EGFR-Targeted Magnetic Nanoparticle Heaters Kill Cancer Cells without a Perceptible Temperature Rise

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ABSTRACT It is currently believed that magnetic nanoparticle heaters (MNHs) can kill cancer cells only when the temperature is raised above 43 °C due to energy dissipation in an alternating magnetic field. On the other hand, simple heat conduction arguments indicate that in small tumors or single cells the relative rates of energy dissipation and heat conduction result in a negligible temperature rise, thus limiting the potential of MNHs in treating small tumors and metastatic cancer. Here we demonstrate that internalized MNHs conjugated to epidermal growth factor (EGF) and which target the epidermal growth factor receptor (EGFR) do result in a significant (up to 99.9%) reduction in cell viability and clonogenic survival in a thermal heat dose dependent manner, without the need for a perceptible temperature rise. The effect appears to be cell type specific and indicates that magnetic nanoparticles in alternating magnetic fields may effectively kill cancer cells under conditions previously considered as not possible.

KEYWORDS: iron oxide · magnetic nanoparticles · intracellular magnetic fluid hyperthermia · epidermal growth factor · epidermal growth factor receptor · alternating magnetic field

Iron oxide magnetic nanoparticles have been widely used for biological applications[1] such as in separation of molecular components,[2] as MRI contrast agents,[3] and for therapy such as in magnetic fluid hyperthermia (MFH)[4] or drug delivery.[5] In magnetic fluid hyperthermia, magnetic nanoparticle heaters (MNHs) dissipate part of the energy of an applied oscillating magnetic field in the form of heat. This energy dissipation leads to a localized temperature rise to the range 43−46 °C, resulting in cancer cell death. MFH has been successfully used both *in vitro*,[6] when using large amounts of nanoparticles surrounding cells, and *in vivo*,[7] when injecting nanoparticles directly into tumors. However, clinically relevant tumors may not be easily accessible to direct injection, and poor tumor accumulation of systemically injected nanoparticles has been reported.[8] In 1979, Gordon[9] suggested that intracellular hyperthermia would be more effective than extracellular hyperthermia in the thermal sense because the cell membrane would act as an insulator. However, heat conduction arguments indicated that the heating effect due to single or small clusters of nanoparticles are negligible to the cell[10] because the rate of heat removal by conduction is much faster than the particle's rate of energy dissipation, precluding a significant rise in temperature, even at the particle surface. This would imply that MFH would have limited utility in treating small tumors and metastatic cancers. On the other hand, it has been recently reported[11] that dispersed single magnetic nanoparticles were able to increase the temperature of their immediate surroundings in a magnetic field amplitude dependent manner, without increasing the temperature of the bulk solution, contradicting the theoretical work and suggesting that intracellular hyperthermia may be possible.

One strategy to improve retention of nanoparticles in tumors and accumulation in cancer cells is active targeting to cell membrane receptors that are overexpressed in cancer cell lines, such as the epidermal growth factor receptor (EGFR).[8] EGFR is upregulated in many cancer cells, resulting in receptor overexpression that correlates with disease progression, survival, stage, and response to therapy.[12] In normal healthy cells EGFR activation is involved in normal cell growth, differentiation, and repair mechanisms. In some cancer cell lines where EGFR is overexpressed, such as in MDA-MB-468, activation of EGFR is involved in apoptosis.[13] The main ligand for EGFR is epidermal growth factor (EGF).[14] In this contribution, we targeted EGFR using iron oxide magnetic nanoparticles conjugated to EGF, and we studied the ability of targeted and nontargeted magnetic nanoparticles to decrease cell viability under the application of a magnetic field. Two well-known adenocarcinoma cell lines were chosen for targeted hyperthermia experiments because of their different

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expression of EGFR. MDA-MB-468 cells overexpress the EGFR (10^6 EGFR/cell)[15] at a level that is 2 orders of magnitude higher than in early stage MCF-7 cells (10^4 EGFR/cell).[16]

RESULTS AND DISCUSSION

The long-term (>10 h) effect of EGF on MCF-7 cells is proliferative, as expected since EGF is a growth factor, but it is apoptotic for MDA-MB-468 when present at supra-physiological concentrations (>10 ng/mL)[13] (Supporting Information, Figure S1). We synthesized iron oxide (IO) nanoparticles by co-precipitation of iron salts and characterized them physically and magnetically (Supporting Information, Figure S2−S4).[17] Carboxymethyldextran (CMDx) was covalently bound to the nanoparticles to make them colloidally stable and biocompatible, and then EGF was conjugated to CMDx by cabodimiide reaction at 4.39 ± 0.71 μg EGF/mg IO (Figure 1a). MDA-MB-468 cells were cultured in contact with nanoparticles to study their localization within the cell using confocal laser scanning microscopy (Figure 1b). Nanoparticles were localized to the cell membrane (red), the cytoplasm, and lysozomes (blue). After 1 h of incubation there was no evidence of CMDx-coated nanoparticles without EGF, whereas EGF-conjugated nanoparticles were found internalized in lysosomes, in vesicles, and attached to the membrane. At 6 and 10 h of incubation with CMDx-coated nanoparticles without EGF some clusters of nanoparticles were visualized in the cytoplasm. When incubated with EGF-conjugated nanoparticles, larger clusters of nanoparticles (green) were observed in the cytoplasm, in small vesicles, in lysosomes (blue), and attached to the cell membrane (red). Both types of nanoparticles were incubated with MCF-7 cells for 1 h. No CMDx-coated particles were observed, but EGF-conjugated nanoparticles were found to be mostly attached to the membrane (see Supporting Information S6).

According to inductively coupled plasma mass spectrometry (ICP-MS), CMDx-coated nanoparticles were internalized at up to 2−5 pg IO/cell in both MDA-MB-468 (Figure 1c) and MCF-7 cells (Supporting Information, Figure S7) after 1, 6, and 10 h of exposure. The internalization of EGF-conjugated magnetic nanoparticles in MDA-MB-468 increased with time. There was not a statistically significant difference in internalization between 1 h (12.74 pg IO/cell) and 6 h (14.53 pg IO/cell) ($p > 0.05$, $\alpha = 0.05$) and between 6 and 10 h (20.86 pg IO/cell) ($p > 0.05$, $\alpha = 0.05$), but there was a statistically significant difference between 1 and 10 h ($p < 0.05$, $\alpha = 0.05$).

Because EGF-conjugated nanoparticles are mostly internalized in MDA-MB-468, we hypothesized that intracellular hyperthermia could be a mechanism for cell death under the application of an oscillating magnetic field. We performed intracellular hyperthermia by incubating both CMDx-coated and EGF-conjugated nanoparticles for 1, 6, and 10 h with MDA-MB-468 (Figure 2) and MCF-7 cells (Supporting Information, Figure S9). Before applying the alternating magnetic field cells were extensively washed after incubation with nanoparticles and prior to treating with the magnetic field to ensure that there were no nanoparticles suspended in the medium during the application of the magnetic field. A magnetic field of 37.5 kA/m at 233 kHz was applied for a period of two hours while monitoring the temperature, and then cells were seeded for cell viability and clonogenic assays. Samples were insulated and placed in a coil inside an incubator at 37 °C. The coil was water cooled. The temperature of the samples was held constant at 37 ± 0.2 °C during the application of the magnetic field for all of the experiments through control of the incubator temperature. Because samples only had internalized nanoparticles, the amount of heat dissipated in the samples was not sufficient to significantly raise the bulk temperature above ambient. A parameter used in hyperthermia studies to compare cell damage under different thermal conditions is the cumulative equivalent minutes (CEM),[18] which standardizes different temperatures and treatment times to a reference temperature of 43 °C. Because there was no measurable increase in temperature during our intracellular hyperthermia experiments, we could not use CEM as a thermal dose parameter. Instead we used the total heat dose (THD)[19] in joules per cell, calculated using the specific absorption rate (SAR) of a known amount of internalized nanoparticles per cell (m_{cell}), at a given amplitude of the magnetic field, for the duration of the treatment (t): $THD = m_{cell} \cdot SAR \cdot t$.

The application of the magnetic field alone under the conditions of our experiments does not result in an effect in cell viability (see Supporting Information S8), as also shown in our previous work.[6] CMDx-coated nanoparticles were not cytotoxic for any of the cell lines, as there was no difference between the cell viability of the control and cell viability of cells incubated with the nanoparticles at any of the studied times ($p > 0.05$, $\alpha = 0.05$). In summary, for the MCF-7 cells a slight decrease to 75−80% in cell viability and survival factor was observed after intracellular MFH with CMDx-coated and EGF-conjugated nanoparticles (Supporting Information, Figure S9). Although this is a statistically significant decrease, it is not a large enough decrease to make the result promising as a form of treatment. MDA-MB-468 cells that underwent treatment with CMDx-coated nanoparticles and the magnetic field (37.5 kA/m, 233 kHz) presented a decrease in cell viability down to 50−60% when compared to the control at all of the times studied ($p < 0.05$, $\alpha = 0.05$), regardless of the incubation time. There is no statistically significant difference between cells incubated for

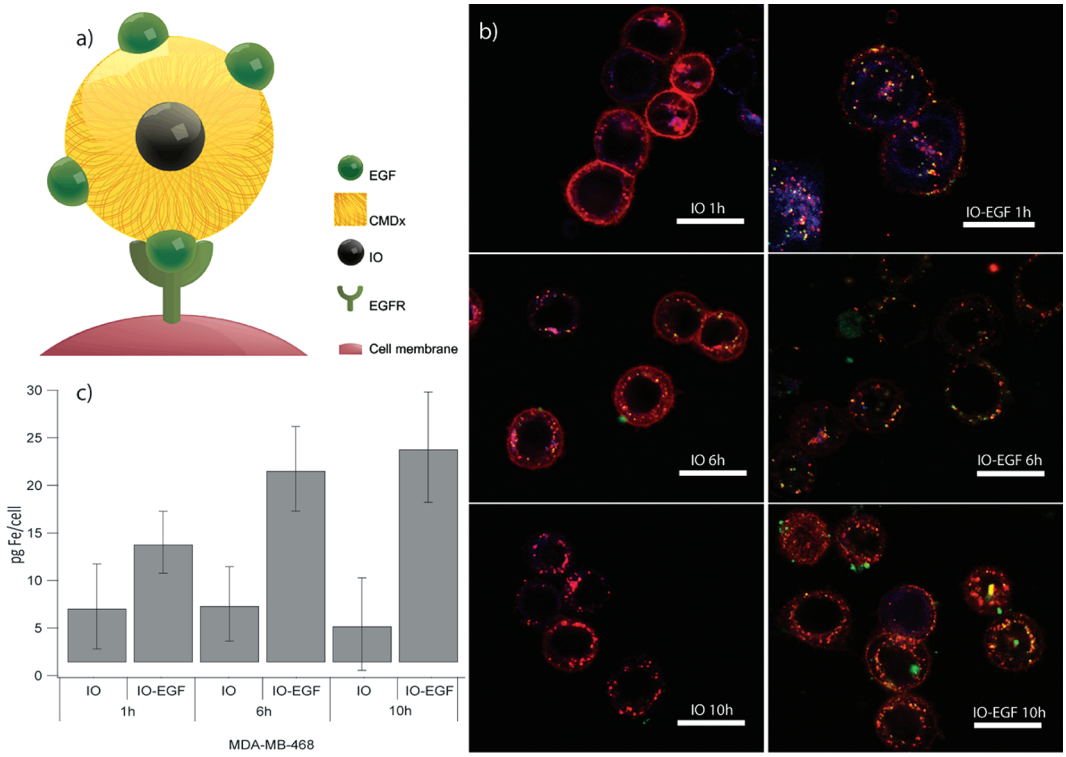

Figure 1. Localization and internalization of nontargeted and targeted nanoparticles. (a) Schematic showing EGF-targeted nanoparticles interacting with the cell membrane through specific ligand−receptor binding (not to scale).[20] (b) Localization of nanoparticles after being in contact with MDA-MB-468 for 1, 6, and 10 h at 37 °C (bar, 20 μm). (c) Uptake of Fe as a function of incubation time by MDA-MB-468 cells (n = 3).

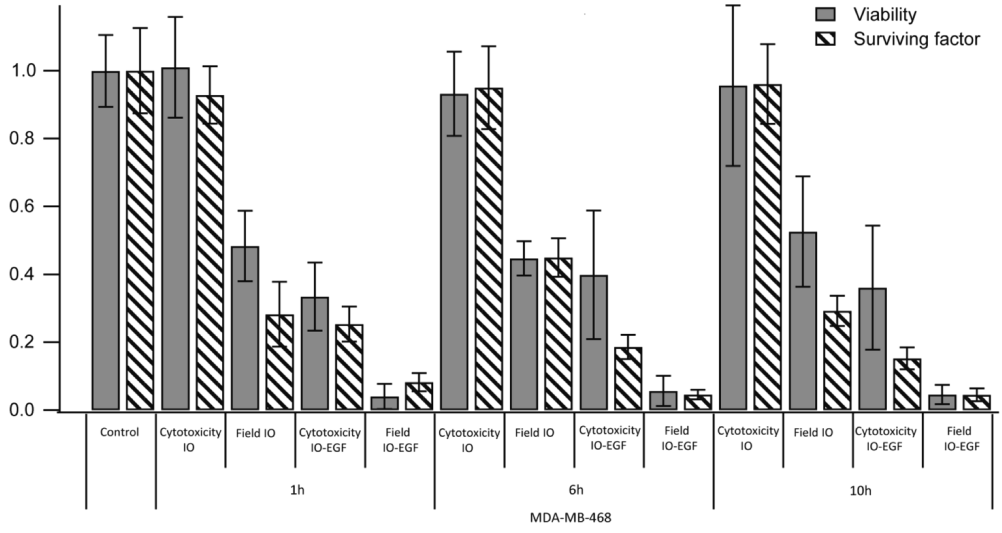

Figure 2. Intracellular hyperthermia using nontargeted and targeted nanoparticles. Cell viability and survival factor of MDA-MB-468 cells after incubation for 1, 6, or 10 h with either CMDx-coated nanoparticles (IO) or EGF-conjugated nanoparticles (IO-EGF) with and without the application of a 37.5 kA/m magnetic field for a period of 2 h (n = 5).

1 h (THD 2.96 μJ/cell), 6 h (THD 4.51 μJ/cell), and 10 h (THD 3.84 μJ/cell) ($p > 0.05$, $\alpha = 0.05$). Cells treated with EGF-conjugated nanoparticles without a magnetic field reduced cell viability down to 30−40% due to the apoptotic effect of the supra-physiological concentration of the EGF bound to the nanoparticles. MDA-MB-468 cells treated with EGF-conjugated nanoparticles and the magnetic field (37.5 kA/m, 233 kHz) reduced cell viability down to 4−6% compared to the control. Hence, the application of the magnetic field on cells incubated with IO-EGF significantly decreases cell viability compared to cells incubated with IO-EGF without application of a magnetic field. The survival factor followed the same pattern as cell

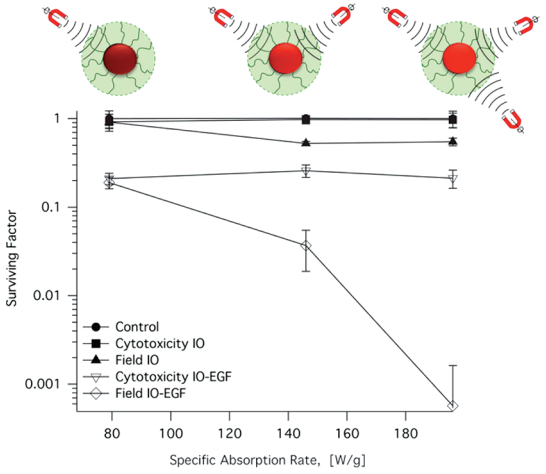

Figure 3. Intracellular hyperthermia at different specific absorption rates. Effect of IO nanoparticle energy dissipation on MDA-MB-468 survival factor at different specific absorption rates after being in contact for 1 h with either CMDx-coated nanoparticles (IO) or EGF-conjugated nanoparticles (IO-EGF) (n = 3).

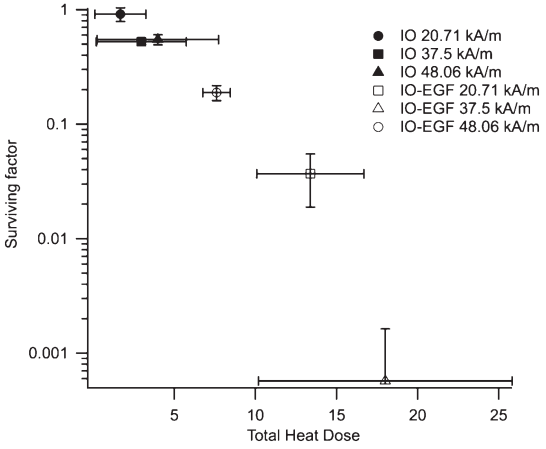

Figure 4. Survival factor of MDA-MB-468 cells at different total heat doses. MDA-MB-468 cells were incubated for 1 h with either CMDx-coated or EGF-conjugated nanoparticles and treated for a period of 2 h at different amplitudes of the magnetic field, parametrized through the total heat dose.

viability, although there was a statistically significant decrease in survival factor compared to cell viability in some cases. This indicates that even though cells are alive after being treated with EGF-conjugated nanoparticles and alternating magnetic field, they lose their ability to form colonies, and probably their ability to regrow as a tumor. In summary, for MDA-MB-468 cells intracellular hyperthermia treatment was improved when using EGF-conjugated nanoparticles compared to CMDx-coated nanoparticles by decreasing both viability and survival factor, not only due to the apoptotic effect of EGF but also because of the application of the alternating magnetic field. Since there was no measurable increase in temperature above 37 $\pm$ 0.2 °C, we tested if the decrease in survival factor depended on the total heat dose deposited within the cells. By increasing the amplitude of the magnetic field, the energy dissipation of the nanoparticle increases, and from SAR values and ICP-MS measurements, the heat dose per cell can be calculated. We thus applied an alternating magnetic field, of different field amplitudes, and thus at different total heat doses to MDA-MB-468 cells incubated with iron oxide and EGF-conjugated iron oxide nanoparticles for 1 h after washing the unbound nanoparticles (Figure 3).

At low SAR (79 W/g IO, 20.71 kA/m, 233 kHz) there was no statistically significant difference in survival factor between cells incubated with CMDx-coated nanoparticles and treated with the magnetic field (THD 1.68 μJ/cell) and the control. At medium (146 W/g IO, 30.5 kA/m, 233 kHz) and high SAR (197 W/g IO, 48.06 kA/m, 233 kHz) the survival factor was reduced to 50% for cells conjugated with CMDx-coated nanoparticles and treated with the magnetic field (THD 2.96 and 3.98 μJ/cell, respectively). There is no statistically significant difference in survival factor between cells treated with CMDx-coated nanoparticles at a medium and high SAR (p = 0.909, α = 0.05). At low SAR there is no difference in survival factor of cells treated with EGF-conjugated nanoparticles with (THD 7.60 μJ/cell) and without the magnetic field (p = 0.170, α = 0.05). At medium SAR, cells incubated with EGF-conjugated nanoparticles and treated with the magnetic field (THD 13.39 μJ/cell) reduced the survival factor to 4% compared to the control. At high SAR, cells incubated with EGF-conjugated nanoparticles and treated with the magnetic field (THD 18.00 μJ/cell) reduced the survival factor to 0.06%. The experiments shown in Figure 3 demonstrate that under similar conditions of incubation time and by varying the nanoparticle's rate of energy dissipation the survival factor of MDA-MB-468 cells decreases in a thermal heat dose dependent manner (Figure 4) to 0.06%, even without a perceptible temperature rise.

The observations of Huang *et al.*[21] of a rise in nanoparticle surface temperature of up to 20 °C relative to the surroundings point to denaturation of particle-bound proteins, such as the EGFR, as a possible mechanism underlying the observed effect. However, further study is needed to test this and other possible mechanisms such as thermal/mechanical actuation of the apoptotic pathway of the EGFR, especially in the case of EGF-conjugated nanoparticles. This latter hypothesis is motivated by observations of mechanical activation of the EGFR[22] and by the recent demonstrations by Mannix *et al.*[23] of the mechanical activation of FcεRI receptors by bound magnetic nanoparticles.

CONCLUSION

We have demonstrated that killing cancer cells using targeted magnetic nanoparticle heaters and alternating

magnetic fields is possible even without a perceptible temperature rise of the medium containing the cells. Our results show that nanoparticles are capable of dramatically decreasing cell viability by targeted intracellular hyperthermia or by some other mechanism that is SAR/field strength dependent. Targeted intracellular hyperthermia therefore seems possible when using EGFR-targeted EGF magnetic nanoparticles heaters on cells that overexpress EGFR and is more effective than nontargeted intracellular hyperthermia. These observations open the possibility of treating small tumors and metastatic cancers using targeted magnetic nanoparticle heaters.

METHODS

Preparation of CMDx-Coated Iron Oxide Nanoparticles. Magnetic nanoparticles were synthesized by the co-precipitation method,[24] which involves the simultaneous precipitation of Fe^{2+} and Fe^{3+} ions in basic aqueous media, according to the procedure reported in ref 17. Briefly, a 2.73 ratio of Fe^{3+}: Fe^{2+} was stirred at 150 rpm at 80 °C for 1 h, adjusting the pH to 8 by adding NH_4OH and adding deionized water to compensate for evaporation. After the reaction magnetic nanoparticles were peptized with $(CH_3)_4NOH$ to provide negative surface charge. Peptized nanoparticles were functionalized with 3-aminopropyltriethoxysilane (APS) to graft functional amine groups ($-NH_2$) onto the nanoparticle surface. For this, 1.125 g of peptized nanoparticles was suspended in a mixture consisting of 75 mL of DMSO, 5 mL of APS, 1.25 mL of water, and 100 μL of acetic acid. The reaction mixture was mechanically stirred at 150 rpm for 36 h at room temperature. Afterward, nanoparticles were washed four times with ethanol and dried at room temperature to obtain a black solid consisting of APS-coated nanoparticles. IO−APS nanoparticles were functionalized with CMDx via reaction with EDC/NHS. Carboxylic groups (−COOH) present in the CMDx reacted with NHS in the presence of EDC, resulting in a semistable ester, which then reacted with primary amines present in the APS-coated nanoparticles, forming a covalent bond. This was achieved by dissolving 1 g of CMDx in 10 mL of deionized water (pH 4.5−5), with 25 mg of EDC and 15 mg of NHS. Finally the CMDx solution was mixed with the IO−APS solution (0.1 g of IO−APS per 10 mL of deionized at pH 4.5−5). The reaction mixture was mechanically stirred at 150 rpm during 36 h at room temperature. Finally, CMDx-coated nanoparticles were washed three times with ethanol and dried at 60 °C in a vacuum oven. The nanoparticles were stored at 4 °C until further experiments. For biological experiments CMDx-coated nanoparticles were sterilized by autoclave at 121 °C for 1 h and then suspended in biological media by ultrasonication for 5 min.

Preparation of EGF-Conjugated Iron Oxide Magnetic Nanoparticles. Sterilized CMDx-coated nanoparticles (60 mg) were suspended in PBS at a concentration of 15 mg/mL by ultrasonication for 5 min at room temperature. Then 15 mg/mL EDC was added, and the solution was adjusted to pH 5. After 30 min at room temperature, the solution was adjusted to pH 8 and brought to 37 °C. A 500 μg amount of EGF suspended in PBS at a concentration of 500 μg/mL was added to the nanoparticle solution and allowed to react for 4 h at 37 °C in a thermoshaker at 200 rpm.[20] EGF-conjugated nanoparticles were dialyzed using 30 K centrifugal tubes against cold PBS. The EGF conjugation reaction was reproducible (4.39 ± 0.71 μg EGF/mg IO) as determined by BCA protein assay.

Characterization of CMDx-Coated Magnetic Nanoparticles. The hydrodynamic diameter of the magnetic nanoparticles in deionized water at a concentration of 0.28 mg IO/mL was measured by dynamic light scattering (DLS) using a Brookhaven Instruments BI-90 Plus particle size analyzer. Particle size and size distribution were determined using a Carl Zeiss LEO 922 energy filtered transmission electron microscope (EFTEM). Ultrathin carbon type A grids were immersed in the colloidal suspensions and then placed on filter paper and dried in a vacuum oven for TEM measurements. The weight percentage of the magnetic core in CMDx-coated nanoparticles was estimated from thermogravimetric analysis using a Mettler Toledo TA TGA-DSC1 thermo gravimetric analyzer, assuming the remnant mass after decomposition of the sample at 800 °C corresponds to the inorganic cores. A Quantum Design MPMS XL-7 SQUID magnetometer was used to determine the magnetic properties of the nanoparticles. The specific absorption rate of the magnetic nanoparticles was estimated by preparing samples of CMDx-coated magnetic nanoparticles in deionized water at a concentration of 0.28 mg IO/mL. For specific absorption rate measurements, samples were placed in a three-turn coil, cooled with water at approximately 20 °C. A magnetic field was generated using a HFI 3 kW RF heating system from RDO Induction Power Supplies, and the temperature of the samples was measured using a Luxotron Fluoroptic Thermometer probe.

MCF-7 and MDA-MB-468 Cell Culture. Cells were cultured on 75 cm^2 cell culture flasks using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% nonessential amino acids, 10 mL of penicillin, 100 μg/mL of streptomycin, 0.876 g/L L-glutamine, and 2.7 g/L sodium bicarbonate. Cells were maintained in a controlled atmosphere at 37 °C, 95% relative humidity, and 5% CO_2. The culture medium was changed twice a week until cells reached approximately 80% confluency.

Internalization and Localization of Nanoparticles. For internalization measurements, CMDx-coated and EGF-conjugated nanoparticles were cultured with MCF-7 and MDA-MB-468 cells for 1, 6, and 10 h at a concentration of 5 mg/mL. After the incubation time, cells were washed twice with warm DMEM, trypsinized, and counted with Trypan Blue. The pellet was collected by centrifugation and washed twice with PBS. The washed pellet was suspended in 200 μL of concentrated HNO_3 for 1 h at 95 °C. Digested solutions were then diluted with deionized water and kept at 4 °C until measurement of iron content using an inductively coupled plasma mass spectrometer.

For localization measurements, CMDx-coated and EGF-conjugated nanoparticles tagged with fluoreiscenamine were cultured with MCF-7 and MDA-MB-468 cells for 1, 6, and 10 h at a concentration of 5 mg/mL. After the incubation time cells were washed twice with warm DMEM and stained using a 5 μL/mL DiD solution for 30 min at 37 °C to stain the membrane and a 0.1 μL/mL LysoTrack Blue solution for 30 min at 37 °C to stain the lysosomes. An Olympus Fluoview FV 300 IX71 confocal laser scanning microscopy was used to visualize nanoparticle interactions with cancer cells.

Intracellular Hyperthermia. MDA-MB-468 and MCF-7 cells were incubated with CMDx-coated or EGF-conjugated nanoparticles for 1, 6, or 10 h. Nanoparticle suspension was decanted and cells were washed twice with DMEM and EDTA prior to trypsinization with trypsine. Cells were manually counted using Trypan Blue and seeded in glass tubes at a concentration of 160 000 cells/mL (2.5 mL DMEM per tube). Controls were placed in an incubator for a period of 2 h. Samples were treated for 2 h at 37 °C with different amplitudes of a magnetic field generated using a HFI 3 kW RF heating system from RDO Induction Power Supplies. The temperature of the samples was measured using a Luxotron Fluoroptic Thermometer probe. After treatment cells were seeded for cell viability and clonogenic assay.

Cell Viability and Clonogenic Assay. Cell viability counts living single cells 5 days after treatment and are reported as viability. Even though some cells are alive after the treatment, their ability to divide and form colonies may be compromised. Hence, we also performed clonogenic assays, to measure the

ability of single cancer cells to form colonies. If cancer cells are unable to form colonies, they may not regrow as a tumor. The results of the clonogenic assay are reported as survival factor.

A total of 100 000 cells were seeded for cell viability in 25 cm^2 flasks at a concentration of 25 000 cells/mL and allowed to grow for 5 days at 37 °C. Cells were then tripsinized and counted manually using Trypan Blue. MDA-MB-468 cells were seeded for clonogenic assay in six-well plates at a concentration of 400 cells/mL (2.5 mL of DMEM per well) for cells treated with CMDx-coated nanoparticles and the controls and at a concentration of 4000 cells/mL (2.5 mL of DMEM per well) for cells treated with EGF-conjugated nanoparticles. MCF-7 cells were seeded for clonogenic assay in six-well plates at a concentration of 400 cells/mL (2.5 mL of DMEM per well). Cells were allowed to grow for 12 days and then fixed, stained with Crystal Violet, and counted following the protocol by Franken *et al.*[25] Only colonies of more than 50 cells were counted.

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Supporting Information Available: Figure S1: Long-term effect on cell viability of EGF-conjugated nanoparticles (IO-EGF) on MCF-7 and MDA-MB-468 cells. Figure S2: Size and polydispersity of single CMDx-coated nanoparticles. Figure S3: Representative thermogravimmetric analysis of CMDx-coated iron oxide nanoparticles. Figure S4: Equilibrium magnetization of CMDx-coated magnetic nanoparticles. Figure S5: Temperature rise measurements of CMDx-coated nanoparticles at different magnetic field amplitudes. Figure S6: Localization of EGF-conjugated nanoparticles after being in contact with MCF-7 for 1 h at 37 °C. Figure S7: Uptake of iron as a function of incubation time by MCF-7 cells. Figure S8: Effect of the application of the magnetic field for 2 h on MCF-7 and MDA-MB-468 cancer cells. Figure S9: Intracellular magnetic fluid hyperthermia using CMDx-coated nanoparticles and EGF-conjugated nanoparticles on MCF-7 cells. Table S1. Data corresponding to Figures 2, 3, and S9. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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