Rapid magnetic heating treatment by highly charged maghemite nanoparticles on Wistar rats exocranial glioma tumors at microliter volume

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One of the most significant challenges implementing colloidal magnetic nanoparticles in medicine is the efficient heating of microliter quantities by applying a low frequency alternating magnetic field. The ultimate goal is to accomplish nonsurgically the treatment of millimeter size tumors. Here, we demonstrate the synthesis, characterization, and the *in vitro* as well as *in vivo* efficiency of a dextran coated maghemite ($\gamma\text{-Fe}_2\text{O}_3$) ferrofluid with an exceptional response to magnetic heating. The difference to previous synthetic attempts is the high charge of the dextran coating, which according to our study maintains the colloidal stability and good dispersion of the ferrofluid during the magnetic heating stage. Specifically, *in vitro* 2 μl of the ferrofluid gives an outstanding temperature rise of 33 °C within 10 min, while *in vivo* treatment, by infusing 150 μl of the ferrofluid in animal model (rat) glioma tumors, causes an impressive cancer tissue dissolution. © 2010 American Institute of Physics. [doi:10.1063/1.3449089]

I. INTRODUCTION

Ferrofluids are stable colloidal dispersions of magnetic nanoparticles in any ordinary liquid typically in aqueous media.^{1–3} The magnetism of such nanoparticles has been an active field of research for over 50 years.^{4–6} Recently, ferrofluids attracted strong interest, as they exhibit unique properties that can be finely tuned by adjusting their composition, coating, size, and shape at the nanoscale. This raises possibilities for important biomedical applications, both *in vitro* and *in vivo*, in the fields of pharmaceuticals, medical diagnosis, and treatment.^{7–12}

A remarkable property of these colloidal systems is the considerable heating effect due to several loss mechanisms when subjected to an alternating (ac) magnetic field. This has been utilized for applications in cancer therapy. Specifically, ferrofluids are selectively deposited in tumors, and subsequently irradiated with an ac magnetic field, in the radio-frequency (RF) range

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of 50 kHz–1 MHz (magnetic heating).¹³ In this way the energy absorption and the respective heat delivery are focused on the tumor without influencing the surrounding healthy tissue. However, by scaling the irradiated volume down to a few millimeters, which is desirable for treating tumors at the limit of the medical diagnosis resolution without affecting the healthy tissue, the efficiency of the method decreases dramatically due to the extremely small quantity of ferrofluid that can be adsorbed in tumors of such small size.¹⁴ It is thus a compulsory task to find ways to increase the efficiency of the magnetic heating of biocompatible ferrofluids that are produced with standard wet chemistry methods.

In this paper, we report the synthetic route, the structural, and the magnetic properties of highly charged dextran coated maghemite ($\gamma\text{-Fe}_2\text{O}_3$) nanoparticles with remarkable energy absorption from an ac magnetic field. An important factor for efficient magnetic heating, especially in microliter quantities, is the nanoparticle concentration in the aqueous solution. In principle, the denser the ferrofluid (in our case 20 mg/ml, see Sec. II), the highest heating effect obtained, providing that surface charges prohibit nanoparticle agglomeration. On the other hand, dense ferrofluids have considerable interparticle interactions, which trend to particle clustering and strong reduction of the magnetic heating efficiency. Hence, surface charge plays a pivotal role in the stability of the ferrofluid, especially at elevated temperatures.

Synthetic efforts have thus been focused on the optimization of the surface charge of the dextran coating, while keeping pH close to the physiological acidity value in the living organisms (pH $\approx$ 7), which is a prerequisite for biological applications. Specifically, 2 μl of the fluid (containing 40 μg of $\gamma\text{-Fe}_2\text{O}_3$) exhibits a temperature rise of 33 °C within 10 min, while 150 μl containing 3 mg of $\gamma\text{-Fe}_2\text{O}_3$ reached to temperatures up to 99 °C within 3 min with no indication of flocculation, when exposed to a moderate magnetic field with amplitude of 11 kA/m and frequency of 150 kHz. We also demonstrate the *in vivo* efficiency of our ferrofluid by inserting 150 μl in small rat glioma tumors, causing significant cancer tissue dissolution, after heating for approximately 20 min.

II. MATERIALS AND METHODS

A. Ferrofluid preparation—structural and magnetic characterization

A dispersion of maghemite nanoparticles was synthesized by the reaction of ferric chloride and ferrous chloride in the presence of KOH using coprecipitation method.^{15,16} Three ferrofluid suspensions were synthesized: One comprised of uncoated maghemite nanoparticles and two comprised of nanoparticles coated with dextran, a biocompatible macromolecule which easily adsorbs on the maghemite surface. The dextran molecule hinders cluster growth after nucleation due to the reaction between iron cations and hydroxide anions. The nominal molecular weight of the dextran coating used was 55 000.

Specifically, maghemite nanoparticles (ferrofluid samples S1, S2, S3) were prepared by coprecipitation mixing 100 ml acidic solutions of 0.66 M FeCl_3 and 0.33 M FeCl_2 . In the case of S2, S3 ferrofluids with 1 wt % of dextran were also added. A 100 ml alkaline solution of KOH (1 M) was then added dropwise over 5–10 min with stirring on a magnetic stirrer while maintaining constant temperature to prevent widening of nanoparticles' size distribution. The stirring continued for a short period of 20 min under a nitrogen-gas atmosphere at 60 °C—a longer period would introduce particle growth. The particles obtained were washed three times with ethanol and water using ultracentrifugation (5000 rpm for 10 min at 10 °C) with nitrogen purged water. The ferrofluids were prepared at high pH and were constantly stirred during this fast one step reaction to avoid the appearance of larger particles. The pH of the final ferrofluid was decreased by carefully numbered titrations of 0.01 M HCl (three washes) to achieve neutrality. In the last titration 0.1% of dextran was added for better dispersion. Last, and of extreme importance, continuous sonication for 10 min and repetitive filtering of the final colloid through a 0.2 μm porous membrane were performed. For comparison and optimization reasons, three ferrofluids with different surface charges were investigated: An ionic one, with no coating (S1) and zeta potential value of 8 mV, and two dextrans coated with zeta potential values of 70 mV (S2) and 350 mV (S3), respectively.

In the case of S1, nanoparticle agglomerations, with average size of ≈ 200 nm according to dynamic light scattering measurements, were formed right after the synthesis at neutral pH. The highest values of zeta potential for S1 (40 and -40 mV, respectively) were observed at low (<4) and high (>8) pH values, most likely due to the hydrodynamic forces introduced by sonication, which were strong enough to prevent the formation of weak bonds. On the other hand, both dextran coated S2 and S3 ferrofluids exhibited positive zeta potential values at neutral pH and were nicely dispersed into the fluid due to strong electrostatic and steric repulsion forces.

In this way, zeta potential values as high as 350 mV (S3) at $\text{pH} \approx 7$ were achieved. The coated maghemite nanoparticle ferrofluid (S3) yield, determined by weighing of the lyophilized sample of the preparation, was 20 mg/ml, a value which characterizes a dense colloidal suspension.

For the structural characterization, the crystal structure, size, and shape of the maghemite nanoparticles were determined by high-resolution transmission electron microscopy (HRTEM) in a Jeol 2011 electron microscope operated at 200 kV with a point resolution of 0.194 nm and $C_s = 0.5$ mm. The maghemite nanoparticles were magnetically characterized on a Quantum Design superconducting quantum interference device magnetometer. Magnetic hyperthermia experiments were performed using an induction heating furnace equipped with water cooled coils, operating at 150 kHz. The method used for temperature measurements is based on a fiber optic thermometry system that allows users to measure temperature in extremely harsh electromagnetic environments. The technology involved is called fluoroptic thermometry (FOT) and is based on probes that are totally immune to electromagnetic interference and of entirely nonmetallic construction. These qualities make FOT probes perfectly suited for measuring temperatures in harsh environments often encountered during biomedical research, such as (a) high static magnetic fields, kilohertz range switching gradients, etc. used in magnetic resonance imaging and (b) alternating magnetic fields at the kilohertz range like induction heating devices. The commercially available system used in this study for accurate temperature measurements is the m3300 Biomedical Laboratory Kit fluoroptic thermometer (Luxtron, LumaSense Technologies, Santa Clara, CA, USA). The probe is protected in a Tefzel jacket and is only 500 μm in diameter, and thus suitable for measuring microliter quantities of ferrofluids.

B. Glioma cell lines—animals

Male Wistar rats weighing 250–350 g were used in this study. The rats were fed ad libitum with standard laboratory food and water. They were individually housed in a controlled environment ($18\text{--}22^\circ\text{C}$; 50%–75% relative humidity) and maintained under a 12 h dark cycle. All animal procedures adhered to standard principles of animal care and were approved by the local animal welfare committee.

C6 glioma cells were maintained in Ham F12 minimum essential medium containing 10% fetal bovine serum, 1% penicillin/streptomycin. The cells were grown to confluency in a humidified atmosphere of 5% CO_2 at 37°C . Exponential growth cultures were harvested with a solution of 0.05% trypsin and 0.02% EDTA and resuspended in Ham F12 medium. C6 cells were washed in Ham F12 medium and viable cells counted by a hemocytometer. Finally, cells were suspended in Ham F12 minimum essential medium to a final concentration of 8×10^6 cells per 10 μl for inoculation.

Rats were anesthetized by IP injection of xylazine (Bayer, Leverkusen, Germany) at 10 mg/kg and ketamine (Parke-Davis, Courbevoie, France) at 100 mg/kg in combination with Atropine at 0.05 mg/kg. Tissue covering the skull was gently elevated and the bregma identified. Approximately 8×10^6 cells were inoculated in the area anterior of the bregma.

After 2 weeks, the development of a tumor was palpable and visible. On the fourth week tumors had an average size of 5–10 mm (Fig. 1), and inoculation of nanoparticles took place. Rats were anesthetized as above.

Tumors were infused with 150 μl of S3 ferrofluid and the rats were subjected to magnetic hyperthermia treatment for 20 min. Following the treatment, the rats were sacrificed and tumors

FIG. 1. T_2 weighted magnetic resonance image of rat head. The glioma tumor area marked with yellow is pointed with a red arrow.

were excised and fixed in formaldehyde. Paraffin-embedded sections were stained with hematoxylin-eosin and examined under a Nikon Eclipse E800 microscope using 2x and 20x lenses.

III. RESULTS AND DISCUSSION

A. Structural and magnetic data

Nanoparticles have been examined by HRTEM and readily compared with the corresponding structural properties of bulk maghemite. In Fig. 2, HRTEM images of the bare (S1) and highly charged coated (S3) samples, together with images of bulk maghemite, are shown, which illustrate the atomic structure of individual nanoparticles and bulk material, oriented along the $[\bar{1}12]$ and $[011]$ low-index zone axes of the cubic system. The experimental interplanar spacings resolved in the studied samples were 0.48 ± 0.01 , 0.41 ± 0.01 , 0.29 ± 0.01 , and 0.25 ± 0.01 nm (Fig. 2), which were unambiguously attributed to the d_{111} (0.482 nm), d_{200} (0.417 nm), d_{220} (0.295 nm), and d_{311} (0.252 nm) d -spacings of maghemite. In all cases defect-free crystalline structures were determined [Figs. 2(a), 2(b), and 2(d)]. No extended defects, i.e., grain boundaries, were observed within nanoparticles, which indicate that the great majority of nanoparticles consisted of single crystalline grains. A few twin boundaries between small uncoated nanoparticles in S1 were detected and analyzed [Fig. 2(d)], while the analysis verified positively the cubic crystal structure of uncoated nanoparticles.

S3 consisted of maghemite crystal particles that exhibited rounded corners and roughly ellipsoid shapes having core sizes between 6 and 20 nm in diameter with the main crystal population in the range of 10–12 nm [Figs. 2(a) and 2(b)]. Moreover, the uncoated maghemite crystal particles (S1) had sizes varying from 3 to 25 nm in diameter, while its main crystal population was measured in the range of 8–12 nm, respectively. The latter exhibited rectangular or cubic shapes, whereas the smaller ones showed a spherical shape as expected for nanoparticles less than 5 nm in diameter. In addition, the practically absolute match between experimental values of interplanar spacings of nanocrystals and the corresponding structural properties of the bulk crystal suggested strain-free coated and uncoated nanoparticles.

FIG. 2. HRTEM micrographs. (a) Dextran coated crystalline particles (S3) of 10–12 nm in diameter (optimally charged). (b) A single crystalline dextran coated nanoparticle (S3) oriented along the $[011]$ zone axis. (c) A part of bulk maghemite crystals viewed along the $[112]$ direction along with the corresponding selected area electron diffraction pattern. (d) Two uncoated (S1) nanoparticles in twin orientation, where arrows signify the twin boundary. Both are oriented along their corresponding $\langle 011 \rangle$ zone axes. The experimental values of the angles between $\{111\}/\{200\}$ (55°) and $\{111\}/\{220\}$ (35°) crystal planes are shown as well.

On the basis of our HRTEM data we thus anticipate that ferrofluids S2 and S3 are monodisperse, well crystalline systems with average size of 10–12 nm. The magnetic moments of samples S1 and S3 were measured at room temperature as a function of the magnetic field and showed superparamagnetic behavior, as expected for maghemite nanoparticles of 10 nm diameter. The obtained saturation magnetizations at room temperature were 61 emu/gr and 39 emu/gr for the S1 and S3 samples, respectively (the S2 sample gave similar results to S3).

B. *In vitro* and *in vivo* magnetically induced hyperthermia

The reason for synthesizing highly charged nanoparticles for magnetic hyperthermia applications is to endure constant heating for several minutes at high temperatures and keep the colloidal stability of the ferrofluid intact. In a colloidal suspension during magnetically induced RF heating, the resulting rising of temperature increases the average numbers of particle collisions. Hence, keeping nanoparticles as far as possible, by increasing electrostatic repulsion, it is possible to minimize the number of particle collisions (and thus prevent agglomeration, which dramatically induces the efficiency of RF heating) for a long period of time.

Figure 3 shows the temperature dependence as a function of the heating time for the three ferrofluids. Heating was performed with a copper coil [inset in Fig. 3(b)], which produced a moderate ac magnetic field with amplitude of 11 kA/m and frequency of 150 kHz, much lower

FIG. 3. Magnetic heating effect on aqueous dispersions of dextran coated maghemite nanoparticles. (a) The influence of the zeta potential on 150 μl of ferrofluid S1 (green line), S2 (blue line), and S3 (black line). (b) The influence of the volume on 150 μl (black line), 10 μl (blue line), and 2 μl (red line) of ferrofluid S3. Solid black lines refer to fitting of the experimental data (see text for details). The green line is the curve for 2 μl water that was used as control sample. In the inset, the coil of the magnetic heating apparatus with the 10 μl sample and the optical fiber used for the temperature monitoring are shown after removing the heat insulating cover.

than the typical value of 400–500 kHz commonly used.^{17–19} Samples were heated from room temperature to the maximum temperature value and then left to cool down back to the initial conditions.

In Fig. 3(a) we observe the strong effect of the zeta potential upon heating: S1 with the lowest zeta potential value does not produce any significant heating effect and started to flocculate right after heating. S2 exhibits a respectable temperature rise, reaching 60 °C within 10 min. Similar to the S1 sample, S2 showed problems on stability and endurance, and it was instantly flocculated after the heating treatment. On the contrary, S3 exhibits an impressive heating effect, reaching 98 °C within 3 min, with no traces of flocculation and a remarkable long-term stability, despite the fact that it reached almost the water boiling temperature. The corresponding specific absorption rate (SAR) is calculated at 286 W/g. This is among the highest SAR values obtained under the specific experimental conditions (see supporting information).²⁰ Most spectacular, by scaling down the quantity of S3, an exceptional heating response is observed [Fig. 3(b)]: 2 μl of this ferrofluid containing 40 μg of $\gamma\text{-Fe}_2\text{O}_3$ (red line) exhibits a temperature rise of 33 °C within 10 min. As far as we know, this result is the highest increase in temperature for such a microgram quantity of maghemite nanoparticles reported in the literature.

The experimental data for sample S3 in Fig. 3(b) have been analyzed according to $T(t) = T_{\text{initial}} + \Delta T_{\text{max}}(1 - \exp(-t/\tau))$, where T_{initial} is the initial temperature, ΔT_{max} the overall temperature change, and τ the time constant of heating.²¹ The pertinent quantity in magnetic hyperthermia is the initial rate $(dT/dt)_{t=0}$ which in our case equals to $\Delta T_{\text{max}}/\tau$. The fits of the above equation to the experimental data during the heating process are shown as solid black lines in Fig. 3(b) and yield similar heating rate $\Delta T_{\text{max}}/\tau \approx 17$ °C/min for the 2 and 10 μl samples. Reliable analysis of the 150 μl sample was not possible due to the fast attainment of the water boiling temperature. However, by fitting the experimental data during the cooling process, the same cooling rate $\Delta T_{\text{max}}/\tau \approx 12$ °C/min is obtained for all three samples. This demonstrates the accuracy and reliability of the experiments. In addition, the 150 μl sample had not been physically or chemically destroyed by reaching the water boiling temperature, a sign of the high stability of the synthesized nanodispersion. Evidently, the principal factor which controls the heating effect in the colloid state is the prevention of agglomeration, i.e., the elimination of interparticle interactions, providing that the size and magnetic properties of the maghemite core particles are “tuned” to maximum energy absorption. The *in vitro* experiments of our ferrofluids markedly showed that it is the surface charge that prevents agglomeration during the heating stage of the ferrofluid by minimizing the collisions between the nanoparticles. It is anticipated that strong interactions are present in the

FIG. 4. For *in vivo* magnetic heating of small rat glioma tumors 150 μl of the S3 ferrofluid was infused into rat glioma tumors with size of 5–10 mm and subsequently subjected to magnetic hyperthermia treatment for 20 min. Panels (a) and (c) (low and high magnifications, respectively) represent sections of control tumor tissue treated without nanoparticles. Panels (b) and (d) (low and high magnifications, respectively) show extensive damage of the tumor tissue after treatment with ferrofluid. White bars=200 μm . The lower inset in panel (b) shows the experimental setup for *in vivo* magnetic heating. The upper inset is an infrared image, which demonstrates the ability of the ferrofluid to produce strong localized heating at the tumor position.

ferrofluids due to the high concentration of maghemite in the suspensions (20 mg/ml), and it is the presence of the charged coating that prevents agglomeration and gives rise to the strong heating effect in the 350 mV S3 sample. Indeed, the less charged 70 mV S2 sample, which has the same high concentration (20 mg/ml) as the S3 sample, presents less magnetic heating than the S3 ferrofluid [Fig. 3(a)] due to the fact that the weak charge cannot compensate the large interparticle interactions and the S2 material exhibits flocculation and no colloidal stability during magnetic heating process.

In order to demonstrate the ability of the S3 ferrofluid to function *in vivo* and assess its medical potential, we infused 150 μl in small rat tumors with average size of 5–10 mm and applied an 11 kA/m ac field at 150 kHz for 20 min. Specifically, C6 rat glioma cells were injected extracranially and allowed to form tumors, which were infused with ferrofluid. The rats' heads were then placed within the induction-heating coil [lower inset in Fig. 4(b)]. Infrared temperature image [upper inset in Fig. 4(b)] shows the ability to heat a precise area of the glioma tumor. Following the treatment, the rats were sacrificed and tumors were excised and paraffin-embedded sections were stained to reveal the nuclei and cytoplasm of cells using hematoxylin and eosin, a standard staining protocol for examining tissue integrity. As can be seen in Figs. 4(b) and 4(d), treatment led to extensive tumor tissue damage and dissolution, indicating that the obtained temperatures were close to boiling temperatures. Control experiments included the infusion of S3 ferrofluid without subsequent treatment as well as exposure to magnetic hyperthermia in the absence of ferrofluid [Figs. 4(a) and 4(c)]. No tumor tissue damage was observed in either case. Thus, the produced surface charged coated ferrofluid S3 performed excellently in the *in vivo* studies due to the preservation of the good dispersion of the ferrofluid within the tumor during the hyperthermia heating.

IV. CONCLUSIONS

To summarize, we have investigated the synthesis, the structural magnetic properties of highly charged coated maghemite nanoparticles with remarkable absorption of an ac magnetic field at very small quantities and demonstrate their *in vivo* efficiency for hyperthermia treatment of small size tumors. The exceptional heating performance of the produced ferrofluid is due to (i) the appropriate size and excellent magnetic properties of the core nanoparticles and (ii) the high surface charge of the dextran coating that produces homogeneous and stable nanoparticle dispersion resulting to minimal interparticle interactions, minimum collision rate, and maximum energy absorption during heating. A balance between density and charge is the key for an effective and stable ferrofluid at elevated temperatures. To the best of our knowledge the microliter ferrofluid quantities used for magnetic heating are the smallest reported in the literature.

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