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Phospholipid micelle encapsulated gadolinium oxide nanoparticles for imaging and gene delivery

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Abstract

We encapsulated gadolinium oxide (Gd₂O₃) nanoparticles within phospholipid micelles as a novel low cytotoxic T₁-weighted MRI imaging contrast agent (MGdNPs) that can also deliver small molecules such as DNA plasmids. MGdNPs show relatively good MRI relaxivity values, negligible cytotoxicity, excellent cellular uptake and expression of DNA plasmids *in vivo*. Biodistribution studies in mice show that intranasal and intraperitoneal administration of MGdNPs can effectively target specific organs.

Introduction

High-contrast, high-resolution imaging of tumor cells is of great importance in the early diagnosis of cancer and for monitoring progression and treatment of the disease.¹ Currently, the most preferred techniques for clinical tumor imaging are X-ray, computed tomography (CT) and magnetic resonance imaging (MRI). MRI is the most common technique due to its low cost, minimal radiation exposure, and its ability to provide extensive anatomical information.¹ At present, two main groups of contrast agents are used in clinical MRI applications. T₂-weighted imaging uses iron oxide nanoparticles as negative contrast agents that cause shortening of the transverse relaxation time of water protons. In contrast, T₁-weighted imaging agents using gadolinium (Gd)-based chelates or nanoparticles act as positive contrast agents by shortening longitudinal relaxation times.^{2,3} T₂-weighted tumor imaging agents give relatively poor contrast because the iron oxide particles are mainly restricted to intravascular compartments.^{4,5} Furthermore, a greater amplification of the signal is necessary at the low concentrations of T₂-weighted contrast agents used for MRI imaging.^{2,3} Superparamagnetic iron oxides (SPIOs) are excreted rapidly from the circulatory system and usually accumulate in the liver or spleen.⁶ Overcoming these difficulties with iron oxide imaging agents can require a several orders of magnitude higher concentration of contrast agent in the tumor cells than usually required.³

T₁-weighted contrast agents are an excellent alternative to T₂ agents for overcoming the problems of low resolution. Gd³⁺ ions in Gd-chelates have seven unpaired electrons, which makes them a good contrast agent but due to their low molecular weight, they are removed rapidly by the kidneys.^{7–9} Also, Gd-chelates are limited in their capacity to be functionalized with various biological molecules for gene delivery or imaging applications and the Gd³⁺ ions are susceptible to leaching leading to cytotoxicity.^{6,10,11} By using Gd₂O₃ nano-particles, a higher spectral sensitivity can be attained because the higher concentration of Gd³⁺ ions produces a longitudinal relaxivity (r₁) of water protons (9–10 s^{−1}mM^{−1}) twice that of Gd-chelates (4–5 s^{−1}mM^{−1}).¹² Also, these nanoparticles can be conjugated with biomolecules like plasmid DNA and peptides, and modified with targeting ligands to produce an effective delivery system.¹⁰ Currently, the biocompatibility of gadolinium-based T₁ contrast agents has been called into question because of the potential for toxicity due to leaching from inadequately coated Gd₂O₃ nanoparticles. T₁-weighted Gd-chelates and Gd₂O₃ nanoparticles for contrast imaging have utilized dendrimers, liposomes, silica and micelle coatings to encapsulate gadolinium ions.^{7,10,13–19} For example, phospholipid micelles modified with polyethylene glycol (PEG) have been previously used safely for coating iron oxide nanoparticles and quantum dots (CdSe/ZnS). Phospholipid micelles have been shown to sequester their cargo for long periods of time without cytotoxic effects.^{20,21} In a different study, these micelles were used as a core for attaching Gd-chelates on the exterior and also for encapsulating Gd-chelates.^{22,23} However, introduction of exogenous and endogenous Gd-chelates drastically changed the biocompatibility and biodegradability of the particles resulting in higher mortality and adverse side-effects in *in vivo* experiments, presumably due to weak binding of Gd³⁺ ions to the chelate structure and leaching of ions.^{1,10,24}

In order to improve biocompatibility and resolution, we have developed an alternative strategy for producing a T₁ contrast agent by encapsulating Gd₂O₃ nanoparticles (average diameter 22 nm) inside phospholipid micelles. In Gd₂O₃ nanoparticles, Gd³⁺ ions are more tightly bound in a lattice compared with Gd-chelates. Further, micelles have a hydrophobic interior which will result in decreased mobility and leaching of Gd³⁺ ions from the core thereby minimizing the diffusion rate to a negligible, nontoxic level. However, the particles still function as an MRI contrast agent. Our results demonstrate that these micelle-coated nanoparticles (MGdNPs) exhibit excellent relaxivity, are not cytotoxic and are efficiently taken up by human embryonic kidney 293 cells (HEK293). HEK293 cells are a particular cell line primarily obtained from human embryonic kidney cells. These cells are easily grown and transfected and have commonly been used for *in vivo* transfection and toxicity studies. MGdNPs were conjugated with R9 peptide, a positively charged cell penetrating peptide consisting of 9 arginine amino acids which facilitate cellular uptake of the loaded cargo. MGdNP-R9 was further coated with a fluorescent reporter plasmid (tDT) for *in vivo* gene delivery and expression in HEK293 cells. *In vivo* biodistribution studies in rats treated with fluorescently-tagged Cy5-MGdNPs *via* the intranasal (i.n.) route show nanoparticle accumulation in the lungs (i.n.) while intraperitoneal administration (i.p.) results in a wider spread involving kidney, liver, spleen and brain.

Experimental section

The following includes the experimental methods used in preparing and characterizing micelles containing Gd₂O₃ NPs and conjugating them with peptides and DNA and the procedures used in *in vivo* and *in vivo* experiments.

The polyol method was utilized to synthesize 22 nm GdNPs.^{7,25} In brief, 2 mmol of gadolinium nitrate hexahydrate (Gd(NO₃)₃·6H₂O) and 6 mmol of sodium hydroxide (NaOH) were dissolved separately in 5 mL of diethylene glycol (DEG). The two solutions

were mixed and heated under nitrogen at 140 °C for 1 h followed by heating at 180 °C for 4 h. Then the temperature was reduced to 140 °C and 254 μL of oleic acid was added to the solution. The mixture was stirred until it formed a viscous, brown solution and 30 mL of methanol was added to precipitate nanoparticles from excess reagents. The precipitate was pelleted by centrifugation at 5000 rpm ($4696\times g$) for 15 min at room temperature. The resulting pellet was washed several times with an excess of methanol and suspended in chloroform. FTIR characterization of GdNPs was performed using a Nicolet IR-100 spectrometer. An aliquot of the chloroform suspension of GdNPs (100 μL of 0.16 mM Gd^{3+} conc.) was pipetted onto a disposable polyethylene IR card and the solution was dried under vacuum before taking the measurements. Micelle encapsulation of GdNPs (MGdNPs) was carried out as described by Dubertret *et al.*²¹ Typically, GdNPs (1 mg) were mixed with 3 mg of amino-PEG-PE (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-poly(ethylene glycol)]), suspended in 1 mL of chloroform and the solvent was evaporated in a vacuum oven for 1 h at room temperature. The pellet obtained after evaporation was heated to 80 °C and dissolved in 1 mL of nanopure water (18 m Ω) to produce 100% amine-functionalized nanoparticles from the water-soluble amino-micelle-coated NPs. To remove the empty micelles the aqueous solution of MGdNPs was centrifuged at 90 000 rpm for 2 h at 4 °C. The supernatant was removed and the pellet obtained was suspended in 1 mL of nanopure water, sonicated (Branson 2510 sonicator) for 20 min and filtered using a 0.2 μm syringe filter. The concentration of Gd^{3+} ions encapsulated in the micelle was determined by inductively-coupled plasma mass spectrometry (ICP-MS) using a Perkin Elmer Elan II DRC instrument. The samples were prepared by diluting 10 μL of stock MGdNP solution (1 mg mL^{-1}) in 10 mL of nanopure water (18 m Ω) containing 2% nitric acid. Transmission electron micrographs (TEM) of MGdNPs were taken by spreading 10 μL of aqueous master solution (1 mg mL^{-1}) on a carbon-coated copper grid. The excess solution was removed with filter paper after ten minutes followed by air drying for 1 h. The sample was visualized with a transmission electron microscope equipped with a digital camera (JEOL 1400) at 80 kV. Dynamic light scattering (DLS) and zeta potential of MGdNPs, MGdNP-R9 and MGdNP-R9-DNA in aqueous solution was performed on a Zetatracer (Microtrac Instruments). To prepare DLS samples, the aqueous master solution (1 mg mL^{-1}) was diluted five-fold and sonicated for 1 h to avoid aggregation. The solution was subsequently filtered using a 0.22 μm syringe filter before taking the measurements.

Various dilutions of MGdNPs were prepared with cell medium (cDMEM). 200 μL aliquots of various micelle solutions were added to a 96 well plate. MR images were obtained using an Agilent ASR 310 7 Tesla MRI high-field scanner. Measurement of T_1 and T_2 values was carried out using Multi-echo longitudinal and transverse relaxation experiments (MEMS) in imaging mode. Nonlinear least square fitting was performed in MATLAB (Mathworks, Inc) on a pixel-by-pixel basis. The images were recorded with Vnmrj 3.0.

For *in vivo* cell uptake and *in vivo* biodistribution experiments MGdNPs were further labeled with NHS-Cy5 using coupling chemistry between amine and NHS groups.²⁹ Typically, 50 μL of MGdNPs (0.16 mM Gd^{3+} conc.) was mixed with 2 μL and 4 μL of NHS-Cy5 (1 mg/200 μL) respectively in two separate vials, and incubated for 1 h. The excess dye was removed *via* dialysis in nanopure water (18 m Ω) using a 7 kDa MWCO cellulose membrane. Water was changed 2–3 times over a period of 24 h to ensure complete removal of unbound dye. MGdNPs were also conjugated to R9 (1 : 5 molar ratio of amine groups on micelle to peptide) *via* the EDC/NHS protocol.³⁰ 5 μL of NHS (10 $\mu\text{g mL}^{-1}$) and 3 μL of EDC (10 $\mu\text{g mL}^{-1}$) were added to 60 μL of R9 peptide (5 $\mu\text{g mL}^{-1}$) in nanopure water (18 m Ω) for 15 min. To this solution, 50 μL of MGdNPs (0.16 mM Gd^{3+} conc.) in PBS (pH 7.2) was added and the mixture was allowed to stand for 2 h at room temperature. The excess reagents (R9, EDC, NHS) were removed by dialyzing the sample overnight in nanopure water (18 m Ω) at 4 °C using a 7 kDa MWCO cellulose membrane.

HEK293 cells were incubated with the Cy5 labeled MGdNPs containing different concentrations of Gd^{3+} for 1 h, washed with sterile PBS three times and viewed under a confocal microscope. Images were taken using appropriate filters. The fluorescence intensity of Cy5 was measured as integrated density using ImageJ software. Photomicrographs captured at 200 $\times$ magnification with an Olympus DP70 camera were used for quantitation. Images were taken at the same exposure and digital gain settings for a given magnification to minimize differential background intensity or false-positivity. The channels of the RGB images were converted to gray-scale and adjusted for brightness and contrast to exclude noise pixels. The images were also adjusted for the threshold to highlight all the positive cells to be counted and a binary version of the image was created with pixel intensities between 0 and 255. Integrated density (IntDen) was calculated and background correction was performed for each image. The percentage changes from the control of the corrected integrated density of at least 3 fields for each experimental condition including control were averaged to represent the IntDen of Cy5 fluorescence and expressed as mean % uptake efficiency $\pm$ S.E.M.

The uptake of Cy5-MGdNPs by HEK293 cells was also visualized using TEM of treated fixed cells. One million HEK293 cells were plated in a petri dish (35 mm) with cDMEM. Next day, the cells were treated with 20 μ L of MGdNPs ($2 \mu g \mu L^{-1}$) for 1 h, detached with 1 mL of Accutase for 2 min and the cell pellet was collected by centrifugation at 1200 rpm for 5 min. Cells were then fixed with 1 mL of 2.5% glutaraldehyde overnight at 4 $^{\circ}C$. Next day, the sample block was warmed up to room temperature, treated with 0.1 M cacodylate buffer (pH 7.2), and washed three times for 5 min each. The cell block was cut into two equal parts, one stained and one unstained. Next, one of the cut cell blocks was post-fixed with 1% osmium tetroxide at 4 $^{\circ}C$ for 1 h. The osmium tetraoxide solution was replaced with 1 mL of 0.1 M cacodylate buffer and after thorough mixing, the cell block was dehydrated in a graded series of 10%, 35%, 50%, 70% and 95% ethanol for 5 min each at room temperature and suspended and washed in 100% acetone three times for 10 min each. Acetone was replaced by 500 μ L of a 1 : 1 mix of acetone : embedding medium (EMBED812 resin) and gradually transferred to 100% resin. Samples were left overnight at 4 $^{\circ}C$ to ensure proper resin infiltration. Next day, after warming up the sample to room temperature, the resin was exchanged by 500 μ L of fresh resin to remove traces of acetone. The sample was heated in the oven at 40 $^{\circ}C$ for 30 min and then at 70 $^{\circ}C$ overnight. The cell block was sectioned with an ultra-microtome producing sections of 110 nm thickness and sections were deposited on a 300 mesh copper grid. Grids were examined using a JEOL 1400 TEM at 80 kV and images were collected with an Orius Gatan (SC 1000) wide angle format camera. Quantification of cellular uptake of Cy5-MGdNPs was performed using a 3i Olympus spinning-disk confocal microscope over a period of 30 min MGdNPs incubation and the images were taken with a Photometrics CoolSNAP HQ CCD camera (Tuscan, AR). The uptake efficiency of Cy5-MGdNP by HEK293 cells was calculated manually by counting around 1000 cells from 100 $\times$ images and plotted as a function of Gd^{3+} ion concentration.

MGdNP-R9 was further conjugated with a reporter plasmid (Td Tomato plasmid, tDT) that encodes a red-fluorescent protein under the control of a CMV promoter. The MGdNP-R9 ($2 \mu g \mu L^{-1}$) and tDT ($1 \mu g \mu L^{-1}$) solutions were prepared separately in Dulbecco's-modified Eagle's medium supplemented with 5% FBS and 1% penicillin/streptomycin (cDMEM). The DNA solution was then added slowly to the MGdNP-R9 solution and vortexed for 30 min. Fig. 1 schematically represents the synthesis of MGdNPs from GdNPs and its conjugation to Cy5 or DNA. HEK293 cells were plated in a 96-well plate at a density of 5000 cells/well and maintained overnight in cDMEM at 37 $^{\circ}C$ in an incubator supplied with 5% CO₂. 24 h after plating, aliquots of MGdNP-R9 containing 200 ng of plasmid expressing the red-fluorescent protein 'Tomato' (tDT) was added to each well of a 96-well cell culture plate with a final volume of 80 μ L. HEK293 cells were transfected with MGdNP-R9-DNA

or MGdNP-R9 (control). Red fluorescent protein (RFP) expression was observed using an Olympus IX71 inverted fluorescence microscope at 24 h, 48 h, and 72 h after transfection. 50 μ L of fresh media was added to the wells every 24 h as nutritional supplementation for the cells. Fluorescent and bright-field images were taken using Olympus DP70 camera attached to the microscope. Transfection efficiency was calculated using ImageJ software (NIH). The red channels of the 200 $\times$ RGB images were adjusted for brightness and contrast and the threshold was adjusted to select all RFP-positive fluorescent cells. The number of RFP-positive cells was then counted using the 'analyze particle' function. The number of cells in the corresponding 200 $\times$ gray-scale images was also counted in the same method. The analysis parameters were kept same to avoid any bias. Transfection efficiency was expressed as the number of RFP-positive cells as a percent of the total number of cells and expressed as mean $\pm$ S.E.M.

Viability of MGdNPs treated HEK293 cells was determined using the WST assay (Roche Laboratory). HEK293 cells were plated at a density of 5000 cells/well in a 96-well cell culture plate. MGdNPs containing different concentrations of Gd^{3+} were added to the wells in triplicate. No MGdNPs were added to the control wells. One hour after the treatment, 10 μ L of WST (water-soluble tetrazolium salt) reagent was added to the cells. Absorbance readings at 450 nm and 630 nm (reference) were taken at 6, 10, 24 and 48 h after MGdNPs treatment using a Synergy H4 plate reader (Biotek). The absorbance readings at 630 nm (control) were subtracted from 450 nm and the average of subtracted triplicate measurements were plotted *versus* time for various Gd^{3+} ion concentrations.

All animal procedures in the *in vivo* experiments were conducted according to the NIH guidelines for the care and use of laboratory animals following a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Male SD rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were housed in a climate-controlled room with 12 h day/night cycle. Water and laboratory chow was available *ad libitum*. Cy5-MGdNPs were administered to mice *via* i.n. and i.p. routes and visualized with the Xenogen fluorescence imager. The biodistribution of Cy5-MGdNPs was monitored following administration of Cy5-MGdNPs. 100 μ L of Cy5-MGdNPs was administered intranasally or intraperitoneally to 8-week-old C57/BL6 mice and three hours after administration, the mice were euthanized and organs were removed, weighed and scanned for Cy5 fluorescence using a Xenogen IVIS imager (Caliper 275 Life Sciences Inc, MA, USA). The intensity of Cy5 fluorescence in different organs is expressed as relative photon counts per mg of tissue compared to naive controls.

For *ex vivo* imaging of lung tissues in mice, male adult (C57/BL-6) mice were intranasally administered with 50 μ L of either MGdNPs containing 0.69 mM Gd^{3+} or Magnevist (positive control) diluted with PBS with a final concentration of Gd^{3+} same as in MGdNPs. The negative control group received 50 μ L of PBS. One hour following administration mice were euthanized by CO₂ asphyxiation and lungs were harvested and inserted in a medical cassette for MR imaging. The cassette was immersed in 50 mL Fluorinert (FC-43, 3 M Corp, Minneapolis) for reducing susceptibility artifacts and eliminating signal from protons that normally would be in the media. The samples were then carried to the Agilent ASR 310 MRI machine. Spin Echo Multi Slice (SEMS) T₁ weighted images of lung tissues were collected using a magnetic field of 7 tesla.

Results and discussions

To produce GdNPs, we used the polyol method and characterized the product by FTIR (Fig. 2A). The additional peaks observed in the GdNPs spectra are the antisymmetric methyl stretch, $\nu_{as}(CH_3)$ at 2915 cm^{-1} , methylene stretches $\nu(CH_2)$ at 2848 cm^{-1} and the rocking

vibration at 719 cm^{-1} $\rho_t(\text{CH}_2)$. The FTIR spectra of pure oleic acid exhibits a peak at 1711 cm^{-1} due to the $\nu(\text{C}=\text{O})$ stretch.²⁵ However, this peak is absent in the FTIR spectra of GdNPs. Also, the peak at 1463 cm^{-1} corresponds to $\nu(\text{COO}^-)$. This indicates that oleic acid is attached to the NPs via its COO^- group. The antisymmetric methyl stretch and methylene stretches demonstrates that the hydrocarbon chains in oleic acid are well-ordered.²⁵ In addition, the rocking vibration at 719 cm^{-1} $\rho_t(\text{CH}_2)$ shows crystalline packing of the alkyl chains of coated oleic acid.²⁶

We further characterized MGdNPs using transmission electron microscopy (TEM) and dynamic light scattering (DLS). Fig. 2B shows a representative TEM of MGdNPs. From the TEMs, the core diameter of MGdNPs was found to be $22 \pm 2\text{ nm}$ (average for 1500 particles). The TEM image exhibited a negligible contrast from the capped oleic acid layer and the particles were well-dispersed and not aggregated.²⁵ As shown in HRTEM inset of Fig. 2B, it clearly reveals the crystalline structure of GdNPs. DLS data (Fig. 2C, Table 1) showed both MGdNPs and MGdNP-R9 have an average hydrodynamic diameter of 27 nm and 28 nm respectively with a polydispersity index (PDI) of 0.01%. However, attachment of DNA dramatically increased the size to 47 nm, as expected. The DLS size distribution is identical to the instrumental response function corresponding to a monodispersed sample, indicating that aggregation is negligible. It is noteworthy that the hydrodynamic value is expected to be larger than the actual diameter because of the counter-ion cloud contributions to particle mobility.²⁷ The zeta potential value of MGdNPs is almost neutral due to the presence of native amine group that remains unaffected in nanopure water. On the other hand, zeta potential of MGdNP-R9 is positive because of the highly basic nature of R9 peptide. Upon attaching DNA to this MGdNP-R9, it imparts a negative charge. (Table 1)

To evaluate the MRI potential of these particles in cellular environments, longitudinal (T_1) and transverse (T_2) relaxation times were measured at various concentrations of MGdNPs in cell medium. Fig. 3A shows the T_1 and T_2 relaxivity maps as a function of Gd^{3+} ion concentration using 0.687 mM, 0.344 mM, 0.172 mM, 0.086 mM, 0.043 mM of Gd^{3+} ions and medium as control. The r_1 and r_2 values were then calculated as 7.39 and 72.58 $\text{s}^{-1}\text{mM}^{-1}$ from the slopes of the $1/T_1$ and $1/T_2$ versus Gd^{3+} ion concentration, respectively (Fig. 3B). The r_2/r_1 ratio was 9.82. T_1 and T_2 relaxivity maps show obvious concentration-dependent color intensity changes due to the increase in relaxivity of water protons as a function of increasing Gd^{3+} concentration. This is an indication of the high sensitivity of MGdNPs and demonstrates their effectiveness as a T_1 MRI contrast agent. Although the lipid layer of micelles will entrap Gd^{3+} ions and prevent their interaction with water, Veggel and Prosser have shown that a direct interaction of Gd^{3+} with water is not required to enhance the relaxation.²⁸ Commercially available Gd-chelates have r_1 values in range of 4–5 $\text{mM}^{-1}\text{s}^{-1}$ while Gd_2O_3 nanoparticles capped with silica and other capping ligands show r_1 values ~ 9 .^{7,10,12} Dubertret *et al.*²¹ showed the micelle encapsulation of quantum dots for cell imaging. These filled micelles were stable for a prolonged period. Based on the long term stability of the micelles containing Gadolinium nanoparticles, it is assumed that micelle encapsulation will prevent cytotoxicity arising from Gd^{3+} ions by reducing the mobility of these ions in the hydrophobic micelle interior. However, the smaller value of 7.39 (r_1) observed in this study is attributed to the strong and stable micelle layer coating the MGdNPs.²¹ The main advantage of using micelle coating for this contrast agent is to achieve an optimal balance between relaxivity and cytotoxicity.

TEM images of thin sections of fixed MGdNPs-treated HEK293 cells clearly demonstrate that cells can take up MGdNPs by endocytosis. Fig. 4 shows cells at different stages of uptake and release of particles into the cytoplasm. Within 1 h after transfection the MGdNPs were observed attaching to the cell membrane, undergoing endocytosis and finally escaping from the endosome into the cytoplasm (Fig. 4A, ad) where free particles were found (Fig.

4f). Nuclear transport is also observed in some cells after 4 h of incubation (Fig. 4B, e). The MGdNPs are then transported into the nucleus through the nuclear pores (Fig. 4B, e). Cells treated with Cy5-labeled MGdNPs also indicate the escape of MGdNPs from the endosomal vesicles into the cytoplasm as Cy5 fluorescence is observed in the cytoplasm only (Fig. 4C, b and c). No uptake of free Cy5 was observed for cells incubated with dye alone at dye concentrations similar to those used for labeling the MGdNPs. On average, 75–80% of the cells internalized Cy5-MGdNPs independent of the concentration of Gd^{3+} ions.

For DNA delivery experiments, MGdNPs were conjugated to R9 and the td ‘Tomato’ plasmid DNA (tDT) which expresses a red-fluorescent protein subsequently electrostatically adsorbed onto MGdNP-R9. HEK293 cells transfected with MGdNP-R9-DNA showed a maximum expression of red-fluorescent protein at 72 h (Fig. 5A, B) with more than 45% of cells expressed RFP as counted using ImageJ software (rsbweb.nih.gov/ij/) (Fig. 5B). Cells treated with empty micelle-R9-DNA conjugates, DNA alone or MGdNPs without DNA did not show any RFP expression. MGdNP-R9-DNA also demonstrated the potential of carrying DNA to the cells and delivering it to the nucleus for subsequent transcription and translation (Fig. 5A, B).

HEK293 cell viability 48 h following the uptake of MGdNPs containing different concentrations of Gd^{3+} ions was determined using the WST assay (Fig. 5C). Even at the highest concentration of Gd^{3+} ions in MGdNPs, the HEK293 cells were viable at 48 h after treatment. Viability of HEK293 cells at 48 h (Fig. 5C) after MGdNPs treatment could be due to the presence of the PEG and the hydrophobic lipid layer surrounding the nanoparticles that prevents the leaching of Gd^{3+} ions from the NP core of the micelle to the intracellular environment. It is worth noting that empty micelle-R9-DNA conjugates were toxic to the cells (data not shown). Empty conjugates are thought to be unstable compared to MGdNPs, which have a filled core and they tend to break in the cellular environment and release the lipid layer of the micelle causing the cytotoxicity.²¹ These results demonstrate that MGdNPs are easily internalized by cells and are nontoxic indicating the potential usefulness of these nanoparticles in designing a combination of MRI contrast and drug or DNA delivery agent.

These *in vivo* observations prompted us to test the efficacy of these particles in a living system. Intranasal administration did not result in systemic absorption or tissue accumulation of Cy5-MGdNPs except in the lung. In contrast, after intraperitoneal (i.p.) administration most of the Cy5 fluorescence was observed in the liver, kidney and spleen. Moreover, i.p. administration of the nanoparticles in a ratio of 5 : 1 (MGdNP : Cy5) showed higher tissue accumulation of NPs in all organs including brain compared to i.p. administration of the nanoparticles at a ratio of 10 : 1 (MGdNP : Cy5) (Fig. 6A, B).

To determine the MR imaging ability of MGdNPs, mice were administered intranasally with particles. Control mice were given PBS (negative), magnevist (positive, Gd-chelates). One hour following administration, lungs were imaged and results are illustrated in Fig. 7. No T_1 contrast was seen in PBS control mice whereas both magnevist or MGdNPs administered mice showed significant T_1 contrast (shown in the yellow inset of Fig. 7). These results demonstrate that the MGdNPs have the capability for MR imaging similar to magnevist.

Conclusions

In this report we show that MGdNPs synthesized by encapsulating GdNPs inside phospholipid micelles functionalized with amine groups have the potential to act as a T_1 -weighted contrast agent for cellular imaging and delivery of DNA without any apparent cytotoxicity. The cell penetrating ability of the particles is enhanced by conjugating them with cationic polyarginine peptides (R9) due to the amine functionality of micelles, while

keeping the size and functionality unaltered. The *in vivo* biodistribution studies show that intraperitoneal administration of MGdNPs results in an accumulation of particles in the liver, kidney and spleen, but only in the lung when they are given intranasally. This means that MGdNPs can be selectively targeted to the various organs to deliver drugs, DNA plasmids, peptides and MRI contrast agent for treating and monitoring tumor development. The particles are nontoxic and are taken up with high efficiency making them useful for both drug/gene delivery and MRI diagnostics.

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Fig. 1.

Schematic representation of the preparation of functionalized micelle-amine-coated gadolinium nanoparticles (MGdNPs). MGdNPs were conjugated with Cy5 or DNA for different experiments. The R9 polyarginine peptide was attached to MGdNPs to increase cell penetration power.

Fig. 2. Characterization of oleic acid-coated Gd_2O_3 . (A) FTIR spectrum of oleic acid-capped Gd_2O_3 showing the stretchings of different functional groups. (B) TEM image of MGdNPs (scale bar, 200 nm); inset showing the HRTEM image of the NP (scale bar, 10 nm). (C) Size distribution as determined by DLS of MGdNPs, MGdNP-R9, MGdNP-R9-DNA in a monodisperse sample. The average particle size is 27 nm.

Fig. 3. MRI characterization of MGdNPs. (A) Plot of r_1 and r_2 versus Gd^{3+} ion concentration of MGd NPs; T_1 (upper panel) and T_2 (lower panel) relaxivity maps as a function of Gd^{3+} ion concentration showing the concentration-dependent color intensity change; a, 0.687 mM, b, 0.344 mM, c, 0.172 mM, d, 0.086 mM, e, 0.043 mM and f, medium. (B) Graphical representation of the r_1 and r_2 values obtained from the slope.

Fig. 4.

Uptake of Cy5-MGdNPs by HEK293 cells. (A, B) Transmission electron microscope images showing uptake of MGdNPs by HEK293 cells. A, low magnification image of a cell showing the attachment of particles to the cytoplasmic membrane and different stages of endocytosis of MGdNPs, (a–d), magnified images of the boxed areas of (A). (a) attachment of the MGdNPs to the plasma membrane; (b, c) stages of endocytosis; (d) escape of MGdNPs from the endosome. (B) low-magnification image of two adjacent cells showing the transport of particles to the nucleus; (e) high-magnification image of the boxed area showing the particles in the nucleus close to nuclear pores; (f) free particles in the cytoplasm of a different cell. Arrows indicate the MGdNPs (scale bar for A, B, 1 μm; a–f, 200 nm); plasma membrane (pm) junction between adjacent cells (j), mitochondrion (m), nucleus (n), nuclear pore (np). (C) Cy5-MGdNPs are taken up by HEK293 cells. Confocal photomicrographs show Cy5 fluorescence inside the cells after 1 h of incubation. (a) bright-field; (b) fluorescence; (c) overlay images; white arrows indicate nuclei; scale bar, 200 μm. (D) Histograms showing the uptake efficiency of HEK293 cells for cy-5-MGdNPs at different concentrations of Gd³⁺ ions.

Fig. 5. (A) Photomicrographs of bright field (BF), fluorescence (FL) or merged images of transfected HEK293 cells showing red fluorescent protein expression 72 h after transfection with MGdNP-R9-DNA. MGdNP, transfection with only micelle-amine coated nanoparticles; MGdNP-R9-DNA, transfection with tDT plasmid-conjugated MGdNPs (scale bar-20 μm). (B) Transfection efficiency was quantitated using ImageJ software and expressed as % transfection efficiency (mean ± S.E.M) as described in the methods section. Highest transfection was observed 72 h after transfection. (C) WST viability assay shows that increasing concentration of Gd³⁺ ions in the MGdNPs does not affect the viability of HEK293 cells.

Fig. 6. Biodistribution of Cy5-MGdNPs in mice imaged using a Xenogen IVIS100 imager. (A) Cy5 fluorescence in different organs of mice given PBS (control) or Cy5-MGdNPs, 5 : 1 (100 uL) or 10 : 1 (100 uL) by i.p. (n = 2) or i.n. (n = 2). Three hours post-administration, fluorescence images of whole organs were acquired using the Xenogen IVIS imaging system. The spectrum gradient bar corresponds to the fluorescence intensity. PBS-treated mice (i.p. or i.n.) showed little or no fluorescence. (B) Histograms showing the relative photon counts per mg of different organs after different treatments.

Fig. 7.
Ex vivo MR imaging of lung tissue in mice with PBS control (negative), magnevist (positive) and MGdNPs. Yellow inset depicts the contrast enhancement by MGdNPs.

Table 1Size and zeta potentials (mean $\pm$ SD) of MGdNPs, MGdNP-R9 and MGdNP-R9-DNA

Sample	Size (nm)	Zeta potential (mv)
MGdNP	27 ± 1.5	1.5 ± 0.2
MGdNP-R9	28 ± 2.1	39.5 ± 0.8
MGdNP-R9-DNA	47 ± 3.5	-17.5 ± 1.1