscope at an operating voltage of 60 kV. Multiple images were taken at high magnification and later combined in a collage to obtain the high resolution image of the whole cell presented in Fig. 4. Cryogenic TEM imaging was performed on a FEI Tecnai Spirit Bio-Twin microscope (FEI, Inc. Hillsboro, OR) at 120 kV at -179 °C.

S2.7 Aggregate quasifractal characterization — image method

Individual aggregates, from both suspensions and those observed in cells, were imaged via (cryo-)EM, and the two-dimensional radius of gyration (R_{g2D}), perimeter (P_e), area (A_p), and longest end-to-end distance (L_{max}) in the aggregate were measured and compared to these four parameters

for computer generated aggregates of prescribed N_p , a_{NP} , k_f , and D_f . This procedure is similar to that employed by Brasil *et al.*²⁷ Computer-generated aggregates were produced via the cluster-cluster algorithm described by Filippov *et al.*, with N ranging from 10–1000, $k_f = 1.0 - 2.0$ and $D_f = 1.5 - 2.5$. For $D_f = 1.3$ and $D_f = 2.7$, the sequential algorithm, also described by Filippov *et al.*, was used to produce aggregates, providing an additional range for structural comparison.²⁵ Over 10,000 aggregates, with random structures (note the employed algorithms produce distinct aggregates each time they are run) but the prescribed k_f , D_f , and N , were constructed for each and 3 orthogonal projections were used as simulated TEM images to determine R_{g2D} , P_e , A_p , and L_{max} . The three noted lengths were normalized by the radius of an IONP in the simulated aggregates, while A_p was normalized by an individual IONP's projected area. The radii of each IONP in each examined aggregate was determined from the cryo-EM images, with the a_{NP} used for normalization taken as the projected area weighted average primary particle radius:

$$a_{NP} = \frac{\sum_{i=1}^{N_0} a_i^3}{\sum_{i=1}^{N_0} a_i^2} \quad (S.1)$$

where a_i is the measured radius of IONP i and N_0 is the observed number of IONPs in an aggregate's cryo-EM image (though not the total number, N , of IONPs bound in the same aggregate). Defining R_{g2D}/a_{NP} , P_e/a_{NP} , L_{max}/a_{NP} , and (A_p/pa_{NP}^2) as C_1 , C_2 , C_3 , and C_4 , respectively, for each measured aggregate and simulated aggregate projection, the square error (E_j) between the aggregate and the image was calculated as:

$$E_j = \sum_{i=1}^4 \left(\frac{C_{i,meas} - C_{i,j}}{C_{i,meas}} \right)^2 \quad (S.2)$$

where the subscripts j and *meas* denote simulated image j and the measured image properties, respectively. With E_j for each simulated aggregate image (over 30,000), number weighted average k_f and D_f were calculated as:

$$k_{f,ave} = \frac{\sum_{j=1}^{j_{max}} N_j \exp(-bE_j) k_{f,j}}{\sum_{j=1}^{j_{max}} N_j \exp(-bE_j)} \quad (S.3a)$$

$$D_{f,ave} = \frac{\sum_{j=1}^{j_{max}} N_j \exp(-bE_j) D_{f,j}}{\sum_{j=1}^{j_{max}} N_j \exp(-bE_j)} \quad (S.3b)$$

where b is the bias factor, taken to be 300 (values between 100–500 are found to change results by 2% at most). The average number of IONPs in an aggregate was calculated as:

$$N_{ave} = \frac{\sum_{j=1}^{j_{max}} N_j \exp(-bE_j)}{\sum_{j=1}^{j_{max}} \exp(-bE_j)} \quad (S.3c)$$

The values reported in Fig. 2 result from calculations with Equations (S.3a–c) for 20 or more aggregates observed under the noted conditions, with the average k_f and D_f noted for all observed aggregates. In addition to average reported values, selected cryo-EM images of aggregates observed in 100% FBS suspension are shown in Supplementary Fig. 7.

S2.8 Aggregate quasifractal characterization — ensemble method

IONP hydrodynamic diameter/radii distribution functions were measured using a NanosightTM, yielding $f(R_H)$, the fraction of particles by number per unit hydrodynamic radius with hydrodynamic radius R_H . Using the average IONP radius (from EM), this distribution was normalized to $f(R_H/a_{NP})$. For the noted simulated quasifractal aggregates,

Supplementary Figure 7 Comparison of 100% FBS aggregate EM images with simulated fractal geometries. The background has been removed in the cryo-EM images (**left**) for clarity and the line segments displayed result from the measurement of individual IONP radii. Included is the computationally generated aggregate with the minimum calculated E_f for each image (**right**).

R_H/a_{NP} was calculated using the Hubbard-Douglas approximation⁴⁸, specifically with the algorithm provided by Gopalakrishnan *et al.*⁴⁹ For each D_f , k_f , N combination, 10 aggregates were randomly generated in the manner noted above, and the resulting average R_H was determined. From these calculations, with a prescribed D_f and k_f (assumed constant for all aggregates in a suspension) $f(R_H/a_{NP})$ can be converted to $f(N, D_f, k_f)$. To then determine the $D_{f,ave}$ and $k_{f,ave}$ which best describe measurements, suspension intrinsic viscosities (which are functions of the sizes and shapes of the suspended aggregates) were inferred from kinematic viscosity measurements with an Ubbelohde 9721-R50 series Viscometer (Cannon Instrument Company Inc., State College, PA) of both the IONP containing suspensions and control PBS and FBS solutions (at identical solute concentrations to those in suspensions). Measurements were made at two shear rates to ensure that the suspensions and solutions were Newtonian; negligible differences between kinematic viscosities were measured at different rates. For intrinsic viscosity inference the densities of all suspensions and baseline solutions were measured gravimetrically (pipetting known volumes onto a calibrated mass balance). Similar to hydrodynamic radius calculation, the expected intrinsic viscosity (η_{agg}) for simulated aggregates of prescribed D_f , k_f , and N were determined using the methods of Mansfield *et al.*⁵⁰, with further details on these calculations provided by Thajudeen & Hogan⁵¹. The difference between the measurement determined intrinsic viscosity (η_{meas}) and that expected from theory (η_{theo}) for a prescribed D_f and k_f was determined, calculating η_{theo} through the relationship:

$$\eta_{theo}(D_f, k_f) = \frac{\int_0^\infty \eta_{agg}(N, D_f, k_f) f(N, D_f, k_f) N dN}{\int_0^\infty f(N, D_f, k_f) N dN} \quad (S.4a)$$

The approximate $D_{f,ave}$ and $k_{f,ave}$ from the ensemble approach (the reported values) were then determined using the equations:

$$D_{f,ave} = \frac{\sum_{i=1}^{i_{max}} \exp(-(\eta_{meas} - \eta_{theo}(D_{f,i}, k_{f,i}))^2) D_{f,i}}{\sum_{i=1}^{i_{max}} \exp(-(\eta_{meas} - \eta_{theo}(D_{f,i}, k_{f,i}))^2)} \quad (S.4b)$$

$$k_{f,ave} = \frac{\sum_{i=1}^{i_{max}} \exp(-(\eta_{meas} - \eta_{theo}(D_{f,i}, k_{f,i}))^2) k_{f,i}}{\sum_{i=1}^{i_{max}} \exp(-(\eta_{meas} - \eta_{theo}(D_{f,i}, k_{f,i}))^2)} \quad (S.4c)$$

where i_{max} denotes the total number of D_f , k_f pairs for which R_H and η_{agg} were calculated. The results of Equations (S.4b–c) as compared to those from Equations (S.3a–b) are shown in **Fig. 2c**.

S2.9 IONP heating (SAR measurements)

A 1 kW Hotshot inductive heating system with a 2.75-turn, water-cooled copper coil (Ameritherm Inc., Scottsville, NY) was used to generate the alternating magnetic field. The resulting field has been previously characterized²⁴ and was adjustable up to 24 kA/m (volume-averaged peak-amplitude across the sample) and the applied frequency was fixed at $190 \pm 10\%$ kHz. A majority of the heating measurements were conducted at 20 kA/m due the high clinical relevance of these field parameters²⁴, but some measurements were also made at 5, 10, and 15 kA/m. Each 1 ml sample in a plastic microcentrifuge tube was insulated and centered in the inductive coil. The samples were then heated for 3 minutes and the temperature was continuously recorded with a Luxtron 3100 fluoroptic thermometry system (Luxtron Inc., Santa Clara, CA). The SAR was estimated from the maximum linear fit within the initial minute of heating (typically the first 15–20 seconds), based on the rate of temperature rise method^{29,52} and a custom fitting routine²⁴.

Supplementary Figure 8 SWIFT T_1 map for well dispersed IONPs in 1% agarose. Measured concentrations ranged from 0.0 to 3.0 mg/ml.

S2.10 T_1 measurement by sweep imaging with Fourier transformation (SWIFT) sequence

The longitudinal relaxation time T_1 was measured by the Look-Locker method with an ultra-short T_2 sensitive MRI sequence (SWIFT) as the read out sequence. The samples were constructed from six 5-mm NMR tubes containing IONPs of different known concentrations and different aggregation status. These tubes were immersed in deionized water within a 3 cm diameter tube. Measurements were performed using a 9.4T magnet equipped with a DirectDrive spectrometer (Agilent Technologies, Santa Clara, CA), with a volume transmit/receive coil having an inner diameter of 3 cm (Agilent Technologies, Santa Clara, CA). Sixty-four total images at $128 \times 128 \times 128$ size along the recovery curve for different recovery times were acquired. The $T_{1\text{eff}}$ map was fitted to a saturation-recovery equation (three parameters fitting) on a pixel-by-pixel basis. The real T_1 map was acquired by doing Look-Locker correction on the $T_{1\text{eff}}$ map (Supplementary Fig. 8)³⁸.