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# Engineered magnetic hybrid nanoparticles with enhanced relaxivity for tumor imaging

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ABSTRACT

Clinically used contrast agents for magnetic resonance imaging (MRI) suffer by the lack of specificity; short circulation time; and insufficient relaxivity. Here, a one-step combinatorial approach is described for the synthesis of magnetic lipid–polymer (hybrid) nanoparticles (MHNPs) encapsulating 5 nm ultra-small super-paramagnetic iron oxide particles (USPIOs) and decorated with $Gd^{3+}$ ions. The MHNPs comprise a hydrophobic poly(lactic acid-co-glycolic acid) (PLGA) core, containing up to ~5% USPIOs (*w*/*w*), stabilized by lipid and polyethylene glycol (PEG). $Gd^{3+}$ ions are directly chelated to the external lipid monolayer. Three different nanoparticle configurations are presented including $Gd^{3+}$ chelates only (Gd-MHNPs); USPIOs only (Fe-MHNPs); and the combination thereof (MHNPs). All three MHNPs exhibit a hydrodynamic diameter of about 150 nm. The Gd-MHNPs present a longitudinal relaxivity ($r_1 = 12.95 \pm 0.53$ (mM s)$^{-1}$) about four times larger than conventional Gd-based contrast agents ($r_1 = 3.4$ (mM s)$^{-1}$); MHNPs have a transversal relaxivity of $r_2 = 164.07 \pm 7.0$ (mM s)$^{-1}$, which is three to four times larger than most conventional systems ($r_2 \sim 50$ (mM s)$^{-1}$). In melanoma bearing mice, elemental analysis for Gd shows about 3% of the injected MHNPs accumulating in the tumor and 2% still circulating in the blood, at 24 h post-injection. In a clinical 3T MRI scanner, MHNPs provide significant contrast confirming the observed tumor deposition. This approach can also accommodate the co-loading of hydrophobic therapeutic compounds in the MHNP core, paving the way for theranostic systems.



## 1. Introduction

Nanoparticles have been proposed for the intravascular administration of imaging and therapeutic agents. As compared to freely administered molecules, nanoparticle can provide multiple functionalities simultaneously, such as imaging, therapy, sensing and targeting [1–3]. In this regard, the design and synthesis of magnetic nanomaterials have attracted the interest of numerous investigators for improving the performance of contrast agents in magnetic resonance imaging (MRI) [4–8], in vitro cell separation and manipulation [9]; controlled and triggered release of therapeutic agents [10,11]; and thermal ablation-based therapies via alternating magnetic fields [12,13]. Super-paramagnetic iron oxide nanoparticles (SPIOs) and gadolinium chelates (Gd-chelates) are the most clinically successful and safe contrast agents for $T_1$- and $T_2$-weigthed MR imaging, respectively [4,6,14–22]. Sufficiently small SPIOs are decomposed by the acidic endo/lisosomal environment and the resulting iron is assimilated by the body for the synthesis of metalloproteins such as hemoglobin [17]; whereas Gd-chelates firmly sequester the metal ions ($Gd^{3+}$) limiting trans-metallation and the consequent possible occurrence of nephrogenic systemic fibrosis [23]. The major limitations of the currently clinically used contrast agents for MR imaging reside in the lack of tissue specificity, inevitably affecting the signal-to-noise ratio; the short circulation time, due to their rapid excretion thought the kidneys and non-specific sequestration by reticulo-endothelial system (RES); and the insufficient relaxivity.

Systemically injected SPIOs are easily recognized and sequestered by macrophages residing within the RES organs, primarily the liver and spleen, so that their circulation half-life is limited to a few minutes and the portion of the injected dose reaching the biological

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target may be insufficient to induce any significant contrast enhancement. A quite successful and well-known technology to minimize RES uptake consists in coating SPIOs with hydrophilic polymers, such as polyethylene glycol (PEG) and dextran [24–27]. For instance Feridex® ($r_2 = 120$ (mM s)$^{-1}$) and Supravist® ($r_2 = 57$ (mM s)$^{-1}$) have their magnetic cores coated with hydrophilic polymeric chains. This has shown to prolong the half-life in the circulation up to ~2 h [28–30], but it also may modulate the relaxation performance [31]. Different nanoparticle formulations engulfing multiple SPIOs have been recently developed in the attempt of extending the circulation half-life and, possibly, enhancing the relaxivity. For instance, 40 nm PEG coated iron oxide nanoparticles induced, at 24 post-injection, a 30% enhancement in contrast as compared to immediately post-injection [32]. This confirms that long circulating and sufficiently small nanoparticles can take advantage of the hyperpermeability of the tumor vessels and accumulate progressively therein [33]. Clusters of SPIOs self-assembled with block copolymers of poly(ethyleneimine) (PEI), poly(caprolactone) (PCL) and PEG have shown in vitro relaxivities up to 300 (mM s)$^{-1}$ at 1.41 T, for complexes of about 80 nm in diameter [6]. Similarly, magnetic micelles with high transversal relaxivity and long circulation half-life were demonstrated by assembling together SPIOs, molecules of paclitaxel and PEG for a total average size of ~180 nm [7].

In this work, we demonstrate the synthesis of lipid coated poly(lactic acid-co-glycolic acid) (PLGA) nanoparticles encapsulating hydrophobic ultra-small SPIOs (USPIOs; with a magnetic core of 5 nm) and directly conjugated to Gd-DOTA (Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) through its superficial lipid coating. The resulting magnetic lipid–polymer (hybrid) nanoparticles (MHNPs) are characterized for their physico-chemical properties and stability under physiological conditions. Three different nanoparticles are synthesized including Gd-DOTA only (Gd-MHNPs); USPIOs only (Fe-MHNPs); and the combination thereof (MHNPs). The loading efficiency and in vitro MRI relaxometric properties are quantified for all three different systems. In mice developing a melanoma tumor on their flank, the MHNPs have been systemically injected and their specific organ accumulation has been measured via inductively coupled plasma mass spectroscopy (ICP-MS) on the element Gd. A clinical 3T MRI scanner has been used for imaging the malignant mass.

## 2. Materials and methods

### 2.1. Materials

DSPE-PEG-COOH (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy-(polyethyleneglycol)-2000), egg PC (L-α-phosphatidylcholine, egg, chicken), DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine), (L-α-phosphatidyl ethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt), PE-NBD) were purchase from Avanti Polar Lipid Inc. and used as received. PLGA (50:50) was purchased from Lactate Absorbable Polymers – DURECT Corporation. N-Hydroxysuccimidyl ester activated 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA-NHS) was purchased from MACROCYCLICS. All other chemicals and solvents were purchased from Sigma–Aldrich and used as received.

### 2.2. Synthesis of Gd–lipid

Gd–lipid was synthesized as illustrated in Supporting information Fig. S1 in two step preparation. In the first step, DSPE was conjugated with DOTA-NHS to form Lipid-DOTA which when reacted with gadolinium nitrate yield Gd–lipid. Briefly, 100 mg (0.13 mmol) of DSPE was hydrated with PBS for 5 h and 100.3 mg (0.2 mmol) of solution of DOTA-NHS in PBS was added and the reaction was carried for 6 h. After the completion of the reaction, the reaction mixture was extensively dialyzed ($M_w$ cutoff = 3.0k) with water to remove excess of DOTA and any water soluble byproducts (Yield 70% by weight). Thus formed lipid-DOTA was further treated with an excess of gadolinium nitrate dissolved in acetate buffer (pH 6.0) for 3 days at 50 °C. Resulting Gd–lipid complex was purified by extensive dialysis against water and was freeze dried to get dry powder. Sample was analyzed by ICP-OES to determine the reaction yield (75% by weight).

### 2.3. Synthesis and characterization of lipid–polymer–inorganic hybrid nanoparticles (MHNPs)

MHNPs with polymeric cores consisting of the 5 nm sized hydrophobic USPIOs and Gd–lipid shell were prepared through a single step nanoprecipitation method. In a typical experiment, 200 μg of Gd–lipid and 260 μg DSPE-PEG-COOH were dissolved in 4% ethanol at 68 °C, to this solution, PLGA (1 mg, $M_n$ ~ 50 kDa) and a calculated amount of USPIOs dissolved in acetonitrile were added drop wise while heating and stirring. Then the vial was vortexes for 3 min followed by the addition of water (1 mL). The solution mixture was stirred at room temperature for 2 h, washed using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) with a molecular-weight cutoff of 10 kDa. Finally, the purified MHNPs were collected in 1 mL water or phosphate buffer saline and stored at 4 °C. MHNP-Gd, MHNP-Fe and control particles were prepared similarly by replacing necessary constituents with bare PLGA, Egg-PC and PLGA/Egg-PC (for control NPs, Gd–lipid was replaced by Egg-PC). The nanoparticle size and surface zeta potential were obtained from nine measurements ($n = 9$) by dynamic light scattering (DLS) (Malvern Zetasizer, ZEN 3600) with a backscattering angle of 173°. The morphology of the particles was characterized by scanning electron microscopy (SEM) (ZEISS NEON 40). Samples for SEM were prepared by dropping nanoparticle suspension (5 μL) onto a polished silicon wafer. After drying the droplet at room temperature overnight, the sample was coated with platinum and then imaged by SEM. Further to understand the internal structure and the distribution of USPIOs into the polymeric core, the TEM measurements were performed. Samples for TEM were prepared by drop casting method over cupper grid using phosphotungustic acid as a negative stain. The quantitative amount of USPIOs per particle was determined by ICP-OES analysis. Time dependent stability of MHNPs was carried for the period of one by measuring the size and PDI using DLS.

### 2.4. In vitro cytotoxicity and cellular internalization studies

First, cells were seeded at $8 \times 10^3$ per well in 96-well plates and incubated for 24 h to reach 50% confluence. Then the culture media were replaced with 150 mL of fresh media and cells were incubated with different concentrations of Fe in MHNPs, cells with out MHNP treatments were taken as a control. After 24 h of incubation, the cells were washed with PBS and incubated in fresh media for 48 h. Cellular viability was then determined using the XTT assay following a protocol provided by the manufacturer. Results were presented in relative viability with respect of control cells. For cellular internalization study, PE-NBD labeled MHNPs were incubated with J-774 cells for 1 h. After 1 h of incubation, cells were washed with PBS three times, nucleus was stained with DAPI and fixed with 4% formalin. Fixed cells were imaged under confocal microscopy.

### 2.5. Relaxometric analysis

In vitro relaxation times were measured in a Bruker Minispec (mq 60) bench-top relaxometer ($B_0 = 1.41$ T) operating at 60 MHz and 37 °C. The longitudinal ($T_1$) relaxation times were obtained using inversion recovery pulse sequence. The transverse ($T_2$) relaxation times were measured using Carr–Purcell–Meiboom–Gill (CPMG) sequence. In vitro $T_2$-weighted MR phantom studies were performed in a clinical 3T scanner (Philisp Ingenia®) using turbo spin echo (TSE) sequence with TR = 2500 ms, TE = 100 ms and a slice thickness of 400 μm. Phosphate buffer saline (PBS) suspension of MHNPs was used for phantom study.

### 2.6. In vivo studies

*In vivo* studies were carried out in accordance with IACUC approved protocol. A male nu/nu mouse (8–10 weeks old) was used. Before the imaging procedure and contrast agent administration, mice were anesthetized with isoflurane and kept under its influence for the duration of the experiment (maximum 4 h) and euthanized by $CO_2$ overdose and cervical dislocation at the end of the imaging session. The contrast agents used were injected intravenously through the tail vein using 0.5 mL insulin syringes. Mice were kept on a 12 h light–dark cycle with food and water ad libitum. All animal experiments in this study were approved by the Institutional Animal Care & Use Committee (IACUC) of The Methodist Hospital Research Institute.

### 2.7. Cell line and tumor model

B16–F10 cells (from ATCC, Rockville, MD, USA) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, and maintained at 37 °C in a 5% $CO_2$ incubator. All the cell culture products were purchased from Invitrogen (Carlsbad, CA, USA). For the tumor model, $10^6$ B16–F10 cells in 200 μL PBS were injected subcutaneously into the flank of 12-weeks old male Nude mice (Nu/Nu) purchased from Charles River (Wilmington, MA, USA). Mice were kept on a 12 h light–dark cycle with food and water ad libitum. All animal experiments in this study were approved by the Institutional Animal Care & Use Committee (IACUC) of The Methodist Hospital Research Institute.