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Manganese-loaded lipid-micellar theranostics for simultaneous drug and gene delivery to lungs

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Abstract

Gadolinium (Gd) contrast agents are predominantly used for T₁ MR imaging. However, the high toxicity of Gd³⁺ and potential side effects including nephrogenic systemic fibrosis have led to the search for alternative T₁ contrast agents. Since manganese (Mn) has paramagnetic properties with five unpaired electrons that permit high spin number, long electronic relaxation times, and labile water exchange, we evaluated Mn as a T₁ magnetic resonance imaging (MRI) contrast agent for lung imaging. Here we report on the design and synthesis of multifunctional lipid-micellar nanoparticles (LMNs) containing Mn oxide (M-LMNs) for MRI that can also be used for DNA and drug delivery. Oleic acid-coated MnO nanoparticles were encapsulated in micelles composed of polyethylene glycol (PEG-2000), phosphatidylethanolamine (PE), DC-cholesterol, and dioleoyl-phosphatidylethanolamine (DOPE). The particles are taken up *in vitro* by human embryonic kidney (HEK293), Lewis lung carcinoma (LLC1), and A549 cells and are devoid of cytotoxicity. When administered to mice intranasally, they preferentially accumulate in the lungs. *In vitro* phantom and *ex vivo* lung MRI results confirmed that M-LMNs are able to enhance T₁ MRI contrast. M-LMNs loaded with plasmid DNA and/or doxorubicin are efficiently taken up by HEK293 cells *in vitro* and by target cells *in vivo*. Taken together, these results demonstrate that M-LMNs are capable of simultaneously providing MRI contrast and DNA and/or drug delivery to target cells in the lung and therefore may prove useful as a lung theranostic, especially for lung cancers.

Keywords

Theranostics; Micelle; Manganese; Drug; Gene; Delivery

1. Introduction

Advances in nanoparticle technology have allowed the development of multifunctional nanoparticles for cancer detection, therapy, and treatment monitoring. Their numerous advantages include cell-targeted delivery to minimize the amount of drug needed to achieve a therapeutic dose [1], increased bioavailability especially for hydrophobic drugs, reduced drug toxicity [2], enhanced mucosal delivery that decreases first-pass metabolism [3],

controllable timing of drug delivery (slow-sustained, pulsatile or stimulus-responsive) [4–6], and the capacity to combine drugs and imaging agents in the same particle [7–9]. Scalability, safety, and cost remain the most formidable challenges in taking multifunctional nanoparticles from the bench to clinical trials.

Magnetic resonance imaging (MRI) is one of the most widely used noninvasive imaging and diagnostic techniques. It provides detailed anatomical images of the body and is excellent for imaging soft tissues. Contrast agents work by altering the T_1 , T_2 , or T_1/T_2 relaxation times of nearby protons. Positive contrast agents appear brighter on the MRI owing to an increase in T_1 signal intensity caused by a reduction in the T_1 relaxation times [10]. Superparamagnetic iron oxide nanoparticles have been extensively studied for use in T_2 contrast imaging in conjunction with a diverse array of nanotherapeutics [11–13]. Previously, we reported a unique formulation of chitosan–polyethyleneimine nanoparticles with iron oxide in the core for imaging together with a plasmid for gene delivery [14]. However, since iron oxide is a relatively poor T_2 -type MRI contrast agent for the lung [15], there is a need to develop nanoparticles containing T_1 contrast agents for better lung imaging that can also be used for drug delivery in lung diseases.

Currently, T_1 MRI utilizes predominantly gadolinium (Gd)-based contrast agents because of the large magnetic moment of Gd^{3+} due to its seven unpaired electrons and slow electronic relaxation time [16,17]. The high toxicity of Gd^{3+} , however, requires that these contrast agents always be given in a chelated form. Despite this, several cases of nephrogenic systemic fibrosis (NSF) have been reported in patients receiving Gd-containing contrast agents [18,19]. Hence, alternatives to Gd-containing T_1 contrast agents are needed.

Since the nonlanthanide metal manganese (Mn) is paramagnetic, has five unpaired electrons in its bivalent state, and is a natural cellular constituent as a cofactor for enzymes and receptors [16,17], we reasoned that Mn may serve as a T_1 MRI contrast agent for lung tissues. The intrinsic properties of Mn include high spin number, long electronic relaxation time, and labile water exchange, all of which make it an attractive contrast agent for MRI. Though, Mn-containing contrast agents are FDA approved for clinical use [16], Mn can be toxic at the high levels required to offset the short plasma half-life of ionic Mn [17,20]. We hypothesized that sequestration of Mn within nanoparticles will reduce the risk of toxicity and overcome the problem of short plasma half-life.

To test the hypothesis, we synthesized ‘core-shell’ cationic lipid micellar nanoparticles (LMNs) in which the core contains hydrophobic drugs or imaging contrast molecules and the shell is composed of phospholipids that can be used for loading DNA or peptide payloads and attaching targeting moieties (Fig. 1). The lipids, 3 β -[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE), are used to facilitate endosomal escape and augment gene/drug delivery [21–25], whereas the polyethylene glycol-phosphatidylethanolamine (PEG-2000-PE) confers biocompatibility and allows for longer blood circulation times [7,26]. The outer surface has a dense layer of PEG polymers that is poorly immunogenic and antigenic, and acts as an excellent repellent for biomolecules. We tested these cationic lipid nanoparticles as a T_1 contrast agent and DNA/drug delivery vehicle using *in vitro* MRI, cellular uptake, transfection, cytotoxicity studies, and *in vivo* experiments in mice. Our results show that these LMNs can be used as a multifunctional vehicle for imaging and treatment of cancer.

2. Experimental section

2.1. Materials

Manganese sulfate, sodium oleate, chloroform, hexane, 1-octadecane, dichloromethane, triethylamine, and acetone were all purchased from Sigma. PEG-2000 PE, DC-cholesterol, DOPE, and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl were all purchased from Avanti Polar Lipids. Cy5.5 NHS was purchased from Invitrogen. All reagents were used without further purification.

2.2. Synthesis of Mn–oleate complexes

Mn–oleate was prepared by the method described previously with some modifications [27]. Manganese sulfate (2 g) and sodium oleate (6.1 g) were dissolved in a combination of ethanol (7.5 ml), hexane (17 ml), and distilled water (10 ml). The solution was heated to 70 °C with vigorous stirring overnight. The solution was then transferred to a separatory funnel and the upper organic layer containing the Mn–oleate complex was washed several times using distilled water. The purified solution was allowed to evaporate, producing a deep red waxy solid that was the manganese–oleate complex.

2.3. Preparation of MnO nanoparticles

Hydrophobic MnO nanoparticles were prepared by the method described previously with some modifications [27]. Manganese–oleate (1.3 g) was dissolved in 1-octadecene (13.5 ml), and degassed at 70 °C for 1 h under vacuum with vigorous stirring. The solution was then purged with argon and heated to 300 °C while stirring under argon. As the temperature reached 300 °C, the initially red solution turned transparent and then to a pale green. The reaction was held at this temperature for 1 h and 15 min, then allowed to cool to room temperature after which dichloromethane (20 ml) was added to improve the dispersibility of the nanoparticles. Acetone (80 ml) was added to precipitate the nanoparticles and the solution was centrifuged at 3000 rpm (835 ×g) at 4 °C for 15 min. Supernatants were discarded and the pellets were reconstituted in 20 ml of dichloromethane. The above purification procedure was repeated two more times to remove excess surfactant and solvent. The purified MnO nanoparticles are dispersible in many organic solvents such as dichloromethane and chloroform.

2.4. Preparation of MnO nanoparticles encapsulated in micelles

MnO nanoparticles were encapsulated inside micelles using a published procedure with some modifications [8,28,29]. PEG-2000 PE (0.1 mg, 2% of total), DC-cholesterol (7.9 μM, 3.95 mg, 66% of total), and DOPE (2.6 μM, 1.95 mg, 32% of total) were added to 1.5 ml of chloroform. 3 mg (0.23 ml of stock solution) of MnO nanoparticles was then added to this solution. To ensure complete solubilization, the reaction solution was sonicated in a Branson 2510 sonicator for 20 min. The solution was then left to evaporate overnight in a vacuum oven at 40 °C. The dry film was heated at 80 °C for 2 min. Then 2 ml of water was added and the solution was again sonicated for 3 h. After the film was dissolved, the solution was centrifuged at 90,000 rpm (334,000 ×g) at 4 °C for 2 h to separate filled micelles from empty ones. The pellet was reconstituted in 1 ml of water and sonicated further for 30 min. The M-LMNs were filtered through a 0.45-micron syringe filter and stored at 4 °C.

2.5. Chemical and physical characterization of nanoparticles

FTIR spectra of oleic acid-coated MnO nanoparticles were obtained using a Nicolet IR-100 spectrometer. A 20 μl aliquot of the oleic acid-coated MnO nanoparticles dispersed in chloroform was dropped onto a disposable polyethylene IR card and the solvent was evaporated under vacuum before taking the measurements. DLS of M-LMNs in aqueous

solution was performed using a DynaPro DLS plate reader. To prepare DLS samples, the M-LMNs stock solution was diluted to a concentration of 0.25 mg/ml and sonicated for 30 min to prevent aggregation. Zeta potential was determined using a MicroTrac ZetaTrac instrument. To prepare the samples, the M-LMN stock solution was diluted to 0.25 mg/ml and sonicated for 30 min to prevent aggregation. TEM of MnO nanoparticles in chloroform and aqueous M-LMNs was performed by pipetting 10 μ l of diluted stock solution (0.25 mg/ml) onto a carbon-coated copper grid. The MnO grid was allowed to air-dry for one hour before visualization and the M-LMN grid was allowed to air-dry overnight. Once dry the M-LMN grid was then negatively stained using a 1% uranyl acetate solution. The sample was visualized with a JEOL 1200 EX transmission electron microscope at 80 kV.

2.6. Preparation and cellular uptake of FM-LMNs

Fluorescent M-LMNs (FM-LMNs) were prepared as previously described with some modifications. The fluorescent-labeled lipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (0.2 mg) was added to the initial lipid mixture in chloroform during micelle preparation. For uptake experiments, cells were seeded 24 h prior to transfection into a 8-well chamber slide at a density of 80,000 cells per well in 300 μ l of complete medium (DMEM containing 10% FBS, 2 mM L-glutamate and 1% penicillin/streptomycin). At the time of FM-LMN addition, the medium in each well was replaced with 250 μ l of fresh DMEM with no FBS. Various amounts of FM-LMNs, diluted in 50 μ l DMEM with no FBS, were added to each well. After 4 h of incubation the cells were washed with PBS and fixed to the slide using a 10% neutral buffered formalin solution. Nuclei of the cells were stained using DAPI. The distribution of FM-LMNs inside the cells was imaged with the multiphoton Olympus BX61W1 confocal microscope.

2.7. Cytotoxicity of M-LMNs, DM-LMNs, and D-LMNs

In vitro cytotoxicity was evaluated in HEK293 cells and LLC1 cells using the PrestoBlue® assay (Roche) according to the manufacturer's specifications. Cells were seeded 24 h prior to transfection into a 96-well plate in 100 μ l of complete medium (DMEM containing 10% FBS, 2 mM L-glutamate and 1% penicillin/streptomycin). At the time of nanoparticle addition, the medium in each well was replaced with 50 μ l of fresh complete DMEM. Various concentrations of the nanoparticles were diluted in 50 μ l DMEM with no FBS and added to the well in triplicate. The cells were cultured in an incubator at 37 °C under 5% CO₂ and viability was determined after 72 h.

2.8. DNA complex formation with M-LMNs, gel retardation assay, and cell transfection

M-LMN complexes with different ratios of micelle:DNA were tested. M-LMNs were diluted with PBS to a final concentration of 2 μ g/ μ l and aliquots of M-LMNs and plasmid DNA solution were diluted separately with an appropriate volume of PBS. The plasmid DNA solution was then added slowly to the M-LMN solution and vortexed for 30 min. The M-LMN:DNA complexes were mixed with loading buffer and loaded into individual wells of a 0.9% agarose gel containing ethidium bromide. Gels were electrophoresed at room temperature in Tris/borate/EDTA buffer at 120 V for 20 min. DNA bands were visualized using a ChemiDoc TM XRS imaging system.

For transfection experiments, cells were seeded 24 h prior to transfection into a 96-well plate at a density of 10,000 cells per well in 100 μ l of complete medium (DMEM containing 10% FBS, 2 mM L-glutamate and 1% penicillin/streptomycin). At the time of transfection, the medium in each well was replaced with 100 μ l of fresh DMEM with no FBS. An amount of M-LMNs equivalent to the desired weight ratio needed for use with 0.2 μ g of DNA plasmid expressing red-fluorescent protein (pCMV-td-Tomato, Invitrogen) was added to each well. Four hours after addition of M-LMNs, 50 μ l of DMEM containing 40% FBS was added to

each well and the plate was incubated for a total of 96 h. All images were made with an Olympus IX71 microscope equipped with an EXFO X-Cite Series 120 fluorescence excitation light source ($\lambda_{\text{ex}}=554$ nm, $\lambda_{\text{em}}=581$ nm) and a DP-70 high-resolution digital camera. Images were taken at 24, 48, and 96 h post-transfection.

2.9. DNase protection assay

To evaluate the resistance to nuclease digestion, M-LMN complexes with different micelle:DNA ratios were tested. M-LMNs complexed with 0.5 μ g plasmid DNA (pCMV-td-Tomato, Invitrogen) at M-LMN: DNA (wt/wt) 5:1 or 2:1 or 0.5 μ g plasmid DNA alone were incubated with 0.5 U DNase I (Roche) for 20 min at 37 °C. To inactivate the DNase I, the solutions were then incubated at 75 °C for 10 min. The samples were then mixed with loading buffer and loaded into individual wells of a 0.8% agarose gel containing ethidium bromide. Gels were electrophoresed at room temperature in Tris/borate/EDTA buffer at 100 V for 40 min. DNA bands were visualized using a ChemiDoc TM XRS imaging system.

2.10. Phantom MRI

Various dilutions of M-LMNs and DM-LMNs were diluted in deionized water and the concentration of manganese in the micelles was determined by ICP-MS. Two hundred μ l aliquots of the various micelle solutions were added to a 96 well plate in duplicate and MR images were obtained using an Agilent ASR 310 7 T MRI high-field scanner. Fast Spin-Echo Multi-Slice (FSEMS) experiments were performed in imaging mode to determine the measure of T_1 values. Nonlinear least-square fitting was performed using the MATLAB program (Mathworks, Inc.) on a pixel-by-pixel basis. A region of interest was drawn for each well, where the mean value was used to determine the longitudinal molar relaxivity R_1 . The image was recorded with VnmrJ 3.0.

2.11. Ex vivo lung MRI

C57Bl/6 mice (n=2) were treated with one intranasal instillation of M-LMNs (50 μ l of a 0.7 mM Mn solution). Control mice (n=4) were given PBS. After one hour, the mice were euthanized; the lungs were collected and placed into a medical cassette to be viewed. MR images were obtained using an Agilent ASR 310 7 T MRI high-field scanner. Gradient Echo Multi-Slice (GEMS) experiments (flip angle=45°; TR=175) were performed in imaging mode. Nonlinear least-square fitting was performed using the MATLAB program (Mathworks, Inc.) on a pixel-by-pixel basis. A region of interest was drawn around each lung and the mean value of the signal intensity was determined in this area. The image was recorded with VnmrJ 3.0.

2.12. Loading and delivery of DOX in vitro by LMNs

Doxorubicin hydrochloride (DOX) along with 4 M equivalents of triethylamine was added to chloroform and the mixture was sonicated for 30 min to dissolve the DOX. Phospholipid micelles encapsulating DOX (referred to as D-LMNs) or DOX and MnO (referred to as DM-LMNs) were prepared as previously described with some modifications [8,28]. The 3 mg of MnO was replaced by 3 mg of DOX in D-LMNs and in DM-LMNs the 3 mg of MnO was replaced by 1.5 mg of DOX and 1.5 mg of MnO. Cellular uptake experiments using D-LMNs and DM-LMNs were performed in the same manner as those of the FM-LMN uptake studies. Cells were seeded 24 h prior to transfection into an 8-well chamber slide at a density of 80,000 cells per well in 300 μ l of complete medium (DMEM containing 10% FBS, 2 mM L-glutamate and 1% penicillin/streptomycin). At the time of FM-LMNs addition, the medium in each well was replaced with 250 μ l of fresh DMEM without FBS. Various amounts of FM-LMNs, diluted in 50 μ l DMEM with no FBS were added to each well. After 4 h of incubation the cells were washed with PBS and fixed with 10% neutral buffered

formalin. Nuclei were stained with DAPI. The distribution of FM-LMNs inside the cells was determined with a multiphoton Olympus BX61W1 confocal microscope.

2.13. In vitro DOX release study

D-LMNs, M-LMNs and free DOX were each dispersed in 1 ml PBS buffer containing 1% Tween 20 (pH 7.3 or pH 5.1) and placed in a dialysis membrane (MWCO of 12,000–14,000 Da). The bag was then immersed in a tube containing 10 ml of the same PBS buffer (pH 7.4 or pH 5.1) and incubated at 37 °C. At specific time intervals the DOX content in the PBS was analyzed using the UV–vis spectrophotometer at 485 nm. Samples were all done in triplicate. M-LMNs were used as a blank.

2.14. Delivery and biodistribution of DOX-loaded LMNs (D-LMNs) or DOX-loaded M-LMNs (DM-LMNs) in vivo

C57BL/6 mice were treated with six rounds of intranasal instillations of D-LMNs (50 μ l containing 0.532 mM DOX solution) over a period of two weeks. Control mice were administered PBS. The mice were then euthanized and the lung, liver, kidney, spleen, and pancreas were collected. The biodistribution of DOX in each organ was viewed using the Xenogen IVIS-200 Optical *In Vivo* Imaging System. The lung, liver, and kidney were then stored in OCT and frozen at –80 °C. These organs were then sectioned, stained with hematoxylin and eosin, and examined for changes in morphology. For biodistribution studies, one round of D-LMNs (50 μ l containing 1.1 mM DOX solution) was administered intranasally (IN) to C57BL/6 mice. Control mice were administered PBS (IN). Twenty-four and 48 h after the administration, the lung, liver, and kidney were excised. The lung, liver, and kidney were then stored in OCT and frozen at –80 °C. These organs were then sectioned and viewed using fluorescence microscopy to determine DOX uptake.

In vitro transfections using DM-LMNs were carried out in the same manner as transfections using M-LMNs, except that the simultaneous GFP/DOX transfection was done using an 8-well chamber slide instead of a 96-well plate for imaging purposes. HEK293 cells were plated with a density of 80,000 cells per well in 300 μ l of complete medium (DMEM containing 10% FBS, 2 mM L-glutamate and 1% penicillin/streptomycin). After 48 h the cells were fixed onto the slide using 10% neutral buffered formalin and viewed for GFP and DOX using fluorescence microscopy. For *in vivo* transfection studies using DM-LMNs, C57BL/6 mice were administered 125 μ g of M-LMNs complexed with 25 μ g EGFP-DNA in 50 μ l PBS (n=2) or 125 μ g of DM-LMNs complexed with 25 μ g GFP-DNA (with 0.25 mM DOX in 50 μ l) (n=2) intranasally. Seventy-two and 96 h after the administration, the lung, liver, and kidney were excised. The lung, liver, and kidney were then stored in OCT and frozen at –80 °C. The organs were sectioned and stained for GFP using anti-GFP antibody, then viewed for GFP expression and DOX uptake using fluorescent microscopy.

2.15. Statistical analysis

Each experiment was repeated at least twice. ImageJ analyses were performed for enumeration of transfection efficiency by cell counting. All statistical analyses were done in GraphPad Prism. Data are presented as means $\pm$ SEMs. The treated and untreated groups were compared using Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of manganese LMNs (M-LMNs)

To prepare M-LMNs, Mn²⁺-oleate complexes were subjected to thermal decomposition in a high boiling-point solvent that produces MnO with a hydrophobic surface layer of oleic

acid. To create a hydrophilic exterior, phospholipid micelles encapsulating these MnO nanoparticles were prepared by the thin-film hydration method in which hydrophobic MnO nanoparticles were added to a mixture of PEG-2000 PE, DC-cholesterol and DOPE dissolved in chloroform. The particles were vacuum dried and the dry film was swelled in water, sonicated and centrifuged to remove uncoated MnO nanoparticles. The micelles coating the MnO nanoparticles are composed of ingredients that have been FDA approved for use in humans or have been used in clinical trials. The lipids DOPE and DC-cholesterol have been used in clinical trials for the nasal delivery of DNA to cystic fibrosis patients [30–33]. Lipid-conjugated PEG-2000 is an essential part of the FDA-approved formulation Doxil® [34].

FTIR spectrometry was done on the MnO–oleate nanoparticles and the bands characteristic of oleic acid-coated hydrophobic MnO nanoparticles are shown in Fig. 2A. The surface-bonded oleic acid is confirmed by the presence of bands in the 2900 and 2850 cm^{-1} range, due to the C–H stretch, and a band at 1461 cm^{-1} (C–H bending) [14]. The morphology and size of nanoparticles were determined using transmission electron micrograph (TEM) and dynamic light scattering (DLS). TEM images of oleate–MnO showed mostly spherical nanoparticles with a size of 10–30 nm (Fig. 2B). DLS analysis showed the hydrodynamic radius for the M-LMNs to be about 100 nm (not shown), which was confirmed by TEM images of M-LMNs where several electron-dense MnO nanoparticles can be seen clustered within an M-LMN particle (Fig. 2C). The surface charge of these micelles was determined by measuring their zeta potential. M-LMNs showed a net positive zeta potential of +37 mV, most likely due to the cationic DC-cholesterol and DOPE with its primary amine head groups. Inductively-coupled plasma-mass spectrometry was used to determine the concentration of Mn encapsulated in the M-LMNs. The MnO loading efficiency was determined to be about 10%.

3.2. Cellular uptake, cytotoxicity and in vivo biodistribution of M-LMNs

To examine the cellular uptake of M-LMNs, the particles were labeled with the fluorescent lipid, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl. Human embryonic kidney (HEK) 293 cells were incubated with labeled M-LMNs for 4 h and the dye was visualized by confocal microscopy (Fig. 3A). M-LMNs were seen in the cytoplasm surrounding the nuclei of the cells. To determine the cytotoxicity of these micelles, cell viability was measured using the Presto Blue assay. HEK293 cells were incubated with different amounts of M-LMNs for 72 h and cells without nanoparticles were used as a control with viability taken as 100%. M-LMNs showed no apparent toxicity when incubated with the HEK cells at any of the concentrations tested (Fig. 3B).

To determine the particles' biodistribution *in vivo*, M-LMNs were labeled with Cy5.5, a near-infrared imaging dye. The resulting particles were administered intravenously (IV) or intranasally (IN) to groups of C57BL/6 mice. Control mice were administered PBS (IN). Twenty-four, 48 and 96 h after treatment, the lung, liver, kidney, and spleen were excised and examined for Cy5.5 using a Xenogen IVIS imager and quantified. Twenty-four hours after IV administration, the Cy5.5-M-LMNs were found mostly distributed in the liver and also in kidney and spleen, but not in the lungs of mice (Fig. 3C). In sharp contrast, intranasally administered Cy5.5-M-LMNs were found preferentially (~85% of total) accumulated in the lungs for up to 48 h (Fig. 3C), demonstrating that M-LMNs have the potential to be used as theranostics for lung disease.

3.3. Gene delivery potential of M-LMNs

In order for the micelles to act as a gene delivery vehicle they must be able to form a stable complex with nucleic acids during transport and entry, to release the DNA within the cells.

The capability of these cationic lipid micelles to form complexes with DNA was evaluated using a gel-retardation assay (Fig. S1). After complexation, the DNA-micelles were pipetted into the wells of an agarose gel and subjected to electrophoresis. DNA that was bound to the micelles remained in the wells, while unbound DNA migrated down the gel. It was observed that M-LMNs were able to completely retard migration of the DNA at weight ratios as low as 5:1 (M-LMNs:DNA). During DNA delivery it is critical to protect the DNA from degradation by nucleases. The absence of ethidium bromide staining in even the wells that contain the M-LMNs:DNA complexes suggests that the M-LMNs are able to fully protect the DNA from the ethidium bromide at weight ratios as low as 5:1 (M-LMNs:DNA).

To further prove that the DNA was protected from nucleases once complexed with the M-LMNs, M-LMNs:ptd-Tomato DNA complexes were exposed to DNase-I (Fig. S1B). The presence of partial DNA bands in the lane and ethidium bromide staining in the well of the 2:1 (M-LMNs:DNA wt/wt) complex suggests that the DNA was only partially complexed to the micelles at this ratio. The DNA that was not fully encapsulated within the micelles was completely degraded, as can be seen from the absence of any DNA bands in this lane. However, the DNA that was fully enclosed in the micelles was protected from DNase I degradation, as can be seen from the ethidium bromide staining present in the well of this lane. At a ratio of 5:1 (M-LMNs: DNA wt/wt) the DNA was fully protected from both DNase I degradation and ethidium bromide staining, as judged by the absence of any bands in the lanes or ethidium bromide staining in these wells. These results suggest that M-LMNs, at a (M-LMNs:DNA wt/wt) ratio of 5:1 or higher are able to completely protect the complexed DNA from nuclease degradation.

We tested the ability of M-LMNs to transduce DNA into cultured cells and achieve protein expression by using a plasmid DNA encoding red-fluorescent protein (RFP) as a reporter. Cells were incubated with various ratios of micelle:pDNA in M-LMN complexes (Fig. 4A, B) and for various times (Fig. 4C, D). The fluorescent images in Fig. 4A and B show that M-LMNs at micelle:pDNA weight ratios of 5:1 or 10:1 readily transfected HEK293 cells. Transfection was less efficient at a ratio of 15:1. Expression of RFP was seen as early as 24 h and was maximal at 96 h (Fig. 4C, D). Also, M-LMN:ptd-Tomato DNA (5:1) induced protein expression levels similar to lipofectamine-transfected DNA (data not shown). These results indicate that M-LMNs may be a useful tool for the delivery of DNA into mammalian cells.

3.4. M-LMNs provide MRI capability in vitro and ex vivo

In addition to delivering nucleic acids and small molecules to target sites, this cationic lipid nanoparticle system was also designed to act as a T_1 MRI contrast agent to allow monitoring of the effects of gene or drug delivery. We recognize that phospholipid-encapsulated oleic acid coated nanoparticles are strongly protected from the outside aqueous environment by a tight hydrophobic layer and that this may prevent water protons from contacting the manganese nanoparticle surface and could therefore lead to a lowering of the relaxivity [35]. However, the DOPE component of the MLNs phospholipid micelle has two unsaturated fatty acid tails, which serve to increase the fluidity of the phospholipid micellar membrane. Since T_1 contrast agents need to have direct interaction with the surrounding water protons to affect their relaxation times [36], this increased fluidity may allow for more interaction of the manganese oxide nanoparticles and water protons. To determine whether M-LMNs were able to act as an effective T_1 MRI contrast agent their relaxation properties were analyzed by MR phantom imaging. Fig. 5 shows the visual (A) and quantitative (B) T_1 MRI contrasts provided by M-LMNs for various Mn concentrations. The R_1 relaxivity of M-LMNs ($1.17 \text{ mM}^{-1} \text{ s}^{-1}$) was larger than the values reported for $\text{MnO-SiO}_2\text{-PEG/NH}_2$ nanoparticles ($0.47 \text{ mM}^{-1} \text{ s}^{-1}$) [37] and comparable to the values reported for PEG-phospholipid-encapsulated HMNs ($1.417 \text{ mM}^{-1} \text{ s}^{-1}$) [9].

Since M-LMNs will preferentially accumulate in the lungs of mice after intranasal administration, we wanted to determine whether M-LMNs would enhance the T₁ MRI contrast of the lungs. One hour after intranasal injection of M-LMNs the mice were euthanized and the lungs were viewed *ex vivo* using MRI. Fig. 5 shows the visual (C) and quantitative (D) T₁ MRI contrasts provided by M-LMNs in mouse lungs. The calculated mean signal intensity for M-LMN-injected lungs was more than 2.5 times higher than that of the PBS-injected lungs. These results demonstrate that in addition to delivering a therapeutic agent, the M-LMNs could potentially act as a T₁ MRI contrast agent to enhance detection and provide a more accurate diagnosis or post-therapy evaluation.

3.5. Cellular uptake, cytotoxicity and biodistribution of doxorubicin (DOX)-loaded LMNs (D-LMNs)

The lipid cores of micelles make them ideal for encapsulating hydrophobic compounds such as DOX for safe transport through the body to the target cells. This is a much more straightforward method than attempting to chemically enhance the hydrophilicity of a water-insoluble drug without changing its activity. To determine the potential of LMNs to deliver small molecular drugs, we replaced MnO in the hydrophobic core with the chemotherapeutic drug DOX. D-LMNs were found to be spherical, as judged by TEM (Fig. 6A) with a hydrodynamic radius of about 100 nm and a positive zeta potential. To evaluate the potential of these nanoparticles for therapeutic delivery, *in vitro* cellular uptake of D-LMNs was evaluated. HEK293 cells were incubated with D-LMNs for 24 h and viewed by confocal fluorescence microscopy. Fluorescence images showed that most of the DOX was distributed in the cytoplasm of the cell (Fig. 6B). This is in contrast to cells incubated with free DOX, where the DOX is found in the nuclei intercalated with DNA [8]. These data suggest that the internalization mechanism of the D-LMNs is different from that of free DOX. Similar results have been reported before by other groups using micellar carriers to deliver DOX to cells [5].

The release of the encapsulated DOX from D-LMNs was determined at pH 7.3, which is the physiological pH and at pH 5.1, which represent the acidic pH inside endosomes, lysosomes, and solid tumors. We used 1% Tween 20 because it forms hydrophobic pockets, which can stabilize the released DOX from the D-LMNs, and help to avoid aggregation of the hydrophobic DOX in the aqueous environment. The release profile of DOX from D-LMNs is shown in Fig. 6C. DOX release occurred with an initial burst during the first 6 h with about 40% of the DOX releasing at pH 7.3 and about 50% of the DOX releasing at pH 5.1. Subsequently, the release occurred more slowly and steadily with more than 90% of the free DOX being released into the solution at either pH after 96 h. However, even after 48 h only 48% of the DOX had been released from the D-LMNs at pH 7.3 and 68% at pH 5.1. These results demonstrate that the DOX is sequestered within the micelles and that the pH of the environment plays a role in DOX release from the micelles. This moderate pH dependent release may be due to the protonation of the amine head group on DOPE in an acidic environment, which could be causing destabilization of the micelle and subsequent release of the contents [38].

To determine whether D-LMNs can deliver active free DOX, LLC1 cells were incubated with D-LMNs containing various concentrations of DOX and cell viability after 72 h was determined (Fig. 6D). The D-LMNs are just as toxic to the cells as free DOX when used at the same DOX concentrations (Fig. 6E). It is also clear that these toxic effects are due solely to the DOX and not the other components of the micelles, as M-LMNs alone exerted no cytotoxic effects (Fig. 6D). These results demonstrate that D-LMNs can deliver DOX as a payload to kill tumor cells.

To study the *in vivo* biodistribution and safety of D-LMNs, C57Bl/6 mice were treated with six rounds of intranasal instillations of D-LMNs over a period of two weeks. Control mice were given PBS. The mice were then euthanized and the lung, liver, kidney, spleen, and pancreas were collected and the amount of DOX in each organ was determined. From Fig. 6F it can be seen that, when administered intranasally, the D-LMNs preferentially accumulate and release DOX in the lungs. The relatively low levels of DOX in the other organs suggest that D-LMNs outside the lung are efficiently cleared from the body. All of these results together demonstrate the potential of D-LMNs for delivering chemotherapeutic agents for the treatment of lung cancer. To evaluate potential toxicity of the D-LMNs *in vivo* organ sections were stained with hematoxylin/eosin (H&E) and examined by light microscopy. Representative images are shown in Fig. S2. It is well known that high levels of free DOX are highly toxic to tissues and can cause ulcerations and necrosis. Despite relatively high levels of DOX accumulation in the lungs, liver, and kidneys, which was confirmed by biodistribution studies (Fig. 6F), no morphological or histological alterations in the organs were observed. The reduction of systemic toxicity can most likely be attributed to the DOX being sequestered within the cationic lipid nanoparticles and not being released in the bloodstream. These studies demonstrate that D-LMNs are able to minimize the chemotherapeutic side effects of DOX on susceptible organs.

3.6. Multifunctional LMNs for simultaneous delivery of MnO, DNA and DOX

To evaluate the potential of LMNs to deliver functional MnO as a T₁ contrast agent, DOX for chemotherapy and plasmid DNA for gene therapy, we synthesized a multifunctional LMN incorporating a mixture of hydrophobic DOX and MnO in the core and the negatively-charged pDNA on the positively-charged surface. The resulting particles, which are referred to as DM-LMNs, had a positive zeta potential and spherical morphology with a diameter similar to M-LMNs (200 nm). To examine whether DM-LMNs were also capable of providing efficient T₁ MRI contrast, the DM-LMNs were analyzed using the same MR phantom imaging as M-LMNs. At a concentration of 1 mM, DM-LMNs were able to provide a T₁ relaxivity that was only slightly less than that of M-LMNs (data not shown). These results suggest that these micelles can provide effective T₁ MRI contrast.

HEK293 cells were incubated with DM-LMNs for 24 h and DOX uptake was determined by analysis of confocal fluorescence microscope images. Similar to D-LMNs, DOX was seen mostly in the cytoplasm of the cells (Fig. 7A). Treatment of LLC1 cells with DM-LMNs for 72 h showed cytotoxicity comparable to that seen with LLC1 cells incubated with free DOX. With DOX concentrations of 1 μ M or higher, over 50% of the cells were killed (Fig. 7B). These results show that DM-LMNs can deliver DOX as efficiently as D-LMNs while still retaining MRI contrast ability.

The next question we wanted to address was whether DM-LMNs could deliver nucleic acids as efficiently as M-LMNs. HEK293 cells were transfected with DM-LMNs at the same DM-LMNs:ptd-Tomato DNA weight ratios as M-LMNs. The results (Fig. 7C–D) show that DM-LMNs were capable of delivering ptd-Tomato DNA to HEK293 cells with slightly less efficiency than M-LMNs. This can most likely be attributed to the loss of cells due to DOX-induced cytotoxicity. To image the simultaneous delivery of DNA and DOX, HEK293 cells were incubated with DM-LMNs complexed with DNA encoding enhanced green fluorescent protein (EGFP) for 48 h. HEK293 cells can be seen with DOX located throughout the cytoplasm, similar to D-LMNs, and EGFP expression throughout the cytoplasm (Fig. 7E). These data show that DM-LMNs can simultaneously deliver nucleic acids and chemotherapeutic agents into cells.

To determine if DM-LMNs are capable of simultaneously delivering DNA and DOX *in vivo* we administered nanoparticles complexed with EGFP-DNA intranasally to mice. At 72 and

96 h after administration, mice were euthanized and the lungs, liver, and kidneys were collected in OCT and frozen at -80°C . Frozen sections were immunostained for EGFP and analyzed for GFP expression and DOX uptake using fluorescence microscopy. EGFP and DOX expression could be seen in the lungs at both 72 and 96 h (Fig. 7F). These results suggest that, regardless of the payload, LMNs are able to preferentially accumulate in the lungs when delivered intranasally and that these nanoparticles are capable of the simultaneous delivery of DNA and DOX *in vivo*. Taken together, these experimental results show that LMNs provide a simple and efficient theranostic micellar formulation that can be used as a multifunctional vehicle for imaging and therapy of cancer *in vitro* and *in vivo*.

4. Conclusions

We have developed a new type of nanoparticle, the M-LMN that can be self-assembled with FDA-approved ingredients [30–34]. The lipids, DC-Chol and DOPE, used in this formulation have been shown by others to be able to encapsulate hydrophobically-coated gold nanoparticles while endowing them with the ability to efficiently deliver siRNA to target cells [22]. The PEG-2000-PE lipid is incorporated into our micelle nanoparticles to confer biocompatibility and allow for longer blood circulation times. These cationic lipid micelles can be loaded with MnO nanoparticles and the chemotherapeutic agent DOX in the core and pDNA on the surface to provide both MRI contrast and DNA/drug delivery to target cells. The synthesis of DM-LMNs is easily scalable, and we found that the unique combination of DOPE, DC-Cholesterol and PEG-2000-PE yielded a high gene transfection efficiency and drug uptake. When administered to mice intranasally as nasal drops, the DM-LMNs were found mostly in the lungs, in marked contrast to other polymers making them an ideal candidate for lung cancer theranostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. A schematic representation of multi-functional lipid-micellar nanoparticles. MnO: manganese oxide; DOX=doxorubicin; PL-1=payload 1; PL-2=payload 2.

Fig. 2. Characterization of M-LMNs. (A) FTIR spectrum of oleic acid-coated hydrophobic MnO nanoparticles; (B) TEM of MnO; scale bar=100 nm. (C) TEM of M-LMNs; scale bar=100 nm.

Fig. 3.

Cell uptake, viability and *in vivo* biodistribution of M-LMNs. (A) HEK293 cells were incubated with M-LMNs (10 µg/ml) for 4 h and cell uptake was determined by laser scanning confocal microscopy; z-stacked images of HEK293 cells showing uptake of rhodamine-conjugated M-LMNs. 1000× magnification is shown. (B) Effects of M-LMN exposure in HEK293 cells. HEK293 cells were incubated for 72 h with various concentrations of M-LMNs and cell viability was assessed. (C) *In vivo* biodistribution of Cy5.5-M-LMNs. Groups of mice (n=4) were injected intravenously (IV) or intranasally (IN) with Cy5.5-M-LMNs and at 24, 48 and 96 h after administration, the Cy 5.5 levels were quantitated by Xenogen imaging. Control mice received PBS alone (IN). Relative fluorescent intensity per mg tissue is shown.

Fig. 4.

Gene delivery potential of M-LMN. HEK293 cells were transfected with M-LMN complexed with ptd DNA encoding red-fluorescent protein (RFP). Transfection efficiency was monitored by fluorescent microscopy. RFP images (*upper panel*) and merge of fluorescent images (200 $\times$ magnification) and phase-contrast (*bottom panel*) are shown. (A–B) Various ratios of M-LMN:DNA (wt/wt) were incubated with HEK293 cells for 48 h. The transfected cells were counted with ImageJ and the percent transfection in groups was compared with GraphPad Prism. 5:1 vs 10:1 * P <0.05. (C–D) Nanocomplexes of M-LMN:DNA wt/wt; 5:1 was incubated for the indicated length of time. The transfected cells were marked and counted using ImageJ software. The groups were statistically compared in GraphPad Prism. 24 h vs 48 h ** P <0.01; 48 h vs 96 h *** P <0.001.

Fig. 5.

MRI potential of M-LMNs containing different concentrations of Mn^{2+} . Two hundred μl aliquots of indicated concentrations of M-LMNs were added to a 96 well plate in duplicate. T_1 relaxometry map derived from the multi-TE T_1 measurements; (A) visual and (B) quantitative T_1 MRI contrasts are shown. 50 μl of 0.7 mM Mn solution of M-LMNs was injected intranasally to mice. After one hour the lungs were collected and imaged *ex vivo* using MRI. (C) Visual and (D) quantitative T_1 MRI contrasts are shown. * $p < 0.05$.

Fig. 6.

Cell uptake, viability and *in vivo* biodistribution of D-LMNs. (A) TEM of D-LMNs; scale bar=100 nm. (B) Laser scanning confocal microscopic images (1000× magnification) (z-stacked) of uptake of D-LMNs by HEK293 cell; (C) Release of DOX from D-MLNs in PBS at pH 7.3 and pH 5.4 as a percentage of total encapsulated DOX. Free DOX was used as control. (D) Effect of D-LMNs on viability of LLC1cells. Cells were incubated for 72 h with various concentrations of M-LMNs or D-LMNs, and viability was assessed by Presto Blue assay. (E) Comparison of exposure of D-LMNs with free DOX in LLC1cells. (F) *In vivo* bio-distribution of D-LMNs. Groups of mice (n=4) were treated intranasally with six rounds of D-LMNs over a 2-week period, the DOX levels in each organ were quantitated by Xenogen imaging. Control mice received PBS. Relative fluorescent intensity per mg tissue is shown.

Fig. 7.

Cell uptake, viability, gene transduction and imaging potential of DM-LMNs. (A) Laser scanning confocal microscopic images (630 $\times$ magnification; z-stacked) of HEK293 cells showing uptake of DM-LMNs. (B) Treatment of LLC1 cells with DM-LMNs compared to free DOX. LLC1 cells were incubated for 72 h with various concentrations of DM-LMNs or free DOX and cell viability was determined. (C–D) Transfection potential of DM-LMNs. HEK293 cells were transfected with DM-LMNs complexed with ptd-Tomato plasmid DNA at wt/wt ratios of 5:1 or 10:1. Transfection efficiency was determined by fluorescence microscopy. Red fluorescent protein (*upper panel*) and the merge of RFP and the phase-contrast image (*bottom panel*) (200 $\times$ magnification) are shown. Transfected cells were counted separately using ImageJ software. The percent of transfected cells was compared with GraphPad Prism. ** $p < 0.01$. (E) Simultaneous green fluorescent protein transfection and DOX delivery by DM-LMNs in HEK293 cells. (F) *In vivo* EGFP-DNA transfection by M-LMNs (a–b) and simultaneous EGFP-DNA transfection and DOX delivery by DM-LMNs (c–e) in mouse lungs (1000 $\times$ magnification) after 72 h.