Published in final edited form as:

Ther Deliv. 2015 October ; 6(10): 1195–1210. doi:10.4155/tde.15.68.

Combining hard and soft magnetism into a single core-shell nanoparticle to achieve both hyperthermia and image contrast

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Abstract

Background—A biocompatible core/shell structured magnetic nanoparticles (MNPs) was developed to mediate simultaneous cancer therapy and imaging.

Methods & results—A 22-nm MNP was first synthesized via magnetically coupling hard (FePt) and soft (Fe₃O₄) materials to produce high relative energy transfer. Colloidal stability of the FePt@Fe₃O₄ MNPs was achieved through surface modification with silane-polyethylene glycol

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/10.2217/tde.15.68

Author contributions

The manuscript was written through contributions of all authors. Q Yang coated the nanoparticles, conducted *in vitro* and *in vivo* studies and led authorship of the manuscript. S Cai, T Zhang, V Chikan and JT Douglas assisted with chemical characterization; NM Davies assisted with animal data analysis. M Gong synthesized the nanoparticles. P Lee and I-Y Choi conducted MRI studies. S Ren and ML Forrest supervised the study. All authors have given approval to the final version of the manuscript.

The authors have no conflicts of interest to declare.

Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

(PEG). Intravenous administration of PEG-MNPs into tumor-bearing mice resulted in a sustained particle accumulation in the tumor region, and the tumor burden of treated mice was a third that of the mice in control groups 2 weeks after a local hyperthermia treatment. *In vivo* magnetic resonance imaging exhibited enhanced T₂ contrast in the tumor region.

Conclusion—This work has demonstrated the feasibility of cancer theranostics with PEG-MNPs.

Engineered **superparamagnetic iron oxide nanoparticles** (SPIONs), including magnetite Fe₃O₄ and γ-Fe₂O₃, with elaborately tailored particle sizes and surface chemistry have been extensively studied as probes for cancer diagnosis using the **MRI** due to their biocompatibility, ease in synthesis and their US FDA approval for clinical usage. Magnetic nanoparticles (MNPs) can also respond to external alternating current magnetic fields and convert magnetic energy into heat energy to induce cancer cell death [1–5], namely **magnetic hyperthermia** treatment (MHT). Therefore, SPIONs represents a powerful tool in the biomedical application to combine cancer diagnosis and therapy [6–12]. An emerging trend in this field is **cancer theranostics**, which, as the name indicates, is an evolving multidisciplinary field that combines diagnostics and therapeutic approaches to the treatment of cancer [13]. The aim of cancer theranostics is to guide pharmacological treatment of cancer based on molecular diagnostic tests. This collaborative platform offers numerous advantages such as single-step procedures, targeted drug delivery and real-time monitoring of early treatment responses. One major challenge of the delivery of the cancer theranostic agents, however, is their insufficient tumoral concentration. The inner region of solid tumor has a diminished blood flow rate and reduced perfusion, which limits the accessibility of chemotherapeutic agents to the tumor [14]. In addition, the dense extracellular due to the cellular hyperplasia hinders the penetration of the anticancer drugs and their intratumoral distribution [15]. The key advantages of cancer theranostic nanoparticles are their *in vivo* stability, large payloads, high surface area to volume ratio, and most importantly, their nanoscale size, which facilitates the nanoparticles to extravasate through the leaky vasculature and preferentially accumulate in the solid tumors, often termed the enhanced permeability and retention (EPR) effect, to achieve tumor-selective imaging/therapy [16–20]. The composition, crystallinity and magnetism of SPIONs can be tailored to enhance magnetic saturation properties [21,22] necessary for theranostic agents. However, relatively moderate heating efficiency and lack of precise control of *in vivo* temperature evolution during the treatment [23] remains major obstacles for the SPIONs to mediate MHT.

In order to achieve strong MRI contrast sensitivity and sufficient heating efficacy for MHT, high magnetization is a prerequisite. Magnetic alloy nanoparticles including FePt [24,25], FeCo [26,27] and FePd [28] have demonstrated enhanced magnetic properties, such as strong magnetism and large coercivity, making them suitable for theranostic application. In addition, compared with FeCo MNPs, FePt MNPs are more chemically stable against rapid oxidation in biological solutions [29]. Novel bimagnetic core/shell nanostructures that incorporate magnetically soft materials (e.g., iron oxide) and magnetically hard magnetic alloy nanoparticles (e.g., FePt, FePd) take advantage of the intrinsic magnetic properties at both ends of the spectrum. These core/shell MNPs combine high magnetocrystalline anisotropy and saturated magnetization via the effective interfacial exchange coupling

interaction between the two phases, affording cooperative magnetic switching and tunable magnetic properties [30–32]. For example, Masala *et al.* [32] and Lee *et al.* [33] synthesized bimagnetic MNPs that combined hard CoFe_2O_4 and soft ZnFe_2O_4 or MnFe_2O_4 , and these core/shell structured bimagnetic MNPs exhibited an increased magnetism compared with the single-phase MNPs of similar size. The enhanced magnetic properties of the core/shell MNPs can be further translated into a high thermal energy transfer capability, which is crucial for generating the high local temperatures needed for thermal ablation of tumor tissues [34] during the magnetically mediated hyperthermia. Compared with a clinically approved Fe_3O_4 MNPs formulation, Feridex[®], a significant enhancement of magnetic heat induction was reported in the exchange-coupled CoFe_2O_4 @ MnFe_2O_4 MNPs, which were intratumorally injected and led to the complete tumor eradication after local hyperthermia [33]. Although high concentration of MNPs within the tumor region can be achieved by local administration, direct injection is invasive and not amenable to suppress small metastatic tumor growth, presenting a severe practical challenge for their biomedical applications. For example, tumor overheating has been found to cause plaque formation over a period of 7 days following the hyperthermia after the local injection of MNPs [33]. In contrast, intravenous (iv.) administration is far less invasive and could offer better coverage for the metastatic tumor regions.

To broaden the potential applications of the bimagnetic MNPs with stable magnetic properties and better physicochemical features, we set out to develop MNPs of core/shell structures with mutual coupling of magnetically hard and soft components to simultaneously achieve high saturated magnetization and coercivity, which could be used as an anticancer theranostic agent for imaging and treatment. In addition, an efficient surface coating strategy was applied to the as-synthesized core/shell MNPs to increase their biocompatibility, aqueous-dispersity and colloidal stability. Surface-coating materials typically include inorganic materials such as gold [35,36] and silica [37,38], and biodegradable polymers such as dextran [39,40], chitosan [41–43], poly (lactide acid) [44,45] and polyethylene glycol (PEG) [46,47]. Among these surface modification methods, PEG coating is one of the most efficient approaches for preventing aggregation of MNPs in physiological conditions due to the steric expulsion between the hydrophilic chains, which extends the *in vivo* circulation and subsequently enhances the accumulation of MNPs in tumors due to its capability to resist reticuloendothelial system clearance [48–52].

The potential of our PEGylated core/shell $\text{FePt}@\text{Fe}_3\text{O}_4$ MNPs (PEG-MNPs) as a robust probe for cancer theranostics was investigated in a mouse model of 4T1.2 Neu breast tumor. The intravenously injected PEG-MNPs were activated by an alternating magnetic field to generate high magnetic heating. Combined with the deep-tissue penetration capacity of the magnetic field, the PEG-MNPs-mediated hyperthermia exhibited significant antitumor efficacy. In addition, the enhanced sensitivity of *in vivo* MRI T_2 maps within the tumor region demonstrated the potential of PEG-MNPs for integrated diagnostics.

Materials & methods

Materials

All chemicals were used as received without further purification unless otherwise noted. Platinum acetylacetonate ($\text{Pt}(\text{acac})_2$) (97%), iron pentacarbonyl ($\text{Fe}(\text{CO})_5$) (>99.99% trace metal basis), iron (III) acetylacetonate ($\text{Fe}(\text{acac})_3$), 1,2-hexadecanediol (Technical grade 90%), dioctylether (99%), oleylamine (technical grade 70%), oleic acid (technical grade 90%), anhydrous hexane (95%), acetone (99.5%) and triethylamine (99.5%) were purchased from Sigma-Aldrich Co. (MO, USA). 2-[methoxy (polyethyleneoxy) propyl] trimethoxysilane (silane-PEG, molecular weight: 460–590D) was purchased from Gelest, Inc (PA, USA). Methanol (99.9%) and phenyl ether (99%) were obtained from Fisher Scientific (PA, USA). Ethanol (200 proof) was purchased from Decon Labs, Inc. (PA, USA). Double distilled water was used in syntheses, characterization, cell culture and animal experiments (sterilized by autoclaving). Metastatic murine breast cancer cell line 4T1.2-Neu was a kind gift from Dr. Zhaoyang You, University of Pittsburgh (PA, USA). Cell culture medium, Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Life Technologies (NY, USA).

Methods

FePt@Fe₃O₄ MNPs synthesis—The core/shell FePt@Fe₃O₄ MNPs synthetic procedure consists of two steps, including FePt core synthesis and Fe₃O₄ coating. Under an argon atmosphere, platinum acetylacetonate ($\text{Pt}(\text{acac})_2$, 0.5 mmol) and 1,2-hexadecanediol (2 mmol) were dissolved in 20 ml of dioctylether, degassed/refilled three cycles and heated to 100°C. Iron pentacarbonyl ($\text{Fe}(\text{CO})_5$, 1 mmol), oleylamine (0.17 ml) and oleic acid (0.16 ml) were injected, degassed/refilled one cycle under argon, and then the solution was heated to 295°C for 30 min. After cooling to room temperature, the FePt MNPs were precipitated using methanol/hexane (5:1 v/v) and washed three-times. The black FePt MNPs were collected and annealed at 450°C for 2 h in a N₂/H₂ annealing atmosphere. The FePt MNPs were dispersed in hexane and stored under N₂. FePt MNPs (25 mg) in hexane, iron (III) acetylacetonate ($\text{Fe}(\text{acac})_3$, 1 mmol), oleylamine (1 mmol), oleic acid (1 mmol) and 1,2-hexadecanediol (5 mmol) were mixed in phenyl ether (20 ml) in a flask equipped with a condenser and degassed/refilled with N₂ for three cycles. The mixture was heated to 100°C and purged under N₂ for 10 min to remove the hexane. The remaining solution was heated to 265°C for 30 min. After cooling to room temperature, the MNP were precipitated using methanol/hexane (5:1 v/v) and washed three-times. The final black FePt@Fe₃O₄ MNPs were dispersed in hexane and stored in the N₂ glovebox.

Specific absorption rate (SAR) measurements of the FePt@Fe₃O₄ MNPs—A series of MNPs sample solutions with different MNP sizes were prepared, and their heating properties were measured using an inductive magnetic field (IMF) generator (field amplitude: 5000 Am⁻¹; frequency: 366 kHz). Briefly, a 2 ml of MNPs dispersed in hexane (~10 mg/ml) was placed in a glass vial and held within the induction heater coil using a Teflon holder. The Teflon holder acted as an insulator and prevented residual heating of the sample from the coil. The temperature change of the colloidal solution was measured using a fiber optic probe (Neoptix Inc., QC, Canada) and the temperature rise of the sample

solutions as a function of time was recorded with a computer. In order to reduce errors due to heat loss to the environment, the initial slope of the time dependent temperature curve (T/t) was taken for the calculation of SAR. The SAR value was calculated with the following equation, $SAR = (C/m_{NP})(T/t)$, where C is the heat capacity of the solution and m_{NP} is the mass of the nanoparticles.

Surface PEGylation of FePt@Fe₃O₄ MNPs—Core/shell FePt@Fe₃O₄ MNPs were modified with 2-methoxy polyethyleneoxy propyltrimethoxysilane (silane-PEG) through a ligand exchange reaction according to the method of Larsen *et. al.* [49,53]. Briefly, 300 μ l of as-synthesized nanoparticles in hexane (30 mg/ml) were washed with 10 ml of methanol / acetone (1:1) and centrifuged at $2000 \times g$ for 30 min. After the supernatant was discarded, precipitated nanoparticles were resuspended in 300 μ l of hexane. The washing step was repeated three-times and the precipitated nanoparticles were then dried under an argon flow to remove residual solvents. The dried nanoparticles were dispersed in 6 ml of toluene, followed by addition of 600 μ l of silane-PEG (1 mg/ml), 3 ml of triethylamine and 60 μ l of double distilled water. The mixture was stirred at 60°C overnight, after which the nanoparticles were precipitated with 25 ml of hexane/ethanol (3:1). The particle suspension was precipitated by centrifugation ($2000 \times g$ for 10 min) and resuspended in 2 ml of toluene. The washing cycle was repeated three times. The washed PEGylated FePt@Fe₃O₄ MNPs (PEG-MNPs) were dried at 35°C overnight with a centrifugal evaporator.

Fourier transform infrared spectroscopy—Fourier transform infrared spectroscopy (FTIR) spectra of approximately 2 mg of dried MNPs were recorded using an IRAffinity-1 FTIR (Shimadzu Scientific Instruments, KS, USA). For each spectrum, 45 scans were collected between 4000 and 400 cm^{-1} at a resolution of 1 cm^{-1} .

Hydrodynamic diameters measurements—PEG-MNPs were dispersed at 1 mg/ml in phosphate buffered saline (PBS), and dynamic light scattering (DLS) measurements of the hydrodynamic diameters of PEG-MNP dispersions were performed at 25°C using a ZetaPlus Particle Size Analyzer (Brookshaven Instrument, NY, USA), and all measurements were repeated five times.

Thermal gravimetric analysis—Thermal gravimetric analysis (TGA) was performed on a Q50 TGA thermal analyzer (TA instruments, PA, USA). Approximately 8 mg of dried PEG-MNPs were heated from room temperature to 900°C at 10°C/min.

Stability of the PEG-MNPs in physiological media—The *in vitro* colloidal stability of PEG-MNPs was tested under model conditions. PEG-MNPs were dispersed at 1 mg/ml in PBS, DMEM and 10% FBS and stored at room temperature (r.t.) or 2–5°C for up to 1 week. Stability of PEG-MNPs in the physiological media was assessed by checking particle aggregation and measuring hydrodynamic diameters by DLS. All measurements were repeated five times.

In vitro cytotoxicity—Metastatic murine breast cancer cell line 4T1.2-Neu was cultured in DMEM supplemented with 10% (v/v) FBS and 1% l -glutamine. Cells were trypsinized and seeded into 96-well plates at 3,000 cells/well ($n = 3$ plates, 12 wells/concentration) and

incubated overnight. PEG-MNPs dispersed in PBS were added to each well with final concentrations of 4 µg/ml to 4 mg/ml. PBS and 10% trichloroacetic acid (TCA) were used as negative and positive controls, respectively. A resazurin-based colorimetric assay was used to measure viability of cell cultures [54], and the IC₅₀ was determined as the midpoint between control groups of positive and negative using GraphPad Prism 6 software (GraphPad Software Inc., CA, USA).

Cellular uptake—To investigate the intracellular uptake of PEG-MNPs, 4T1.2-Neu cells were seeded in 12-well plates at 3×10^5 cells/well and then incubated with PEG-MNP suspensions in PBS at 0.3 mg/ml for 24 h. The same volume of the PBS without PEG-MNPs was added to cell cultures at the same cell density as a negative control. After three washes with PBS, the cells were detached with trypsin and then resuspended in DMEM for cell counting using a hemocytometer. To measure the intracellular uptake of PEG-MNPs, the cell suspensions were centrifuged at $8000 \times g$ for 10 min, and cell pellets were digested in 30% HCl at 70°C for 1 h [55]. PEG-MNPs in cells were quantified on an inductively coupled plasma mass spectrometer (ICP-MS) (7500, Agilent Technologies, CA, USA) for iron content, and samples collected from untreated cells were used as a negative control. All measurements were repeated four times.

PEG-MNPs accumulation in tumors—The animal study was approved by the Institutional Animal Care and Use Committee of the University of Kansas. To determine the dosage of PEG-MNPs used for the tumoral uptake, antitumor efficacy and MRI experiments, PEG-MNPs suspended in sterile saline were injected into tumor-free female Balb/c mice via the tail vein at three different dosages, 100, 50 and 20 mg/kg. Body weights of the mice were recorded every other day, and the animals were monitored for toxic reactions for 10 days after the injection. Female Balb/c mice were anesthetized with 2% isoflurane in O₂, and 3×10^6 4T1.2-Neu breast cancer cells in PBS were injected into the right mammary fat pad under the second nipple along the lateral line. Tumor sizes were measured three- to four-times weekly with a digital caliper in two perpendicular dimensions, and the tumor volume was calculated as: tumor volume = $0.52 \times (\text{width})^2 \times (\text{length})$. Once tumor volumes reached 150 mm³, 100 mg/kg of PEG-MNPs suspended in sterile saline was injected intravenously via the tail vein. Animals were euthanized at 0.5, 4, 24, 48, 72 and 96 h post injection (n = 3 per time point), and tumors were excised and blood samples were drawn into Ethylenediaminetetraacetic acid-treated vials through cardiac puncture followed by centrifugation for 5 min at $2000 \times g$ to collect plasma. Biological samples were digested using aqua regia at 130°C for 1 h and diluted with 1% HNO₃ prior to platinum analysis by ICP-MS.

Therapeutic efficacy—Female Balb/c mice bearing 4T1.2-Neu breast tumors were randomly divided into four groups, and the minimum number of animals was used in each group for significance (n = 3–5 per group), including animals receiving repeated PEG-MNPs-induced hyperthermia (PEG-MNPs plus IMF), animals injected with MNP-injected (PEG-MNPs) but not subjected to the IMF, animals injected with saline and placed into IMF for 5 min (Saline plus IMF) and animals injected with saline as untreated controls (Saline). When tumors reached 150 mm³ in size, 100 mg/kg of PEG-MNPs dispersed in sterile saline

was injected via the tail vein. After 24 h, the mice were placed under anesthesia in a water-cooled magnetic induction coil with the tumor region positioned in the central part of the coil. An inductive magnetic field of 400 kHz at 25.6 kAm^{-1} was applied for 5 min for two consecutive days using a SI-7KWHF induction heater (Superior Induction Company, CA, USA) and a 2.5-cm copper coil. Following the inductive heating treatment, tumor growth was monitored until it reached an end point of 1500 mm^3 .

MRI—*In vivo* MRI was performed using a 9.4-Tesla horizontal MR system (Agilent Technologies, CA, USA), in the Hoglund Brain Imaging Center at the University of Kansas Medical Center. Tumor bearing mice ($n = 2\text{--}3$ per group) were injected intravenously with PEG-MNPs as stated in the previous section. MRI T_2 maps (echo times: 9.3, 14, 20, 30 and 40 ms) of mouse whole bodies were obtained at predetermined time points after injection. MR images acquired from animals without any injections were used as controls. MR images were processed using the Stimulate software [56]. A mono-exponential model of signal decay was applied to calculate T_2 values within the tumor region on a pixel-by-pixel basis.

Statistical analyses—Tumor size ratio for the four experimental groups were analyzed and expressed at average $\pm$ SD. Statistical significance was determined by the unpaired t-test with $p < 0.05$ using the GraphPad Prism 6 software.

Results & discussion

Synthesis & physicochemical characterization of core/shell FePt@Fe₃O₄ MNPs

Coreduction of $\text{Pt}(\text{acac})_2$ and $\text{Fe}(\text{CO})_5$, followed by annealing at 450°C for 2 h formed the hard FePt core. Then the soft Fe₃O₄ shell was controllably hybridized via thermal decomposition from $\text{Fe}(\text{acac})_3$ in the presence of oleylamine, oleic acid and 1,2-hexadecanediol in phenyl ether. Transmission electron microscopy (TEM) images of the core/shell FePt@Fe₃O₄ MNPs (Figure 1A) clearly revealed the spherical shape of the core/shell MNPs, and the detailed structure of the core/shell structure was characterized by the high-resolution TEM (HRTEM) in the inset that shows the coating of 5-nm Fe₃O₄ (lighter ring) on the initial 12-nm FePt core (darker region) to render a homogenous 22-nm core/shell structure. The highly uniform size distribution of the core/shell MNPs in Figure 1A confirmed that the MNPs formed is solely the core/shell FePt@Fe₃O₄ and not a mixture of FePt and Fe₃O₄ MNPs with much smaller sizes.

The exchange-coupled behavior of the FePt@Fe₃O₄ MNPs was compared with two different MNPs coupling schemes, including randomly mixed hard and soft MNPs and aggregated hard and soft MNPs. In comparison, the aggregated FePt and Fe₃O₄ MNPs were prepared by removing the oleic acid used to isolate and stabilize the MNPs during the synthesis. The M-H hysteresis measurement of the MNPs conducted with a superconducting quantum interference device (Figure 1B) shows that the coercivity values (H_c) for Fe₃O₄ and FePt MNPs are 50 Oe and 800 Oe at ambient temperature, respectively. Several research groups have investigated the influence of annealing temperature on the microstructures and magnetic properties of the FePt MNPs [57–60]. It has been shown that higher annealing temperature could provide increased magnetic coercivities. For example, conversion of the soft face-centered cubic (fcc) phase FePt with a single SiO₂ [57] or a TaO_x [58] capping

layer to the hard face-centered tetragonal (fct) phase have been found after annealing at 400°C, and a highest coercivity (4.2 kOe) of FePt/TaO_x was reached as the annealing temperature increased to 550°C [58]. However, further increases in annealing temperature above 580–600°C could cause severe MNPs aggregation, increasing the particles size and size distribution [58,60]. In this study, annealing was performed at 450°C and a partial conversion from chemically disordered fcc into the ordered centered fct structure was achieved. The coercivity of our FePt MNPs is 800 Oe, which is consistent with the data reported by Kang *et al.* [61]. Comparatively, the core/shell FePt@Fe₃O₄ MNPs have an H_c of 600 Oe with a single-phase-like loop due to the smooth magnetization transition, indicating the adjacent moments effectively aligning with each other due to the magnetic exchange coupling at the interface. In addition, a uniform size dispersion of the core/shell MNPs without any particle aggregation was illustrated in Figure 1A.

The effectiveness of hybrid FePt@Fe₃O₄ magnetically coupled nanoparticles as an MRI contrast agent was examined by measuring the dependence of the longitudinal ($1/T_1 = R_1$) and transverse ($1/T_2 = R_2$) relaxation rate constant of FePt@Fe₃O₄ MNPs solution. First, we measured R_1 and R_2 of the soft Fe₃O₄ MNPs, hard FePt MNPs, 1:1 mixture and core/shell FePt@Fe₃O₄ MNPs as a function of MNP concentration using the inversion recovery and spin echo pulse sequences, respectively, on a 9.4 T nuclear magnetic resonance (NMR) spectrometer [62] (Supplementary Figure 1A & B). The relaxivities (r_1 (or r_2)) were determined from the slope of the plot of R_1 and R_2 versus MNP concentration. The values for r_1 equals 2.0, 0.2 and 1.2 Hz ml mg⁻¹ for Fe₃O₄ MNPs, FePt MNPs and a 1:1 mixture MNPs, respectively, and the values for r_2 equals 20.1, 3.6 and 17.4 Hz ml mg⁻¹ for Fe₃O₄ MNPs, FePt MNPs and the 1:1 mixture MNPs, respectively. For FePt@Fe₃O₄, r_1 equals 4.4 Hz ml mg⁻¹ and r_2 equals 99.2 Hz ml mg⁻¹. The larger relaxivity of the FePt@Fe₃O₄ MNPs relative to Fe₃O₄ and FePt MNPs is hypothesized to stem from the favorable combination of coercively and saturation magnetization of the Fe₃O₄ shell and the FePt core structure. Comparing R_1 and R_2 of Fe₃O₄ MNPs, FePt MNPs, the 1:1 mixture of Fe₃O₄: FePt MNPs, a 1:1 mixture prepared by ligand exchange and core/shell FePt@Fe₃O₄ MNPs at 1 mg/ml, the FePt@Fe₃O₄ MNPs have the largest effect upon the relaxation rate constant (Supplementary Figure 1C & D), indicating their enhanced efficiency as contrast agents for MRI to promote spin-lattice relaxation. Therefore, the core/shell FePt@Fe₃O₄ MNPs were beneficial in both high oxidation resistance and maximum magnetic coupling to surrounding spins.

Measurement of SAR of the core/shell FePt@Fe₃O₄ MNPs

During a local hyperthermia treatment, the MNPs dispersed in the biological fluids or tumor tissues are subjected to a magnetic alternating current field and then induce a gradual alignment of the magnetic moments towards the external field. When the magnetic moments of MNPs relax to their equilibrium orientation states, the magnetic energy is dissipated and converted into heating effects that are related to the maximum power loss during the magnetization reversal process. SAR is a key parameter to describe the energy amount converted into thermal energy material per gram [63]. In order to achieve efficient MHT, the MNPs should heat rapidly within a short period of treatment time. Therefore, a maximized

SAR is required as MNPs possessing the high SAR value enable a smaller injection dose due to their high heat-generation capability, thus reducing the toxic effects.

The SAR values of core-shell nanoparticles were compared with single-component MNPs, randomly mixed MNPs and aggregated MNPs (Figure 2A). While the SARs of single-component MNPs range from 20 (FePt MNPs) to 300 Wg⁻¹ (Fe₃O₄ MNPs), the SAR of core-shell MNPs was of 550 Wg⁻¹. The significantly increased SAR value results from the mutual coupling between the magnetically hard (FePt) and soft (Fe₃O₄) materials, which enhances the efficiency of magnetic thermal induction and reduces the required dose of MNPs for hyperthermia. The heating effect depends on the particle geometry as well as the anisotropy. The SAR of core-shell nanoparticles can be precisely tuned by varying the combination of the differently sized core and shell components.

Using an adopted theoretical model, we then calculated SAR values as a function of particle size at varying polydispersity indices (σ). During the calculation, the values of the magnetic anisotropy constant and the saturation magnetization of FePt MNPs ($M_d = 1140$ kA/m, $K = 206$ kJ/m³) and Fe₃O₄ MNPs ($M_d = 446$ kA/m, $K = 9$ kJ/m³) were taken from the published data [64]. The K values have been reported to vary from 206 to 7000 kJ/m³ for FePt MNPs with the fcc structure and fct structure after annealing at 500°C for 5 h [65]. As our FePt MNPs were annealed at a lower temperature and a shorter time, the unannealed value was used in the theoretical calculation. The calculated SAR of both MNP types increased with particle sizes up to a maximum of 183 W/g for 16.2 nm FePt MNPs and 132 W/g for 18.3 nm Fe₃O₄ MNPs. These MNPs are single domain superparamagnetic particles that produce heat in alternating magnetic fields via loss mechanisms due to Néel and Brownian relaxation of the particle magnetic moment [34,66]. Néel relaxation refers to an internal magnetic moment vector rotation along the crystal easy axis, and the power loss corresponding to this process is exponentially proportional to the magnetic anisotropy constant and the MNPs volume. In the Brownian mode, however, MNPs undergo oscillation as a whole toward the external field, and the corresponding power loss correlates linearly with the hydrodynamic volume of the MNPs. Due to the exponential correlation between the relaxation time versus the product of the magnetic anisotropy constant and the particle size in the Néel mode, the Néel relaxation typically dominates the effective relaxation process for the MNPs with smaller size [34]. In our study, the optimal size is 16.2 nm for FePt MNPs and 18.3 nm for Fe₃O₄ MNPs (Figure 2B). Beyond this point, the dominant relaxation process changed to the Brownian relaxation, which is independent of the magnetic anisotropy and only relies on the particle size. Size distribution of the MNPs also appeared to play a critical role to achieve high SAR. As shown in Figure 2B, when the polydispersity index (σ) increased from zero to 40%, the SAR values decreased accordingly by 32.74% for FePt MNPs and 62.17% for Fe₃O₄ MNPs. We have also observed that the measured SAR value of FePt MNPs is much lower than the calculated value using the theoretical model, probably due to the achieved partial fct structure using the relatively lower annealing temperatures during the synthesis.

Surface PEGylation & physicochemical characterization of core/shell FePt@Fe₃O₄ MNPs

Colloidal stability and biocompatibility are two essential requirements for clinical application of exchange-coupled MNPs. The core/shell FePt@Fe₃O₄ MNPs synthesized in organic solvent and coated with oleic acid are not easily dispersed in water. These as-made MNPs are hydrophobic and they are subject to rapid elimination from the blood circulation before reaching tumor sites by macrophages of the mononuclear phagocyte system. Modification of the core/shell FePt@Fe₃O₄ MNPs was achieved by exchanging oleic acid with hydrophilic PEG molecules using silane-PEG, and the success of PEGylation was confirmed by FTIR. The three FTIR peaks resolved between 840 and 1040 cm⁻¹ in the purified PEG-MNPs dry power (Figure 3) were assigned to the Si-O-Fe stretching. The peaks at 1100 cm⁻¹ and 1360 cm⁻¹ corresponded to the ether group C-O-C stretch band and the vibration band, respectively.

Detection of these characteristic peaks confirmed the presence of a silane-PEG coating on the surface of MNPs. The weight percentage of silane-PEG was estimated to be 21.34% using TGA (Figure 4), based on a 21.34% mass loss from 17.28 to 455.01°C. In addition, the longitudinal relaxivity value for PEG-MNPs was determined to be 0.2084 ml (s⁻¹) (mg⁻¹) with a good linear correlation ($R_2 = 0.99629$) (Supplementary Figure 2).

PEGylated FePt@Fe₃O₄ MNPs were well dispersed in aqueous solutions with a hydrodynamic diameter of 25.9 ± 2.8 nm and a narrow colloid size distribution (Figure 5). The stability of monodisperse PEG-MNPs was investigated in PBS, DMEM with 10% (v/v) FBS and PBS with 10% (v/v) FBS, at both room temperature and refrigerated temperature for a week. Although hydrodynamic diameters of all six of the PEG-MNPs dispersions increased (Table 1), from 9.26% in PBS at 4°C to 29.7% in DMEM at room temperature, no agglomeration was observed during this period of time (Supplementary Figure 3), indicating high colloidal stability due to the steric repulsion between the surface hydrophilic PEG chains.

Intracellular uptake & *in vitro* cytotoxicity of PEG-MNPs

The PEG-MNPs had low toxicity and high uptake in cells. The IC₅₀ of silane-PEG-coated was determined as 0.487 ± 0.073 mg/ml in murine breast cancer 4T1.2 Neu cells, and the cells were approximately 85–100% viable at the concentrations of 4–100 µg/ml after incubation for 72 h (Figure 6A). The ICP-MS iron analysis of digested cell pellets after a 24-h PEG-MNPs treatment demonstrated significant intracellular uptake of PEG-MNPs with 4.98 ± 1.05 pg/cell (Figure 6B), which is probably due to the enhanced nanoparticles internalization of the particles provided by the PEG chain [67].

Dose-finding & PEG-MNPs accumulation in tumors

The toxicity of the PEG-MNPs was first assessed in the healthy lab mice as described in similar studies [68,69]. Healthy Balb/c mice were dose escalated by intravenously administration of PEG-MNPs into the tail veins. After the PEG-MNPs injection, the mice were monitored for 10 days and checked for signs of toxic reactions, including lethargy, paralysis, weight loss or respiratory depression. All of the mice remained in good condition and their eating habits did not change over the time of the experiment. In addition, the body

scores of the mice remained above 2.5 and body weight losses were less than 20% (Figure 7). No toxic reactions were observed during the study. Therefore, a dosage of 100 mg/kg was chosen for subsequent animal experiments.

It has been reported that nano-sized bare SPIONs are susceptible to opsonization by plasma proteins, followed by rapid clearance from systemic circulation by macrophages of the reticuloendothelial system [70]. For example, the plasma half-life of SPIONs sized at 40 nm was less than 10 min after intravenous injection, and approximately 90% of the injected particles accumulated in mononuclear phagocyte system organs including the liver, spleen and bone marrow [71]. Therefore, the amount of MNPs that reached the target tissues was substantially reduced, limiting their biomedical applications. In this study, the surface of as-synthesized core/shell MNPs was grafted with hydrophilic and steric PEG chains to minimize their clearance by macrophages [53] and thus prolong their retention time. Figure 8A shows that PEG-MNPs exhibited a half-life of 1.35 h after intravenous injection into tumor-bearing Balb/c mice. Pharmacokinetic analysis of PEG-MNPs was performed by fitting data (0–24 h) to a two-compartment model (Table 2). The plasma PEGMNPs concentration decreased by $98.24 \pm 0.33\%$ in the first 4 h as PEG-MNPs distributed from the central compartment to peripheral compartments, followed by slow clearance in the beta phase.

Grafting PEG molecules onto the surface of the MNPs stabilizes the MNPs due to the steric interactions, and PEGylation has been demonstrated to extend the systemic circulation times of MNPs after the intravenous administration [72,73]. For example, 40 nm PEGylated MNPs-encapsulating liposomes exhibited a blood half-life of approximately 1 h in rats. Shown in the Figure 8 and Table 3 are the PEGylated PEG-MNPs accumulation in the leaky vasculature of the breast tumor region from 4 h to 96 h; this accumulation and retention in tumor vasculature of approximately 1% of the injected dose is consistent with the EPR effect. The leaky tumor vasculature and the reduction in functional lymphatic vessels collectively allow nanocarriers within 10–100 nm to diffuse from blood stream and selectively accumulate in the tumor tissues. The EPR effect, reported in 1980s [74], has been extensively exploited by researchers to target anticancer drug-loaded nanoparticles to tumor regions [19,75–77], and in the absence of targeting moieties, for example, peptides or antibodies, EPR is believed to be the primary driver of nanoparticle accumulation in tumors and subsequent therapeutic effect. An MRI study of MNPs PEGylated with a similar molecule weight of PEG found the particles distributed evenly throughout the volume of subcutaneous squamous cell carcinomas and C3H mammary carcinomas, 24 h after intravenously administration [50]. In addition to the possible EPR effect, PEGylation addresses certain key issues with the conventional uncoated MNPs, such as poor water dispersity, low colloidal stability and short plasma residence time. Further improvements in intratumoral accumulation and uniform distribution may require combination strategies, including modifying the PEG-MNPs with a targeting moiety to improve cancer cell internalization, for example, folic acid or TNF [55,78], or manipulating the tumor blood flow and vascular permeability by local hyperthermia [79,80].

PEG-MNPs-mediated hyperthermia

Hyperthermia may cause the shrinkage or sometimes a complete eradication of tumors by killing cancer cells and damaging proteins within the cancer cells [33,81,82]. The effectiveness of hyperthermia treatment mainly depends on the degree of temperature increase during a defined length of treatment. The thermal energy transfer capability of the PEG-MNPs was demonstrated in a phantom test using PEG-MNPs in saline solution (25 mg/ml) of a similar volume as the breast tumor at the starting point for the treatment. After applying an inductive magnetic field for 5 min, the temperature of the PEG-MNP solution increased from 22.5 to 64.5°C.

Repeated hyperthermia treatment in a rabbit liver cancer model was reported to inhibit growth by over 92%, compared with less than 80% inhibition after a single treatment. The superior tumor reduction with repeated treatment was due in part to the coagulation-induced necrosis of tumor in the heated region [82]. A murine study observed complete regression of large, >15 mm, mammary carcinomas after repeated MNPs-mediated hyperthermia [81]. Based on our finding that the MNPs were retained in the tumor for at least 48 h, we chose to repeat the 5-min hyperthermia treatment on the day following the initial treatment. For the animals that received PEG-MNPs plus inductive magnetic field, the repeated hyperthermia was well tolerated and the tumor size increased 2.32-fold 2 weeks after the treatment (Figure 9). In comparison, for the animals in the saline group, PEG-MNPs only group, and saline plus IMF group, the tumor size increased more than ninefold in the same time period. In addition, the tumor size was reduced to 30% in PEG-MNPs-induced hyperthermia treated (PEGs + IMF) mice when compared with tumor size of the saline-treated animals, while no significant difference in tumor volume was observed among saline group, PEG-MNPs only group and inductive magnetic field only group. PEG-MNPs-mediated hyperthermia demonstrated a significantly enhanced suppression of the tumor growth, and this is likely due to thermal ablation of the cancer cells by PEG-MNP mediated magnetic- heat energy transfer, achieving improved local tumor control.

MRI of breast tumor

The potential application of PEG-MNPs as an MRI contrast agent for cancer diagnosis was evaluated by MRI T_2 maps on Balb/c mice implanted with 4T1.2-Neu breast tumors. Mice injected with the intravenously PEG-MNPs were randomly divided into three groups: MRI at 24 h post injection ($n = 3$), 48 h post injection ($n = 2$) and 48 h post injection with hyperthermia at 24 h post injection ($n = 3$). MRI T_2 maps of animals in all three of the PEG-MNP injected groups showed significant signal reduction within the tumor region when compared with the untreated animals (Figure 10). Region of interest analysis showed T_2 values in the tumor were remarkably lower (Table 4) in animals with PEG-MNPs injection compared with controls.

Conclusion

In conclusion, we developed biocompatible MNPs through surface-PEGylation of a core/shell exchanged-coupled FePt@Fe₃O₄ MNPs. The PEG-MNPs carry attractive magnetic and physicochemical properties that may be tailored to enhance the biomedical application

PEG-MNPs, including mediating local hyperthermia combined with diagnostic imaging. PEGMNPs- induced hyperthermia in a breast orthotopic tumor model in immune-competent animals demonstrated the enhanced antitumor effectiveness due to the high magnetic-thermal energy transfer capability of PEG-MNPs. In addition, augmented MRI contrast in the tumor region suggested application potential of PEG-MNPs for cancer diagnosis. Our further studies will focus on developing targeted core/shell PEGMNPs to expand the scope of their bio-application in theranostics.

Future perspective

The recent progress in the synthetic and material chemistry has allowed the researches to precisely control the key parameters and to prepare multifunctional bi-MNPs with enhanced magnetic properties and physicochemical stability. This opens a new avenue in the development of more advanced delivery systems for cancer detection and treatment, which require sufficient concentrations of therapeutic and diagnostic agents in the tumor sites. Therapeutic DNA, cytotoxic drugs and imaging contrast agents can be covalently conjugated to, or physically incorporated into the MNPs. Under the influence of an external magnetic field, the multifunctional MNPs can transport the theranostic moiety to tumors for sustained drug release. Drug-loaded MNPs may also be linked with ligands such as antibodies and peptides to target their related receptors that are overexpressed in the tumor cells, thus achieving tumor-specific delivery. The bimagnetic MNPs hold tremendous promise as therapeutic delivery platforms. However, seeking out strategies to maintain the magnetism of MNPs during the multistep reaction, to enhance drug-loading efficiency, and to enhance the target ability with appropriate ligands may continue to be an obstacle that needs to be addressed in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the NIH (1R01CA173292, PI: ML Forrest; 1P30CA168524-01; PI Jensen) and support from the Hoglund Brain Imaging Center. The Hoglund Brain Imaging Center is supported by NIH (P30 AG035982, PI Swerdlow) and the Hoglund Family Foundation. S Ren thanks the financial support from the National Science Foundation (NSF) under Award No. NSF-CMMI-1332658 for magnetic nanoparticle synthesis and assembly, NSF-DMR-1451326 for magnetically hard nanocrystals and US Department of Energy award (DE-FG02-13ER46937) for the physical property characterization. V Chikan would like to acknowledge the support of NSF through grants NSF-1128570 for the SAR characterization and the Terry Johnson Cancer Center at KSU for funding the induction heater.

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Key terms

Superparamagnetic iron oxide nanoparticles: Synthetic iron oxide particles with diameters between 10 and 100 nm. The main forms are γ -Fe₂O₃ (maghemite), Fe₃O₄ (magnetite) or α -Fe₂O₃ (hermatite) particles.

MRI: A noninvasive technique that uses a strong magnetic field and radio frequency pulses to construct detailed and cross-sectioned images of organs, tissues and other internal body structures on a computer, providing important information for cancer diagnosis, staging and treatment planning.

Magnetic hyperthermia: A magnetic nanoparticles-mediated cancer therapy that increases the temperature in tumor tissue above 43°C to cause the necrosis of cancer cells without damaging the surrounding normal tissues.

Cancer theranostics: A combination of diagnostic agents and therapeutic entities, aiming to guide pharmacological treatment of cancer based on molecular diagnostic tests.

Executive summary

Preparation of hard/soft exchanged-coupled core/shell MNPs

- The exchanged-coupled FePt@Fe₃O₄ magnetic nanoparticles (MNPs) with highly uniform size distribution were successfully synthesized via a two-step reaction.
- The enhanced magnetism of the core/shell FePt@Fe₃O₄ MNPs was determined with higher specific absorption rate and larger relaxivity.

Surface PEGylation of as-synthesized core/shell FePt@Fe₃O₄ MNPs

- Hydrophilic silane-PEG segments were conjugated on the surface of the FePt@Fe₃O₄ MNPs, and the 26-nm PEGylated MNPs exhibited enhanced dispersity and stability in the aqueous media.
- The biocompatible polyethylene glycol-magnetic nanoparticles (PEG-MNPs) showed low cytotoxicity, and they can be effectively internalized by cancer cells.

In vivo hyperthermia treatment & magnetic resonance imaging of breast tumor

- The intravenously injected PEG-MNPs were well tolerated by the animals, and no side effects or any behavioral abnormalities were observed during the course of the experiment.
- Two weeks after the completion of PEG-MNPs induced hyperthermia, the breast tumor volume was reduced to 30% when compared with the untreated animals.
- Twenty-four hours after the intravenous injection of PEG-MNPs, the MRI contrast of the breast tumor area was enhanced by approximately twofold due to the accumulation of PEG-MNPs in the tumor area.

Figure 1. Nanostructures and magnetic characteristics of exchange coupled magnetic nanoparticles

(A) FePt@Fe₃O₄ (core/shell) magnetic nanoparticles; (B) The magnetic hysteresis loop of hard, soft and core/shell magnetic nanoparticles.

Figure 2. Hyperthermia characteristics of exchange coupled magnetic nanoparticles

(A) Magnetic field dependence of SAR for FePt hard MNPs, Fe₃O₄ soft MNPs, 1:1 mixed FePt and Fe₃O₄ MNPs before ligand exchange, 1:1 mixed FePt and Fe₃O₄ MNPs after ligand exchange and core/shell FePt@Fe₃O₄ MNPs. (B) Calculated FePt and Fe₃O₄ MNP diameter dependence of SAR (field amplitude: 5000 Am⁻¹; frequency: 366 kHz). The σ corresponds to the particle distribution parameter in the lognormal distribution used in the calculation to simulate the effect of polydispersity of the sample. The arrows in (B) indicate that σ values increase from 0 to 40% in a 10% increment.

Figure 3. Fourier transform infrared spectroscopy of (A) as-made FePt@Fe₃O₄ MNPs, (B) PEGylated FePt@Fe₃O₄ MNPs

Figure 4. Thermogravimetric analysis of the PEG-MNPs

Figure 5. Dynamic light scattering of the freshly prepared PEG-MNPs in PBS at 25°C (size distribution in number)

Hydrodynamic diameter: 25.9 ± 2.8 nm.

Figure 6.

(A) Effect of the PEG-MNPs on the proliferation of murine breast cancer 4T1.2-Neu cells.

After 72-h incubation, the relative number of the viable cells was determined by a resazurin assay. $IC_{50} = 0.487 \pm 0.073$ mg/ml; (B) Intracellular uptake of the PEG-MNPs by 4T1.2-

Neu cells after 24-h incubation at the IC_{50} concentration. (Iron analysis by ICP-MS:

detection limits = $0.00379 \mu\text{g/ml}$; $R^2 = 0.9982$).

PEG-MNPs: Polyethylene glycol-magnetic nanoparticles.

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Figure 7. Balb/c mice were intravenously injected with 100, 50 and 20 mg/kg of PEG-MNPs via the tail vein

Figure 8.

After intravenous administration, PEG-MNPs were (A) cleared from the systemic circulation, and (B) accumulated in the 4T1.2-Neu breast tumors. $n = 3$ per time point. (Platinum analysis by ICP-MS: detection limits = 10.01 ppt; $r^2 = 0.9998$).

PEG-MNP: Polyethylene glycol-magnetic nanoparticle.

Figure 9. Tumor volume (V/V_{initial}) after treatment, 3 n 5

PEG-MNPs were injected intravenously once tumors reached approximately 150 mm^3 , and an inductive magnetic field of 400 kHz at 25.6 kAm^{-1} was applied for 5 min in the following 2 days. Suppression of tumor growth was only observed in the mice treated with PEG-MNPs hyperthermia (*p < 0.05, **p < 0.01 and ***p < 0.001). Inset shows a mouse with a 150 mm^3 -sized breast tumor (red circle) in a water-cooled coil receiving hyperthermia treatment.

Figure 10. Representative axial T_2 Maps of Balb/c mice with 4T1.2-Neu breast tumors (circled) without PEG-MNPs injection, and at 24 and 48 h after PEG-MNPs injection (iv.) Signal (T_2) decrease was observed in the tumor region at 24 h and 48 h post injection.

Table 1

Dynamic light scattering measurements of PEG-MNPs dispersion stored in physiological media for 1 week (size of freshly prepared polyethylene glycol-magnetic nanoparticles: 25.9 ± 2.8 nm).

	2–5°C, nm	r.t., nm
PBS	28.3 ± 3.2	31.2 ± 3.3
DMEM	30.3 ± 3.0	33.6 ± 3.2
10% FBS	29.9 ± 3.3	31.3 ± 3.2

DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal bovine serum; PBS: Phosphate buffered saline.

Table 2

Pharmacokinetics of 2.5-mg intravenous PEG-MNPs in Balb/c mice with 4T1.2-Neu breast tumors (average $\pm$ SD; n = 3).

Parameters (unit)	PEG-MNPs iv.
V_d (ml)	0.768 ± 0.489
C_p^0 (mg ml ⁻¹)	131.609 ± 51.074
$AUC_{0-24\text{ h}}$ (mg h ml ⁻¹)	154.467 ± 31.428
Cl (ml h ⁻¹)	0.017 ± 0.003
k (h ⁻¹) (β)	0.033 ± 0.026

Table 3

Tumor tissue area under the curve (average $\pm$ SD) and $C_{\max}$ (average $\pm$ SD; n = 3) of 2.5-mg intravenous PEG-MNPs.

Parameters (unit)	PEG-MNPs iv.
AUC _{0-96 h} (mg h ml ⁻¹)	58.870 $\pm$ 12.953
C _{max} (μ g mg ⁻¹)	0.932 $\pm$ 0.279

iv.: Intravenous; PEG-MNPs: Polyethylene glycol-magnetic nanoparticle.

Table 4

T_2 values of the breast tumor regions.

Group	Average T_2 (ms)	Standard deviation (ms)
Untreated	37.07	13.97
24 h post injection	20.77	0.34
48 h post injection	26.65	1.28
48 h post injection with hyperthermia	31.21	2.61