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A small MRI contrast agent library of gadolinium(III)-encapsulated supramolecular nanoparticles for improved relaxivity and sensitivity**

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Abstract

We introduce a new category of nanoparticle-based T_1 MRI contrast agents (CAs) by encapsulating paramagnetic chelated gadolinium(III), i.e., Gd^{3+} -DOTA, through supramolecular assembly of molecular building blocks that carry complementary molecular recognition motifs, including adamantane (Ad) and β -cyclodextrin (CD). A small library of Gd^{3+} -DOTA-encapsulated supramolecular nanoparticles (Gd^{3+} -DOTACSNPs) was produced by systematically altering the molecular building block mixing ratios. A broad spectrum of relaxation rates was correlated to the resulting Gd^{3+} -DOTACSNP library. Consequently, an optimal synthetic formulation of Gd^{3+} -DOTACSNPs with an r_1 of $17.3 \text{ s}^{-1} \text{ mM}^{-1}$ (ca. 4-fold higher than clinical Gd^{3+} chelated complexes at high field strengths) was identified. T_1 -weighted imaging of Gd^{3+} -DOTACSNPs exhibits an enhanced sensitivity with a contrast-to-noise ratio (C/N ratio) ca. 3.6 times greater than that observed for free Gd^{3+} -DTPA. A Gd^{3+} -DOTACSNPs solution was injected into foot pads of mice, and MRI was employed to monitor dynamic lymphatic drainage of the Gd^{3+} -DOTACSNPs-based CA. We observe an increase in signal intensity of the brachial lymph node in T_1 -weighted imaging after injecting Gd^{3+} -DOTACSNPs but not after injecting Gd^{3+} -DTPA. The MRI results are supported by ICP-MS analysis *ex vivo*. These results show that Gd^{3+} -DOTACSNPs not only exhibits enhanced relaxivity and high sensitivity but also can serve as a potential tool for diagnosis of cancer metastasis.

1. Introduction

Magnetic resonance imaging (MRI) is a medical imaging technique capable of three-dimensional (3D), high-resolution visualization of specific tissues or organs in the body and has been extensively used in neurological, cardiovascular, and oncological diagnosis[1–3]. Paramagnetic MRI contrast agents (CAs), usually composed of gadolinium(III) ion (Gd^{3+}), are capable of reducing the longitudinal relaxation time (T_1) of surrounding water protons, thus increasing signal intensity in T_1 -weighted MRI. In general, the performance of T_1 -weighted MRI CAs is determined by the relaxivity (r_1), which can be correlated to several parameters, including the loading capacity (Gd^{3+} quantity in the intraparticle space) and the water accessibility (the distance of closest approach between water and Gd^{3+} , the number of water molecules associated with Gd^{3+} , and/or the diffusion of water) as described by relaxation theory [4]. Based on this fundamental understanding, researchers have incorporated Gd^{3+} onto or into nanoscale carriers to enhance r_1 of paramagnetic CAs. Compared to small Gd^{3+} chelated complexes alone, nanocarriers (e.g., dendrimers [5–8], polymers [9–14], liposomes [15,16], and inorganic nanoparticles (NPs) [17–20]) allow

dramatically increased Gd^{3+} loading. In some cases, poor water accessibility to the intraparticular Gd^{3+} buried inside the carriers can compromise the performance in T_1 -weighted MRI [21] and this challenge can be tackled by, for example, using porous nanocarriers [8,22] where a water molecule can travel from the bulk water into the interior space. Nevertheless, fine-tuning the balance of the relevant parameters in a synergistic fashion is challenging due to the lack of direct correlations among the parameters, not to mention the unsatisfactory synthetic approach employed for the development of Gd^{3+} -incorporated nanocarriers that are considered to be time-consuming and cumbersome [23]. Therefore, there is a need to establish a method capable of effective optimization of all parameters to achieve high relaxivity enhancement of T_1 -weighted MRI CAs.

Previously, we introduced a convenient, flexible, and modular self-assembly approach [24] for the preparation of supramolecular NPs (SNPs) by mixing three molecular building blocks, including an adamantane-grafted (Ad-grafted) polyamidoamine dendrimer (Ad-PAMAM), a β -cyclodextrin-grafted (CD-grafted) branched polyethylenimine (CD-PEI), and an Ad-grafted polyethylene glycol (Ad-PEG). A variety of SNPs with controllable sizes, surface chemistry, and cross-linking degree of the Ad-PAMAM/CD-PEI hydrogel network in the SNP cores were prepared. We were able to demonstrate that the uniqueness of this supramolecular synthetic approach facilitates the preparations of different SNPs for applications in PET imaging [24], gene delivery [25], and photothermal treatment [26]. We set out to further explore such a self-assembled approach to develop Gd^{3+} ·DOTA-encapsulated SNPs (Gd^{3+} ·DOTACSNPs, Fig. 1) that can be utilized as highly efficient T_1 -weighted MRI CAs. In our design, Gd^{3+} chelated complex (Gd^{3+} ·DOTA) is covalently conjugated onto CD-PEI building block to give Gd^{3+} ·DOTA-CD-PEI, which enhances r_1 due to a distinct chemical environment with a high Gd^{3+} loading capacity inside the pseudo porous polymer-dendrimer hydrogel network and good water accessibility to the intraparticular space. By altering the mixing ratios among Gd^{3+} ·DOTA-CD-PEI and the other two molecular building blocks (Ad-PAMAM and Ad-PEG), a small library of Gd^{3+} ·DOTACSNPs can be produced with different sizes and cross-linking degrees of the corresponding hydrogel networks. A systematic variation of SNP sizes and cross-linking degrees results in a broad performance diversity of r_1 in the Gd^{3+} ·DOTACSNP library due to the respective changes in relaxation parameters. By finding the balance between the material's properties and r_1 performance of the resulting library, Gd^{3+} ·DOTACSNPs could serve as a powerful and efficient SNP-based T_1 -weighted MRI CA for dynamic imaging of biological processes *in vivo*.

Herein, we demonstrate the self-assembly of a small library of Gd^{3+} ·DOTACSNPs from three molecular building blocks – namely, Gd^{3+} ·DOTA-CD-PEI, Ad-PAMAM, and Ad-PEG as depicted in Fig. 1. By systematically mixing the molecular building blocks, a broad spectrum of r_1 is correlated to the resulting Gd^{3+} ·DOTACSNP library by evaluating the relaxation rate. In addition, we use T_1 -weighted imaging to detect Gd^{3+} ·DOTACSNP *in vitro*. We monitored lymphatic system uptake of Gd^{3+} ·DOTACSNPs *in vivo* with MRI over time and compared results with ICP-MS analysis of brachial lymph node tissue *ex vivo*. The results show that Gd^{3+} ·DOTACSNPs not only exhibits enhanced relaxivity and high sensitivity but can also serve as a potential tool for diagnosis of cancer metastasis.

2. Materials and Methods

2.1. Materials

Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification otherwise noted. 1-Adamantanamine (Ad) hydrochloride and β -cyclodextrin (β -CD) were purchased from TCI America (San Francisco, CA). DOTA/CD-grafted branched polyethylenimine (DOTA-CD-PEI) and Ad-

grafted polyethylene glycol (Ad-PEG) were prepared via the method previously reported by our group [24].

2.2. Synthesis of Gd³⁺·DOTA-CD-PEI

Various quantities of GdCl₃·6H₂O (0.1, 0.2, 0.5, 1.0, 1.2 and 2.0 mg, verified with ICP-MS) were added to a 750-μL solution of DOTA-CD-PEI and the reaction mixture was stirred at rt for 20 min to obtain Gd³⁺·DOTA-CD-PEI with different Gd³⁺ loading concentration.

2.3. Synthesis of Gd³⁺·DOTA-CD-SNPs

To a 750-μL solution of Ad-PEG (9mg/mL), various concentrations of Ad-PAMAM (22.5, 15, 11.3, 7.5 mg/mL) in 15-μL DMSO were slowly injected under vigorous stirring. A 750-μL Gd³⁺·DOTA-CD-PEI solution containing different Gd³⁺ loading concentration (0.32, 0.63, 1.61, 3.22, 3.9 and 6.44 mM, verified with ICP-MS) was sequentially added into the mixture to obtain a collection of Gd³⁺·DOTA-CD-SNPs.

2.4. Dynamic light scattering (DLS)

DLS experiments were performed with a Zetasizer Nano instrument (Malvern Instruments Ltd., United Kingdom) equipped with a 10-mW helium-neon laser ($\lambda = 632.8$ nm) and thermoelectric temperature controller. Measurements were taken at a 90° scattering angle.

2.5. Transmission electron microscope (TEM)

The morphology and sizes of Gd³⁺·DOTA-CD-SNPs were examined on a Philips CM 120 transmission electron microscope (TEM), operating at an acceleration voltage of 120 kV. The TEM samples were prepared by drop-coating 2 μL of Gd³⁺·DOTA-CD-SNPs solutions onto carbon-coated copper grids. Excess amounts of droplets were removed by filter papers after 45 s. Subsequently, the surface-deposited Gd³⁺·DOTA-CD-SNPs were negatively stained with 2% uranyl acetate for 45 s before the TEM studies.

2.6. Zeta potential measurements

Zeta potentials of Gd³⁺·DOTA-CD-SNPs were determined by photon correlation spectroscopy using a Zetasizer Nano instrument, (Malvern Instruments, Malvern, Worcestershire, UK). The measurements were performed at 25°C with a detection angle of 90°, and the raw data were subsequently correlated to Z average mean size using a cumulative analysis by the Zetasizer software package.

2.7. ICP-MS Analysis

Samples (20 μL) were diluted to a total volume of 2.00 mL using aqueous 2% nitric acid. 20 μL of a 5-ppm aqueous indium ion solution (ICP-MS grade) was then added to each tube as an internal standard and the contents vortexed vigorously. ICP measurements were then conducted on an Agilent 7500 Series ICP-MS in helium collision gas mode. Reported measurements of gadolinium in each sample represent the average of 3 measurements, and are back-calculated to offset the initial dilution. The individual measurements are typically obtained with a relative standard deviation of approximately 3% or less per sample.

2.8. Encapsulation Efficiency of Gd³⁺·DOTA-CD-SNPs

The encapsulation efficiency is defined as the amount of Gd³⁺ within the SNPs divided by the total amount of Gd³⁺. 400 μL of Gd³⁺·DOTA-CD-SNPs prepared at various concentrations of Gd³⁺ (0.7, 1.4, 2.1, 3.5, 6.0, 6.7, 9.2, 11, 12, 14 and 17 mM) was added into a centrifugal filter at 10K rpm for 30 min. After recovering filtrate containing free Gd³⁺ and supernatant containing encapsulated Gd³⁺, the quantity of Gd³⁺ in both solutions was quantified by ICP-

MS. The encapsulation efficiencies of each resulting Gd^{3+} -DOTACSNPs are summarized in Fig. S1.

2.9. Relaxation measurements

T_1 relaxation rate measurements were performed on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) equipped with a narrow-bore (54 mm) 14.1 T magnet and a broadband probe at 295 K. Both inversion recovery (IR) and saturation recovery (SR) pulse sequences were acquired.

2.10. Phantom preparation and imaging

For the dilution phantom imaging study, 90 mm capillaries were filled with the diluted solution at concentrations ranging from 4.88 μM to 195 μM and placed together in an 8mm NMR tube containing D_2O . Imaging was performed on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) equipped with a Micro 5 gradient system (maximal gradient strength of 192 G/cm in three orthogonal directions). A saturation recovery pulse sequence was used for phantom T_1 -weighted imaging and the magnetization evolved 2.5 s before applying a spin echo pulse sequence to acquire the image.

2.11. *In vivo* imaging

In vivo imaging studies used 4–6 week-old female NOD CB17-Prkd^{scid}/IcrCr1B1tw (NOD/SCID), weighing 25–30g, obtained from BioLASCO, Taiwan. All animal procedures were in accordance with the regulations approved by the Institution Animal Care and Utilization Committee at National Taiwan University. All the MR data were acquired on a Varian INOVA 7-T NMR spectrometer (Varian Inc., CA, USA) with microimaging capability. The images were obtained using a microimaging probehead and a 30 mm I.D. quadrature birdcage imaging RF coil (Varian Inc., CA, USA) with self-shielded gradient systems and a maximum strength of 100 gauss/cm in each of the x-, y- and z-directions (Resonance Research Inc., Billerica, MA, USA). T_1 -weighted images were acquired along the coronal plane using a multiple-slice spin echo pulse sequence with the following parameters: TR/TE = 50/12 ms, matrix = 256×128 , FOV (field of view) = 51.2×25.6 mm, slice thickness = 0.25 mm, NT = 4. We acquired 2 slices during each TR and total number of slices = 96. The total acquisition time was about 25 min. The three dimensional T_1 -weighted images were acquired before injection of Gd^{3+} -DOTACSNPs and at the following times after injecting 20 μL of Gd^{3+} -DOTACSNPs in the right foot pad of the NOD/SCID mouse: 40–65 min, 100–125 min, 160–185 min, 220–245 min, 280–305 min, and 340–365 min. Additionally, we acquired images 12 h and 24 h after injecting Gd^{3+} -DOTACSNPs. No gross side effects were observed during or after injection of Gd^{3+} -DOTACSNPs. For quantitative analysis of acquired images, we take the average signal in a $11 \times 9 \times 8$ voxel volume containing the lymph nodes indicated by the circles, the region of interest (ROI).

3. Results and Discussion

The three molecular building blocks, Gd^{3+} -DOTA-CD-PEI, Ad-PAMAM, and Ad-PEG (Fig. 1), were prepared and characterized according to literature [24]. By systematically changing (i) the concentration of encapsulated Gd^{3+} (0.32, 0.63, 1.61, 3.22, 3.90 and 6.44 mM) and (ii) the Ad-PAMAM/DOTA-CD-PEI ratios (1:1, 2:3, 1:2 and 1:3), a small library composed of 24 different formulations of Gd^{3+} -DOTACSNPs (Fig. 2) was prepared. The size and cross-linking degree of the hydrogel core are dependent on the Ad-PAMAM/DOTA-CD-PEI ratio. The 24 different formulations of Gd^{3+} -DOTACSNPs result from mixing Gd^{3+} -DOTA-CD-PEI, prepared at one of six Gd^{3+} concentrations, with solutions of Ad-PAMAM/Ad-PEG prepared at one of four Ad-PAMAM concentrations. The dynamic nature of the supramolecular synthetic approach leads to 80–95% of Gd^{3+} encapsulated into

Gd³⁺·DOTACSNPs, which was quantified by ICP-MS (see Supporting Information, Fig. S1). A broad spectrum of r_1 emerges from the resulting Gd³⁺·DOTACSNPs library with r_1 ranging from 4.19 s⁻¹mM⁻¹ to 17.3 s⁻¹mM⁻¹. Measurements were performed in water on a 600 MHz NMR spectrometer and are presented in a mesh plot in Fig. 2. The results highlight an optimal synthetic formulation of Gd³⁺·DOTACSNPs (1:2 molar ratio and 3.9 mM of Gd³⁺) with a relaxivity of 17.3 s⁻¹mM⁻¹ (proposed attribution of relaxation enhancement described in Supporting Information, Fig. S4). We do not measure a difference in Gd³⁺·DOTACSNPs relaxation between 300 and 600 MHz (results not shown). This is significantly higher than clinical Gd³⁺ chelated complexes, i.e. Gd³⁺·DTPA, which has r_1 ~4.0 s⁻¹mM⁻¹ at 300 MHz and 600 MHz. We characterize the resulting Gd³⁺·DOTACSNPs by measuring the hydrodynamic size of Gd³⁺·DOTACSNPs with DLS (Fig. 3A) and the results are consistent with TEM images. As shown in Fig. 3B, the TEM images indicate that Gd³⁺·DOTACSNPs have an average diameter of 103 ± 10 nm with a spherical morphology and narrow size distribution. The hydrodynamic size of Gd³⁺·DOTACSNPs (124 nm) measured by DLS analysis was larger than the value obtained from TEM, probably due to the swelling of the PEG shell layer on the Gd³⁺·DOTACSNPs in water. The zeta-potential of the resulting optimal Gd³⁺·DOTACSNPs is 22.5 ± 1.4 mV. The stability tests of Gd³⁺·DOTACSNPs were carried out under two conditions: (i) 400 times dilution in water and (ii) 10% serum. The solutions were prepared from Gd³⁺·DOTACSNPs with 3.9 mM of Gd³⁺ and then incubated under the conditions for 24 h. The sizes of both solutions were characterized by TEM and summarized in Fig. 3C. The original size of Gd³⁺·DOTACSNPs is 103 ± 10 nm (Fig. 3C, left). The size after 400 times dilution is 112 ± 15 nm (Fig. 3C, center) and in 10 % serum is 101 ± 9 nm (Fig. 3C, right). There are no distinct changes in the average sizes of Gd³⁺·DOTACSNPs after dilution and in 10% serum; therefore, we conclude that the Gd³⁺·DOTACSNPs remain stable under these conditions.

To test feasibility of Gd³⁺·DOTACSNPs as a sensitive MRI CA, we performed a series dilution study of Gd³⁺·DOTACSNPs with deionized (DI) water to establish the minimum Gd³⁺ concentration detectable by MRI. Fig. 4 shows T₁-weighted imaging results of the series dilution for Gd³⁺·DOTACSNPs solution and Gd³⁺·DTPA (Fig. 4A) at seven concentrations (from 195 to 4.88 μM) and Fig. 4B plots the C/N ratios as a function of Gd³⁺ concentration for Gd³⁺·DOTACSNPs (circles) and Gd³⁺·DTPA (squares). The Gd³⁺·DOTACSNPs and phantoms were imaged separately (note different scale bars) and the C/N ratio between Gd³⁺·DOTACSNPs and DI water are higher than the C/N ratio between Gd³⁺·DTPA and DI water at all concentrations. The results indicate that the significant sensitivity improvement and relaxivity enhancement of Gd³⁺·DOTACSNPs compared to Gd³⁺·DTPA is maintained after diluting the CA solutions down to μM concentrations of Gd³⁺.

The lymphatic system is a common route for metastatic spread of cancer [27–29]. Dynamic imaging of lymphatic drainage has been regarded as a powerful diagnostic protocol for monitoring cancer metastasis. We attempted to explore the use of Gd³⁺·DOTACSNPs as a new type of NPs for dynamic imaging of lymphatic drainage compared with conventional Gd³⁺ chelated complexes. Fig. 5A shows 3D maximum intensity projection (MIP) [30] T₁-weighted *in vivo* imaging results before and after injecting Gd³⁺·DOTACSNPs into NOD/SCID mice (experimental details in Supporting Information). The yellow circles contain the right brachial lymph node of the mouse. We injected 20 μL of a 3.9 mM MRI CA solution into the front right foot pad of a NOD/SCID mouse and monitored the changes with MRI. The draining of the lymph nodes was clearly visualized with the Gd³⁺·DOTACSNPs. The average voxel signal intensity increases in the right brachial lymph node after injecting Gd³⁺·DOTACSNPs but not after injecting Gd³⁺·DTPA (Fig. 5B and also see Supporting Information Fig. S2). Meanwhile, the average signal intensity in the left brachial lymph

node remains around 20% less than the right lymph node (see Supporting Information, Fig. S3). After 12 h, the average signal intensity in both lymph nodes decreases close to the values before injecting the Gd^{3+} ·DOTACSNPs. To support detection with MRI of Gd^{3+} ·DOTACSNPs drainage in the brachial lymph node, we use ICP-MS analysis on *ex vivo* tissue removed at two time points after injecting MRI CA, i.e., Gd^{3+} ·DOTACSNPs and Gd^{3+} ·DTPA, into the front foot pads of the mice following the same procedure used for *in vivo* imaging. We removed the brachial lymph nodes of mice after 30 min and 2.5 h. The brachial lymph nodes were dissected and underwent ICP-MS measurement. The ICP-MS analysis revealed that Gd^{3+} concentration decreased by approximately 6% after 2.5 h. As a comparison, the Gd^{3+} concentration from mice treated with Gd^{3+} ·DTPA was negligible and close to the baseline measurement on untreated brachial lymph node tissue. The results show that the ICP-MS measurements are in good agreement and validate the T_1 -weighted MRI results (experimental details of ICP-MS analysis are in Supporting Information).

4. Conclusion

We have successfully demonstrated the systematic synthesis of a small library of Gd^{3+} ·DOTACSNPs by a supramolecular self-assembly approach. The results highlight an optimal synthetic formulation of Gd^{3+} ·DOTACSNPs with an r_1 of $17.3 \text{ s}^{-1}\text{mM}^{-1}$, which is ca. 4-fold higher than clinical Gd^{3+} chelated complexes at high field strengths. In addition, the resulting Gd^{3+} ·DOTACSNPs were detected by T_1 -weighted imaging at a concentration of $4.88 \mu\text{M}$ Gd^{3+} with a contrast-to-noise ratio (C/N ratio) of 9.46, ca. 3.6 times greater than the C/N ratio for Gd^{3+} ·DTPA at the same concentration, signaling a higher sensitivity than conventional MRI CAs. Moreover, the dynamic lymph node drainage MRI demonstrated the applicability of Gd^{3+} ·DOTACSNPs *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic representation of self-assembly approach for preparation of Gd³⁺·DOTA-encapsulated supramolecular nanoparticles (Gd³⁺·DOTA-SNPs).

Fig. 2.

A mesh plot of r_1 relaxivity performance of Gd^{3+} -DOTACSNPs with variation of (i) ratio of two molecular building blocks, i.e. Ad-PAMAM and DOTA-CD-PEI, and (ii) loading of Gd^{3+} (24 data points). The peak value indicates the highest r_1 relaxivity (Gd^{3+} -DOTACSNPs with a ratio of 1:2 and at a Gd^{3+} concentration of 3.9 mM).

Fig. 3. (A) Dynamic light scattering (DLS) results and (B) transmission electron microscopy (TEM) image show the narrow size distribution and spherical morphology of the resulting Gd³⁺.DOTACSNPs (formulate with a ratio of 1:2 and at a Gd³⁺ concentration of 3.9 mM). (C) Stability studies of Gd³⁺.DOTACSNPs before (left) and after 24 h incubation under different conditions: 400 times dilution in water (center) and 10% serum (right). All the sizes of Gd³⁺.DOTACSNPs were characterized by TEM after 24 h incubation. (Each sample has at least 20 counts.)

Fig. 4.

(A) Phantom images for capillaries containing Gd^{3+} -DOTAC-SNPs (formulate with a ratio of 1:2 and at a Gd^{3+} concentration of 3.9 mM, top) and Gd^{3+} -DTPA diluted with deionized water (bottom). The capillary on the right contains deionized water (labeled 0 μM) and the concentration of Gd^{3+} in the capillaries increases from 4.88 μM to 195 μM from right to left. (B) Plot of contrast-to-noise (C/N) ratios as a function of Gd^{3+} concentration for Gd^{3+} -DOTAC-SNPs (circles) and Gd^{3+} -DTPA (squares) of the phantom images. The C/N ratio between Gd^{3+} -DOTAC-SNPs and DI water is higher than the C/N ratio between Gd^{3+} -DTPA and DI water at all concentrations.

Fig. 5.

(A) Three-dimensional (3D) T_1 -weighted MR images show dynamic lymphatic drainage of Gd^{3+} -DOTACSNPs. The yellow circle contains the right brachial lymph node. The five images were acquired before injecting and at four times after injecting 20 μ L of Gd^{3+} -DOTACSNPs (formulate with a ratio of 1:2 and at a Gd^{3+} concentration of 3.9 mM) as labeled. (B) Average voxel signal intensity plotted as a function of image acquisition time (0 h represents data acquired before injecting the MRI CA). (C) Gd^{3+} concentration from ICP-MS measurements on lymph node tissue removed 30 minutes and 2.5 h after injecting the MRI CA. Dashed line represents the baseline measurement on untreated brachial lymph node tissue. Note the log scale for the Gd^{3+} concentration axis.