In Vivo Potential of Manganese Chelated Porphysomes as MRI Contrast Agents

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Abstract

Porphysome nanoparticles are composed of porphyrin-conjugated lipids. The attachment of the porphyrin moiety to each phospholipid confers novel properties to the liposome-like nanoparticle, allowing it to perform a variety of diagnostic and therapeutic applications. The metal chelating properties of porphyrin can be used to bind manganese (Mn), transforming the porphysome into a contrast agent for magnetic resonance imaging (MRI). Previous work has extensively characterized the properties of the Mn-porphysome. Herein, we build upon that work by demonstrating the bio-interactions of Mn-porphysomes *in vitro* to validate their study *in vivo*. Particle stability in serum was inferred from fluorescence quenching efficiency, and tolerability to cells was measured using an MTT assay. Mn-porphysomes remained >80% quenched after 14h and showed no toxicity to cells at concentrations below 125 mM. These preliminary results suggest that the porphysome may be used to enhance MRI contrast *in vivo*.

Key Words

Porphysome, MRI contrast agent, Manganese, Nanoparticles, Porphyrin

Introduction

The porphysome is a liposome-like nanoparticle made from porphyrin-lipid monomers with diverse potential for cancer nanomedicine. The unique conjugation of results in a combination of properties of liposomes (phospholipids) and porphyrins, as well as some emerging properties resulting from synergistic interactions. The porphysome has many applications for cancer nanomedicine including the ability to act as a fluorophore for tumour delineation [1], activatable photosensitizer [2], photothermal agent [3], and radiotracer [4].

The porphysome also has the potential to act as a contrast agent for magnetic resonance imaging (MRI), but this remains to

be explored. MRI is a non-invasive, non-ionizing, high-resolution imaging modality that can be enhanced through the use of contrast agents, which function by locally affecting the magnetic moments of hydrogen nuclei. Manganese (Mn) is a paramagnetic metal, and its permanent net dipole allows it to exert a T_1 and T_2 shortening effect. Free ionic manganese cannot be used due to issues with neurodegenerative toxicity and poor bioavailability [5]. These barriers can be overcome by the stable chelation of the metal to the porphyrin moiety of porphyrin-lipid to create Mn-porphyrin-lipid. The nanoparticles formed from Mn-porphyrin-lipid monomers have different properties from free-base (non-chelated) porphysomes. In addition to paramagnetic effects conferred by Mn, the Mn-porphysome is non-photobleachable, displays quenching of fluorescence even in its monomeric form, and exhibits only non-radiative relaxation following excitation [6].

Previous work has extensively characterized the MR properties of Mn-porphysomes *in vitro* and reported T_1 and T_2 relaxivity values (r_1 and r_2) of $1.2 \text{ mM}^{-1}\text{s}^{-1}$ and $7.0 \text{ mM}^{-1}\text{s}^{-1}$ respectively [7]. This study builds on previous work by focusing on the bio-interactions of these particles. These preliminary results enable the study of the Mn-porphysome as an MRI contrast agent for enhanced tumour detection *in vivo*, which contributes to the inherent multimodality of the porphysome. Nano-sized MRI contrast agents may help increase the limit of detection, which can aid tumour screening and lead to early intervention.

Materials and Methods

Manganese chelation

A solution of 1 mole equivalent porphyrin-lipid (pyropheophorbide- α conjugated to 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine), 5 mol equivalent MnCl_2 , 20 mol equivalent NaOAc in glacial acetic acid at a concentration of 4 mM porphyrin-lipid was heated under reflux for 2 hours at 60°C . The solvent was dried using a centrifugal evaporator and dissolved in chloroform. A liquid-liquid

extraction was performed using 1:9 MeOH:H₂O as the aqueous phase. Mn-porphyrin-lipid was retained in the organic phase.

Particle formulation

Mn-porphysome nanoparticles were composed of 40 mol% Mn-porphyrin-lipid, 15 mol% free-base porphyrin-lipid, 40 mol% cholesterol (Spectrum) and 5 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (CordenPharma). Components were prepared in chloroform and dried to a lipid film under a stream of nitrogen gas. Lipid films were hydrated using deoxygenated HEPES buffer (pH 7.4). Solution was sonicated for 30 minutes at 60°C in a 40 kHz bath sonicator (Branson 2510).

Particle characterization

Chelation of manganese by porphyrin-lipid was monitored using reverse-phase analytical uPLC-MS performed on a ACQUITY UPLC CSHM Phenyl-Hexyl 1.7µm column, using a Waters uPLC system with a photodiode array detector, and a Waters TQ mass detector using electrospray ionization. The conditions were as follows: solvent A) 0.1% formic acid in water, and solvent B) 0.1% formic acid in acetonitrile; gradient, from 60% A + 40% B to 100% B in the first 3 min, keeping 100% B for 1min, then to 60% A + 40% B in 0.1 min and keeping for 2min; flow rate 0.6mL/min. Particle size was characterized by dynamic light scattering using a Zetasizer ZS90 (Malvern).

Serum stability

0.013 mM free-base porphyrin-lipid prepared in a 60 v/v % solution of fetal bovine serum. Fluorescence quenching efficiency of the particles was measured against identical samples disrupted with 0.5 v/v % Triton X-100. Fluorescence measurements were performed using a CLARIOstar (Mandel) plate reader once every hour for 14 hours while incubated at 37°C. Porphysomes were excited at 410 nm and the area under the curve (AUC) of the emission spectrum from 600-750nm was calculated to compare the fluorescence intensity between Mn-porphysomes and the extent of fluorescence activation upon nanostructure dissociation.

Particle tolerability to cells

MAT-LyLu cells were chosen for their known biocompatibility with the Copenhagen rat strain, which will be used for in vivo studies. 2000 MAT-LyLu cells/well were seeded in a 96 well plate using RPMI 1640 medium and incubated at 37°C. 24 hours after seeding,

the cells were incubated with 200 µL of either medium containing treatment or control untreated medium at 37°C. After 24 hours, medium for different treatments was removed and replaced with medium containing 0.5 mg/mL MTT Reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Invitrogen) and incubated for 2 hours at 37°C. MTT reagent medium was removed and replaced with 100 µL DMSO and incubated at room temperature for 2 hours. Absorbance was measured at 540 nm using a SpectraMax M2 plate reader (Molecular Devices).

Results

We were able to synthesize and characterize manganese chelated, nano-sized porphysomes. The Mn-porphyrin-lipid monomers were identified by uPLC-MS. The expected m/z for our monomer (C₅₇H₈₀N₅O₉PMn) was 1065.2; we observed a molecular ion [M]²⁺ of 1065.1 m/z, and a [M]²⁺ of 533.2 m/z (Fig. 1A). Using dynamic light scattering to characterize the porphysome nanoparticles formed from these monomers, we observed a z-average size of 93.2 ± 0.2 nm and a polydispersity index of 0.130 (Fig. 1B). The Mn-porphysome's T₁ and T₂ relaxivity (r₁ and r₂) have been previously reported to be 1.2 ± 0.2 mM⁻¹s⁻¹ and 7.0 ± 0.5 mM⁻¹s⁻¹ respectively [7].

Efficiency of porphysome fluorescence quenching is used to evaluate nanoparticle intactness because only intact porphysome nanostructures display fluorescence quenching. Mn-porphysomes show a decrease in quenching efficiency from 0.98 to 0.83 over a 14 hour period in 60 v/v % fetal bovine serum at 37°C (Fig. 2A). The reduction of quenching efficiency is greater for Mn-porphysomes than free-base porphysomes which show a decrease in quenching efficiency from 0.98 to 0.93 over the same period.

Mn-porphysomes were tested for their tolerability to cells across a range of concentrations estimated to be physiologically relevant based on calculations of expected % injected dose/gram accumulation at tumour. Mn-porphysomes display toxicity to MAT-LyLu cells at concentrations above 125 mM as measured by an MTT assay (Fig. 2B). Both components of the Mn-porphysomes (free-base porphysomes and MnCl₂) were found to be non-toxic across the same range. However, the observed difference in toxicity between Mn-porphysomes and controls above 125 mM was not statistically significant (p>0.05).

Discussion

Mn-porphysomes are manganese chelated nanoparticles, which makes them ideal candidates to act as MRI contrast agents. Their nano-size (≤100nm) is suited for preferential tumour accumulation.

Figure 1: Characterization of Mn-porphysome nanoparticles.

A) Mass spectrum of Mn-porphyrin-lipid monomers. Molecular ion at 1065.1 m/z closely matches theoretical sum of manganese and free-base porphyrin-lipid (1065.2 m/z). B) Representative particle size distribution by intensity determined using dynamic light scattering. Z-average size = 93.2 ± 0.2 nm, PDI = 0.130.

The Mn-porphysome is small enough to avoid significant detection by the mononuclear phagocytic system [8], but large enough to avoid renal filtration [9], thus allowing for prolonged circulation times and greater probability of extravasation to the tumour site. This is believed to occur by a mechanism called the enhanced permeability and retention (EPR) effect [10], though the ubiquity of this mechanism and the role of other factors has made it a controversial topic in recent years [11,12].

Stability in serum is important for nanoparticles to achieve long circulation times to facilitate tumour accumulation. Adsorption of, and interactions with serum proteins can destabilize the porphy-

some, which will alter pharmacokinetics. We infer particle stability from quenching efficiency because the fluorescent porphyrin moieties demonstrate fluorescence quenching as a result of tight packing and interactions between molecules when assembled as a porphysome. Fluorescence is recovered upon dissociation into monomers. Particles that are not stable in serum display rapid decay of quenching efficiency [13].

We believe particle stability to be inversely related to Mn content. Chelation of Mn may limit the packing of monomers in the porphyrin bilayer, resulting in the observed decreased serum stability of Mn-porphysomes relative to free-base porphysomes. Particle

Figure 2: Bio-interactions of Mn-porphysome nanoparticles.

A) Stability of free-base porphysomes (FB-PPS) and Mn-porphysomes (Mn-PPS) in 60 v/v % fetal bovine serum over a 14 hour period (mean of replicates, SD error bars, $n=1$). Fluorescence quenching efficiency of porphysome nanoparticles used as a measure of particle structural stability. B) Viability of MAT-LyLu rat prostate tumour cells following treatment with various groups (mean, SEM error bars, $n=3$). Viability determined using an MTT assay measuring absorbance at 540 nm.

stability may be improved by increasing free-base porphyrin lipid, but high Mn content is desirable to provide greater MRI contrast. An optimal formulation must balance these two factors.

The chelation of Mn to the porphysome nanoparticle is able to intrinsically overcome the dopamine-dependent neurodegenerative effects [14,15] since nanoparticles cannot readily cross the blood-brain barrier [16]. Our study focused on a dopamine-free system to study particle tolerability to cells. Mn-porphysomes were an-

ticipated to be completely tolerable, so the observed toxicity was surprising despite lack of statistical significance. Both free-base porphysomes and free MnCl₂ did not show toxicity, which suggests the observed Mn-porphysome toxicity was likely a synthetic artefact due to excess reagents from chelation reaction. We hypothesize that this challenge can be overcome with additional purification.

Future work should be focused on investigating alternative methods to purify the Mn-porphyrin-lipid following chelation and con-

firming the recovery of cell viability. Once this is established, the Mn-porphysome can proceed *in vivo* to validate its ability to act as an MRI contrast agent for tumour detection.

Conclusions

Appropriate bio-interactions are necessary for nanoparticles to function *in vivo*. The preliminary results of this study suggest that Mn-porphysome is stable in serum for 14H and is tolerable to cells up to 125mM. These results complement the previous characterization of Mn-porphysomes and affirm the need for future *in vivo* studies. The limitation of particle purity may confound *in vitro* particle tolerability results and requires additional refinement to the synthesis and purification of the particle before use *in vivo*. This work helps to validate the use of the porphysome as a contrast agent

for MRI, which will contribute to the inherent multimodality of this particle.

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This is an interesting report on using porphyrin nanoparticles as contrast agents to improve MRI scans. The authors mention that the Mn-porphyrins are slightly more toxic than controls above 125 mM. However, they do not provide us with the context we need to understand this. What are the controls, and how are they showing whether the results are valid? What concentration of porphyrin is required to observe improvement in MRI contrast? Is 125 mM above or below the concentration needed to observe differences in the MRI?

With regards to this paper (Zhang et al.), yes, I am recommending it for publication after the comments/suggestions are addressed.

Supplementary Information

Materials and Methods

Animal models

All animal work was performed in adherence to the Animal Use Protocol approved by University Health Network. 5 million GFP-Luc-MAT-LyLu cells in 200 μ L RPMI 1640 medium was subcutaneously injected in the right hind limb flank of an adult male Copenhagen rat and allowed to grow for about 2 weeks. A dose of Mn-porphyrin-lipid equivalent to 5.5 μ mol/kg Mn was injected intravenously in PBS buffer and 30% DMSO as an excipient.

MR Imaging

For *in vivo* rat studies, scanning used the B-GA12 gradient coil insert and 7.2 cm inner diameter RF volume coil. Rats were positioned in prone orientation on a slider bed with 1.8% anaesthesia delivered via nose cone. Respiration was monitored using the SA Instruments system (Stony Brook, NY). T_1 and T_2 maps were acquired as stacks of 2D axial slices with matching geometric features (60 x 48 mm field-of-view, 200 x 160 matrix, 300 x 300 micron in-plane resolution, 1.5 mm slice thickness). T_2 mapping used a multi-spin-echo technique (36 echoes, 12 ms echo time, echo times ranging from 12 to 432 ms; 6 second repetition time, 12 min scan time). T_1 mapping used a variable repetition time technique (8 repetition times of 500, 750, 1000, 1500, 2000, 2500, 3000, 5000 ms; 16 min 15 sec). T_2 and T_1 maps were generated using in-line image sequence analysis. T_1 and T_2 values within tumor regions-of-interest were evaluated using MIPAV software (National Institutes of Health, Bethesda, MD).

Results

Mn-porphyrin-lipid enhances tumour contrast on T_1 relaxation maps of a subcutaneous tumour after 4 hours. The T_1 effect disappears 24 hours post injection and tumour contrast returns to base-

line. Mn-porphyrin-lipid displays no noticeable T_2 tumour enhancing effect.

Discussion

These preliminary results are the first exploration of Mn-porphyrin-lipid *in vivo* and demonstrate a proof of principle of Mn-porphyrin-lipid based nanoparticles. Injection of Mn-porphyrin-lipid monomers serves as a control for behaviour of completely dissociated particles *in vivo*. We believe the mechanism for monomer tumour accumulation is through the enhance permeability and retention effect [1]. Tumour enhancement at earlier time points (4H) and loss of enhancement at later times (24H) can be explained with our understanding of pharmacokinetics of Mn-porphyrin-lipid sized monomers. Smaller particles (>10nm) have shorter circulation half lives due to renal clearance [2], and small size also limits the ability of the particle to be retained at the tumour site [3]. The Mn-porphyrin's size makes it better suited for high yield delivery to the tumour. With the injection of the Mn-porphyrin nanoparticle, we expect greater accumulation and therefore greater contrast enhancement of the tumour at both the 4 and 24 hour time points.

The subtle T_1 effect at 4H may also be explained by a relatively small injected dose. Comparable liposome/porphyrin based manganese systems use an injected dose of 30 μ mol/kg [4,5]. To avoid potential toxicity effects observed *in vitro*, our system utilized a reduced dose. We anticipate once the toxicity issue is resolved and the intact nanoparticle is injected we will observe an even greater T_1 effect.

These preliminary results are validation of the ability of Mn-porphyrin-lipid to act as an MRI contrast agent for tumour detection. Toxicity must be addressed before a full dose can be explored. These results are promising evidence that nanoparticles constructed from these Mn-porphyrin-lipid monomers will be able to enhance tumour detection by MRI.

Supplementary Figure 1: T_1 relaxation map of subcutaneous tumour following Mn-porphyrin lipid injection.

Tumour site indicated with yellow arrow. Scale bar in ms. Left) Pre-injection. Centre) 4H post injection. Right) 24H post injection.

Supplementary Figure 2: T_2 relaxation map of subcutaneous tumour following Mn-porphyrin lipid injection.

Tumour site indicated with yellow arrow. Scale bar in ms. Left) Pre-injection. Centre) 4H post injection. Right) 24H post injection.

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